BIOLOGY OF WET BUBBLE DISEASE
OF CULTIVATED MUSHROOMS

by

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A thesis presented for the degree of
Doctor of Philosophy

University of Stirling

1985

C O N T E N T S

ABBREVIATIONS AND CHEMICAL FORMULAE

1. Abbreviations

c.	circa
C.M.I.	Commonwealth Mycological Institute
d	days
G	gravitational force
G.C.R.I.	Glasshouse Crops Research Institute
h	hours
HMW	high molecular weight
LMW	low molecular weight
LSD	least significant difference
M	Molar
m	metre
mhc	moisture holding capacity
mM	milliMolar
min.	minutes
NCPPB	National Collection of Plant Pathogenic Bacteria
NTI	Numerical Threshold for Infection
PAF	Pseudomonas Agar F
rpm	revolutions per minute
s	seconds
SDeW	Sterile de-ionised water
SDG	Sucrose density gradient
SDW	Sterile distilled water
SEM	Standard Error of the Mean
S.E.M.	Scanning Electron Microscope
sp.	species
S.U.C.C.	Stirling University culture collection
syn.	synonym

TCI	Theoretical Colonisation Index
T.E.M.	Transmission electron microscope
U.V.	Ultra violet
TLC	Thin layer chromatography
w/v	weight/volume
%	percentage
μm	1×10^{-6} metres
λ	wavelength

2.   Chemical formulae

$Al\ Cl_3$	Aluminium chloride
CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
DHBA	Di-hydroxybenzoic acid
DpNa	p-nitroaniline
EDTA	Ethylene-diamine tetra acetic acid
EtOH	Ethanol
FDA	Fluorescein diacetate
H_2SO_4	Sulphuric acid
$Na_3(C_6H_5O_7).2H_2O$	Sodium citrate
NaOH	Sodium hydroxide
OsO_4	Osmium tetroxide
$Pb(NO_3)_2$	Lead nitrate
TCH	Thiocarbohydrazide

ABSTRACT

The study examined the physical and biotic factors which affect the host-parasite relationship between the necrotrophic fungus Mycogone perniciosa (Magnus) and its host Agaricus bisporus (Lange) Sing. in vivo and in vitro and the relevance of these factors to the conditions under which A.bisporus is commercially cultivated.

The effect of environmental variables on growth of M.perniciosa varied depending on its developmental stage. Mycelial growth in vitro on media developed specifically for this work was optimum at $25^{o}C$, pH 4.1 and C : N ratio 55 : 1. Growth of the fungus occurred on media containing Na-carboxymethylcellulose, sucrose, chitin or galactomannan. The environmental conditions found to be optimum for M.perniciosa in vitro closely paralleled the conditions under which A.bisporus is cultivated commercially. Germination trials showed that verticillate conidia germinate most readily at $18^{o}C$, chlamydospores at $25^{o}C$. Relative proportions and overall production of the two types of M.perniciosa spores was also temperature dependant. Conidia were air dispersed.

M.perniciosa exhibits biotrophic tendencies in the presence of A.bisporus. Saprophytic ability of M.perniciosa appeared greater in the absence than in the presence of the host, with a high susceptibility to fungistasis in composts and casing. Inoculation tests in vitro and in vivo identified a range of fungi as being potential hosts of M.perniciosa.

A structural study was made of infected host tissue. M. perniciosa

appeared to cause highly localised host cell wall breakdown.   The hypothesis that enzymic degradation of host cell walls is an important part of disease development was confirmed by ultrastructural observations and by the assay of wall hydrolase enzymes.

The development of infected tissue appeared to be dependant on toxins and growth promoting substances originating from both the host and the pathogen.   Disease development was also dependant on antibiosis resulting from other organisms colonising the compost and casing layers.

Attempts were made to control M.perniciosa in cropping beds using such antagonistic bacteria.   Isolates showing most potential were Pseudomonas bacteria of species group 3 (P.fluorescens complex). Disease control was expressed as both (i) a reduction in the development of infected sporophore tissue, and (ii) an increase in the yield of healthy sporophores in the presence of the pathogen.   Correlation between antagonism of M.perniciosa in vitro and disease control was greatest in the former case.

The level of disease control achieved by inoculating antagonistic bacteria to A.bisporus casing heavily infected with M.perniciosa was significant but was highly variable.

INTRODUCTION AND LITERATURE REVIEW

Cultivation of edible mushrooms in the U.K.

Production of edible mushrooms in the U.K. is, with few exceptions, concerned with culture of one species, Agaricus bisporus (Lange) Sing.

Mushroom growing, as opposed to the picking of fruit bodies appearing in the wild was first started in France, c. 1650, using spent manure and straw from hot beds used in the cultivation of melons and cucumbers. This was followed by a period in which mushrooms were produced in increasingly regulated conditions in the 18th and 19th centuries, both in caves and later in specially designed buildings.

Typically mushrooms were grown on a substrate rich in horse manure and wheat straw which had been stacked in a yard and left for c. 4 weeks to enable 'conditioning' to occur. The composting process was found to be aided by turning of the stack by a number of times during the course of fermentation. When the grower decided that the compost was ready it was laid out in beds on the floor, (usually bare earth), and inoculated with colonised compost from the previous crop. The beds were then left for a period of time to enable the mycelium to colonise the new substrate. The length of time for the complete colonisation of composts under such conditions would have been variable depending on the growing conditions encountered.

The beds were subsequently covered with a layer of soil or marl, (known as the casing layer). This last treatment was found to be essential for the successful initiation of fruit bodies.

Modern mushroom production, comprehensively reviewed by Atkins (1974) and Vedder, (1978), is increasingly sophisticated with the introduction of

computerised environmental control within purpose built cropping houses. Furthermore the use of mass pasteurisation and conditioning techniques within closely monitored 'tunnels' and the introduction of semi-synthetic composts with a reduction in the use of manure, together with the development of rapid composting techniques has resulted in modern mushroom production being more akin to an industrial process than is any other horticultural system. The operation of casing as a means of initiating fructification is still required however. At present this is done with the application of a layer (c. 3.5-4 cm thick) of material to the compost surface. The exact composition of this layer varies from country to country and from farm to farm. In the U.K. most growers use a mixture of ground chalk, or limestone, and moss peat at pH 7.0 - 7.5. This casing layer is essential for consistent production of sporophores. The nature of this initiation phenomenon has been extensively researched by a number of authors.

The microflora of the casing layer, (particularly <u>Pseudomonad.</u> bacteria) has been shown to be essential for fructification to occur using standard casing material, (Flegg, 1979, Hayes, Randle and Last, 1969) and it was shown that in particular <u>P. putida</u> can be responsible for fruit body initiation.

The concentration of CO_2 in the beds and in the air is also critical in determining the point at which vegetative growth will cease and fruit body initiation will occur, assuming that other conditions are conducive to the process, (Long and Jacobs, 1974).

It has been suggested that air and compost temperatures and nutrient levels in the casing layer are major factors in fruit body initiation, (Paranjpe and Chen, 1979, Flegg, 1980). However the exact nature of any

of these influences is far from clear. Initiation of sporophores was shown to be possible in the absence of conducive micro-organisms with a nutritionally inert casing layer of activated charcoal in sterile conditions by Long and Jacobs, (1974).

Humidity and temperature are also influential in the successful initiation of sporophores. (Vedder, 1978).

The effect of each of these factors on the process of initiation in A.bisporus will vary between individual strains but the requirement of a casing layer for fructification under commercial conditions is inescapable.

Disease problems associated with mushroom culture

From the early days of mushroom production growers have been faced, until recently not only with the unpredictable nature of the crop itself, but to this day with a number of pests and diseases affecting the mushrooms and the substrate on which they are grown.

Notably a disease known as 'La Molle' was recognised as probably the growers' most serious problem. Smith (1924) refers to earlier records of 1888 and 1889 in which the parasitic fungus associated with infected mushrooms was named as Hypomyces perniciosus. The fungus was renamed Mycogone perniciosa (Magnus) by Constantin and Dufour (1892).

'La Molle', later known as Wet Bubble disease, of Agaricus bisporus was first accurately described by Smith (1924).

The appearance of the disease was seen not to be related to any particular stage in the crop, in some instances infection occurring at the start of cropping, at other times when the crop is well advanced.

Mushrooms affected by the disease show deformity of the gills with the presence of a white mycelial covering, in addition deformity of the cap with the appearance of irregular bubbling of the surface may occur. In some cases a dense covering of mycelium may be present on the stipe.

In extreme cases the infection may cause total disruption of the mushroom tissue, resulting in the formation of 'sclerodermoid' masses, or near spherical tissue masses bearing no outward resemblance to a typical fruit body of A.bisporus. It was considered that the exact nature of the infected material produced is related to the stage in sporophore differentiation at which it was infected. Characteristically the sclerodermoid tissue exudes droplets of amber coloured liquid (Plate I1) while producing a characteristically unpleasant smell. It was assumed that this exudation was due to enzymic action by the pathogen on the host tissue, (Smith, 1924).

The fungus associated with Wet Bubble disease of A.bisporus, Mycogone perniciosa, (Magnus) was identified as a separate species from Mycogone rosea by Constantin and Dufour, (1892), the latter species later being shown to parasitize Agaricus hortensis.

A member of the fungi imperfectii M.perniciosa has no known sexual stage, although it is assumed to be the imperfect stage of a member of the Hypomyces genus, (Brady, 1978).

Both in pure culture and on infected sporophore tissue it produces two types of conidia, a phialidic or verticillium like state, and a blastic chlamydospore state. The former are produced singly on whorled verticillate conidiophores and are typically uni-septate and thin walled, (11-15 x 2-3μm), (Plate I2). The latter are typically produced on the older regions of plate cultures. They are bi-cellular with an upper warty

PLATE I.1.   Sclerodermoid tissue produced on A.bisporus
beds infected with M.perniciosa.   The tissue surface is
covered with white M.perniciosa mycelium.
Note the presence of droplets of liquid, which are amber
in colour, on the tissue surface (arrowed).
Bar represents 1 cm.

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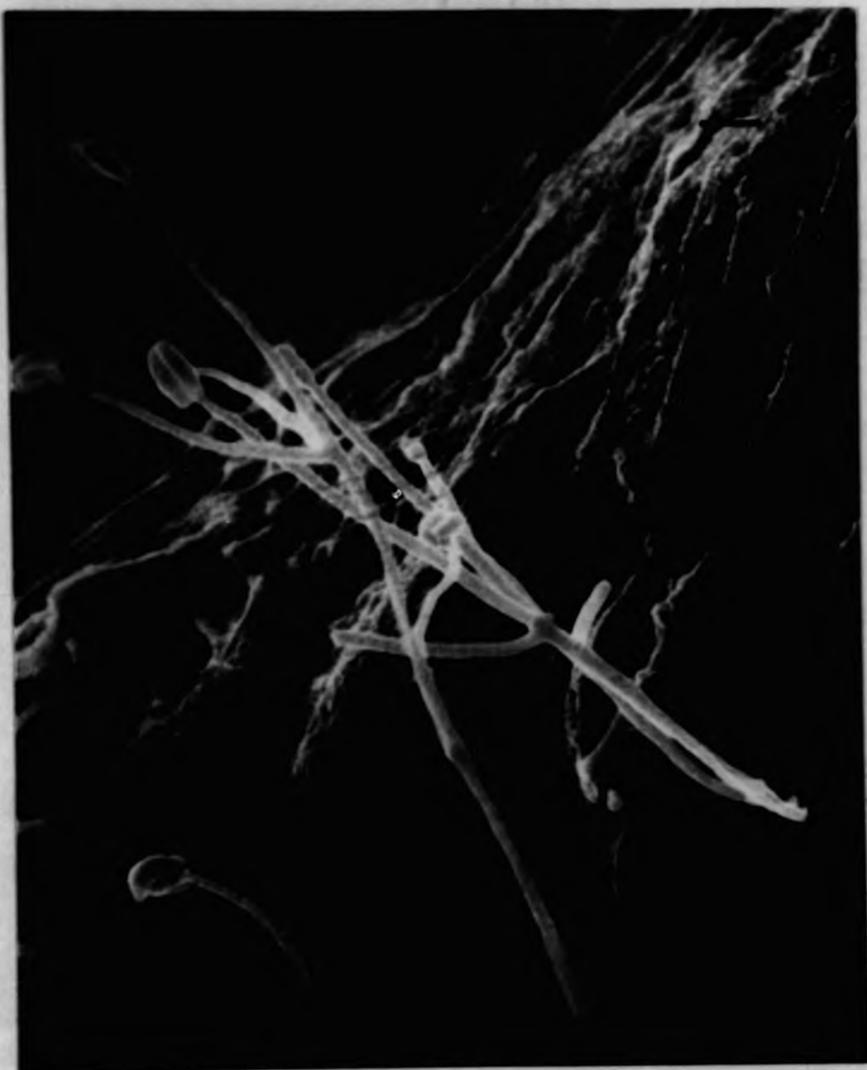
PLATE I. 2.   Scanning electron micrograph of <u>M.perniciosa</u>
verticillate conididiophores arising from <u>A.bisporus</u> sporophore
cap tissue.   Spores are uniseptate.

