Project Title	A Study of the Taxonomy, Biology and Epidemiology of Cobweb Causing Pathogens, primarily *Dactylium (Cladobotryum) dendroides*
Project No	M 29
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Background

Cobweb disease has occurred for many years on mushroom farms in Britain and elsewhere. Its occurrence was sporadic and uncommon with an apparent tendency when it did occur, to be an irritating autumnal problem on a small number of farms. This situation changed slowly and, largely unremarked upon, in the late 1980's and early 1990's until in the mid 1990's it had become a serious, threatening epidemic.

The reasons for this are complex and remain imperfectly understood. At the time of the epidemic, however, it became abundantly clear that there was only sparse information available concerning the pathogens responsible for Cobweb and, more importantly, the associated cap spotting which accompanied it. The objective of this project has been to fill this vacuum of knowledge and understanding with the aim of improving control strategies. The part played by pathogen-resistance to pesticides, the persistence of pesticides in casing and the successful search for alternative chemicals have been the subjects of other HDC projects running concurrently and complimentarily with this one.

Project M 29 has been specifically designed to throw light upon the exact identity of the disease causing pathogens (Taxonomy), the biology of the pathogen, and its epidemiology on mushroom farms, and finally to interpret this information to provide improved control methods.

The disease, Cobweb, is caused by several species of *Cladobotryum* and it is this name that will be used throughout this report. These organisms are widely referred to by growers and research workers alike as *Dactylium* spp. to the point where Dactylium has largely become the colloquial name for disease and pathogen alike.

The results of this major investigation are numerous and complex and are divided into three major sections, each summarised separately. The report is completed by a brief summary of the practical implications to commercial growers.

Summary

Taxonomy. Four methods were used to categorise *Cladobotryum* isolates. Growth rate data was variable both within and between apparent groupings so this is unlikely to be a stable characteristic to identify isolates to species level. The number of cells per conidium is often considered to be a good taxonomic character and three distinct groups of isolates were identified based on the mean cell number (MCN) of conidia. However, the genetic data from the RAPD studies identified only one group of genetically-similar isolates. This group included (i) isolates whose conidia had MCN values of <2 and also 2-3; (ii) all the *C. mycophilum* isolates (with a characteristic odour) that had been obtained either from culture collections or the British mushroom industry; and (iii) the large group of fast-growing, thiabendazole-resistant isolates collected during the HDC survey of 1995 (M14a), which lacked the distinct odour and low MCN associated with *C. mycophilum*. No *C. dendroides* isolates occurred in this group. Thus, the RAPD data suggests that these genetically related isolates display considerably morphological variation. It also suggests that the thiabendazole-resistant isolates, which dominated the cobweb epidemic in the early 1990's, are genetically

more similar to *C. mycophilum* than *C. dendroides*. Mating interactions also suggest that the thiabendazole-resistant isolates are not *C. dendroides* as they failed to mate with any of the *C. dendroides* isolates. Some of the isolates obtained during the HDC survey of 1995 (M14a) did mate with other *C. dendroides* isolates from around the world indicating that "true" *C. dendroides* was also present in the British mushroom industry during the cobweb epidemic but these isolates were not thiabendazole resistant.

Pathogenicity tests indicated that there was some variation in the intensity of symptoms associated with the various different isolates tested. The thiabendazole-resistant isolates were the most pathogenic, sporulating earlier and heavier than any others. In contrast, the *C. dendroides* (mating group) isolates all sporulated more sparsely than all other pathogenic isolates. Some non-pathogenic isolates were also identified and these tended to have been isolated from substrates other than *Agaricus*, including wood, soil and other species of fungi. While this may suggest that isolates from non-*Agaricus* sources are not a threat to *Agaricus* mushroom production, most of the non-pathogenic isolates had been in culture collections for many years and may have lost their pathogenicity in storage. Pathogenicity tests using freshly isolated cultures from diverse substrates would establish if non-*Agaricus* isolates were capable of causing cobweb symptoms on *Agaricus*.

Conclusions

- *Cladobotryum* isolates show significant variation in terms of their growth rate and conidial morphology.
- There is no relationship between the morphological data and the genetic RAPD data suggesting that genetically similar isolates show a range of morphologies.
- The thiabendazole-resistant isolates associated with the cobweb epidemic in Britain in the early 1990's were genetically similar to *C. mycophilum*, but they were also morphologically different from current descriptions of this species.
- True *Cladobotryum dendroides* appears to be less pathogenic than the thiabendazole-resistant isolates which dominated the cobweb epidemic in the early 1990's. However, both *C. dendroides* and *C. mycophilum* were also encountered during the epidemic.

Biology. The work described in this section on the biology of *Cladobotryum* in mushroom casing has revealed that *Cladobotryum* growth is not severely affected by either casing formulation or casing moisture content or casing water tension (matric potential). Despite a wide variety of experimental conditions, ranging from different peat sources and rates of sugar beet lime, to very wet or very dry growing conditions, Cobweb colonies still developed in all cases. Some minor effects on growth were detected, such as a 30% sugar beet lime rate and wetter growing conditions can result in larger cobweb colonies. However, results were not clear-cut and some ambiguity occurred. In a second experiment the establishment of cobweb colonies was quicker in wetter casings but subsequent colony growth was faster in drier casings.

Cladobotryum growth on casing was shown to be dependent on *Agaricus* with practically no growth or sporulation occurring in its absence. Thus, *Cladobotryum* spores landing on casing materials would not be capable of further growth. Such contamination of casing ingredients could however result in subsequent development of cobweb disease and it would be useful to know how long any such spores can remain viable and potentially infectious. This is an aspect of *Cladobotryum* biology that future work could focus on.

Conclusions

- Statistical analysis of cobweb colony diameters at the end of the first flush suggest that the rate of sugar beet lime inclusion has a significant effect on the establishment of cobweb, with larger colonies occurring when high (30%) rates of sugar beet lime are used.
- Cobweb colonies were capable of significant growth over a wide range of matric potentials in casings that were very dry to periodically waterlogged.
- Drier casings slowed down the establishment of cobweb while wetter casings led to rapid establishment but subsequent growth rates were marginally higher in the drier casings (41 mm/day in the wetter casings as compared with 34 mm/day in the drier ones).
- Management of casing matric potential in a bulk peat and sugar beet lime casing mix is unlikely to have any major effect on cobweb control.
- Casing is unable to support good growth and sporulation of *Cladobotryum* in the absence of developing mushrooms.

Epidemiology. The work described in this section on the dispersal of *Cladobotryum* conidia within a mushroom house clearly demonstrates that standard watering operations applied to a cobweb disease colony, or a straightforward salting of that colony, will result in a major release of *Cladobotryum* conidia. These conidia will then proceed to be dispersed throughout the house where they can cause spotting symptoms or new disease colonies to occur. The pattern of dispersal will vary depending on whether the air circulation fan is switched on or off, with conidia being more uniformly distributed throughout the house if the fan is on, or more locally distributed around the source, when the fan is off. Although conidial distribution can be more localised if the fan is switched off, small numbers of conidia will still succeed in moving to mushroom beds both above and below the source, as well as moving some distance from the original source. This widespread airborne dispersal of conidia following their disturbance emphasises the importance of early disease identification and isolation if the spread of the disease is to be controlled.

Straightforward salting was shown to be virtually ineffective in preventing the spread of the disease. The technique MUST be used in conjunction with either (a) a tissue, which covers and contains the disease colony prior to salting, or (b) a hand held dust extractor fitted with a fine air-filtration unit. If either of these techniques is used, then extremely few conidia are released into the air resulting in virtually no dispersal of disease propagules throughout the house.

Conclusions

- *Cladobotryum* conidia are liberated into the air in great abundance when disease colonies are disturbed by watering or straightforward salting (Figure A).
- Picking operations and other activities in the cropping chamber do not result in significant numbers of conidia being released.
- The vast majority of conidia settle out within 15 minutes of liberation.
- Even low numbers of conidia/m^3 of air can cause significant spotting symptoms.
- *Cladobotryum* conidia become quickly distributed throughout the house whether or not the system is open (i.e. single layer of bags) or contains barriers to movement (i.e. full shelves or stacked trays).
- When cobweb is prevalent, switching air circulation fans off during watering should concentrate the incidence of subsequent disease or spotting symptoms to the areas closest to the initial location of the disease.

- ❖ Straightforward salting operations WILL result in *Cladobotryum* conidia being disturbed and distributed around the cropping house and MUST NOT be practised.
- ❖ Salting in conjunction with the use of a tissue to initially cover areas of disease will dramatically reduce the dispersal of *Cladobotryum* conidia around the house (Figure B).

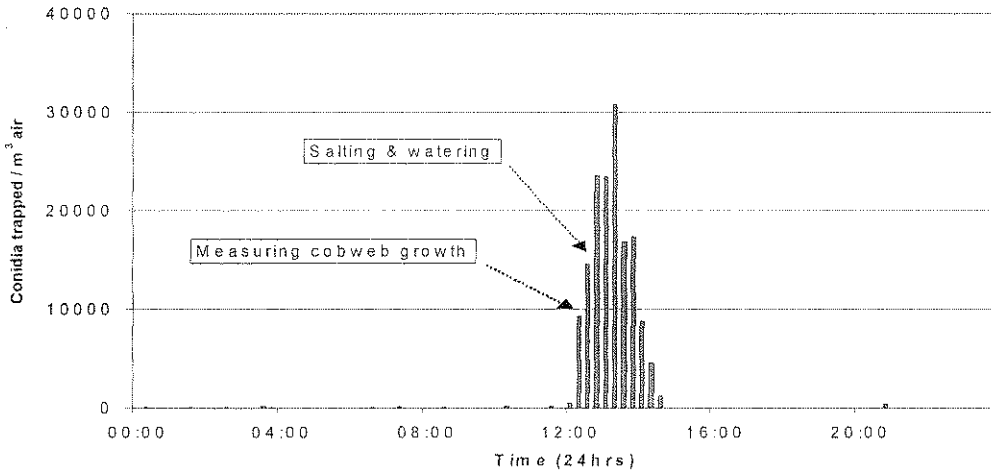


Figure A. Number of *Cladobotryum* conidia in the air following salting, then watering of cobweb infected crop.

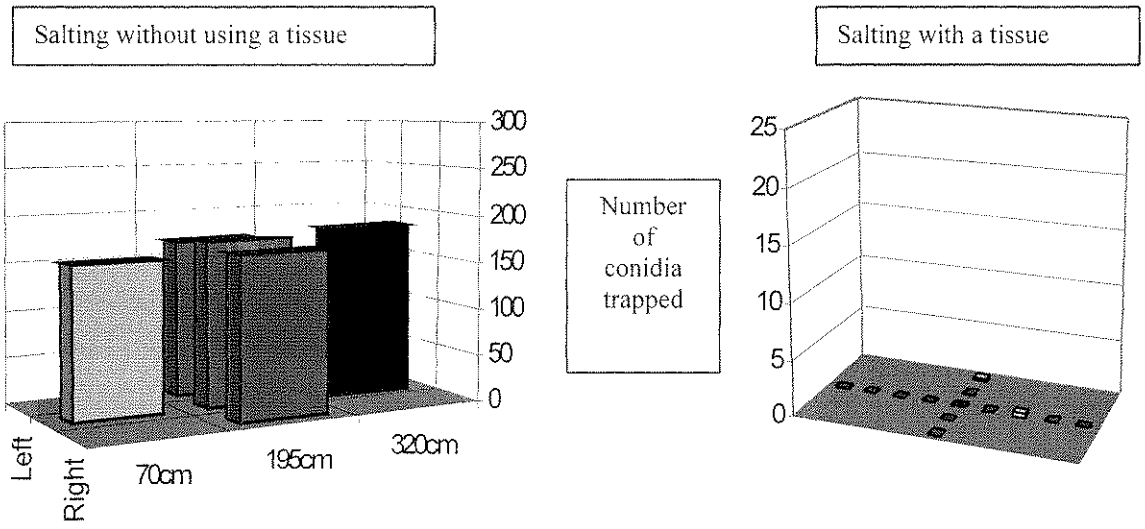


Figure B. Number of *Cladobotryum* conidia trapped at various locations on a single shelf within a mushroom house following different salting techniques.

Action points for growers

This project has defined the major aspects of non-chemical disease control of Cobweb:

- Identify any patches of Cobweb disease as early as possible.
- Do not disturb the patches by watering or straightforward salting.
- Salting should be done in conjunction with a tissue to cover areas of disease. This practice has been shown to be a highly effective means of eliminating disease spread within a mushroom house.
- Switch fans off when watering if there is a risk of undetected Cobweb on the beds. This will minimise disease spread to areas around any undetected Cobweb patches.
- There is little evidence to suggest that different casing materials or cropping conditions will have much of an effect in reducing Cobweb disease symptoms.

Science Section

1 General Introduction

1.1 *Cladobotryum* spp. - Cobweb disease of *Agaricus bisporus*

The UK mushroom industry, worth £170m/annum, was shaken during the mid 1990's by an epidemic of cobweb disease (Anon, 1998; Gaze, 1995a). *Cladobotryum dendroides,* commonly considered the major causal agent of cobweb disease, has always been present in the mushroom industry worldwide but it had never before had such devastating effects (Gaze, 1996a). Forer *et al* (1974) estimated that *C. dendroides,* along with two other pathogens (*Verticillium fungicola* and *Mycogone perniciosa*), cost the Pennsylvanian industry over $9,000,000 alone. Although *C. dendroides* made up only *c.* $400,000 of that loss, it was consistently the third most problematic pathogen of mushrooms after *V. fungicola* and *M. perniciosa*, dry and wet bubble disease, respectively (Fletcher, Hims, & Hall, 1983). Gaze and Fletcher (1975) during a national survey of mushroom diseases within the UK concluded the same three diseases whilst not uncommon caused fewer losses. It was estimated total losses from all three diseases amounted to less than 5% of total yield.

During 1995, cobweb disease was elevated to the status of a major disease problem in Britain (Gaze, 1996b). Although losses were not quantified, it was considered at the time the most problematic disease affecting mushroom cultivation in the United Kingdom (Gaze, 1996a). Not only did it become more difficult to control than before, but it was also entering the crop at an earlier stage in the cycle (Gaze, 1995b). This lack of control and early establishment within a crop often resulted in the crop being terminated early so as to prevent disease levels becoming too high.

The reason for such an increase in severity and frequency of the disease remains a matter for a certain amount of conjecture but resistance to benzimidazole fungicides was implicated (McKay et al., 1998). However, a survey of British isolates of *Cladobotryum* showed that benzimidazole resistance was present in 75% of isolates tested (Grogan and Gaze, 1996). Isolates collected from around the UK could be distinguished by their growth rate and sensitivity to thiabendazole resulting in the formation of three subgroups. Type A isolates were slow-growing and sensitive to thiabendazole; Type B1 isolates were faster growing and resistant to thiabendazole and Type B2 isolates were fast-growing but sensitive to thiabendazole. The majority of the British isolates tested (75%) were of the B1 type and showed little variation, while the less common types A and B2 accounted for 25% of all isolates collected, and were more variable.

Cladobotryum dendroides has the potential to cause both quantitative and qualitative losses to the mushroom industry. Quantitative losses are caused by the characteristic grey, coarse, cobweb like mycelial growth on casing, reducing cropping area and causing a wet rot of any mushrooms in its path, whereas qualitative losses are attributable to cap spotting (Plate 1). Spotting of the mushroom caps is particularly problematic (Gaze, 1996a). If an area of mycelial growth on mushroom casing remains unchecked, sporulation will occur. The release and dissemination of conidia within a mushroom cropping house will facilitate the establishment of further colonies on the casing and spotting to develop on mushroom caps rendering them unsalable (Gaze, 1996a; Lane, *et al.*, 1991). Spotting on mushroom caps is simply the initial stages of infection, which, if allowed to develop, would eventually engulf the entire cap, ultimately leading to further sporulation (Sinden and Hauser, 1953). It is therefore essential that spotted mushrooms are

identified and discarded at picking. If overlooked, development during storage and transit is possible, which can result in mushrooms being rejected by the buyer.
Airborne conidial dissemination is arguably the most common form of disease dispersal within a mushroom farm (Sinden & Hauser, 1953; Sinden, 1971; Atkins, 1974; Lane *et al.*, 1991; Gaze, 1995(b); Gaze, 1995(c); Dar, 1997). Other mechanisms include contamination of casing materials, drainage water, machinery, and pickers with mycelial fragments, microsclerotia and conidia. Watersplash and fly-dispersal have also been suggested and remain plausible mechanisms of disease spread (Fletcher *et al.*, 1986; Lane *et al.*, 1991; Gaze, 1995d; Gaze, 1995a; Dar, 1997). The source of primary infection on an uninfected mushroom farm is however not yet understood, although several speculative theories have been proposed (Sinden, 1971; Gaze, 1995a).

1.2 Mycoparasitism

The term mycoparasitism is most simply defined as the parasitism of one fungus by another (Ainsworth, 1971), a phenomenon recognised by scientists for over 100 years (Barnett, 1964). Implicit within that statement is the process of nutrient transference from the host to the parasite, to the detriment of the former and benefit to the latter. As is often the case, this simple definition inadequately describes what is an extensive subject area and a review of the topic is given by Jeffries and Young (1994).

Cladobotryum dendroides is an example of a mycoparasite and has been isolated from a wide variety of sources. It has reportedly been isolated from several species of fungi including; *Coriolus versicolor, Physisporinus sanguinolentus, Piptoporus betulinus, Polyporus squamosus, Stereum hirsutum, Armillaria mellea, Pleurotus ostreatus, Polyporus badinus, Russula* sp., *Lactarius* sp., *Lepista nuda, Ramaria corrugata, Botrytis cinerea, Cantharellus* sp., *Lenzites* sp., *Polyporus tomentosus* (Petch, 1938; Rudakov, 1978; Anon, 1951; Sutton, 1973; CBS list of cultures; Jeffries and Young, 1994). In addition it has been isolated from several non-fungal substrata such as woodland soil, decaying wood (*Pinus* sp.), moss (*Polytrichum* sp., IMI list of cultures), and a diseased wheat spike (ATCC list of cultures).

The extensive host range demonstrated by *C. dendroides* might be due in part to its ability to parasitise using various mechanisms. *Cladobotryum dendroides* is not only considered an exogenous parasite but is also capable of intrahyphal invasion and antibiotic activity (Nair, 1976; Rudakov, 1978; Turner and Aldridge, 1983). Depending on the host species, *C. dendroides* has been described as biotrophic (Rudakov, 1978), necrotrophic (Rudakov, 1978; Dar and Seth, 1991; Lane; 1993), and saprotrophic (von Arx, 1974). It is considered biotrophic when parasitising *Botrytis cinerea*. Its hyphae first compete with the host for substratum before causing lysis of the mycelium. It then proceeds to penetrate the host hyphae where it survives biotrophically (Rudakov, 1978). When parasitising basidiomycetes it is generally considered to be necrotrophic as it causes a wet rot which completely decomposes the fruiting body (Forer *et al.*, 1974; Dar and Seth, 1992; Jeffries & Young, 1994). Where this fungus has been isolated from decaying wood it is described as saprophytic (Lane, 1993; Jeffries & Young, 1994). Despite this organisms inherent ability to parasitise a plethora of host fungi it remains best known for its parasitism of only one - *Agaricus bisporus* – the cultivated mushroom.

1.3 Taxonomic nomenclature

Cladobotryum dendroides, the asexual (anamorphic) state of *Hypomyces rosellus*, was first proposed as *Dactylium dendroides* by Fries (1832) and is commonly considered the primary

causal agent of cobweb disease. In assigning this species to the genus *Dactylium* Nees (1817), Fries was extending the characteristics of this genus that had been previously occupied solely by *Dactylium candidum*. *Dactylium candidum* possessed unbranched conidiophores whereas several species added to the genus by Fries in 1832 possessed branched conidiophores, including *C. dendroides*. Fries expansion of *Dactylium* Nees was widely accepted throughout the mid 19th century and the type of the genus remained as *D. candidum*.

Saccardo (1886) however redefined the genus by differentiating between species with unbranched and branched conidiophores. Seemingly ignoring historical protocol which dictated that Nees's original type, *D. candidum*, which was not challenged by Fries, should remain as the type for the genus *Dactylium*, Saccardo referred it and all other species possessing unbranched conidiophores to a new genus, *Dactylaria* Sacc.

The next major reclassification of *Dactylium* occurred in 1962 when De Vries analysed the type material of *Cladobotryum varium* Nees. When compared to the type material of *D. candidum* Nees it was concluded that the two should fall into the same genus i.e. *Cladobotryum*. However the problem remained, should *C. dendroides* be associated with *Cladobotryum* (thus following Fries classification), or not (thereby accepting Saccardo's classification).

The debate continued into the late 1960's and early 1970's. The difficulty in finding a well-defined classification for *D. dendroides* and other *Dactylium* spp. with branched conidiophores was illustrated and confounded by Barron (1968). He described the popular concept of *Dactylium* as being somewhat vague and the genus composed of a heterogeneous assemblage of species. He continued to state that "*Dactylium dendroides* the conidial state of *Hypomyces rosellus* is not a *Cladobotryum* and is not readily classified into any form genus known to me." Cole and Kendrick were later to agree with Barron when in 1970 they proposed that *C. dendroides* be expelled from the genus *Cladobotryum*. Their conclusions were based on a comparison of time lapse photography of *C. variospermum* conidiogenesis and studies and sketches of *C. mycophilum* and *C. dendroides* performed by Gams and Hoozemans (1970). Cole and Kendrick's conclusions conflicted with those of Gams and Hoozemans who earlier that year (1970) revised the genus *Cladobotryum*. Gams and Hoozeman's reworking of the *Cladobotryum* Nees genus was based on conidiophore, phialide, and conidial characteristics and they concluded that *C. dendroides* was indeed a member of this genus.

Several authors subsequently studied and classified the *Cladobotryum* genus to include *C. dendroides*, thereby accepting Gams and Hoozemans inclusion and strengthening it's admission into the genus (De Hoog, 1978; Bridge Cooke, 1986; Ellis and Ellis, 1988). The most recent classifications/analyses of the genus concur that the inclusion of *C. dendroides* within the genus *Cladobotryum* is quite valid. Rogerson and Samuels took several years to formulate one of the most comprehensive studies of *Hypomyces* and their anamorphs undertaken in recent times. They encompassed species occurring on discomycetes, boletes, Aphyllophorales, and Agaricales (Rogerson and Samuels, 1985; Rogerson and Samuels; 1989; Rogerson and Samuels, 1993; Rogerson and Samuels, 1994). The anamorphic characteristics used in their classification included the branching of the conidiophores, proliferation of the conidiogenous cell and conidial septation. A strong argument was also put forward to justify the amalgamation of all (except one - *H. chrysostomus*) the anamorphic forms of aphyllophoricolous *Hypomyces*. This includes species from the genera, *Cladobotryum*, *Helminthophora*, *Sepedonium*, *Sibirina*, and *Mycogone*. Rogerson and Samuels proposed in that paper that the new larger genus adopts the name *Cladobotryum*. This was considered the oldest name for that group of species constituting the anamorphic forms of polyporicolous *Hypomyces*.

The anamorphic state of *Hypomyces rosellus* originally classified as *Dactylium dendroides* by Fries in 1832 is therefore now widely considered a member of the genus *Cladobotryum*. It's position however as primary causal agent of cobweb disease has recently been questioned.

For many years it had been appreciated that several morphologically similar species produced cobweb disease symptoms although *C. dendroides* was always considered the most common. For example Fletcher *et al.* (1989) named *H. auranteus* as another cobweb causing species. Similarly, *Cladobotryum mycophilum,* with its characteristic camphor odour, has been sporadically implicated in the causing of cobweb disease symptoms (Geels *et al,* 1988). A recent report however has shown that benzimidazole-resistant isolates, which played a fundamental role in the 1995 epidemic, were more closely related to *C. mycophilum* than *C. dendroides* (McKay *et al*, 1999). Whilst these benzimidazole-resistant isolates did not conform exactly to descriptions of either *C. dendroides* or *C. mycophilum,* nucleotide sequence data for the internal transcribed spacer (ITS) regions of these isolates suggested they were more similar to *C. mycophilum*. The suggestion that benzimidazole-resistant isolates are more closely related to *C. mycophilum,* also therefore suggests, that for the period of the epidemic, *C. mycophilum* was the most common cobweb causing pathogen.

1.4 Scientific and commercial objectives

There is very little information available concerning the taxonomy, biology and epidemiology of *Cladobotryum* spp. that are pathogens of *A. bisporus*. The aim of this study is to provide some of this information so that effective disease control strategies can be formulated. Prophylactic and curative fungicidal treatments exist with can control the initiation and spread of the disease, provided isolates are sensitive, however chemical treatments are no panacea. The literature is scattered with cases of fungal pathogens, including *Cladobotryum* spp., developing resistance to chemical treatments (Fletcher & Yarham, 1976; Fletcher *et al*, 1980; Grogan & Gaze, 1995; Whitehead, 1995; Grogan & Gaze, 1996). There is also a growing movement towards pesticide free or 'organic' produce (Lelley, J., & Straetmans, U., 1987). Knowledge about the biology and epidemiology of *Cladobotryum* spp. will hopefully provide a solid framework around which a more holistic control strategy may evolve for use within the mushroom industry.

The objectives of this project are briefly summarised under the following three subject areas:

1. **Taxonomy:** To categorise *Cladobotryum* isolates according to:
    a) Morphology (growth rate, conidial characteristics)
    b) Molecular variability (Random Amplified Polymorphic DNA (RAPD) analysis
    c) Mating characteristics
    d) Pathogenicity
2. **Biology:** To investigate the effects of the following factors on the development of cobweb:
    a) Casing formulation
    b) Matric potential
    c) Presence of *Agaricus*
3. **Epidemiology:** To investigate the role of conidia in disease epidemiology with respect to:
    a) Conidial release over time
    b) Spatial dispersal of conidia
    c) Controlling conidial release

These objectives are dealt with in detail in individual sections within each chapter.

2 Taxonomy

The confusion surrounding not only the *Cladobotryum* genus but more specifically cobweb causing pathogens was highlighted in section 1.3. The disorder regarding the classification of *C. dendroides* and it's morphological similarity to a number of other species has lead to misidentification in the past (Nobles & Madhosingh, 1963; Ogel *et al*, 1994). It is important therefore to resolve this confusion, at least among those isolates collected from the British mushroom growing industry.

The objective of this section is to attempt to categorise a large number of *Cladobotryum* isolates with respect to (i) growth and conidial characteristics, (ii) molecular variability using RAPD analysis, (iii) mating characteristics and (iv) pathogenicity. Up to 82 isolates were examined in total but not all isolates were examined in each experiment. Full details of all isolates are given in Appendix 1.

2.1 Morphology: Growth & conidial characteristics

2.1.1. Introduction

The vast majority of taxonomic accounts pertaining to cobweb causing pathogens relate to traditional taxonomic methodologies. These commonly include information regarding both anamorphic (asexual) and teleomorphic (sexual) structure, conidium development and structure, growth rate, and growth characteristics (Gams & Hoozemans, 1970; Cole & Kendrick, 1970; de Hoog, 1978; Rogerson & Samuels, 1993 & 1994). Rogerson and Samuels (1985, 1989, 1993, & 1994) have even divided Hypomycetes according to their substrata. Despite the current trend for molecular taxonomy, which will be discussed in more detail in section 2.2, traditional taxonomic methods should not be dismissed as archaic and consequently overlooked. They can be a rapid, low technology, and low cost means of identification, as well as allow the determination of growth rate, sporulation intensity and other important pathological characteristics.

One of the earliest references mentioning the growth rate of cobweb disease in pure culture (2% malt extract) proposed a growth rate of between 7–8 mm/day (Anon, 1951). A single isolate of this pathogen was grown at 60°F (15.5°C) on various pH-amended media. Two forms of growth were recognised; staling and non-staling. Staling growth was described as growth which 'falls off' from the maximum as the accumulation of waste products given out by the fungus renders the medium unsuitable for further growth. Non-staling growth is that which maintains the maximum.

Later studies have published similar growth rates. Gams & Hoozemans (1970) reported a growth rate of 7mm/day for *C. mycophilum* at 20°C and 10.7mm/day for *C. dendroides* whilst Lane *et al* (1991) reported a growth rate of 7.5mm/day. Lane (1993) subsequently recorded the growth rate of seven different isolates of *Dactylium dendroides* growing on 3% MEA incubated at 25° and noted highly variable results. Growth rate ranged from as little as 1mm/day up to 8.5mm/day with two isolates from *Agaricus* spp. having growth rates of 6.5 and 6.6mm/day.

The most recent study of isolates from the British mushroom industry, collected during the 1995 epidemic, identified three distinct *Cladobotryum* types with regards to growth rate and fungicide resistance (Grogan & Gaze, 1996). Additionally, all isolates grew more rapidly than those previously described by other workers as shown in the following table:

Details of *Cladobotryum* types collected from Britain in 1995 (Grogan & Gaze, 1996).

Subgroup type	Radial growth rate	Resistance status to thiabendazole
Type A	Slow 11-14mm/day	Sensitive
Type B1	Fast 18-23mm/day	Resistant
Type B2	Fast 18-23mm/day	Sensitive

Descriptions of conidial characteristics of *Cladobotryum* spp. have been more variable than growth rates, although remain a means of discrimination. Descriptions of the length, width, and septa number of conidia can vary for the same species between authors (Gams & Hoozemans, 1970; De Hoog, 1978; Ellis & Ellis, 1988; Lane, 1993; Rogerson & Samuels, 1993). It is known however that nutritional and environmental conditions can alter the size of conidia, which, may explain in part why descriptions vary as they do (Ruppel, 1974). Different authors grew the fungi from which conidia were taken on different media with various nutritional values and / or disparate environmental conditions. The variability of conidial form observed among these descriptive studies may therefore be in part due to the divergent nutritional / environmental conditions. Even when nutritional and environmental conditions concur, conidial size and form can not always be used to distinguish between species. De Hoog (1978) noted that whilst markedly different types of conidiation were present within the *Hyphomycetes*, these characters by themselves were not sufficient to distinguish between species. Individual strains of *C. mycophilum* and *C. varium* had such variable conidial characteristics that differences between the two species were only gradational. However, Lees *et al* (1995) found it possible to distinguish between two different strains of *Microdochium nivale* by the assessment of a combination of conidial characteristics when average number of septa or conidial length alone were found to be inadequate.

Experiments were carried out to establish the range of growth rates and conidial characteristics that occur within the British population of cobweb causing pathogens. All group A & B2 isolates described by Grogan and Gaze (1996) as more variable were included in this study whilst only a representative sample of the less diverse B1 type isolates were selected. In addition, the data were subjected to statistical analysis to see if certain groups of characteristics could be used to define specific groups of isolates.

2.1.2 Materials and methods

2.1.2.1 Sources of fungal isolates.

Thirty-six of the 82 isolates listed in Appendix 1 were studied for their growth and conidial characteristics (Table 1). Thirty-three of these, were originally collected by Grogan & Gaze (1996). Two of these isolates (241C and 202A) have since been identified as *C. mycophilum* and two (5A and 192B1) as *C. dendroides* by taxonomists at International Mycological Institute (IMI). Most isolates were recovered from diseased mushrooms and were geographically well distributed throughout the UK. In addition, three strains of *Hypomyces rosellus(C. dendroides)*, CC1, CC2, and CC3, were obtained from The International Mycological Institute (IMI) culture collection. Summary details of isolates are presented in Table 1.

Table 1. Details of *Cladobotryum* isolates used in growth rate and conidial studies.

Type	Isolate Number	Place of Origin
A#	187	W. Sussex
A	195A	Devon
A	202A (*C. mycophilum*)	Kent
A	202B	Kent
A	220B	France (INRA)
A	220D	France (INRA)
A	222	Wiltshire
A	233A	Sussex
A	238A	N. Humberside
A	238B	N. Humberside
A	257	Kent
A	289	Devon
B1	192B1 (*C. dendroides*)	Sussex
B1	245	Ireland
B1	214	Devon
B1	215B	Fife
B1	239	Horley
B1	5A (*C. dendroides*)	Lancashire
B1	288*	Oxfordshire
B1	298*	Humberside
B1	215A*	Fife
B1	247B*	Northumberland
B1	166*	Dorset
B1	235*	Norfolk
B1	165*	Worcestershire
B1	180*	Bridgend
B1	192A*	Sussex
B1	249C*	Lancashire
B2	193A	Suffolk
B2	193B	Suffolk
B2	193C	Suffolk
B2	220C	France (INRA)
B2	241C (*C. mycophilum*)	East Lothian
-	CC1*	U.K.
-	CC2*	Unknown
-	CC3*	Canada

Isolate type according to Grogan & Gaze (1996); Type a and B2 are thiabendazole sensitive; type B1 is thiabendazole resistant

* Isolates for which only conidial data was obtained.

2.1.2.2 Maintenance of cultures.

Samples of all isolates were kept in long term storage in liquid nitrogen following the method devised by Challen & Elliott (1986). When required, a sample was recovered from liquid-nitrogen-storage and subcultured onto 3% malt extract agar (MEA) (Oxoid) in 90mm diameter Petri dishes.

Working cultures were also maintained on MEA slopes and held at 4°C. Every six months these were subcultured onto fresh slopes, incubated at 25°C over a period of 48hrs and then maintained at 4°C until needed. MEA was considered the most appropriate growth media as it had been used

previously in several studies of *Cladobotryum dendroides* (Anon, 1951; Hsu & Han, 1981; Lane 1993; Grogan & Gaze, 1996).

2.1.2.3 Growth rate test and conidial sampling.

For each isolate to be tested, 10 MEA Petri dishes were inoculated with a 5mm plug of mycelium taken from the growing edge of a fresh culture. The plugs were placed 10mm from the edge of the Petri dish, mycelium side uppermost, with hyphal tips pointing towards the centre of the dish.
Five replicate cultures for each isolate were incubated at 18°C and five at 25°C. These temperatures were selected to reflect those at which *Agaricus* is grown commercially. All replicates were randomly distributed within each incubator for the duration of the experiment. Growth was recorded at 24-hour intervals from the leading edge of the plug to the colony margin along the longest axis of the Petri dish. After 14 days incubation, or when the Petri dish was completely covered with *Cladobotryum* mycelium, all plates were stored at 4°C to prevent further growth of the fungus until conidial characteristics could be measured (Wood, 1958).

Conidia characteristics were measured using a light microscope with calibrated eyepiece at 400x magnification. Using a sterile needle, conidia were taken from the maturest areas of hyphal growth, in order to ensure that a disproportionate number of immature conidia were not selected. Conidia were mounted in a water droplet on a glass microscope slide, covered with a glass cover slip, then observed. The maximum length, maximum diameter and cell number for ten randomly selected conidia were recorded from each replicate which resulted in a total of 50 conidia sampled for each strain at each temperature.

2.1.2.4 Statistical analysis.

Statistical analyses were performed using the Genstat 5 computer package. Individual variates were subjected to analysis of variance to test for differences between strains. In addition a hierarchical cluster analysis was performed on a similarity matrix constructed from six measures on each strain – conidia length, width and cell number at both temperatures (18°C and 25°C). Similarity matrices were constructed using the Euclidean metric, and the dendrograms constructed using the complete link or furthest neighbour algorithm.

2.1.3 Results.

2.1.3.1 Growth rate.

Considerable variation was recorded in radial growth rate between isolates. Variation was also observed within the same isolate grown at different temperatures (Figure 1). Type B isolates demonstrated consistently higher mean growth rates than Type A isolates when incubated at 25°C. Type B isolates had mean growth rates of from 17.9 to 20.8mm/day, a range of less than 3mm/day whereas Type A isolates had mean growth rates of from 10.9 to 16.3mm/day, a range of 5.4mm/day (excluding the anomalous isolate 195 with a growth rate of 4.8mm/day).

This difference was not observed between the two groups when incubated at 18°C where mean growth rates were more similar throughout Types A and B. However, mean growth rates for Type B isolates were more homogenous (range - 4.8mm/day) than type A isolates (range - 8mm/day), with the exception of isolate 220C which demonstrated an anomalous low mean growth rate.

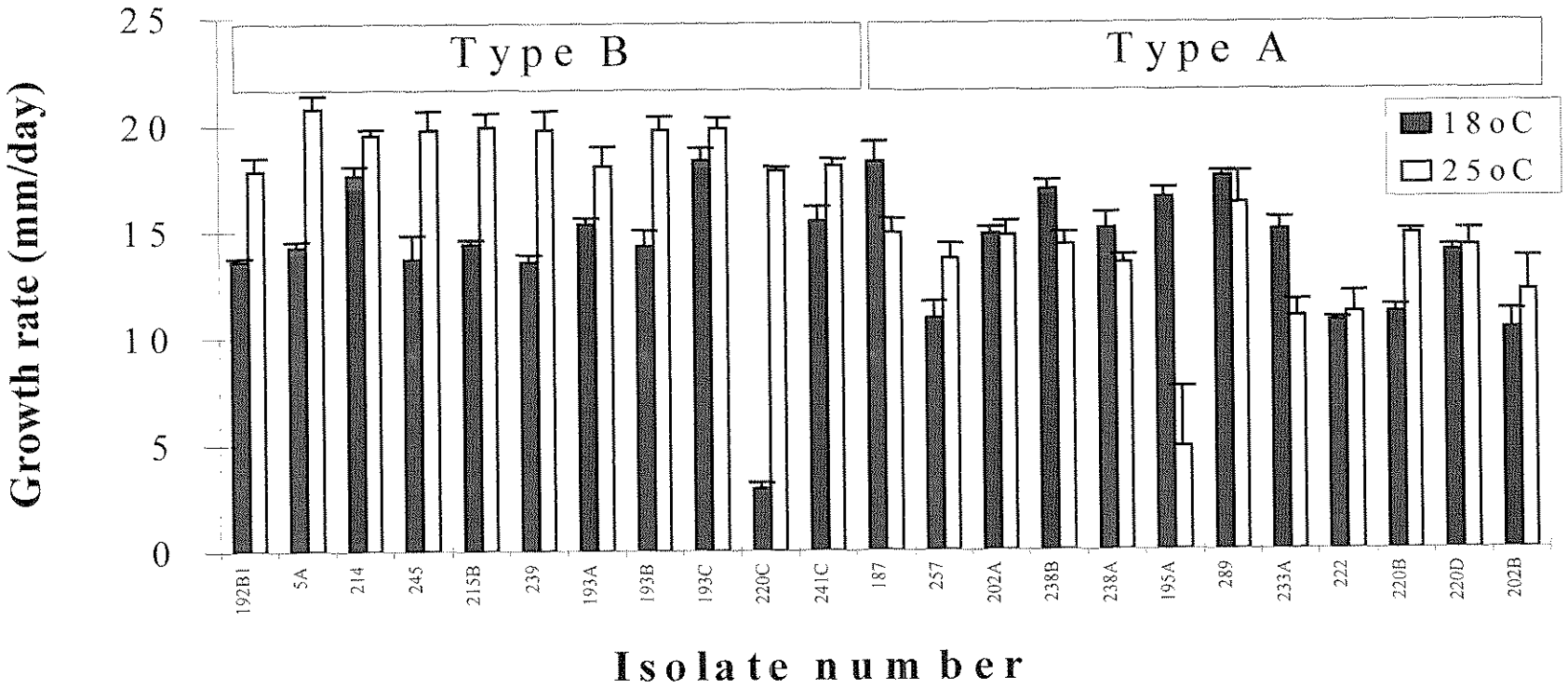


Figure 1. Mean growth rate of 23 *Cladobotryum* spp. isolates cultured on malt extract agar (Oxoid) at 18°C and 25°C. Error bars indicate the variation between the five replicates used to calculate the mean.

