Project Title:	Studies on fungicides in mushroom casing in relation to disease control
Project Number:	M 41a
Project Leader:	Dr Helen Grogan, HRI-Wellesbourne
Second Interim Report:	8th April 2004
Key Workers:	Mr. Georgios Papadopoulos, HRI-Wellesbourne Prof. Allan Walker, HRI-Wellesbourne Dr Martin Wood, Reading University
Location of Project:	HRI-Wellesbourne Warwick CV35 9EF
Project Co-ordinator	Mr Peter Davies Shackleford Mushrooms Ltd. Shackleford, Godalming, Surrey GU8 6AE
Date Project commenced:	1st October 2002
Date Project Completed:	30th September 2005
Key words:	Prochloraz, Sporgon 50WP, *Verticillium fungicola, Agaricus bisporus*, mushroom, disease

Contents

M 41a: Studies on fungicides in mushroom casing in relation to disease control - 2nd interim report

PRACTICAL SECTION FOR GROWERS

Headline

Sporgon 50WP still gives good control of dry bubble disease, provided it is applied well and disease levels are kept in check with good disease management.

Background and expected deliverables

The fungicide Sporgon 50WP is the only chemical available for the control of dry bubble disease of mushrooms, caused by the pathogen *Verticillium fungicola*. Recent HDC funded work (project report M 14b) showed that in Britain over 60% of isolates tested were moderately tolerant of Sporgon 50WP. Cropping experiments have shown that Sporgon 50WP still gives good control of these isolates (project report M 14c) but analysis of casing throughout the cropping period showed that prochloraz levels (active ingredient in Sporgon 50WP) decreased with time. Thus control of dry bubble with Sporgon 50WP in later flushes may be less effective. Good disease management is therefore still very important in order to keep this disease under control. Expected deliverables from this project include:

1. information on the persistence of Sporgon 50WP in mushroom casing over time
2. information on the key environmental and cultural factors affecting Sporgon 50WP persistence
3. information on the most effective timing of Sporgon 50WP applications for the control of dry bubble disease

Summary of the project and main conclusions

This project is studying the factors that affect the activity and persistence of the fungicide prochloraz (Sporgon 50WP) in mushroom casing. Factors such as casing ingredients, casing microbiology (including *Agaricus* activity) and environmental conditions such as temperature and moisture content will be studied to determine their role in controlling fungicide activity and persistence. Studies will be carried out to

investigate ways to optimise fungicidal activity, and modifications to current management techniques will be studied in casing microcosms to determine whether they may be effective in commercial practice. Any positive results arising from laboratory studies will be tested on mushroom crops.

This report covers the first 18 months of a three-year PhD project. In that time we have completed several experiments looking at:

- the degradation of prochloraz (a.i. in Sporgon 50WP) in casing soil under laboratory conditions and also under standard cropping conditions
- the effect of *Agaricus bisporus* on prochloraz degradation in casing in a small scale and a large scale study
- the effect of casing moisture content on the recovery of prochloraz from casing

The main **conclusions** of the first 18 months work are:

- Prochloraz was relatively stable in casing under laboratory conditions but not under standard growing conditions
- *A. bisporus* did not degrade or remove prochloraz from casing in either a small scale or large scale study
- Casing moisture content did not have any significant effect on prochloraz recovery in either small or large scale studies
- Some factor associated with standard growing conditions appears to affect prochloraz persistence

Future work

The results to date suggest that prochloraz disappears rapidly from casing under commercial growing conditions although it is more persistent under laboratory conditions. This loss of activity is not affected by either *A. bisporus* or fluctuating moisture content. The observed pattern of loss is similar to what might be expected as a result of microbial breakdown. Areas of future work include:

- Microbial breakdown of prochloraz
  - We will determine whether microbial breakdown is in fact occurring by carrying out a series of microbial enrichment experiments to select out prochoraz-degrading microbes from a sample of prochloraz-treated casing.

- We will identify, where realistically possible, the nature of any prochloraz-degrading populations isolated.
- We will screen casing-ingredients, casing-handling-equipment and fungicide-application-equipment to locate the natural habitat of putative prochloraz-degrading microbes.
- We will endeavour to identify control measures to reduce or eliminate prochloraz-degrading microbes from commercial mushroom production.

- Optimising the use of prochloraz to control *Verticillium*
  - We will determine the germination response of *Verticillium* spores to (a) increasing concentrations of prochloraz, and (b) different times of prochloraz application using a casing microcosm.
  - We will identify the most effective way to use prochloraz to control *Verticillium*.

Financial benefits

It is important to maximise the disease-controlling effects of costly fungicides by identifying the important factors that influence efficacy. This project aims to deliver knowledge that will help to identify the most effective way to use Sporgon 50WP to control dry bubble disease.

Action points for growers

At this point in time there are no recommendations for change of practice as a result of this project, though we aim to be in a position to do so when the project is completed. Contamination of casing with *Verticillium* spores during casing preparation and/or application is likely to be the most important route for *Verticillium* infections on a farm so that a high standard of general hygiene along with minimum dust generation will be best aids to disease control. The following points highlight the best practice for the control of dry bubble:

- Ensure Sporgon 50WP is applied correctly and evenly at the rates specified on the label

- Do not apply fungicide to very dry casing (i.e. between flushes) if there is a risk of run-off, and therefore under dosing; pre-water very dry casing before fungicide treatment
- Do not dry-sweep any areas or raise dust, ESPECIALLY when casing is being prepared and crops are being cased
- Ensure filters for fresh air at airing are in good condition
- Ensure diseasing-teams identify and treat dry bubble pieces quickly BEFORE any watering is done
- Do not dry-sweep cropping rooms, especially when there is disease in a crop
- Terminate badly-infected third flushes early to minimise the build up of background disease levels on the site
- Keep fly-numbers down

1 Introduction and literature review

1.1 General introduction

Little is known about what happens to fungicide active ingredients once they have been applied to mushroom casing. Results from HDC funded research (Grogan and Jukes 2003) suggests that prochloraz (Sporgon 50WP) concentrations in casing decrease with time during the cropping cycle so that fungicide levels are much reduced when threats from diseases are at their highest. The mushroom industry has very few approved fungicides for use and some of these are compromised as a result of fungicide resistance among pathogen populations

The use of Sporgon 50WP (prochloraz) in the British mushroom industry in1995 was 1,797 kg active substance (Pesticide Usage Survey Report 135n, MAFF 1997). At today's prices this is equivalent to around £120K, which represents approx. 0.1% of the £122m value of British mushrooms in 2000 (DEFRA National Statistics). Despite this spend, there is still considerable loss of production due to disease which, for dry bubble disease (*Verticillium*) has been estimated to stand at £2-3million. If fungicide efficacy can be enhanced then this loss due to disease could be reduced. This project aims to understand the reasons behind the loss of fungicide activity in mushroom casing and to identify factors that may enhance persistence. A successful outcome could reduce fungicide costs and increase the level of disease control. The objectives of the project are:

- To determine what factors and conditions affect prochloraz persistence and activity in mushroom casing
- To examine the potential to manipulate those factors that appear to be important in order to maximise prochloraz levels throughout the duration of the crop
- To investigate if manipulating fungicide persistence and activity will enhance the efficacy of products under practical cropping conditions
- To examine if the effects of casing ingredients (wet dug peat, dried sphagnum peat, sugar beat lime, chalk, ground limestone) and casing micro-organisms (including reported prochloraz degraders and *Agaricus bisporus*) on prochloraz activity and degradation.

1.2 The casing layer in the cultivation of mushrooms

It has been known for at least two hundred years that mushroom beds or trays must be covered with a layer of "soil" to induce fruiting and it was also known that those soil based casing materials varied in their performance, some giving good yields while other did not (Hayes 1973). A compost, full grown with mycelium, produces hardly any mushrooms while a layer of casing soil on the top of the compost initiates the production of mushrooms in reasonable quantities.

In relation to the compost, the casing soil is seen as a protective, covering layer which stimulates the production of mushrooms and which acts as a water reservoir with sponge-like properties retaining large amounts of water and releasing it again as required. Generally speaking, the casing will keep fully-grown or spawn-run compost, in good condition preventing it from drying.

More specifically, Visscher (1988) refers a number of different functions the casing has:

- To protect the compost layer from drying-out and from too rapid disappearance of metabolic products
- To supply water for the growth and development of mycelium and fruit bodies
- To regulate water evaporation in such way that the climate in the growing room meets the minimum air humidity standards
- To provide certain bacterial species which will stimulate fructification.
- To create a low osmotic value environment as the compost itself has too high osmotic value to produce enough mushrooms, even after ventilation.

