Project Title:	Mushrooms: Oviposition substrate selection in sciarid and phorid fly pests.
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The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiment was carried out and the results obtained have been reported with detail and accuracy. However because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of these results especially if they are used as the basis for commercial product recommendations.

CONTENTS

PRACTICAL SECTION FOR GROWERS

Commercial benefits of the project

This project aims to identify the olfactory (smell) and chemical stimuli, associated with mushrooms and their cultivation, to which female sciarid and phorid flies respond. This information will underpin the production of non-insecticidal methods of mushroom fly control, and may provide additional or improved methods of monitoring and trapping sciarid and phorid flies on mushroom farms.

Background and Objectives

The total cost of pest infestations to the UK mushroom industry is currently about £11-12 million per annum including direct loss to insect attack, transmission of pathogens and insecticide costs. The two most important mushroom fly pests are sciarid and phorid flies. There is a requirement to develop alternatives to chemical insecticides, driven by consumer concerns about pesticide residues and the increasing withdrawal of chemicals available to the growers. Identification of the volatile chemicals that attract sciarid and phorid flies to mushroom crops should lead to improved methods of monitoring fly populations or for controlling flies using baits impregnated with the attractant chemicals. However little is known about the response of phorids and sciarids to the volatiles produced during mushroom cultivation.

The objectives of this project are as follows:

1. To determine which substrates in mushroom houses attract female phorid and sciarid flies and elicit oviposition
2. To determine which component chemicals of the substrates have potential to elicit attraction and oviposition
3. To determine which volatiles are bioactive
4. To apply knowledge gained from (1-3) to the development of targets / traps / monitoring devices
5. To test the devices in the laboratory and mushroom house environment.

Targets for 2000/01

1. Carry out behavioural response experiments to determine the attractiveness to flies of different potential oviposition substrates.
2. Devise a method for the collection of air surrounding the various substrates.
3. Analyse the chemical composition of the air surrounding the various substrates.

Summary of results and conclusions

The behavioural response of phorid flies to cultivation substrates (phase II compost, 4 day spawned compost, 14 day spawned compost and button mushrooms) was investigated using a static air olfactometer. The response of phorid flies was found to vary significantly depending on the substrate. Fully spawned compost attracted phorid flies the most and pasteurised compost attracted them the least. Four-day spawned compost and button mushrooms were intermediates. These results are consistent with the use of fungal volatiles by phorid flies to detect egg-laying sites (HDC project M 32). The oviposition of phorid females was found to vary with the cultivation substrates, with oviposition increasing with the mycelial load of the compost. However, more adults emerged from the 4 day spawned compost than the other substrates. Research into the behavioural response of sciarid flies is ongoing.

A method for analysing the air surrounding the cultivation substrates was tested and a suitable protocol developed. The air was sampled using a micro-extraction technique and then analysed using gas chromatography. This enables the air to be separated into its constituent chemicals, which are then be identified using mass spectroscopy. Analysis showed that growth of *Agaricus bisporus* through compost has a significant effect on the intensity of the production of volatile chemicals in the substrate. A comprehensive experiment into the differences in volatile production between substrates is ongoing.

Action points for growers

- Mushroom flies respond to volatile compounds produced during mushroom cultivation.
- Prevent access of flies during the spawn running and case running growth phase.
- Ensure air vents are securely fly proof.

Anticipated practical and financial benefits

- Provide improved methods of monitoring and trapping sciarid and phorid flies.
- Reduce the cost to growers of insect pest control.
- Reduced insecticide use.

SCIENCE SECTION

BEHAVIOURAL RESPONSE EXPERIMENTS

INTRODUCTION

Megaselia halterata (Wood) (phorid fly) and *Lycorellia castanescens* (Lengersdorf) (sciarid fly) are important pests in production of the commercial mushroom, *Agaricus bisporus* (Lange) Sing, (Scheepmaker, Geels, Van Griensven *et al*., 1996; Scheepmaker, Geels, Smits et al., 1998). The total cost of these pest infestations to the UK mushroom industry is currently about £11-12 million per annum including direct loss to insect attack, transmission of pathogens and insecticide costs.

Megaselia halterata adults are small hump-backed flies, 2-3 mm in length, that posses short antennae, which are visible at low magnification. Gravid females are attracted to growing mycelium (Hussey, 1959) and only oviposit where mycelium is actively growing (Hussey & Wyatt, 1962; Richardson & Hesling, 1978). Consequently, compost and casing with fungal mycelium growing through it are the only stages of mushroom cultivation at which phorid infestations are likely to occur. Each female lays about 40-60 eggs, normally at an active mycelial front. Eggs hatch rapidly and larvae complete their three instars in 9-10 days (Hussey, 1959; no temperature details given). Females are sexually receptive 24 hours after emergence, males copulate when they are 4 d old (Ondraschek, 1953, cited by Hussey, 1959). Adults require a period of flight prior to mating (Davies, 1941, cited by Hussey, 1959) and females require carbohydrate for egg maturation (Hussey, 1959). Oviposition occurs 2-3 d after insemination (Ondraschek, 1953, cited by Hussey, 1959).

Despite their economic importance to the industry, little is known about the behaviour of the adult sciarid and phorid flies. Research into the preferences of the larvae and the possible use of nematodes for biological control is advanced, with several commercial nematode applications available. However, the reasons for oviposition by females into the mushroom compost are unknown. Olfactory cues are thought to play an important role in the search for oviposition sites, though the precise details are still unknown. This research aims to determine what olfactory cues may be used for identification of oviposition sites the ultimate aim being to harness whatever cues may be in use by *M. halterata* to produce non-insecticidal methods for control.

The objectives of this project are as follows:

1. To determine which substrates in mushroom houses attract female phorid and sciarid flies and elicit oviposition
2. To determine which component chemicals of the substrates have potential to elicit attraction and oviposition
3. To determine which volatiles are bioactive
4. To apply knowledge gained from (1-3) to the development of targets / traps / monitoring devices.
5. To test the devices in the laboratory and mushroom house environment.

This project is using laboratory bioassays to identify the attraction of females to different cultivation substrates as follows : pasteurised compost, compost seeded with *A. bisporus*, and sporophores. A static air olfactometer was designed, tested and used for this purpose. The design was based on that of Pfeil (1993) to evaluate the attraction of *M. halterata* to compost colonised by *A. bisporus*. This earlier system employed a release chamber, with three test cups attached to the base. Samples were placed into two of the test pots while the third acted as a control pot, and after a certain time the number of flies within the test pots and the release chamber were determined. Statistical analysis, (Pfeil, 1993) assumed that the chance of finding flies within the release chamber and the three test pots would be equal and a chi-squared test was applied. However, this approach was flawed in a number of ways: (1) If flies remained within the release arena, this would not necessarily indicate a choice by the fly. Only flies that had made a choice to move to one of the test pots should have been included in the statistical analysis. (2) The presence of two test pots but only one control pot introduced a bias within the experimental design towards the test substrates: there were 6 entrances leading to the test samples and only 3 to the control. For an unbiased experimental design, there should be an equal chance of the flies encountering a control pot, as there would be to a test pot. Thus Pfeil's (1990), static air olfactometer, was modified to negate these problems.