2.1.3.2 Conidial characteristics.

Analysis of conidial characteristics demonstrated the presence of variation among isolates, but also within the same isolate when grown at different temperatures. Significant differences ($P<0.001$) existed in the mean cell number, conidial length, and conidial diameter of different isolates. Conidial length and conidial diameter also differed significantly ($P<0.001$) when an isolate was grown under different temperatures. Mean cell number was the least variable characteristic and did not differ significantly when isolates were grown at either 18°C or 25°C.

Analysis of Mean Cell Number (MCN) (Figure 2) showed that the isolates could be divided into three distinct groups;

	Mean Cell Number
Group 1	> 3
Group 2	2-3
Group 3	< 2

All type B isolates except 241C (MCN = 1.51) were shown to have a MCN of between 2 and 3, and were relatively homogenous in comparison to type A (Figure 2). Type A isolates were more heterogeneous. For example, type A isolates 187, 289, and 195A showed a MCN in excess of 3, type A isolates 220B, 238B, and 238A had a MCN between 2 - 3, and type A isolates 220D, 202A, 257, 202B, 233A, and 222 revealed a MCN of less than 2 (Figure 2).

Cluster analysis of all the conidial data for each of the temperatures 18°C and 25°C also gave three groupings of isolates but they were different to each other (Figures 3 & 4) and also to the three groups based on mean cell number alone (Figure 2). The characteristics of these clusters are presented in Tables 2 & 3.

Table 2. Clustering of *Cladobotryum* spp. isolates based on conidial characteristics when grown at 18°C.

Cluster	Mean Cell Number Range	Mean Conidia Length Range (µm)	Mean Conidia Diameter Range (µm)	Descriptive summary
I – 18°C	2.3 - 3.4	24.8 - 28.1	9.4 - 11.1	**Long & Thin**
II - 18°C	1.5 - 1.7	19.8 - 21.8	11.7 - 12.8	**Low cell number & Wide**
III - 18°C	2.0 - 3.0	20.9 - 24.0	9.7 - 11.7	**Average**

Table 3. Clustering of *Cladobotryum* spp. isolates based on conidial characteristics when grown at 25°C.

Cluster	Mean Cell Number Range	Mean Conidia Length Range (µm)	Mean Conidia Diameter Range (µm)	Descriptive summary
I 25	> 3.2	> 24.9	8.4 - 11.0	**High cell number & long**
II 25	1.5 - 2.3	19.8 - 23.0	10.7 - 12.7	**Wide, low cell number**
III 25	2.0 - 3.2	20.0 - 23.3	8.8 - 10.0	**Average**

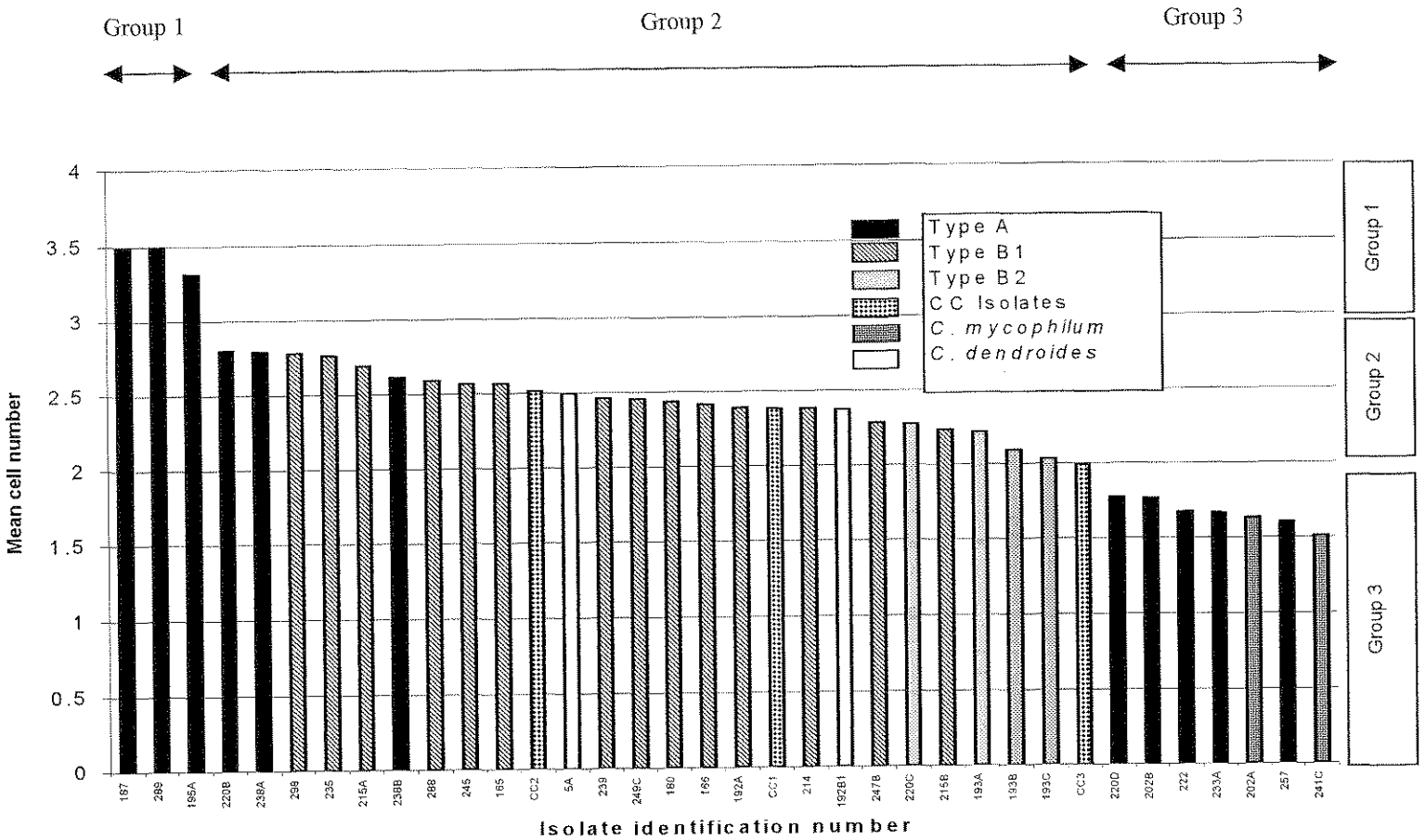


Figure 2. Conidium mean cell number of 33 *Cladobotryum* spp. isolates. Grouping structure and type categories are indicated.

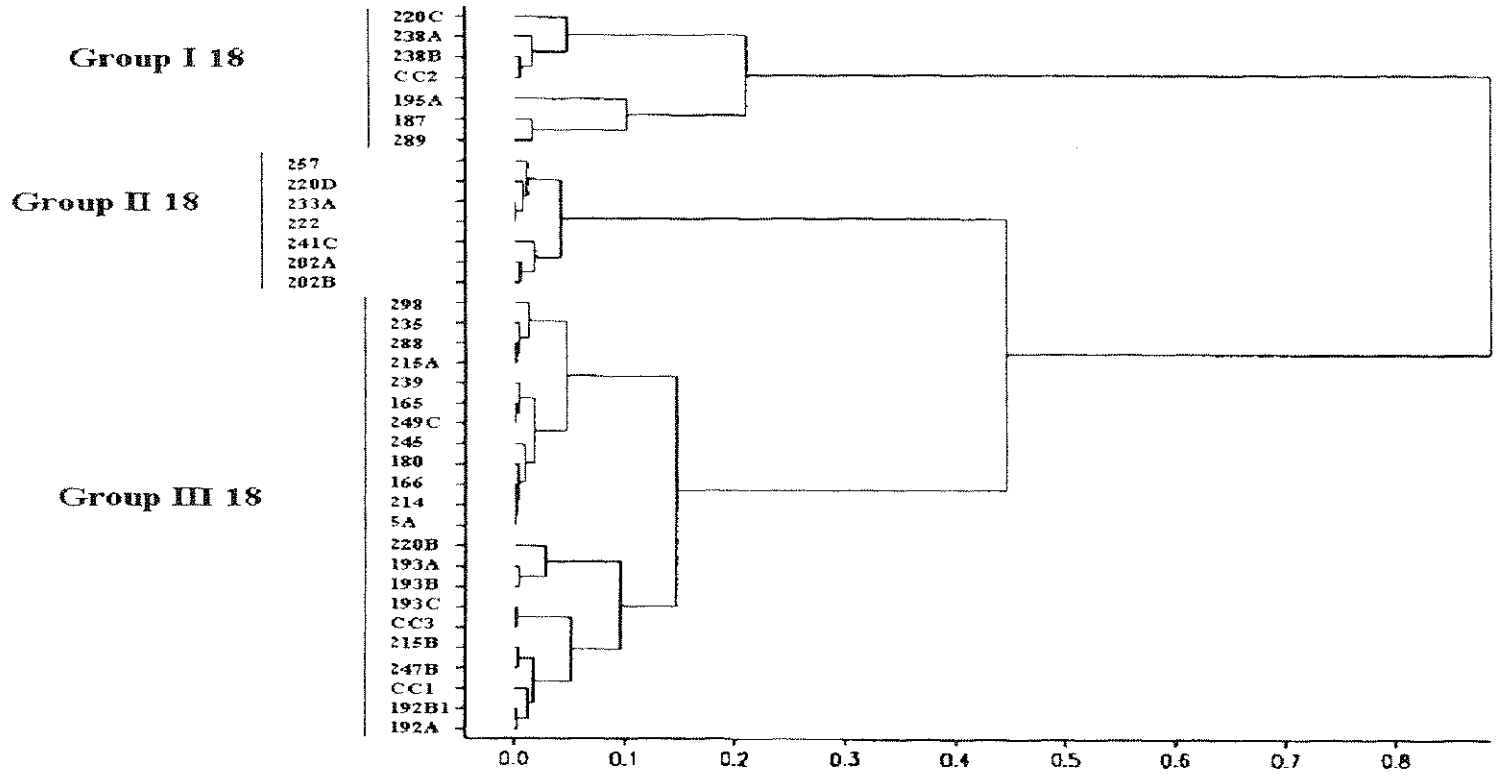


Figure 3. The distance between 36 *Cladobotryum* spp. isolates grown at 18°C, based on conidial characteristics; length, diameter, and average cell number.

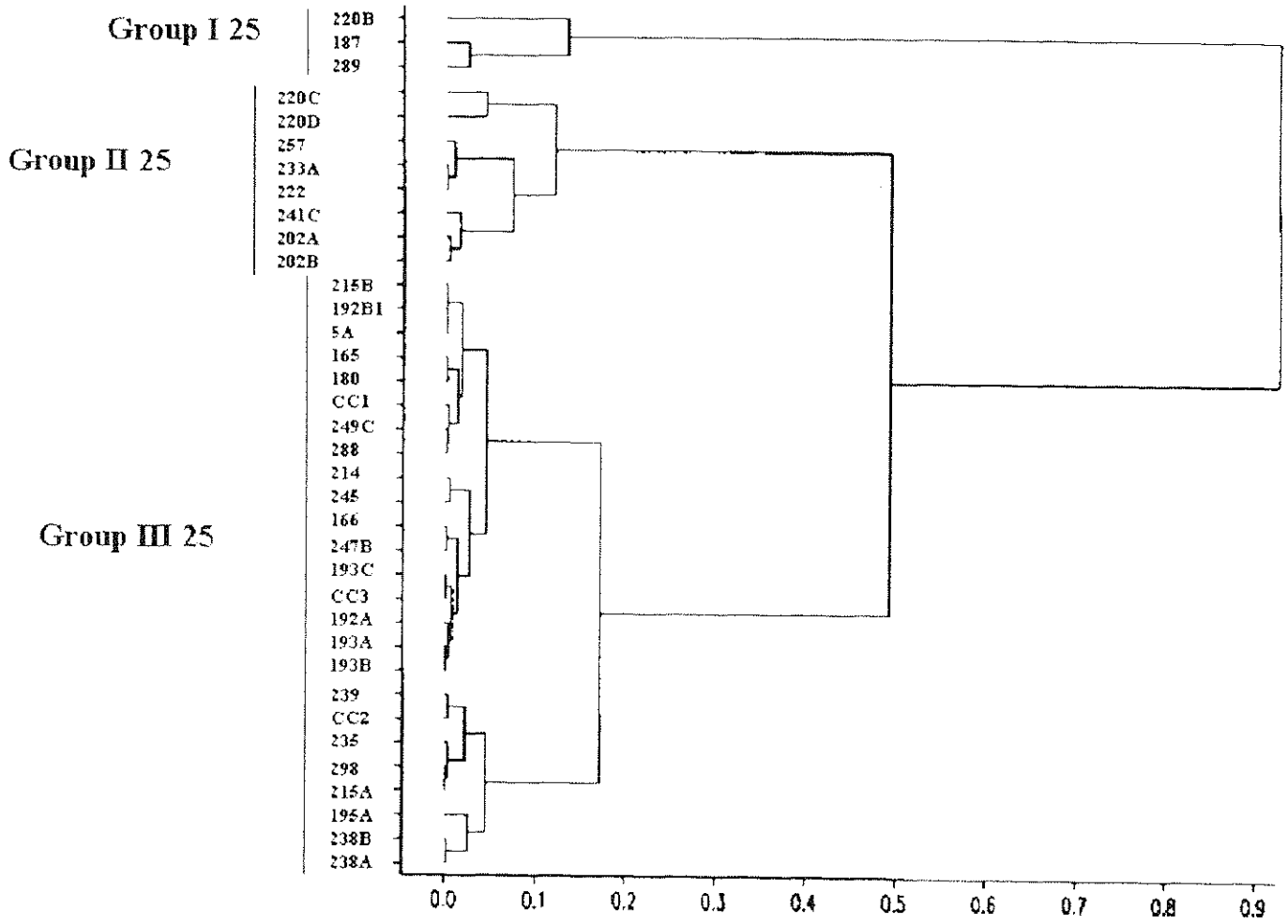


Figure 4. The distance between 36 *Cladobotryum* spp. isolates grown at 25°C, based on conidial characteristics; length, diameter, and average cell number.

2.1.4 Discussion

2.1.4.1 Growth rate.

The present study has confirmed that there are differences in the radial growth rate of different cobweb causing pathogens as suggested by Grogan and Gaze (1996) but it has established that this difference appears to be a function of temperature. Whilst all type B isolates consistently grew faster than type A isolates at 25°C, this was not the case at 18°C. The differences in growth rates between different groups of isolates observed in this study may explain previous contradictory reports relating to optimum temperature for the growth of *C. dendroides* (Wood, 1958; Lane *et al*, 1991). The homogeneity in the growth rates of type B isolates and the heterogeneity of type A isolates has also been demonstrated at both 18°C and 25°C for the first time, further strengthening the groupings made by Grogan and Gaze (1996). In a similar study on *Sclerotium* spp., Punja & Damiani (1996) differentiated between three unknown isolates on the basis of growth rates at various temperatures. It was concluded that these were three separate species. The growth rate differences observed between isolates during the present study were not as distinct as those recorded by Punja & Damiani (1996) for their *Sclerotium* isolates. However, further studies of *Cladobotryum* isolate groups, in conjunction with the growth rate data, may help to assign them to distinct taxonomic groups.

2.1.4.2 Conidial characteristics.

Conidial length, diameter, and level of septation encountered during this study were all in general concurrence with previous reports for *Cladobotryum* spp. (Gams & Hoozemans, 1970; De Hoog, 1978; Ellis & Ellis, 1988; Lane, 1993; Rogerson & Samuels, 1993). However, distinction between groups of isolates was made difficult, not only by the often-gradational nature of the results for any single characteristic but also because previous descriptions are so variable. For example, De Hoog (1978) considered *C. dendroides* conidia to be usually four celled, as did Ellis and Ellis (1988), whereas the findings of Gams & Hoozemans (1970), Rogerson & Samuels (1993) and Lane (1993) all agreed that cell number varied from 1 - 4.

Previously, conidial morphology has been used not only to differentiate between different species, but also to differentiate between isolates of the same species (Christensen & Latch, 1991; Senaratna *et al.*, 1991; Tedford *et al.*, 1994; and Lees *et al.*, 1995). Conidial length and diameter have been the most common characteristics used to differentiate between isolates during previous studies, although number of septa (which governs mean cell number) has also been used. For example, Lees *et al.* (1995) attempted to use the average number of septa as well as length and width to differentiate between isolates of *Microdochium nivale*.

Whereas Lees *et al.* (1995) found that conidial width was the most useful conidial characteristic for the differentiation of *Microdochium nivale* isolates, the study of *Cladobotryum* isolates described in this report found that mean cell number was the most useful characteristic to distinguish between isolates. Other conidial characteristics were observed to vary quite considerably, both between and within isolates, and also when grown at different temperatures. Mean conidial cell number was the only characteristic that did not significantly alter with temperature. This characteristic being the least temperature dependant, and therefore possibly the most reliable single characteristic to differentiate between the isolates, was chosen for further investigation.

Lane (1993) studied the percentage distribution of septation between different isolates and concluded there was no relationship between isolate host origin and level of septation, although

he looked at a relatively small number of isolates. The present study has shown however that mean cell number can be used to distinguish between isolates. Type B isolates, with the exception of isolate 241C (which was identified as *C. mycophilum* and had characteristics which were different to other type B isolates), all occurred in mean cell number group 2 (Figure 2). Type A isolates however, occurred in all three mean cell number groups (1, 2 & 3). This reinforces the homogeneity of type B isolates and the heterogeneity of type A isolates that was also observed with growth rate data. However, for the first time subgrouping of A type isolates was evident. Isolate 241C (more correctly classified as a type A isolate) and 202A, identified by IMI as *C. mycophilum*, occurred, along with five other isolates in mean cell number group 3 (figure 2), suggesting that all seven isolates may be *C. mycophilum*. Three type A isolates (195A, 187, and 289) remained separate from all other isolates and formed a distinct mean cell number group of their own (group 1). Conidia of group 1 isolates had a considerably higher mean cell number than any of the other isolates examined. The remaining three type A isolates (220B, 238A & 238B) fell into mean cell number group 2 along with all the type B isolates, two of which were identified as *C. dendroides* and thus it was hypothesised that this mean cell number group may all be *C. dendroides*.

When the groupings based on mean cell number were correlated with growth rate data and cluster analysis of conidial characteristics, no two analyses produced the same pattern of groupings, however the cluster groupings at 25°C were very similar to the mean cell number groupings (Figures 2 & 4)

2.1.5 Conclusions

The gradational distribution of growth and conidial characteristics from one extreme to another makes it difficult to group isolates on the basis of these characteristics. This diversity may well serve to explain why different descriptions of cobweb causing pathogens occur leading to confusion surrounding their identification. A few general conclusions may be drawn from the data presented in this section as follows:

- *Cladobotryum* isolates from Britain show significant variation in terms of their growth rate with type B isolates growing faster than type A isolates at 25°C but not at 18°C.
- Conidial mean cell number was the least variable of the characteristics measured and three distinct groups of isolates were identified as follows:
    - ❖ Group 1: mean cell number >3; (3 type A isolates)
    - ❖ Group 2: mean cell number 2-3; (3 type A and 23 type B isolates)
    - ❖ Group 3: mean cell number <2 (6 type A isolates + 241C)
- Three distinct groups of isolates also occurred based on cluster analysis of conidial data (length, diameter and cell number) but isolates clustered differently depending on whether they were grown at 25°C or 18°C. The groups formed from the 25°C data were very similar to those formed based on mean cell numbers alone.

2.2 Random Amplified Polymorphic DNA (RAPD) analysis.

2.2.1 Introduction.

Random Amplified Polymorphic DNA (RAPD) analysis or Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) is an increasingly used technique to determine intraspecific and interspecific molecular variation among isolates of diverse populations (Muthumeenakshi *et al.*, 1994; Kelly *et al.*, 1994; Nicholson & Rezanoor, 1994; Assigbetse *et al.*, 1994; Theodore *et al.*, 1995; Moller *et al.*, 1995; Voigt *et al.*, 1995; Sequerra *et al.*, 1997). The technique utilises a short primer, about 10 bases long, which arbitrarily binds to the genomic DNA thereby negating the need for specific primers which requires knowledge of the genomic sequence.

Although RAPD remains generally accepted as a quick, economical and highly reproducible method for the study of genetic diversity within a population, variation - created by mismatch during Polymerase Chain Reaction (PCR) cycles - has been identified as a problem with this technique (Ellesworth *et al.*, 1993).

Application of this technique will facilitate the measure of molecular relatedness of selected British *Cladobotryum* isolates. It will also enable comparisons to be made with *ex* type *Cladobotryum* spp. obtained from culture collections and other research workers from around the world (table 4).

2.2.2 Materials and methods.

2.2.2.1 Subculturing of isolates and mycelial filtration.

Isolates were grown in malt broth (Oxoid) and the flasks incubated at 25°C in an orbital shaker (200rpm). When sufficient growth had taken place the mycelium was filtered using a buchner and flash frozen in liquid nitrogen. The frozen mycelium was and then freeze-dried for at least 24 hours prior to storage at -20°C.

2.2.2.2 DNA extraction.

Freeze dried mycelium was ground using a sterile mortar and pestle. When fully powdered 400mg was placed in a sterile 10ml MSE tube and 3ml biologically sterile extraction buffer (Raeder & Broda, 1985) added. Using a sterile Pasteur pipette, the buffer was thoroughly mixed with the mycelial powder into a homogenous paste and incubated for 30mins in a 37°C water bath. Phenol (pH 8.0, Sigma) (2.1ml) and chloroform (0.9ml) were then added to the paste and mixed well by gentle inversions.

Centrifugation of the mixture (MSE Hi spin - 10 ml rotor) at 11,000g (20°C) was performed for 60min. The aqueous phase, containing the DNA was transferred to a new tube. 150µl of RNase (20mg/ml stock) was added and incubated at 37°C for 15min. Phenol - chloroform extraction was repeated twice, followed by a final chloroform extraction to remove any trace of phenol.

Upon transference to a 50ml 'Sterilin' tube, isopropanol (0.54 volumes) was added to the aqueous phase and mixed well by tapping. The mixture was centrifuged at 6000g in a bench top centrifuge for five minutes to collect a pellet of DNA. The supernatant was removed and 3ml ice cold 70% ethanol added. The pellet was dislodged to assist the washing. The mixture was centrifuged for a second time at 6000g for five minutes and the ethanol drained off. The wash was repeated. The pellet was freeze dried for 2 - 3 minutes to remove traces of ethanol.

The DNA pellet was dissolved in a suitable volume of TE8 (500 - 700µl) and incubated at 37°C. When fully dissolved the mixture was centrifuged briefly to collect all the solution together and was transferred to an eppendorf (1.5ml) for storage at -20°C.

2.2.2.3 Quantification and quality analysis of extracted DNA

Two methods of quantification were employed; visual and spectrophotometric.

Visual: An aliquot (5µl) of extracted DNA sample was electrophoresed on 0.8% agarose gel. A visual estimation was then made of the quantity and quality of the sample by comparing ethidium bromide fluorescence under a U.V. light transilluminator with a series of standards (λDNA Hind III). This method also facilitated the detection of sheered DNA. A compact clear single band indicated DNA of high quality whereas a smeared band indicated sheering of the DNA had taken place.

Spectrophotometric: Spectrophotometry allowed the quantification of DNA or RNA as well as an analysis of quality. Reading absorption of ultraviolet irradiation at 260nm allowed DNA quantification whilst the ratio of absorption at 260nm and 280nm allowed the purity of the sample to be determined.

On the basis of both analyses a dilution (with sterile water) of an aliquot of the genomic DNA was performed. This ensured the concentration of DNA within all the PCR stock solutions would be equal at 4ng/µl.

2.2.2.4 Standardisation of Polymerase Chain Reaction (PCR) conditions

PCR conditions were standardised using primer A11. The optimised conditions for thermal cycling and quantities of components used in each reaction are given in tables 4 and 5 respectively.

Table 4. Standardised thermal cycling conditions.

Temperature (°C)	**Time (minutes)**	**Number of Cycles**
95	2	1
94	1	35
40	0.5	
72	1	
72	7	1

Table 5. Standardised component quantities for a 50µl PCR.

Component	Quantity (µl)
Genomic DNA	5
Sterile water	36.125
Buffer	5
dNTP	1
Primer	2.5
Dynazyme	0.375

2.2.2.5 Choice of primers for analysis.

Two random Operon primer kits (A & B) were purchased. These primers (Table 6) were screened under the standardised conditions of PCR in order to assess the RAPD patterns produced. Five isolates (187, 193A, 166, 235, and 238A) were chosen for the screening procedure. These were selected to give a representative range of growth rate, fungicide resistance profile, geographical location, and spore morphology. Primers that resulted in amplification of a moderate number of fragments, and also those which amplified only one or two fragments, were chosen for further study (Table 6).

Table 6. Nucleotide sequences of the 30 primers screened during this study.

Primer	Sequence 5' to 3'	Primer	Sequence 5' to 3'
A-01	CAGGCCCTTC	B-01	GTTTCGCTCC
A-02	TGCCGAGCTG	B-02	TGATCCCTGG
A-03	AGTCAGCCAC	B-03	CATCCCCCTG
A-04*	AATCGGGCTG	B-04	GGACTGGAGT
A-05	AGGGGTCTTG	B-05	TGCGCCCTTC
A-06	GGTCCCTGAC	B-06	TGCTCTGCCC
A-07	GAAACGGGTG	B-07	GGTGACGCAG
A-08	GTGACGTAGG	B-08*	GTCCACACGG
A-09*	GGGTAACGCC	B-09	TGGGGGACTC
A-10	GTGATCGCAG	B-10*	CTGCTGGGAC
A-11*	CAATCGCCGT	B-11	GTAGACCCGT
A-12	TCGGCGATAG	B-12	CCTTGACGCA
A-13*	CAGCACCCAC	B-13*	TTCCCCCGCT
A-14	TCTGTGCTGG	B-14*	TCCGCTCTGG
A-15*	TTCCGAACCC	B-15*	GGAGGGTGTT

*Primers selected for further study.

2.2.2.6 Screening of Cladobotryum spp. isolates

Seventy-six *Cladobotryum* spp. isolates (Table 7) including several from outside the U.K. and four species other than *Cladobotryum dendroides* were screened against each of the ten primers selected for the study. In addition to these 76 isolates of *Cladobotryum* spp. two outgroups (i.e. different genera) were included in each test in order that a definitive level of similarity could be established between *Cladobotryum* and different genera. The two genera selected were *Trichoderma* and *Coniothyrium*. Two control reactions devoid of template DNA were also included within each test.

Two 25µl polymerase chain reactions were performed for every primer isolate combination the component quantities for each being half that described in Table 5. The thermal conditions of the PCR were as outlined in Table 4.

PCR products after the addition of 10µl of gel loading dye were electrophoresed at 100 mV for 150 minutes in 1.4% agarose gels containing 4µl ethidium bromide (10mg/ml) / 100ml gel. Two DNA molecular weight markers (Pharmacia Biotech 100 Base – Pair Ladder and Boehringer Mannheim VI) were included alongside each row of PCR products to enable accurate sizing of the amplified bands.

Following electrophoresis each gel was placed on a U.V. light transilluminator in order to fluoresce the ethidium bromide stained DNA molecules. At this time the banding pattern of each isolate was photographically recorded using Polaroid, black and white, positive-negative, print film – type 665.

By the comparison of distances travelled by the PCR product and the marker bands of known molecular weight it was possible to estimate the fragment / bands molecular weight using a computer package – Gel.exe. Fragment sizes were rounded up to the nearest 25bp. PCR products not amplified and therefore not visible as bands when fluoresced in both replicate reactions, were not analysed, nor were product bands in excess of 2000bp.

Each fungal isolate was scored for the presence or absence of every unique fragment produced by any one particular primer and a binomial matrix developed. Hierarchical cluster analysis was then performed using the Genstat 5 computer package (Payne, R.W. *et al*, 1993). Similarity matrices used in the hierarchical cluster analysis were constructed using the simple matching and Jaccard's coefficient methods and composed of all ten primers with all 76 fungal isolates. Dendrograms were then constructed using the group average algorithm (UPGMA) and drawn using the 'dendrogram' option.

Table 7. *Cladobotryum* spp. isolates screened against all ten primers (✓).

	Isolate number	Name	Country of origin
✓	5A	*C. dendroides*	GB
✓	164	*C. dendroides*	GB
✓	165	*C. dendroides*	GB
✓	166	*C. dendroides*	GB
✓	167	*C. dendroides*	GB
✓	169	*C. dendroides*	Ireland (Rep.)
✓	174	*C. dendroides*	GB
✓	187	*C. dendroides*	GB
✓	192A	*C. dendroides*	GB
✓	192B1	*C. dendroides*	GB
✓	192C	*C. dendroides*	GB
✓	193A	*C. dendroides*	GB
✓	193B	*C. dendroides*	GB
✓	193C	*C. dendroides*	GB
✓	195A	*C. dendroides*	GB
✓	196A	*C. dendroides*	GB
✓	202A	*C. mycophilum*	GB
✓	202B	*C. mycophilum*	GB
✓	209	*C. dendroides*	GB
✓	213	*C. dendroides*	GB
✓	214	*C. dendroides*	GB
✓	215A	*C. dendroides*	GB
✓	215B	*C. dendroides*	GB
✓	217A	*C. dendroides*	GB
✓	217B	*C. dendroides*	GB
✓	217C	*C. dendroides*	GB
✓	220B	*C. dendroides*	France
✓	220C	*C. dendroides*	France
x	220D	*C. dendroides*	France
✓	222	*C. dendroides*	GB
✓	229B	*C. dendroides*	GB
✓	231	*C. dendroides*	GB
✓	232B	*C. dendroides*	GB
✓	233A	*C. dendroides*	GB
✓	235	*C. dendroides*	GB
✓	238A	*C. dendroides*	GB
✓	238B	*C. dendroides*	GB
✓	239	*C. dendroides*	GB
✓	240	*C. dendroides*	GB
✓	241C	*C. mycophilum*	GB

	Isolate number	Name	Country of origin
✓	243B	*C. dendroides*	GB
✓	245	*C. dendroides*	GB
✓	247B	*C. dendroides*	GB
✓	249C	*C. dendroides*	GB
✓	257	*C. dendroides*	GB
✓	260A	*C. dendroides*	GB
✓	273	*C. dendroides*	GB
✓	281	*C. dendroides*	GB
✓	288	*C. dendroides*	GB
✓	289	*C. dendroides*	GB
✓	297	*C. dendroides*	GB
✓	298	*C. dendroides*	GB
✓	358	*C. dendroides*	GB
✓	CC1	*C. dendroides*	UK
✓	CC2	*C. dendroides*	UK
✓	CC3	*C. dendroides*	Canada
✓	CC4	*C. dendroides*	UK
✓	CC5	*C. varium*	India
✓	CC6	*C. dendroides*	Canada
✓	CC7	*C. dendroides*	Netherlands
✓	CC8	*C. dendroides*	Netherlands
✓	CC9	*C. dendroides*	USA
✓	CC10	*C. dendroides*	Italy
✓	CC12	*C. varium*	France
✓	CC13	*C. varium*	Canada
✓	CC14	*C. varium*	Japan
✓	CC15	*H. dactylaroides*	NZ
✓	CC16	*C. mycophilum*	Netherlands
✓	CC17	*C. mycophilum*	Germany (East)
✓	CC18	*C. multiseptatum*	NZ
x	CC19	*C. dendroides*	GB
✓	CC20	*C. dendroides*	USA
✓	CC21	*C. dendroides*	USA
✓	CC22	*Cladobotryum* spp.	USA
✓	CC23	*Cladobotryum* spp.	USA
✓	CC24	*C. dendroides*	Northern Ireland
✓	CC25	*C. dendroides*	Ireland (Rep.)
✓	CC26	*C. dendroides*	Ireland (Rep.)
✓	CC27	*C. dendroides*	Northern Ireland
x	CC28	*C. dendroides*	Ireland (Rep.)
x	CC29	*C. dendroides*	Ireland (Rep.)
x	CC30	*C. dendroides*	Australia

2.2.3 Results

Variation in similarity (%) among the isolates tested was greater when analysis was performed using the Jaccard method than when using the simple matching method, as expected. Similarity varied from 100% to around 10% when using Jaccard's coefficient method (Figure 5) or from 100% to around 90% when using the simple matching method (Figure 6). Both methods gave very similar groupings of isolates which indicates that the banding patterns were stable. Unless otherwise stated all similarity measurement readings given below will relate to the Jaccard's coefficient method of analysis and not the simple matching.

Fifty-one out of the 76 isolates showed a high degree of similarity and were classified as Group 1 (Figure 5). This group consists of isolates with greater than 50% similarity. Isolates in this group included slow-growing and fast-growing thiabendazole-sensitive isolates as well as fast-growing thiabendazole-resistant isolates. This group was also apparent, without omission or addition, when the simple matching method of analysis was employed (Figure 6). No definitive morphological characteristic was immediately apparent for this group of genetically similar isolates. It included one French isolate (220C), one British isolate collected in 1982 (cc1), several Irish isolates (cc24, 25, 26, & 27 and isolate 169) and the majority of British isolates with a mean cell number of 2-3 and <2. Isolates with a common place and time of isolation, such as 202a & 202b, 215a & 215b, frequently showed a high level of similarity.

Three American isolates (cc20, cc21, and cc22) formed a closely related group with around 85% similarity, and two European *C. mycophilum* isolates (cc16 & cc17) also formed a close group with around 70% similarity. Neither fell within group 1, but they were more closely related to Group 1 than to any other isolates.

The two isolates identified as *C. dendroides* by Gams and Hoozemans (1970), and known to be capable of mating (cc7 and cc8), showed a similarity of approximately 55%. This was considerably less than many isolates within group 1. There was a loose grouping of isolates around this mating pair but levels of similarity were low, approaching that with the outlier species *Coniotherium*, so that nothing significant can be concluded from the data. Similarly, four *C. varium* isolates from France (cc12), India (cc5), Canada (cc13) and Japan (cc14) showed very little similarity.

2.2.4 Discussion

Genetic relatedness among 76 *Cladobotryum* isolates, as determined by RAPD analysis, indicated a high degree of variability between *Cladobotryum* isolates with only one clear group of genetically-similar isolates emerging. This high variability suggests that RAPD analysis may be too sensitive a test to discriminate between different species within the genus *Cladobotryum*. The one group of genetically related isolates contained two *C. mycophilum* isolates identified by IMI which agreed with published descriptions of this species (De Hoog, 1978, Rogerson and Samuels, 1994). Species characteristics include the presence of a camphor like odour (the perfect state of *C. mycophilum* is *H. odoratus*) and predominantly 1-2 celled conidia. However, the majority of the isolates in the RAPD group did not have a camphor-like odour and conidia mean cell number was often between 2 and 3, rather than <2. Thus the RAPD grouping implies that this species may be more variable than existing descriptions indicate. This view is backed by recent research on genetic relatedness using ITS sequences. McKay et al. (1999) illustrated that the majority of *Cladobotryum* isolates from Britain and Ireland examined by them were genetically more similar to *C. mycophilum* than *C. dendroides*, but they also indicated that *C.*

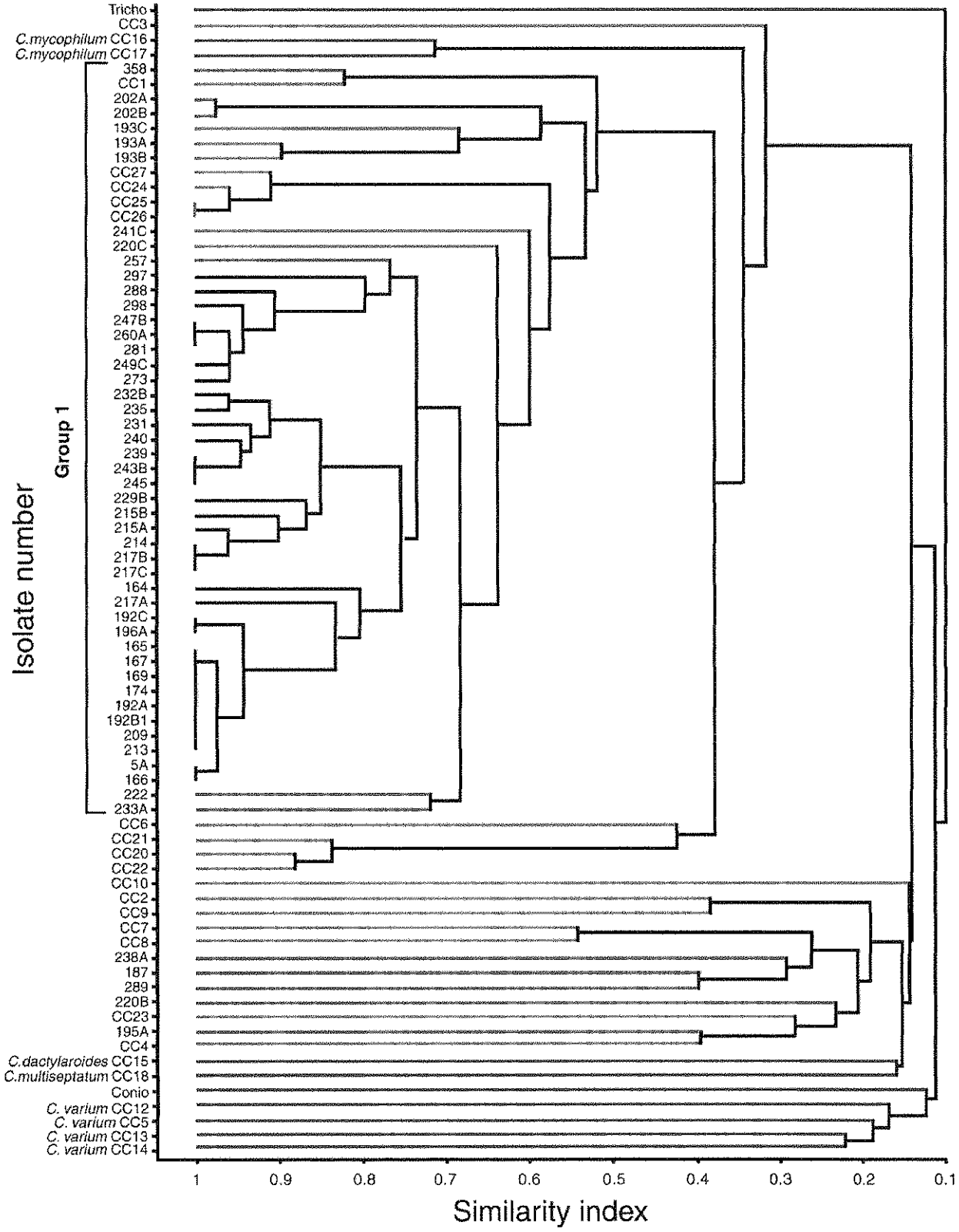


Figure 5. Genetic relatedness of 76 *Cladobotryum* spp. and two outgroups when tested with RAPD using ten primers; dendrogram constructed using Jaccard's coefficient and the group average algorithm.