1.3 Pesticides in the soil

1.3.1 The fate of pesticides in the soil

Ideally any chemical used in crop protection, should persist long enough to control target organisms and then degrade into inert products. Leaching and run-off losses, however, can lead to inadequate pest control as well as pollution of surface and ground waters (Vink, 1997).

Cornejo et al. (2000) suggested that soil functions as an active filter, where the chemicals are degraded by biological and non biological processes, and as a selective filter because it is able to retain some chemicals to prevent their leaching to ground

water. Both the fate of the agrochemicals in the soil and their dispersion in the environment mainly depend on the characteristics and the overall functioning of the ecosystem.

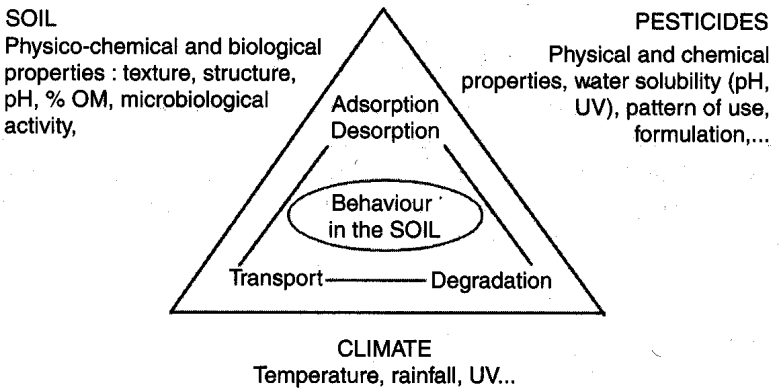
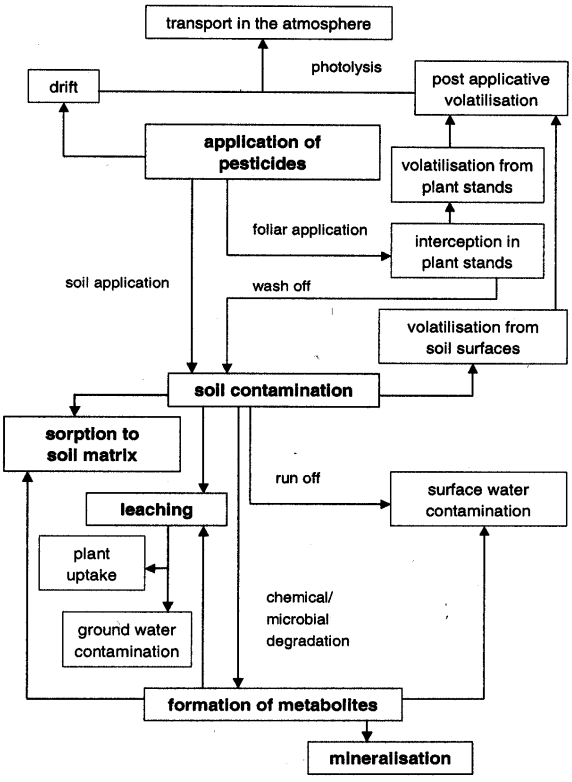


Figure 1. Factors and processes affecting the behaviour of pesticides in the soil (Cornejo et al. 2000).

In the soil, pesticides are affected by the simultaneous influence of transfer, adsorption-desorption, and physico-chemical degradation and biodegradation phenomena. All these processes are dynamic and non-linear.

A more detailed presentation of the fate of pesticides in agro-ecosystems was given by Kreuzig and Bahadir (1998) (Figure 2.):



When a chemical is applied, it should stay in the target region long enough to produce the desired effect, and then degrade into harmless materials. Progress toward this ideal is only possible with an increased understanding if the various pathways by which a compound disappears from the site of application and the related effects of different environmental conditions. The processes of sorption, leaching, microbial and chemical degradation, volatilisation are not independent, they are closely interrelated and a successful availability assessment depends on an understanding of how they affect one another.

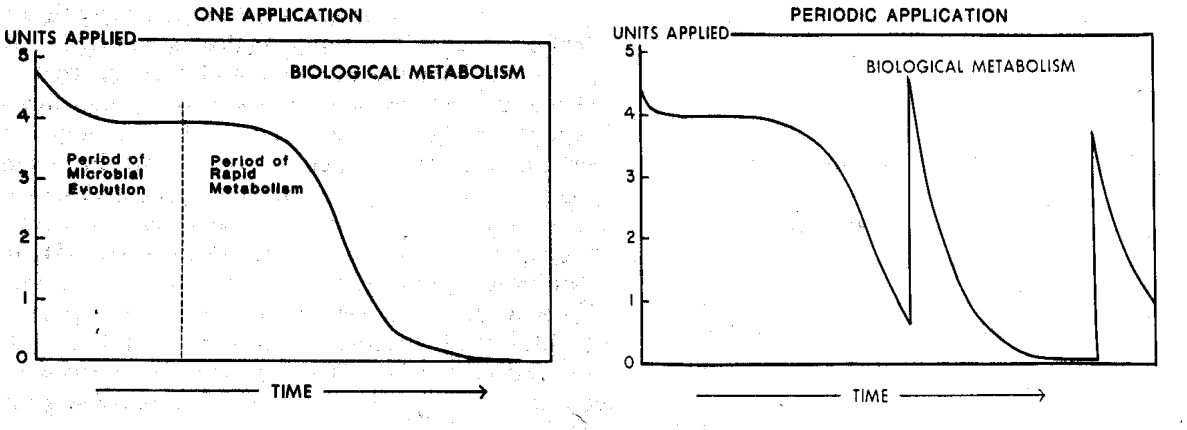
1.3.2 Sorption

Sorption and degradation are the main concentration determining processes for pesticides used in soil. Roy et al. (2000) defined sorption as a key process governing the distribution of pesticides between soil solution (available fraction) and soil particles (non-available fraction). The origin of adsorption-desorption phenoma is the molecular attraction by the surface of the mineral and organic components which is affected by a number of different factors such as water solubility, ionisation or polarity of the molecule, composition (clay, organic matter) of the soil, soil pH. An adsorbed molecule is not generally bio-available, is not carried away by the movement of water in the soil, and is often less rapidly degraded. On the other hand, desorbed molecules are bio-available and are easily degraded and carried away by run-off water (Cornejo et al., 2000).

1.3.3 Degradation

Soil is an ecosystem endowed with high degradation potential. In principle pesticides can be subject to degradation processes in all soil phases: biochemical processes in the biophase and purely chemical processes in the solid, liquid and gaseous phases. However microbial metabolism is the most important degradation process for pesticides in soil environments and it usually results in production of more or less toxic metabolites and mineral compounds (H_20, CO_2, NH_3…). The replacement of many persistent active ingredients with molecules that are more biodegradable makes microbial metabolism the most important degradation process for the vast majority of control agents in use today. Biodegradation is effectively a decomposition of the chemical by the soil microbial population (mainly bacteria and fungi). Often, when a microorganism is exposed to an unfamiliar chemical, new enzymes must be produced

if the chemical is to be utilised as a preferred source of energy. In this situation a characteristic lag phase occurs (Figure 3.) in the degradation curve because a certain



period of time is required for production to get under way. If the lag phase is long compared to the time required for a significant degree of non biological reaction to occur, then microbial activity should have little impact on the persistence of the pesticide (Farmer and Yukiko1987).

1.3.4 Sorption vs. degradation

For highly sorbed chemicals, the sorption process can significantly affect the rates of chemical and microbiological degradation. Sorption reduces the concentration of pesticides in the solution phase, resulting in a reduction in the amount of degradation occurring there. Thus, sorption on soil organic matter or clay surfaces can result in protection of the chemical from degradation. Burkhard and Guth (1981) reported, as sorption of triazine herbicides increased, their half-lives were also increased. However it becomes more complex when sorption, instead of protection of a chemical from degradation by making it less available, actually enhances the process because the soil surfaces themselves can act as catalytic agents for degradative reactions (Moyer et al. 1972)

1.4 Prochloraz in the soil

1.4.1 Physicochemical properties

Prochloraz [N-propyl-N-{2-(2,4,6-trichlorophenoxy)-ethyl}imidazole-1-carboxamide] belongs to the group of imidazole fungicides that inhibit ergosterol biosynthesis. It is widely used to control eyespot disease and powdery mildew on cereals and it is also

effective against a broad spectrum of fungal diseases on fruits as well as vegetables (Kapteyn et al. 1992; Tomlin 2000). A minor use of the compound is for disease control in mushroom growing systems. Prochloraz is weakly basic, with pK_a 3.8. It exhibits a low water solubility of 34.4 mg/l and a high lipophilicity with a $logP_{ow}$ of 4.38. It is of short persistence in aqueous solutions, and its half-life in soil ranges from 5 and 37 days under field conditions. In the laboratory, soil half-lives range between 92 and 171 days (Hollrigl-Rosta et al., 1999). The main metabolic pathway starts with prochloraz-formylurea, which is then hydrolysed to prochloraz-urea. Both substances have been isolated in mammalian and soil degradation studies.