Bar represents 10 μm.
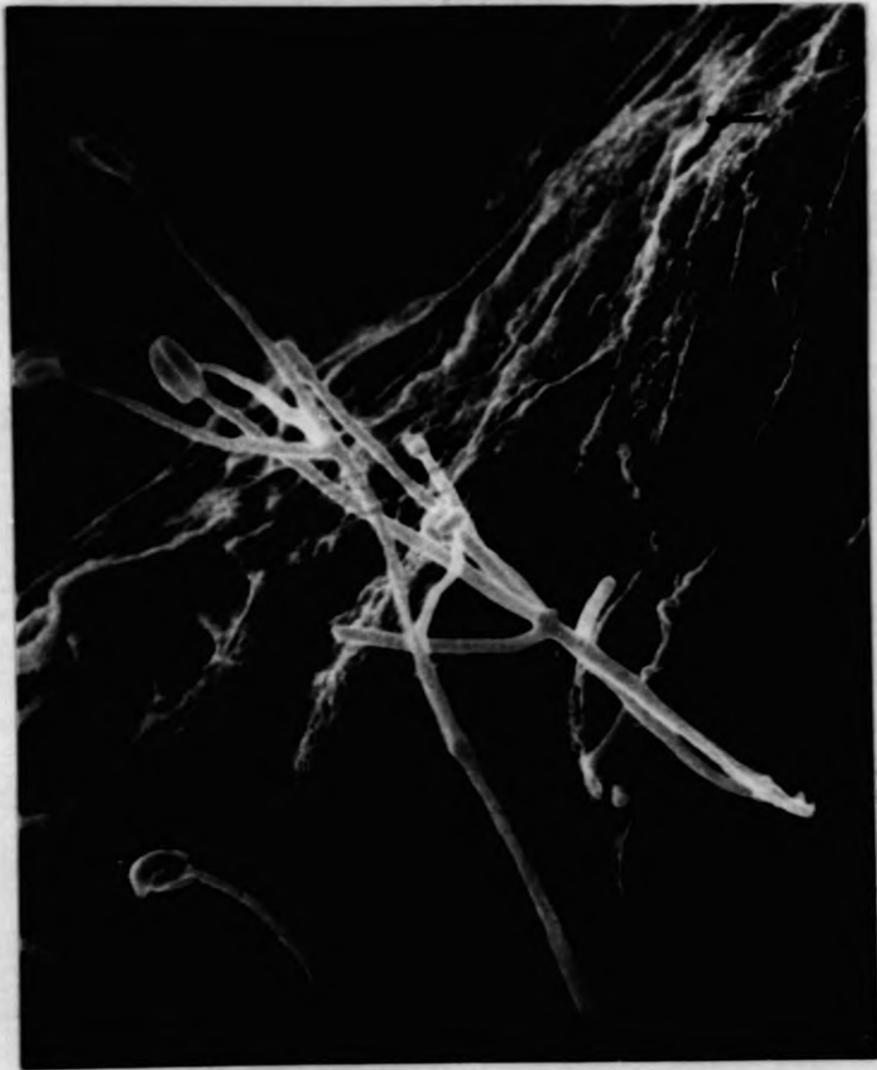
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and a lower hyaline cell, (13-3 x 16-31 μm and 8-13 x 8-17 μm respectively). The presence of the chlamydospore stage, giving both cultures and infected sporophores a light brown velvety appearance is generally regarded as being diagnostic of Wet Bubble. Vegetative hyphae are typically narrow, (3-4 μm), frequently septate and with dense multinucleate cytoplasm, (Smith, 1924).

There are no reports of marked differences between isolates of M.perniciosa in terms of pathogenicity, (Gandy, 1979). Differences do occur between isolates in terms of levels of viral infection in M.perniciosa affecting growth rate in pure culture, but this has no apparent effect on pathogenicity, (Atkey and Barton, 1976, Vincent-Davies, 1972).

The host range of M.perniciosa has not been extensively investigated. Brady and Gibson (1976b) lists five basidiomycete fungi as being potential hosts in the wild. Parasitation of fungi other than A.bisporus by M.perniciosa has only been recorded in two other instances (Barron and Fletcher, 1972, Moore, 1959).

The production of mushrooms in the U.K. has risen from 11,000 tonnes in 1950 to a level of 50,000 tonnes in 1977. The current turnover of the industry is c.£63 million, the most economically important protected crop in the U.K., (Hinton, 1982).

There have been a number of attempts to estimate the value of crop losses due to pests and disease. Smith (1924) stated that Wet Bubble was the single most important disease problem affecting mushrooms, occurring in all districts of England where mushrooms were cultivated.

Forer, Wuest and Wagner (1974) looked at the effect of the disease on returns for mushrooms crops in Pennsylvania (USA) and estimated a loss of 4% due to Wet Bubble disease, second only to losses caused by Verticillium malthousei (syn.V.fungicola), (10.3%). In the U.K. Wet Bubble disease

was, until recently, the principal fungal disease. A questionnaire sent to growers revealed that the majority of farms with no Wet Bubble had less than 10 cropping houses, with an overall occurrence of 73% of farms infected. Of farms with over 100,000 sq.ft. of cropped area/annum, 84% were infected with M.perniciosa, more than with any other fungal pathogen. The present situation is likely to be somewhat different with Dry Bubble, (Verticillium fungicola) apparently causing the greatest losses, although there is no recent survey data published. (Gaze, 1985). It is apparent however, that losses due to Wet Bubble remain significantly high both in the U.K., Western Europe and America, as well as in developing countries, (Forer, Wuest and Wagner, 1974).

While there are reports that brown and off-white strains of A.bisporus are more resistant to Wet Bubble that white strains, (Brady and Gibson, 1976b), there is no indication that individual strains of 'white' mushrooms,as grown predominantly in the U.K.,show any variation in resistance to the disease. So long as consumers demand white mushrooms for fresh consumption, and the increased resistance to virus infection by such strains is an important consideration, the relevance of such resistance to fungal infection is of no practical importance.

Infection of A.bisporus by M.perniciosa

Smith (1924) stated that M.perniciosa is unable to grow alongside vegetative hyphae of A.bisporus and that the pathogen was rarely found in cropping beds below the level of the casing layer. He also noted that M.perniciosa was capable of growing and surviving in a range of soil types.

This is in contradiction to observations by other workers, (Fletcher and Ganney, 1968, Vincent-Davies, 1972), who observed that many verticillate conidia were lysed within 72h in non-sterile soils, and that M.perniciosa has a generally low competitive saprophytic ability. Furthermore, it has been shown that M.perniciosa is unable to grow through the casing layer prior to infection, requiring direct contact with A.bisporus initials or sporophore tissue before germination of chlamydospores and subsequent infection can occur. The presence of an unidentified 'germination factor' in sporophore tissue, and its absence in vegetative mycelium, was therefore inferred, (Vincent-Davies, 1972).

Despite the evidence of Smith, (1924), it seems likely that infection, on the basis of present knowledge, is the result of the introduction of M.perniciosa chlamydospores to the crop. The role of verticillate conidia appears to be that of building up existing infection as they are apparently susceptible to lysis in soils, including casing soil, and therefore are assumed to have no role in 'carrying over' infection between crops. Infection of sporophores by actively growing vegetative mycelium is unlikely, (Vincent-Davies, 1972).

The application of M.perniciosa conidia to composts prior to casing of the crop was shown to have little or no effect in producing disease symptoms, (Smith, 1924, Fletcher and Ganney, 1968). Only where introduction of M.perniciosa conidia occurs at casing or subsequent to it does the production of infected material occur to any great extent. There is no particular size or stage of sporophore development at which infection preferentially occurs, (Smith, 1924).

The role of substrate and environment in growth and spread of M.perniciosa has not been fully investigated. Smith, (1924), determined the

optimum temperature for growth in vitro as between 22°-25^oC, with a
minimum temperature of 10°-17^oC, and maximum of 28°-32^oC.

Optimum pH was shown to be 5.5-6.5, with glucose the best carbon
source for vegetative growth, (Espinasse and Touze-Soulet, 1968).

Spread of inoculum was shown to occur principally in run-off water
from infected beds, (Fletcher and Ganney, 1968). Re-inoculation to beds
was also seen to occur with dust from previous crops and on the hands and
cutting knives of workers. Cross and Jacobs (1968) considered that casing
material could become contaminated during storage as a result of contact
with contaminated dust and water, after steam sterilisation and prior to
usage.

Aerial spread of conidia was considered a possibility by Zoberi,
(1961). Cross and Jacobs (1968) similarly found evidence of aerial
dispersal from pure cultures of M.perniciosa. The importance of this
was questioned by Fletcher and Ganney (1968) who found little evidence
to suggest that spores could be released from infected beds in vivo,
and concluded that such dispersal was unlikely to contribute to the
spread of the disease.

The dispersal of spores by flies was examined by Fletcher and Ganney,
(1968) who found no evidence to suggest that spread of the disease could
occur in this manner.

M.perniciosa appears to have a poor ability to colonise compost
and casing soil, (Fletcher and Ganney, 1968) and non-cropping soils,
(Vincent-Davies, 1972). It seems unlikely however, that spread of
the disease with water as the sole means of dispersal is sufficient
to account for the rapid spread of the disease that is possible
in some circumstances, as has been apparent in Taiwan,
(Forer et al, 1974). The means by which M.perniciosa

may be dispersed is in need of re-appraisal.

Control measures suggested by Smith, (1924), were based on disinfection of areas of disease on the beds and of treatment of the cropping house with lysol, (1.5-2%), formalin, (2%) and coal-tar, (1.5-2%). Similarly, the boots and cutting knives of workers should be disinfected at regular intervals.

Brady and Gibson (1976b), considered that maintaining a low temperature (< 16°C) in cropping houses was important in reducing development of the disease. Treatment of casing material prior to usage, chlorinated water, formalin (2%), or fumigation with methyl bromide was also suggested as a means of reducing the incidence of disease.

The use of chemical fungicides for the control of Wet Bubble in mushrooms was reviewed by Gandy and Spencer, (1978). They concluded that the best control could be achieved using Chlorthalonil (flowable formulation) or more recently Prochloraz manganese may be satisfactory (Van Zaayen and Van Adrichem, 1982, Van Zaayen, 1983). Benlate, Zineb and Mancozeb can also be effective although instances of Zineb tolerance and Benlate tolerance have been noted for M.perniciosa and other fungal pathogens of A.bisporus, particularly V.fungicola, (Brady and Gibson 1976b) Fletcher and Yarham (1976), Gandy and Spencer, (1976), Samuels and Johnston, (1980).

That the mushroom crop is itself a fungus leads to problems associated with the use of broad spectrum fungicides, leading to a balance between possible damage to the crop and the need for effective control of fungal diseases having to be struck.

That control of fungal diseases can be achieved using non-chemical means has been considered a possibility.

The whole process of mushroom cultivation has been likened to a complete

biological control system by Baker and Cook, (1974), who considered the highly specific nature of the composting and cropping system to have been inadvertently developed as a model for the production of an environment suitable for the crop and with the exclusion of non-beneficial organisms, with the exception of a small number of pests and diseases. That the system may be further modified to widen the scope of such exclusion has been recently shown with the isolation of strains of Verticillium lecanii parasitic on sciarid flies, commonly affecting mushrooms resulting in successful control of the pests within glasshouse environments (Hall, 1980, Spencer, 1980).

The extension of such biological control to the control of fungal diseases of mushrooms was considered a possibility using Trichoderma viride and Acremonium strictum (Gandy, 1979, De Troghoff and Ricard, 1976); however, both of these fungi may under some circumstances, adversely affect mushroom crops.

Mechanisms of pathogenesis by M.perniciosa

Vincent-Davies, (1972), observed that chlamydospores only germinated in the immediate vicinity of sporophore tissue. Subsequent growth within infected tissue is between the cells of A.bisporus, (Smith, 1924) and is seldom intra-cellular, (Vincent-Davies, 1972). Infection of tissue resulting in sclerodermoid mass formation induced the death of A.bisporus cells at the outer surface, (Smith, 1924). Sclerodermoid mass formation was considered to be the result of induced hypertophy and hyperplasia in

infected tissue with subsequent degradation by a range of enzymes resulting in the typical odour and liquid exudation in advanced sclerodermoid masses, (Smith, 1924, Vincent-Davies, 1972). Vincent-Davies (1972), suggested that the expansion phase of sclerodermoid mass development was in some way regulated by vitamins, or by hormones of an unknown nature, or more simply by the loss of host cell / cell contact and therefore control of cell division by the host. The Agaricus / Mycogone system was considered to be the result of interaction between two unidentified factors, ultimately symptom expression being the result of interaction between such mutually contradictory 'factors'. There is no record of M.perniciosa producing hormone-like substances or antibiotics, although M.japii, a mycoparasite of Inocybe rimosa has been shown to produce a biologically active red pigment, Mycogonin, (syn. Bikaverin), (Terashima Ishida, Hamasaki and Hatsuda, 1972). Bikaverins are a group of fungal secondary metabolites with noted anti-protozoal action and have also been shown to affect cell vacuolation in some instances, (Cornforth, Ryback, Robinson and Park, 1971, Bdlock, Detroy, Hostalek and Munim-al-Shakarchi, 1974), although there is no record of such compounds having antifungal activity.

The occurrence of toxins in both saprophytic and parasitic fungi has been extensively reported, notably in members of the Eurotiaceae, (Penicillium spp. and Aspergillus spp.) where fungi have been shown to produce a number of antifungal toxins (Berdy, 1974, Ciegler, Kadis and Ajl, 1971).

Mechanisms of disease resistance

While the success of A.bisporus in colonising commercial substrates is self evident, this is due in the main to the extreme specificity of the compost to the needs of A.bisporus rather than any identified means by which the fungus can preferentially compete with other saprophytes. However, there are indications that A.bisporus is capable of antagonism towards other organisms, (Vedder, 1978). Brady and Gibson (1976b) as previously noted, states that brown and off-white strains of A.bisporus are more resistant to fungal diseases than are white strains, although the nature of such differences is undetermined. Berdy, (1974), noted that antibiotics have been isolated from a number of basidiomycetes, and it is widely known that a number of fleshy fungi contain a number of extremely potent mammalian toxins. The occurrence of an antibiotic, tentatively named psalliotin was observed by Atkinson, (1954), as occurring in Psalliota xanthoderma (syn. Agaricus xanthoderma). It is apparent therefore that A.bisporus may produce some form of antibiotic which could be of importance in determining the competitive saprophytic ability within a healthy crop and may ultimately be of significance in indicating any possibility of selecting for strains showing resistance to pathogens.

In the present study attempts have been made to further investigate the nature of mycoparasitism in the M.perniciosa / A.bisporus antagonism, in an effort to determine the exact method of pathogenesis.

Survival and growth of M.perniciosa in mushroom substrates and in the wild has been investigated and further information relating to alternative hosts in the wild obtained.

The nature of the host / parasite interface has been examined using a range of techniques in microscopy, together with the examination of the role of toxins in pathogenesis as a possible means of antagonism and disease resistance in the pathogen and in the host respectively.

The possibility of disease control using non-chemical means was investigated. The efficacy of micro-organisms antagonistic to the pathogen introduced into the casing layer as a means of reducing the incidence of disease was examined as part of working towards an integrated approach to control of pests and disease within mushroom crops.

MATERIALS AND METHODS

I GENERAL

1. Source of Agaricus bisporus sporophores

Sporophores of A.bisporus were supplied by Messrs. Dumbreck, (Freuchie, Fife), Glasshouse Crops Research Institute (G.C.R.I.) (Littlehampton, W. Sussex), or produced at Stirling University. Isolates used were all commercially available strains.

A6	-	Sinden
D621	-	Darlington
D649	-	Darlington
S22	-	Sinden

2. Culture of mushrooms

Mushrooms produced at Glasshouse Crops Research Institute were grown on "rapidly prepared compost", cased with peat and chalk, in containment rooms. (Smith, 1976).

Mushrooms produced at Stirling University were in controlled environment growth rooms on/one phase commercial short cycle compost supplied by Messrs. Dumbreck, and cased with moss peat/grit/ground limestone, (3 : 1 : 1, by volume) pH 7.0. (Vedder, 1978).