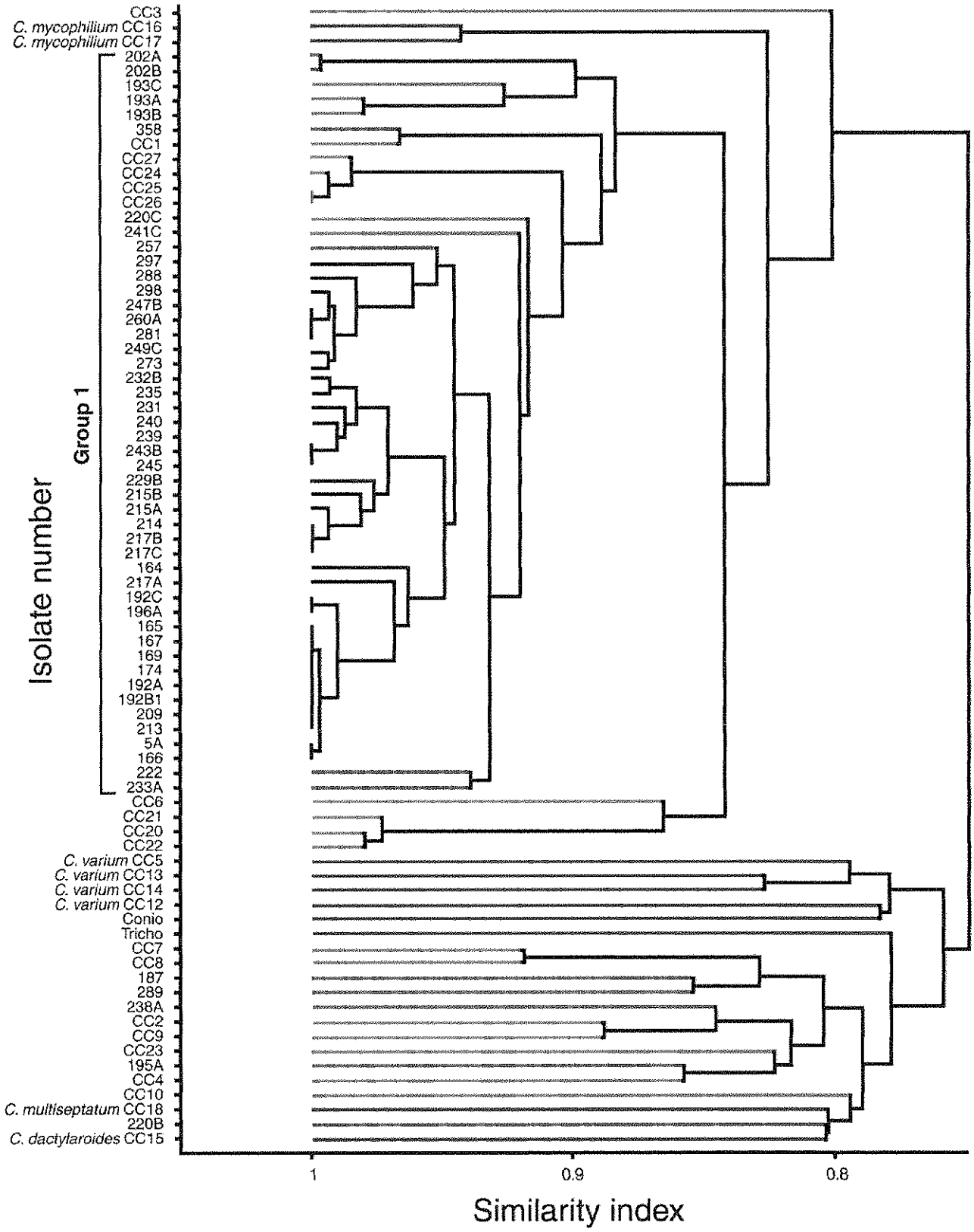


Figure 6. Genetic relatedness of 76 *Cladobotryum* spp. and two outgroups (*Coniothirium minitans* & *Trichoderma harzianum*) when tested with Random Amplified Polymorphic DNA analysis using ten primers; dendrogram constructed using simple matching method and the group average algorithm.

mycophilum itself contained three subgroups. The RAPD group described in this chapter would support this finding.

2.2.5 Conclusions

- A large number of *Cladobotryum* isolates collected from Britain and Ireland, with a variety of growth and conidial characteristics as well as different sensitivities to fungicides, appear to be genetically more similar to *C. mycophilum* than *C. dendroides*.
- Current descriptions of *C. mycophilum* do not encompass isolates that have been shown to be genetically similar to this species.
- RAPD analysis on its own did not closely group isolates identified as *C. dendroides* or *C. varium* indicating that a different measure of genetic relatedness may be required to identify groups of similar isolates.

2.3 Mating tests

2.3.1 Introduction

Differentiating between fungal species can be achieved using several different criteria that can be categorised as follows:

1) morpho, based on morphological characteristics,
2) phylogenetic, based on biochemical or molecular variations,
3) ecological, based on different ecological niches,
4) polythetic, based on several characters,
5) biological, based on exclusive interbreeding groups.

Traditionally morphospecies have been most common, however, biological and phylogenetic means of differentiation have recently become more popular (Hawksworth *et al*, 1995). The individuals belonging to a biological species are, if compatible, able to sexually reproduce and thereby increase genetic variation within the progeny. It is this ability to exchange genetic information, giving rise to viable progeny with different characteristics from the parents (but formed from a combination of the parent's genetic information), which delimits biological species. Blakeslee (1904) first published a report describing the occurrence of (+) and (-) mating strains although sexual reproduction within fungi had been appreciated prior to this. Edgerton (1912) was first to demonstrate the occurrence of (+) and (-) strains within the *Ascomycetes* whilst working with a species of *Glomerella*.

Fungal mating mechanisms can be divided into two main types; Homothallic where isolates are self-fertile and heterothallic where isolates are self-sterile. Homothallic strains are able to sexually reproduce without the involvement of a complementary strain whereas heterothallic strains require the presence of an opposing mating type, either + or - (Cisar & TeBeest, 1999).

C. dendroides and *C. mycophilum* are both considered heterothallic, and thus, require the presence of two opposing mating type strains, belonging to the same biological species, before sexual recombination can occur (Rogerson & Samuels, 1993; 1994). The sexual fruit body (perithecia) and spore (ascospore) morphology of both species have been described as *Hypomyces rosellus (C. dendroides)* and *H. odoratus (C. mycophilum*), Rogerson and Samuels (1994) considered them to be indistinguishable from one another. The two species can only be distinguished from each other by their asexual *Cladobotryum* forms, which will mate with complimentary strains of the same species but not with each other.

The objective of this study is to examine the mating interactions of various *Cladobotryum* isolates collected from the British mushroom industry and abroad, which appear to include several distinct species based on morphological characteristics. Sexual compatibility should facilitate the determination of biological species groupings as well as mating types. A further impression of the degree of genetic relatedness between these isolates may thus be formed to complement earlier findings from morphological and phylogenetic studies (sections 2.1 & 2.2).

2.3.2 Materials and methods.

Fifty-one isolates from different sources around the world were used in this study, which included representative isolates from several species including *C. dendroides, C. mycophilum,*

C. varium as well as two unidentified *Cladobotryum* sp. from USA and single isolates of *H. dactylaroides, C. multiseptatum* and *C. asterophorum* (Table 8).

Table 8. Identification number, putative species name, and place of origin of all isolates included within the mating compatibility study. IMI = International Mycological Institute, Egham, Surrey, UK; CBS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Identification number	Name	Identification authority	Place of origin
187	*C. dendroides*		GB
195A	*C. dendroides*		GB
202A	*C. myconhilum*	Confirmed by IMI	GB
202B	*C. myconhilum*		GB
220B	*C. dendroides*		France
220D	*C. dendroides*		France
222	*C. dendroides*		GB
233A	*C. dendroides*		GB
238A	*C. dendroides*		GB
257	*C. dendroides*		GB
358	*C. dendroides*		GB
289	*C. dendroides*		GB
193A	*C. dendroides*		GB
193B	*C. dendroides*		GB
193C	*C. dendroides*		GB
220C	*C. dendroides*		France
241C	*C. myconhilum*	Confirmed by IMI	GB
CC1	*C. dendroides*	IMI	UK
CC2	*C. dendroides*	IMI	UK
CC3	*C. dendroides*	IMI	Canada
CC4	*C. dendroides*	IMI	UK
CC5	*C. varium*	IMI	India
CC6	*C. dendroides*	CBS	Canada
CC7	*C. dendroides*	Mating type "a"	Netherlands
CC8	*C. dendroides*	Mating type "b"	Netherlands
CC9	*C. dendroides*	CBS	USA
CC10	*C. dendroides*	CBS	Italy
CC12	*C. varium*	CBS	France
CC13	*C. varium*	CBS	Canada
CC14	*C. varium*	CBS	Japan
CC15	*H. dactylaroides*	CBS	New Zealand
CC16	*C. myconhilum*	CBS	Netherlands
CC17	*C. myconhilum*	CBS	Germany
CC18	*C. multiseptatum*	CBS	New Zealand
CC19	*C. asterophorum*	CBS	Japan
CC20	*C. dendroides*		USA
CC21	*C. dendroides*		USA
CC22	*Cladobotryum* spp.		USA
CC23	*Cladobotryum* spp.		USA
164	*C. dendroides*		GB
192B1	*C. dendroides*	Confirmed by IMI	GB
217c	*C. dendroides*		GB
245	*C. dendroides*		GB
247b			GB
CC24	*C. dendroides*		Northern Ireland
CC25	*C. dendroides*		Ireland
CC26	*C. dendroides*		Ireland
CC27	*C. dendroides*		Northern Ireland
CC28	*C. dendroides*		Ireland
CC29	*C. dendroides*		Ireland
CC30	*C. dendroides*		Australia

Fresh cultures of each isolate were prepared and grown at 25°C on MEA plates to provide a mother culture from which fifty 5-mm plugs were taken. Mating tests were conducted following the method described by Rogerson and Samuels (1993) for *Hypomyces* spp. Isolates were crossed with one another on oatmeal agar plates (formulated according to Booth, 1971). Plugs for each pair of isolates to be tested were placed on opposing sides of the oatmeal plate, 10mm from the outer edge. Plugs were positioned mycelium-side up and with hyphal tips pointing towards the centre of the plate. The plates were marked clearly to show the position of each isolate prior to being incubated at 20°C for a minimum of eight weeks.

The interaction of each isolate pairing was assessed visually. A dissection microscope (Zeiss – Stemi 2000-C) was used to search the zone of contact for perithecia – sexual fruit bodies produced as a result of sexual compatibility between two isolates (Plate 2). When perithecia were found, the production of ascospores was confirmed by examining either the droplet formed at the tip of the perithecia or a squashed perithecium mounted on a glass slide and examined at x400 magnification, (Zeiss – Axiolab) (Plate 3).

2.3.3 Results

Positive mating reactions, involving the production of perithecia and ascospores, were observed between several, but not all, isolates. The positive mating reactions highlighted two discrete populations that would mate among themselves but not with individuals from the other population (Figure 7). Population I consisted of mating types a and b of *C. dendroides* and included isolates described as *C. asterophorum, C. multiseptatum, H. dactylaroides,* and a small number of isolates sampled during the UK epidemic. Population II consisted of (+) and (-) mating types and only contained two isolates which reliably mated with one another (Table 9). Although mating reactions were occasionally observed between several pairs of isolates, these interactions were considered unreliable when a positive mating reaction during one replicate was not confirmed in either of the other two replicates. The remaining 34 isolates either did not mate or did not mate more than once with another isolate and could not therefore be reliably allocated to a mating group.

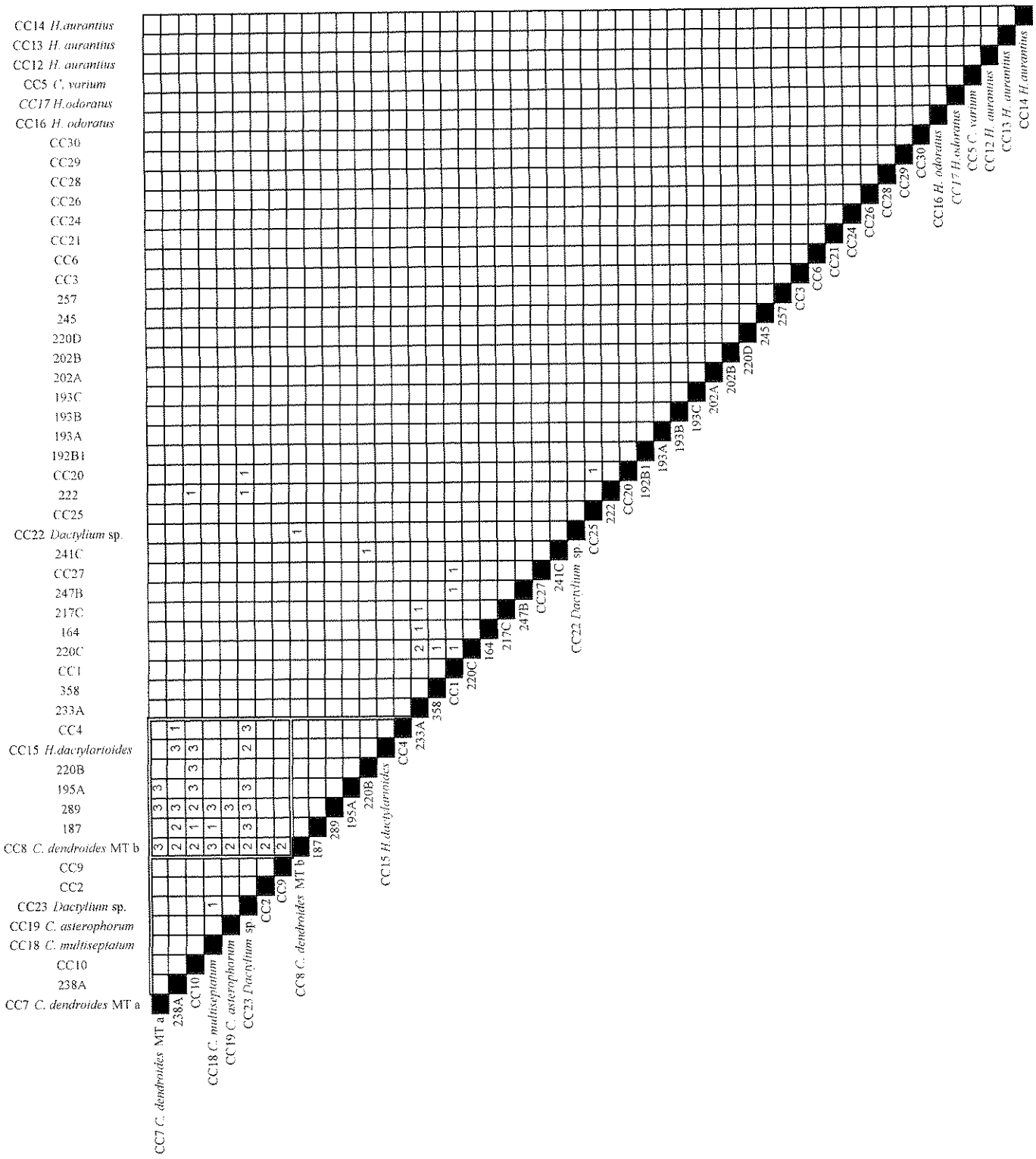


Figure 7. Mating reactions of 51 *Cladobotryum* spp. when crossed with one another on oatmeal agar plates at 20oC. Each digit reflects the number of replicate plates (maximum of 3) where a possitive mating reaction was observed to occur .

Table 9. Mating types of the isolates that comprised populations I and II. Other details also given.

Population	Mating type	Name	I.D. Number	Place of origin *	Substrate#
I	a	*C. dendroides*	238A	UK	*A.b.*
I	a	*C. dendroides*	CC10	Italy	o.f.
I	a	*C. multiseptatum*	CC18	NZ	*A.b.*
I	a	*C. asterophorum*	CC19	Japan	o.f.
I	a	*Dactylium* spp.	CC23	USA	*A.b.*
I	a **	*C. dendroides*	CC7	Neth.	o.f.
I	a	*C. dendroides*	CC2	UK	o.f.
I	a	*C. dendroides*	CC9	USA	Dead wood
I	b	*C. dendroides*	195A	UK	*A.b.*
I	b	*C. dendroides*	220B	France	*A.b.*
I	b	*H. dactylaroides*	CC15	NZ	o.f.
I	b	*C. dendroides*	CC4	UK	Dead *Pinus*
I	b **	*C. dendroides*	CC8	Neth.	o.f.
I	b	*C. dendroides*	187	UK	*A.b.*
I	b	*C. dendroides*	289	UK	*A.b.*
II	(-)	*C. dendroides*	233A	UK	*A.b.*
II	(+)	*C. dendroides*	220C	France	*A.b.*

* UK = United Kingdom; NZ = New Zealand; USA = United States of America; Neth. = The Netherlands;

** Isolates CC7 and CC8 were obtained as a mating pair of *C. dendroides* and were used to categorise isolates as either mating type a or b

\# *A.b.* = *Agaricus bisporus*; o.f. = other fungal species (see Appendix 1); *Ag.* sp. = *Agaricus* species.

2.3.4 Discussion

One clear biological group (Population I) has been identified from this work composed, not only of isolates from *Agaricus bisporus* from Britain, New Zealand, Japan and America, but also including isolates from fruit bodies of other fungal species and dead wood. These isolates can be categorised as *C. dendroides* mating type a or mating type b. Additional work not reported here indicates that these isolates are relatively slow growing and are sensitive to benzimidazole fungicides; they also include a few isolates obtained during the British epidemic (isolates 238A, 195A, 187, 289). Additionally, this mating group has been shown to be molecularly distinct from all other isolates tested including both *C. varium* and fast-growing fungicide-resistant isolates (section 2.2).

Unfortunately, the most problematic isolates within the industry did not mate reliably with any other isolates. It is therefore difficult to gain an impression using these results alone of how these fast-growing, fungicide-resistant isolates are related to either one another, or isolates collected from culture collections. However, the fact that they did not reliably mate

with population I suggests that at least two biological species are capable of causing cobweb disease symptoms within the mushroom industry.

In addition to suggesting more than one biological species may cause cobweb disease, these results highlight the confusion surrounding the identification of species within this genus. Four morphologically defined species have been shown to sexually reproduce with one another. The majority of isolates within population I have been identified by various workers as *C. dendroides*. However, three isolates, *C. asterophorum, C. multiseptatum,* and *H. dactylaroides*, were regarded by de Hoog (1978) to be sufficiently morphologically distinct from *C. dendroides* to be considered different species. Thus, whilst one method of speciation (morpho) has divided this population another (biological) has united them.

With such confusion surrounding the identification of individuals within this genus the mating compatibility tests described above have been of great value in the elucidation of cobweb disease pathogen diversity. Further work, concentrating on trying to produce mating reactions under different conditions between the fungicide resistant isolates may help to clarify their position within this somewhat confused genus.

2.3.5 Conclusions

- *C. dendroides* isolates can be identified by their mating reactions with a known mating pair. These isolates are relatively slow growing and are sensitive to benzimidazole fungicides. This type of isolate was not common during the 1995 cobweb epidemic in Britain.
- The majority of isolates collected during the 1995 cobweb epidemic did not mate reliably with each other, nor with *C. dendroides* or any other species. This suggests that these isolates are **not** *C. dendroides* and their original identification as *C. dendroides* is likely to be incorrect.
- Mating reactions were not recorded for a group of C. varium isolates or a group of C. *mycophilum* isolates. This indicates either that the isolates are all of the one mating type or the conditions used in the experiment were not suitable for mating reactions to occur for these species.

2.4 Pathogenicity.

2.4.1 Introduction.

Variability among *Cladobotryum* isolates, obtained from different sources, has been established in the previous sections for factors such as growth rate, conidial characteristics, RAPD profile, and mating compatibility. It was decided to also test the pathogenicity of isolates with respect to *Agaricus bisporus*, especially for *Cladobotryum* isolates that were obtained from non-*Agaricus* substrates, as different pathotypes can exist within a population. This information may help to clarify differences between isolates.

The objective of this section is to establish whether or not selected *Cladobotryum* isolates are capable of causing cobweb disease of *Agaricus bisporus*. This will be determined by a small scale cropping experiment in which containers of mushroom compost are cased, then subsequently inoculated with a mycelial plug of one of 37 different *Cladobotryum* isolates.

2.4.2 Materials and methods

2.4.2.1 Selection and preparation of test isolates

Thirty-seven isolates were selected for study (Table 10). These included all type A, type B2 isolates collected during the cobweb epidemic, four selected isolates of the molecularly similar type B1 isolates and the majority of the culture collection isolates. Isolates were selected to give as much geographical and molecular variety as possible.

Each isolate was sub-cultured from MEA slopes onto MEA plates and grown at 25°C to allow regeneration and assessment of viability. Each isolate was then transferred onto a mushroom agar (MA) plate and grown at 25°C until colonies were well grown but not completely filling the plate. These cultures on nutrient-poor plates provided the 5mm plugs for inoculation of mushroom casing.

Cultures were set up according to their radial growth rate in such a way that all cultures were ready on the day of inoculation.

2.4.2.2 Mushroom cultivation

Spawn-run compost was prepared according to current commercial practice at the Experimental Mushroom Unit, HRI, Wellesbourne, Warwickshire using *A. bisporus* strain A12 (Sylvan). Four replicate inoculation experiments were carried out each using the following protocol. A 480g quantity of compost was weighed into each of 40 pots, measuring 105mm square by 145mm deep, and gently compressed. Casing made from a deep-dug black-peat and sugar beet lime (TunnelTech English) was used to case each pot to the recommended depth of 45mm (Noble, 1995). Commercial casing inoculum was included in the casing at a rate of 4kg / m^3 of peat to aid casing run.

All forty pots were transferred to a growth cabinet (Fisons, patent no. 812417) where the cropping cycle was completed. Temperature and relative humidity (RH) were maintained at 25 °C, and 96%, respectively, for the first 10-12 days to allow rapid and complete *A. bisporus* colonisation of the casing layer. At the end of this period *A. bisporus* mycelium was evident on the upper surface of the casing layer. The temperature and RH were reduced to 18°C and 89% over three days (airing), which stimulates the development of sporophore initials

(pinning). The lower temperature and RH were then maintained throughout the rest of the cropping period. Water was applied as required using a hand held plant sprayer. This allowed the gentle watering of the pots by way of a fine mist and thereby reduced water splash contamination between inoculated pots. Water requirements were determined at least once every day by the visual assessment of the casing layer of each pot. Where volumes in excess of 10ml/pot were required, water was applied in stages to reduce water logging of the casing layer.

Table 10. Details of isolates used in pathogenicity tests.

Isolate Number	Origin	Substrate	Source of culture	Year of isolation	Growth on agar mm/day	Fungicide resistance status
187	GB	*A. bisporus*	HRI	1995	15	Sensitive
195A	GB	*A. bisporus*	HRI	1995	15	Sensitive
202A	GB	*A. bisporus*	HRI	1995	10	Sensitive
220B	France	*A. bisporus*	INRA	1995	10	Sensitive
220D	France	*A. bisporus*	INRA	1995	15	Sensitive
222	GB	*A. bisporus*	HRI	1995	10	Sensitive
233A	GB	*A. bisporus*	HRI	1995	15	Sensitive
238A	GB	*A. bisporus*	HRI	1995	15	Sensitive
257	GB	*A. bisporus*	HRI	1995	10	Sensitive
358	GB	*A. bisporus*	HRI	1995	15	Sensitive
289	GB	*A. bisporus*	HRI	1995	15	Sensitive
165	GB	*A. bisporus*	HRI	1995	20	Resistant
215A	GB	*A. bisporus*	HRI	1995	20	Resistant
192B1	GB	*A. bisporus*	HRI	1995	20	Resistant
CC25	Ireland	*A. bisporus*	UCD	1990's	20	Resistant
193A	GB	*A. bisporus*	HRI	1995	20	Sensitive
220C	France	*A. bisporus*	HRI	1995	20	Sensitive
241C	GB	*A. bisporus*	HRI	1995	20	Sensitive
CC1	U.K.	*A. bisporus*	IMI	1982	22.5	Sensitive
CC2	?	?	IMI	1970	5	Sensitive
CC3	Canada	Soil	IMI	1963	11	Sensitive
CC4	U.K.	*Pinus* sp.	IMI	1955	3	Sensitive
CC5 *C. varium*	India	*C. versicolor*	IMI	1980	5	(not tested)
CC6	Canada	*A. bisporus*	CBS	1946	9	Sensitive
CC7	Netherlands	*A. mellea*	CBS	1969	3	Sensitive
CC8	Netherlands	*A. mellea*	CBS	1969	10	Sensitive
CC9	U.S.A.	Dead wood	CBS	1934	6	Sensitive
CC10	Italy	*S. hirsutum*	CBS	1981	15	Sensitive
CC12 *H. aurantius*	France	*Agaricus* sp.	CBS	1977	5	(not tested)
CC13 *H. aurantius*	Canada	Soil	CBS	1965	5	(not tested)
CC14 *H. aurantius*	Japan	*T. versicolor*	CBS	1977	5	(not tested)
CC15 *H. dactylarioides*	N.Z.	Polyporus sp.	CBS	1978	5	(not tested)
CC16 *H. odoratus*	Netherlands	*A. mellea*	CBS	1969	15	Sensitive
CC17 H. odoratus	Germany	*L. pyriforme*	CBS	1980	15	(not tested)
CC18 *C. multiseptatum*	N.Z.	*A. bisporus*	CBS	1971	5	(not tested)
CC22 *Dactylium* sp.	U.S.A	*A. bisporus*	PSU	1970	22	Sensitive
CC23 *Dactylium* sp.	U.S.A	*A. bisporus*	PSU	1982	10	Sensitive

2.4.2.3 Inoculation technique.

Inoculation of the casing was carried out once pinning had occurred and this procedure was done in a Class II laminar flow cabinet to prevent cross contamination between pots inoculated with different isolates. Using a sterile spatula, a small area of casing was lifted and placed to one side to leave a hole about 15mm deep. Three 5mm MA plugs taken from the growing edge of the test isolate were then placed in the hole, mycelium-side up, in a triangular pattern. The removed casing was replaced, covering the plugs, and gentle pressure applied. Inoculated pots were then returned to the cabinet and placed position according to the statistical design described below.

Disease growth was monitored at daily for the duration of the cropping period. Each pot was carefully removed from the cabinet and the longest colony diameter and the diameter perpendicular to that were measured and recorded. The date on which sporulation was first evident was also recorded for each isolate.

2.4.2.4 Statistical design and analysis.

The test was replicated four times using the same strain of *A. bisporus* and 37 disease isolates. Each isolate was repeated only once within any single replicate, except 192B1, which was used as a standard and as such occurred four times in each replicate. A four-replicate alpha design for 40 plots per replicate divided into 10 blocks of four was therefore used. The alpha design ensured that each test isolate occurred at least once in a block with the standard isolate, and that no pair of test isolates appear together in a block more than once. An analysis of variance was carried out on the colony diameter data.

2.4.3 Results

2.4.3.1 Colony growth

Nine days after inoculation, the average colony diameter for the standard isolate 192B1 was 99mm (Figure 8). Twenty-four isolates produced colony diameters which were not significantly different to 192B1 but which ranged in size from 122mm (isolate 193A) to 67 mm (isolate CC2). All these isolates formed significant cobweb patches on the casing, engulfing mushrooms in their path, and producing conidia within six to nine days (Plate 4a & b). These isolates can all be considered to be pathogenic to *A. bisporus*. Three of them had been originally isolated from other fungal species (CC16, CC10 & CC8) and one had been isolated from soil (CC3) but all remaining isolates had been isolated from *A. bisporus*. Three culture collection isolates, originally taken from *A. bisporus*, produced visible colonies that were significantly smaller in size and did not produce conidia (CC18, CC22 & CC6) but nonetheless, mycelium had grown away from the point of inoculation (Plate 4c). Since they failed to produce conidia during the course of the experiment, they should be considered to be only mildly pathogenic. This lack of pathogenic vigour may be a result of long-term storage and subculturing of the isolates in a culture collection, a factor that is known to reduce the pathogenicity of some species. The remaining nine isolates grew very poorly or not all (Plate 4d), producing no conidia and with mycelial growth very sparse and colony diameters often not much more than the inoculated area. These isolates should be considered to be non-pathogenic. Eight of these nine isolates had been isolated from non-*Agaricus* substrates such as wood, soil or other fungi, which would further substantiate this conclusion. The ninth isolate, which had been isolated from an *Agaricus* sp. (CC12), may have lost its pathogenicity as a result of long-term storage and subculturing of the isolate in a culture collection.

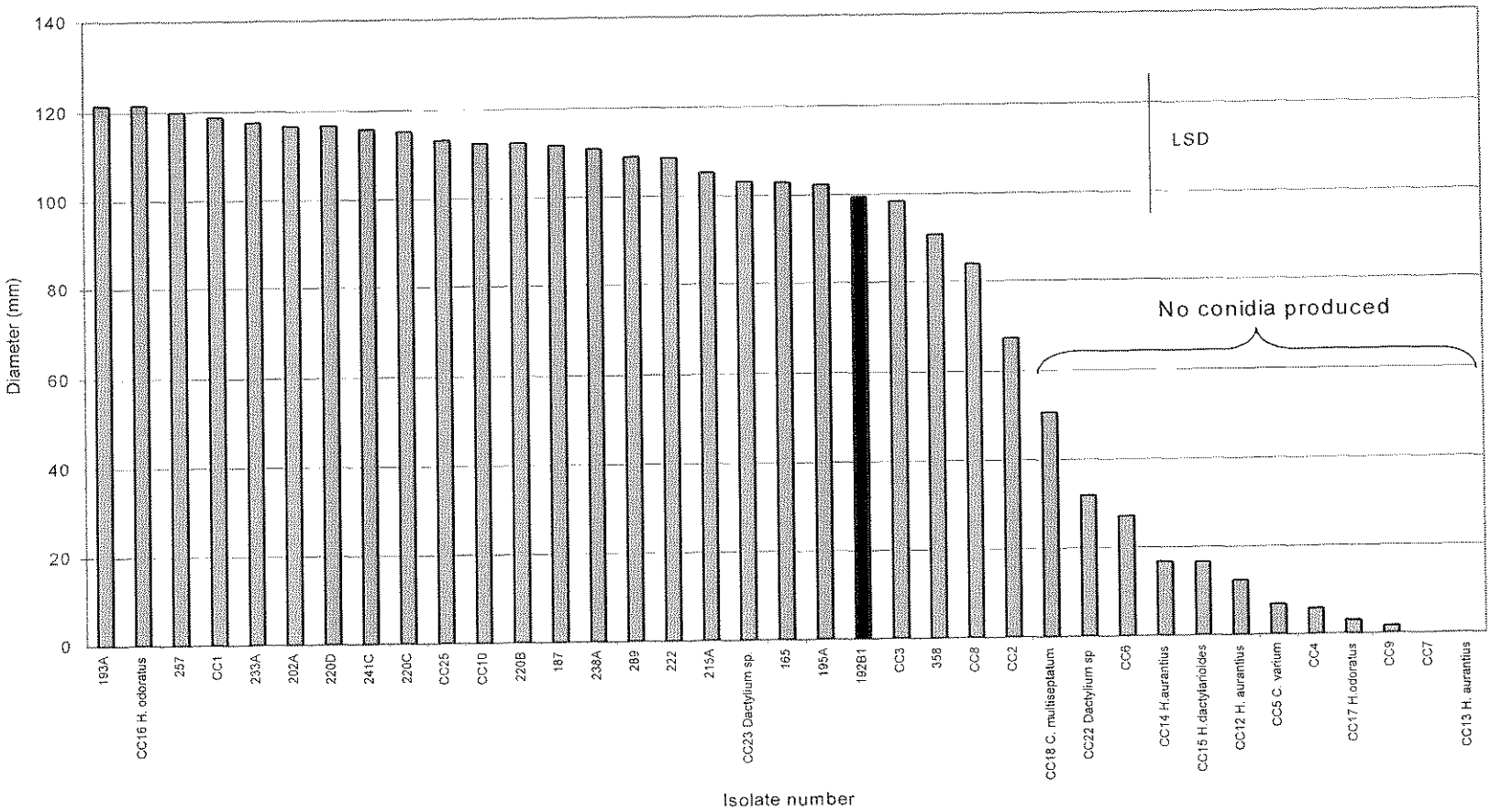


Figure 8. Mean colony diameter on mushroom casing of 37 isolates of *Cladobotryum* spp. measured nine days after inoculation. Isolates with the prefix 'CC' were collected from culture collections. All other isolates were collected during a HDC survey of cobweb causing pathogens (M14a). Unless stated otherwise isolates from culture collections were catalogued as *Cladobotryum dendroides/Hypomyces rosellus*.

2.4.3.2 Sporulation

All isolates that formed good colonies on mushroom casing also produced conidia. However, the four fungicide resistant isolates tested, such as 192B1, sporulated more heavily and earlier (Day 6 to 7 after inoculation) than all the fungicide-sensitive *C. dendroides* mating group isolates, such as CC10, which sporulated more sparsely and later (Day 8 to 10 after inoculation) (Plate 4a & b). The remaining fungicide-sensitive isolates showed a range of sporulation intensities but mainly poor to moderate.

2.4.4 Discussion

Pathogenicity testing of 37 *Cladobotryum* isolates indicated that the most pathogenic isolates, as indicated by good cobweb growth on the casing in conjunction with heavy sporulation, starting 6 to 7 days after inoculation, was recorded for the four benzimidazole-resistant isolates tested. Twenty-one isolates were also quite pathogenic, producing good cobweb growth, but sporulation intensity was generally lighter and started later, between days 7 and 10 after inoculation. Pathogenic isolates which belonged to the *C. dendroides* mating group (Table 9), tended to sporulate latest, between days 8 and 10 after inoculation. All pathogenic isolates however were capable of parasitising any mushrooms in their path.

Pathogenicity testing of isolates indicated that eight out of 12 isolates from non-*Agaricus* substrates, including wood, soil and other fungi, failed to develop any cobweb symptoms on *Agaricus*, despite their growing well on culture plates. This suggests that only certain *Cladobotryum* isolates are pathogenic to *Agaricus*. Pathogenicity usually requires the pathogen to have enzyme systems that are adapted for attacking the host tissue (Jefferies & Young, 1994). *Cladobotryum* isolates from substrates other than *Agaricus* may therefore have enzyme systems different to those required by pathogens of *Agaricus*. However, some pathogens may be able to parasitise more than one host, if the mechanisms of pathogenicity for different hosts are similar. This would explain how four isolates from non-*Agaricus* substrates (CC3, CC8, CC10 & CC16), which included two apparently different *Cladobotryum* species, were also capable of colonising and parasitising *Agaricus*. This feature would also mean that conidia from *Cladobotryum* isolates parasitising various fungi in the wild could serve as the initial inoculum for a cobweb outbreak on a mushroom farm.

An alternative explanation for the lack of pathogenicity among *Cladobotryum* isolates from non-*Agaricus* substrates could be that all these isolates were from culture collections. Loss of pathogenicity can be a feature of culture collection isolates, particularly if isolates are subcultures extensively (IMI, *pers comm*). However, all the pathogenic isolates used in this study were subcultured frequently, and a number of older culture collection isolates performed no differently to younger isolates in terms of their pathogenicity. Therefore, there is a good likelihood that the non-pathogenicity of some isolates reflects their different substrate specificity rather than a loss of pathogenicity. In contrast, however, four culture collection isolates, originally isolated from *Agaricus* (CC6, CC12, CC18 & CC22) and which were only weakly or not at all pathogenic, may reflect a loss of pathogenicity as a result of long term storage. However, it is also quite likely that there is a range of pathogenicity intensity among any population of a given organism. Freshly collected isolates from diverse substrates would need to be examined in order to verify this hypothesis.

The most interesting result from the pathogenicity tests is the fact that the benzimidazole-resistant strains all sporulated very heavily and earlier than all other isolates. This goes some

way towards explaining why the epidemic in 1994/95 was so difficult to control compared with previous experiences of cobweb.

2.4.5 Conclusions

- Thiabendazole-resistant isolates consistently sporulated earlier, and generally more profusely, than all other pathogenic *Cladobotryum* isolates.
- Isolates identified as *Cladobotryum dendroides* by mating interactions and which produced cobweb symptoms on *Agaricus*, tended to sporulate more sparsely, and later, than other pathogenic isolates.
- *Cladobotryum dendroides* isolates that were non-pathogenic to *Agaricus*, had been originally isolated from non-*Agaricus* substrates.
- Four *Cladobotryum* isolates, originally isolated from either other fungi or soil were equally as pathogenic as isolates from infected mushroom crops. This suggests that *Cladobotryum* present on wild mushrooms can be a potential source of *Cladobotryum* on a mushroom farm.
- *Cladobotryum varium /Hypomyces aurantius* failed to parasitise *Agaricus*.

2.5 Summary

There was not much overlap between the four methods used to categorise *Cladobotryum* isolates. Growth rate data was variable both within and between apparent groupings but this is unlikely to be a stable characteristic from a taxonomic point of view. The number of cells per conidium is often considered to be a good taxonomic character and three distinct groups were identified based on the mean cell number (MCN) of conidia. The genetic data from the RAPD studies identified only one group of genetically-similar isolates at the level at which the analyses were carried out. However, this group included isolates whose conidia had MCN values of <2 and also 2-3. It included all the *C. mycophilum* isolates (with a characteristic odour) obtained either from culture collections or the British mushroom industry. It also contained the large group of fast-growing, thiabendazole-resistant isolates collected during the HDC survey of 1995 (M14a) that lacked the distinct odour and low MCN of associated with C. *mycophilum*. Thus, the RAPD data suggests that these genetically related isolates display considerably morphological variation. It also suggests that the thiabendazole-resistant isolates which dominated the cobweb epidemic in the early 1990's are genetically more similar to C. *mycophilum* than C. *dendroides*. The mating interactions also suggest that the thiabendazole-resistant isolates are not C. *dendroides* as they failed to produce any sexual fruitbodies when crossed with each other. A distinct group of C. *dendroides* isolates were identified from the mating interactions between isolates from throughout the world and it included isolates obtained during the HDC survey of 1995 (M14a) indicating that "true" C. *dendroides* was also present in the British mushroom industry during the cobweb epidemic. The absence of conclusive positive matings between any of the *C. mycophilum* or other *Cladobotryum* species tested suggests however that perhaps the conditions for mating interactions were not suitable for those species during the course of these experiments. Further work should be carried out to try and produce mating interactions within these groups of isolates so that biological species groups can be identified, if they exist.

The pathogenicity tests indicated that there was some variation in the intensity of symptoms associated with the various different isolates tested. The thiabendazole-resistant isolates were the most pathogenic, sporulating earlier and heavier than any others. In contrast, the C. *dendroides* (mating group) isolates all sporulated more sparsely than all other pathogenic isolates. Some non-pathogenic isolates were also identified and these tended to have been isolated from substrates other than *Agaricus*, including wood, soil and other species of fungi. While this may suggest that isolates from non-*Agaricus* sources are not a threat to *Agaricus* mushroom production, most of the non-pathogenic isolates had been in culture collections for many years and may have lost their pathogenicity in storage. Pathogenicity tests using freshly isolated cultures from diverse substrates are needed to establish if non-*Agaricus* derived isolates are capable of causing cobweb symptoms on *Agaricus*.