SCIENTIFIC AND TECHNICAL PROGRESS FOR YEAR 2

Materials and Methods

Fly culture

Phorid larvae were reared on actively growing fungal mycelium. Aliquots (20g) of fully spawned compost (1% spawn, by weight Phase II compost incubated at 25ºC for 3 weeks) were mixed with 80 g of Phase II compost (Mushroom Unit, HRI, Wellesbourne). The compost mixture was then pressed firmly into polyethylene pots (No 16 clear pots, with white polythene lids, AW Gregory & Co. Ltd., London). Pots that had emerging adults were then paired with the new compost mixtures within large propagators (36 cm seed tray, Richard Sankey & Sons Ltd., Nottingham), with air holes (1 cm diameter) burnt into the top, sealed with dental plugs and a strip of foam insulation tape (0.5 cm draught seal, Woolworth's PLC, London) around the rim. The base of the propagator was covered with moist absorbent paper. Syrup (Lyles Golden Syrup, Tate & Lyle, London) was dabbed onto the inside surface of the propagator lid as a carbohydrate source for the female flies. The pot lids from the emerging fly cultures were removed and the propagator lid and base sealed and held firmly in place with elastic bands. Propagators were stored at room temperature within the lab for 5d, sufficient for mating, egg maturation and oviposition to occur. After this time CO_2 was introduced to the propagators to narcotise any remaining flies and the pots of new compost were sealed with lids containing a 1 cm ventilation hole in the middle sealed with a cotton wool dental bung. Larvae developed within the mycelium-seeded compost and adults emerged approximately 2-3 weeks later, depending on temperature.

Olfactometer Design

The static air olfactometer consisted of a 20 x 12 x 8 cm release arena with two 2 cm diameter ventilation holes, covered with fine mesh (Figure 1). A fly release device was centrally mounted on a rubber stopper (No 25, Fisher scientific, UK) within the arena. This comprised a 9cm diameter Petri dish with a 15 cm length of string attached to the lid of the Petri dish and run through an aperture in the lid of the release arena. Flies were placed within the Petri dish and released by pulling the string through the aperture, which was then secured by taping it to the external surface of the release arena lid.

Test pots (No 8 clear pots, with white polythene lids, AW Gregory & Co. Ltd., London) with lids were positioned underneath the release arena and connected by glass tubes (6cm x 0.6 cm

i.d.). There were four tubes to each test pot positioned equidistant from the centre of the lid and flush to the arena floor and glued in place creating a pit fall trap from which flies could not easily escape. The two test pots were positioned equidistant from each other and centrally with respect to the arena floor. This created an unbiased uniform olfactometer design. Sixteen static air olfactometers were used.

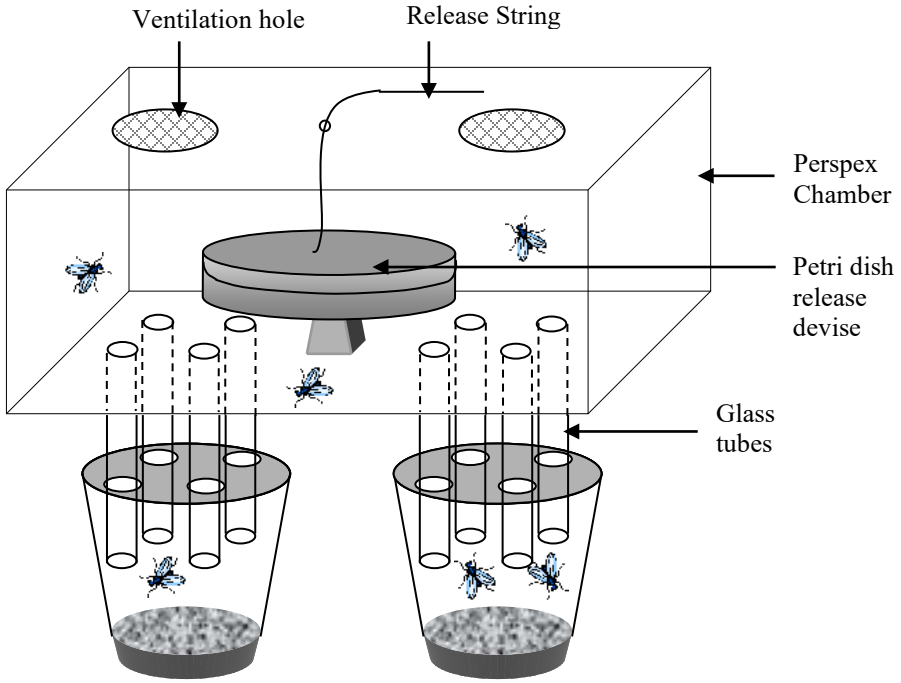


Figure 1. Static air olfactometer.

Substrate Preparation

Four substrates were examined: pasteurised compost, four day spawned compost, fully spawned compost and button mushrooms. They were produced in the laboratory, within test pots, using pasteurised compost from the HRI Mushroom Unit. Compost was collected and stored at 4ºC. Fifty grams of pasteurised compost was compressed into test pots prior to the experiment. All compost substrates were prepared within the test pots. For spawned substrates 50g of pasteurised compost was top- and middle- spawned (0.5% w/w grain spawn) with an average of six spawn grains per pot. The fully spawned compost was

incubated for 14-15d at 25ºC, while the four day spawned compost was prepared and incubated for 4 d at 25ºC prior to the experiment. The sporophores were cultured at the mushroom unit (HRI) using the same strain of *Agaricus bisporus* that was used in the spawned substrates. Twenty grams of whole button mushrooms were used in each test pot (approximately two sporophores).

Untreated pots (control) comprised of a 30 g layer of Plaster of Paris (3:4 ratio of water to plaster (w/w)). To ensure that the control pots had the same relative humidity as the treated pots moistened cotton wool was placed on the layer of plaster. Where the test substrate was compost, 5 g of cotton wool was moistened with 37.5g of water (compost moisture content is 75%); and where the test substrate was sporophores 2 g of cotton wool were moistened with 19 g of water (sporophore moisture content is 95%).

Preliminary experiments

Preliminary experiments were conducted to verify that the olfactometers were suitable for the fly species and that the design of the olfactometers and the controlled temperature (CT) room, in which experiments took place, had no influence on fly distribution. Both test pots contained a 1 cm layer of moistened plaster of Paris in the base. Sixteen olfactometers were positioned at random within a 4 x 4 grid in a CT room, and 20 gravid females were released into each. The experiment was conducted in total darkness for 16 hours after which the position of the flies within the olfactometers was recorded. The experiment was repeated three times.

The distribution of flies within the two test pots was analysed using a generalised linear model, assuming Poisson distribution, with a log link function. The preliminary experiment showed that there was no significant influence of position (row or column) on fly distribution, nor any bias towards either side of the olfactometer. However, there was a significant effect of occasion on the fly distribution, and this was taken into account in the design of the substrate tests.

Substrate Experiment

Since fly behaviour varied between the preliminary experiments the substrate experiments were repeated. The substrate experiments were designed according to a Latin cube

randomisation. In each experiment, the treatments were replicated four times within the 16 olfactometers. The experiments were repeated on four different occasions. The location of olfactometers within the 4 x 4 grid, as well as the position of substrates and their left or right orientation, were fully randomised. All experiments were conducted in darkness in a controlled temperature room at 25°C. Twenty gravid flies were introduced into each olfactometer via the release device.

After 16 hours, the number of flies in the treated and untreated pots, and in the release arena were recorded. Treated pots were covered and retained, those from two experiments being covered with a lid, containing a ventilation hole, in order to trap the flies within the pot. The lid was then replaced with a sticky trap and flies caught on the trap were observed under the microscope to determine if oviposition had occurred. Emergence experiments were conducted using treated pots from all 4 occasions. Treated pots were again covered, with a lid containing a ventilation hole, and stored at 25°C for one week before replacing the lid with a sticky trap, again containing a ventilation hole. The treated pots were retained at 25°C for a further two weeks and then adult emergence was recorded, since adults fly to the top of the pot and become trapped on the sticky trap. The mushroom substrate could not be included in the emergence test, since cut mushrooms would before the next generation of flies emerges.