Culture was in 16 cm diameter plastic pots or 18 litre plastic troughs. In all cases temperature regimes were similar to those used under commercial conditions, except where experimental requirements dictated otherwise.

3. Source of Fungal isolates

Mycogone perniciosa isolates were initially supplied by Central Bureau für Schimmellcultur, CBS 323.52, and by Miss D.G. Gandy (G.C.R.I.).

Reisolation of M.perniciosa was carried out from infected sporophore material at approximately 10 week intervals.

The following isolates were obtained from the Stirling University Culture Collection, (S.U.C.C.).

Botrytis cinerea,   Pers. ex Pers.

Cladosporium herbarum,   (Pers.) Link ex S.F. Gray

Chaetomium olivaceum,   Cooke & Ellis

Chaetomium globosum,   Kunze. ex Fries

Geotrichum candidum,   Link ex Pers.

Rhizopus tritici,   Saito

Mucor hiemalis,   Wehmer S.L.

Penicillium expansum,   Link ex Thom

P. frequentens,   Westling

Trichoderma viride,   Pers. ex Fr.

Isolates kindly supplied by Dr. T. Fermor, Glasshouse Crops Research Institute (G.C.R.I.) were as follows.

Coprinus sp $H_2 \times H_5$

Flammulina velutipes,   (Curt. ex Fr.). Karst.

Aqaricus silvicola,   (Vitt.) Peck.

Volvaria volvacea,   (Bull. ex Fr.) Sing.

Schizophyllum commune,   Fr.

Pleurotus ostreatus,   (Jacq. ex Fr.) Kummer (PH)

A. bitorquis,   (Quel) Sacc. W2, W4 and K32

Psilocybe merdaria,   (Fr.) Kummer

A. bisporus,   (Lange) Sing 522, D621, A6

A. macrosporus,   (Møller & Schaeff.) Pilat (B)

Fulvia fulvum,   Cooke

Oedocephalus sp.

Stropharia merdaria, (Fr.) Quel.

Isolates of Verticillium fungicola G3 and S1 were kindly supplied by
Mr. C. Matthews, Glasshouse Crops Research Institute, (G.C.R.I.)
V. psallioatae and V. fungicola 188930 were obtained from the
Commonwealth Mycological Institute, (C.M.I.).

Bacterial Isolates

Bacterial isolates of Pseudomonas tolaasi and Ps. fluorescens, 1311,
2325, 387 and 1116 were obtained from the National Collection of Plant
Pathogenic Bacteria (NCPPB).

Ps.tolaasi 2312 and Bacillus megaterium 9376 were kindly supplied by
Dr. J. Mansfield, (Wye College).

Other bacteria used were isolated by the author at Stirling, Freuchie
and G.C.R.I.

4. Culture and storage of isolates

Isolates of M.perniciosa were routinely cultured on Malt agar or
Mushroom/Malt agar.

Cultures-of A.bisporus were routinely maintained on Malt agar.

Other fungi were cultured on either Malt agar or Czapek Dox agar.

Storage was on suitable agar slopes in universal bottles, at 4°C.

Cultures of Cladosporium herbarum were maintained in 250 cm^3 conical
flasks containing c. 30 cm^3 Czapek Dox agar, inoculated with a conidial

suspension in sterile distilled water (SDW) excess liquid being decanted off after wetting of the agar surface. Incubation was under long wave ultraviolet radiation (Phillips 'Black light' fluorescent tubes, 16h photoperiod) at 18°C for 6-10 days.

5. **Preparation of spore suspensions**

Spores were harvested by flooding sporulating cultures with SDW and lightly scraping the surface with a sterile glass rod.

The resulting suspension was filtered through muslin and the spores washed to remove nutrients carried over from the agar by three cycles of centrifuging (1000 G for 2 min.), in SDW.

Dry spore harvesting was achieved by inverting sporulating cultures in 9 cm. Petri dishes over sterile filter paper and repeatedly tapping the plate base to dislodge the spores.

Specific areas of sporulating cultures were harvested with a dry sterile paint-brush.

6. **Adjustment of spore suspensions**

Spore concentration was measured using a haemocytometer, and the concentration adjusted to specific needs with SDW.

C. herbarum for thin layer chromatography (TLC) plate bioassays were adjusted to a concentration of c. 1×10^5 spores cm^{-3}.

The ratio chlamydospores / verticillate conidia of M.perniciosa was altered by centrifuging spore suspensions through a sucrose density gradient (SDG). 10-35% (MSE superspeed centrifuge for 15 min., 9000 r.p.m., 10,000 G, 6 x 16.5 swing out rotor), (Hames, 1978).

PH measurement

Values of pH were measured using a Pye Unicam model p w 9418 pH meter.

Spectral analysis

Ultraviolet (U.V.) absorption spectra were obtained with a Pye Unicam SP 1800 spectrophotometer.

7. Production of Axenic sporophores

A.bisporus was cultured under axenic conditions using a modification of the method adopted by Long and Jacobs, (1974).

Growth chambers consisted of 3.5 or 5 l glass jars fitted with air inlet and outlet ports. These contained 550 g (c. 2 l) of compost, over 2 cm of coarse grit to assist air circulation. The chambers were sterilised for two 1 h periods at 1.4 kg cm^{-2}, sterility being assessed by plating out samples of compost on to Nutrient agar and Malt agar and incubating at 25°C.

Inoculation of the flasks was with four 1 x 1 cm pieces of Malt agar cultures of A.bisporus, (strain D621), to the compost surface.

Incubation was at 22°C $\pm$ 2°C, in the dark until the compost was fully colonised. Flasks were cased with a layer of sterile activated charcoal (BDH) c. 1.5 cm thick, 250 g. / flask, when the compost was seen to be fully colonised. At casing 35 cm^3 of SDW, bacterial or spore suspension, prepared as previously described, were added to each flask as necessary. Subsequent incubation was at ambient temperature, 17°C $\pm$ 6°C.   Five days after casing, a sterile humidified air flow was connected to each flask to induce fructification (Figure M1).
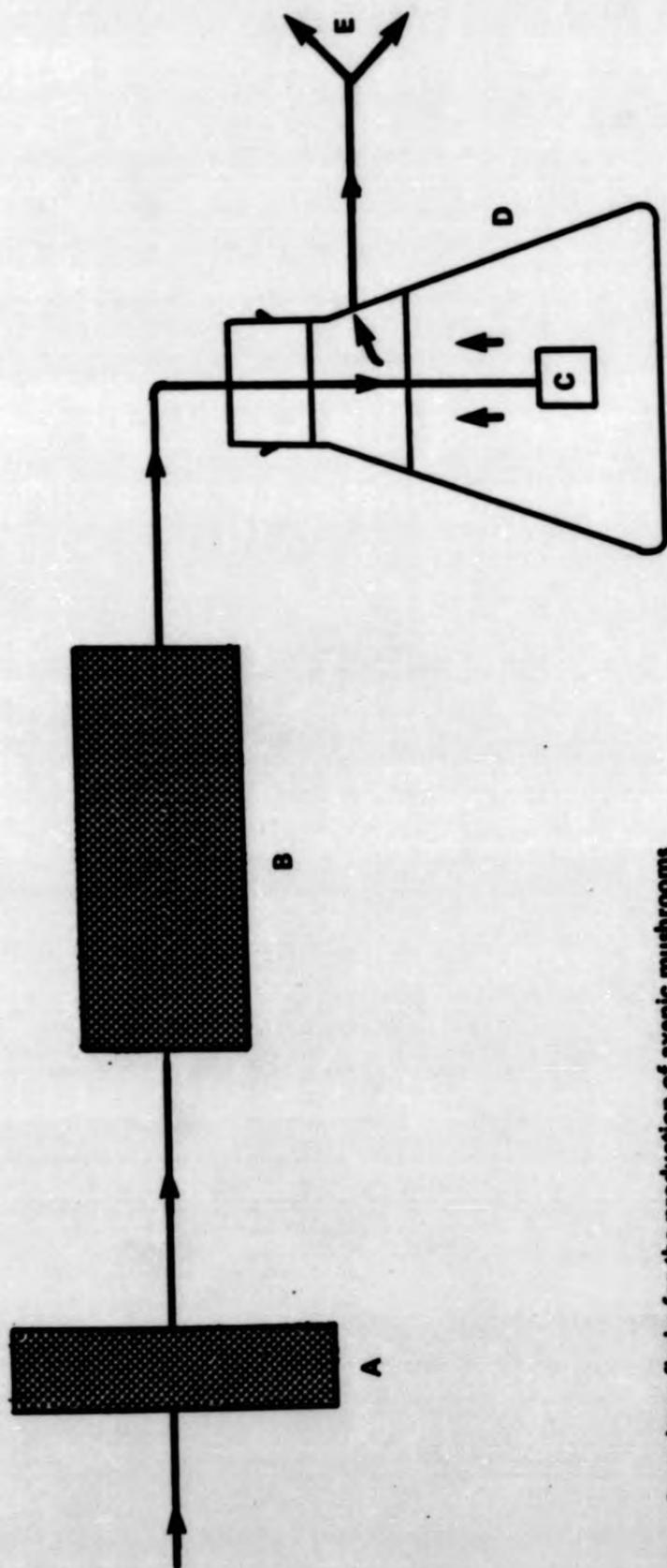
Aeration system for culture flasks for the production of axenic mushrooms

(A) - Pre-filter (MS Type 681)
(B) - Sterilising cotton fibre filter
(C) - Aeration stone
(D) - Aeration flask containing S.D.W.
(E) - Supply to culture flasks

→ - Direction of air flow

FIGURE M1

8. **Culture Media**

Malt Extract Agar

25 g Malt extract agar, (Oxoid CM59)

1000 cm^3 distilled water

Mushroom Malt Agar   (MM Agar)

25 g Malt extract   (Oxoid L39)

25 g Healthy sporophores (pressed)

15 g Agar, (Oxoid CM3)

1000 cm^3 distilled water

Czapex Dox Agar

45.4 g Czapek Dox agar   (Oxoid CM97)

1000 cm^3 distilled water

Czapek Dox liquid medium

33.4 g Czapek Dox liquid medium, (Oxoid CM95)

N.B.   Half strength = 16.7 g

1000 cm^3 distilled water

Nutrient Agar

28 g Nutrient agar (Oxoid CM3)

1000 cm^3 distilled water.

Where necessary, streptomycin sulphate (Sigma) was added to flasks of

cooled media to give a concentration of 100 µg/cm^{-3}.

All culture media were sterilised by autoclaving for 15 min. at

1 kg cm^{-2}.

Specialised media for specific experimental procedures are

described under the relevant heading.

9. Isolation of Mycogone perniciosa from infected sporophores

Routine re-isolation of M.perniciosa was by dissecting out small pieces (c. 2 x 2 mm) of tissue from sporophore cap or sclerodermoid mass tissue, under aseptic conditions, and plating out on to Malt agar or Mushroom/Malt agar in 9 cm Petri dishes, with streptomycin, and incubating at 20°C.

10. Artificial infection of healthy sporophores

Healthy button stage sporophore caps of A.bisporus were inoculated with small pieces (c. 2 x 2 mm) of an agar culture of M.perniciosa, (c. 21 days old) placed beneath the flap of a V-shaped incision cut with a sterile scalpel.

Inoculated sporophores were placed on plastic grids over moist tissue paper in closed plastic sandwich boxes and incubated at 20°C $\pm$ 1°C in the dark.

11. Germination tests

Before use, glass microscope slides were placed in stainless steel racks and thoroughly cleaned. After soaking in a surface active detergent (Haemosol) for 18 h they were rinsed under running tap water and finally in three changes of distilled water, followed by drying at 120°C for 1 h. After wrapping in aluminium foil they were subsequently heated to 160°C for 2 h. This cleaning technique gave slides which had no deleterious effects on spore germination or spread of bioassay droplets, (O'Neill, 1981).

Clean slides were supported on test tubes in closed plastic sandwich boxes lined with moist tissue paper. ->

Three 20 µl droplets of spore suspensions and/or test solutions were pipetted on to each slide and incubated at 18°C $\pm$ 2°C in moist chambers, prior to microscopic examination.

II. FACTORS AFFECTING GROWTH OF M.PERNICIOSA IN VITRO AND IN VIVO

1. Growth and survival of M.perniciosa

A. Growth of M.perniciosa in liquid cultures

Liquid cultures of M.perniciosa were grown in 100 cm^3 of medium in 250 cm^3 conical flasks. The media used varied according to the experimental procedure adopted. Inoculation was with three discs (4 mm diameter) per flask from the margin of a 6 day old culture of M.perniciosa on MM agar. PH of autoclaved media was determined using a Pye Unicam PW9418 pH meter.

M.perniciosa was grown on a range of media for the determination of growth optima. Media consisted of optima determination media (OD media) as outlined in Table M1, with the addition of carbon or based on the media of Fergus, (1969) and Park (1975), nitrogen sources, and buffered to specific requirements as outlined under individual headings. Replicated flasks were normally incubated in randomised blocks at 20°C $\pm$ 2°C for 10 days, unless otherwise stated. Mycelium was collected on tared dry filter papers (Whatman No. 1) and dried at 60°C for 18 h prior to weighing.

B. Optimum temperature

Optimum temperature for growth of H.perniciosa was determined

Table M1

in OD cultures with the addition of sucrose (20 g l⁻¹) and ammonium sulphate (0.5 g l⁻¹) as carbon and nitrogen sources respectively.

Optima Determination media (OD Media)	
Potassium phosphate KH_2PO_4	1.0 g
Potassium chloride KCl	0.5 g
Magnesium sulphate $MgSO_4$ $7H_2O$	0.2 g
Calcium chloride $CaCl_2$ $2H_2O$	0.1 g
Yeast extract (Difco)	0.5 g
Distilled water/citrate-phosphate buffer	1000 cm^3

C. Optimum pH for growth of H.perniciosa

Determination of the optimum pH for growth of H.perniciosa was determined in OD media with the addition of 0.5 g l⁻¹ ammonium sulphate $((NH_4)_2SO_4)$ and carboxymethylcellulose (Sodium salt) - High viscosity (1500 ± 400 cP) (BDH) (10 g l⁻¹).

pH was adjusted using citrate-phosphate buffer, as shown in table M2. (Male, 1965).

Optimum pH was determined on the basis of dry weight of fungus produced after incubation for 10 days at 20°C ± 2°C.

D. Optimum C:N ratio

Optimum C:N ratio for vegetative growth was determined in OD media with sucrose as the sole carbon source (20.192 g l⁻¹) with the addition of varying amounts of calcium nitrate as the sole nitrogen

B. Optimum temperature

Optimum temperature for growth of M.perniciosa was determined in liquid OD cultures with the addition of sucrose (20 g 1^{-1}) and ammonium sulphate (0.5 g 1^{-1}) as carbon and nitrogen sources respectively.