2.6 General Conclusions

- *Cladobotryum* isolates show significant variation in terms of their growth rate and conidial morphology
- There is no relationship between the morphological data and the genetic RAPD data suggesting that genetically similar isolates show a range of morphologies
- The thiabendazole-resistant isolates associated with the cobweb epidemic in Britain in the early 1990's was genetically similar to C. *mycophilum*, but was morphologically different from current descriptions of this species.

- *Cladobotryum dendroides* appears to be less pathogenic than the thiabendazole-resistant isolates encountered during the cobweb epidemic in the early 1990's.

3 Biology of *Cladobotryum* in mushroom casing

The physical properties of mushroom casing, such as water holding capacity, pH, air filled porosity, texture and nutrient status, are known to be affected by the type and quantity of the two major ingredients, peat and lime (Flegg, 1954; Edwards and Flegg, 1954; De Kleermaeker, 1954; Visscher, 1988; Stoller, 1952a; Stoller, 1952b). In turn, variation in the physical properties of casings have been demonstrated to affect *Agaricus bisporus* growth, yield, dry matter content, and the cleanliness (Reeve *et al*, 1959a; Reeve *et al*, 1959b; Kalberer, 1985; Kalberer, 1987; Kalberer, 1990; Kalberer, 1991; Noble and Gaze, 1995; Noble, 1995; Noble, 1996; Noble *et al*, 1999).

Some of the most recent and most comprehensive studies conducted on the effects of casing materials on mushroom yield and quality were those undertaken by Noble (1995 & 1996), and Noble *et al* (1999). Generally these studies demonstrated the importance of casing formulation to mushroom yield and quality. However, more specifically they highlighted several key points;

1) peat type affected the yield and cleanliness of mushrooms,
2) sugar beet lime (SBL) gave rise to a higher yield than chalk lime,
3) higher SBL rates reduced the water retention of the casing and imparted no beneficial effect on yield, dry matter content, or cleanliness of the mushrooms,
4) the optimum Ψ_m (matric potential) for mushroom yield lay between –7.9 and –9.4kPa with wetter and drier treatments resulting in yield reductions.

Whilst the effects of casing properties on *A. bisporus* have been studied in some depth, no literature was found which considered the effects of casing properties on disease organisms. Since casing properties are known to affect the mycelial structure of *A. bisporus* (Nobel *et al*, 1999), this may in turn affect disease development in terms of the response of *Agaricus* to the pathogen. It is also possible that casing properties may have a direct influence on disease expression.

The objectives of this section are (i) to examine the effects of several casing formulations on the development of cobweb disease expression; (ii) to examine the effect of casing matric potential on cobweb disease expression and (iii) to examine the influence of *Agaricus* on cobweb development.

3.1 Casing formulation

3.1.1 Introduction

The most popular peat type used for casing in the UK until 1990 was mechanically harvested milled peat, which is partially dried prior to transportation and baling (e.g. Shamrock Irish Peat, Horticultural Grade, from Bord na Mona). The grower then rewetted the peat during casing preparation. This preference for baled milled peat dwindled between 1990 and 1995 with the arrival on the casing market of undried, bulk-extracted peat. Although studies have shown that milled and bulk peats differ only very slightly with regards to mushroom yield (Noble, 1995; Noble, 1996), bulk peats are more robust. Milled peats not only have a higher propensity to pan than bulk peats, thereby reducing the absorption of water, but they also have a lower water retention than bulk peats (Noble, 1995). So, whilst the yield from milled peats

may not be less than bulk peats *per se* the more robust character of bulk peats has ensured there are fewer casing problems associated with watering.

Since casing components and quantities had the ability to alter the growth of *A. bisporus* (Noble, 1995), and water potential and pH affected cobweb growth and spore germination, *in vitro* (Lane, 1993), it was decided to test the hypothesis that different casing types affected cobweb disease expression. As mentioned above, there was a change in the type of casing used within the UK mushroom industry around 1990-95 (Gaze, *pers com*) which coincided with the cobweb epidemic. It has therefore been suggested that casing type might have been a contributing factor in the development of the disease.

A cropping experiment was designed whereby spawn-run compost was cased with a variety of casing types which were then subjected to either a wet or dry watering regime, giving a range of casing types with a range of moisture contents. Casings were then inoculated with the disease to test the hypothesis that expression was affected by casing type and/or watering regime.

3.1.2 Materials and methods.

3.1.2.1 Compost preparation.

Phase two compost (Batch No. 19/97) was prepared according to current standard commercial practice at HRI experimental mushroom unit Wellesbourne. The compost was spawned with *A. bisporus* strain A12 (Sylvan) and spawn run for 23 days at 25°C and 95% relative humidity (RH). This is slightly longer than the usual 14 -17 day spawn. Steamed pots (80°C for 8hrs) measuring 265mm in diameter were each filled with 3.5kg of spawn-run compost which was then compressed using the base of an empty pot before the casing layer was applied. A total of 96 pots were prepared in total.

3.1.2.2 Casing preparation.

Four casing formulations were prepared using either milled or bulk peat types, each with two rates of SBL inclusion (Table 11); all four casings included casing spawn at a rate of 4kg/m^3 casing. Each casing formulation was used to cover 24 pots of spawn-run compost to the commercially recommended depth of 4.5cm (Noble, 1995).

Table 11. Four casing formulations utilised - percentages calculated by volume.

Casing number	Peat type	Sugar beet lime (SBL)
1	Bulk	15%
2	Bulk	30%
3	Milled	15%
4	Milled	30%

3.1.2.3 Cropping procedures.

Cased pots were labelled and placed in a three-shelf cropping chamber according to the experimental design described in section 3.1.2.6. The cropping chamber was then subjected to standard environmental conditions employed at HRI for case-run, airing, and cropping. Temperature, RH and CO_2 concentration were automatically monitored and controlled by computer. During case run, temperature was maintained at 25 °C and RH at 96%. This facilitated rapid and complete hyphal colonisation of the casing layer and after 10 days mycelium was evident on the upper surface of the casing layer. The crop was aired at this stage by gradually reducing the temperature to 18 °C and the RH to 89% over a period of four days in order to stimulate pinning. These conditions of temperature and RH were then maintained throughout the cropping period. The risk of disease outbreaks was minimised by using the commercial practice of salting disease areas, and by only harvesting two flushes. Salting involves the application of salt granules to patches of disease. The complete covering of the fungus with salt is intended to kill off the disease colony prior to watering.

3.1.2.4 Watering treatments and moisture content analysis.

Each casing type was subjected to two different watering regimes during the cropping cycle to give 4 x 2 = 8 casing/watering combinations. Thus, eight treatments were established in total; four casing formulations (Table 11) each with two watering regimes. The watering regimes consisted of a wet treatment similar to standard commercial practice, and a dry treatment, which was approximately half the commercial application rate. 12 pots of each casing formulation received a wet treatment and 12 pots received a dry treatment.

Casing moisture content (MC) was measured nine times during the cropping cycle at strategically important points i.e. start, finish, before and after watering and before and after harvesting. Casing samples were taken from six of the 12 pots prepared for each casing/watering combination using a length of copper tubing measuring 26mm in diameter. This tube, which had been sharpened at one end to cut more easily through the casing, was pushed vertically through the entire casing layer of the pot, retracted and the core contained within then pushed into a marked foil tray using a plastic plunger. The fresh weight of the sample was recorded immediately, and the sample was dried at 75°C for 48hrs. The MC of the sample was then calculated by transforming the weight differential into a percentage of the wet sample.

$$100 - \left(\frac{100}{WW} \times DW \right) = MC(\%)$$

Where: WW = Wet Weight, DW = Dry Weight, MC = Moisture Content

Moisture content was then converted into matric potential (Ψ_m) using a matric potential curve produced for each particular casing type. Matric potential is widely regarded as a better measure of water availability to micro-organisms than MC (Dix and Webster, 1995). The matric potential curve was formed using a technique similar to that first described by Noble *et al* (1999) which had been modified and developed from the methodologies of Montagne *et al* (1992) and Anon (1982).

3.1.2.5 Inoculation technique and data recording

Six of the 12 pots for each of the casing/watering treatments were inoculated. Inoculation of pots was conducted after the airing process was complete and pins had formed but before sporophore development. Inoculation of pots followed the protocol outlined in section 2.4.2.3 however only isolate 192B1 was used and inoculation took place in the cropping house, although care was still taken not to contaminate non-target pots. This isolate was singled out for further study because it was typical of the fungicide resistant group that was prolific during the epidemic.

The diameter of the cobweb disease colonies was recorded on a daily basis. For each colony, the longest diameter and the diameter perpendicular to that were measured and the average calculated. Increase in average colony diameter was plotted against time for each casing treatment and the rate of colony extension was calculated in mm/day. Mushroom yield was also recorded on a daily basis for two flushes. Mushroom weight was recorded with each day's harvest being split into spotted and unspotted mushrooms. This gave an indication of mushroom quality and also allowed an estimation to be made as to the influence of the disease on mushroom quality with time.

3.1.2.6 Statistical design and analysis

This experiment contained four casing treatments, each receiving two different watering treatments to give eight casing/watering combinations. Six replicate pairs of pots were prepared for each treatment combination, one of which was inoculated and used to estimate cobweb growth, with the second pot of each pair being used to estimate moisture content (moisture content analysis required destructive sampling of the casing so a separate pot was used for this). For the purpose of the statistical design each pair of pots was allocated a single position. This gave "4 casing formulations" x "2 watering regimes" x "(6 x 2) replicates" = 48 pairs of pots. These were arranged in an incomplete Trojan square design which allowed for environmental variation in three dimensions; front to back, left to right, and top to bottom. Statistical analyses were performed using the Genstat 5 computer package. Individual variates were subjected to analysis of variance to test for differences between treatments, i.e. peat type, SBL rate, watering regime, etc, with regards to mushroom yield and cobweb expression.

3.1.3 Results & Discussion

3.1.3.1 Moisture content

Moisture contents of four different casing formulations subjected to two different watering regimes varied considerably throughout the cropping period (Figure 9). Readings varied from in excess of 77% to below 50%. Commercial cropping practice typically maintains casing moisture content between 70-72% w/w (Noble & Gaze, 1995). The treatment that most closely resembled commercial conditions throughout the trial was bulk peat containing a low SBL rate with either a standard or dry watering regime (Figure 9).

Generally the MC of the casings increased or was maintained during the first 11 days of the crop when regular watering was taking place and the mycelium colonised the casing layer (Figure 9). The MC fell during pin set and development as watering ceased and cropping began. This reflects the absorption of water by the developing mushrooms (composed from approximately 90-95% water) from the casing layer during sporophore production (Kalberer,

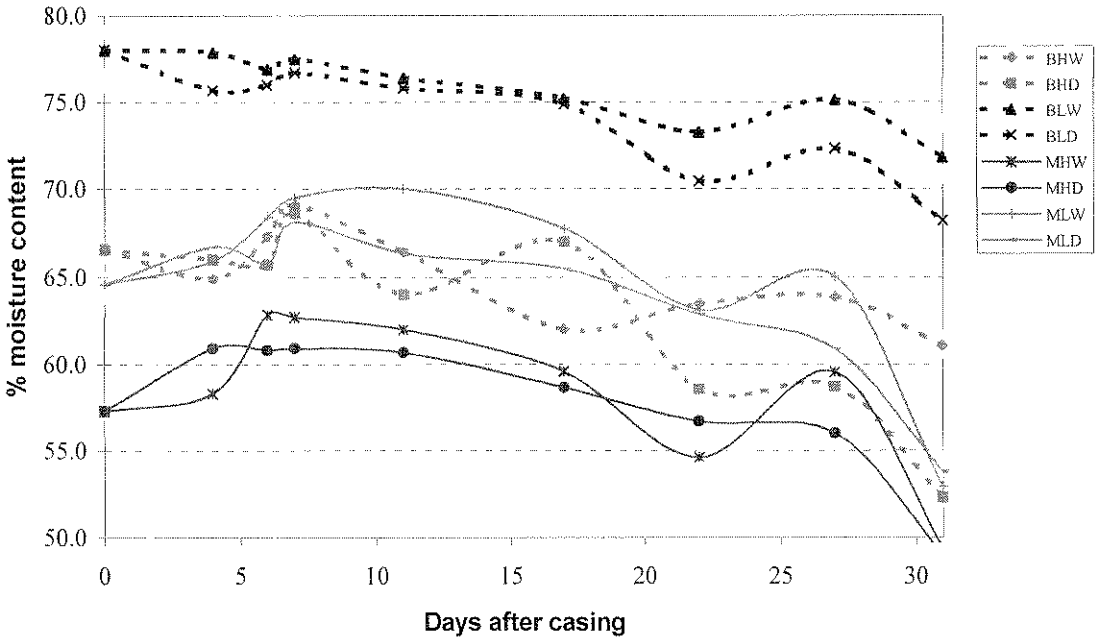


Figure 9. Moisture content of four different casing types combined with two different watering regimes from the time of casing to the end of the second flush. B/M = bulk or milled peat type; H/L = high or low sugar beet lime rate; W/D = wet or dry watering regime.

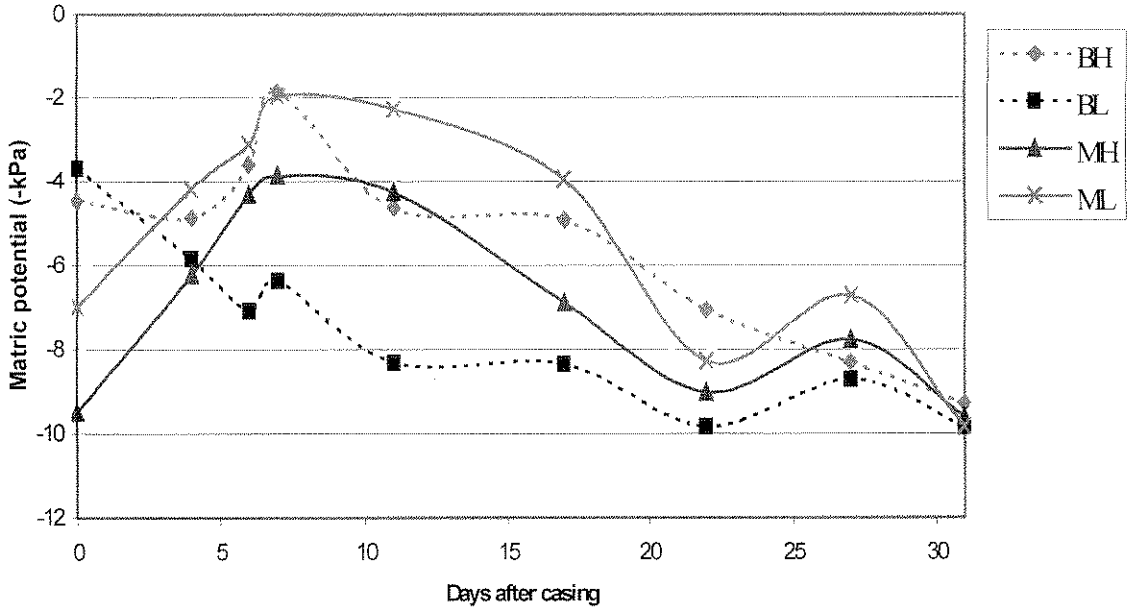


Figure 10. Matric potential of four different casing types from the time of casing to the end of the second flush. B/M = bulk or milled peat type; H/L = high or low sugar beet lime rate.

1990). The casing MC increased briefly during a period of intercrop watering before falling to even lower levels again after the second flush.

Casing formulation had a much greater effect on casing moisture content than either a wet or dry watering regime, despite a near twofold difference in the volume of water applied (Figure 9). This is most evident for the casing type bulk peat with low SBL rate (BLW & BLD; Figure 9). Disparity in MC between the wet and dry treatments of this casing type never varied more than about 4% whereas the disparity between different casing formulations was a minimum of about 6%.

Peat type and the inclusion rate of SBL appeared to have the greatest affect on the MC of the casing treatments. Bulk peat formulations consistently demonstrated a higher MC than their milled peat counterparts. For example, bulk peat with a low rate of SBL (BL) consistently demonstrated a higher MC than milled peat with a low SBL rate (ML). An increase in SBL rate was shown to decrease the MC of the casing. Double the rate of SBL significantly reduced the MC of both bulk peat and milled peat-based casings (Figure 9).

3.1.3.2 Matric potential (Ψ_m)

Because MC was shown to vary more between casing type than between watering regimes, Ψ_m was calculated for each casing formulation based on the MC data from both wet and dry watering regimes (Figure 10). Matric potential is measured on a negative scale where "0" represents the highest matric potential possible (water is freely available). The relationship between MC and Ψ_m is not simple. Substrates with a high MC may have a low Ψ_m due to water retaining properties of the substrate. Bulk peat with a low SBL content had the highest MC of all casings throughout the crop, however, when converted into Ψ_m this casing type was shown to have the lowest Ψ_m from the sixth day after casing to the completion of the experiment.

Matric potential for all casing types varied throughout the duration of the crop from –1.8 kPa to –9.8 kPa (Figure 10). Generally, the Ψ_m of casings increased during the first week as water was applied, followed by a steady reduction during the cropping phase with all casing types showing a Ψ_m of between –9 and –10 kPa on the final day of testing. Bulk peat with low SBL however did not show this pattern. This casing type showed the greatest reduction in Ψ_m during the first week of the study followed by a more gradual reduction during the remainder of the experiment.

At the start of the study the casings containing milled peat had low Ψ_m compared to those containing bulk peat. However, during the first week of the study the Ψ_m of milled peat casings increased at a greater rate than that of the bulk peat casings, so that by the end of the first week their Ψ_m were comparable to bulk peat with high SBL and greater than bulk peat with low SBL.

The casings composed of milled peat showed greater homogeneity than those composed of bulk peats. Whilst the Ψ_m of the two milled peat casings mirrored each other closely throughout the study the Ψ_m of the of the two bulk peat casings varied during the first and last weeks of the experiment.

In addition to the Ψ_m curve shape, the inclusion rate of SBL had different effects on the two different peat types. The milled peat with low SBL had a consistently higher Ψ_m than the milled peat with high SBL, whereas, the bulk peat with low SBL had a consistently lower Ψ_m than the bulk peat with high SBL.

3.1.3.3 Mushroom yield.

Control yields for the first flush varied from approximately 138 g/kg compost (bulk peat with a high SBL rate) to 164 g/kg compost (milled peat with a high SBL rate) (Figure 11). Neither peat type nor SBL rate alone appeared to have an effect on yield, as no patterns were immediately apparent. When yield was correlated with the average matric potential for each casing from day 0 (at casing) to day 22 (at end of first flush), there was no relationship detected (Figure 12). No significant correlation existed therefore between mushroom yield and Ψ_m.

3.1.3.4 Cobweb radial growth rate.

A significant number of inoculated pots failed to develop any cobweb symptoms. There was no distinct pattern to the failure of colony development with no one casing type being affected more than any other. As a result the statistical analysis of the data reveal few significant effects of treatments. The high rate of sugar beet lime appeared to result in significantly larger colony diameters at the end of the first flush independently of all other treatments (Table 12). However, despite the absence of significant relationships, some trends in the data are apparent by the end of the first flush with colony diameters tending to be larger in the wet treatments compared to the dry treatments. The smallest colonies overall occurred in the milled peat with low sugar beat lime rate (Table 12).

Table 12. Cobweb colony diameters (mm) for various treatments by the end of the first flush. Treatment means from analysis of variances. Data include zero values.

Peat type	Sugar Beet Lime rate	Watering treatment		Average	Least significant difference (P = 0.05)
		Dry	Wet (standard)		
Bulk	High	93	122	107	Not significant
Bulk	Low	62	50	56	
Milled	High	73	130	101	
Milled	Low	9	32	20	
Average		59	83	**104 38**	
Least significant difference ($P = 0.05$)		Not significant		68.9	

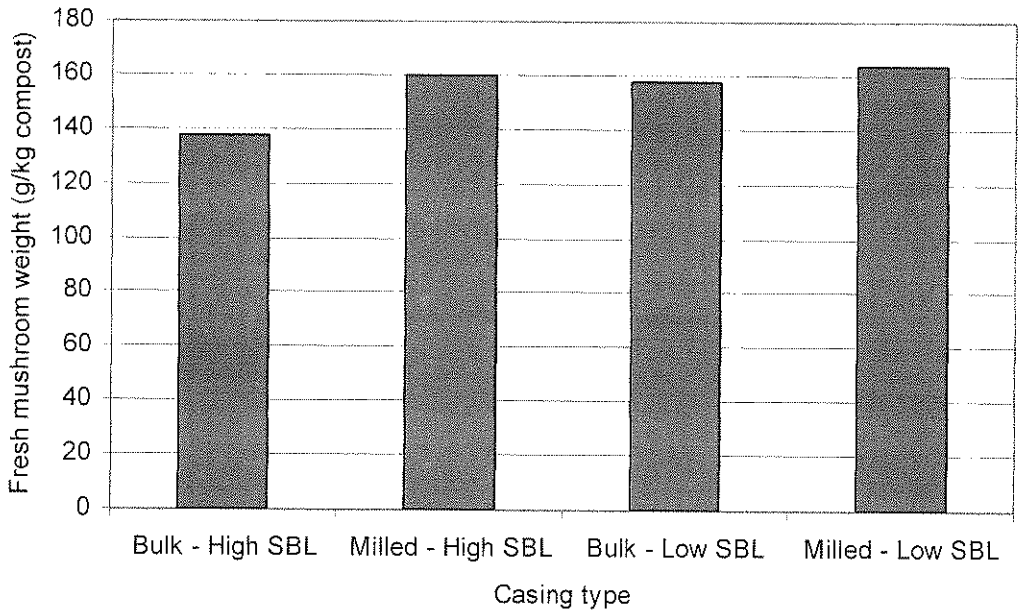


Figure 11. Yield of first flush mushrooms harvested from the control pots of each casing type.

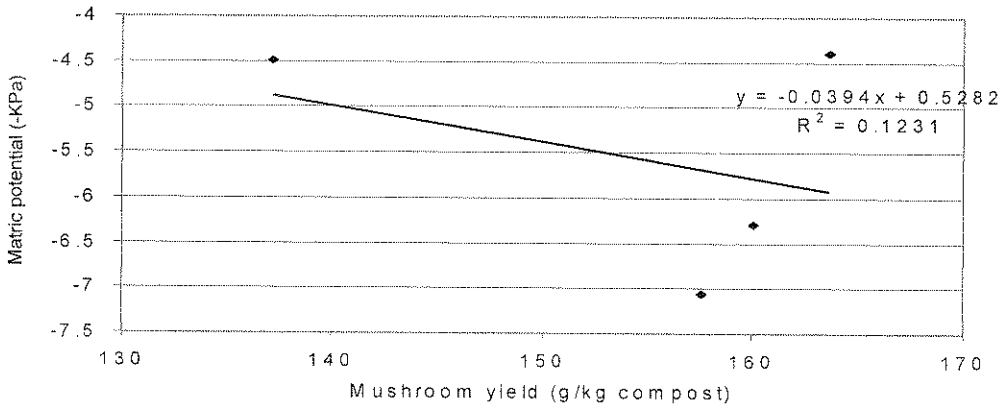


Figure 12. The effect of casing matric potential on mushroom yield. Matric potential calculated from the mean of all recordings between day 0 and day 22 after casing. Mushroom yield only included those mushrooms harvested from the control pots first flush.

With time most colonies grew quite well and colony growth rate was also calculated for each treatment. Mean growth rates varied from approximately 12 mm/day up to 24 mm/day (Figure 13). These values are likely to be underestimated as the data included many zero values for colonies that failed to grow. Again the statistical significance of differences between individual treatment means was low with the best level of significance (P = 0.08) being for the combinations of peat type and sugar beet lime rate (Figure 13). Although statistical analysis of all the data suggested that Bulk peat with a low SBL rate produced the lowest mean growth rate of 12.3 mm/day, if zero values were omitted than mean colony growths rates for all treatments were similar, between 28.6 and 37.0 mm/day, with the higher growth rates being recorded from the standard wet treatments.

Thus, no statistical correlation appeared to exist between the components of the casings (peat or SBL inclusion rate) and the rate of disease growth. However, a correlation was evident between average matric potential and average colony growth rate. As matric potential increased towards zero, growth rate also increased (Figure 14). Bulk peat with low SBL rate had a mean Ψ_m of –8.8kPa and a disease growth rate of only 12 mm/day. In contrast, bulk peat with a high SBL rate had a Ψ_m of –5.5kPa and a growth rate of 24.5 mm/day. This relationship must be interpreted with caution however as they also include zero values. If zero values are omitted than mean colony growths rates for all treatments are more similar.

3.1.4 Discussion

The primary objective of this work was to establish if casing had an influence upon the expression of cobweb disease. The results suggested that colony diameters were smaller when either a dry watering regime or low (15%) rate of sugar beet lime was used, but the occurrence of many zero values in the data reduced the significance of any relationships. Average colony growth-rate correlated with the average matric potential of the casing, which in turn is correlated to the physical properties of the casing and it's component parts. Further work is necessary to define these relationships more clearly.

The effects of Ψ_m on *A. bisporus* growth and subsequent yield during this study did not concur with those of Nobel *et al* (1999). Within the range studied neither casing type or Ψ_m had any discernible effect on the yield of mushrooms. Not only was this study smaller than that conducted by Nobel *et al* (1999) but it also followed a different experimental design. Previous experience has demonstrated that atypical mushroom yield results are not uncommon from pot experiments (Grogan, *pers com*).

Another point requiring comment is the importance of the conversion of moisture content (MC) into matric potential (Ψ_m). Interpretation of results using moisture content alone would have lead to different conclusions. For example, MC data indicated bulk peat with a low rate of SBL inclusion gave rise to the wettest casing type. When converted into Ψ_m it became apparent that whilst this casing type contained the most water, this water was actually the least available to both the *A. bisporus* mycelium and cobweb disease pathogen.

An interesting finding from this study was the negligible influence of watering on the moisture content of the casing layer. An almost two fold difference in the volume of water applied to each casing type as either a wet (standard) or dry treatment, had very little effect on

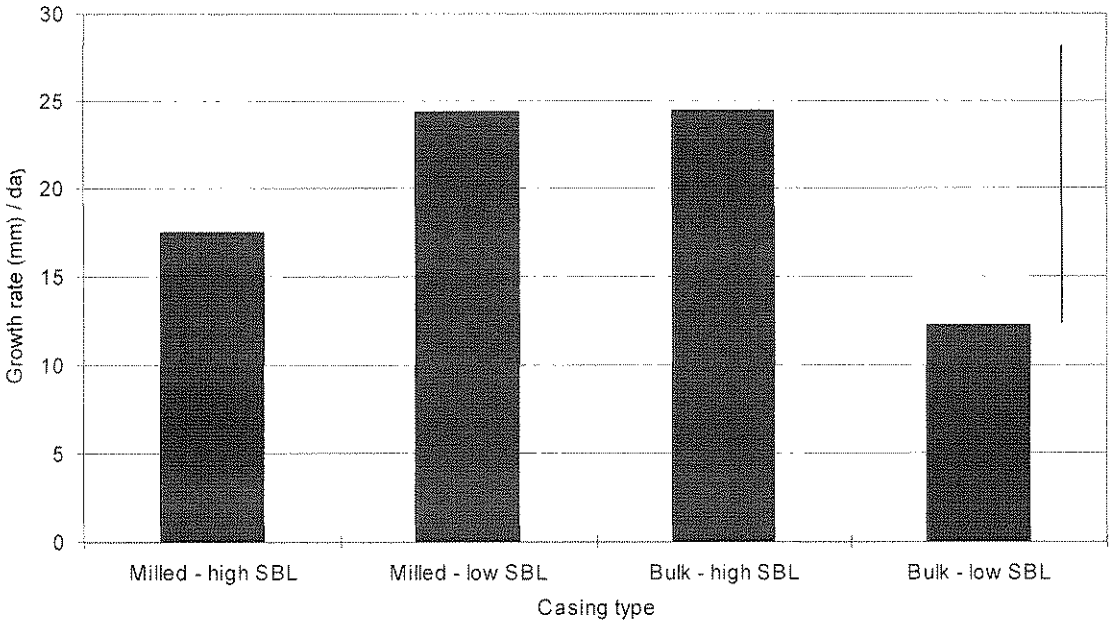


Figure 13. Radial growth rate of cobweb (isolate 192B1) on four different casing types. Growth rate calculated as mean linear growth rate of all inoculated pots, including zero values. Interaction effect between peat type (milled or bulk) and sugar beet lime rate (high or low SBL) significant at $P = 0.08$.

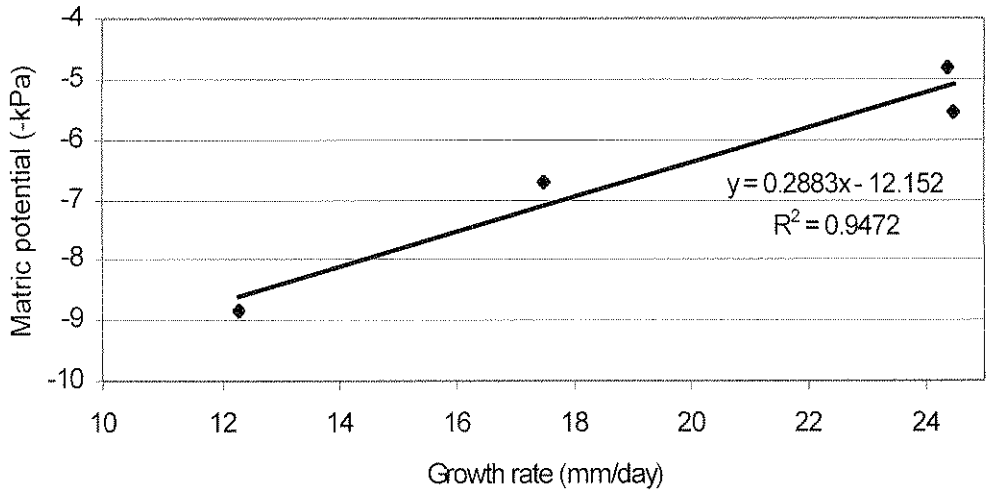


Figure 14. The effect of average casing matric potential on the average growth rate of cobweb disease. Growth rate calculated from mean linear growth rate of all inoculated pots (including zero values). Matric potential calculated from mean of all measurements taken during the linear growth period of the disease (11 – 22 days after casing).

the moisture content of that casing type. However, there is some suggestion in the data that the establishment of cobweb colonies may have been favoured by the wet treatment, as colony diameters were larger more quickly in all the wet treatments.

3.1.5 Conclusions

- Casing formulation affected the moisture content of each casing type. High rates of sugar beet lime (30%), and the use of milled rather than bulk peat, resulted in casings with lower moisture contents.

- Although initially there was a good correlation between casing moisture content and matric potential, with the wettest casing having the highest matric potential, this relationship did not persist during cropping. From Day 6 onwards the wettest casing consistently had the lowest matric potential, while the driest casing had an intermediate matric potential.

- Wet and dry watering regimes did not affect the moisture content of any given casing formulation.

- Statistical analysis of cobweb colony growth rate suggested that different casing formulations (peat type and sugar beet lime rate) had had different colony growth rates and this effect could be attributed to the matric potential of the casing. However, the presence of many zero values in the data prompts the use of caution in interpreting these results as when zero values were excluded, all growth rates were similar.

- Statistical analysis of cobweb colony diameters at the end of the first flush also suggest that the rate of sugar beet lime inclusion has a significant effect on the establishment of cobweb, with larger colonies occurring when high (30%) rates of sugar beet lime are used.

- Cobweb colony diameters appeared to be larger at the end of the first flush following a standard (wet) watering regime compared with the dryer treatment which received half the standard amount of water. This trend was not statistically significant at P=0.05, but this may reflect the presence of many zero values in the data.

- Further work is needed to more fully understand the relationship between cobweb growth, casing formulation and casing matric potential.

3.2 Matric potential

3.2.1 Introduction

Following on from previous work, which suggested that cobweb colony growth rate could be affected by the matric potential, the specific effect of altering casing matric potential upon the disease and mushroom was studied.

Mushroom sporophore development has been known for several years to be closely linked to casing water availability (Kalberer, 1985; Bels-Koning, 1950). Many studies have examined the effect of casing moisture on mushroom yield and quality (Reeve, *et al*, 1959; Schroeder & Schisler, 1981; Visscher, 1988; Kalberer, 1985; Kalberer, 1990; Kalberer, 1991; Noble & Gaze, 1995; Noble *et al*, 1999). Additionally, matric potential is known to alter the growth of *A. bisporus in vitro* (Magan *et al*, 1995). The most recent of these studies identified the optimum matric potential of casing for *A. bisporus* to be in the range -7.9 to -9.4 kPa (Noble *et al*, 1999).

Matric potential has also been demonstrated to affect the growth, *in vivo,* of many filamentous fungi including *Cladobotryum* spp. (Magan, 1988; Clarke, *et al,* 1980; Boddy, 1983; Wilson & Griffin, 1979; Hocking & Pitt, 1979; Lane *et al*, 1991). *In vitro*, the hyphal growth rate of *C. dendroides* was shown to be progressively inhibited by a decreasing matric potential (Lane, 1993). In other words, the dryer the substrate the slower *C. dendroides* hyphae grew. Growth of *C. dendroides* was observed to cease at -4.97MPa. However, direct comparisons between *in vitro* and *in vivo* studies should not be drawn. In studies of *Trichoderma and Penicillium* spp., Magan (1988) found that laboratory based studies using osmotic or matric potential control systems did not directly simulate the conditions of the natural substratum. It is therefore important to establish a true *in vivo* impression of the effect of casing matric potential on the growth of the cobweb disease pathogen.

The objective of this section is to examine the effect of casing matric potential on cobweb disease expression. This will be done by monitoring the growth of cobweb in casings that have a range of casing matric potentials.

3.2.2 Materials and methods

3.2.2.1 Compost preparation.

Compost (Batch No. 18/98) was prepared according to current standard commercial practice at HRI experimental mushroom unit at Wellesbourne, Warwick. Ninety-six pots of spawn-run compost were prepared as described in section 3.1.2.1.

3.2.2.2 Casing preparation.

A quantity of bulk, deep-dug peat was acquired from Blue Prince mushroom farm, Woking. Sugar beet lime (SBL) was incorporated at a rate of 15% and *A. bisporus* caccing (A12 strain) was added at 0.5% (by volume). The resultant casing was sieved through an 11mm wire mesh before being applied to the compost filled pots. The depth of casing was checked using a dipstick and adjusted to the recommended 4.5cm (Noble, 1995). This casing formulation had been previously shown to demonstrate a wide variability with regards to matric potential (section 3.1) and was a mixture commonly used by commercial growers.

Casing was applied to 96 pots of spawn-run compost, which were then subdivided into 8 groups of 12 replicates (6 x 2 replicate pairs) with each group of 12 representing a single watering treatment. The pots comprising the four wetter treatments were cased four days before the four dryer treatments so that all pots would be ready for airing on the same day. Wet casing is colonised more slowly by *A. bisporus* mycelium than dry casing and it was therefore decided to allow the wetter treatments three extra days to colonise the casing (Noble, *pers com*).

3.2.2.3 Cropping procedures

Cropping conditions and procedures were the same as those outlined in section 3.1.2.3. Mushrooms were harvested from two flushes.

3.2.2.4 Watering regime.

Eight different watering regimes were applied to pots ranging from very dry to very wet so that the matric potential of each treatment differed. Watering regimes were all based around the standard watering regime. In the first days after casing watering of treatments consisted of applying x 0.25, x 0.5, x 0.75, (x 1), x 1.25, x 1.5, x 1.75 and x 2 the amount of water applied to the standard treatment (x 1). The water requirement of the standard treatment was assessed on a daily basis by both a visual and tactile inspection. In addition information from moisture content analysis was used to decide whether more or less water was needed. Watering was continued so as to provide casings with a wide range of casing moisture contents, and hence a wide range of casing matric potentials. Later in the cropping cycle, watering was done with reference to moisture content analyses in order to maintain the casings at a range of moisture contents. Thus at a certain point the drier treatments received no further watering, while the standard and wetter continued to be watered. Twelve replicate pots were prepared for each water treatment and set up as 6 x 2 replicate pairs. One of each replicate pair was used to provide casing samples throughout the crop for moisture content analysis with remaining pot of each pair being inoculated with cobweb.

Daily watering of each treatment was based on the requirements of the standard (x1) treatment. The required volumes were applied in either 30 or 15 ml aliquots. The daily volume was applied spread throughout the day, when volumes were large. This minimised water run off by giving the casing time to absorb one application before the next was applied. The water was applied as gently as possible to minimise disturbance of the casing structure. The aliquots of water were measured using a 50ml syringe before being transferred to 500ml water bottles that allowed the water to be dispensed as a fine jet. This was not only a gentle means of application but also allowed the water to be directed accurately to ensure even distribution without spillage.

3.2.2.5 Moisture content/matric potential

Casing moisture content was measured regularly throughout the cropping period. Moisture content was calculated for the casing as described in section 3.1.2.4. However, because the range of matric potentials of the dryer samples were predicted to be greater than those of Noble *et al*, (1999) the technique for determining the matric potential curve had to be modified to exert greater tension upon the test sample. This was achieved by lengthening the suspended water column. However in order to maintain the integrity of the 'water column-casing sample' contact, powdered sand was used as a bed within the funnel onto which the saturated sample could be placed. A matric potential curve was prepared for the casing. The

corresponding matric potential for each moisture content was calculated using the equation that described the matric potential curve.

3.2.2.6 Inoculation technique

Inoculation of pots with *Cladobotryum* isolate 192B1 was conducted as described in section 3.1.2.5. The four wettest treatments were inoculated with cobweb disease (isolate 192B1) 12 days after casing and the four drier treatments were inoculated 13 days after casing.

3.2.2.7 Disease pathogen growth

Cobweb colonies were measured on a daily basis and growth rates were determined as described in section 3.1.2.5.

3.2.2.8 Statistical design and analysis

This experiment contained eight watering treatments, each with six replicate pairs of pots to give 8 x (6 pairs of pots) = 96 pots in total. Pots were positioned in a three shelf cropping house with 32 pots on each shelf with 4 pots (2 replicate pairs) of each treatment occurring on each shelf in an 8 x 4 (2 pairs) array. The experiment was designed formally as two (4X4)/2 Trojan squares with one row missing. This ensured the eight pairs on each half-shelf formed a complete set of treatments. Additionally, each treatment appeared only once at each horizontal position along the shelf and pairs of treatments did not occur together more than once within each end of the house.

Statistical analyses were performed using the Genstat 5 computer package. Analyses of variance and regression analyses were performed on the data to identify relationships between the watering treatments and matric potential, as well as, matric potential and mushroom yield, and, matric potential and disease growth.