Statistical Analysis

The count data were analysed using a generalised linear model, assuming Poisson distribution, and a log link function. This analysed the interaction of the different substrates and the treated or untreated pot. Analysis of proportion data was done using a generalised linear model, assuming a binomial distribution and a logit link function. This logit analysis, unlike the log-linear analysis, enabled the inclusion of flies that remained within the release arena, i.e. those that did not respond to the bioassay. Several models were designed: Model 1 considered the number of flies within the treated pot as a proportion of the flies that responded to the test; Model 2 considered these flies as a proportion of the total number of flies; in Model 3 the number of flies within the untreated pots was analysed as a proportion of the total number of flies; Model 4 analysed the number of flies that responded to the bioassay as a proportion of the total number of flies.

Some of the variation within the data could have been due to the influence of occasion or positional effects of the olfactometers. However, the design of the experiment (Latin cube) was such that the effect of the substrate was independent of these other influences. Therefore, the models could be designed so that a differential response between occasion and position would not alter the treatment effect.

For both Poisson and binomial distributions, the deviance ratios were based on a denominator deviance of 1.0. However, the residual deviance can be used to assess the fit of the model, by comparison to a chi-squared distribution, using the residual degrees of freedom. An apparent lack of fit may indicate that explanatory variables have been omitted from the model or that the data are over-dispersed. Over-dispersion would occur where the behaviour of flies was not independent, for example if there was a degree of aggregation within the olfactometer. If over-dispersion occurs then a dispersion parameter, estimated from the residual deviance, can be incorporated within the model to overcome the apparent lack of fit. The inclusion of a dispersion parameter inflates the standard error, reducing the t-value and the significance of the effect so weaker effects may disappear at the 5% significance level. Therefore, each model was applied twice: firstly, the model had dispersion parameter of 1.0, based on the denominator deviance; the model was then re-applied incorporating an over-dispersion parameter, estimated from the residual deviance.

The models were used to determine the effects of the different treatments in terms of proportional change from a baseline or index treatment. Phorid larvae are mycetobiont, hence it was expected that pasteurised compost would elicit a minimal response from the gravid females. Pasteurised compost was considered a baseline treatment and the effects of other treatments were compared with this index level. The fully spawned compost was also used as an index level, since this should represent the maximal response. Fitted proportions were also produced for all the models. The standard error values for these predictions were taken from the over-dispersed model. Models that were based on a fixed dispersion of 1.0 have a reduced standard error, however, the inclusion of a dispersion parameter into the over-dispersed model, ensured a better fit for the model. Hence, these inflated error terms are provided.

Results

Log-linear model

Data for the distribution of *M. halterata* females in response to substrate are given in Table 1. Analysis with log linear and logit-models revealed similar trends. In the log-linear model, where the dispersion was fixed at 1.0, there was a significant substrate treatment interaction ($p=0.004$), indicating that the fly choice was not random between the treated and untreated pots and that this reaction was variable dependent on the substrate (Table2). The fly choice between the fully spawned and pasteurised compost was significantly different (Table 2) ($p<0.001$) as was 4d and fully spawned compost ($p=0.025$). Where the dispersion was estimated (2.24) there was no significant substrate treatment interaction ($p=0.121$) and the fly choice between 4 d and fully spawned compost was not significant ($p=0.256$). The difference between the fully spawned and pasteurised compost remained significant, but at a lower probability ($p=0.019$). The fitted proportions (Table 3) illustrate the direction of the fly choice between the two test pots. The only instance where the model predicts more flies within the untreated pot is where pasteurised compost is the substrate. The numbers of flies within the treated pot increased with mycelial growth through the substrate and the mushrooms proved slightly more attractive than the 4d spawned compost.

Model 1

When the proportion of flies within the treated pots was compared with the total number of flies that responded, using a logit model, there was a highly significant substrate effect, both when the dispersion was fixed at 1 ($p=<0.001$) and when the dispersion was estimated (1.39, $p=0.016$). This indicated that the flies that responded to a treated pot varied with the substrate. The number of flies responding to fully spawned compost was significantly different from pasteurised compost ($p<0.001$, dispersion factor fixed at one) and those responding to the mushroom and four day spawned compost varied significantly from fully spawned compost ($p=0.034$, 0.009 respectively, dispersion factor fixed at one). The fitted proportions for this model (Table 4) mirror those of the log-linear model.

Table 1. Distribution of gravid female *Megaselia halterata* within the treated (T) and untreated (U) pots and the release arena (RA).

Substrate	Occasion 1			Occasion 2			Occasion 3			Occasion 4		
	T	**U**	**RA**	**T**	**U**	**RA**	**T**	**U**	**RA**	**T**	**U**	**RA**
Pasteurised	5	10	5	3	3	14	6	3	11	3	5	12
	2	6	12	4	6	10	17	2	1	2	15	3
	5	9	6	8	3	9	7	2	11	2	12	6
	4	8	8	7	6	7	8	4	8	7	9	4
4 day spawned	6	10	4	7	3	10	5	6	9	6	13	1
	10	7	3	11	4	5	8	4	8	2	12	6
	9	9	2	8	4	8	8	1	11	6	6	8
	8	5	7	9	5	6	6	4	10	7	7	6
Fully spawned	12	4	4	15	1	4	8	2	10	7	12	1
	17	2	1	8	3	9	7	8	5	6	10	4
	9	11	0	8	1	11	6	9	5	8	7	5
	12	4	4	15	2	3	5	3	12	7	5	8
Mushrooms	5	5	10	5	5	10	10	7	3	6	6	8
	6	8	6	6	4	10	13	5	2	6	11	3
	7	3	10	5	6	9	7	8	5	9	7	4
	10	4	6	11	3	6	8	4	8	5	7	8

Table 2. Probablity (*p*) and t values (t) for a t-test of the difference between treatments and index levels for both log-linear and logit models. Figures in parentheses indicate the dispersion factor. The t values were calculated as the estimate of parameter divided by its standard error.

Index	Pasteurised		4 Day spawned		Mushrooms		Fully spawned	
	p	t	*p*	t	*p*	t	*p*	t
Log-linear Analysis								
Pasteurised (1)			0.154	1.43	0.056	1.91	<.001	3.60
Pasteurised (2.24)			0.344	0.95	0.207	1.28	0.019	2.41
Fully spawned (1)	<.001	-3.60	0.025	-2.24	0.086	-1.72		
Fully spawned (2.24)	0.019	-2.41	0.140	-1.50	0.256	-1.15		
Logit Analysis								
Treated/respond								
Pasteurised (1)			0.156	1.42	0.061	1.87	<.001	3.93
Pasteurised (1.39)			0.237	1.20	0.122	1.59	0.002	3.33
Fully spawned (1)	<.001	-3.93	0.009	-2.60	0.034	-2.12		
Fully spawned (1.39)	0.002	-3.33	0.034	-2.21	0.081	-1.80		
Treated/total								
Pasteurised (1)			0.024	2.26	0.012	2.52	<.001	5.01
Pasteurised (1.86)			0.107	1.65	0.073	1.85	<.001	3.97
Fully spawned (1)	<.001	-5.01	0.005	-2.83	0.011	-2.54		
Fully spawned (1.86)	<.001	-3.67	0.046	-2.07	0.072	-1.86		
Untreated/total								
Pasteurised (1)			0.846	-0.19	0.369	-0.90	0.066	-1.84
Pasteurised (1.65)			0.881	-0.15	0.489	-0.70	0.162	-1.43
Fully spawned (1)	0.066	1.84	0.102	1.63	0.341	0.95		
Fully spawned (1.65)	0.162	1.43	0.212	1.27	0.464	0.74		
Respond/total								
Pasteurised (1)			0.055	1.92	0.095	1.67	<.001	3.68
Pasteurised (2.57)			0.240	1.20	0.305	1.04	0.028	2.29
Fully spawned (1)	<.001	-3.68	0.070	-1.81	0.042	-2.03		
Fully spawned (2.57)	0.028	-2.29	0.267	-1.13	0.214	-1.27		

Table 3. Predictions of fly distribution from log-linear model. Figures in parentheses show the count data as proportions. Standard errors are based on over-dispersed model.