1.4.2 Soil moisture content and sorption of prochloraz

Sorption of organic chemicals on soils, substantially influences their fate because mechanisms such as transport, microbial uptake or metabolization, are operative only on the fraction of the chemical that is present in the soil solution (Pignatello, 1989).
Roy et al. (2000) studied the effect of soil moisture content of a loam soil (46.3% sand, 39% silt, 17.4% clay and 2.44% organic matter), on the sorption of five sterol biosynthesis inhibitors (SBIs) including prochloraz. In this study the Wershaw's humus model (humic substances have a membrane-like structure) was adapted. Based on this, it was assumed that low soil moisture content would modify the structure of humic substances and generate hydrophobic surfaces, which favour sorption of hydrophobic fungicides. The effect becomes adverse for more hydrophilic compounds, which are more sorbed at high soil moisture due to their higher affinity for hydrophilic regions of humus.

Specifically prochloraz was found to partition rapidly into the liquid-like interior of humus at low soil moisture content, but increased diffusion at high soil moisture caused additional sorption by intra-aggregate colloid surfaces. The mechanism of sorption of prochloraz involved both physical and ionic processes depending on the pH surrounding the chemical. The neutral form of prochloraz could first partition into the hydrophobic interior of the membrane-like humic substances, especially at low soil moisture. As molecules diffuse throughout the soil particles, they come into contact with water of lower pH at the colloid surface and a small portion can protonate. The ionised species might then be strongly adsorbed to clay and humus cation exchange sites. The diffusion process could be responsible for the highest level

of long-term sorption observed at the high soil moisture by increasing the probability of ionisation of molecules at colloid surfaces. The changes in prochloraz concentrations at different soil moisture contents are shown in the figure below:

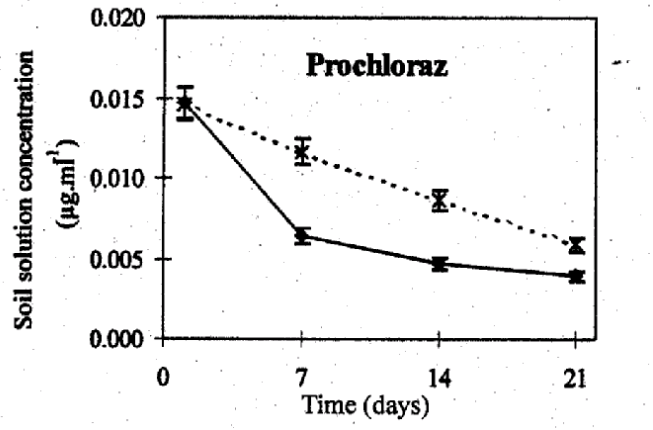


Figure 4. Changes over time in the concentrations of prochloraz in the soil solution at (x) 26.1% and (♦) 46.6% soil moisture content (Roy et al., 2000)

It was concluded that all five SBI fungicides are strongly and rapidly sorbed onto soil and the bioavailable fungicide concentrations for soil organisms (earthworms, micro-organisms and plant roots) are very low. Finally, no degradation was reported for any of the five fungicides (prochloraz included), during the 3-week period of equilibration and the lowest rate of recovery of all fungicide tested was 95% (Roy et al., 2000).

1.4.3 Sorption of prochloraz in different soils

Sorption of prochloraz was also studied by Hollrigl-Rosta et al. (1999) in order to characterise the behaviour of this compound in six different soils (two silt loams, three loamy sands and one sand) and to examine the influence of pH, organic carbon and clay content to sorption isotherms.

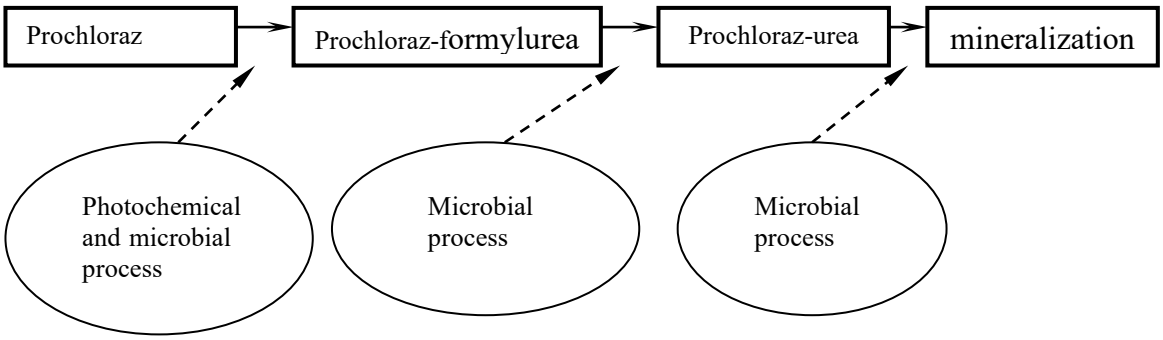
Experimental results in this study confirmed that prochloraz is sorbed on soil through a two-step process as rates of sorption were highest within the first hour and significantly lower over the subsequent course of time. This was interpreted as a weak binding of the molecules to outer sorption sites in the first stage, and subsequent diffusion to inner sorption sites in the second stage (Hollrigl-Rosta et al., 1999). Higher clay content appeared to have no enhancing influence on the sorption of prochloraz while distribution coefficients (K_d) were positively correlated with organic

carbon content. In general, the sorption of non-ionised poorly water soluble lipophilic compounds is governed by organic carbon content rather than clay content. This may be due to the fact that organic matter often covers the surfaces of clay minerals, and exposed clay surfaces are shielded by a lipophobic hydration shell and hence are not directly accessible for lipohilic organic compounds.

Hollrigl-Rosta et al. (1999) also studied the influence of soil pH to the sorption of prochloraz and found that K_d increases when pH is decreased. In laboratory degradation experiments in the dark, it was found that there was slightly slower dissipation of prochloraz in acidic than in neutral to basic soils. Stronger sorption of the molecules at lower pH suggests a reduction in bioavailability of prochloraz to degrading micro-organisms. In conclusion it was reported that this compound is strongly adsorbed on all soils tested and that it is not likely to leach into deeper soil layers despite its relatively long persistence.

1.4.4 Degradation of prochloraz

Degradation studies by Hollrigl-Rosta et al. (1999) demonstrated that both biotic and abiotic degradation occur concurrently for prochloraz. The overall fate of this chemical in soil is determined by a combination of photochemical and microbial processes. It is suggested that a photolytically activated microbial degradation, which was observed for chloramben, may be also occur with prochloraz. The importance of the microbial activity of soil in prochloraz degradation was also stressed by Bock et al. (1996) as they reported the formation of prochloraz metabolites (prochloraz-formylurea) after incubating a medium containing the compound and a strain of *Aureobacterium* spp. Finally, the later steps of prochloraz degradation, from the hydrolysis of prochloraz-formylurea to prochloraz-urea through to its mineralization depend only on microbial metabolism. The breakdown pathway of prochloraz is shown below:



1.5 Aim of this study

The fate of pesticides in agricultural soils has been studied extensively in last decades. However, there is limited knowledge about the fate of prochloraz in mineral soils and few researchers have looked at prochloraz-soil interactions (Hollrigl-Rosta et al. (1999, Roy et al. 2000). Furthermore, in mushroom crops where prochloraz is applied on a peat-based casing little is known about sorption, biodegradation, availability for disease control and effectiveness of this fungicide against *V. fungicola*. The overall aim of this study is:

- To understand the factors affecting the persistence of prochloraz in the casing material in relation to disease control

2 Small scale laboratory experiments

2.1 Introduction

A batch of small-scale experiments was set up to explore the behaviour of prochloraz under defined laboratory conditions. The objectives of these *in-vitro* experiments were:

- To compare prochloraz behaviour at 2 different temperatures and in 2 soil types (mushroom casing and a sandy loam)
- to investigate the effect of wetting and drying cycles of casing on prochloraz recoveries
- to investigate the effect of the presence/absence of *A. bisporus* on prochloraz behaviour in a small-scale cropping experiment
- to explore the influence different casing moisture levels on prochloraz recoveries

2.2 Materials and methods

2.2.1 Prochloraz behaviour in mushroom casing (MC) and in sandy loam (SL) at 15ºC and 25ºC

25g of peat-based fresh mushroom casing (Tunnel Tech Ltd) and of a sandy loam (Deep slade field, Wellesbourne) were placed in glass jars with screw-top lids and received 30ppm prochloraz. The fungicide was applied with a 5ml Pasteur pipette and the jars were incubated at 15ºC and 25ºC for 56 days in total. Samples were taken for extraction and analysis once every week. Extraction of prochloraz residues was achieved after mixing the 25g of soil/casing with 50ml of acetonitrile ACN and shaking for 1h on wrist-shaker. The samples were centrifuged at 5000rpm for 3 min to remove solid wastes. The samples were injected into a High performance liquid chromatography (HPLC), Kontron series 300 with a Pinnacle II C-8 (5µm, 150x4.6mm, Restek) column. The mobile phase used was acetonitrile: water: orthophosphoric acid (85: 15: 0.25 v/v) at a flow rate of 1 ml/min. Prochloraz was quantified by UV absorbance at 220nm. The software used for processing the results was KromaSystem 2000.

2.2.2 The effect of wetting and drying cycles of casing on prochloraz recoveries

24 metal columns with 100g of casing each and 24 glass jars with 25g of casing each were prepared. The glass jars were the same as described in 2.2.1. The dimensions of

the columns were 6cm diameter x 4.5cm height. The 4.5cm height of the columns is similar to the depth of the casing layer in mushroom crops. The casing in the jars received no water throughout the experiment and the lids were kept closed during the experiment but opened once every week for a few seconds to allow aeration but also to avoid moisture losses. In contrast, the columns remained open for the duration of the experiment. The columns were weighed and watered every day to make up the losses of moisture. The watering schedule followed the same pattern as for a commercial crop (early heavy waterings for 7 days, no water before 1st flush, regular waterings between flushes of mushrooms).

Both jars and columns were incubated at 25ºC until Day 8 when the temperature was gradually reduced to 18 ºC between Day 8 to Day 11. From Day 11 and onwards temperature was kept steady at 18 ºC. The temperature regime as well as the watering schedule was the same as in commercial mushroom units. The fungicide (Sporgon WP, prochloraz 46%) was applied on Day 5 to give a concentration in the casing of 15ppm. A second dose of prochloraz was applied on Day 21. This experiment included 2 treatments (Jars and Columns). Triplicated samples were taken for extraction and analysis on a weekly basis from Day 0 up to Day 42 (8 sampling points). Extraction and analysis was the same as in 2.2.1

2.2.3 The effect of the presence/absence of *A. bisporus* on prcohloraz behaviour in small scale cropping experiment

48 plastic pots (10x10x15cm) were used containing approx. 450g of mushroom compost on the bottom and exactly 150g of fresh casing forming a 4.5cm thick layer on top. Half of the pots (24) contained *A. bisporus*-spawned compost and the rest (24) contained compost with the same ingredients but with no *A. bisporus*. 3 replicate pots were prepared for each of 8 sampling times. The 48 pots were then arranged in 3 randomised blocks each containing 16 pots. Throughout the experiment the pots were kept in a growth cabinet with controlled temperature and relative humidity. The day when the casing layer was applied was considered as Day 0 and the day when the crop ended, after three flushes of mushrooms, as Day 42. Between Day 0 and Day 8 all pots received 22ml of water per pot every day which is equivalent to 2L/m^2, and the temperature in the cabinet was kept at 25ºC with relative humidity, at 95%. After Day 8 the waterings stopped and the temperature gradually reduced from 25 ºC to 18 ºC.

The pots with *A. bisporus* spawned compost produced mushrooms in three flushes with regular waterings (22ml/pot) between them.

Prochloraz was applied (Sporgon WP 46%) in 20ml of water using a Pasteur pipette, on Day 3 and Day 21 to give a concentration in casing of 15ppm fresh weight. Three pots were destructively sampled every sampling time for each treatment. For each sample, the whole casing layer (150g on preparation) was removed, mixed with 100ml acetonitrile in glass jars and shaken for 1h. Samples were purified using Solid Phase Extraction (SPE) cartridges. This procedure is described in 2.2.5. Residues analysis was same as in 3.2.1

2.2.4 The influence different casing moisture levels to prochloraz recoveries

Four plastic open-top containers received 100g of fresh casing each and left for 24h to dry at room temperature, creating four different moisture levels: 50, 60, 70, 80 % water content. The moisture content of the fresh casing was 77.86%. The moisture of the casing in each plate was monitored every hour and when it reached the desired level (ie 50, 60, 70, 80 %) the casing material of the container was divided into 4 identical replicates. The 80% water content was created by adding water to casing. Each replicate was placed in a glass jar with screw-top lid and Prochloraz was applied to give a concentration of 15mg/kg of fresh weight. Extraction and analysis were the same as in 3.2.1 and 3.2.2.

2.3 Results

2.3.1 Prochloraz behaviour in mushroom casing (MC) and in sandy loam (SL) at 15°C and 25°C

At 15°C (Figure 5.) the initial recovery obtained from casing was low as only 19.48mg/kg prochloraz out of 30mg/kg was recovered (i.e. 64.9% recovery) with the recovery from sandy loam slightly better (76.1% initial recovery). In both substrates prochloraz degraded slowly with calculated half-lives of 85.5 days for casing (MC) and 108.2 days for sandy loam (SL). At 25°C (Figure 6.) prochloraz disappeared more rapidly from both casing and sandy loam, in comparison to 15°C, with calculated half-lives of 48.1 days for the casing and 79.6 for the sandy loam.

The disappearance of prochloraz from mushroom casing (MC) was enhanced by the higher temperature (Figure 7.) and similarly the fungicide degraded faster when sandy loam (SL) was incubated at 25ºC (Figure 8.)

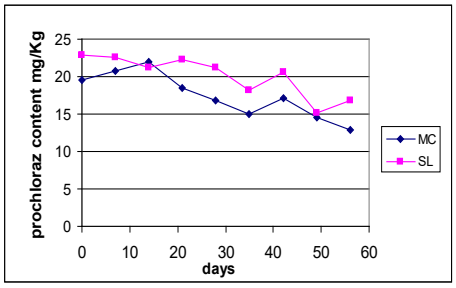


Figure 5. Prochloraz recoveries from casing and sandy loam at 15ºC

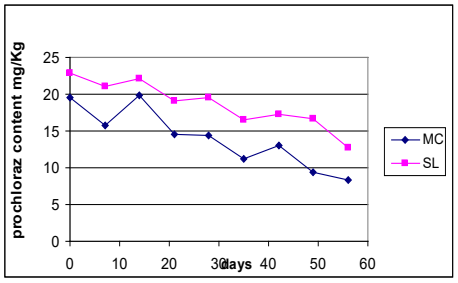


Figure 6. Prochloraz recoveries from casing and sandy loam at 25ºC

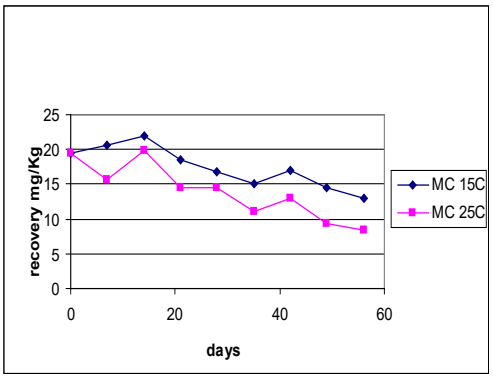


Figure 7 . Prochloraz recoveries from mushroom casing at 15ºC and 25ºC

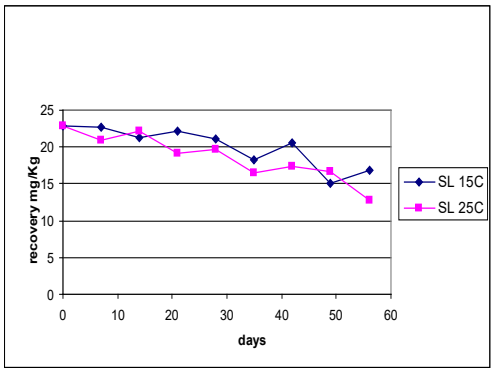


Figure 8. Prochloraz recoveries from sandy loam at 15ºC and 25ºC

Table 1.