3.2.3 Results

3.2.3.1 Watering treatment effect on matric potential.

A range of casing moisture contents was successfully obtained following the eight different watering treatments (Figure 15a) giving rise to a directly comparable range of matric potentials (graphs not shown). The two driest watering treatments (x 0.25 & x 0.5) consistently had the lowest moisture contents (and matric potentials) throughout the duration of the experiment. The standard (x 1) and wetter treatments showed only small differences in moisture content up to Day 14 after casing, but larger differences from then on to the end of the crop (Figure 15a). The average matric potentials during the entire period of the experiment are given for each of the eight watering treatments in Figure 15b. The standard watering treatment (x 1), which was intended to provided an optimum matric potential (for mushroom growth) of between -9.4 and -7.9kPa, gave rise to a mean matric potential of –9.0 kPa. The x 1.5 watering treatment also) gave rise a matric potential close to the optimum (according to Noble *et al.*, 1999), of –9.0 kPa. Therefore, four watering treatments (x 0.25, 0.5, 0.75 and 1.25) generated matric potentials lower than the *A. bisporus* optimum, two treatments (x 1 and 1.5) matched the optimum, and two treatments (x 1.75 and x 2) produced higher matric potentials (i.e. wetter) than the *A. bisporus* optimum.

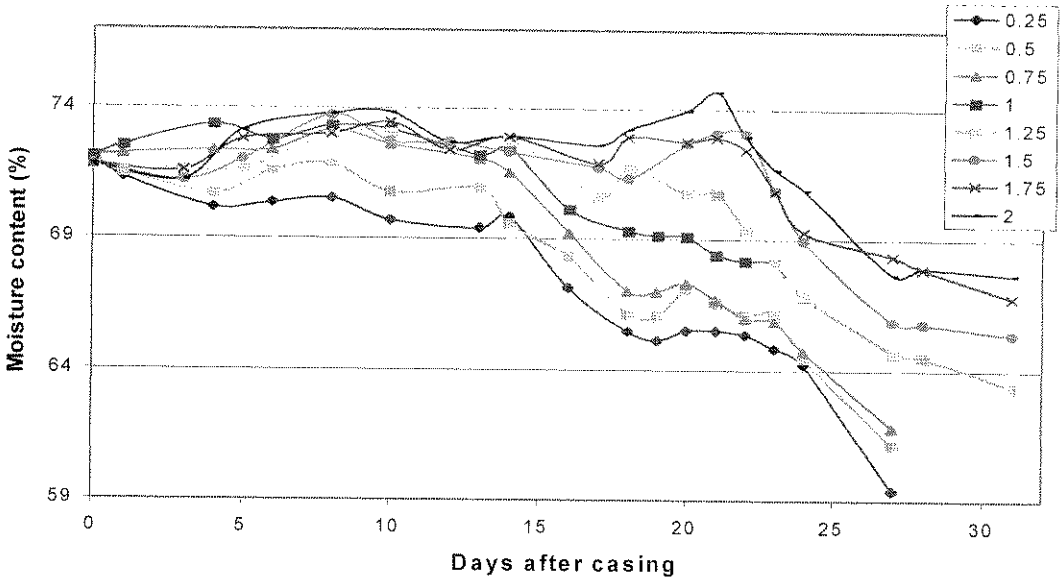


Figure 15a. Moisture content (%) of identical casing materials applied to spawn run compost but subjected to eight different watering regimes during a cropping period.

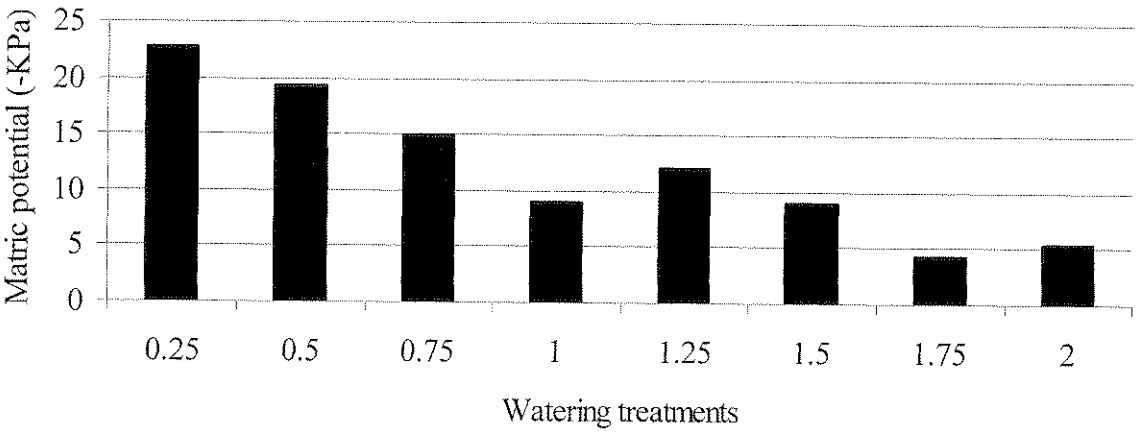


Figure 15b. Average casing matric potentials for the entire period of the experiment for casing receiving eight different watering treatments from very dry (0.25) to very wet (2).

3.2.3.2 Mushroom yield response to matric potential

The best average yield of 844 g/pot (241 g/kg from two flushes) was obtained from the x 1.75 treatment, which had an average matric potential of –4.4 kPa. The lowest yield of 644 g/pot (184 g/kg) was obtained from the x 0.5 treatment, which had a matric potential of –19.3 kPa (Figure 16a). These results firmly suggest a significant relationship between matric potential and yield and when individual yields for each replicate are plotted against individual matric potential values, there is a significant linear regression between the two variables (Figure 16b). The regression analysis indicated that 19.7% of yield could be accounted for directly by matric potential, however, the optimum matric potential appeared to be closer to –4.35 kPa than between –9.4 and 7.9 kPa as suggested by Noble *et al.*, (1999).

3.2.3.3 Growth of cobweb in response to matric potential.

Cobweb colonies developed in all inoculated pots during this experiment. Colonies began to establish more quickly in the four wetter treatments compared to the four drier treatments in the three days following inoculation. However, once colonies had become established by days 4 & 5 after inoculation, growth in the drier pots proceeded at a slightly faster rate than growth in the wetter pots (Figure 17a).

Average diameter growth rates of cobweb colonies between days 5 & 10 after inoculation ranged from 34.3 mm/day for the wettest treatment to 41.1 mm/day for the driest treatment (Figure 17b). Regression analysis of the relationship between average matric potential during the period of maximum cobweb growth (days 17 to 23) and growth rate indicated that 40.8% of the reduction in colony diameter growth rate could be explained by an increase in matric potential (Figure 17c). In other words, the wetter casings with relatively high matric potentials resulted in slower growth rates of the pathogen than drier casings with relatively low matric potentials.

3.2.4 Discussion

Using one casing formulation, and a range of watering treatments, it was possible to carry out an experiment on cobweb growth where the only variable was casing moisture content (and hence casing matric potential). The casing matric potentials of individual during the entire period of the crop ranged from –22.9 kPa in the driest treatment to –5.5 in the wettest treatment.

A significant relationship was found between casing matric potential and mushroom yield, supporting the findings of Noble *et al.* (1999). They suggested that the matric potential for optimum *Agaricus* yield was between –7.9 and –9.4 with higher and lower matric potentials resulting in yield reductions. The results from the experiment presented here indicated that optimum *Agaricus* yield was obtained at a matric potential of –4.4 kPa but this may be as a result of the small scale nature of this experiment.

Cobweb colonies developed on all casings irrespective of casing matric potential, which ranged from –32.0 to –0.7 kPa during the period of cobweb growth. However, the different watering treatments did have some effect on the growth and establishment of the pathogen. Cobweb colonies were slower to establish in the drier treatments with very low matric potentials compared with the wetter ones, but once established, they tended to grow at a slightly faster rate. Lane *et al* (1991) examined the growth of cobweb *in vitro* over a range of matric potentials and found that growth rate decreased steadily from 9.4 to 0 mm/day over the matric potential range of –0.48 to –4.97. This is in contrast to the quite substantial growth

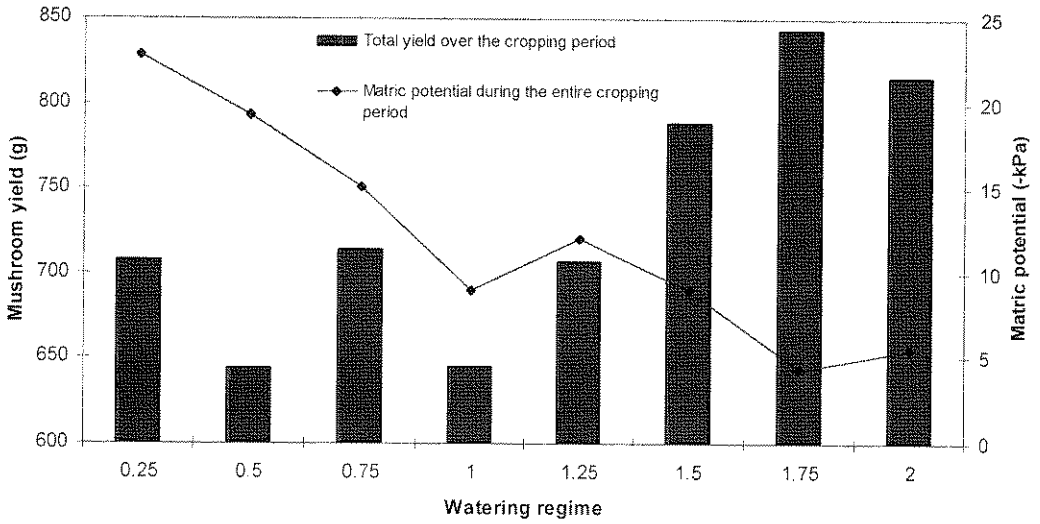


Figure 16a Mean mushroom yield (g) per control pot over the first and second flush of the crop for each watering regime. Mean matric potential (calculated over the entire experimental period) of each regime is also presented.

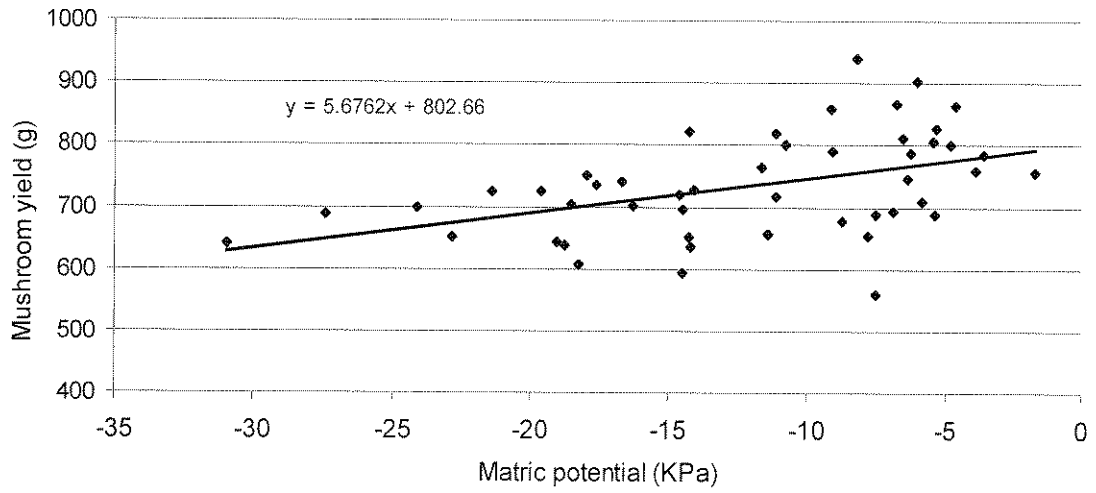


Figure 16b. Linear regression analysis of the relationship between mushroom yield and average casing matric potential for the entire duration of the crop. $r^2 = 0.20$.

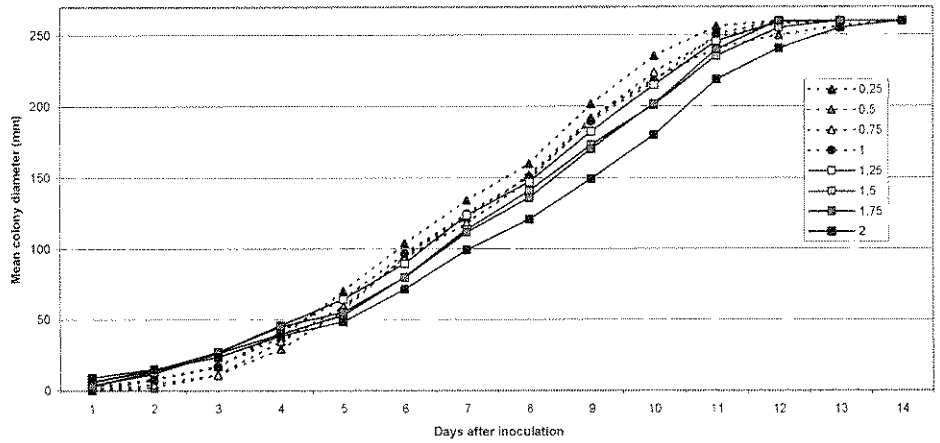


Figure 17a. Mean cobweb pathogen colony (isolate 192B1) diameter growing on each of eight identical casing layers receiving different quantities of water over a period of two weeks.

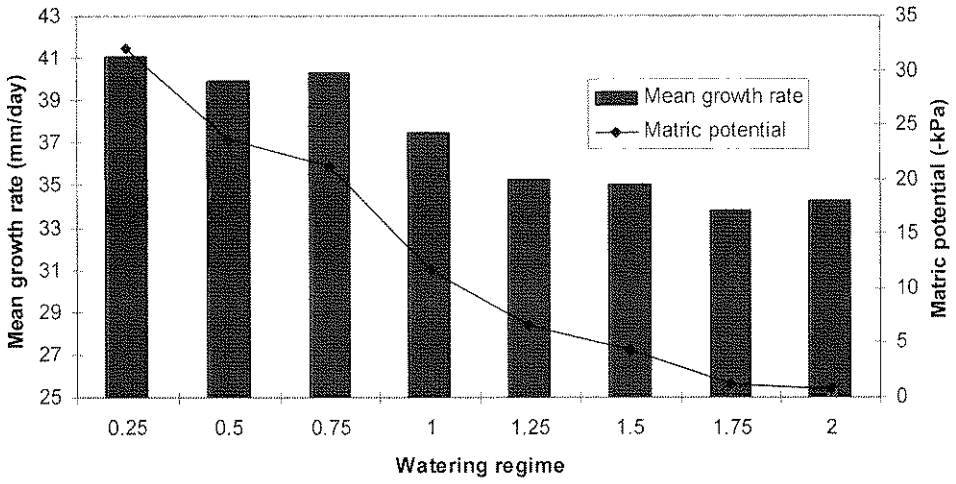


Figure 17b Mean cobweb pathogen colony (isolate 192B1) growth rate (diameter extension) for each watering regime. Mean matric potential of each treatment during the linear stage of colony hyphal extension is also presented.

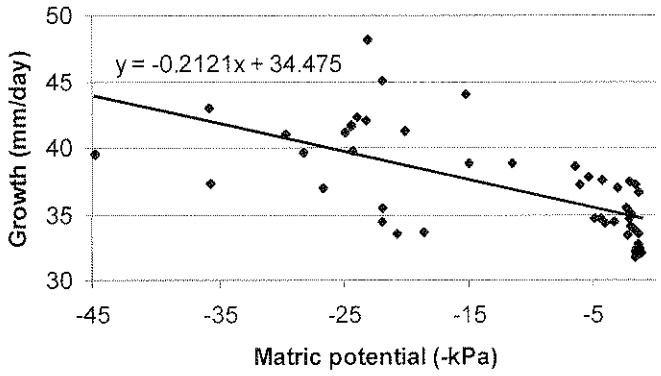


Figure 17c. Linear regression analysis of the relationship between *Cladobotryum* growth and average casing matric potential during period of maximum growth rate (between days 17 and 23). $r^2 = 0.41$

rates at much lower matric potentials reported here for *in vivo* conditions and confirms the conclusions of Magan (1988) who highlighted the incommensurable nature of *in vitro* and *in vivo* studies. Reducing the matric potential in casing to very negative values did not have the desired effect of preventing cobweb growth, as was suggested by the *in vitro* data.

The results presented in the previous section suggested that lower growth rates occurred in the drier casings with more negative matric potentials (Figure 14), a finding that was not confirmed in the experiment reported here. However, cobweb colonies did not establish very well in the previous experiment so any conclusions drawn would be less reliable as a result. In addition, that experiment was examining the effect of casing formulation on cobweb growth rather than matric potential, *per se*, so that results would not necessarily translate. Furthermore, a much smaller range of matric potentials were obtained in the previous study (–4.4 to –7.1 kPa) compared to this one so that again, extrapolation is unsound beyond that range.

The reduction of *in vivo* pathogen growth with increasing water availability demonstrated during this study, which contradicts the *in vitro* work of Lane *et al.* (1991), may be explained by water-logging (Dix and Webster, 1995). Whilst increasing matric potential can often facilitate more rapid fungal growth, excessive water can effectively 'drown' or 'suffocate' mycelium by impairing gaseous exchange. Although measures were taken to avoid water logging of the upper casing surface, such as applying the water in small doses throughout the day and evenly distributing the water over the entire casing surface, it was unavoidable in some of the wetter treatments. Some of these had so much water applied during the day that water logging inevitably occurred on the surface before all the water could be absorbed. Thus, pathogen hyphae colonising wetter treatments were subjected to periods of water logging that would have hindered growth. The wettest treatments obviously received the most water and thus the longest cumulative period of water logging. Disease colonies growing on the wettest casings were thereby knocked back most often and their growth was hindered the most as a result. However, the act of watering to the point of saturation has been shown to spread cobweb disease (Dar, 1997), therefore, whilst watering may slow cobweb disease growth it would ensure more disease colonies were established and thus counteract any benefits gained. Additionally, the application of excessive water might not only hinder cobweb disease growth but also, reduce mushroom yield.

3.2.5. Conclusions

- Cobweb colonies were capable of significant growth over a wide range of matric potentials in casings that were very dry to casings that were periodically waterlogged.
- Drier casings slowed down the establishment of cobweb while wetter casings led to rapid establishment
- Colony diameter growth rates were marginally higher in the driest casing, at 41 mm/day, compared to that in the wettest casing, at 34 mm/day.
- There is a significant negative relationship between casing matric potential and colony growth rate but only 40.1% of any decrease in colony diameter growth can be attributed to an increase in casing matric potential.
- Management of casing matric potential in a bulk peat and sugar beet lime casing mix is unlikely to have any major effect on cobweb control.

3.3 Dependence of cobweb disease pathogen growth on the presence of *Agaricus bisporus* in casing

3.3.1 Introduction

Cladobotryum dendroides has been described as both mycoparasitic, and saprophytic (Jeffries & Young, 1994). It has been isolated from many different organic substrates such as woodland soil, decaying wood, and moss, where it grows saprophytically. It is important therefore to establish whether or not *Cladobotryum* is capable of saprophytic growth on casing in the absence of *Agaricus*. Such information would establish whether or not stored casing could support the growth of cobweb, and hence serve as a source of contamination on-farm.

The objective of this section is to establish whether or not *Cladobotryum* can grow on mushroom casing in the absence of any *Agaricus*.

3.3.2 Materials and method

3.3.2.1 Experimental conditions

Three experimental treatments were set up consisting of the following:

Treatment 1: No *Agaricus* - unspawned phase II compost; No caccing in casing;
Treatment 2: Limited *Agaricus* – unspawned Phase II compost; Caccing in casing
Treatment 3: Normal *Agaricus* – spawn-run Phase II compost, Caccing in casing

Agaricus strain A12 (Sylvan) was used throughout. Either standard phase II compost or spawn-run phase II compost from the HRI mushroom unit was used. For each treatment, sixteen square pots measuring 110mm x 110mm across by 140mm deep were filled with 480g of compost, which was gently compacted, using the base of a spare pot. Casing was prepared using deep dug black peat supplemented with 15% sugar beet lime and sieved to remove large lumps. The 16 pots for treatment 1 were cased with this mixture, which contained no caccing. Caccing (Sylvan A12 casing inoculum) was added to the remaining casing at a rate of 42 ml for 20 litres. All pots were cased to the recommended depth of 45mm (Noble & Gaze, 1995).

3.3.2.2 Cropping procedure

All pots were placed in a Fisons growth cabinet (patent No. 812417) which allowed the control of temperature and RH. These parameters were set at 25°C and 96% RH. Two different temperature and RH probes were used to confirm the cabinet settings – Vaisala (Humidity and temperature indicator HMI 31) and Squirrel data logger (SQ8-4U) with Vaisala probe (HMP 314TH). After seven days, when *A. bisporus* mycelium was visible on the upper surface of the casing in Normal *Agaricus* treatment, the temperature and RH were reduced to 18°C and 89%, respectively, over a period of three days. Although the temperature was reduced during this period the RH within the cabinet failed to fall as predicted and would not drop below 92%. Despite this, pinning occurred as expected four days later. Water was applied to all pots when necessary according to visual and tactile assessment. Whilst the system was designed to mimic full scale production conditions wherever possible, higher than normal air speeds ensured casing dried more quickly, and, thus watering had to occur more

frequently. Water was applied using a plant sprayer that delivered 1ml per spray in the form of a fine mist. Mushrooms were harvested from one flush only.

3.3.2.3 Inoculation technique

When pinning in the Normal *Agaricus* treatment was complete, on the 13th day after casing, eight pots for each treatment were inoculated using isolate 192B1, following the technique outlined in section 2.4.2.3. The remaining eight pots in each treatment were uninoculated controls. These pots indicated the establishment of secondary disease colonies, which would mark the end of reliable primary disease colony measurements.

3.3.2.4 Data recording

Both disease examination and mushroom harvesting were conducted on a daily basis. Mushrooms were harvested when they had reached closed cup stage of development. Care was taken during harvesting not to disturb any areas of disease. Upon completion of harvest disease was assessed. As with previous experiments the longest diameter and the diameter perpendicular to that were recorded for each inoculated and control pot. The date sporulation was first observed was recorded for each disease colony. A visual assessment of sporulation intensity on a scale of 1, very light to 7, very heavy, was also made.

3.3.2.5 Statistical design

Within the growth cabinet the pots were arranged in a balanced incomplete block design following an extended 3x3(4) Latin Square to take into account any environmental fluctuations within the cabinet from back to front and from right to left. Thus, four pairs of pots (an inoculated and control) from each treatment were placed on the top shelf and four on the bottom. The inoculated pot of each pair was always positioned to the left of the control.

3.3.3 Results

3.3.3.1 Mushroom yield.

Only Treatment 3, containing spawn run compost and casing with caccing, produced any mushrooms (Figure 18). Treatments 1 & 2 produced no mushrooms during the experiment, as might be expected, since these treatments contained unspawned compost. Spotting symptoms were not observed on any of the harvested mushrooms until the last day of the first flush. The mushroom yield from inoculated pots for the first flush was less than half that of control pots indicating the effect of cobweb on yield.

3.3.3.2 Disease pathogen growth.

Only Treatment 3, with Normal *Agaricus* levels present, produced significant cobweb colonies following inoculation (Figure 19). Treatments 1 & 2, containing limited or no *Agaricus*, produced only very small colonies after inoculation, which failed to develop any further (Plate 5). These most likely developed as a result of residual nutrients in the inoculum plugs. Colony growth rate in Treatment 3 continued to increase attaining a maximum of 32mm/day between five and six days after inoculation. Growth then slowed as the confinements of the pots impeded further hyphal extension. Every colony which formed on casing, regardless of treatment, sporulated to some extent, even if only very lightly. Sporulation occurred earlier when no *Agaricus* or limited *Agaricus* was present, compared to

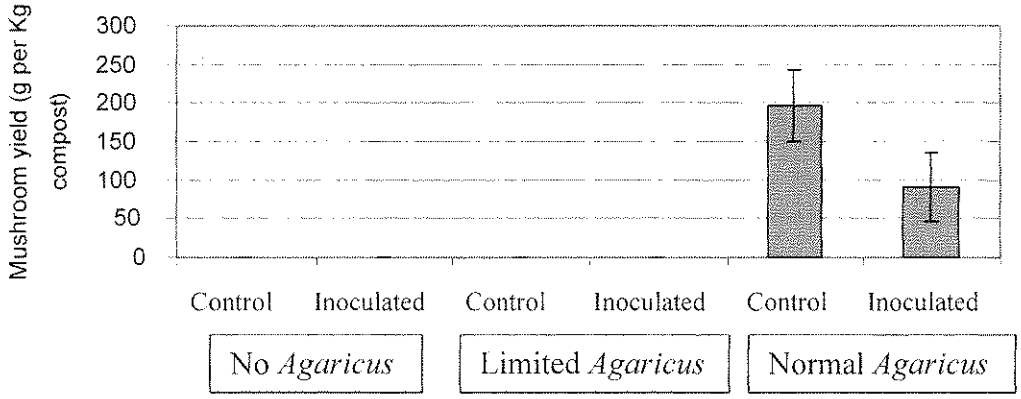


Figure 18. Mushroom Yield from control and inoculated pots from each treatment.

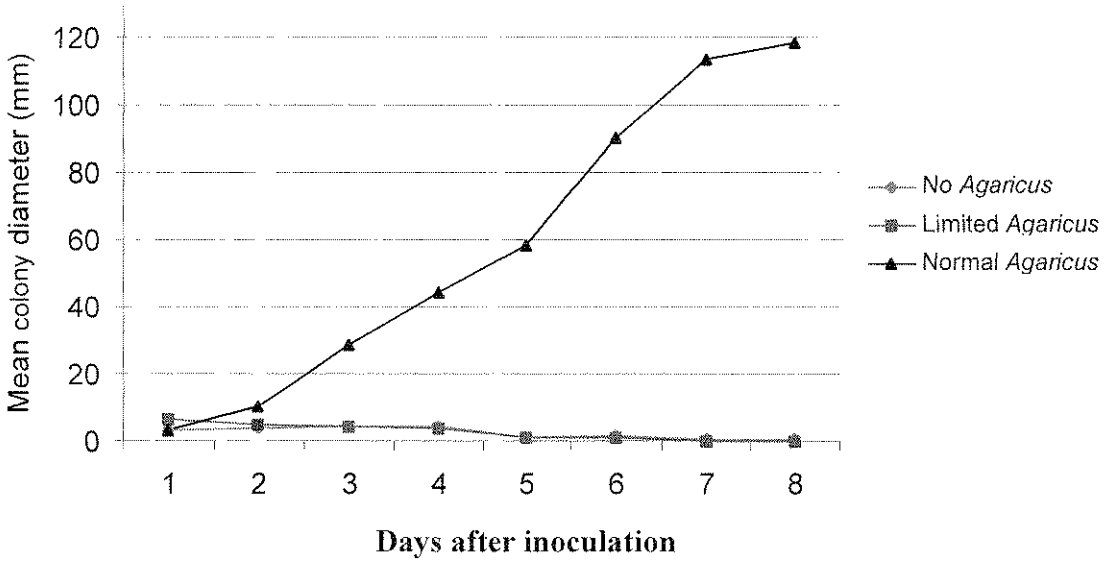


Figure 19. Cobweb growth on casing with different levels of *Agaricus bisporus*.

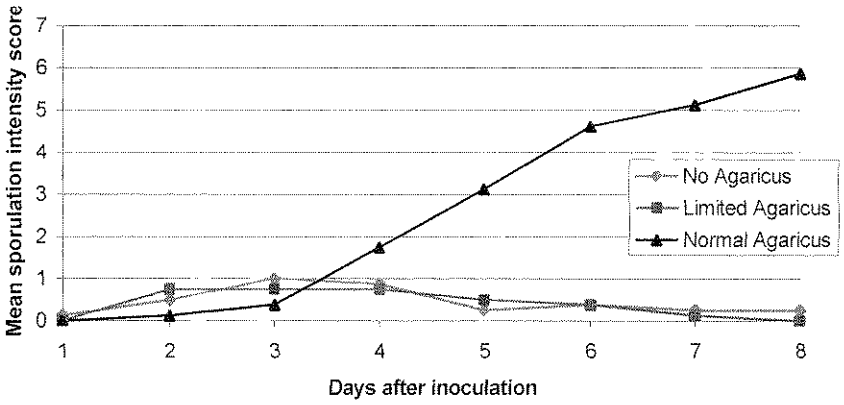


Figure 20. Sporulation intensity of cobweb colonies following inoculation of casing, containing different levels of *Agaricus*, with *Cladobotryum* isolate 192B1 (0-1= very light, 1-2 = light, 2-3 = light – moderate, 3-4 = moderate, 4-5 = moderate – heavy, 5-6 = heavy, 6-7 = very heavy).

Treatment 3, which had normal levels of *Agaricus* mycelium in the casing. However, sporulation intensity in the limited or no-*Agaricus* treatments was always very light (Figure 20). In addition, many of the colonies had disappeared by the end of the study, and were no longer sporulating. In contrast, when normal *Agaricus* levels were present, sporulation was initially delayed while colonies were growing, but once sporulation began, it became more intense as time progressed. Despite high levels of sporulation throughout the cabinet, strong air movements and continued watering of the pots, no colonies developed on the control pots. Spores were however liberated towards the end of the study as spotted mushrooms were harvested from control pots at the end of the first flush.

3.3.4 Discussion

Whilst other casing factors have been shown to have little effect on the development of disease symptoms through the casing layer the presence of *A. bisporus* mycelium has been shown to have a very significant effect. The results from this study suggest that the growth of cobweb colonies within the casing layer is greatly dependent upon the presence of *A. bisporus*. Cobweb growth over casing was rapid, and continued throughout the entire study period, only under the conditions associated with a normal *Agaricus* crop, however, *Cladobotryum* was unable to grow saprophytically when *A. bisporus* was absent. The small amount of pathogen growth on treatments with little or no *A. bisporus,* observed one or two days after inoculation, should not be confused with weakly saprophytic growth. This can be explained by the nutrient reserves held in the agar plugs used to inoculate the casing.

These results indicate that the relationship between *Cladobotryum* and *A. bisporus* is a parasitic one where *Cladobotryum* growth is dependent on *A. bisporus,* resulting in a reduction in mushroom yield.

3.3.5 Conclusions

- Casing is unable to support the growth of *Cladobotryum* in the absence of developing mushrooms.

3.4 Summary

The work described in this section on the biology of *Cladobotryum* in mushroom casing has revealed that *Cladobotryum* growth is not severely affected by either casing formulation or casing moisture content or matric potential. Despite a wide variety of experimental conditions, ranging from different peat sources and rates of sugar beet lime, to very wet or very dry growing conditions, Cobweb colonies still developed in all cases. Some minor effects on growth were detected such as 30% sugar beet lime rates and wetter growing conditions resulting in larger cobweb colonies. However, results were not clearcut and some ambiguity occurred. In a second experiment the establishement of cobweb colonies was quicker in wetter casings but subsequent colony growth was faster in drier casings.

Cladobotryum growth on casing was shown to be dependent on *Agaricus* with practically no growth or sporulation occurring in its absence. Thus, *Cladobotryum* spores landing on casing materials would not be capable of further growth. Such contamination of casing ingredients could however result in subsequent development of cobweb disease and it would be useful to know how long any such spores can remain viable and potentially infectious. This is an aspect of *Cladobotryum* biology that future work could focus on.

3.5 General Conclusions

- Statistical analysis of cobweb colony diameters at the end of the first flush suggest that the rate of sugar beet lime inclusion has a significant effect on the establishment of cobweb, with larger colonies occurring when high (30%) rates of sugar beet lime are used.
- Cobweb colonies were capable of significant growth over a wide range of matric potentials in casings that were very dry to periodically waterlogged.
- Drier casings slowed down the establishment of cobweb while wetter casings led to rapid establishment but subsequent growth rates were marginally higher in the drier casings (41 mm/day as compared with 34 mm/day).
- Management of casing matric potential in a bulk peat and sugar beet lime casing mix is unlikely to have any major effect on cobweb control.
- Casing is unable to support good growth and sporulation of *Cladobotryum* in the absence of developing mushrooms.

4 Conidia dispersal

Conidia are a common asexual means of propagation within the Ascomycetes. As exact copies of the parent they perform the role of a dissemination tool and are free to be dispersed by whatever means applicable as soon as mature (Soper, R, 1986). In many cases conidia are the predominant means by which an ascomycete disease spreads itself to infect new hosts or tissue. Cobweb disease of mushrooms, caused by *Cladobotryum* spp., (asexual forms of *Hypomyces* spp.), is no exception. *Cladobotryum* conidia are generally considered to be the major means of dispersal of this disease although other means of dispersal have been considered and remain possible means of disease transmission (Sinden, 1971; Atkins, 1974; Lane *et al*, 1991; Gaze, 1995(b); Gaze, 1995(c); Dar, 1997).

There is a general lack of knowledge regarding the epidemiology of cobweb disease, in particular knowledge regarding conidial dispersal and movement. Many studies have described the distinctive morphological characteristics of the conidia (Plate 6) in order to aid classification, but few have examined their biological significance or the manner in which they are liberated. Dar (1997) assessed water splash, water run off, sciarid flies, and air currents for their ability to disperse *Cladobotryum* conidia. He concluded that all four were capable of dispersing conidia but to various distances: water splash and air currents were considered to disperse conidia locally, to 40 and 75cm maximum respectively, whilst water run off and flies were considered capable of spreading disease further.

4.1 Conidial release pattern

4.1.1 Introduction

The importance of conidia to many plant pathogens as a means of dispersal has ensured that several have been studied in depth. Not only have dispersal mechanisms been investigated thoroughly but also movement of conidia within contained environments similar to mushroom cropping houses such as glass houses (Frinking, 1991; McCartney, 1991; Kerssies, 1993a; Kerssies, 1993b; Jenkinson & Parry, 1994; Pederson *et al*, 1994; Rodriguez *et al*, 1996; Madden *et al*, 1996; Lacey, 1996; Ntahimpera *et al*, 1997; Williams *et al*, 1998). However, few studies exist relating to either the cobweb pathogen conidial liberation or even general conidial liberation within the somewhat unique environment of a mushroom cropping house. Gandy (1972) briefly investigated the dispersal of *Verticillium malthousei* conidia within a mushroom crop, and as previously described, Dar (1997) highlighted potential means of cobweb disease dissemination within a growth room.

Because conidia are passively liberated, that is to say the fungus does not actively eject them, dispersal vectors such as air currents, water splash, invertebrates and vertebrates are required to liberate and/or disseminate them. One of the most common dispersal vectors is air movement. Airborne dissemination was chosen for study during this investigation over other dispersal vectors highlighted by Dar (1997) for several reasons.

Most importantly, experience of working with the disease suggested airborne dispersal was possibly an important means of conidial dissemination. Not only were conidia produced terminally on erect hyphae, but also conidia have been observed as being released into the atmosphere when a sporulating area of disease was disturbed. A significant release and dispersal of conidia by air currents would explain the occurrence of severe spotting symptoms

in the absence of heavy fly infestation. Additionally, conidia from many other pathogens have been shown to be both liberated and dispersed by air currents. For example, Chastanger *et al* (1978) found wind speeds of 0.11 m/sec (comparable to those recommended for mushroom cultivation) were sufficient to disperse conidia of *Botrytis cinerea*.

It was the aim of this study therefore to monitor conidial liberation and airborne dispersal within a mushroom crop infected with cobweb disease. Continuous monitoring of the conidial load in the air should identify patterns of conidial dispersal that can be cross-referenced to records of cropping operations and movements within the house.

4.1.2 Materials and methods

4.1.2.1 Spore trapping

Cobweb conidial movements within a crop of mushrooms was studied with the aid of a Burkard Seven-Day Recording Volumetric Spore Trap. This style of spore trap draws air through a fixed aperture (2x14mm) and over a strip of adhesive cellophane tape attached to a cylinder that revolves once every seven days (2mm/hour). Particles contained in the air, i.e. conidia, dust, etc, are impacted onto the tape where they remain fixed in the adhesive, providing a chronological record of air contaminants.

The advantages of the Burkard spore trap over many other styles include:-

- it allows continuous sampling of the atmosphere over a seven day period
- conidial numbers/m^3 of air can be calculated because airflow and aperture dimensions are known
- the occurrence of any one spore can be attributed to a specific period of time enabling its occurrence to be correlated to other activities
- trapped spores on the cellophane tape can be permanently slide-mounted and therefore examined at leisure.

During the course of the experiments described in this section, the airflow through the aperture of the Burkard spore trap was set at 10 litres/min. The spore trap was placed on the floor of the mushroom house, facing the back wall, immediately after the crop was aired and prior to inoculation of the crop with cobweb. The spore trap was maintained throughout the cropping period, which involved changing the battery, monitoring and adjusting airflow, winding the clockwork mechanism, and changing the tape every seventh day.

The cellophane strip was removed from the cylinder after seven days and cut into daily sections measuring 48 mm in length. Each daily section was mounted between glass slides and cover slips in a mounting gel containing 100 ml distilled water, 50 ml glycerol, 35 g 'Gelvatol', and 2 g phenol. Sections were then examined at 400x magnification. Several passes, each 0.5 mm wide, were made along the length of every slide and the distance of all *Cladobotryum* conidia from the start of the tape was recorded. The distance (mm) from the start of the tape was then converted into time with 2 mm equating exactly to 1 hour. For a given period of time (e.g. 15 minutes, 24 hours) the total number of conidia trapped was calculated as follows:

$$O^n \times \left(\frac{W^t}{W^s} \right) = T^n$$

where O^n = number of conidia observed for a given time period; W^t = total width of tape (mm); W^s = width of tape sampled (mm); T^n = total number of conidia trapped for a given time period.

This figure can then be further transformed to show the number of conidia/m^3 air as follows:

$$T^n \times \left(\frac{1}{V} \right) = N$$

where T^n = total number of conidia trapped for a given time period; V = volume of air sampled during that time period (m^3); N = number of conidia/m^3 of sampled air.

The number of conidia/m^3 of air was calculated for each 15 minute interval, or averaged for every 24 hour period.

4.1.2.2 Crop management

A mushroom crop was established and managed as outlined in sections 3.1.2.1, 3.1.2.2, 3.1.2.3, and 3.1.2.4. Half the pots were inoculated with the thiabendazole-resistant *Cladobotryum* isolate 192B1. Disease measurements were taken as outlined in section 3.1.2.5. In addition, a record was kept of entry and exit times, and the time and duration of every cropping operation.

4.1.3 Results

The mean number of conidia trapped/m^3 air varied on a daily basis (Figure 21). The first conidia were trapped on the 15th day after casing, the same day that disease was first observed and only four days after inoculation with the disease. During the early stages of cropping the mean numbers of conidia trapped were very low at <10 conidia/m^3, however, this increased dramatically after the 1st flush. On the first day after the 1st flush (the 23rd day after casing), and coinciding with a salting operation to reduce the disease area on the casing, over 1000 conidia/m^3 of air were trapped. The numbers of conidia trapped/m^3 of air remained high until the onset of the second flush when again the numbers fell to <10 conidia/m^3, despite a significant increase in the area of disease present on the casing. Interestingly, no conidia were recorded at all on the 31st day after casing - the final day of the 2nd flush.