Flasks were incubated at a range of temperatures (4°, 10°, 15°, 20°, 25°, 30°, 35°C) in the dark for 10 days, and dry weight of fungus produced determined as previously described.

C. Optimum pH for growth of M.perniciosa

Determination of the optimum pH for growth of M.perniciosa was determined in liquid culture, using OD medium with the addition of 0.5 g 1^{-1} ammonium sulphate ((NH_4)$_2$$SO_4$) and carboxymethylcellulose (Sodium salt) - High viscosity (1500 ± 400 cP) (BDH) (10 g 1^{-1}).

pH was adjusted using citrate-phosphate buffer, as shown in table M2. (Hale, 1965).

Optimum pH was determined on the basis of dry weight of fungus produced after incubation for 10 days at 20°C $\pm$ 2°C.

D. Optimum C:N ratio

Optimum C:N ratio for vegetative growth was determined in OD media with sucrose as the sole carbon source (20.192 g 1^{-1}) with the addition of varying amounts of calcium nitrate as the sole nitrogen

Table M2

Citrate - Phosphate buffer used for the determination of the optimum pH for growth of M.perniciosa (After Hale, 1965).

Vol. l^{-1} 0.2M Na_2HPO_4	Vol. l^{-1} 0.1M Citric Acid	pH before autoclaving	pH after autoclaving
441.0	559.0	4.4	4.50
493.0	507.0	4.8	4.85
515.0	485.0	5.0	5.05
536.0	494.0	5.2	5.20
557.5	442.5	5.4	5.40
580.0	420.0	5.6	5.60
604.5	395.5	5.8	5.725
692.5	307.5	6.4	6.35

source to give C:N ratios of 1:1, 10:1, 21:1, 40:1, 55:1, 77:1, 99:1, 117:1, 146:1, 160:1, at pH 4.1 after autoclaving.

Optimum C:N ratio for growth of M.perniciosa was determined on the basis of dry weight fungus produced as previously outlined.

E.   Substrate utilisation

The ability of M.perniciosa in vitro to utilise a range of artificial substrates containing a range of carbon and nitrogen sources was examined.

i)   Carbon source utilisation

The ability of M.perniciosa to grow on agar plates containing 1% available carbon was determined.   Plates containing either sucrose, Na-carboxymethyl-cellulose (high viscosity, 1500 $\pm$ 400 cP), pectin (apple, 250 grade) supplied by BDH Chemicals Ltd. or glactomannan polysaccharide (gum, locust bean), xylan (larchwood), D-xylose or chitin (crab shell poly-N-acetylglucosamine) supplied by Sigma Chemical Co. Ltd., as sole carbon sources were inoculated with single 3 mm diameter discs cut from the margin of 10 day old M.perniciosa MM agar cultures and incubated at 20°C $\pm$ 2°C.   Growth was determined by measuring the extent of colonisation of the agar surface after 10 days.

Utilisation of native cellulose was determined as the ability of M.perniciosa to colonise sterile filter paper (Whatman No. 1) soaked in OD media containing 0.5 g l^{-1} ammonium sulphate.

ii) Nitrogen source utilisation

Optimum nitrogen source for growth of M.perniciosa in liquid culture was determined as dry weight of fungus produced in OD media containing sucrose as the sole carbon source (20 g l^{-1}) with 0.12 g l^{-1} of available nitrogen, using a range of nitrogen sources as outlined in Table M3.

Table M3

Nitrogen sources for the determination of optimum for growth of

M.perniciosa

Nitrogen source	$g \, l^{-1}$
Ammonium sulphate $(NH_4)_2 \, SO_4$	0.50
Urea	0.24
Calcium nitrate Ca $(NO_3)_2 \, 4H_2O$	0.885
Sodium nitrate Na NO_3	0.6525
Ammonium nitrate $NH_4 \, NO_3$	0.315

2. **Enzymic activity of M.perniciosa**

A. **Production of amylase enzymes**

The presence of amylase enzymes in culture filtrates from
M.perniciosa was detected by enzymic breakdown of wheat starch in
agar plates.

Droplets, (50 µl) of filtrates of M.perniciosa cultures, (Czapek
Dox liquid medium and Czapek Dox liquid medium with the addition of
0.2 µl cm^{-3} sterile filtered juice from pressed healthy A.bisporus
sporophores were placed on the surface of agar plates containing
1.5% wheat starch and incubated for 4 h at 18°C prior to the agar
surface being flooded with iodine solution (1% w/v). Amylase
activity was indicated by the presence of a clear zone in the medium
at the site of sample application.

Controls were of droplets of uninoculated sterile medium placed
on the agar surface.

B. **Cellulase activity**

The presence of cellulose activity in M.perniciosa liquid cultures
were determined viscometrically, using the method of Domsch and Gams (1969),

M.perniciosa cultures, grown in OD liquid medium with
carboxymethylcellulose (1%) as the sole carbon source with the addition
of 0.5 g l^{-1} of ammonium sulphate, were filtered and the supernatant
sterilised by filtration.

Sterile supernatant was tested for cellulase activity by mixing
2 cm^3 of the sample with 8 cm^3 of 1% Na-carboxymethylcellulose
(1500 ± 400 cP) buffered at pH 5.2 with 0.1N sodium acetate :
0.1N acetic acid buffer at 30°C and

viscosity determined immediately, and subsequently at regular intervals using a 10 cm^3 capillary viscometer, (Technico, Cannon-Fenske, Gallenkamp, Loughborough). Results were expressed as percentages relative to controls of SDW (0% viscosity) and untreated 1% buffered carboxymethylcellulose (100% viscosity).

C. Enzyme spectrum

The range of enzymes produced by M.perniciosa in vitro was investigated using the API ZYM pre-packaged diagnostic system (API System S.A., Paris) consisting of prepared galleries containing 19 assay cupules each containing a specific substrate for the identification of a particular enzyme (Table M4).

Samples of mycelium from M.perniciosa cultures grown in Czapek Dox liquid medium, and from those grown in Czapek Dox liquid medium with the addition of 0.2 µl cm^{-3} sterile filtered juice from pressed healthy 'button stage' sporophores, after incubation at 25°C $\pm$ 1°C for 10 days, together with sterile filtered samples of M.perniciosa culture supernatant from Czapek Dox liquid medium with the addition of sporophore filtrates, as described above, together with uninoculated media as controls were each placed in the cupules of separate galleries, (c. 65 µl / cupule) and incubated for 4 h at 37°C $\pm$ 1°C.

Table M4

Enzymes tested for using the API ZYM Diagnostic System

Enzyme assayed for	Substrate
Phosphatase (alkaline)	2-naphthyl phosphate
Esterase (C4)	2-naphthyl butyrate
Esterase lipase (C8)	2-naphthyl caprylate
Lipase (C14)	2-naphthyl myristate
Leucine arylamidase	L-leucyl-2-naphthylamide
Valine arylamidase	L-valyl-2-naphthylamide
Cystine arylamidase	L-cystyl-2-naphthylamide
Trypsin	N-benzoyl-DL-arginine-2-naphthylamide
Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide
Phosphatase (acid)	2-naphthyl phosphate
Phosphoamidase	Naphthol-AS-BI-phosphodiamide
α galactosidase	6-Br-2-naphthyl-αD-galactopyranoside
β galactosidase	2-naphthyl-βD-galactopyranoside
β glucuronidase	Naphthol-AS-BI-βD-glucuronic acid
α glucosidase	2-naphthyl-αD-glucopyranoside
β glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
N-acetyl-B glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide
α mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
α fucosidase	2-naphthyl-αL-fucopyranoside

After incubation each gallery was 'developed' by the addition
of one drop of each of two reagents, 25% Tris (hydroxymethyl)
aminomethane + 10% laurylsulphate in 3.4% hydrochloric acid and
0.35% Fast blue in 2-methoxyethanol. After exposing the
incubated galleries to bright light, the colour of individual cupules
was compared to a standard chart and the presence of specific enzymes
consequently determined.

3.   Factors affecting germination of conidia

A.   Substrate

The influence of substrate on germination of M.perniciosa
conidia was investigated by incubating spores, harvested as
previously outlined, in 20 μl droplets of liquid substrate
(Table M5) on clean glass slides and incubating in moist chambers
for either 72 h (chlamydospores) or 24 h (verticillate conidia) at
$20°C \pm 2°C$ prior to staining with cotton blue in lactophenol and
microscopic examination.

Table M5

Liquid substrates utilised in the examination of the influence of substrate on germination of M.perniciosa conidia

1.	Sterile de-ionised water (SDeW)
2.	Sterile distilled water (SDW) pH 6.7
3.	Sterile filtered juice from pressed healthy A.bisporus sporophores. (button stage)
4.	Chloroform extract of 3
5.	Methanol extract of 3
6.	Czapek Dox liquid medium (half strength)
7.	0.4 M dihydroxybenzoic acid (DHBA)
8.	0.4 ethylene-diamine tetra acetic acid (EDTA)

Organic extracts (Samples 4 and 5) were applied directly to the glass surface and dried in a sterile air flow immediately prior to the application of spores in SDW. Other treatments were applied as double strength droplets (10 μl) of substrate and SDW or SDeW spore suspension where appropriate.

In addition to the effect of substrate on germination of conidia the effects of substrate on subsequent germ tube growth was determined by measuring the length of germ tubes of conidia incubated in substrates as outlined above, using a camera lucida microscope attachment, (Leitz).

B. Temperature

The effect of temperature on germination of M.perniciosa conidia was determined by incubating spores harvested from 10 day old MM agar cultures in half strength sterile filtered juice from pressed healthy 'button stage' A.bisporus sporophores (c. 1 x 10^5 spores cm^{-3}) on glass slides in the dark in moist chambers at various temperatures, (4°, 15°, 18°, 20°, 25°, 30° and 35°C). Percentage germination was determined after 24 h incubation for verticillate conidia and after 72 h incubation for chlamydospores.

C. Light

The effect of light on germination of conidia was determined by placing spores harvested from 14 day old MM agar cultures in 20 μl droplets of half strength sterile filtered juice from pressed healthy 'button stage' A.bisporus sporophores (c. 1 x 10^5 spores cm^{-3}) on

clean glass slides and incubating samples in moist chambers in the dark, Tungsten light (135 Watts m^{-2}) or under ultraviolet light (Phillips 'black light' fluorescent tubes).

D. Age of conidia

Variation in germination potential of M.perniciosa conidia of differing ages was examined by harvesting dry spores from 14 day old MM agar cultures incubated at 20°C $\pm$ 2°C and storing in sterile Petri dishes at ambient humidity for varying lengths of time at 10°C $\pm$ 1°C, (10, 21, 28, 42, 54, 62 and 69 days).

Germination potential of conidia was subsequently determined by incubating 20 µl droplets of a spore suspension (c. 1 x 10^5 spores cm^{-3}) in half strength sterile filtered juice from pressed healthy 'button stage' A.bisporus sporophores on clean glass slides in moist chambers at 18°C and 25°C for verticillate conidia and chlamydospores respectively. The level of germination was determined microscopically after 24 h (verticillate conidia) and after 72 h (chlamydospores) following staining of samples with one drop of cotton blue in lactophenol.

4. Environmental factors affecting sporulation of M.perniciosa

A. The effect of temperature

250 cm^3 flasks each containing 100 cm^3 of Czapek Dox liquid medium were inoculated with a single 5 mm diameter disc cut from the margin of a 10 day old culture of M.perniciosa grown on MM agar.

Flasks were incubated at ambient, (16°C $\pm$ 3°C), 20°C and 30°C in adjacent water baths. When growth of the fungus had extended to the flask edge, one drop of Tween 80 was added to each flask and spores were removed by shaking on a wrist action shaker for 30 minutes. Samples were removed from the flasks and numbers of spores in suspension determined using a haemocytometer.

B. The effect of light

250 cm^3 flasks each containing 30 cm^3 of Malt agar were inoculated with a single disc (3 mm diameter) of M.perniciosa cut from the margin of 10 day old culture on MM agar. Flasks were incubated for 42 days at 20°C $\pm$ 2°C under adjacent, screened lighting conditions, Tungsten light (135 Watts m^{-2}), U.V. light (Phillips 'black light')16 h photo-period, and total darkness.

100 cm^3 of SDW + 1 drop of Tween '80 was subsequently added to each flask and this was then shaken for 30 minutes on a wrist action shaker.

Samples of the resulting suspension were subsequently withdrawn and examined microscopically, and spores counted using a haemocytometer.

5. Aerial dispersal of M.perniciosa spores

Aerial dispersal of M.perniciosa conidia was examined using a Cascade impactor (Cassella Ltd.) (London) with a vacuum pump operating at a drawn air flow of 17.5 l min^{-1}, according to the manufacturer's recommendations. Impaction was on to four glass discs pre-coated with a thin layer of melted petroleum jelly, the discs being stained with a single drop of cotton blue in lactophenol prior to

microscopic observation. Impaction of conidia was preliminarily confirmed by misting a conidial suspension (c. 1×10^5 spores cm^{-3} in SDW) into the air immediately surrounding the trap using a chromatography reagent spray.

Spore release from M.perniciosa cultures was examined by positioning the trap orifice 5 cm from the edge of 12 day old MM agar cultures in an air flow of 0.1 m sec^{-1} at 55% relative humidity.

Spore dispersal from infected mushroom beds was examined at G.C.R.I. using a severely infected crop grown in trays of 0.554 m^2 surface area and infected with M.perniciosa at the time of casing. The impactor was positioned 0.6 m above the cropping house floor and 0.5 m from the nearest infected tray. Sampling was for 1 h at intervals of 24 h.

6. Determination of theoretical colonisation indices of M.perniciosa

The theoretical colonisation index (TCI) for M.perniciosa conidia was determined for 10 day old verticillate conidia and 35 day old chlamydospores on a range of substrates (Table M6). Droplets (20 μl) of spore suspensions (c. 1×10^5 spores cm^{-3}) were applied to 600 μm thick water agar strips placed on the substrate surface in closed plastic 'sandwich' boxes and incubated at 25°C $\pm$ 1°C. Samples were removed after 24 h and stained with cotton blue in lactophenol.

Measurements were of % germination and of germ tube length. Controls were of inoculated agar strips placed on sterile clean glass microscope slides placed on the substrate surface alongside the experimental samples.

The TCI as a comparative unit indicative of the ability of the fungus to colonise various substrates is defined as the product of percentage germination after 24 h incubation and the mean germ tube length at that time, (Mitchell and Dix, 1975a).