	Untreated	s.e	Treated	s.e
Pasteurised	5.14 (0.534)	1.75	4.49 (0.466)	1.55
Mushrooms	7.66 (0.439)	4.42	9.80 (0.561)	5.61
4 Day spawned	6.43 (0.463)	1.68	7.46 (0.537)	1.90
Fully spawned	5.16 (0.359)	1.77	9.22 (0.641)	3.00

Model 2

There were highly significant substrate effects in the second logit model, analysing the number of flies within treated pots as a proportion of the total number of flies, (dispersion fixed at 1, $p<0.001$; dispersion estimated at 1.86, $p=0.008$). In this model, all the substrates were significantly different from both fully spawned and pasteurised compost (Table 2). The fitted proportions for this model, although lower, follow the same pattern of fly behaviour as previous models with attraction to the treatment increasing with mycelial growth through the compost (Table 4).

Model 3

There were no significant, overall substrate effects when the flies responding to the untreated pot, as a proportion of the total number of flies, were analysed (dispersion fixed at 1, $p=0.250$; dispersion estimated at 1.65, $p=0.486$). In addition, no significant differences were found when the substrates were compared to both pasteurised and fully spawned compost (Table 2). The fitted proportions for this model are consistent, ranging from 0.259 – 0.322, over the four substrates (Table 4).

Model 4

A final logit model was designed to analyse the number of flies responding to the bioassay (choosing either a treated or untreated pot) as a proportion of the total number of flies. Where the dispersion was fixed at one, there were overall substrate effects ($p=0.003$) and there were significantly different results for the fully spawned and pasteurised compost ($p=0.003$) and mushroom and fully spawned compost ($p=0.042$) (Table 2). Where the dispersion factor was estimated (2.57) the significant substrate effect was lost ($p=0.168$) but the other significant differences remained.

Table 4. Fitted proportions based on the logit models. Standard errors are based on over-dispersed models. Logit models were designed: the first considered the number of flies within the treated pot as a proportion of the flies that responded to the test; the second considered these flies as a proportion of the total number of flies; the number of flies within the untreated pots was analysed as a proportion of the total number of flies; the final model analysed the number of flies that responded to the bioassay as a proportion of the total number of flies.

	Treated/Respond		Treated/Total		Untreated/Total		Respond/Total	
	proportion	s.e	proportion	s.e	proportion	s.e	proportion	s.e
Pasteurised	0.464	0.041	0.280	0.034	0.322	0.032	0.601	0.042
Mushrooms	0.554	0.039	0.372	0.036	0.291	0.031	0.663	0.042
4 Day spawned	0.532	0.038	0.362	0.036	0.315	0.032	0.672	0.041
Fully spawned	0.649	0.035	0.469	0.037	0.259	0.031	0.736	0.039

although there was a slight increase in the t values. The fitted proportions for this model again increase with mycelial growth through the compost with the button mushrooms being slightly more attractive than the four-day spawned compost (Table 4).

True response

Using the fitted proportions from the logit models it is possible to estimate the true proportion of flies that are responding to the substrates (RS). The number of flies trapped within the untreated pots was consistent for all treatments (U). Since the preliminary experiments showed there to be no bias toward either of the test pots and confirmed the uniformity of the olfactometers it can be assumed that these flies did not respond to the substrate, and became trapped by chance, due to the pitfall design of the olfactometer. Therefore of those flies found in the treated pot (T) it can be assumed that a similar proportion did not actively chose this pot, but also became trapped by chance. Subtracting the proportion of flies within the untreated pot (U) from the proportion of flies within the treated pot (T) would provide an indication of the proportion of flies that responded to the substrate (T-U). The proportion of flies that responded to the experimental set-up (R), by choosing a treated or untreated pot was calculated in the final logit model. Therefore, by subtracting these proportions from 1 will provide the proportion of flies that did not respond to the substrates (1-R or NRS). The addition of this proportion (NRS) and the proportion of flies that did respond to the substrate (T-U) provides a value for the total number of flies whilst ignoring those that did respond, but not to the stimulus. Therefore, dividing the proportion of flies that responded to the stimulus, by the total proportion of flies ((T-U)/[(T-U)+NRS]) provides an accurate account of the proportion of flies within the olfactometer that responded to the different treatments

The calculation for the estimate of flies responding to the substrates (Table 5) mirrors the previous predictions from the logit models. There is an obvious substrate effect, though this has yet to be analysed statistically so any significant differences are not yet known. The response of flies to pasteurised compost is a negative value indicating no response of flies to this substrate. The response increases from four-day spawned, to button mushrooms, to fully spawned compost.

Table 5. Estimate of the true proportion of flies that responded to the substrates. Where: T proportion of flies in treated pots; U proportion of flies in untreated pots; T-U proportion of flies responding to substrates; NRS proportion of flies not responding to the substrate; RS proportion of flies responding to the substrates, derived from T-U / [(T-U) + NRS].

	T	U	1-NRS	T-U	NRS	RS
Pasteurised	0.280	0.322	0.601	-0.042	0.399	-0.118
Mushrooms	0.372	0.291	0.663	0.081	0.337	0.194
4 Day spawned	0.362	0.315	0.672	0.047	0.328	0.125
Fully spawned	0.469	0.259	0.736	0.210	0.264	0.443

Oviposition and Emergence

Table 6. Oviposition of M. halterata in treated pot substrates, where T is the number of flies on the trap, O is the number of flies on the trap that had oviposited and P is the total number of flies within the test pot.

Substrate	Occasion	T	O	P	T/P	Average T/P	O/P	Average O/P
PC	3	2	1	7	0.286	0.346	0.500	0.208
PC	3	3	1	6	0.500		0.333	
PC	3	3	0	17	0.176		0.000	
PC	3	3	1	8	0.375		0.333	
PC	4	0	0	3	0.000		-	
PC	4	2	1	2	1.000		0.500	
PC	4	3	0	7	0.429		0.000	
PC	4	0	0	2	0		-!	
4 day	3	1	1	6	0.167	0.343	1.000	0.538
4 day	3	1	1	8	0.125		1.000	
4 day	3	5	4	8	0.625		0.800	
4 day	3	2	1	5	0.400		0.500	
4 day	4	1	0	2	0.500		0.000	
4 day	4	3	0	7	0.429		0.000	
4 day	4	1	0	6	0.167		0.000	
4 day	4	2	2	6	0.333		1.000	
FS	3	3	2	5	0.600	0.466	0.667	0.667
FS	3	6	4	8	0.750		0.667	
FS	3	2	1	7	0.286		0.500	
FS	3	2	2	6	0.333		1.000	
FS	4	3	1	7	0.429		0.333	
FS	4	3	2	8	0.375		0.667	
FS	4	4	2	6	0.667		0.500	
FS	4	2	2	7	0.286		1.000	
BM	3	6	3	10	0.600	0.456	0.500	0.576
BM	3	7	6	13	0.538		0.857	
BM	3	4	1	7	0.571		0.250	
BM	3	3	3	8	0.375		1.000	
BM	4	3	1	9	0.333		0.333	
BM	4	2	1	5	0.400		0.500	
BM	4	3	2	6	0.500		0.667	
BM	4	2	1	6	0.333		0.500	

A logit model will be employed to analyse the proportion of trapped flies that had oviposited and the proportion of flies that were trapped. On crude analysis of the average proportions, the number of flies within the pots captured on the sticky trap appears to be consistent. However, the number of captured flies that oviposit does appear to be affected by treatment. It will not be known if these results are significant until the logit analysis has been performed. Means were calculated for the data (Table 7) using a transformation, Log(n+0.375), to take account of the many zero data points and the data distribution (Table 8). Although the data are inconsistent, with obvious differences between occasions, it appears that more adults emerged from the four day spawned compost than the fully spawned whilst none emerged from the pasteurised compost.