Half-lives*	**15ºC**	**25ºC**
Mushroom Casing (MC)	85.5	48.1
Sandy Loam (SL)	108.2	79.6

* in Days

2.3.2 The effect of wetting and drying cycles of casing on prochloraz recoveries

The prochloraz residues recovered from, both from jars and columns, showed that prochloraz was relatively stable with a slow degradation rate. After the 2nd prochloraz application (Day 21) the results from columns exhibited inconsistency (Figure 9.)

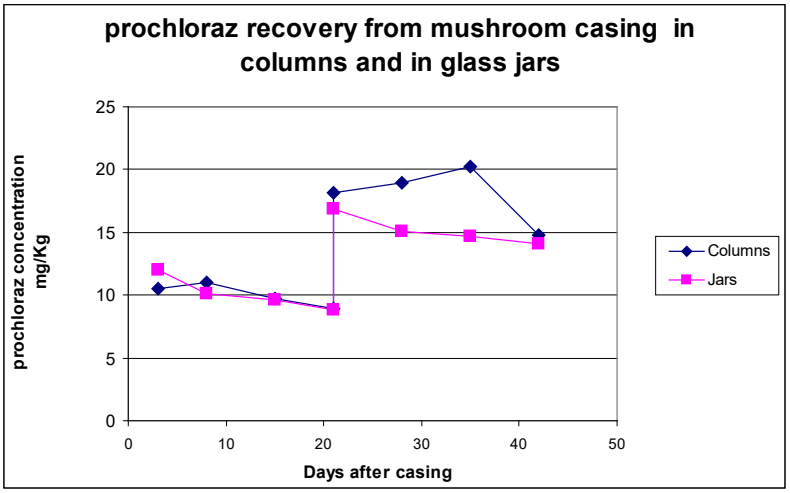


Figure 9. Recoveries from jars and columns investigating the effects of wetting and drying cycles on prochloraz

All calculated half-lives of prochloraz in this experiment were greater than the cropping period.

2.3.3 The effect of the presence/absence of *A. bisporus* on prochloraz behaviour in a small scale cropping experiment

82% of the applied prochloraz was recovered from casing at Day 5. Between Day 5 to Day 11 prochloraz levels were fairly stable for both treatments but there was a slight decline between days 11 and 21. After the second prochloraz application on Day 21 the residues of the fungicide in casing were more stable for pots containing *Agaricus* while there was a slight decrease of prochloraz levels in the controls (Figure 10).

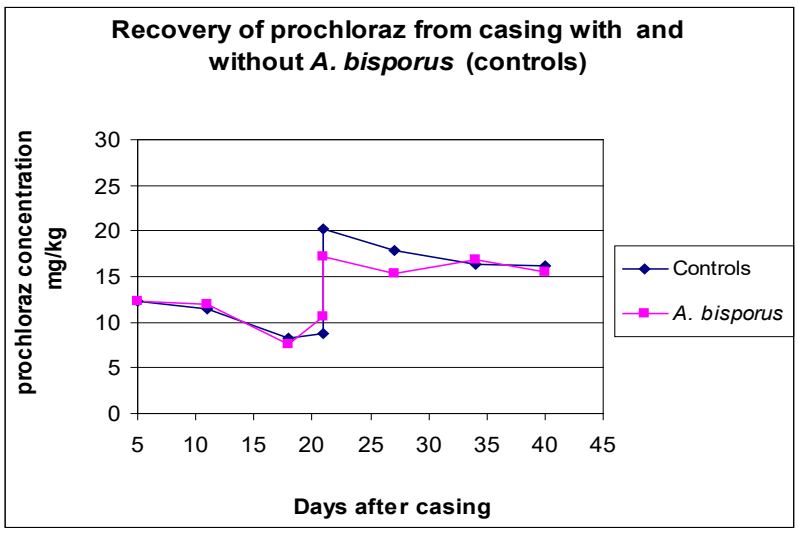


Figure 10. The recovery of prochloraz in a small scale cropping experiment.

Half-lives were calculated for both treatments from Day 5 to Day 21 and from Day 21 to Day 42 as shown in the table below (Table 2.):

Table 2. Half-lives (in days) of prochloraz in casing spawned with *A. bisporus* and casing without *A. bisporus* (controls)

Treatment	Day 5 to Day 21	Day 21 to Day 42
A. bisporus	35	198
Controls	27	58

2.3.4 The influence of different casing moisture contents on prochloraz recovery

The recoveries were not affected by the moisture content of the casing. However, the drier casing (50 and 60% water content) gave more consistent results in comparison to the wetter (70 and 80%) casing as STDV error bars indicate (Figure 11).

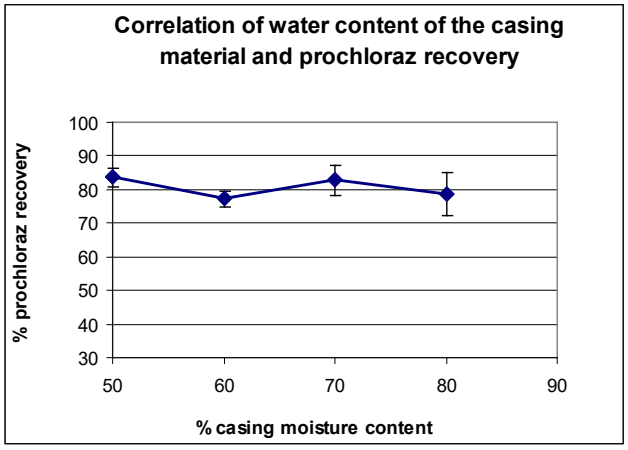


Figure 11.The effect of casing moisture on the recoveries obtained

2.4 Conclusions-Discussion

Although prochloraz disappeared more rapidly at 25ºC than at 15ºC, the levels in mushroom casing and the sandy loam were relative high even after 50 days. It was fairly persistent in both peat-based casing and in the mineral soil (sandy loam) and there was no evidence of biological or chemical degradation. Sorption processes could play a role in this behaviour in both soil types. Sorption reduces the concentration of pesticides in the solution phase which protects the pesticides from potential biological degradation in the soil solution. The humic substances of peat (the main ingredients of

the casing) have very a high exchange capacity and internal/external surface area, which cause strong sorption.

Wetting and drying cycles are standard practice in a mushroom growing unit and the water content influences the physico-chemical properties of the soil (casing) and consequently its relationship with pesticides (prochloraz). In this experiment, we investigated whether or not the constant wetting and drying cycles could be responsible for the rapid disappearance of prochloraz from casing reported by Grogan and Jukes (2003). We found no evidence of rapid disappearance of prochloraz or any effect of wetting and drying cycles on the rate of loss. Prochloraz losses from leaching and run-off have not been investigated in this study, but Grogan and Jukes (2003) showed that prochloraz does not move through the casing layer towards the compost.

Since wetting and drying cycles did not have any impact on prochloraz recoveries and did not cause any appreciable prochloraz loss, *A. bisporus* was the next factor to be investigated for possible effects on degradation. However, in small scale studies, the residue levels in the *A. bisporus* spawned pots were not significantly different from the residues extracted from casing without *Agaricus bisporus*. Mushrooms did not therefore exhibit any ability to remove or breakdown the fungicide.

The moisture content of casing did not influence the extraction, detection and measurement of prochloraz residues. Roy et al. (2000) found that higher moisture levels had adverse effects on the recoveries by increasing the proportion of fungicide sorbed in the solid phase. That is not the case in our experiments as no evidence has been found to suggest that high levels of moisture in casing increase prochloraz sorption and decrease its recovery. It should be stressed that in Roy's study the maximum soil moisture was 46.6% and only 2.44% organic matter whereas we have used a peat based casing soil with moisture range 50-80%.

Finally the introduction of the SPE cartridges improved the purification of the samples and eventually the detection the prochloraz residues by HPLC. Prochloraz peaks were sharp with identical retention times with the standards.

3 Prochloraz behaviour in a large scale cropping experiment

3.1 Introduction

The small-scale experiments suggested that prochloraz behaviour was quite stable and largely unaffected by either the wetting/drying regime of the crop management practices or by the presence of *Agaricus*. This is in contrast to published results for large-scale experiments in a mushroom unit (Grogan and Jukes 2003). An experiment was set up in HRI's mushroom Unit using all standard cultural techniques and equipment that are used in most commercial units in UK. The specific objectives were to investigate the effect of *A. bisporus* on the persistence of prochloraz with 2 different prochloraz application rates under *in-vivo* conditions, and to compare these results with earlier *in vitro* studies.