Table M6

Substrates used for the determination of comparative TCI values for M.perniciosa on host and non-host substrates

1.	Control (sterile glass slides)
2.	Woodland soil, mixed deciduous woodland, Hermitage wood Stirling University campus.
3.	Clay soil, cultivated, University gardens, Stirling.
4.	Unused casing soil, non-sterile.
5.	Casing / compost, mixed. Stored for 9 months post cropping.
6.	Mushroom casing soil - fresh, post cropping.
7.	Mushroom compost - fresh, post cropping.

7. Growth of M.perniciosa in complex substrates

A. Survival of M.perniciosa mycelium in soil

The ability of M.perniciosa to survive in casing soil and compost was investigated. Barley seeds were soaked in SDW for 48 h prior to autoclaving (1 kg cm^{-2}, 30 min.), and subsequently inoculated with an M.perniciosa spore suspension and incubated for 21 days at 18°C $\pm$ 2°C.

Colonised seeds were removed at 1 week intervals, surface sterilised with mercuric chloride (HgCl$_2$) and plated on to MM agar (+ streptomycin) and incubated at 20°C.

B. Germination and growth of conidia in composts

The effect of diffusates from compost on germination and growth of conidia was investigated in vitro.

Mushroom compost, colonised by A.bisporus was steeped in SDW (pH 6.4) for 18 h at 4°C and the resultant supernatant separated into high molecular weight (HMW) and low molecular weight (LMW) fractions by dialysis through cellophane (visking dialysis tubing) for 48 h at 4°C with two changes of diluant. All fractions were concentrated by freeze drying and resuspended in SDW to a concentration equivalent to the diffusate from 10 g of compost cm^{-3}. Osmotic potential of each solution was measured using an osmometer. (Fiske Associates Inc. Uxbridge, Mass. Model 330D).

The effect of diffusates and dialysis fractions on germination and growth of M.perniciosa and Cladosporium herbarum conidia was examined by placing droplets (10 µl) of a conidial suspension (from a 14 day

old and 8 day old culture respectively) together with one 20 µl droplet of each of the test fractions on clean glass slides in moist chambers and incubating samples for 48 h at 18°C $\pm$ 1°C. Germination levels and germ tube growth was determined after killing sporelings by the addition of droplets of cotton blue stain in lactophenol.

C. Spectrophotometric assessment of fungal growth

Quantitative assessment of fungal growth was made by fluorescein diacetate determination of esterase activity using the method of Swisher and Carroll (1980).

In order to produce a calibration curve for M.perniciosa samples of M.perniciosa mycelium were shaken for 1.5 h at 20°C $\pm$ 1°C (Gallenkamp orbital incubator, 100 r.p.m.) in 25 cm^3 SDW containing 8 µg cm^{-3} of fluorescein diacetate (FDA) from a 2 mg cm^{-3} stock solution of FDA in acetone, in 100 cm^3 conical flasks. After incubation the contents of the flasks were filtered (0.22 µm pore size) through tared filters and the absorbance (at 490 nm) of the resulting supernatant determined on a Cecil CE 272 UV spectrophotometer. The dry weight of the fungus used was then determined by weighing the dried filter membranes and a calibration of dry weight of fungus against absorbance of resulting fluorescein substrate constructed,

(Figure M2)

D. Growth of M.perniciosa in compost

The ability of M.perniciosa to colonise mushroom compost was determined quantitatively using the fluorescein diacetate technique.
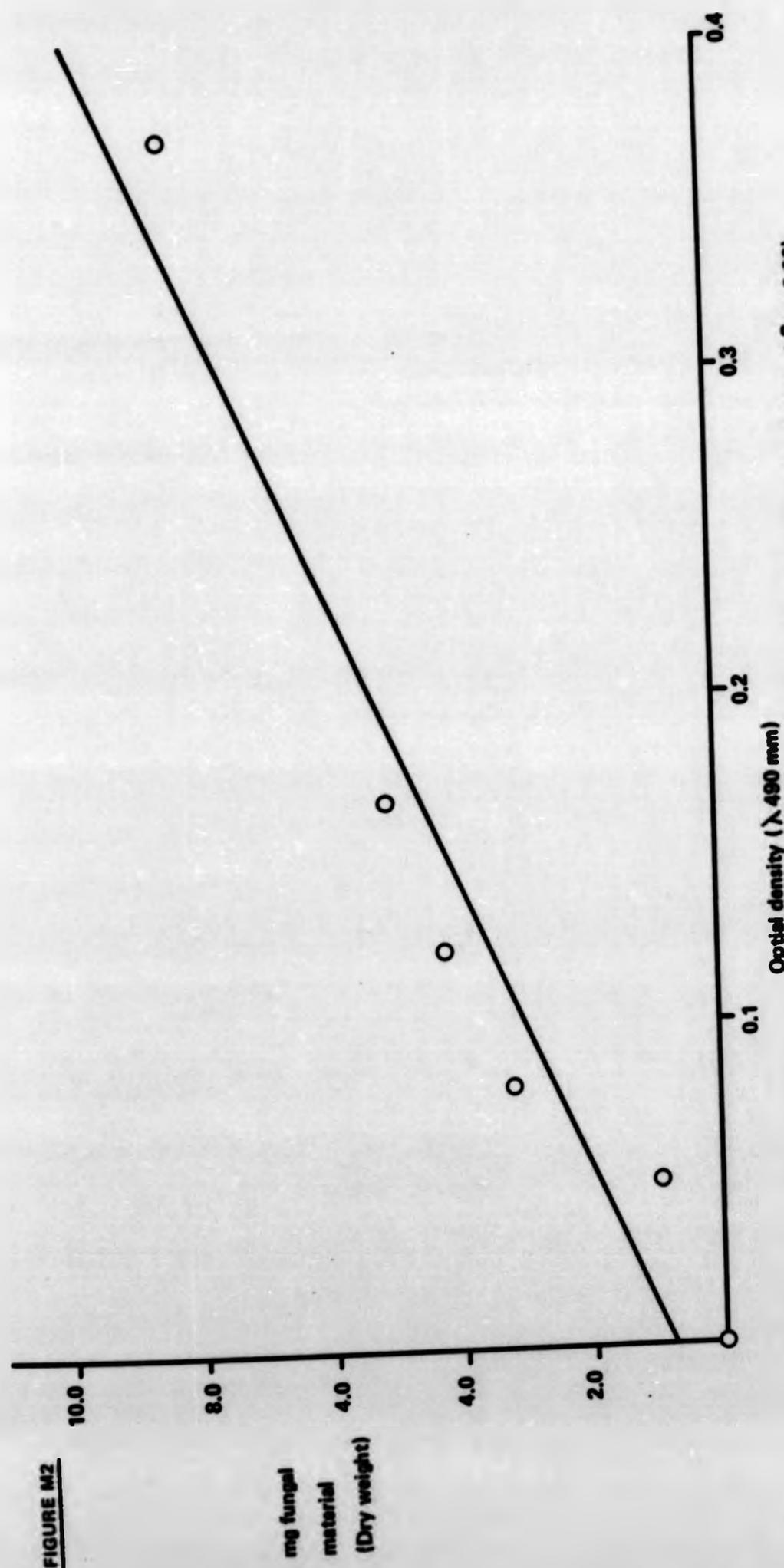
FIGURE M2

mg fungal
material
(Dry weight)

Optial density (λ 490 mm)

Regression of fungal dry weight against F.D.A. hydrolytic activity for _Mycogone perniciose_ (r² = 0.90) determined using the method of Swisher and Carroll (1980).

Flasks containing 250g of sterile compost were inoculated with a conidial suspension (c. 4 x $10^4 g^{-1}$ of compost) from a 14 day old M.perniciosa MM agar culture. Compost samples were subsequently supplemented with SDW, diffusates and dialysis fractions from colonised compost at the rate of 0.25 $cm^3 g^{-1}$ of compost, prepared as previously described, and incubated at $20^\circ C \pm 2^\circ C$ for 7 weeks. In addition flasks of compost previously colonised by A.bisporus and sterilised with ethylene oxide were similarly inoculated with M.perniciosa. Sterilisation procedure using ethylene oxide was according to manufacturers' recommendations (Pern Engineering Ltd., Northfleet).

Following incubation samples (c. 1.0 g) were withdrawn from each flask and esterase activity determined as described above. Controls were of uninoculated compost and absorbance values at 490 nm deducted from experimental values giving an absorbance which could then be directly related to the quantity of M.perniciosa mycelium present in the compost, using the previously prepared calibration data.

In addition, non-sterile compost inoculated with M.perniciosa in a manner similar to those flasks described above was observed, although determination of M.perniciosa mycelium by esterase activity was not possible for this treatment.

8. Infection of A.bisporus initials

The growth stage of A.bisporus sporophores at which infection by M.perniciosa can first occur was examined.

Sporophore initials (strain D621) grown under axenic conditions in 3.5 l flasks, as previously outlined, were excised under sterile conditions and each placed in a 20 µl droplet of an M.perniciosa

conidial suspension (c. 1×10^5 spores cm^{-3}) in Sorensens buffer (Hale, 1965

at pH 6.3, 7.2 or 7.6. on clean glass cavity slides.

The slides were placed in moist chambers and following incubation for 5 days at 23°C $\pm$ 1°C in the dark stained with cotton blue in lactophenol for microscopic examination. Results were expressed as percentage germination of M.perniciosa conidia and related to the size of A.bisporus initials with which they were incubated.

9. **Determination of Numerical Threshold for Infection (NTI)**

NTI was determined for chlamydospores of M.perniciosa using untreated mature sporophores of A.bisporus (strain D629 ('cup stage').

A chlamydospore suspension was prepared as previously described and the concentration determined using a haemocytometer. Serial dilutions of this spore suspension stock were diluted to give a range of concentrations of between 0 and 250 spores $10 \mu l^{-1}$. Single droplets (10 μl) of suspension were applied to the centre of freshly picked 'button stage' sporophore caps which were subsequently incubated in moist chambers (6°C $\pm$ 1°C) for 10 days.

Inoculated caps were examined after incubation under a dissecting microscope and assessed for growth and sporulation of M.perniciosa.

10. **Interaction of M.perniciosa with other fungi**

The sensitivity of M.perniciosa to other fungi was investigated using dual cultures on agar plates.

M.perniciosa and one of a number of fungi were inoculated on to

Czapek Dox agar, 3.5 cm apart in 9 cm Petri dishes and incubated at either $16^{\circ}C(\pm 2^{\circ}C)$ or $20^{\circ}C\pm 2^{\circ}C$. Growth of M.perniciosa from the point of inoculat was measured at regular intervals and compared to controls of M.perniciosa grown on Malt agar in the absence of other fungi. Where fungi grew more slowly or more quickly than M.perniciosa these were inoculated on to agar prior to, and after, inoculation with M.perniciosa respectively.

In addition to growth on agar plates, the growth of M.perniciosa in the presence of A.bisporus was investigated in mushroom compost by inoculating sterile compost (50 g) in 15 cm diameter glass Petri dishes with a single 5 mm diameter disc cut from the margin of a 10 day old M.perniciosa MM agar culture at the same time as inoculation with similar discs cut from the margin of 14 day old A.bisporus (strain D621, A6, GCRI 431) cultures, 6 cm apart. Plates were incubated at $16^{\circ}C \pm 4^{\circ}C$ for 66 days and growth of M.perniciosa determined microscopically. Controls were of sterile compost inoculated with M.perniciosa in the absence of A.bisporus.

Assessment of growth of M.perniciosa in the presence of other fungi was based on the conclusions of Gray and Morgan-Jones, (1981). They considered that the pattern of growth of mycoparasites in the presence of the vegetative mycelium of specific fungi was directly related to the relationship between the two fungi. They described four patterns of growth shown in vitro by mycoparasites in the presence of non-host fungi, a potential host, a mutually antagonistic fungus and the natural host, (Table M7, Figure M3).

Table M7

In vitro relationship between mycoparasites and host and non-host fungi

Growth pattern (category)	Relationship of fungus to the mycoparasite	Growth pattern of the mycoparasite
A	non-host	Growth is at a uniform rate both before and after contact between the two fungi.
B	potential host	Growth of the mycoparasite is reduced gradually as the two fungi come into contact, following contact growth is further reduced but not stopped.
C	mutual antagonist	Following an initial uniform growth rate prior to contact between the two fungi growth of the mycoparasite ceases.
D	natural host	Following an initial high growth rate, growth of the mycoparasite is slowly reduced and eventually stops altogether following contact between the two fungi.

(After Gray and Morgan-Jones, (1981)).