Table 7. Number of adult *M. halterata* that emerged from the test substrates

Occasion	Pasteurised Compost	4 day Spawned	Fully Spawned
One	0, 0, 0, 0,	0, 8, 0, 0	0, 0, 0, 0
Two	0, 0, 0, 0,	23, 0, 0, 0	18, 0, 5, 0
Three	0, 0, 0, 0,	0, 0, 19, 0	0, 0, 0, 0
Four	0, 0, 0, 0,	40, 36, 31, 40	50, 0, 52, 18

Table 8. Mean emergence of *M. halterata* from test substrates. Number in parenthesis indicates the standard error.

Occasion	Pasteurised	Four day	Fully Spawned	Total
One	0 (0)	0.434 (0.337)	0 (0)	0.110 (0.112)
Two	0 (0)	0.679 (0.449)	1.557 (0.425)	0.539 (0.206)
Three	0 (0)	0.630 (0.428)	0 (0)	0.146 (0.143)
Four	0 (0)	36.56 (0.026)	30.03 (0.508)	5.058 (0.298)
Total	0 (0)	2.002 (0.238)	0.959 (0.217)	

Discussion

This study indicates that *M. halterata* gravid females respond differently to different substrata associated with mushroom cultivation. The logit models provided better descriptions of the data since: (1) they analysed the proportion of flies within the olfactometer, including flies that remained in the release area (rather than just taking into account flies that had responded

to a treatment); (2) they had lower dispersion factors, with the exception of flies responding to test substrata as a proportion of the total number of flies. While over-dispersion may be a consequence of fly aggregation, it may also indicate that explanatory variables have been omitted from the models.

Both models showed that there was a positive response to compost substrata and that attraction increased with increase in fungal mycelium. There was highly significant attraction to fully spawned compost. Interestingly, the proportion of flies in the control pot was not reduced (logit models), therefore the increase in the number of flies in the pot containing fully spawned compost reflected an increase in the total number of flies responding, which is borne out by the estimate of true response to substratum (Table 1). Though the log-linear model indicated that more flies chose the control in the presence of pasteurised compost, implying a deterrent effect, this was not confirmed by the logit model, which detected no significant difference.

Following mating and egg maturation, gravid females need to find oviposition sites suitable for larval development and this will presumably be the overriding factor in the behaviour of these females. Previous studies have shown that *M. halterata* larvae feed on mycelium, and that attractiveness of compost to females increased with the volume of actively growing mycelium (Hussey, 1961). This was based on larval number, within different substrates, after their exposure to a phorid infested mushroom house. Further, eggs are not laid in unspawned compost even if surrounded by spawned material (Hussey, 1961), but are laid preferentially adjacent to the growing mycelial front (Hussey, 1959). Thus, it might be expected that 4 d spawned compost would be preferable for oviposition, since mycelium is actively growing. However, there was no evidence in the present study indicating preferential attraction by gravid females to this substratum, rather they were more attracted to fully spawned compost. It is likely that olfactory cues are involved in locating oviposition sites. Early observations (Moreton, 1954; Hussey, 1959) indicated that *M. halterata* collect around newly opened spawn cartons. Interactions between actively growing *A. bisporus* and compost have been implicated in attraction (Moreton, 1954; Hussey, 1959), though not proved (Pfeil & Mumma, 1993). Nor has there yet been a consistent result for the attraction of *M. halterata* to fungal volatiles (Burrage, 1981; Grove & Blight, 1983; Pfeil & Mumma, 1993). Nonetheless, the present study also points to olfactory cues - humidity and visual factors were the same in controls and treatments. If olfactory attractants are involved, then the larger mycelial biomass

and presumably larger amounts of volatiles in fully spawned compost might be more attractive and elicit a greater response than other substrata. The results on oviposition in the present study are consistent with this, though more adults emerged from 4d spawned compost. The involvement of olfactory cues is also consistent with the increase in fly activity when fully spawned compost was a treatment. Knowledge of the volatiles released by different substrata may give an indication of which chemicals may be involved in oviposition substratum selection by *M. halterata*. Such research is ongoing in our laboratory.

CHEMICAL ANALYSIS OF AIR SURROUNDING SUBSTRATES

INTRODUCTION

Various methods have been used to analyse the volatiles produced by *Agaricus bisporus* and other commercially cultivated mushrooms, most of this research has been concerned with sporophore volatiles and food quality. Few studies have attempted to analyse the volatiles produced by fungal mycelium, and previous work into the mycelial volatiles may have been limited by the extraction methodology and detection techniques available at the time (Grove, 1981; Grove & Blight, 1983; Pfeil. & Mumma, 1992). Therefore, prior to analysis of compost volatiles it was necessary to investigate possible techniques and develop a suitable methodology. Solid-phase microextraction (SPME), developed in 1990 by Arthur & Pawliszyn, is an extraction method that removes the need for solvents and concentration procedures. The process of microextraction involves exposing a silicone-based fibre to the headspace above a substrate. Chemicals within the substrate will partition between the headspace and the fibre. The compounds adsorbed onto the fibre are then thermally desorbed in the injection port of a gas chromatograph (GC). The compounds are then carried onto the GC column where retention time is recorded. Where mass spectrometry (MS) is coupled to the GC, the fractionation patterns of the compounds can then be used to identify their chemical structure. Solid-phase microextraction fibres and sampling devices are commercially available, and with 27 different fibres available, this method has many different applications. Since this method has been successfully utilised in detecting low levels of flavour compounds in food and beverages (Marsili, 1999; Yang & Peppard, 1994), its possible application to detection of fungal volatiles was investigated.

SCIENTIFIC AND TECHNICAL PROGRESS FOR YEAR 2

Method development

SPME manual sampler

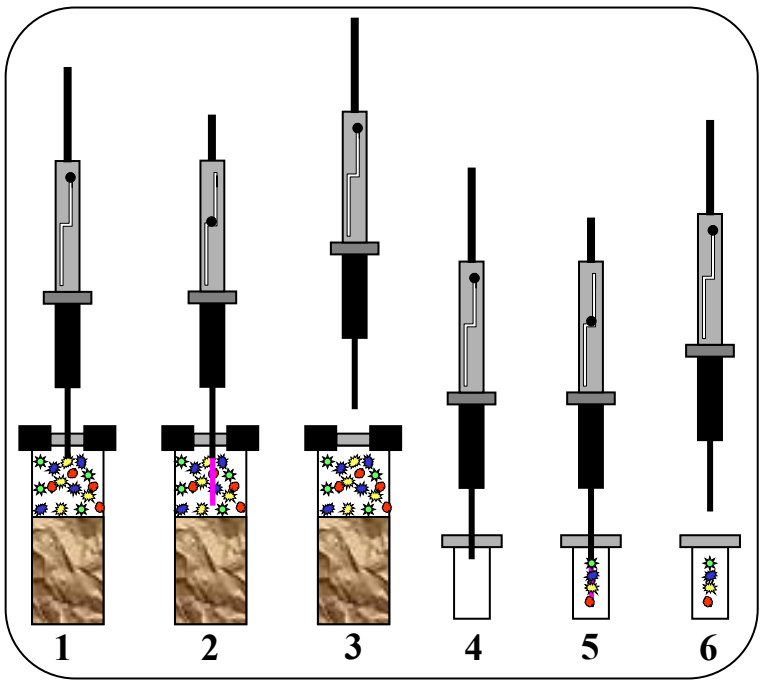


Figure 2. Solid phase micro-extraction sample preparation. 1: Pierce septum of sample tube; 2: Expose the fibre to headspace, to extract volatiles; 3: Retract the fibre and remove the sampler; 4: Pierce GC injection port septum; 5: Expose fibre to thermally desorb volatiles; 6: Retract fibre and remove sampler from the injection port.