3.2 Materials and methods

3.2.1 Mushroom crop details

Trays with spawned mushroom compost, colonised by live *Agaricus* culture, were prepared as described by Grogan and Jukes (2003). In addition trays of compost without live *Agaricus* culture were also prepared.

A commercially available casing (Tunnel Tech Ltd) was used in these experiments. A breakdown of the ingredients is listed in Table 3. All mushroom trays received a 45-50mm deep layer of casing. The day when the casing was applied is designated "Day 0".

Table 3. Percentage by volume of ingredients

Ingredient	%v/v
Black deep-dug peat	42
Brown surface peat	42
Sugar beat lime	16*

*Sugar beet lime average, can fluctuate from12-20%

**Small amount of water was also added

3.2.2 Growing conditions

Mushrooms were grown in wooden trays (0.9 x 0.6 x 0.2 m) containing the compost and the casing. The trays were arranged inside the growing room in 2 stacks, each 3 trays high. Heavy water drenches (2 l/m^2) were applied up to twice a day every day, until Day 7. During this time environmental conditions were maintained at 25ºC temperature and 95% RH. From Day 8 and afterwards the trays received no water and between Day 8-11 temperature gradually decreased to 18ºC and relative humidity to 87%, to stimulate the formation of mushrooms. The first flush was picked between Days 18 and 20, the second flush between Days 26-28 and the third between Day 34-36. Up to four waterings, each about 2L/m^2 were applied between flushes.

3.2.3 Prochloraz application

The fungicide was applied as Sporgon 50 WP 08802 (Sylvan Spawn Ltd, BASF plc) with active ingredient prochloraz 46%. The recommended rate for the mushroom crop is 120g/180L water/100m^2 casing area applied with the first watering around Day 4 with the same amount applied again after the 1st flush, around Day 21-22. A second treatment consisted of applying 240g/180L water/100m^2 in one dose on Day 10 (This rate has been used in Europe in the past but is not approved in UK)

Table 4. Prochloraz rates and application time schedule

Sporgon rates* in g/100m^2	Day 4	Day 10	Day 21
120	✓	—	✓
240	—	✓	—

* mixed with 180 L of water

The fungicide application equipment consisted of a 25L tank and a lance with No1 rose, connected to an electric pump. On each application day, the volume of spraying liquid was calibrated to ensure delivery of the required amount of fungicide to each tray.

3.2.4 Sampling

Samples were taken throughout the duration of the crop from Day 4 up to Day 45. Each sample, taken randomly from the trays, was made up of 5 casing cores, 26mm diameter (45-50 mm deep). Samples were frozen (-18ºC) on the day they were taken Prior to extraction the cores were evenly mixed before they were analysed. Dry and fresh weights were also determined for each sample. From the total of each 5-core sample, 50g were allocated for residue analysis and the rest for moisture determination

3.2.5 Prochloraz extraction and analysis

Samples (50g of casing) were mixed with acetonitrile (ACN) in glass jars with plastic screw lids. The samples were shaken for 1h and centrifuged (MSE Mistral 2000) at 5000 rpm for 3 min to remove solid wastes. To purify prochloraz residues, 6ml Solid Phase Extraction cartridges (SPE tubes, ENVI-18, Supelclean™) were used. Crude extracts containing the pesticides residues were mixed with distilled water (5 : 95 v/v) passed through the SPE cartridges. The pesticide was retained in the SPE column and was eluted with 5ml ACN: H_2O: H_3PO_4 (85:15:0.25 v/v) before High Performance Liquid Chromatography (HPLC) procedure was followed.

3.2.6 Experimental design-statistical analysis

The experiment was designed as an incomplete Latin square with 4 treatments (2x2 factorial), 6 replicates and 24 plots. The 4 treatments consisted of 2 fungicide doses each with and without (control) live *Agaricus*. Trays were arranged inside the growing room according to standard practices (2 stacks, each 3 trays high). The arrangement can be seen in Table 5. The statistical analysis was ANOVA using GenStat software.

Table 5. Arrangement of plots and treatments in the growing room

	Column 1	Column 2	Column 3	Column 4
top	Plot 1 *Agaricus*-120+120	Plot 4 Control-120+120	Plot 7 Control - 240	Plot 10 *Agaricus*-240
middle	Plot 2 Control-120+120	Plot 5 *Agaricus*-120+120	Plot 8 *Agaricus*-240	Plot 11 Control-240
bottom	Plot 3 *Agaricus*-240	Plot 6 Control-240	Plot 9 Control-120+120	Plot 12 *Agaricus*-120+120

Column 1	Column 2	Column 3	Column 4
Plot 13 *Agaricus*-120+120	Plot 16 Control-240	Plot 19 Control-120+120	Plot 22 *Agaricus*-240
Plot 14 *Agaricus*-240	Plot 17 Control-120+120	Plot 20 Control-240	Plot 23 *Agaricus*-120+120
Plot 15 Control-240	Plot 18 *Agaricus*-120+120	Plot 21 *Agaricus*-240	Plot 24 Control-120+120

3.3 Results

For the 120+120 g/100m^2 rate, the initial recoveries of prochloraz residues after the 1st application (Day 4) were very good. From the trays containing *Agaricus*, 95% of the initial prochloraz amount was recovered (114 g/100m^2) and from the trays without *Agaricus* (control trays) the recovery reached 96.6% (116 g/100m^2). From Day 4 onwards, prochloraz levels in the casing fell in both *Agaricus* and non- *Agaricus* (control) trays.

After the 2nd application (Day 21), the recovered prochloraz residues followed a decline similar to that reported by Grogan and Jukes (2003). The fall in prochloraz levels between Day 23 and Day 28 was sharp for both treatments and continued until Day 45 but at a slower rate. On Day 45, prochloraz levels in the *Agaricus* were to 14% of the applied dose for the 2nd application (Day 23). Similarly, in the non-*Agaricus* (control) trays the levels the fungicide were only 15.5% of the total prochloraz amount in casing on Day 23. Statistically, there was no difference between the treatments in the 1st application (Day 4 to Day 21) and in the 2nd (Day 23 to Day 45) weak evidence was to support that *Agaricus* (A) gave smaller recoveries from Control trays (C). Both prochloraz recovery curves for the *Agaricus* and non-*Agaricus* plots, followed similar patterns throughout the crop and are shown in the figure below:

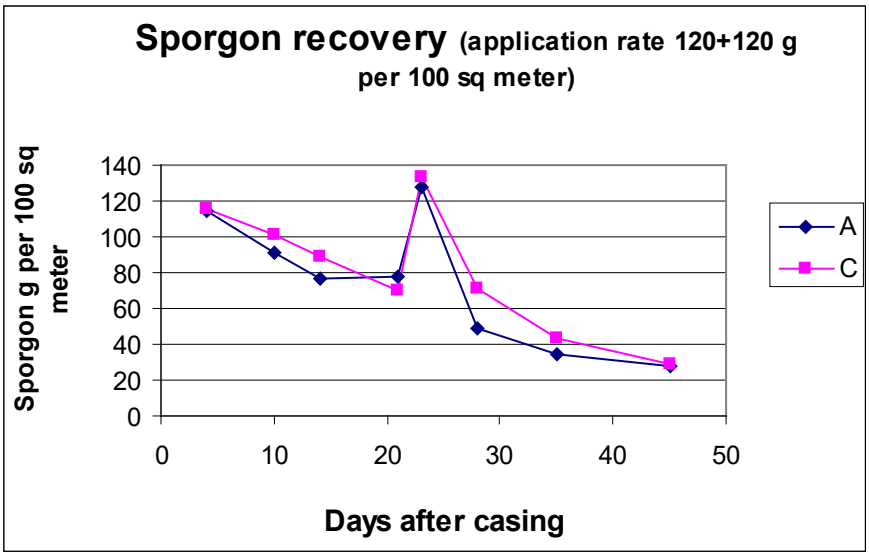


Figure 12. Levels of Sporgon (prochloraz a.i.) after 2 applications on Day 4 and Day 21, in casing spawned with *Agaricus* (A) and in casing without *Agaricus* (C)

Half-lives of prochloraz were also calculated following fitting of an exponential trend line to the curves for the1st application (Day 4 to Day 21) and 2nd application (Day 23 to Day 45). The half-lives (Table 6) following the first application were relatively short and following the second application, they were short still (Table 6.).