FIGURE M3
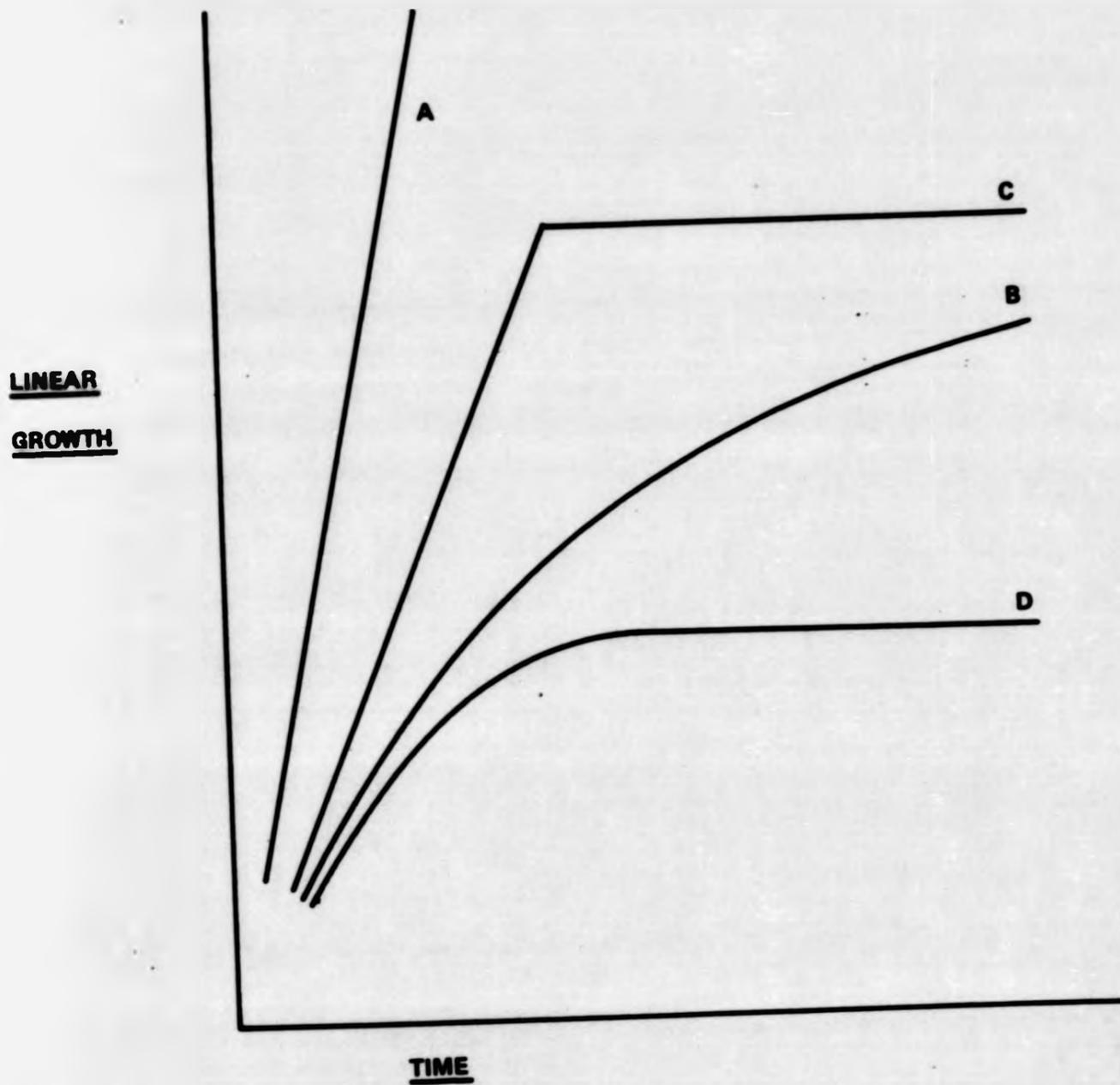
Characteristic growth reactions of Hypomycetes when grown in the presence of host and non host fungi. (After Gray & Morgan-Jones (1981))

A. In the presence of a non-host species

B. In the presence of a potential host

C. In the presence of a mutually antagonistic fungus

D. In the presence of the natural host

11. Alternative hosts of M.perniciosa

The ability of M.perniciosa to infect fleshy fungi other than A.bisporus was investigated. Mature fruit bodies of a range of fungi were collected from Stirling University Campus (mixed deciduous woodland) and inoculated artificially with M.perniciosa.

Tissue blocks (c. 1.5 x 1.5 cm) were excised aseptically from cap tissue of selected sporophores and placed on clean glass slides in sandwich boxes lined with moist tissue paper. Inoculation was with one 20 µl droplet of an M.perniciosa SDW spore suspension (c. 1 x 10^5 spores cm^{-3}) on to the block surface prior to incubation at 18°C $\pm$ 2°C. Blocks were examined at regular intervals using a dissecting microscope for evidence of germination and growth of M.perniciosa.

Infection of sporophores in the wild was attempted by applying single droplets (20 µl) of an M.perniciosa conidial suspension (c. 1 x 10^5 spores cm^{-3}) directly to the cap surface of selected young sporophores and subsequently examining samples in situ during development, and subsequently using a dissecting microscope, for evidence of germination and growth of M.perniciosa.

III. MORPHOLOGY OF INFECTED TISSUE

The structure of A.bisporus tissue infected by M.perniciosa was examined using sporophores cultured in trial plots at G.C.R.I., or in trays or pots at Stirling University, as previously outlined (Section 12).

Tissue samples (c. 1 x 1 mm) were routinely excised from healthy and infected tissue and plated out on to Malt agar, Nutrient agar or Kings B agar (Table M11), and incubated at 20°C or 23°C in order to determine the fungi or bacteria present in the tissue.

Measurements of the resistance of sclerodermoid masses to penetration (rheological strength) were made with a hand-held penetrometer (3 mm diameter probe), (J. Chatillon & Sons, Kew Gardens, N.Y. USA).

1. Antagonism of bacteria towards M.perniciosa

Bacteria were screened for antagonism towards M.perniciosa using a dual agar culture technique.

Bacteria isolates were each streaked on to Czapek Dox agar in 9 cm Petri dishes and the plates immediately re-inoculated with a single 3 mm disc of M.perniciosa cut from the margin of a 10 day old MM agar culture, 3.5 cm and at rightangles to the bacterial streak. Plates were incubated for 10 days at 23°C $\pm$ 1°C and growth of M.perniciosa towards the bacteria measured. Controls were of M.perniciosa Czapek Dox agar cultures incubated under identical conditions in the absence of bacteria.

2. Antagonism of bacteria towards A.bisporus

Bacteria were screened for antagonism towards A.bisporus using a modification of the technique of Gandy, (1968). Blocks (c. 1 x 1 cm x 0.5 cm) of healthy A.bisporus (strain A6) cap tissue were excised under aseptic conditions and placed on clean glass slides in moist chambers and inoculated with a single 10 µl droplet of a suspension

of bacteria from a 24 h old Nutrient agar culture suspended in SDW (c. 1×10^8 bacteria cm^{-3}). Blocks were then incubated for 72 h at $23°C \pm 1°C$ prior to examination. Antagonism of bacteria was expressed as a browning of the tissue surface compared to controls of A.bisporus tissue blocks inoculated with SDW.

3. Infection of A.bisporus under axenic conditions

In order to investigate the ability of M.perniciosa to infect A.bisporus under axenic conditions, cultures produced as previously outlined (Section I7) were infected with an M.perniciosa conidial suspension (c. 1×10^5 spores g^{-1} of casing) at the time of casing with sterile activated charcoal. Flasks were observed daily and the appearance of cultures noted.

In order to determine the effect of bacteria on infection of A.bisporus by M.perniciosa, healthy and infected A.bisporus axenic cultures described above were also inoculated with an SDW suspension of specific bacteria (c. 1×10^6 bacteria g^{-1} of casing) from a 24 h old nutrient broth culture at the time of casing.

4. Microscopic preparations

A. Differentiation of A.bisporus and M.perniciosa

Initial preparation of infected A.bisporus tissue was by hand-cut sectioning using a sharp razor blade, and by 'squash' preparation of small (c. 1×1 mm) tissue samples on clean glass slides, mounted in water, or a range of stains, in order to assess differential staining, hyphal dimensions and interactions between host and pathogen hyphae.

Fresh tissue preparations were immersed in a range of stains, as

detailed in Table M8, for c. 1 min., and examined under the light
microscope to determine whether it was possible to differentiate
between hyphae of A.bisporus and M.perniciosa in infected material
and in dual cultures.

TABLE M8

Water soluble stains used for examination of fresh sections

Water

Clear lactophenol (Laundon, 1968)

Cotton blue/Lactophenol (1% w/v) (Laundon, 1968)

Neutral red 0.06% aqueous (Ikeduigwu and Webster 1970a)

Toluidine blue. (0.05% w/v in 0.1 M phosphate buffer, (pH 6.8)
(O'Brien, Feder and McCully, 1964).

Trypan blue. (1% w/v) (Rossall, Mansfield and Hutson, 1980)

Aniline blue 0.005% in Ethanol (50%) (Currier, 1957).

Acetic Aniline blue, 1% in 7% Acetic Acid, (Jensen, 1962)

Lugol's iodine 1% aqueous iodine in 2% aqueous potassium
iodide. (Gurr, 1960).

Methylene blue. (0.13% aqueous)(Sirockin and Cullimore, 1969).

B. Autoflourescence

Fresh tissue sections were mounted in water and viewed using
transmission flourescence microscopy (Reichert Fluoropan microscope,
excitation filter BG 12, emission filters OG 1, and GG9), (Harris
and Hartley, 1976).

C. Tissue fixation

Permanent preparations were prepared using a modification of the method of Sabatini, Bensch and Barnett, (1963). Pieces of tissue (c. 2 x 2 mm) were fixed in 2% glutaraldehyde in 25 mM sodium cacodylate buffer, pH 7.2. Infiltration of fixative was under intermittent vacuum for 18 h at 0°C. Dehydration was with three changes of 2-methoxy ethanol for 24 h, followed by substitution with three changes of absolute alcohol in 24 h.

Embedding was in JB4 Methacrylate resin (Polyscience Inc. Warrington, Pennsylvania) in rubberised moulds, under oxygen-free nitrogen.

Sections were cut on an LKB pyramitome 11800 using glass knives, and mounted in water on clean glass microscope slides, and fixed by drying on a hot plate.

D. Periodic acid Schiffs stain

Methacrylate embedded sections mounted on clean glass slides were placed in di nitro phenyl hydrazine (DNPH) 0.5% w/v in 15% Acetic acid for 10 min. Slides were then transferred to 1% periodic acid (w/v aqueous) for 10 min. and subsequently rinsed in running tap water for 10 min. This was followed by placing the slides in Schiffs reagent for 25 min. and subsequently transferring quickly and directly to three successive baths, 2 min. in each, of 0.5% sodium metabisulphite. The slides were then rinsed in running water for 10 min. and dried on a hot plate, a cover slip being positioned over the section prior to examination, starch and more complex polysaccharides, staining deep red. (O'Brien and McCully, 1981).

E. Toluidine blue

Methacrylate embedded sections were flooded with Toluidine blue

1% w/v in 5% aqueous boric acid and warmed on a hot plate to 50°C
for 1 min., care being taken not to allow the sections to dry out.
The slides were then rinsed in running distilled water for c. 1 min.
to remove excess stain. After partial drying the sections were
covered with a cover slip and examined microscopically.
Cytoplasm and walls of fungal material stain dark blue with no
noted tissue specificity.

5. **Host/Parasite interactions**

A. **Assessment of cellular interaction and cell death**

Healthy and infected sporophore tissue and sclerodermoid
material was assessed for host cell death using the Trypan blue
method.

Fresh preparations of sample material were mounted in a drop
(c. 10 µl) of 1% w/v Trypan blue (aqueous) and assessed for uptake of
the stain in intact cells after c. 1 min. Dead cytoplasm stains
dark blue. (Rossall, Mansfield and Hutson, 1980).

B. **Microscopic assessment of A.bisporus / M.perniciosa biomass**

Methacrylate sections of a number of mature sclerodermoid mass
tissue samples were stained in Toluidine blue (1%) in 1% Borax.
Drawings of sections were made using a camera lucida microscope
attachment (Leitz) and assessed for % M.perniciosa, A.bisporus and
air space in order to estimate the biomass of individual component
fungi.

Not changed.

C. Spatial arrangement and branching within sclerodermoid material

Methacrylate sections (0.5 μm) *(5 μm)* of mature sclerodermoid material, from a point 0.5 cm below the tissue surface, were prepared as previously described. Fifty successive sections were stained with 1% Toluidine blue in 5% Borax and one area of the tissue was selected and drawn with the aid of a camera lucida attachment, (Leitz).

The nature of the spatial relationship between A.bisporus and M.perniciosa cells was assessed in terms of the juxtaposition of the two hyphal types, and the form of the individual cells, and the positioning of any penetration points.

6. Ultrastructural studies

Preparation of material for electron microscopy

A. Chemicals

Chemicals were either of electron microscopy or analytical grade. Correct procedures were adopted in the handling and storage of the highly toxic chemicals used, (Muir, 1977, Anonymous 1982).

B. Transmission electron microscopy, (TEM)

i Fixation, dehydration and embedding

Material for transmission electron microscopy (TEM) was cultured as outlined in Sections I1 and I2.

Small tissue samples, (c. 1-2 x 1-2 mm) were cut using a sharp razor blade. The fixative used was either 1% potassium permanganate (Manocha, 1965), or 2% glutaraldehyde in 50 mM sodium cacodylate

buffer, pH 7.2 (Sabatini, Bensch and Barrnett, 1963). The tissue was infiltrated with fixative by intermittent vacuum for 6 h then left at c. 20°C for a further 18 h. Following fixation, the tissue was rinsed in 50 mM sodium cacodylate buffer, pH 7.2 for 1 h. The tissue was then fixed in 2% OsO_4 in 50 mM sodium cacodylate buffer, pH 7.2 overnight, (c. 18 h).

Following fixation, tissue was rinsed in 50 mM sodium cacodylate buffer, pH 7.2 for 1 h prior to dehydration in a series of increasing EtOH (BDH chemicals) concentrations. Propylene oxide was used as the linking substitution agent between dehydration and embedding of the tissue.

Tissue was finally embedded in epoxy (low viscosity) resin (EMscope Laboratories Ltd., EMIX resin kit).

The procedures adopted are outlined in Figure M4.
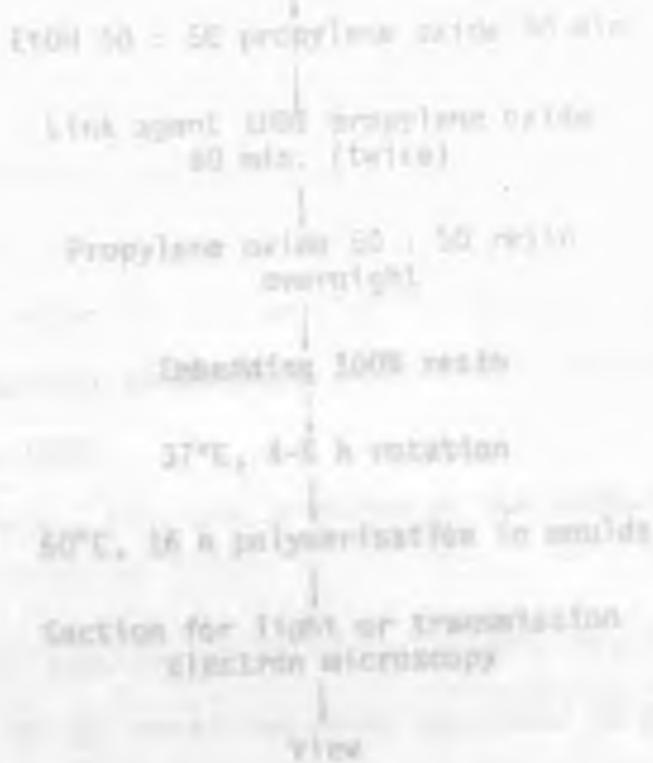
Figure M4

Preparation of tissue for transmission electron microscopy

Tissue sampled

Fixation in 1%
potassium permanganate
20 min.

Fixation in 2%
glutaraldehyde
2 h.

Intermittent vacuum c. 6 h

Normal pressure   c. 18 h

Rinse in buffer   c. 4 h.

Post-fix in 2% OsO$_4$ overnight

Rinse in buffer, 1 h

Rinse in water, 1 h.
(twice)

Dehydration series in EtOH
10, 20, 30, 50% 30 min. each

75, 90, 100% (twice) 60 min. each

EtOH 50 : 50 propylene oxide 30 min.