To use the fibres they were held within a manual sampler (Figure 2). The septum-piercing needle was used to pierce the septum of the sample container. The fibre was then exposed directly to the headspace of the sample. The fibre was retracted into the manual sampler. The fibre was thermally desorbed in the injection port of the GC. Compounds were separated on the analytical GC column and detected by MS.

Substrate Preparation

Four substrates were investigated: pasteurised compost, four day spawned compost, and fully spawned compost. Substrates were produced in the laboratory using freshly pasteurised compost from the Mushroom Unit (HRI). Compost was collected and stored at 4ºC. All substrates were prepared within No 8 clear pots, with white polythene lids, (AW Gregory & Co. Ltd., London). For spawned substrates 50g of pasteurised compost was top and middle spawned (0.5% w/w grain spawn). The average number of grains required was six; therefore, three grains of spawn were positioned on top of 25 g of compost compressed in to the test pot. The remaining 25 g covered the grains and remaining three grains were compressed onto the compost. The fully spawned compost was incubated for 14-15 days at 25ºC and the four day spawned compost was prepared and incubated for four days at 25ºC prior to analysis. Fifty grams of pasteurised compost was compressed into a test pot prior to analysis.

Fibre Selection

Of the 27 commercially available fibres, three were recommended for use in analysis of volatiles / odours: polydimethylsiloxane (PDMS); Carboxen™/ Polydimethyl-siloxane (CAR/PDMS); poly-dimethylsiloxane / divinylbenzene (PDMS/DVB). Fibre coatings are designed for analytes of different molecular weight and polarity: PDMS is recommended for volatiles (MW 60-275); CAR/PDMS is suitable for gases and low molecular weight compounds (MW 30-225); PDMS/DVB is recommended for volatiles amines and nitroaromatic compounds (MW 50-300). A preliminary experiment was designed to assess which of the three fibres would be most suitable for analysis of cultivation substrates.

Prior to use, the fibres were conditioned to the temperatures of the GC injection port. During this process, any loose coating on the fibre is removed, thus reducing the background interference when the fibres are used in analysis.

Table 9. Temperature and conditioning recommendations for GC use:

Stationary phase	Film thickness	Max temperature	Recommended temperature	Conditioning temperature	Time (Hours)
CAR/PDMS	75µm	320 ºC	240-300 ºC	280 ºC	0.5
PDMS/DVB	65µm	270 ºC	200-270 ºC	260 ºC	0.5
PDMS	100µm	280 ºC	200-270 ºC	250 ºC	1.0

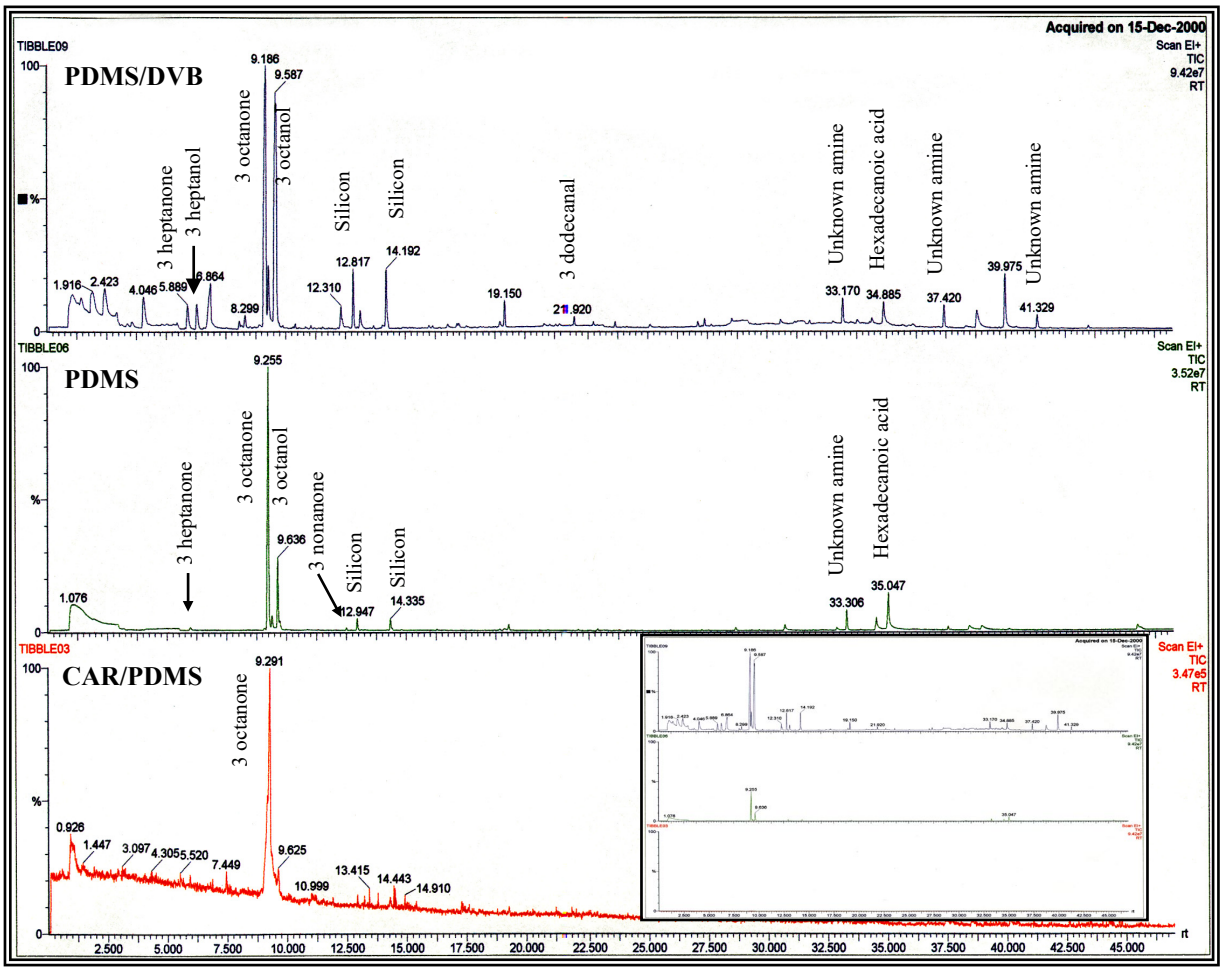
After the appropriate conditioning time the fibre was analysed to assess the level of background interference. The GC temperature program used was the same as would be used in sample analysis. The initial column temperature was 35ºC for 3 minutes, heated to 240ºC at a rate of 5ºC/minute and held at 240ºC for 3 minutes. The injection port temperature was set at 260ºC and fibres were thermally desorbed for two minutes. The analytical column was a 30m x 0.25mm i.d. DB5 fused silica capillary column with a 1µm film thickness. The mass detection range of the MS was set at *m/z* 35-400. The fibre would be re-conditioned and analysed if the interference levels were too high. The process of running a fibre blank was also repeated at the beginning and end of each sample run to ensure that there was no carry-over, of volatiles, between samples.