The effect the number of airborne conidia had on the incidence of spotting can be observed in figure 22. When studied in conjunction with figure 21, it shows how the number of conidia trapped/m^3 of air related to the relative number of spotted mushrooms harvested. Spotted mushrooms were not observed in any numbers until the final two days of the flush (21 and 22 days after casing). For example, on the final day of the 1st flush, approximately 50 % of the mushrooms harvested from both inoculated and control pots were spotted. Thus, it appeared the low levels of airborne conidia witnessed prior to the 1st flush were sufficient to cause sizeable losses during the later stages of the 1st flush. This suggested a lag period of about

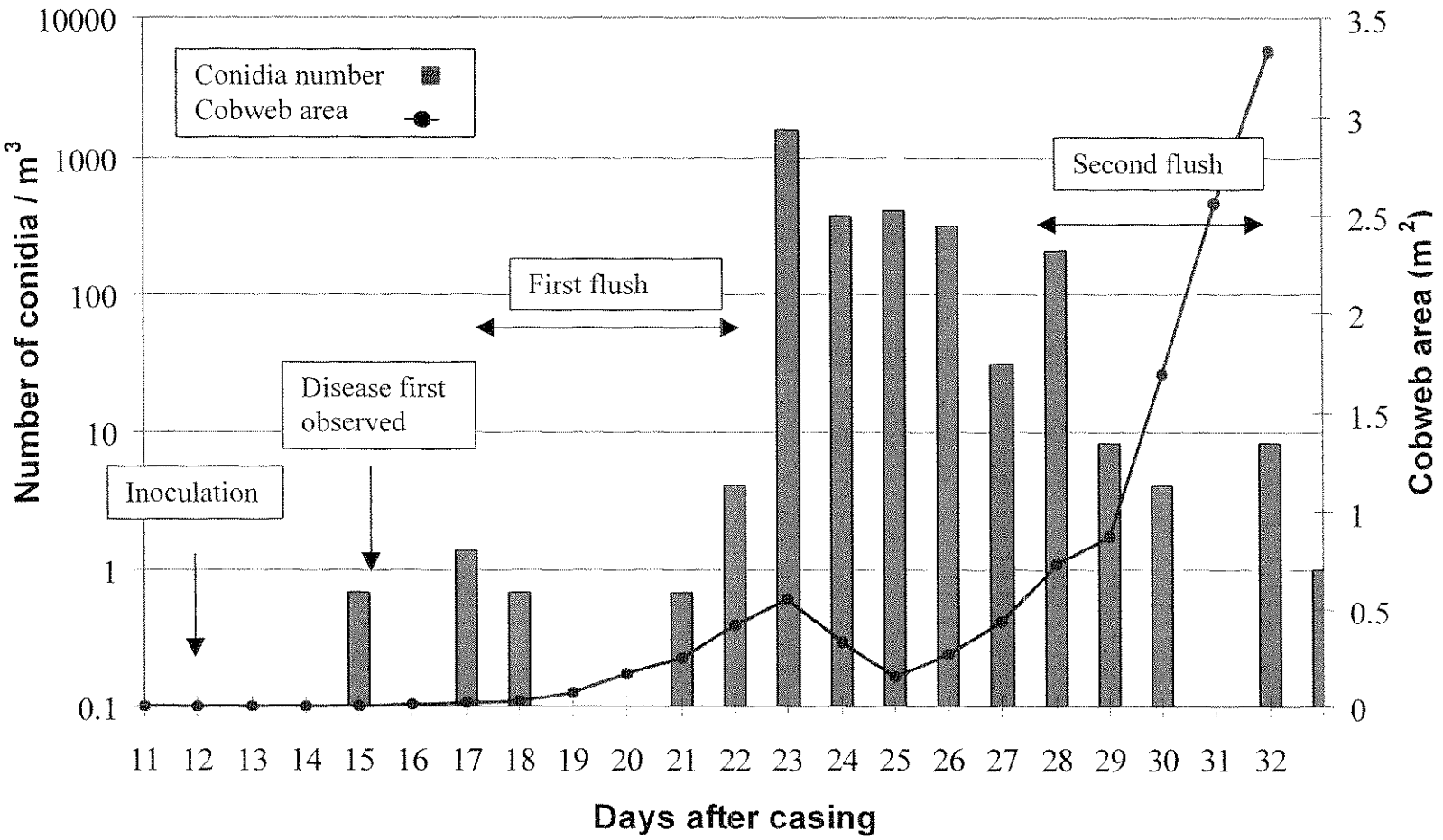


Figure 21. Number of *Cladobotryum* conidia trapped using a Burkard spore trap, and area of cobweb disease developing on casing following inoculation of a mushroom crop.

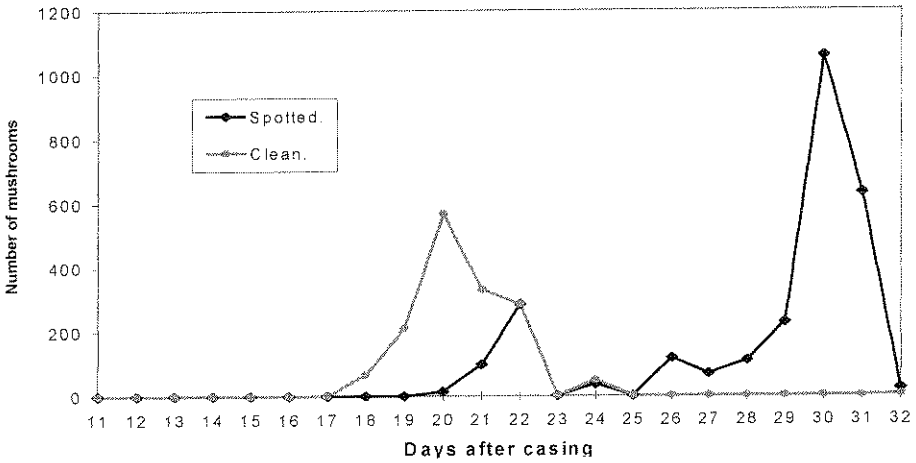


Figure 22. Number of clean and spotted mushrooms harvested over two flushes from an intentionally cobweb infected crop.

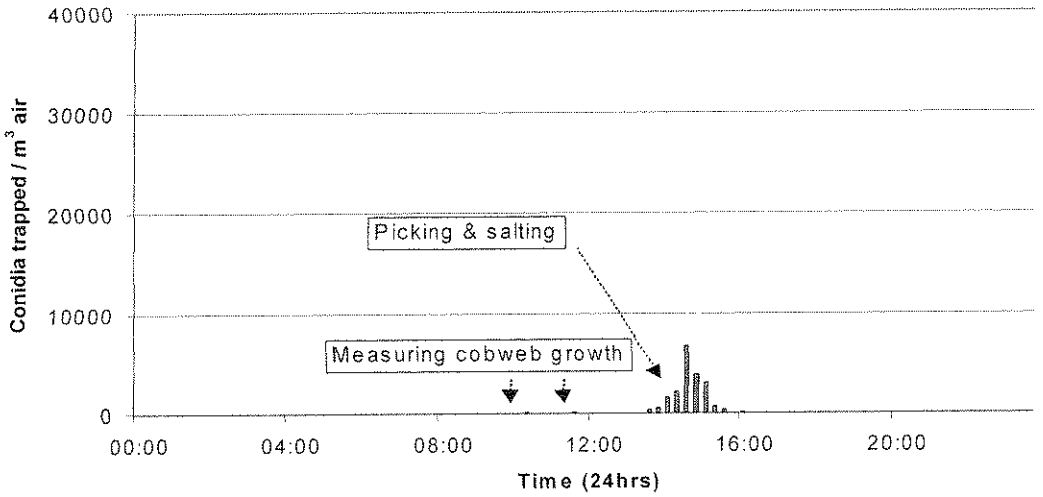


Figure 23. Number of cobweb pathogen conidia (strain 192b1) trapped per m^3 of air every 15 minutes on the 28th day after casing.

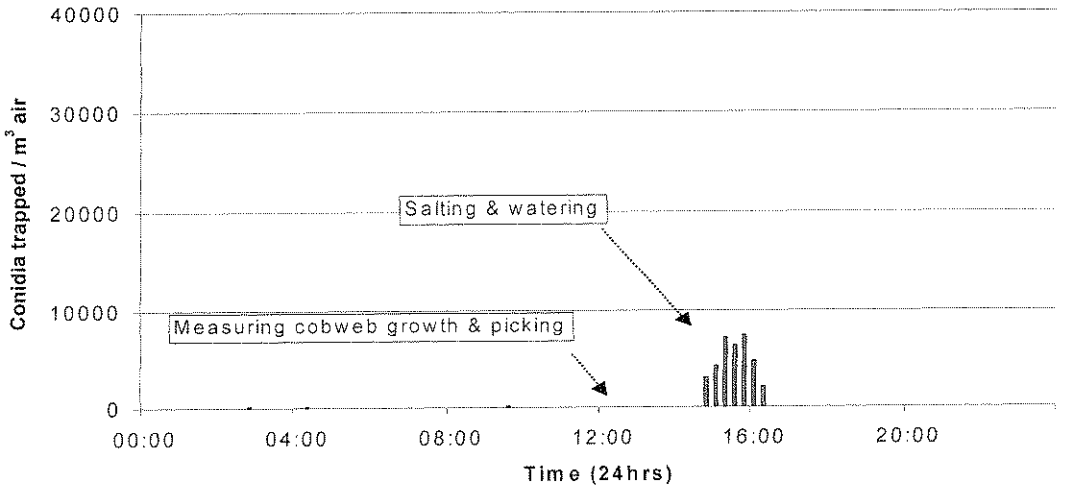


Figure 24. Number of cobweb pathogen conidia (strain 192b1) trapped per m^3 of air every 15 minutes on the 24th day after casing.

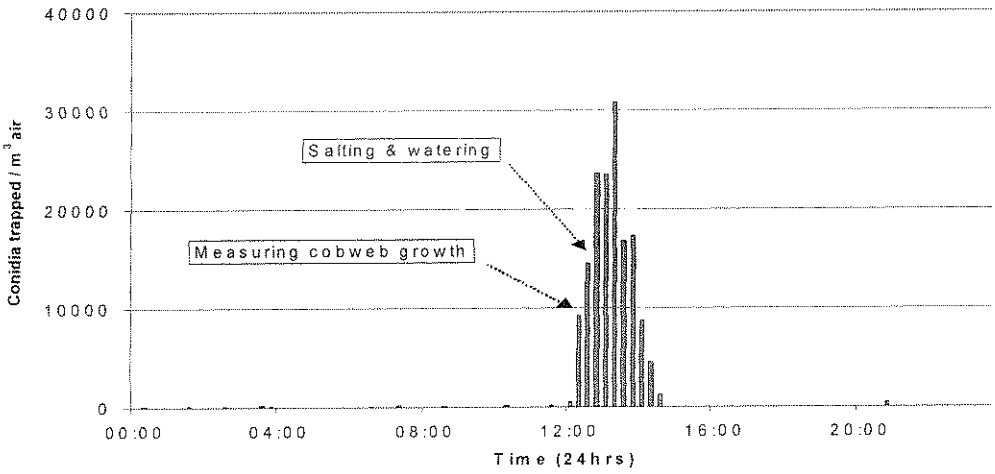


Figure 25. Number of cobweb pathogen conidia (strain 192b1) trapped per m^3 of air every 15 minutes on the 23rd day after casing.

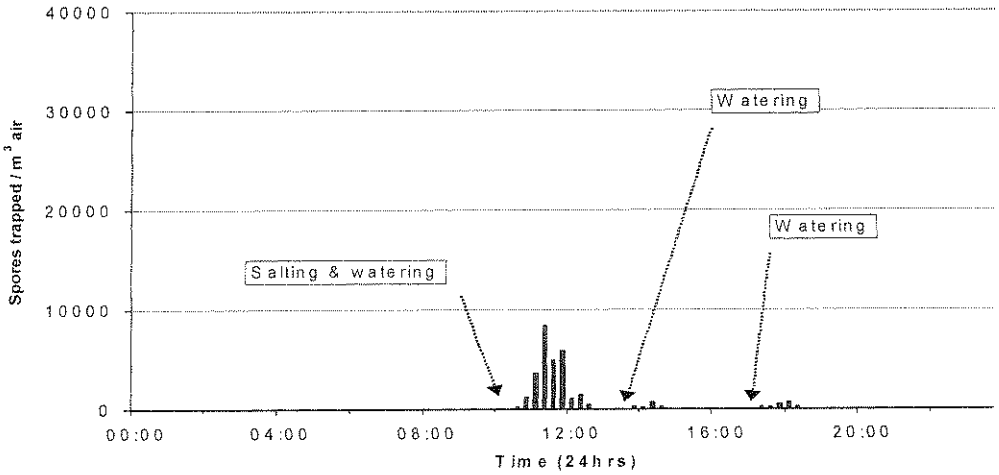


Figure 26. Number of cobweb pathogen conidia (strain 192b1) trapped per m^3 of air every 15 minutes on the 26th day after casing.

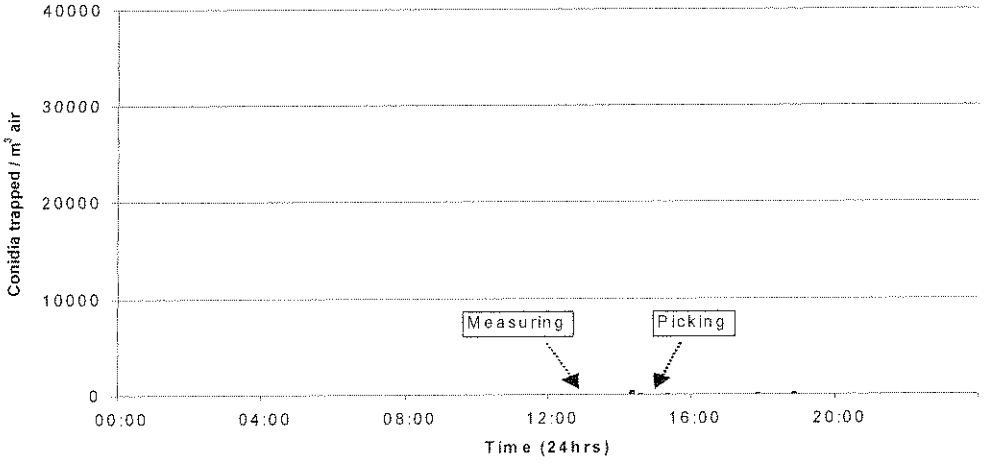


Figure 27. Number of cobweb pathogen conidia (strain 192b1) trapped per m^3 of air every 15 minutes on the 29th day after casing.

four days existed from the time the conidia were liberated to the development of spotting symptoms.

All mushrooms harvested during the 2nd flush were spotted. This followed six days of heavy conidial loading of the air (Figure 21 & 22). In addition, spotting symptoms were more intense. Whereas spotted mushrooms from the 1st flush had only one or two spots per cap, 2nd flush mushrooms had far more (10+), indicating the higher spore load in the air during the interflush period.

When the spore-trap data was presented on a 15-minute basis rather than a 24-hour basis it was evident that the conidial loading of the air was not constant. For the best part of each day the air was relatively free of conidia, however, occasionally large numbers of conidia were liberated into the air (Figures 23, 24, 25, 26). The number of conidia trapped rose sharply, peaking after around 1 hour before falling with equal rapidity. The whole period of conidia build up and settling out took no more than three hours and often less than two (Figure 24). Conidia numbers during one of these events increased to over 30,000/m^3 which equated to around 2.5 million within the cropping house (80m^3 total) during that 15 minute period (Figure 25). Although such high numbers of conidia were unusual, 5,000 - 10,000 conidia/m^3 was not (Figure 23).

The peaks of airborne conidia occurred when certain cropping operations were carried out. Whilst the relatively low background conidia loading of the air didn't seem to follow any discernible pattern, the large peaks of loading always coincided with a period of salting, watering, or both. This is most obvious on day 26; one period of salting and watering and two periods of watering alone were each closely followed by a period of conidial loading of the air (Figure 26). The first application of salt, followed by watering, liberated the majority of conidia observed that day, with the subsequent two applications of water liberating fewer conidia.

Measuring and picking did not liberate as many spores as salting and watering. Relatively few conidia were liberated during the 29th day after casing when measuring and picking took place but salting and watering did not (Figure 27).

4.1.4 Discussion

In contrast to the findings of Gandy (1972) when studying *Verticillium malthousei* the seven-day volumetric spore trap has proved to be a useful tool for the monitoring of *Cladobotryum* conidia within a mushroom cropping house. Due to the relatively contained nature of the mushroom cropping house a limited range of spores was trapped (Plate 7). Only *A. bisporus* spores, *Penicillium* spp., and cobweb pathogen conidia were trapped in any great numbers. Thus, identification was simplified. Identification was also helped by the distinct nature of cobweb pathogen conidia. Cobweb pathogen conidia are relatively large and septate, whereas *A. bisporus* and *Penicillium* spp. spores are smaller and non-septate. Identification of cobweb pathogen conidia, despite their distinct appearance, was however not always simple. During cropping periods *A. bisporus* spores were liberated, and trapped in such numbers they made identification of any other spores difficult, regardless of size or distinct appearance (Plate 8). One further point to be made with regards to this technique's suitability for this purpose is that conidial viability cannot be assessed. It can therefore not be categorically stated that each conidium observed had the ability to produce a new colony or spotting symptoms.

The importance of early disease-identification and containment in reducing the severity of disease symptoms was demonstrated clearly. Small, young disease colonies were shown to liberate enough conidia to cause quite significant spotting symptoms in the first flush. Also quite evident is the fact that if larger areas of sporulating disease colonies are watered over, it can cause the liberation of literally millions of conidia, leading to massive spotting. However, somewhat more worrying, is the discovery that salting, a process employed to contain this disease has a propensity to exacerbate the problem. The conidia liberating capabilities of this operation were highlighted by peaks of trapped conidia following salting, but also by a lack of airborne conidia if no salting was carried out. For example, during the 2nd flush when salting and watering ceased, a large reduction in the number of conidia liberated into the atmosphere was observed, despite the fact that the area of cobweb on the casing was increasing considerably. Consequently, the development of a non-disruptive but effective means of treating disease colonies on the casing is required.

4.1.5 Conclusions

- *Cladobotryum* conidia are liberated into the air in great abundance when disease colonies are disturbed by watering or salting without any protection
- Picking operations and other activities in the cropping chamber do not result in significant numbers of conidia being released.
- The vast majority of conidia settle out within a few hours of liberation
- Even low numbers of conidia/m^3 of air can cause significant spotting symptoms

4.2 Spatial dispersal of cobweb disease pathogen conidia

4.2.1 Introduction

Several factors will affect the distribution of conidia within a mushroom cropping house including strength and direction of air movements, density of conidia, and aerodynamics of the conidia (Ingold, 1965). Of these factors it is the strength and direction of air currents which may vary most significantly during a crop and thus having the greatest effect on the distribution of conidia. For example, cobweb conidia of any given strain will remain relatively consistent with regards to size and shape whereas air currents can be far more variable. The strength and direction of air currents can not only change with an alteration of the fan speed but also with an alteration of house layout such as the movement of shelves or other equipment, the opening of doors, etc.

The objective of this study is to elucidate spatial patterns of cobweb conidial dispersal within a mushroom house. Several alternative means of trapping conidia will be examined and the effect of shelving on spore dispersal will also be investigated.

4.2.2. Materials and methods

4.2.2.1 Trapping techniques

Four trapping techniques were used to monitor conidial numbers in a mushroom cropping house where *Cladobotryum* conidia were released from a single cobweb colony. The house contained a three-shelf unit and was laid out as shown in Figure 28.

a) Burkard Seven-Day Recording Volumetric Spore Trap
The setting up, maintenance and analysis of the spore-trapping tape from the Burkard spore trap followed the protocol outlined in section 4.1.2.1. The trap was activated the day before conidial liberation and allowed to run for the duration of the experiments (3 days).

b) Rotorods
Rotorod traps were developed by Perkins in 1957 and are based on the principle that small airborne particles are deposited with high efficiency on narrow cylinders (Gregory, 1951). The trap relies on two narrow vertical metal arms rotating quickly around an axis (Figure 29). The leading edge of each arm is covered with transparent tape coated with a petroleum jelly based adhesive (similar to that used for the Burkard spore trap) that traps small airborne particles and which can be later removed for microscopic examination.

Revolving at a constant 3100 rpm, the elastic properties of the brass arms ensures the vertical uprights rotate at 90° to the horizontal arms. After three hours of sampling the rotorods were stopped and the adhesive tape mounted onto a glass slide. Sterile forceps and scalpel were used to carefully remove the adhesive tape covering the leading edge of each arm, which because still tacky could be mounted directly onto a glass slide. Each length of tape was then covered with mounting gel (section 4.1.2.1) and cover slip. Following drying of the mounting gel the slides were microscopically examined. The number of conidia trapped per unit area of tape was calculated and recorded for each trap position.

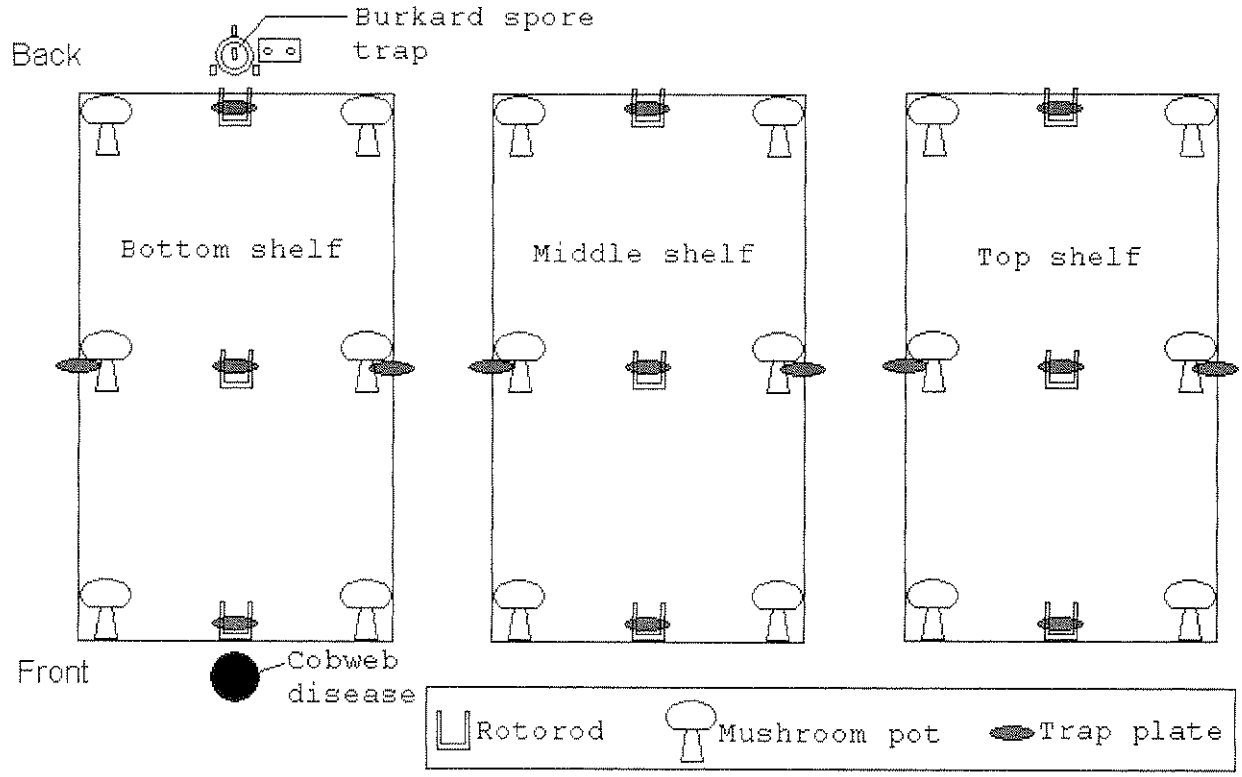


Figure 28. Distribution of conidial traps on each of three shelves and position of cobweb disease and Burkard spore trap within the experimental mushroom house.

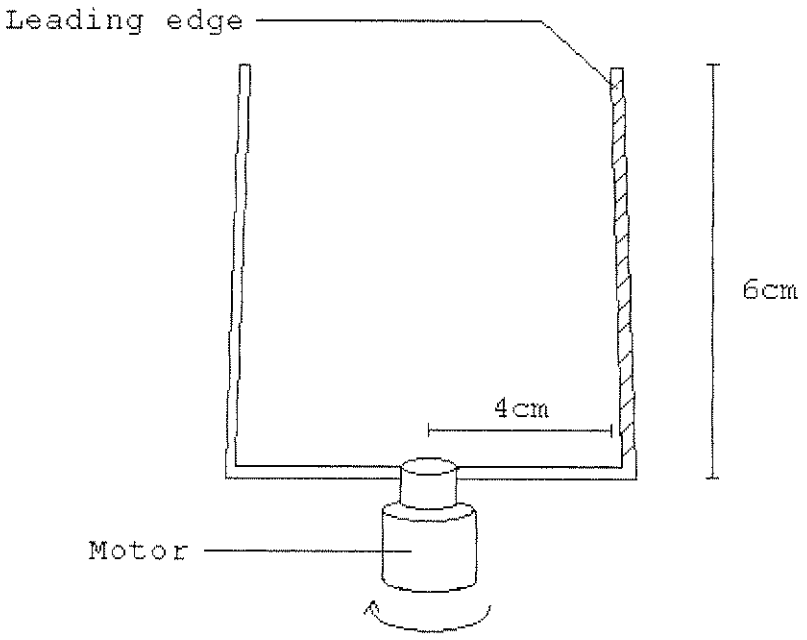


Figure 29. Rotorod apparatus used to trap cobweb conidia from the air at several trap positions within a mushroom house.

c) Mushrooms

Pots containing first-flush mushrooms at the small closed-cup stage of development, were used to provide information on the pattern of spotting symptoms which would develop, following the liberation of conidia within a cropping chamber. Immediately prior to the liberation of conidia, six pots of growing mushrooms were positioned on each of the three shelves as indicated in Figure 28. The diameters of all developing mushrooms at each position on each shelf were recorded and the mushroom surface-area for that position was calculated. The number of spots that subsequently developed was expressed per unit area of mushroom at the time that conidia were liberated.

d) Trap plates

Malt Extract Agar (MEA)(3% Oxoid) was prepared and sterilised according to the manufacturers recommendations. Upon cooling to 50°C, an aliquot of 1000 ppm liquid thiabendazole was added to the MEA to give a final concentration of amended MEA of 100 ppm thiabendazole. The amended MEA was then dispensed into Petri dishes and allowed to solidify overnight.

Before liberation of the cobweb conidia took place, 15 thiabendazole-amended plates were placed on the shelves as indicated in Figure 28 and marked with the position and time of exposure. Immediately prior to liberation of the cobweb conidia the plates were uncovered in a specific order and the time noted. Exactly 15 minutes later the exposed plates were covered (following the same order as for uncovering) and replaced with fresh thiabendazole-amended plates. These were also exposed immediately in the same order as the first plates and had also been marked with the position and time. Fresh plates were exposed every 15 minutes for three hours, giving12 trap plates for each position. All exposed plates were incubated at 25°C. Any emerging fungal colonies were recorded on a daily basis and identified.

4.2.2.2 Conidial release

The liberation of cobweb conidia was brought about by introducing a pot of mushroom compost with developing first flush mushrooms, on which had been established a colony of cobweb measuring 150 mm diameter. The conidia were released by watering the cobweb colony using a tri-nozzle hose attachment that dispensed 40ml of water over a period of 4 seconds from a height of 30cm.

4.2.2.3 Shelving effects

Two experiments were carried out in order to ascertain the effects of shelving on the movement of air, and hence conidia, around the house. In the first experiment, conidia were released in an empty house with open shelves, allowing the free movement of air through the aluminium mesh shelves. In the second experiment, each shelf was covered with plastic sheeting simulating the presence of a mushroom crop. The various traps were positioned for each experiment as described in 4.2.2.1 and the two experiments were carried out on consecutive days.

4.2.3 Results

4.2.3.1 Individual trapping technique results

a) Burkard spore trap

Conidial peaks similar to those described in the previous section were clearly distinguishable at around the time of the controlled conidial liberation for both the open and closed shelf systems i.e. 08:10hrs on both days (Figure 30). Whilst the open-shelf peak coincided exactly with the liberation of conidia the closed-shelf peak appeared to occur slightly after the conidia were liberated (about 15-30 minutes). Additionally a smaller peak occurred just before the main peak for the closed-shelf experiment. This smaller peak occurred about 30-60mins before the controlled release of conidia and it coincides with the time when the pot containing the cobweb colony was brought into the house.

b) Rotorods

Conidial numbers trapped on each shelf from the open-shelf system followed the same broad pattern. The highest numbers of trapped conidia occurred close to the source with the bottom shelf trapping the highest number of all. As the distance from the source increased however, there was virtually no difference between shelves in the number of conidia trapped (Figure 31). This contrasted with the closed-shelf system, where there was very little difference in the number of conidia trapped with increasing distance from the source of liberation (Figure 32).

c) Mushroom trap crop

Mushroom spotting was heavy throughout the house following liberation of conidia in both open-shelf and closed-shelf systems (Figures 33 – 38). The number of spots per cm^2 of mushroom tissue in any single trap position was never less than 1.5 and frequently greater than 4.

d) Trap plates

The vast majority of conidia (≅90%) were trapped in first 15 minutes after liberation in both the open-shelf and closed-shelf systems (Figure 39). Each subsequent 15-minute period demonstrated an approximately ten-fold reduction in the number of conidia trapped. An hour and a half after liberation almost all conidia had settled out of the air in both systems. Numbers of conidia trapped in the open-shelf system were consistently lower in comparison to the closed-shelf system. This may be a reflection of the colony from which conidia were liberated rather than an effect of the two shelf systems examined.

Conidia were trapped at all positions on all shelves in both the open-shelf and closed-shelf systems (Figures 40 – 45). In the open-shelf system, there was a clear, though sometimes small, reduction in the number of conidia trapped with increasing distance from the source (Figures 40, 41 & 42), similar to the rotorod data in Figure 31. The number of conidia trapped with increasing distance from the source in the closed-shelf system was more homogenous (Figures 43, 44 & 45), again similar to the rotorod data.

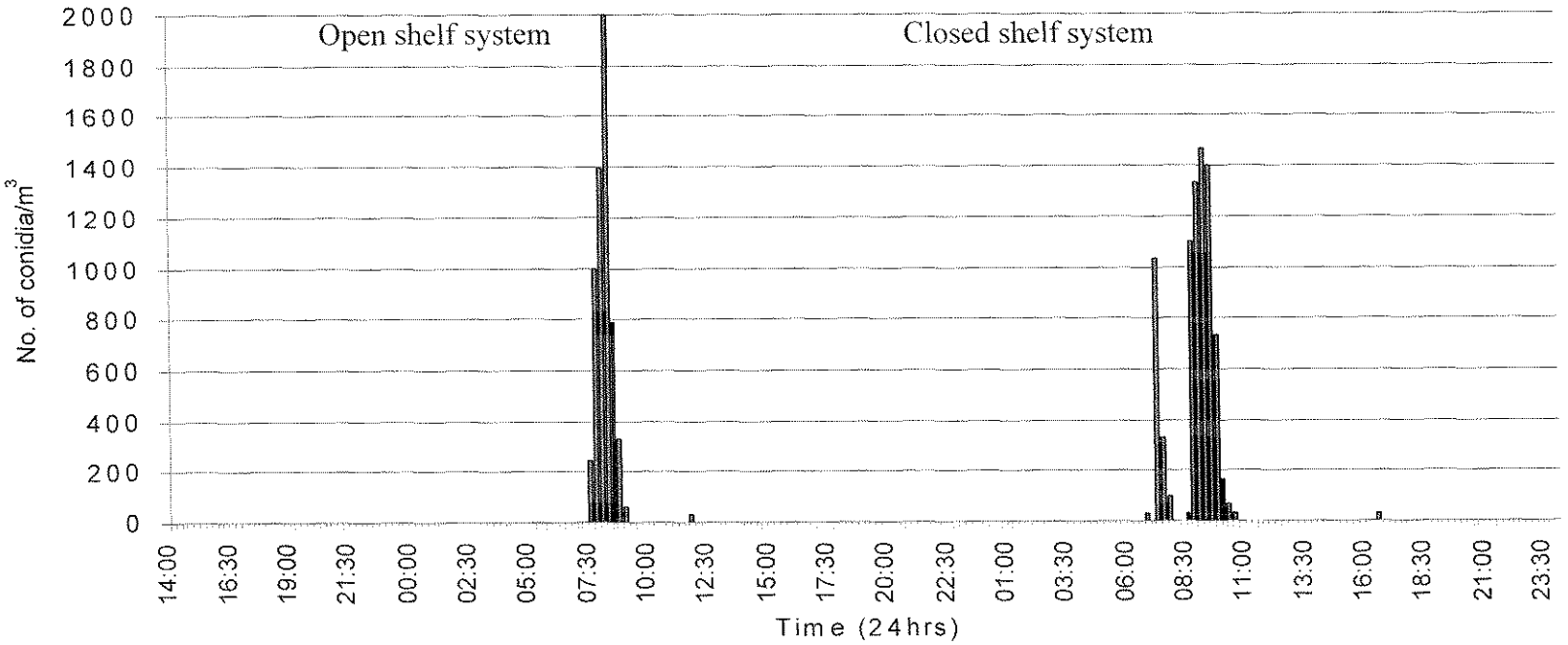


Figure 30. Number of cobweb pathogen conidia (isolate 192b1) trapped in 15 minute periods by the Burkard Seven-Day Recording Volumetric Spore Trap following two controlled liberations at 08:10hrs on two consecutive days.

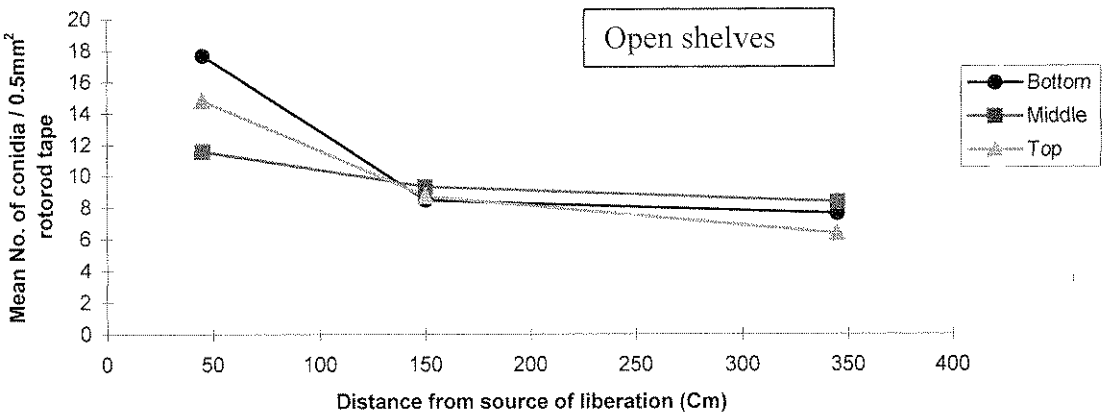


Figure 31. Number of cobweb pathogen conidia trapped using rotorods at different distances and heights from the point of conidial liberation in an open shelf system.

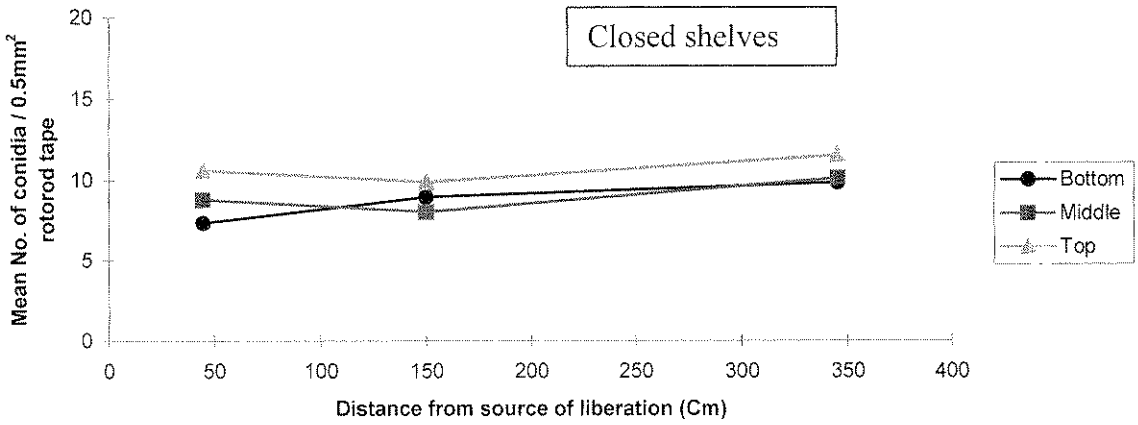
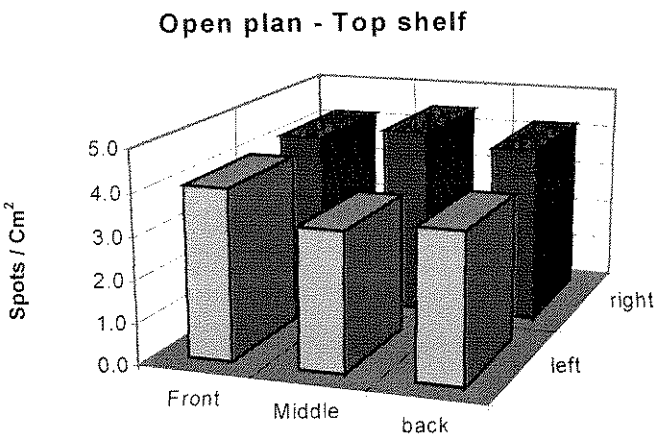
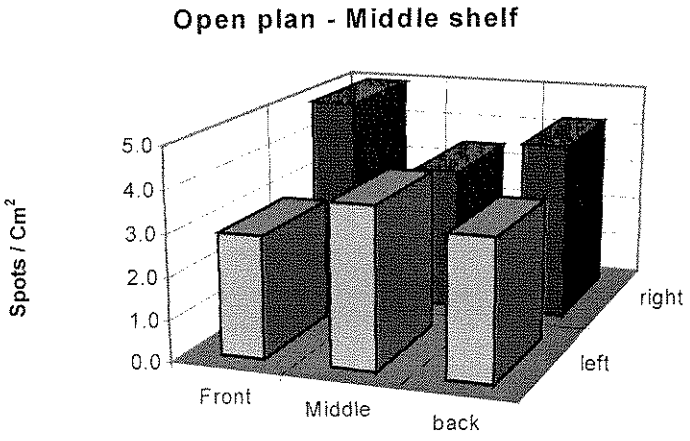
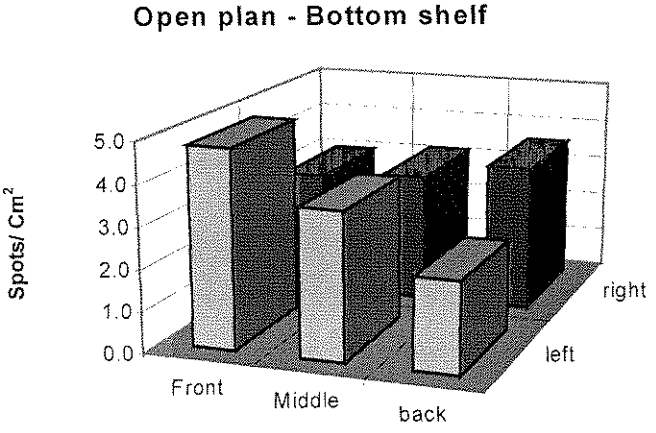


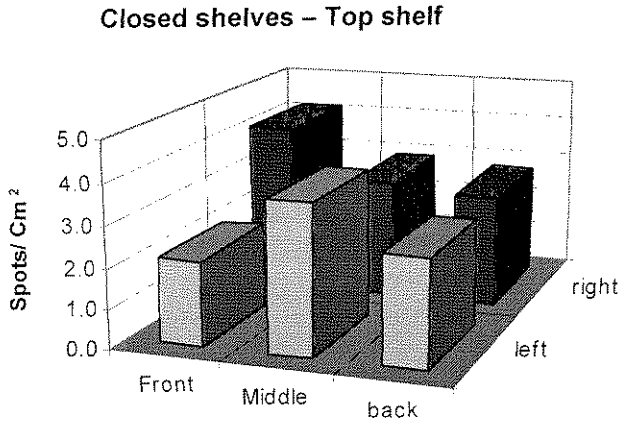
Figure 32. Number of cobweb pathogen conidia trapped using rotorods at different distances and heights from the point of conidial liberation in a closed shelf system.