Table 6. Prochloraz half-lives for *Agaricus* and Control (without *Agaricus*) CASING for the 120+120 g/100m^2 application rate

Treatment	Half-life in days (Day 4 to Day 21)	Half-life in days (Day 23 to Day 45)
Agaricus (A)	30.5	11.1
Without *Agaricus* (C)	22.7	10.5

In accordance to crop management practices, heavy waterings were applied to the casing during the early days of the crop, followed by intermittent watering between the flushes. Part of that water was utilised by the growing mushrooms that were harvested in 3 flushes. The differences in casing moisture contents between the *Agaricus* and non-*Agaricus* (Control) treatments are shown in the figure below (Figure 13.):

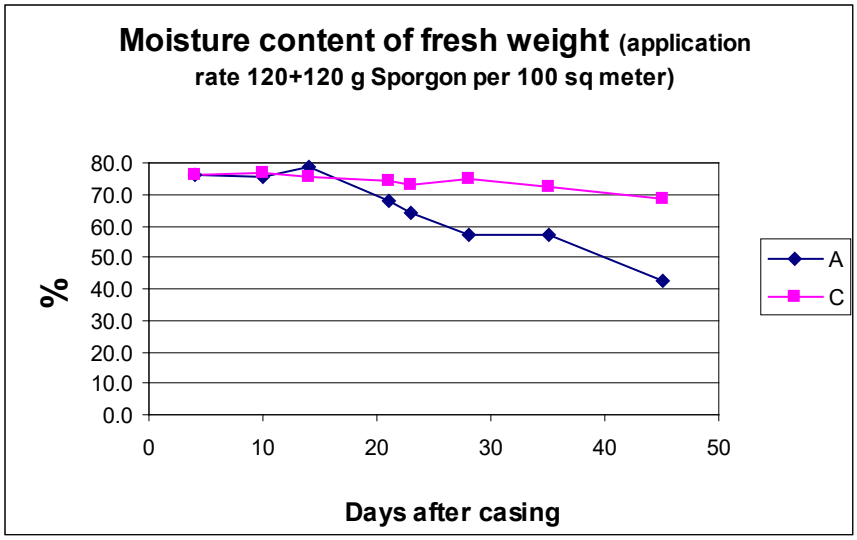


Figure 13. Moisture content of casing in trays with *Agaricus* (A) and without *Agaricus* (C) for the 120+120 g/100m^2 prochloraz rate.

The single 240 g/100m^2 prochloraz dose was applied on Day 10 and the initial recoveries obtained from the casing were 90.9% for the *Agaricus* (A) plots and 109.1% for the Control (C) plots. Prochloraz disappeared rapidly from casing until

Day 28 when the decline of fungicide levelled off up to the end of the crop Day 45. At the end of the crop (Day 45) only 12.5% of the initial prochloraz amount was found in the *Agaricus* (A) plots and 16.25% in the plots without mushrooms (C). The curves for *Agaricus* (A) and non-*Agaricus* (C) followed similar patterns as shown (Figure 14.):

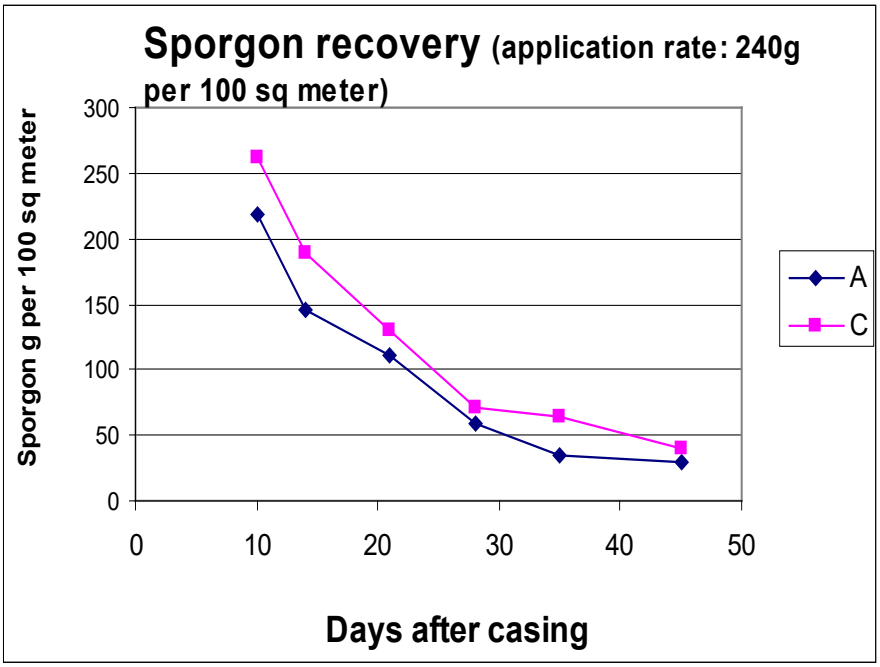


Figure 14. Levels of Sporgon (prochloraz a.i.) after a single application on Day 11, in casing spawned with *Agaricus* (A) and in casing without *Agaricus* (C)

Statistically, there is weak evidence suggesting differences between the treatments but both curves had the same degradation rate. On a Log scale, both curves transformed to straight parallel lines following a 1st order kinetic equation. The calculated slope parameter was –0.06884

Exponential trend lines were fitted to the data and the calculated half-lives are shown below (Table 7.):

Table 7. Prochloraz half-lives for casing spawned with *Agaricus* (A) and Control (C) (without *Agaricus*) plots in the single 240 g/100m^2 application rate

Treatment	Half-life in days (Day 11 to Day 45)
Agaricus (A)	11.7
Without *Agaricus* (C)	12.8

Finally, the water content of the casing for the single 240 g/100m^2 application rate (Figure 15.) gradually reduced as the crop progressed, in a similar manner to that in the 120+120 g/100m^2 (Figure 13.)

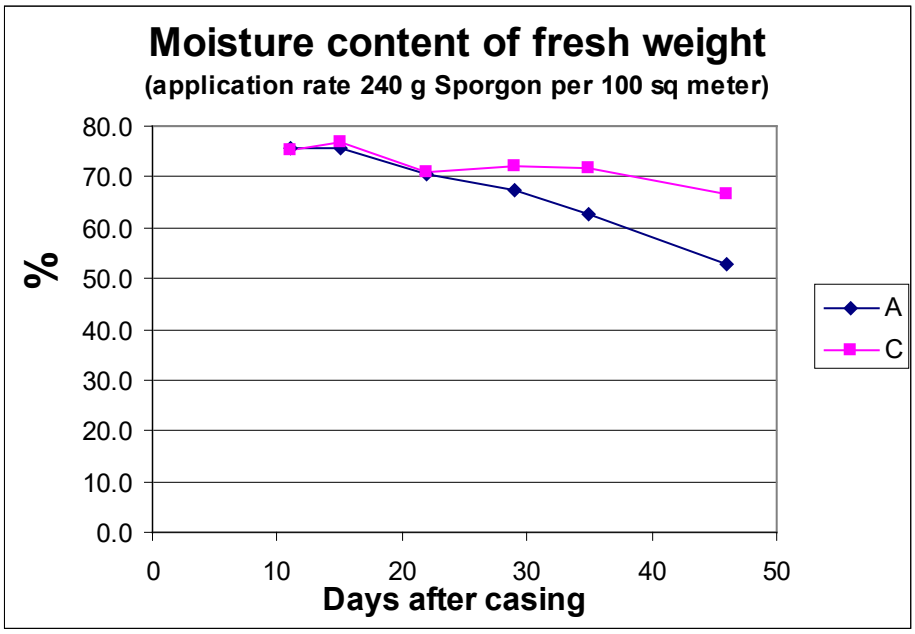


Figure 15. Moisture content of casing in trays with *Agaricus* (A) and without *Agaricus* (C) for the single 240 g/100m^2 prochloraz rate.

3.4 Discussion

Unlike in the small scale experiments, in this study a significant decrease in prochloraz levels in the casing was observed at both application rates. As expected from the small scale studies *A. bisporus* did not have any significant effect on prochloraz residues. The findings of this *in situ* experiment agree with these of Grogan and Jukes (2003). With the 120+120 g/100m^2 application rate the decline was dramatic between Day 21 and Day 28 suggesting a possible microbial degradation Bock et al. (1996). With the single 240 g/100m^2 prochloraz rate, both treatments (*Agaricus* and Controls) the rate of disappearance was the same, suggesting that the factors responsible for prochloraz degradation –or dissipation- is independent of the presence of *A. bisporus*. The lower moisture levels found in the *Agaricus* trays compared to the Control trays, did not affect prochloraz recoveries significantly. This agrees with the results from our earlier *in-vitro* work.