The fibre was then used for sampling after successful conditioning. Gas tight glass vials were filled to 2/3 capacity with fully spawned compost and left to equilibrate for 30 minutes. The fibre was then exposed to the headspace for 30 minutes, then analysed. This was repeated for all three fibres.

The chromatograms for the three different fibres were compared (Figure 3). The mass spectrum of any detected peaks were analysed, to identify their source, either the sample or fibre. The detection of silica within an analysed peak indicated that its origin was the silica-fused fibre and not the sample of compost. The number and intensity of peaks detected by the fibres, as well as the proportion of silicon based peaks, would determine the most appropriate fibre for analysis of the cultivation substrates.

The PDMS/DVB fibre had the highest total ion count (9.42e7) followed by the PDMS (3.52e7) and CAR/PDMS (3.47e5) (Figure 3). This can be observed in the insert, Figure 3. Not only did the PDMS/DVB detect the highest peak intensities, but also this fibre detected more peaks. The CAR/PDMS was only able to detect a single chemical present in the headspace of the fully spawned compost. Therefore, the CAR/PDMS was deemed unsuitable for this analysis. The PDMS fibre did not contain many silicon impurities, however within the first 10 minutes few chemicals were detected. It is during the first 10 minutes of the temperature program that most volatiles would elute from the column. It is therefore crucial to have good detection for this period. The PDMS/DVB fibre did contain many silicon impurities, but due to its excellent detection in the first 10 minutes, it was clearly the most suitable fibre for this analysis.

Figure 3. GCMS chromatograms from fibre analysis. All fibres were exposed to fully spawned compost for 30 minutes.



Initial Compost Analysis

The PDMS/DVB fibre performed well for fully spawned compost, but is was necessary to test it with all the substrates. This would provide information as to what volatiles are present within the different substrates. The GC temperature programme was modified in an attempt to reduce the background interference from the fibre. The final temperature was increased to 250ºC and the injection port was set to 250ºC. The fibre exposure time was increased to 1 hour to maximise chemical adsorption.

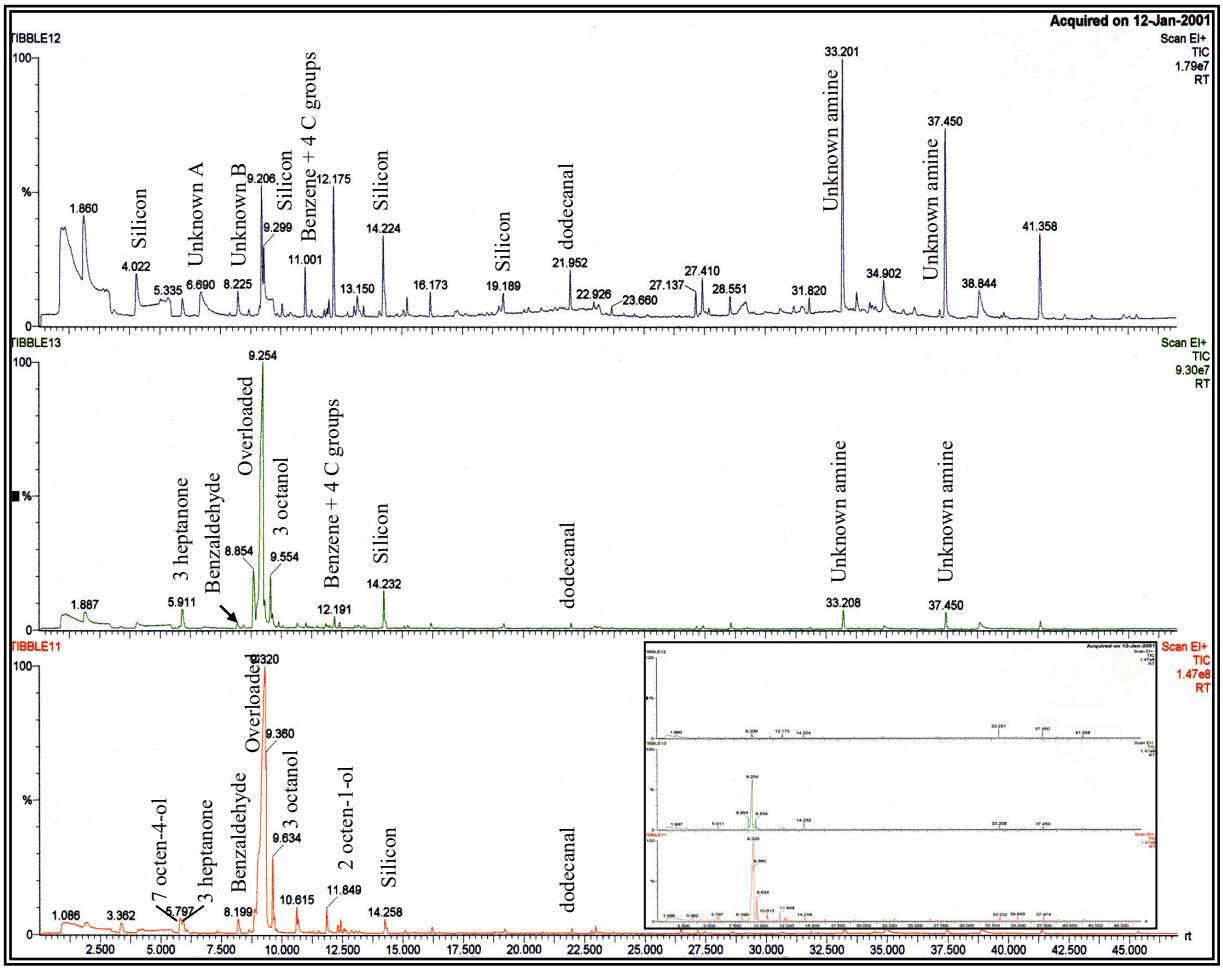
Phase II compost possesses a very different volatile profile compared to compost with growing mycelium (Figure 4). When chromatograms are compared at the same intensity (Figure 4, insert) it is clear that as mycelium grows through the compost there is an increase in the concentration of the volatiles with retention times of 5-12 minutes. Alkalised benzene compounds, those containing carbon side groups, were not found in fully spawned compost, though they were present in phase II compost and 3-day-old spawned compost.

Solid-phase microextraction using a PDMS / DVB fibre is suitable for analysing the volatile constituents of the compost substrates. However, volatile profiles from several samples of each substrate need to be compared, since any volatiles involved in fly attraction would be consistently present. The changes made to the temperature program and the increase in sampling time appears to have reduced the background noise and provide a suitable method for sampling and analysis.

Standard Preparation

Chemical standards can be used to enable comparison between analyses performed on different occasions. There are two possible approaches to the use of chemical standards. Either a sample can be 'spiked' with a chemical that is known to be absent within the sample, or a mixture of chemicals can be analysed independently. The inclusion of a standard of known concentration will highlight any variation of column conditions on different occasions. The retention time and peak height and area for a known concentration of a known chemical should remain constant. However, on different occasions, the column conditions may vary, along with detector response, affecting these parameters. The inclusion of a standard highlights these differences and any deviation between occasions can be used to normalise the data, enabling both qualitative and quantitative statistical analysis. Ideally, the standard should be similar to the analyte of interest, however in this instance the analyte of interest is unknown.

Figure 4. GCMS chromatograms for cultivation substrates. SPME fibres were exposed for 1 hour.