Link agent 100% propylene oxide
60 min. (twice)

Propylene oxide 50 : 50 resin
overnight

Embedding 100% resin

37°C, 4-5 h rotation

60°C, 16 h polymerisation in moulds

Section for light or transmission
electron microscopy

View

ii   Sectioning

Resin blocks containing tissue were trimmed using a small
hacksaw blade and razor blade prior to sectioning. Areas for
viewing in the transmission electron microscope were selected and
the block face trimmed with a sharp razor blade to leave the desired
area on a raised 'mesa' to facilitate the cutting of ultrathin
sections.

Ultrathin sections c. 90 mμ thick were cut with glass knives
using an LKB Ultratome III. The sections collected as ribbons in a
plastic trough filled with clean distilled water fitted to the knife
using dental wax. The ribbons of ultrathin sections were collected
on coated 80 mesh copper grids (unless otherwise stated) and allowed
to dry.

After staining, sections were viewed using a Jeol JEM 100C
transmission electron microscope (60-80 kV accelerating voltage).

C.   Staining

i   Uranyl acetate / Lead citrate

Uranyl acetate. 2% uranyl acetate (w/v) was dissolved in clean
distilled water and stored at 4°C. Used by itself uranyl acetate
gives a general enhancement of contrast in tissues fixed with OsO_4
(Glauert, 1965).

Lead citrate. This was prepared by the method of Reynolds (1963) as
follows:

a) A solution of $Pb(NO_3)_2$ (1.33g), $Na_3(C_6H_5O_7).2H_2O$ (1.76g) in
30 cm^3 distilled water was mixed in a 50 cm^3 volumetric flask.

b) The solution was shaken vigorously for 1 min. and then
allowed to stand for 30 min. to ensure the complete
conversion of lead nitrate to lead citrate.

c) Carbonate free 1N-NaOH (8 cm^3) was then added and the
solution made up to 50 cm^3 with distilled water and mixed
by inversion. The lead citrate dissolved and the
solution was ready for use (pH 12.0).

Lead citrate singularly, or in conjunction with uranyl acetate
provides a general increase in contrast.

Staining of sections was achieved by placing the grids
(section on to the stain) on a drop of uranyl acetate maintained on
a grease-free Parafilm strip in a closed Petri dish for 30 min. The
grids were then removed, flushed with distilled water and dried
before being placed on a drop of lead citrate stain maintained in
the same manner as above. Pellets of NaOH were included in the Petri
dish with lead citrate in order to absorb free carbonate which would
otherwise be readily absorbed by the lead citrate and thus impair
staining. After 30 min. on lead citrate the grids were removed and
flushed sequentially with distilled water, 0.02M NaOH and finally
distilled water before drying.

ii Silver proteinate

The method described by Roland (1978) was used.
Ultrathin sections were mounted on uncoated 400 mesh gold grids
and immersed in 1% periodic acid for 30 min., washed with distilled
water and placed in 0.2% (w/v) thiocarbohydrazide (TCH, Agar Aids)

in 20% acetic acid for 24 h. After washing in series of 15, 10 and 5% acetic acid solutions, (10 min. each), the grids were then washed in distilled water for 30 min. before placing in an aqueous 1% solution of silver proteinate (Agar Aids) in the dark for 30 min. The sections were finally washed twice for 10 min. in distilled water before drying and viewing.

Silver proteinate staining is derived from the periodic acid-Schiff stain (PAS) and emphasises staining of polysaccharide groups (Roland, 1978).

iii Ruthenium Red

The staining procedure adopted was a modification of that described by Luft, (1971). Tissue was fixed as previously described in 2% glutaraldehyde in buffer containing 0.05% Ruthenium red (5 mg/10 cm^{-3}) and then rinsed in buffer containing 0.05% Ruthenium red.

The tissue was post-fixed in OsO_4 containing 0.05% Ruthenium red prior to dehydration and embedding in the way previously described. Sections were stained with uranyl acetate / lead citrate prior to viewing.

Ruthenium red has an affinity for polysaccharides which is enhanced by the presence of OsO_4, (Luft, 1971).

D. Scanning electron microscopy, (S.E.M.)

Material for scanning electron microscopy was cultured as outlined in Sections I1 and I2.

Tissue samples (c. 5 x 5 mm) were cut using a sharp razor blade.

The fixative used was either 2% glutaraldehyde in sodium cacodylate buffer, pH 7.2, 2% glutaraldehyde in sodium cacodylate buffer, pH 7.2, with post-fixing in 2% OsO_4, OsO_4 vapour or OsO_4 vapour with post-fixing stabilisation in 0.05% thiocarbohydrazide (TCH) in sodium cacodylate buffer, pH 7.2. In addition, unfixed material was prepared for examination. (Hayat, 1978, O'Brien and McCully, 1981).

Samples were washed in sodium cacodylate buffer (3 changes) and dehydrated through a graded EtOH series. Samples were substituted with amyl acetate through a graded EtOH/amyl acetate series using a modification of the method of O'Brien and McCully (1981), prior to critical point drying with liquid CO_2, (Polaron model E3000 (Polaron Ltd. Watford)).

Fresh samples were also prepared by immersion in liquid nitrogen. These were then 'freeze fractured' to expose internal areas of tissue.

Samples were mounted on stubs with silver paint or double sided tape and sputter coated with gold, (S150 sputter coater, (Edwards Crawley)). Examination was at 15 kV accelerating voltage using a Cambridge Mark I or Jeol T20 scanning electron microscope.

The procedures adopted are outlined in Figure M5.

Preparation of tissue for scanning electron microscopy

Tissue sampled

↓

Fixation in:-

2% glutaraldehyde   2% glutaraldehyde OsO_4 OsO_4 unfixed
    2.h   2.h   Vapour   Vapour
                           ↓   6 h   16 h

Rinse in buffer, Post-fixation
in
2% OsO_4 ⟵   0.05% TCH
3 h

Rinse in buffer (x3)

↓

Dehydration series in EtOH

↓

10, 20, 30, 50%, 30 min. each

75, 90, 100% (twice) 60 min. each

Substitution with amyl acetate

↓

10, 25, 50, 75% amyl acetate in EtOH
100% amyl acetate, 30 min. each

↓

CO_2 critical point drying   Samples frozen in
                                liquid nitrogen

↓

Mount on microscope stubs

↓

Sputter coat with gold

↓

View

IV. THE PRODUCTION OF ANTIFUNGAL, ANTIBACTERIAL AND GROWTH PROMOTING
SUBSTANCES BY M.PERNICIOSA AND A.BISPORUS

1. Source of material

Experiments on the production of toxic and growth promoting
substances by either the host or the pathogen were performed using
isolates of M.perniciosa made at Stirling by the author and with
A.bisporus strain D621, unless otherwise stated. Healthy and
infected material was produced using the methods previously
described (Sections I1 and I2).

2. Investigation of presence of toxins

A. Bioassay of extracts from M.perniciosa

M.perniciosa, cultures in static liquid malt extract medium,
(200 cm^3 medium in 500 cm^3 conical flasks) were incubated at 18°C
for 10 days. At 2 day intervals after inoculation cultures were
centrifuged (10 min., 4000G) and filtered (Whatman No. 1). The
supernatant was then shell frozen in round bottomed flasks in
liquid nitrogen and freeze dried, (Chemlab SB3 freeze drier). Dried
filtrates were re-suspended in 20 cm^3 of sterile distilled water and
assayed for presence of toxins using the glass slide bioassay
technique (Section I11).

Incubation of slides was at 20°C for 18 h in moist chambers, at
which point spores were killed by the addition of one drop of cotton
blue in lactophenol to each sample droplet. Measurement was of spore
germination and germ tube growth, expressed as a percentage relative
to controls.

B. **Conductiometric assessment of antagonism**

Tissue samples (0.5 x 1.0 x 0.1 cm) were excised from surface sterilised healthy A.bisporus ('button stage' strain D621) and placed in 250 cm^3 conical flasks containing 100 cm^3 Czapek yeast medium consisting of:-

> Czapek Dox liquid medium (Oxoid CM95), 16.7 g
>
> Yeast Extract Difco-Bacto 5 g
>
> Streptomycin sulphate (Sigma) 0.5 g
>
> Distilled water   1 litre

The flasks were incubated for 5 days at 22°C $\pm$ 1°C in a Gallenkamp (Loughborough) orbital incubator at 100 r.p.m.

M.perniciosa cultures were of either Czapek Dox liquid medium or of Czapek Dox liquid medium supplemented with sterile filtered juice from pressed healthy A.bisporus sporophores (0.3 μl cm^{-3}) in 250 cm^3 conical flasks each containing 50 cm^3 of medium. Flasks were each inoculated with two 3 mm diameter discs cut from the margin of a 14 day old MM agar culture and incubated for 8 days at 20°C $\pm$ 1°C.

Prepared A.bisporus tissue, after incubation, was rinsed in sterile de-ionised water (SDeW) twice, and placed in 50 cm^3 SDeW in chromic acid cleaned glass flasks. Sterile filtrates from M.perniciosa cultures, prepared as described above were added to separate flasks of A.bisporus tissue in SDeW (1.0 μl cm^{-3}) and solution conductivity measured over a 48 h period, using a conductivity meter (Portland Electronics Ltd., Model P335 (Bolton)), with samples maintained at 20°C $\pm$ 1°C in an orbital incubator (25 r.p.m.). Sample

blanks were of M.perniciosa culture filtrates with or without host filtrates in the culture medium added to SDeW without A.bisporus tissue. Controls were of A.bisporus tissue in SDeW with the addition of uninoculated Czapek Dox liquid medium or of uninoculated Czapek Dox liquid medium containing host filtrates, as used for the culture of M.perniciosa test samples.

C. Microscopic observation of antagonism

Sterile filtrates of M.perniciosa cultures, prepared as described above, were added directly to A.bisporus Czapek yeast tissue cultures, grown as described above, $(1.0 \; \mu l$ filtrate $cm^{-3})$ and samples of host mycelium examined at regular intervals for changes relative to controls of flasks of A.bisporus tissue cultures with the addition of either uninoculated Czapek Dox liquid medium or of Czapek Dox liquid medium supplemented with A.bisporus sporophore filtrates.

Cell death was determined using the Trypan Blue (1% w/v) vital staining technique with uptake of the large molecular weight indicating membrane disruption in killed cells, (Rosall, Mansfield and Hutson, 1980). Comparisons between A.bisporus tissue in control samples and in samples in the presence of M.perniciosa culture filtrates were of hyphal width, the number of cells exhibiting vacuolation and cytoplasmic granuolation when observed microscopically.

3. Solvents for tissue extraction, gel filtration and thin layer chromatography

All solvents were ANALAR grade, precooled to 4°C before use in

tissue extraction procedures.

A.   Chemicals: TLC spray reagents

Reagents for the identification of compounds directly on TLC
plates (Table M8), were applied by spraying prepared plates with
reagents using an aerosol spray (Shandon).

B.   Spectral analyses

Ultraviolet (U.V.) absorption spectra were obtained with a Pye
Unicam (Cambridge) SP 1800 spectrophotometer.

C.   Measurement of pH

Values of pH were measured with a Pye Unicam (Cambridge) model
PW 9418 pH meter.

4.   Preparation of fungal material

Extraction procedures were carried out on a range of healthy
and infected material.

Healthy sporophores were produced by the methods previously
described, or supplied by Messrs. Dumbreck, Freuchie. Material was
picked and placed immediately in plastic bags.

Fungi were cultured in Czapek Dox liquid medium with or without
the addition of sterile filtered juice from pressed healthy
A.bisporus sporophores ($0.5 \text{ cm}^3 \text{ } 100 \text{ cm}^{-3}$), ($0.22 \mu m$ pore size,
Millipore).

Table M8

Reactions used to identify various chemical groups on TLC plates according to the methods of Christie (1976) and Merck (1974)

Test reagent	Chemical group visualised	Reaction on the TLC plates
DpNA p-nitroaniline	Phenolics	Various colours
$AlCl_3$ Aluminium chloride	Flavonoids	Fluorescent yellow under longwave U.V. light
Vanillin	Higher alcohols	Blue colour
Hydroxylamine hydrochloride	Carboxylic acid derivatives	Show up as purplish spots
Dinitrophenyl hydrazine	Ketone derivatives	Blue colour immediately
	Aldehyde derivatives	Green, slowly
	Unsaturated carbonyl derivatives	Rarely colour

Fungal material and media were separated prior to solvent extraction by centrifugation (5500 G) for 5 min. and filtration (0.22μm pore size). Fungal material was subsequently rinsed thrice in sterile distilled water and re-centrifuged. Extraction was begun within two hours of harvesting. Samples not used immediately were stored at -20°C.

5. Extraction procedures

Precautions were taken to minimise exposing extracts to light.

Material was initially extracted in ice-cold methanol in a pre-cooled pestle and mortar and filtered through four layers of muslin, and squeezed to dryness. The resultant liquid was centrifuged for 5 min. at 5500 G and the supernatant filtered under vacuum, (Whatman No. 1). Samples were routinely evaporated to dryness on a rotary evaporator (Buchi Rotavapor), small quantities of extracts (less than 2 cm^3) were evaporated to dryness in a stream of oxygen-free nitrogen.

Where applicable, liquid samples were shell frozen in round bottomed flasks in liquid nitrogen and freeze dried prior to solvent extraction.

Preparative column chromatographic purification of solvent extracts was on 31 cm columns of Sephadex LH 20, eluting with methanol at a flow rate of 2 cm^3 min^{-1}.

Detection of antifungal activity in solvent fractions was by spotting samples directly on to TLC plates and assaying using the Cladosporium spray technique, (Section II 7A).

Where applicable, extraction of material was also in methanol :
chloroform, (1:1) or directly in chloroform (Figures M6 and M7).

Lipidic substances were extracted from tissue and cultures
using the method outlined by Deven and Manocha, (1975), (Figure M8).

Lipidic compounds were visualised on TLC plates using a range
of reagents (Figure M9).   (Christie, 1976)

Figure M6

Solvent extraction procedure adopted in the preparation of samples
from healthy A.bisporus sporophores prior to chromatographic
separation and bioassay.

Extraction of tissue in

X10 volumes of methanol, (4°C)

Re-extraction of tissue in X 10

volumes of chloroform, three times,

(4°C), on anhydrous sodium sulphate,

(1 gram/gram. fresh wt. tissue).