4 Conclusions

The overall conclusion of this study can be summarised as follows:

- Prochloraz was relatively stable in casing under *in-vitro* conditions,
- *A. bisporus* did not degrade or remove prochloraz from casing,
- Casing moisture content did not have any significant effect on prochloraz recovery,
- Some factors associated with *in-vivo* cultivation appears to affect prochloraz persistence

5 Future work

The results suggest that although prochloraz is stable under laboratory conditions, it disappears rapidly from casing in standard growing conditions. Since no significant evidence has been found that *A. bisporus* influences fungicide concentrations in casing, microbial degradation should be investigated. Microbial enrichment experiments to isolate potential prochloraz degraders will be useful. Further sorption studies with ^{14}C prochloraz will clarify what portion of the fungicide is adsorbed, what is exposed to degrading microbes and what is available for disease control. The pathogen, *Verticillium fungicola,* will be introduced into our studies in order to test prochloraz effectiveness against the disease. Four *questions* have been identified which will be investigated in the future as follows:

1. Are there prochloraz-degrading microbes in casing or equipment (casing mixer, spraying tank, other farms)? (March-Sept 2004)
2. What is the proportion of prochloraz sorbed in casing solid phase/ water phase (Kd value)? (March-July 2004)
3. Can we increase prochloraz persistence? (April-Dec 2004)
4. Is there a link between prochloraz persistence and control of *Verticillium*? (Jan-July 2005)

6 References

1. Bock C, Diekmann H., Kroppenstedt R. M., Schmidt U., (1996). Degradation of prochloraz and 2,3,6-trichlorophenol by environmental bacteria strains. Applied Microbiology and Biotechnology. 45: 257-262 1996.
2. Burkhard N Guth J A (1981). Chemical hydrolysis of 2-chloro-4, 6-bis-(alkylamino)-1,3,5-triazine herbicides and their breakdown in soil under the influence of adsorption. Pesticide Science 12: 45-52.
3. Cornejo J., Jamet P., Lobnik F. (2000) Pesticide/soil interaction. Introduction. INRA, Paris 2000.
4. Farmer W, Yukiko A (1987) Fate of pesticides in the environment. Chemical conversion of pesticides in the soil-water environment. Division of agriculture and natural resources, university of California. 1987.
5. Grogan H Jukes A (2003). Persistence of the fungicides thiabenazole, carbendazim and prochloraz in mushroom casing soil. Pest Managament Science 59:1225-1231
6. Hayes, W. A., (1973). The casing layer. Proceedings of the 1st Seminar in Mushroom Science held in the Department of Biological Science, The University of Aston in Birmingham, November.
7. Hollrigl-Rosta A., Kreuzig R., Bahadir M., Rutters H., 1999). Sorption of prochloraz in different soils. Journal of Agricultural Food Chemistry. 1999. 47, 1242-1246.
8. Kapteyn J. C., Pillmoor J. B., De Waard M. A., (1992). Biochemical mechanisms involved in selective fungitoxicity of two sterol 14-α-demethylation inhibitors, prochloraz and quinconazole: Accumulation and metabolism studies. Pesticide Science. 1992, 36, 85-93.
9. Kreuzig R, Bahadir M (1998). Pesticides in soils- Experimental design of laboratory, lysimeter and field studies. Landschaftsokologie und Umweltforschung 38. 99-100
10. Moyer JR, Hance RJ, McKone CE, (1972). The effect of adsorbents on the rate of degradation of herbicides incubated with soil. Soil Biology and Biochemistry 4:307-311

11. Pignatello J., (1989). Sorption dynamics of organic compounds in soils and sediments. Reactions and Movements of Organic Chemicals in Soils, 2nd ed.; Sawhney B. L., Brown K., Eds.; SSSA Special Publication 22; SSSA: Madison, WI 1989; 45-50.
12. Roy C, Gaillardon P, Montfort F, (2000) The effect of soil moisture content on the sorption of five sterol biosynthesis inhibiting fungicides as a function of their physicochemical properties. Pest Management Science. 2000. 56: 9, 795-803. 24 ref.
13. Tomlin C. (2000) The Pesticide Manual, A world compendium. 12th ed. Farnham: British Crop Protection Council (BCPC), 2000.
14. Vink J 1997. Pesticide biotransformation and fate in heterogenous environments. PhD Thesis 1007, University of Wagenigen, Netherlands.
15. Visscher, H. R., (1988). Cultivation of mushrooms. Rustington, W Sussex: Darlington Mushroom Laboratories.

7 Poster presented at HRI Student Symposium 1st - 2nd March 2004.

Studies on fungicide behaviour in mushroom casing in relation to disease control

[1,2]Georgios Papadopoulos, [2]Helen Grogan, [1]Martin Wood

[1]The University of Reading, Department of Soil Science, Whiteknights, Reading RG6 6DW, UK

[2]Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK

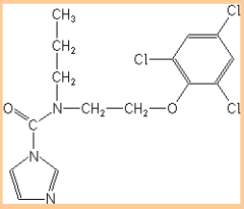
Background

Control of dry bubble disease (*V. fungicola*) relies heavily on prochloraz (Sporgon 50 WP)

Recent work (Grogan *et al* 2003) showed that prochloraz levels in casing were significantly reduced by the end of the second flush and this may make disease control more difficult

Objectives

- To determine the factors affecting the persistence of prochloraz in mushroom casing
- To manipulate certain factors in order to maximise prochloraz activity throughout the duration of the crop



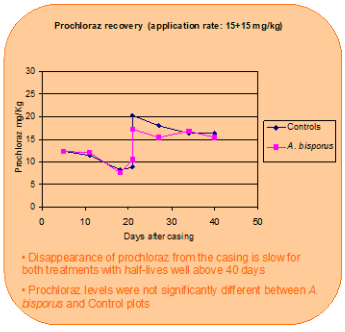
Prochloraz: [N-propyl-N-{2-(2,4,6-trichlorophenoxy)-ethyl}imidazole-1-carboxamide]

Dry bubble disease (*V. fungicola*) on mushroom crops

Laboratory studies

- In a batch of small scale experiments, the behaviour of prochloraz was studied in the casing material in glass jars, in 4cm high columns and in pots inoculated with *A. bisporus* (cultivated mushroom). Prochloraz was applied as Sporgon 50 WP on Day 3 and Day 21 to give concentration of 15ppm to each treatment (equivalent to 120g/100m²)

Experiment to determine the effect of *A. bisporus* on prochloraz degradation in a controlled environment cabinet



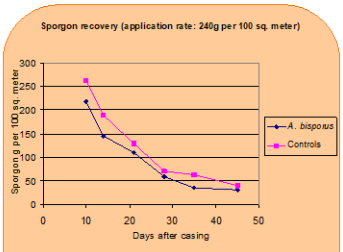
- Disappearance of prochloraz from the casing is slow for both treatments with half-lives well above 40 days
- Prochloraz levels were not significantly different between *A. bisporus* and Control plots

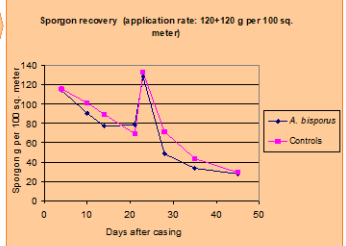
- Prochloraz was relatively stable in casing under *in-vitro* conditions,
- *A. bisporus* did not exhibit any ability to remove or breakdown the fungicide
- The fate of prochloraz in casing was not influenced by the wetting and drying cycles of a mushroom crop

In-vivo studies

- In a large-scale experiment on a mushroom unit, prochloraz was applied at two different rates using a fungicide spray tank. Prochloraz was applied as Sprogon ® 50 WP either on day 5 and day 21 at the rate of 120g/100m² or on day 11 at the rate of 240g/100m².
- Fungicide residues were extracted with acetonitrile and measured by HPLC. Solid Phase Extraction (SPE) cartridges were used to improve the separation of prochloraz residues

Large-scale experiment in the Mushroom Unit at HRI





- The levels of prochloraz fell rapidly in both application rates
- At the 120+120 g per 100 sq. m, prochloraz disappeared dramatically after the 1st flush (Day 21)

- Significant and rapid decrease in prochloraz levels in the casing at both application rates
- Casing moisture content did not have any significant effect on prochloraz recovery
- At the 120+120 g/100m² application rate the decline was dramatic between Day 21 and Day 28 suggesting a possible microbial degradation