Previous research has highlighted the presence of carbonyl compounds and alcohols containing eight carbon atoms (Pysalo & Suihko, 1976), for this reason a ketone series was used. Two different concentrations were made, 10µg/ml and 2µg/ml, from a 1mg/ml stock solution. The density of the solutions (ρ=g/ml) was used to calculate the concentrations for the solutions. Initially, three ketones were used, 2-hexanone (ρ=0.81), 2-heptanone (ρ=0.8197) and 2-octanone (ρ=0.820). The standard solutions were prepared using n-hexane (bp=69ºC) as the solvent (GC grade) and a 1µl injection was analysed. The solvent front masked the 2-hexanone, so the solutions were made again using dichloromethane (bp=39.75ºC) (GC grade) as the solvent, and 2-nonanone (ρ=0.832) was included. The dichloromethane was still smothered in the solvent front; despite its decreased boiling point and elution time, however fewer impurities were present. When the 2µg/ml solution was analysed 2-nonanoe was not detected, so two other standards were made, with concentrations

of five and 20µg/ml and analysed to determine which concentrations should be used. A retention time shift occurred with the 5µg/ml standard, therefore the 10 and 20µg/ml standards were deemed the most suitable for normalising the experimental data (Table 9). The two standards would be analysed on each occasions to enable comparison of data collected on different days. All standards were refrigerated to prevent evaporation of the solvent and maintain the known concentrations.

Table 9. Ketone standard, retention times (RT) and peak dimensions.

	10µg/ml			**20µg/ml**		
	RT	Peak Area	Peak Height	RT	Peak Area	Peak Height
2-heptanone	6.007	132019792	10939	6.081	350880800	25338
2-octanone	9.188	185804560	95387	9.261	55355168	28458
2-nonanone	12.502	3807945	27635	12.542	350766464	14811

Sample Containers

In previous experiments, substrates were grown in ventilated pots compost and transferred to a gas tight vial for sampling. However, where mycelium was growing through the substrate, transfer of material may release hyphal contents through mechanical breakage. This may release volatiles into the headspace that would not occur during natural growth. This would cause detection of volatiles that, under normal circumstances, would not be present. In addition, in the instance of the 4 day spawned compost, it is possible that during transfer of material from growth to sampling containers that no growing mycelium would be transferred. It would not be possible to grow the substrates within the sampling containers, as they are gas tight. Instead, the possibility of sampling the headspace in the growth pots was assessed. The growth containers were sealed with lid that had 1cm hole, filled with a cotton wool plug, for ventilation. It was possible to insert the sampling needle through this hole, without removal of the plug, enabling headspace sampling without disturbance of the growing substrate. However, since the containers were plastic it was necessary to ensure that there were no volatiles released and detected within the headspace that would interfere with analysis of the substrate volatiles. This was achieved by sampling and analysing a container blank, followed by pasteurised compost, within the container. This was repeated with a 30minute and a one-hour sampling time. The results showed a large amount of silica impurities, present in all analyses, presumed to be either fibre or column artefact. However, there did not appear to be any obvious contamination of the substrate volatiles, caused by the

plastic container. Therefore, the use of plastic containers was deemed the most suitable method for sampling volatiles.

Experimental Design

The results of the initial investigations were used to provide a suitable method for analysis of the volatiles released by the cultivation substrates. Volatiles would be sampled using solid-phase microextraction, and a PDMS/DVB fibre. Pasteurised compost, four day spawned and fully spawned compost would be analysed. To increase the confidence of results gained it would be necessary to repeat any analyses, also increasing the reproducibility of the experiments. It is likely that variation between different batches of phase II compost, produced at the mushroom unit, will occur. Therefore, three different batches of compost will be tested. Three samples will be taken from each compost batch and the three substrates produced, within the laboratory, from each sample. The samples will be taken from the front, middle and rear of the pasteurisation tunnel. This will provide sufficient replicates for the substrates and the compost batches. Analysis of a sample takes approximately 50 minutes and, prior to the next analysis, the oven must cool down to 35°C (45 minutes). This places a time constraint on the number of samples that can be processed in a day. The fibre must be condition at the start of a sampling day and a fibre blank performed at the beginning and end of the day. This will highlight any problems associated with the fibre and the column condition. In addition, the two ketone standards must also be analysed on each sampling day. This means that it is only feasible to analyse three samples per day (Table 10). The sample delivery was randomised, using Genstat (Table 11).

Table 10. Typical analytical day.

	Analysis	Time	Sample
1	Condition Fibre	30 mins 260°C	
2	Cool Down	45 mins	
3	Fibre Blank	50 mins	
4	Cool Down	45 mins	
5	20 µg/ml standard	50 mins	Sample 1 expose,
6	Cool Down	45 mins	1 hour
7	Sample One	50 mins	Sample 2, expose
8	Cool Down	45 mins	1 hour
9	Sample Two	50 mins	Sample 3, expose
10	Cool Down	45 mins	1 hour
11	Sample Three	50 mins	
12	Cool Down	45 mins	
13	10 µg/ml standard	50 mins	
14	Cool Down	45 mins	
15	Fibre Blank	50 mins	

It was important to ensure that all sample preparation and analyses are consistent to ensure statistical validity of the results. The growth containers used throughout were No 8 clear pots, with white polythene lids, (AW Gregory & Co. Ltd., London). Fifty grams of compost were used for each of the substrates, compressed to a uniform depth of 5cm, ensuring uniform headspace volume. Fibre were exposed to the sample for 1 hour and then desorbed for 2 minutes.

Table 11. Design for SPME substrate experiments.

Substrate	Compost batch	Sample	Analysis
Pasteurised	1	front	1
Pasteurised	1	rear	2
Pasteurised	1	middle	3
Four Day	1	middle	1
Four Day	1	front	2
Four Day	1	rear	3
Fully Spawned	1	middle	1
Fully Spawned	1	front	2
Fully Spawned	1	rear	3
Pasteurised	2	middle	1
Pasteurised	2	rear	2
Pasteurised	2	front	3
Four Day	2	front	1
Four Day	2	middle	2
Four Day	2	rear	3
Fully Spawned	2	rear	1
Fully Spawned	2	middle	2
Fully Spawned	2	front	3
Pasteurised	3	middle	1
Pasteurised	3	rear	2
Pasteurised	3	front	3
Four Day	3	rear	1
Four Day	3	middle	2
Four Day	3	front	3
Fully Spawned	3	rear	1
Fully Spawned	3	middle	2
Fully Spawned	3	front	3

Statistical Analysis

For each analysis the number, retention time, height and area of peaks will be recorded. These parameters will be normalised, according to the ketone standard. Multivariate analysis will be used to determine any differences between the substrates and compost batches.

Analysis of mushroom volatiles

The manual sampler used in the laboratory experiments will not be suitable for the analysis of volatiles from mushrooms. The mushrooms are grown at the Experimental Mushroom Unit, HRI. The manual sampler used is not suitable for remote sampling, however a field sampler is available, specifically designed for sampling and storage prior to analysis. When the fibre is retracted into the sampler, it passes through a gas tight septum, preventing the release of volatiles, which would occur with a manual sampler. This would enable sampling in-situ at the mushroom unit and prevent volatile release during transportation to the analytical laboratory. Before this sampler can be used to analyse the mushroom volatiles, it must be tested to ensure that volatiles will not degrade over the storage period. These experiments are still in progress, and on their completion, a method for the analysis of mushroom volatiles will be developed.

CONCLUSIONS

The behavioural experiments have indicated that phorid females are more attracted to fully spawned compost than other cultivation substrates. Attraction to the cultivation substrates increased with the mycelial load of the substrate. This result indicates that the phorids are responding to volatiles associated with fungal growth when searching for oviposition sites.

The success of the initial experiments has shown SPME to be a suitable method for sampling the substrate volatiles. In addition, further experiments have provided a protocol for this analysis. The analysis of air surrounding the cultivation substrates should provide an indication of which chemicals are acting as oviposition stimulants.

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