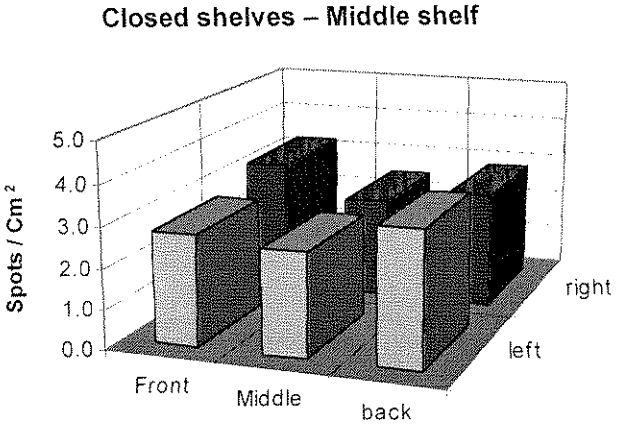


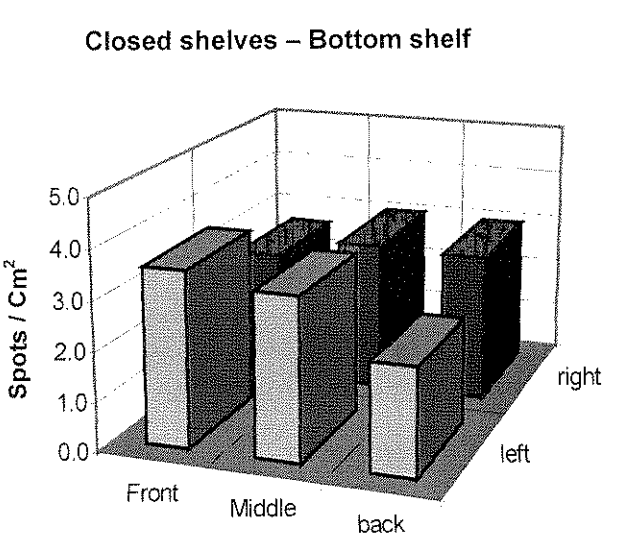




Figures 33 (bottom), 34 (middle), & 35 (top). Distribution and intensity of spotting symptoms developing on mushrooms placed on three open shelves within a mushroom house following a controlled liberation from a single disease colony located at the front of the house.







Figures 36 (bottom), 37 (middle), & 38 (top). Distribution and intensity of spotting symptoms developing on mushrooms placed on three closed shelves within a mushroom house following a controlled liberation from a single disease colony located at the front of the house

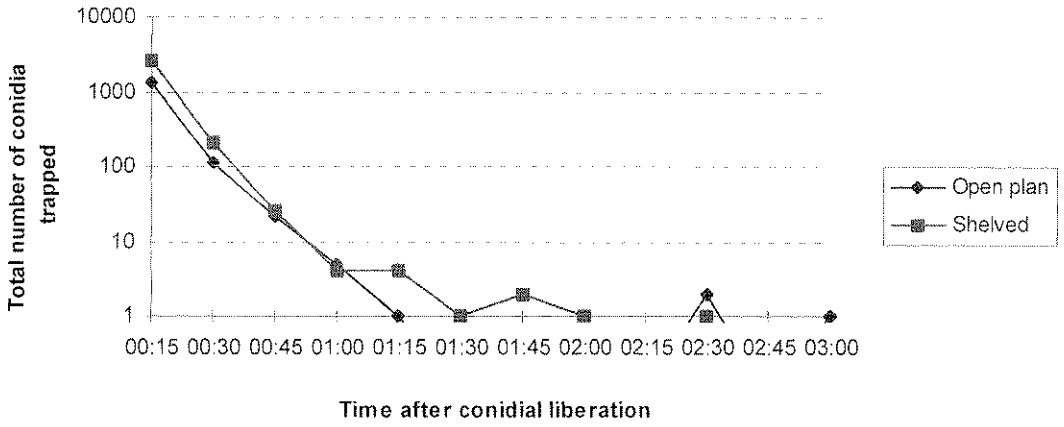


Figure 39. Total number of *Cladobotryum* conidia trapped in 1 hour on trap-plates distributed throughout a mushroom house following a controlled release of conidia in either an open-shelf or closed shelved system.

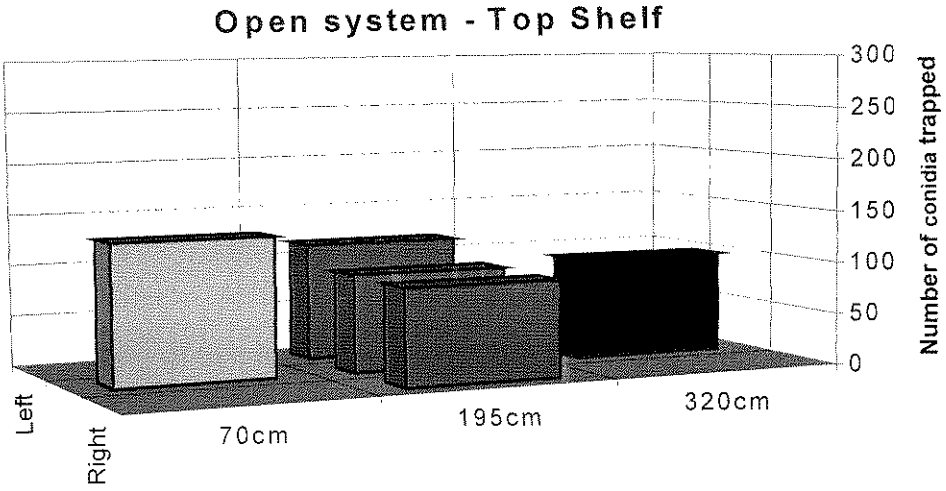
Conidia were trapped at all positions on all shelves in both the open-shelf and closed-shelf systems (Figures 40 – 45). In the open-shelf system, there was a clear, though sometimes small, reduction in the number of conidia trapped with increasing distance from the source (Figures 40, 41 & 42), similar to the rotorod data in Figure 31. The number of conidia trapped with increasing distance from the source in the closed-shelf system was more homogenous (Figures 43, 44 & 45), again similar to the rotorod data.

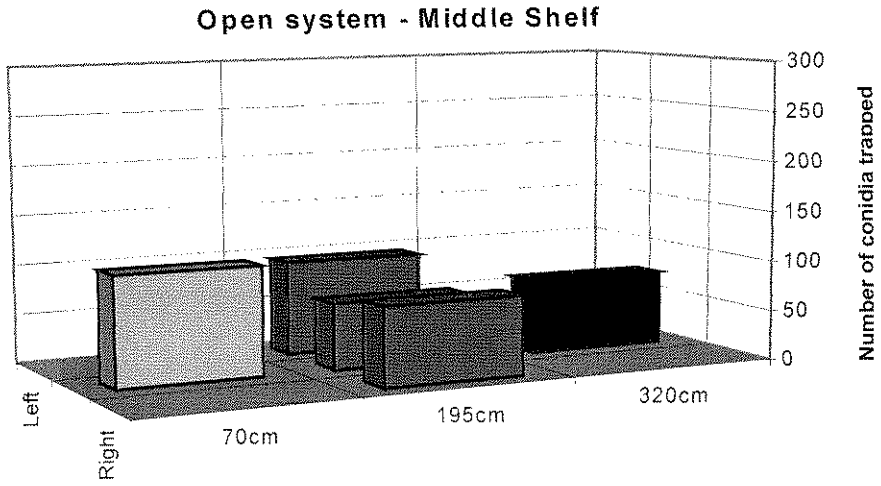
4.2.4 Discussion

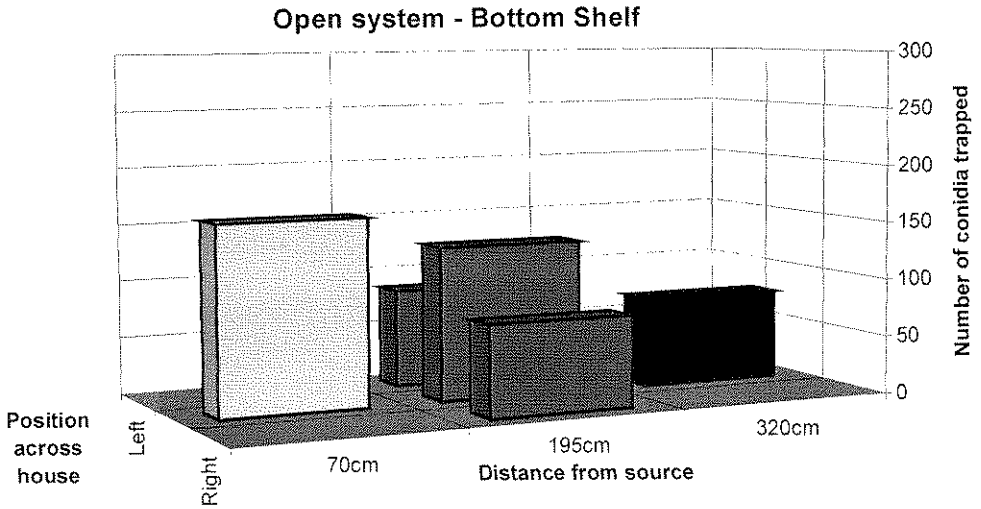
The most significant result to emerge from these data is that very high numbers of *Cladobotryum* conidia were trapped throughout a cropping house following disturbance of a single patch of cobweb. All four trapping techniques demonstrated this. The mushroom spotting results clearly demonstrated the devastating effect of a single spore liberation event on mushroom quality with over four spots per cm^2 of mushroom cap area developing. These results therefore do not agree with Dar (1997) who suggested that air currents were only capable of dispersing cobweb conidia relatively short distances (75cm). A standard commercial airflow pattern within the growing room used in these experiments was clearly sufficient to distribute cobweb conidia throughout the room, up to at least 4 m away from the source.

A few dispersal patterns were apparent although the high number of conidia trapped throughout the house may have made the observation of distinct patterns more difficult. However, there was a distinct impression that the partitioning of the house by the inclusion of closed shelving resulted in a more even distribution of conidia throughout the house. When the shelves were open, and conidia allowed to fall freely to the ground, more conidia settled out close to the source as might be expected. However, a great many conidia were still carried throughout the house, up to 4 m away from the source to where the burkard spore trap was positioned.

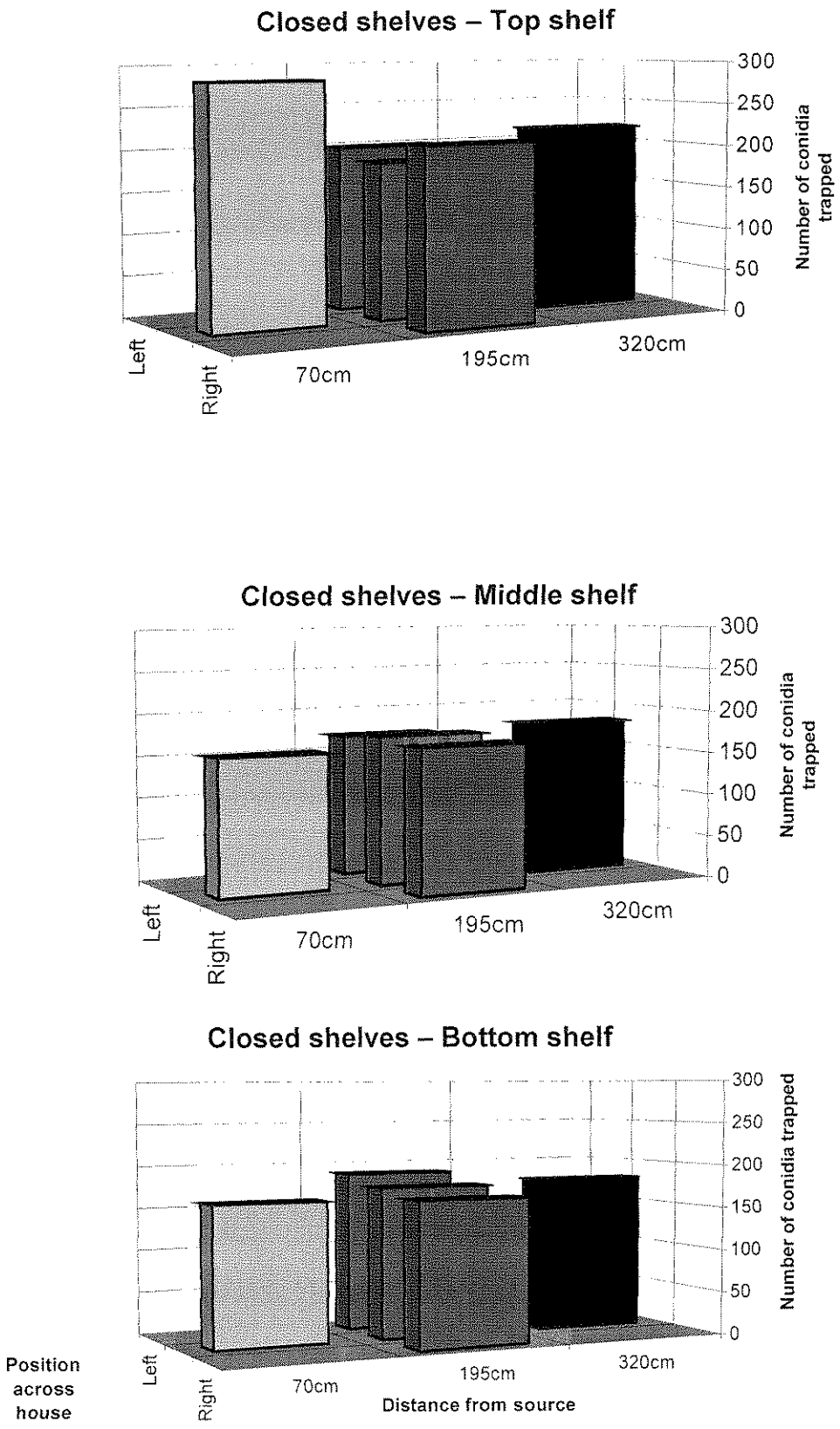
The burkard and trap plates were deemed the most suitable techniques for further investigations of this type. The Burkard trap, despite small problems associated with its







Figures 40 (bottom), 41 (middle), & 42 (top). Number and distribution of conidia growing on trap plates positioned on three open shelves within a mushroom house following a controlled liberation from a single disease colony positioned at the front of the house.



Figures 43 (bottom), 44 (middle), & 45 (top). Number and distribution of conidia growing on trap plates positioned on three closed shelves within a mushroom house following a controlled liberation from a single disease colony positioned at the front of the house.

imprecise clockwork mechanism, allowed continuous monitoring of the atmosphere. Analysis of the spore-trap tape revealed an unexpected release of conidia when the cobweb colony was brought into the cropping house prior to the start of the closed-shelf experiment. This minor liberation of cobweb conidia, which was associated with moving the disease colony, would have been undetected by any of the other techniques. The ability to divide the recording tape into 15-minute periods also gives this technique a sensitivity that only trap plates can easily better. However, dividing the tape into 15-minute sections is also misleading as in reality each 15-minute section of tape is exposed for one hour as it passes the aperture through which conidia are drawn. Thus, the apparent gradual build up of conidia over a 45-minute period prior to the main peaks in Figure 30, although apparent on the Burkard tape, does not actually occur. This was highlighted by the results from the trap plates, which demonstrated that the majority of conidia settled out during the first 15 minutes following their release.

Previous results (section 4.1) regarding conidial release demonstrated the ease with which conidia are released by watering and salting activities. The results presented above now compound this problem by demonstrating that once released, conidia are distributed widely and in large numbers by the air-flow normally employed in commercial mushroom cultivation.

Thus, two target areas for further investigation have been highlighted. The first is to reduce the liberation of conidia and the second to limit the dissemination of conidia should liberation occur.

4.2.5 Conclusions

- *Cladobotryum* conidia that are released from a single source are quickly distributed throughout the cropping house.
- *Cladobotryum* conidia become quickly distributed throughout the house whether or not the system is open (i.e. single layer of bags) or contains barriers to movement (i.e. full shelves or stacked trays).
- Three methods of measuring conidial dispersal within a cropping house all gave comparative results.

4.3 Preventing conidial liberation and spread

4.3.1 Introduction

The results presented in the previous two sections indicate that *Cladobotryum* conidia are liberated most frequently and in greatest numbers when disease colonies are either accidentally watered over or intentionally salted. Whilst only more thorough disease-detection could reduce the impact of unintentionally watering over disease patches, perhaps the method of salting disease patches, used to kill off disease, could be modified to reduce the extent of conidial release. More gentle application of the salt and/or containment of the conidia once liberated are considered to be the possible techniques to reduce the magnitude of conidial release. Not treating disease patches is not really an option, although if they are left alone and undisturbed they will liberate only few conidia. An unchecked disease colony would rapidly grow and sporulate heavily making the accidental disturbance and concomitant conidial release a real possibility.

As demonstrated in the preceeding two sections, *Cladobotryum* conidia are distributed throughout cropping houses in high numbers by the air currents routinely employed in commercial mushroom production. Velocities in the order of 10-20cm/second are used to mix the air within the mushroom house and thereby prevent stratification of the atmosphere and associated temperature and relative humidity variation (van den Boomen, 1988; Noble & Gaze, 1993). These air currents are essential to homogenous environmental conditions within the mushroom house and are produced by high volume fans. The air is normally introduced into the cropping house through perforated ducting running along the length of the ceiling. The objective of this section is to discover if switching the fans off for a short period of time during, and after a controlled liberation, has any effect on the patterns of conidial dispersal already described.

4.3.2 Materials and methods

4.3.2.1 Experiment 1 – Effect of fan speed on conidial dispersal

The computer controlled air-circulation fans within mushroom cropping houses at HRI operate at about 25% capacity to give an air speed of 10-20 cm/second, which is fairly standard within the mushroom industry. Conidial liberation experiments were conducted with the fans set at normal speed (Fan on) or with the fans switched off (Fan off). The Burkard spore trap and trap plate methods were selected to trap conidia in these experiments. Each experiment was replicated three times.

A pot of mushrooms with a growing *Cladobotryum* colony, measuring on average 158 x 154 mm, was located on the middle shelf of a 3-shelf unit in a mushroom cropping house. Each shelf was covered with plastic to simulate the conditions when the shelves are full of mushroom compost, which would provide physical barriers to the movement of conidia. A Burkard spore trap was placed at the back of the house on the floor, and monitored and recorded as described in section 4.1.2.1. Trap plates were placed in 4 trapping positions on each shelf, 50 to 100 cm from the source of conidia, to compare conidial distribution between shelves (Figure 46). In addition 4 trap plates were positioned at increasing distance from the source on the middle shelf only to determine relative movement of conidia from the source (Figure 46). All trap plates were replaced at 15 minute intervals for a period of 1 hour after conidial release. Trap plates were prepared and recorded as described in section 4.2.2.1(d).

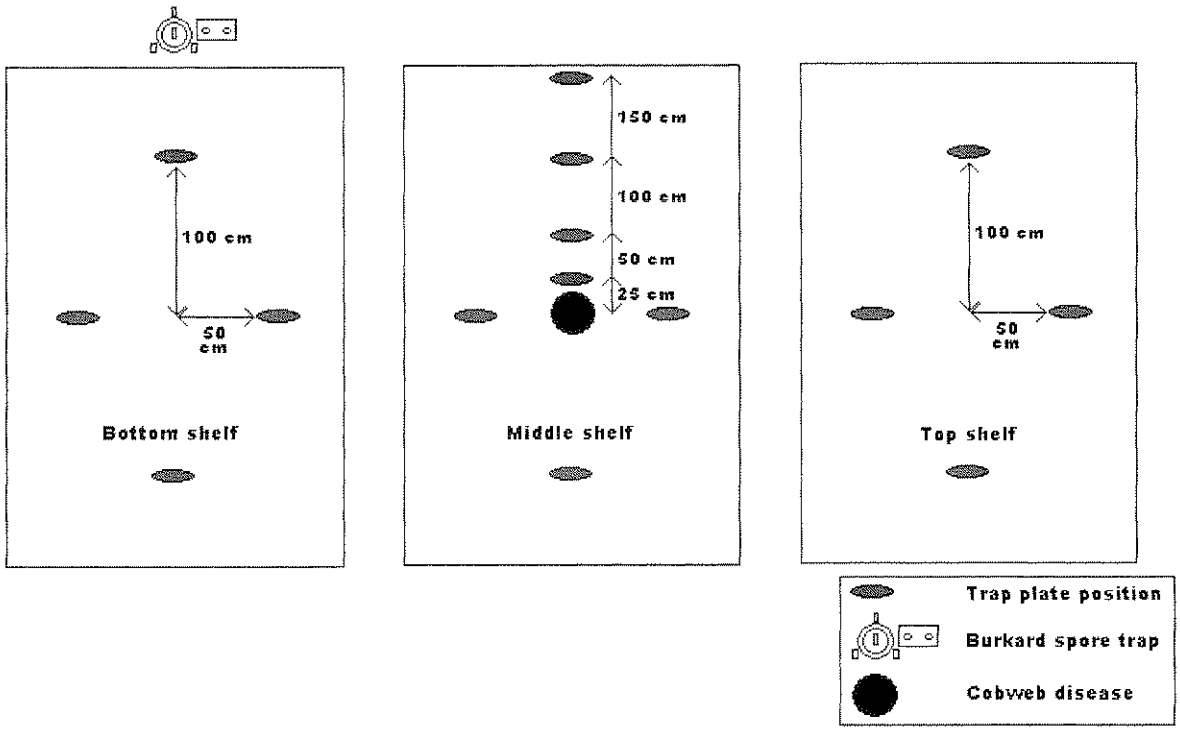


Figure 46. Position of trap plates on three shelves in a mushroom cropping house in order to study the dissemination of conidia within the house when the air circulation fan is on and off.

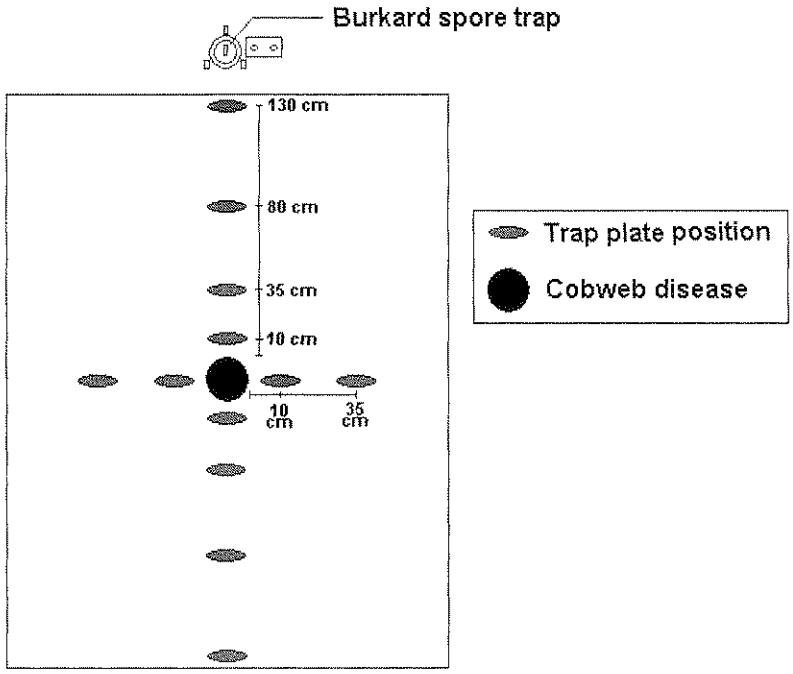


Figure 47. Position of trap plates on the middle shelf of a three shelf unit examining the dissemination of conidia following four different salting techniques.

The controlled liberation of conidia was achieved by watering over a cobweb colony with a tri-nozzled lance. A volume of 40ml of water was applied in four seconds from a distance of 30cm. Average cobweb colony diameters were 158 x 154 mm, but some variation occurred. All colonies used were sporulating heavily.

4.3.2.2 Experiment 2 – Effect of different salting techniques on conidial dispersal

Four different salting techniques were tested to determine their ability to liberate cobweb pathogen conidia:

I. **Salting** – Regular table salt applied directly to the disease, either using a trowel or by hand.
II. **Tissue salting** – Damp tissue (at least 3 cm larger in all directions than the colony area) placed over the disease colony before salting. The tissue is then pinned down around the edges with salt before the central area of tissue is gently covered.
III. **Air filtration** – Salting is conducted as in technique I but in addition the air above the salted area is constantly drawn through a fine filtration unit (Draper dust extractor – reference No. 2097D050N; Plate 10).
IV. **Tissue salting & dust extractor** – Tissue and salt are applied as in technique II in conjunction with air filtration as in technique III.

All four salting techniques were tested three times with the fan off and techniques II, III & IV were tested three times with the fan on. It was not considered necessary to re-test the simple salting technique (I) with the fan on as previous experiments have shown that liberation and distribution is both severe and widespread with this salting technique (see section 4.2.3).

As in the previous experiment, a pot of mushrooms with a growing *Cladobotryum* colony, measuring on average 158 x 154 mm, was located on the middle shelf of a 3-shelf unit in a mushroom cropping house. Each shelf was covered with plastic to simulate the conditions when the shelves are full of mushroom compost, which would provide physical barriers to the movement of conidia. A Burkard spore trap was placed at the back of the house on the floor, and monitored and recorded as described in section 4.1.2.1. Twelve trap plates were placed on the middle shelf only, at various distances from the source, to give an impression of conidial distribution around the source following the various salting techniques (Figure 47). Trap plates were exposed for 20 minutes in order to trap the majority of conidia following conidial release (see 4.3.2.1(d) & Figure 39). Trap plates were prepared and recorded as described in section 4.2.2.1(d).

4.3.3 Results

4.3.3.1 Experiment 1. Effect of the air circulation fan on conidial dispersal

The concentration of conidia in the air sampled by the Burkard spore trap at the back of the cropping house was much higher when the fan was on compared with when the fan was off (Figure 48). The vast majority of the conidia were trapped within the first two hours following conidial liberation, with very few conidia being trapped after this time.

More conidia were trapped at various positions on the shelves by trap plates when the fan was off than when it was on (Figure 49). The vast majority of these conidia were confined to the middle shelf where the source of conidia was located (Figure 50). In addition, 90% of them were deposited within 50 cm from the source of the conidia compared with > 30% when the

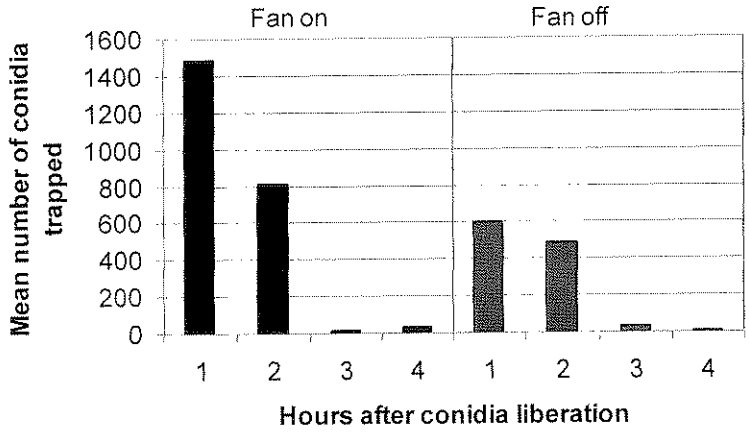


Figure 48. Number of conidia trapped/m^3 of air during a four-hour period following controlled liberation of conidia in a mushroom cropping house with the air circulation fan switched on or off. Spores trapped using the Burkard spore trap. Data are means of three replicate experiments.

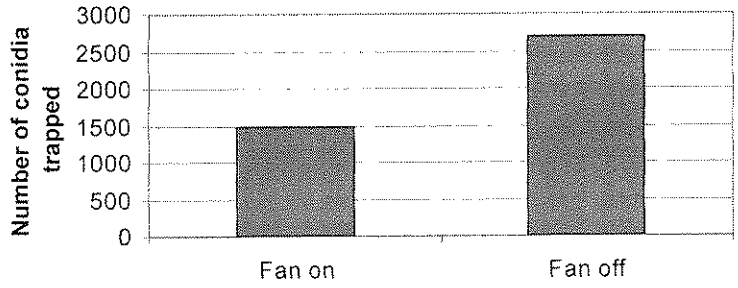


Figure 49. Number of conidia trapped at 12 positions (4 on each shelf) in a 1 hour period following a controlled liberation of conidia with the air circulation fan switched on or off. Data are means of three replicate experiments.

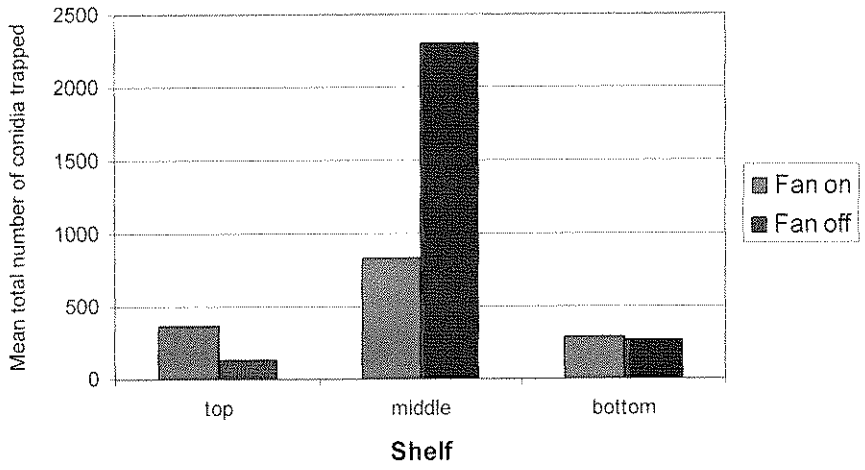


Figure 50. Number of conidia trapped in 1 hour at four positions for each shelf following a controlled liberation of conidia with the air circulation fan switched on or off. Data are means of three replicate experiments.

fan was on (Figure 51). This more concentrated deposition around the source when the fan is off, would have reduced the concentration of conidia in the air that was to be eventually sampled by the Burkard spore trap located at the back of the room. Thus, when the fans are off, conidia are deposited in high numbers close to the source, which results in a lower concentration of conidia in the air throughout the rest of the house.

Whilst switching the fan off affected the dissemination of conidia it did not affect the rate at which conidia settled out of the air. As previous experiments had shown, settling out of conidia was rapid, with the majority (>90%) deposited on trap plates within 15 minutes of the controlled liberation (Figure 52).

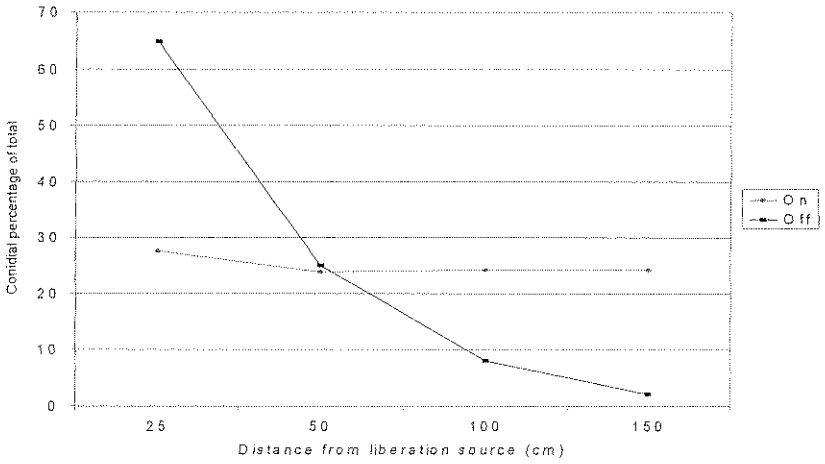


Figure 51. Conidia trapped on trap plates at increasing distances from the source expressed as a percentage of the total number of conidia trapped (on the middle shelf only). Data are means of three replicate experiments.

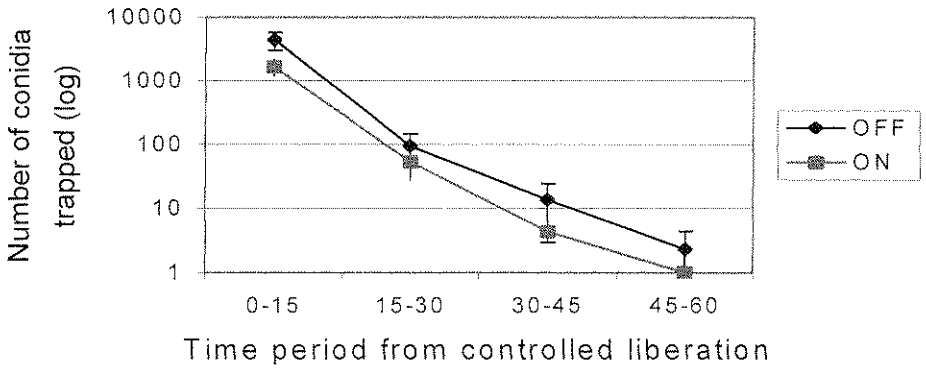


Figure 52. Number of conidia trapped on trap plates at 15 minute intervals at 12 trap positions (4 on each shelf) following a controlled liberation of conidia with the air circulation fan switched on or off. Data are means of three replicate experiments.

4.3.3.2 Experiment 2. Effects of different salting techniques on conidial dispersal

Straightforward salting did not prevent conidia being liberated into the air throughout the cropping house whether the fan was off (Figure 53) or on (see Figure 23). However, very few conidia were trapped when salting of cobweb colonies was done in conjunction with tissue or air filtration (Figures 54, 55 & 56). With these techniques, results were similar when the fan was switched on or off.

Salting of a cobweb colony with the fan off resulted in the majority of conidia being deposited close to the source (Figure 57 and Plate 9), similar to the pattern described earlier for a controlled liberation of conidia (see Figure 51). However, very few conidia were trapped on trap plates when salting of cobweb colonies was done in conjunction with tissue or air filtration (Figures 58, 59 & 60). Results were also very good for these treatments when the fan was switched on (Figures 61, 62 & 63).

4.3.4 Discussion

It has been demonstrated in this section that if a cobweb colony is disturbed when the air circulation fan is on, the concentration of *Cladobotryum* conidia in the air is fairly uniform throughout the house. If the fan is switched off, the majority of conidia will precipitate out close to the source but a small number will still get carried to other shelves as well as some distance (at least 1.5 m) from the source. Switching the fans off will therefore not prevent the dissemination of conidia within a house, and new colonies and spotting symptoms would be expected to occur with decreasing frequency at a distance from the original source. Similarly, straightforward salting of cobweb colonies will also result in significant numbers of conidia being dispersed throughout the house, which will also be concentrated around the source if the fan is switched off during this operation. However, a dramatic and almost total reduction in conidia distribution was achieved when salting was done in conjunction with either the use of tissues or air filtration irrespective of whether or not the fan was on or off.

Whilst the benefits from reducing the number of conidia liberated are obvious, the benefits to the grower of minimising dissemination by turning the fan off are more subtle. Firstly, localised dispersal ensures secondary colonies are more likely to develop closer to the original source, which in turn can makes their detection more efficient, and likely. By increasing the probability that secondary colonies will develop close to the original, efforts to detect them can be concentrated in these areas. Secondly, the ubiquitous dispersal of conidia when the fan is on results in the ubiquitous spotting of mushrooms; a phenomenon demonstrated clearly in section 4.2. If dispersal of liberated conidia can be minimised by switching the fan off during salting and/or watering, it is possible that the spotting damage inflicted upon that crop may also be minimised.

As well as showing that switching the fan off can reduce dispersal generally around the house these data also suggest that this action may give greater protection to upper shelves with no disease. Airborne conidia liberated on the middle shelf will, through the process of gravity, be deposited on the bottom shelf. However, without updrafts conidia will not be deposited on the upper shelf. The fact that some conidia were trapped on the top shelf implies that updrafts carrying conidia had occurred, even though the fan was off.

Fan OFF – Burkard spore trap

Figure 53

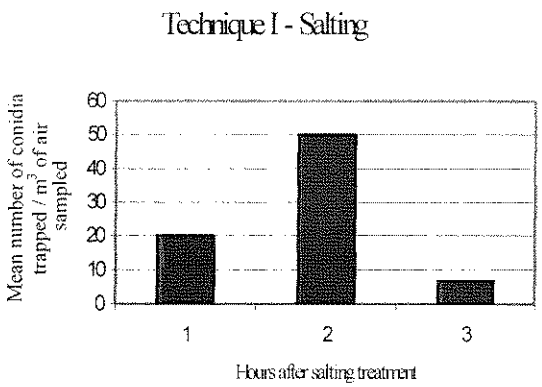


Figure 54

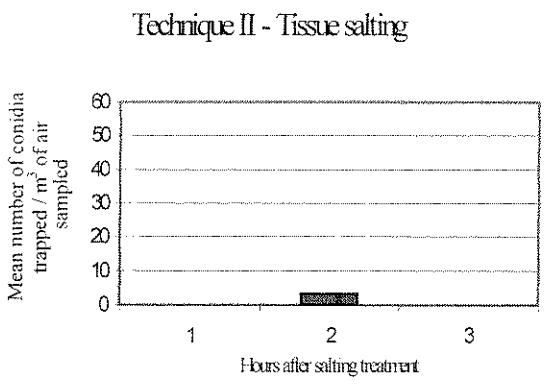


Figure 55

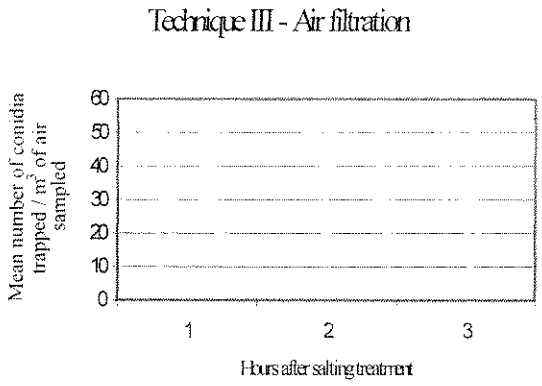
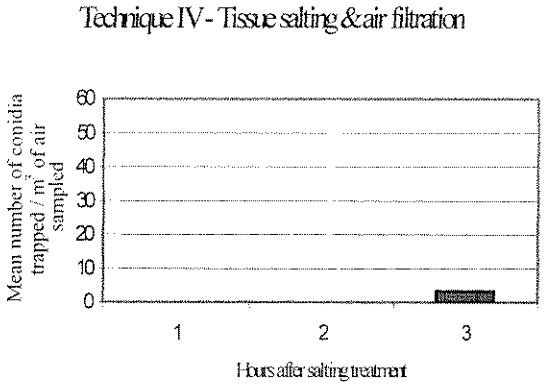


Figure 56



Figures 53 - 56. Number of cobweb conidia trapped using a Burkard spore trap following four different salting techniques when the air circulation fan was switched off. Data are means of three replicates.

Fan OFF – Trap plates

Figure 57

Technique I - Salting

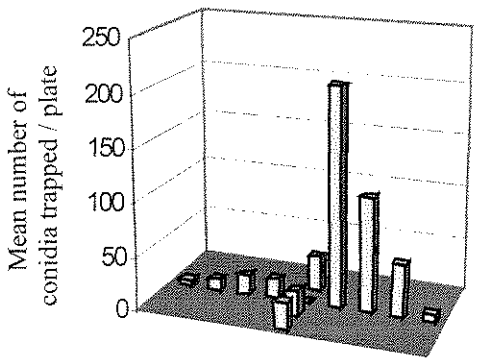


Figure 58

Technique II - Tissue salting

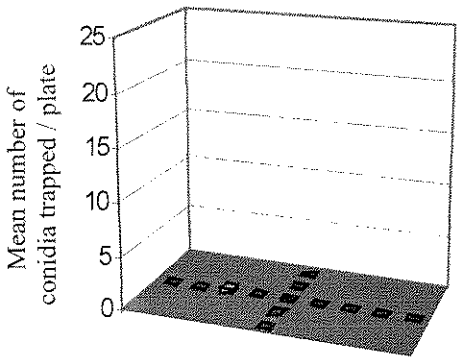


Figure 59

Technique III - Air filtration

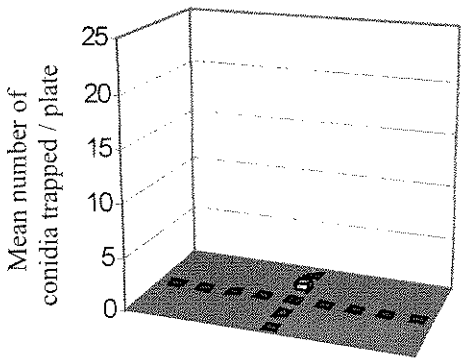


Figure 60

Technique IV - Tissue salting & air filtration

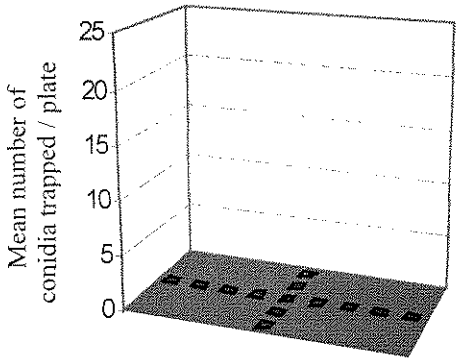


Figure 57 – 60. Number of cobweb conidia trapped on trap plates positioned on the middle shelf of a three shelf unit following four different salting techniques in conjunction with the air circulation being switched off. Cobweb colony was positioned in the centre of the shelf (see Figure 47). Data are means of three replicates.

Fan ON – Trap plates

Figure 61.

Technique I – Salting

(Distribution similar to Figure 44)

Technique II - Tissue salting

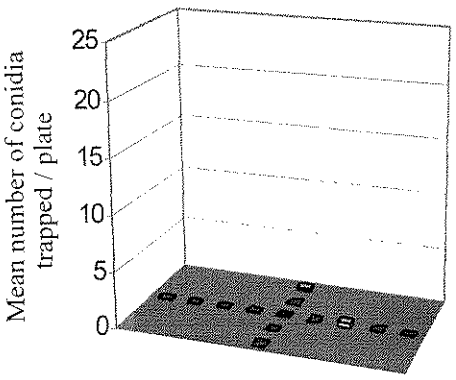


Figure 62

Technique III - Air filtration

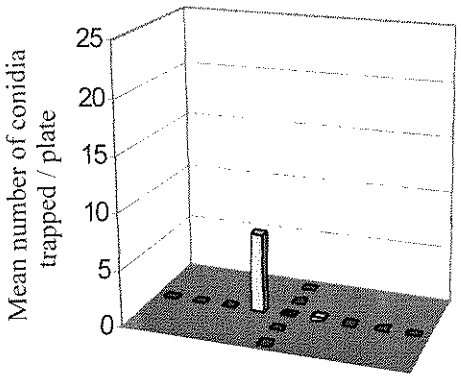
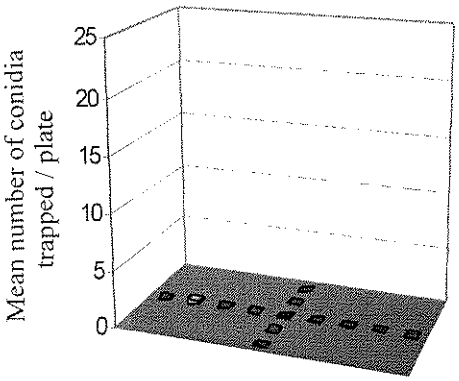


Figure 63

Technique IV - Tissue salting & air filtration



Figures 61 - 63. Number of cobweb conidia trapped on trap plates positioned on the middle shelf of a three shelf unit, following three different salting techniques in conjunction with the air circulation fan being switched on. Cobweb colony was positioned in the centre of the shelf (see Figure 47). Data are means of three replicates.

The most suitable technique for application within the mushroom should be decided by practicality. Using this criterion it is felt that tissue salting is the most appropriate. This technique is effective, cheap, simple to use, and relatively rapid. Air filtration, although effective, simple to use, and even more rapid than tissue salting, would require the purchase and maintenance of a dust extractor, costing in excess of £400.

4.3.5 Conclusions

- When cobweb is prevalent, switching air circulation fans off during watering and straightforward salting operations should concentrate the incidence of subsequent disease or spotting symptoms to the areas closest to the initial location of the disease.
- Straightforward salting operations will result in conidia being disturbed and distributed around the cropping house.
- Salting in conjunction with the use of a tissue to initially cover areas of disease will dramatically reduce the dispersal of *Cladobotryum* conidia around the house.

4.4 Summary

The work described in this section on the dispersal of *Cladobotryum* conidia within a mushroom house clearly demonstrates that standard watering operations applied to a cobweb disease colony, or a straightforward salting of that colony, will result in a major release of *Cladobotryum* conidia. These conidia will then proceed to be dispersed throughout the house where they can cause spotting symptoms or new disease colonies to occur. The pattern of dispersal will vary depending on whether the air circulation fan is switched on or off, with conidia being more uniformly distributed throughout the house if the fan is on, or more locally distributed around the source, when the fan is off. Although conidial distribution can be more localised if the fan is switched off, small numbers of conidia will still succeed in moving to shelves above and below the source, as well as moving some distance from the original source. This widespread airborne dispersal of conidia following their disturbance emphasises the importance of early disease identification and isolation, if the spread of the disease is to be controlled.

Straightforward salting was shown to be virtually ineffective in preventing the spread of the disease. The technique MUST be used in conjunction with either (a) a tissue, which covers and contains the disease colony prior to salting, or (b) a hand held dust extractor fitted with a fine air filtration unit. If either of these techniques is used, then extremely few conidia are released into the air resulting in virtually no dispersal of disease propagules throughout the house.

4.5 General Conclusions

- *Cladobotryum* conidia are liberated into the air in great abundance when disease colonies are disturbed by watering or salting without any protection
- Picking operations and other activities in the cropping chamber do not result in significant numbers of conidia being released.
- The vast majority of conidia settle out within a few hours of liberation
- Even low numbers of conidia/m^3 of air can cause significant spotting symptoms
- *Cladobotryum* conidia become quickly distributed throughout the house whether or not the system is open (i.e. single layer of bags) or contains barriers to movement (i.e. full shelves or stacked trays).
- When cobweb is prevalent, switching air circulation fans off during watering and straightforward salting operations should concentrate the incidence of subsequent disease or spotting symptoms to the areas closest to the initial location of the disease.
- Straightforward salting operations WILL result in conidia being disturbed and distributed around the cropping house.
- Salting in conjunction with the use of a tissue to initially cover areas of disease will dramatically reduce the dispersal of *Cladobotryum* conidia around the house.

5 Conclusions

Taxonomy:

- *Cladobotryum* isolates show significant variation in terms of their growth rate and conidial morphology
- There is no relationship between the morphological data and the genetic RAPD data, suggesting that genetically similar isolates show a range of morphologies
- The thiabendazole-resistant isolates associated with the cobweb epidemic in Britain in the early 1990's was genetically similar to *C. mycophilum*, but was morphologically different from current descriptions of this species.
- *Cladobotryum dendroides* appears to be less pathogenic than the thiabendazole-resistant isolates, the dominant isolate encountered during the cobweb epidemic in the early 1990's.

Biology:

- Statistical analysis of cobweb colony diameters at the end of the first flush suggest that the rate of sugar beet lime inclusion has a significant effect on the establishment of cobweb, with larger colonies occurring when high (30%) rates of sugar beet lime are used.
- Cobweb colonies were capable of significant growth over a wide range of matric potentials in casings that were very dry to periodically waterlogged.
- Drier casings slowed down the establishment of cobweb while wetter casings led to rapid establishment but subsequent growth rates were marginally higher in the drier casings (41 mm/day as compared with 34 mm/day).
- Management of casing matric potential in a bulk peat and sugar beet lime casing mix is unlikely to have any major effect on cobweb control.
- Casing is unable to support good growth and sporulation of *Cladobotryum* in the absence of developing mushrooms.

Epidemiology:

- When cobweb is prevalent, switching air circulation fans off during watering and straightforward salting operations should concentrate the incidence of subsequent disease or spotting symptoms to the areas closest to the initial location of the disease.
- Straightforward salting operations will result in conidia being disturbed and distributed around the cropping house.
- Salting in conjunction with the use of a tissue to initially cover areas of disease will dramatically reduce the dispersal of *Cladobotryum* conidia around the house.

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8 Appendix

Plate 1
Plate 2
Plate 3
Plate 4
Plate 5
Plate 6
Plate 7
Plate 8
Plate 9
Plate 10

Plate 1. Mushroom cap spotting caused by cobweb pathogen conidia.

Plate 2. Perithecia produced following the mating of isolate CC8 (*C. dendroides* Mt. b) with CC23 (*C. dendroides* Mt. a) under the conditions outlined in section 2.3.2. (Bar = 300μm).

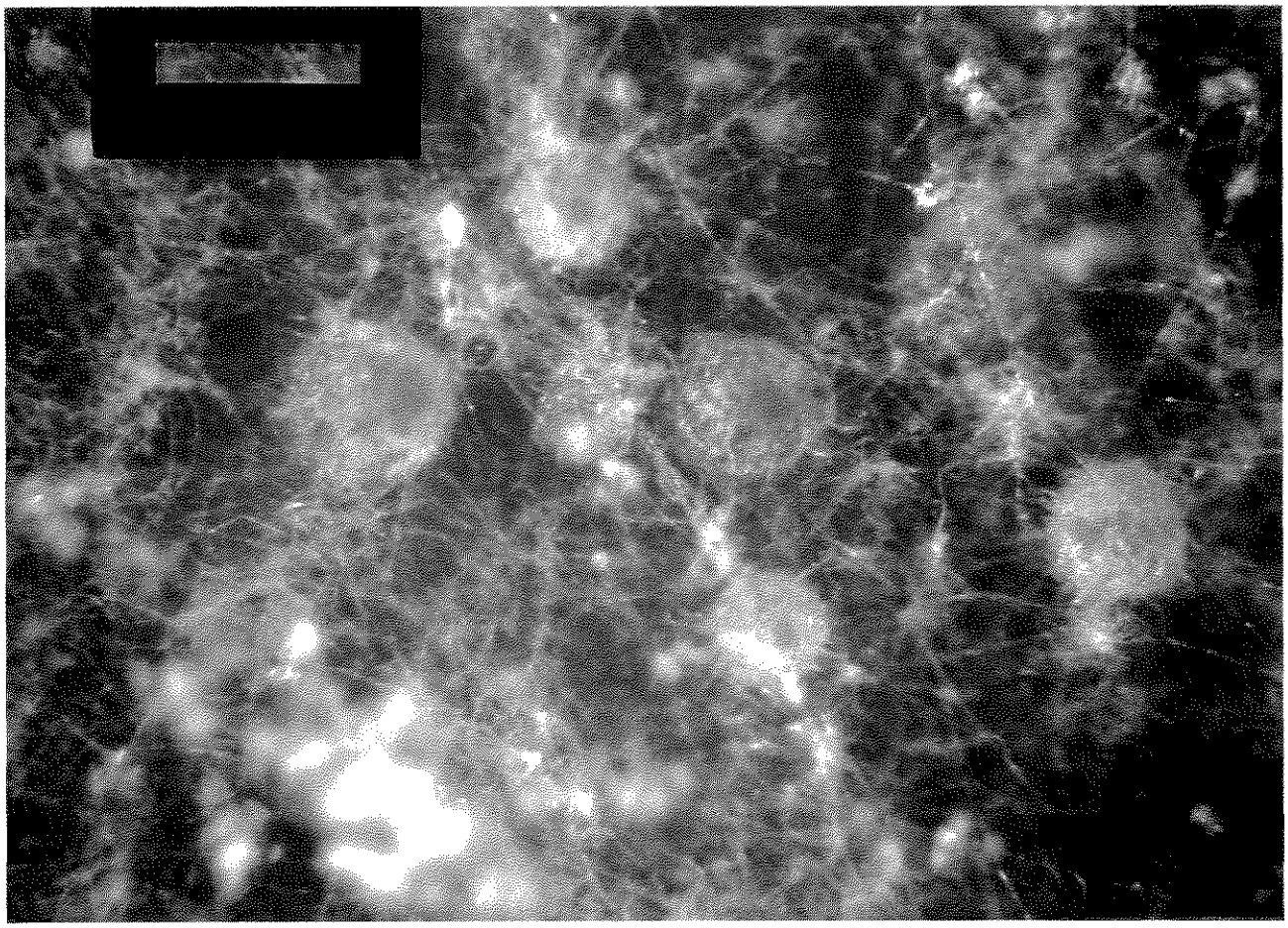

Plate 3. Ascospore produced following the mating of isolate CC8 (*C. dendroides* Mt. b) with CC23 (*C. dendroides* Mt. a) under the conditions outlined in section 2.3.2. (Bar = 20μm).

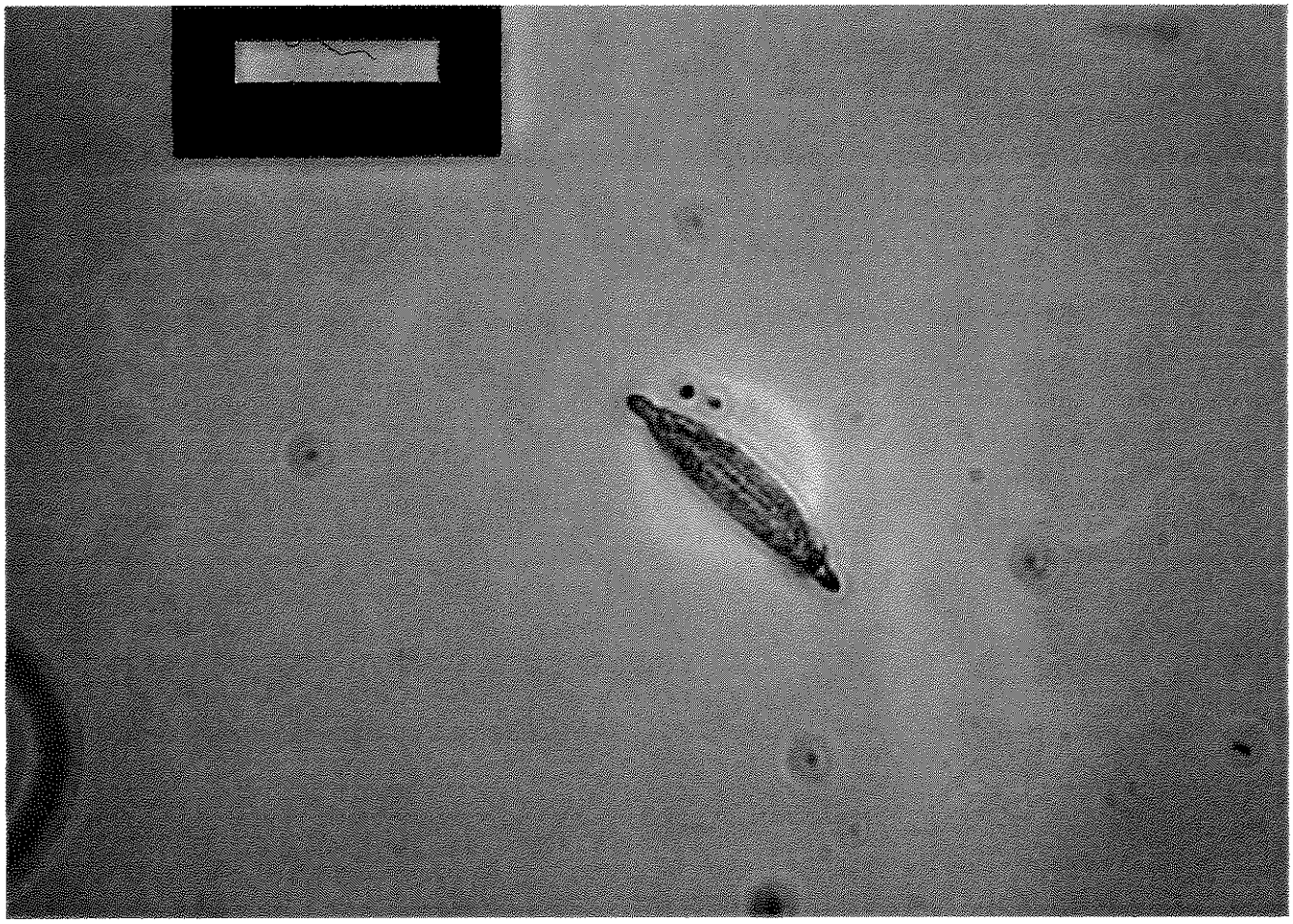

Plate 4. Pathogenicity of four *Cladobotryum* spp. isolates recorded nine days after inoculation of an *Agaricus bisporus* crop (a = isolate 192B1, b = isolate CC10, c = isolate CC18, d = isolate CC4). Note extensive cobweb growth and sporulation in 'a', extensive growth but poor sporulation in 'b', poor growth and no sporulation in 'c', and no apparent growth or sporulation in 'd'.

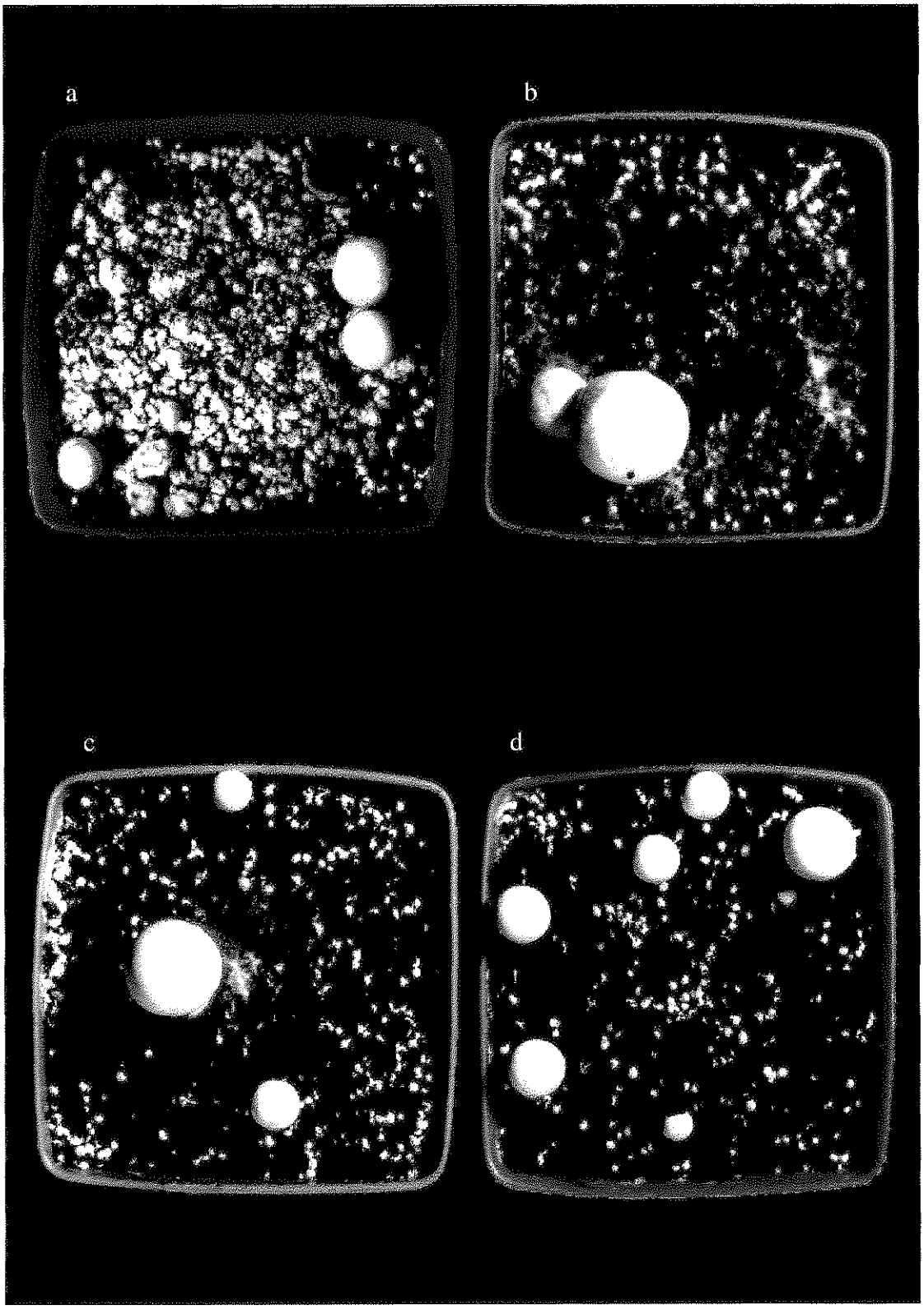

Plate 5. Cobweb colonisation of three formulations of compost and casing containing various amounts of *A. bisporus* (strain A12) mycelium. Formulation 1 = No *A. bisporus*; Formulation 2 = caccing in casing layer only; Formulation 3 = Fully spawn run compost with caccing in casing layer.

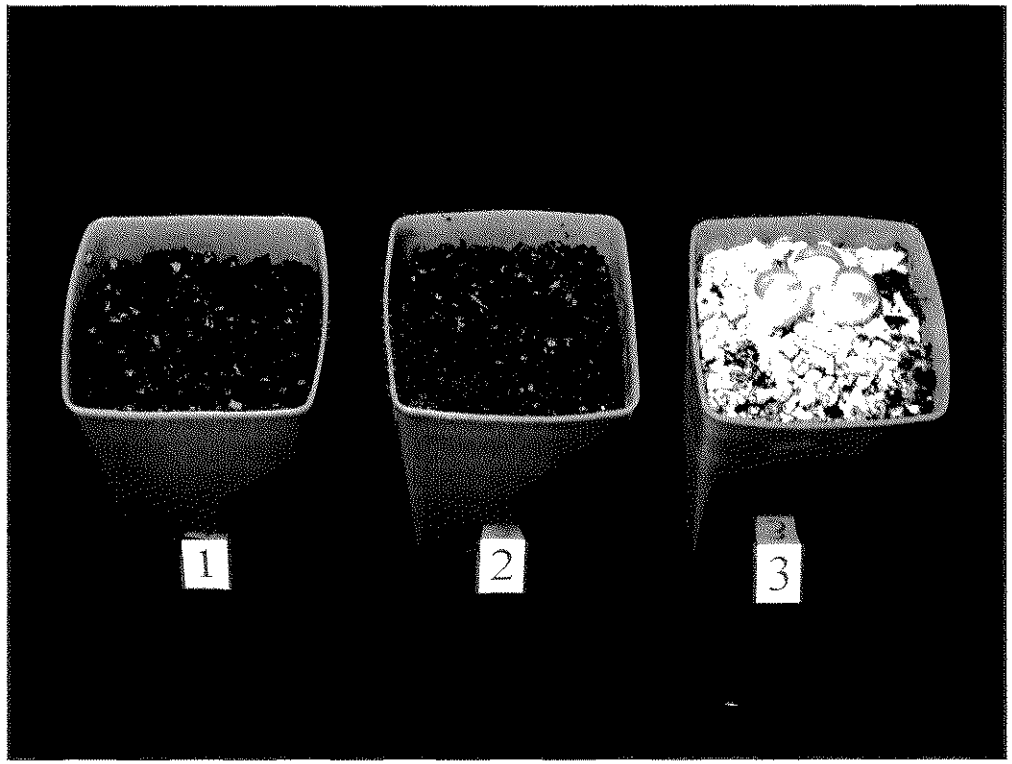

Plate 6. Cobweb pathogen conidia and conidiophores (isolate 192B1). (Bar = 20μm).

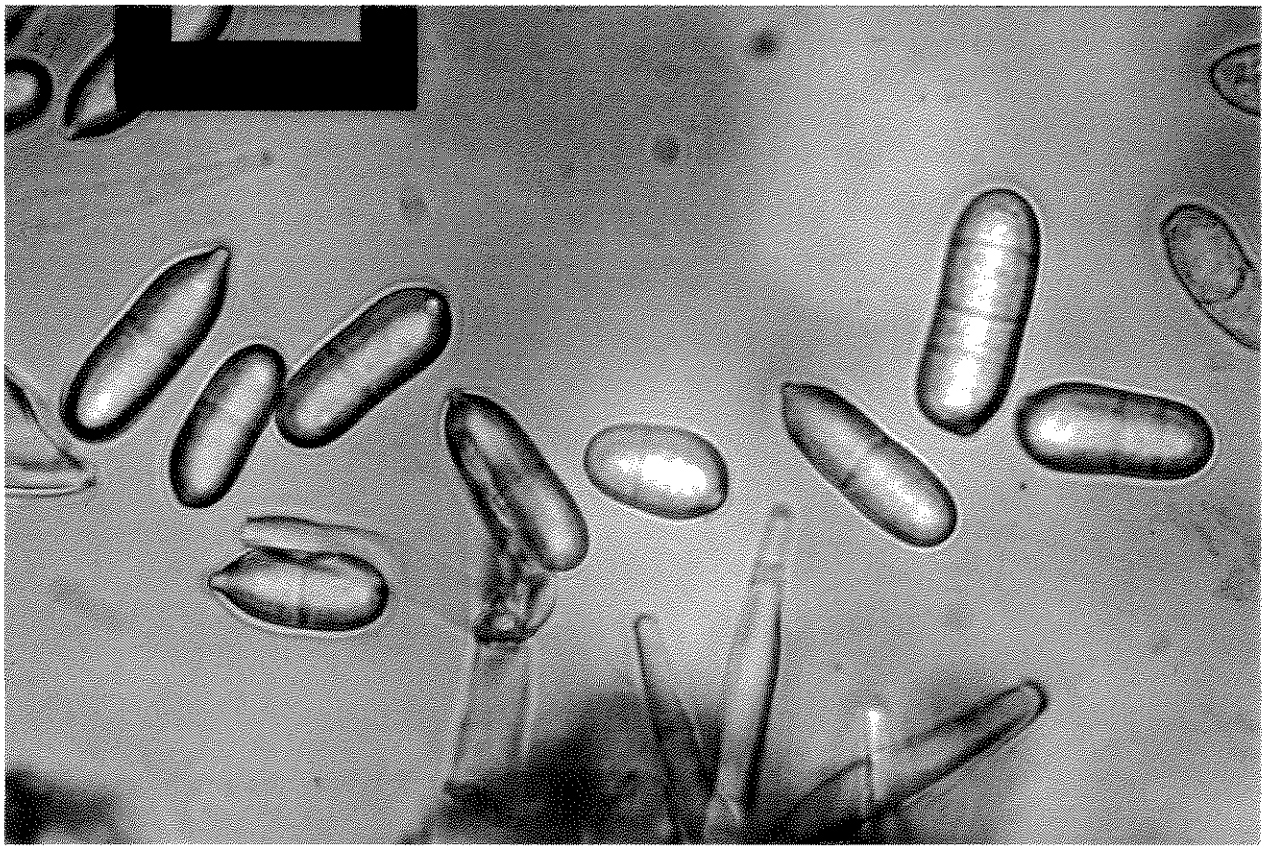

Plate 7. Limited range of particles trapped within a mushroom cropping house infected with a cobweb pathogen (isolate 192B1) when using a seven-day recording volumetric spore trap (Burkard). (Bar = 50µm).

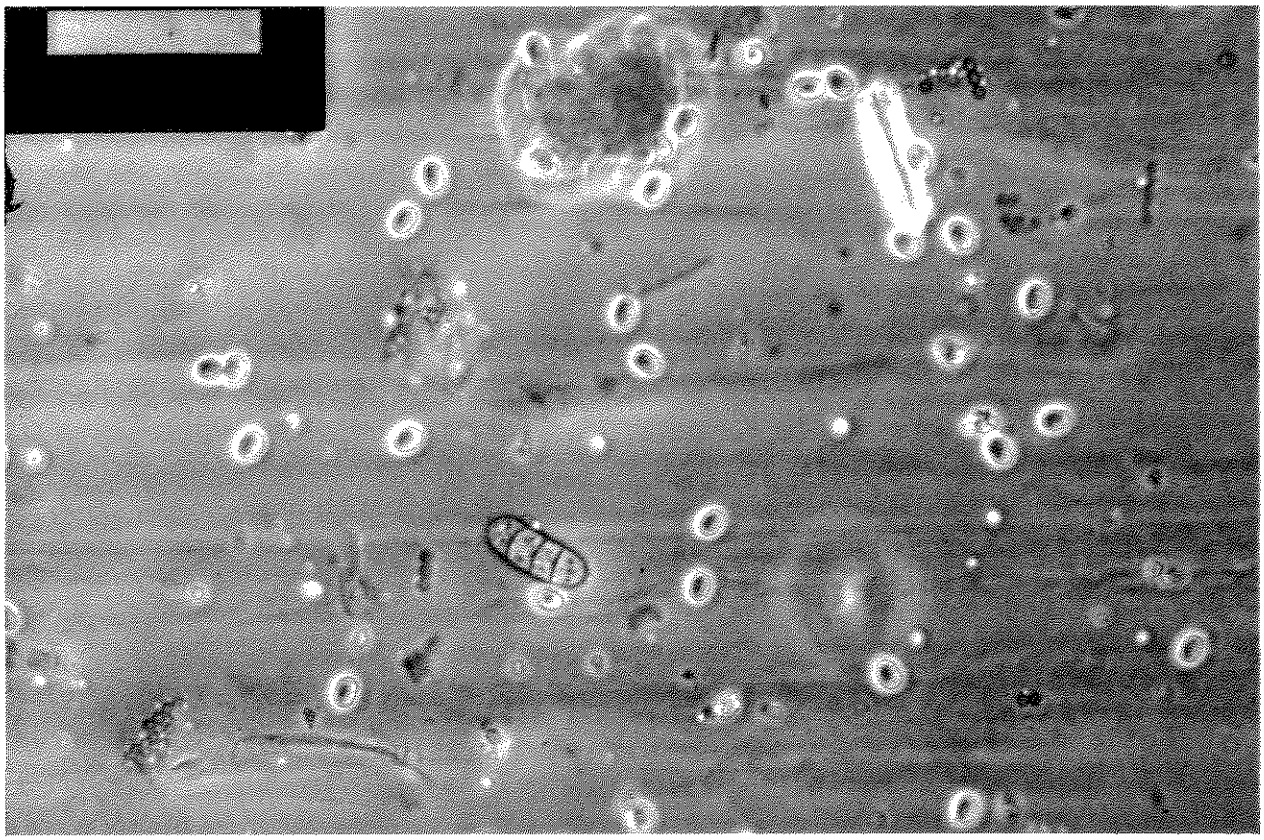

Plate 8. High numbers of *A. bisporus* spores obscuring other particles making identification of those particles more difficult. (Bar = 50μm).

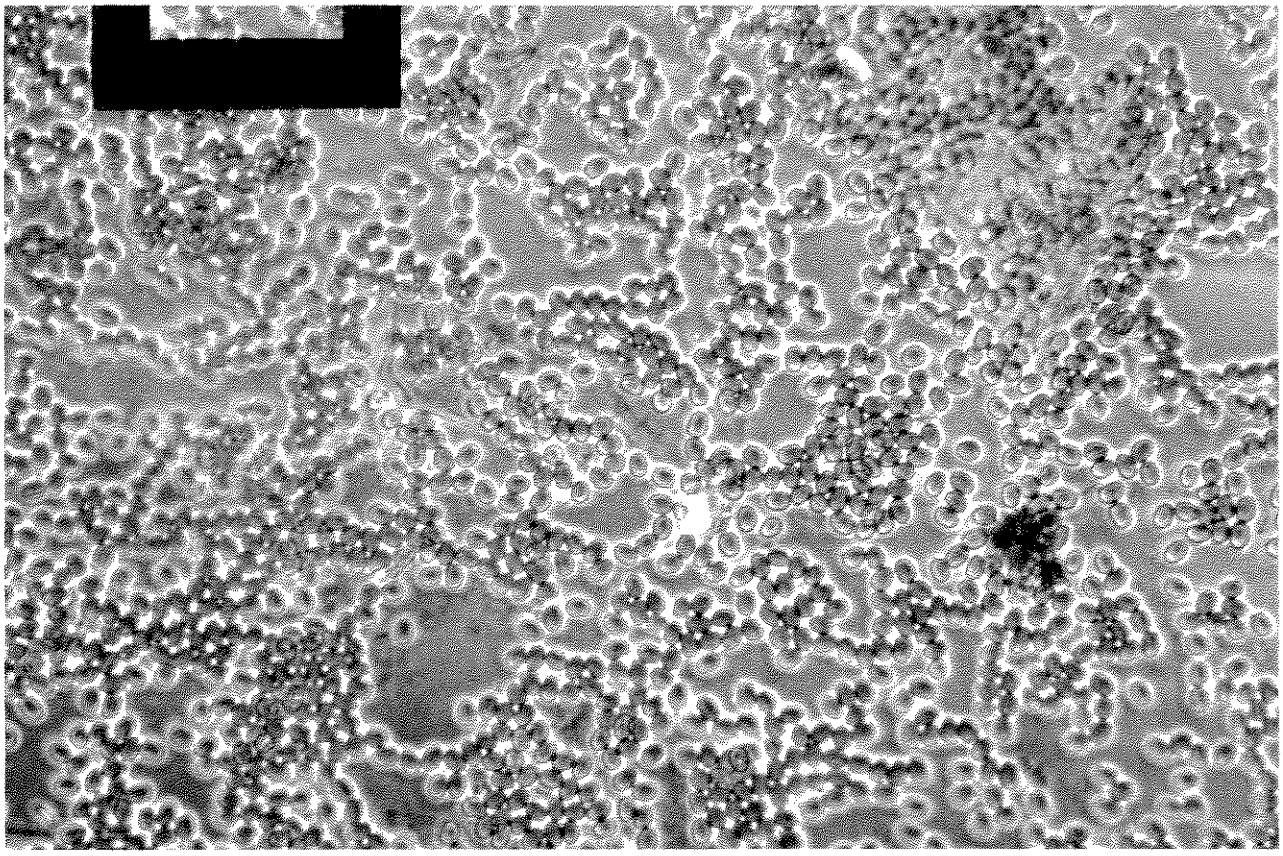

Plate 9. The physical disturbance of cobweb pathogen (isolate 192B1) conidia using salt granules such as those commonly used to cover cobweb colonies in a commercial situation.

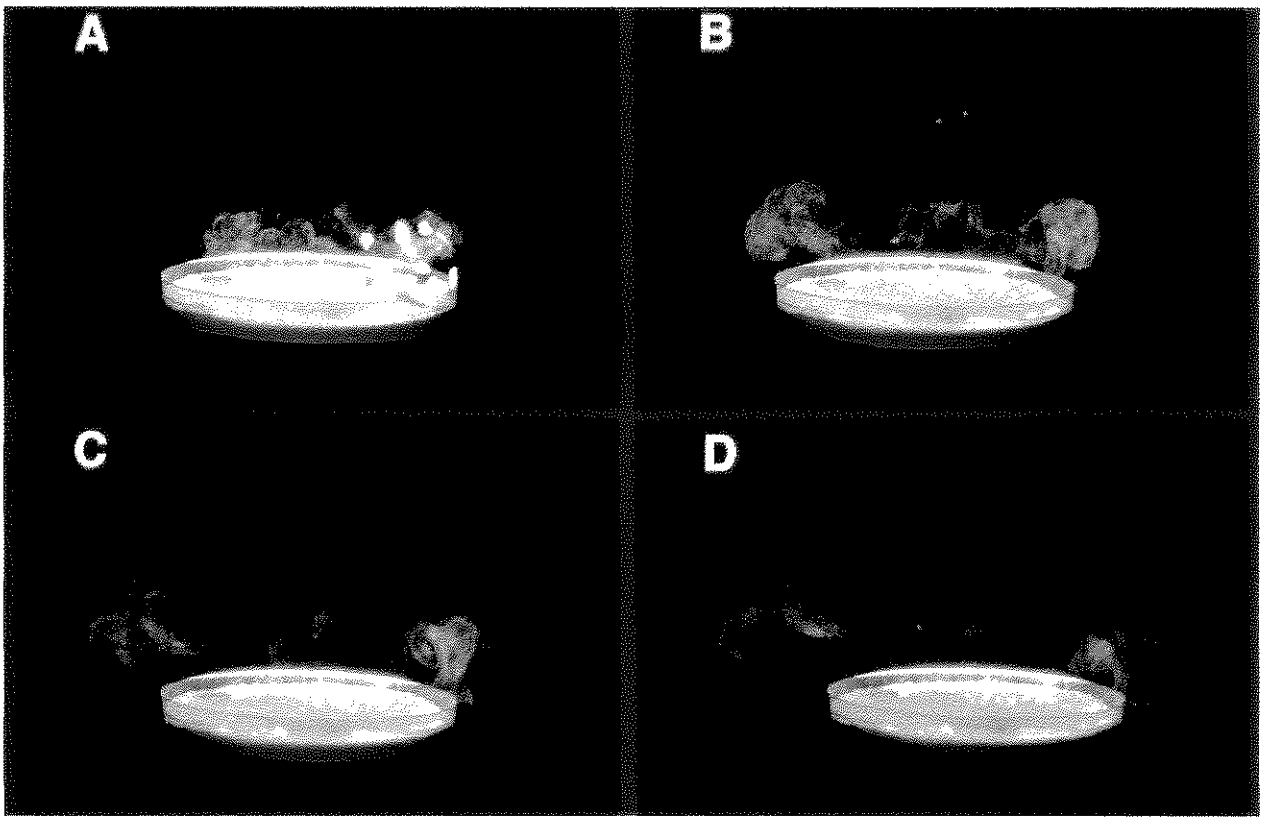

Plate 10. Air filtration technique employed to reduce the number of cobweb pathogen conidia released into a mushroom house atmosphere during the salting process.