Combine solvent fractions   Discard residue

Concentrate retained chloroform fraction on

a rotary evaporator.
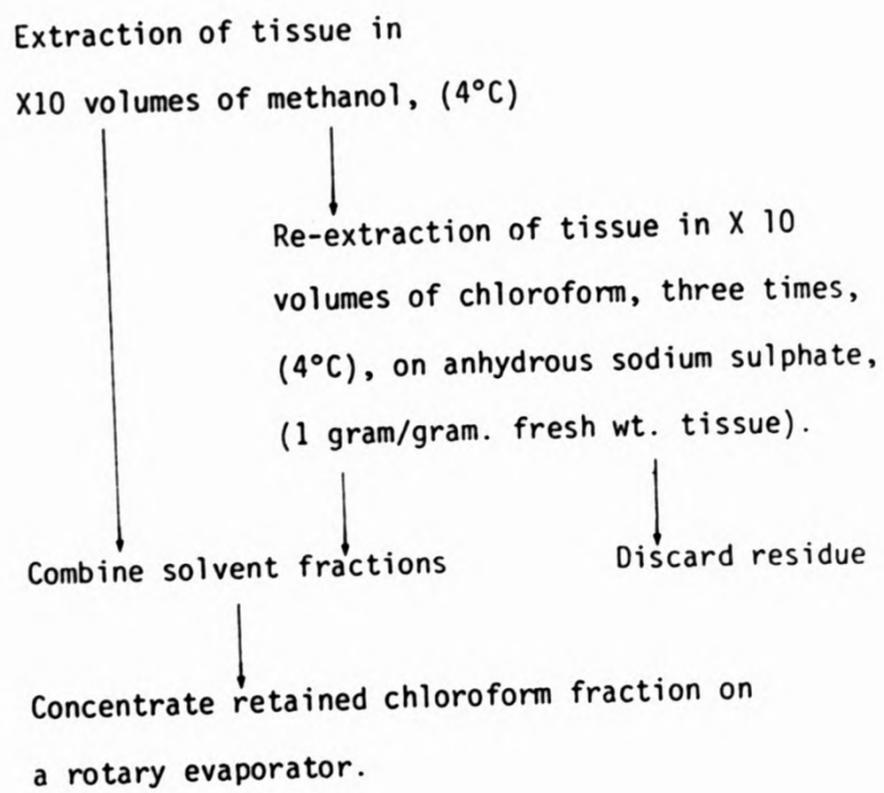
Figure M7

Chloroform extraction procedure adopted in the preparation of samples
from healthy A.bisporus sporophores prior to chromatographic separation
and bioassay.

Extraction of tissue in X10 volume of chloroform,

(4°C), three times.

↓

Tissue filtered through four layers of muslin

↓

Supernatant filtered, (Whatman No. 1)

↓

Chloroform phase shaken for 30 min. over anhydrous
sodium sulphate (2.3 g cm^{-3}).

↓

Solvent extract concentrated on a rotary evaporator.

Figure M8

Solvent extraction procedure adopted in the preparation of lipid
samples from healthy and infected A.bisporus tissue prior to
chromatographic separation and bioassay. (After Deven and Manocha (1975)).

Extraction of tissue in a homogeniser,

with X20 volume chloroform : methanol,

(2:1).

———————————————————— supernatant

Extraction of tissue residue with X20

volume chloroform : methanol, (1:2).

———————————————————— supernatant

Extraction of tissue residue with X20

volume chloroform : methanol, (2:1).

———————————————————— supernatant

Discard residue

Combine solvent fractions.

Wash fractions with X0.5 volume distilled

water, and separated, (6 times).

Lower lipid phase retained and concentrated

on a rotary evaporator.

Figure M9

Test regime adopted for the identification of lipid and related substances in solvent extracts of healthy and infected A.bisporus tissue on TLC plates.   (After Christie, (1976)).

Spray with 50% H_2SO_4
heat to c. 180°C for 30 min.,
lipid compounds char black.

Zinadze reagent

Phospholipids colour blue

If +ve   If -ve

Ninhydrin reagent   Dragendorffs
                    reagent

NH_2 group shows   Choline groups
red   give +ve reaction

Orcinol reagent

heat to 100°C for 15 min.

Glycolipid give purple reaction.

Ganglioside stain

heat to 110°C for 2 min.

Glycolipids show yellow,

Gangliosides show violet blue.

6. Fractionation of extracts and isolation of toxins

A. Thin layer chromatography (TLC)

i Analytical

Extracts were applied (2.5 cm origin) to precoated TLC plates (Merck 5715 Si Gel F_{254}, 0.25 cm thick), using drawn out Pasteur pipettes. For two dimensional chromatography, extracts were spotted on to a plate 2 cm from one corner. Chromatograms were developed in a range of solvents by ascending chromatography. Plates were air dried and examined under U.V. light (254 nm and 366 nm) (Universal lamp, Camag) and bands observed marked with a pencil.

ii Preparative

Preparative thin layer chromatography was carried out on precoated TLC plates (as above). Extracts of tissue were applied to the origin at a concentration equivalent to 1g fresh wt. cm^{-1} and chromatograms developed in chloroform:methanol (8:1 or 6:1). Bands were scraped from plates with a scalpel blade and compounds eluted with chloroform.

All chromatograms were developed in closed glass chromatography tanks lined with tissue paper soaked in solvent.

7. Bioassay techniques

A. Chromatogram bioassays

i Detection of antifungal toxins

The method devised by Klarman and Sanford (1968) was used to detect antifungal compounds in thin layer chromatograms of extracts.

Developed chromatograms were sprayed with a dense suspension
of Cladosporium herbarum spores in Czapek Dox liquid medium (half
strength) and incubated at 20°C in moist chambers for 4 days. The
fungus had been grown on Czapek Dox agar for 8-10 days at 18°C
under U.V. illumination. Inhibitory compounds were revealed as
areas of white silica gel where the dark green fungus failed to grow.
Presence of toxins in sample extracts separated using paper
chromatography was detected using Cladosporium herbarum in a similar
manner.

Solutions of separated compounds in chloroform were spotted on
to TLC plates using drawn out Pasteur pipettes. Their antifungal
activity was assessed on a semi-quantitative basis according to the
clarity and extent of inhibition zones.

In addition, developed chromatograms were sprayed with
suspensions of either M.perniciosa or A.bisporus spores, in half
strength Czapek Dox liquid medium and incubated at 23°C for 5 days
in moist chambers. After incubation, plates were stood in closed
chromatography tanks containing iodine vapour. Actively growing
fungus was browned by the iodine vapour, while the presence of
inhibitory antifungal compounds was seen as white bands of silica gel
where the fungus had failed to grow.

ii Detection of antibacterial toxins

Developed chromatograms were assessed for presence of anti-
bacterial toxins using the method developed by Lund and Lyon (1975).
Plates were sprayed with a suspension of Bacillus megaterium from a

24 h old culture grown in nutrient broth. The culture was
centrifuged (5 min., 5000 G) and re-suspended in fresh broth prior
to application. Test chromatograms were incubated at 23°C for 24 h
in moist chambers prior to spraying with Aesculin, (Aesculin 0.2%
w/v, Ammonium ferric citrate 0.1% w/v, yeast extract 0.5% w/v, in
distilled water).

On plates sprayed with the bacterium and subsequently with the
Aesculin spray, hydrolysis of the Aesculin resulted in the
development of a brown colour. Zones of inhibition were identified
by the absence of colour.

B. Sporeling bioassays

A microscope slide bioassay technique was adopted for the
examination of toxicity of samples derived from chromatographic
separation.

Extracts were assayed against spore germination and germ tube
length of Cladosporium herbarum, Botrytis cinerea, M.perniciosa and
A.bisporus spores. Tissue extracts were usually tested at a
concentration equivalent to 0.2 g fresh wt. of tissue cm^{-3}.

Glass slides were cleaned, as previously described, (Section
I 11).

The required amount of extract to be assayed was dissolved in
chloroform and three 20 µl droplets (replicates) were pipetted on to
each glass slide. The slides were dried in a sterile air flow for
c. 20 min. and the point of extract application overlain with a
further 20 µl droplet of a spore suspension of the test fungus,

(c. 5×10^4 spores cm^{-3} in sterile distilled water). As a control, primary droplets of chloroform only were included. After incubation, at 18°C for 18-24 h in moist chambers, sporelings were killed and stained by adding a single drop of cotton blue in lactophenol.

Germination was assessed and expressed as a percentage relative to controls. Where appropriate, the germ tube lengths of at least 24 sporelings per droplet were measured with a calibrated micrometer eyepiece or with a map measurer from camera lucida drawings. The total length of all germ tubes produced by each spore were recorded.

8. The effect of agar type on toxin diffusability

In preliminary investigations, the suitability of a range of proprietary agar preparations for testing the effect of toxins in solid media on fungal growth was assessed. A range of commonly available agar types were included in Nutrient broth, (Oxoid CM1), as detailed in Table M9. The surface of agar plates were seeded with Bacillus megaterium (c. 5×10^3 cm^{-2}) and air dried in a sterile air flow. Droplets (10 µl) of streptomycin sulphate (50 µg cm^{-3}) were subsequently placed on to the agar surface and plates incubated for 36 h at 25°C. The diameter of zones of inhibition at the site of antibiotic application were measured and agar types compared directly for restriction of antibiotic diffusion.

Antibiotic activity was significantly better ($p < 0.01$) in plates solidified using either Oxoid agar No. 3 (L13) or Oxoid

purified agar (L28) when compared to other agar types tested.

Oxoid agar No. 3 (L13) was selected as the most suitable agar for tests carried out in relation to diffusion in vivo.

Table M9

Suitability of agar types for diffusion of antibiotics based on inhibition of B.megaterium by streptomycin sulphate applied to seeded agar plates, after incubation at $25^{o}C$ for 36 h.

(a) = Mean $\pm$ SEM

Agar type	Diameter of inhibition zone (a)
Oxoid L28 1.2%	35.0 $\pm$ 0.41
Oxoid L13 1.5%	34.2 $\pm$ 0.46
Oxoid L12 1.2%	28.7 $\pm$ 0.46
Oxoid L33 1.5%	24.5 $\pm$ 0.67
Oxoid CM409 1.2%	19.1 $\pm$ 0.45

9. Investigation of the effect of A.bisporus tissue on growth of
    M.perniciosa

    The effect of diffusates from A.bisporus sporophore tissue was
    investigated using 'replacement' agar cultures.

    Tissue from healthy 'button stage' A.bisporus sporophores was
    excised under sterile conditions (c. 3 g) and placed on sterile
    cellophane discs, (5 cm diameter) on Czapek Dox agar (L13) in 9 cm
    Petri dishes for 72 h at 4°C. Plates were stored during this period
    in sealed jars containing either air or oxygen-free nitrogen. The
    tissue pieces were subsequently removed together with the cellophane
    and the agar surface inoculated with M.perniciosa from a 14 day old
    Malt agar culture; cultures were then incubated at 23°C $\pm$ 2°C and
    linear growth recorded. Controls were of M.perniciosa Czapek Dox
    agar (L13) cultures, previously overlain with sterile cellophane.

10. Disc bioassay technique for assessment of toxin activity

    Antibiotic assay discs (Whatman AA, 1 cm diameter) were loaded
    with extracts from either A.bisporus or M.perniciosa mycelium
    (c. 0.2 gm. fresh wt.) in 100 μl chloroform. Discs were then dried
    for at least 1 h in a sterile air flow before transfer to Petri dishes
    containing either Czapek Dox or Malt extract agar (L13). Inoculation
    was either with 200 μl of an M.perniciosa spore suspension (c. 1×10^6
    spores cm^{-3}) spread over the agar surface with a sterile rod, followed
    by partial drying of the gel surface, prior to placement of the assay
    disc, or by inoculating A.bisporus mycelium to a single point on the
    agar surface. Inhibition zones were measured after incubation for
    either 14 or 21 days at 18°C.

V. THE UTILISATION OF ANTAGONISTIC BACTERIA AS A METHOD OF DISEASE
CONTROL

1. Screening techniques

A. Antagonism towards M.perniciosa

Bacteria isolated from a range of sources were examined for
antagonism towards M.perniciosa in vitro.

Czapek Dox agar in 9 cm diameter Petri dishes was inoculated with
M.perniciosa (2 x 2 mm) from the margin of a 14 day old MM agar
culture to a point 1 cm off centre. Plates were each immediately
re-inoculated with a bacterial isolate by applying a single streak,
(7 cm long) 3.5 cm from the M.perniciosa inoculum and at right angles
to it.

Plates were subsequently incubated at 23°C $\pm$ 1°C and growth of
M.perniciosa towards the bacteria measured at periodic intervals.

Results were expressed as a percentage of growth of M.perniciosa
relative to controls of M.perniciosa grown on Czapek Dox agar in the
absence of competitors, under identical conditions.

B. Microscopic observation of antagonism

The nature of antagonism towards M.perniciosa by bacteria was
examined microscopically by excising small pieces of mycelium
(c. 1 x 1 mm) from the interaction zone of agar cultures and mounting
on clean glass slides. Samples were stained with Trypan Blue and 1%
crystal violet, and examined with a Zeiss Universal microscope.

C. Antagonism towards A.bisporus

Bacterial isolates showing antagonism towards M.perniciosa were

screened for browning of healthy sporophore tissue using a modification of the techniques adopted by Gandy, (1968).

Blocks of tissue (c. 1.0 x 1.0 cm) were excised aseptically from healthy A.bisporus sporophore caps, (Strain A6), and placed on clean glass slides. Droplets (c. 20 μl) of an SDW bacterial suspension, (c. 1 x 10^8 cells cm^{-3}) from a 24 h old Nutrient broth culture, were applied to the upper surface of individual blocks, and samples incubated at $25^{0}C \pm 1^{0}C$.

Blocks were observed with a dissecting microscope after 10 min. and after 24 h. Antagonism of bacteria towards A.bisporus was taken as pitting of the block surface relative to controls inoculated with SDW, when observed after 10 min. incubation and of browning of the block surface after 24 h.

2. The effect of selected bacteria on mushroom production in infected beds

Commercial mushroom compost (short cycle) was inoculated with grain spawn (Sinden A6) and incubated in 16 cm diameter plastic pots, as previously described.

Pots were cased with 454 $\pm$ 4 g of a moss peat/lime mixture (7:3 w/v) to which coarse grit was added, (7:3 w/v) moisture content was initially adjusted to c. 70% with a final pH of 7.4 after storage.

After steaming (1.5 h, 93-53°C temperature gradient), the casing material was inoculated with a spore suspension of M.perniciosa conidia in SDW from 15 day old Malt agar cuttings to give a concentration of 1.32 x 10^3 spores cm^{-2} in test pots.

Bacteria were applied at a concentration of c. 2.3×10^5 bacteria g^{-1} of soil at the time of casing or at the first sign of disease in previously untreated diseased pots.

Controls were of pots inoculated with M.perniciosa in the absence of test bacteria, and pots to which bacteria were applied in the absence of the pathogen.

Experimental design was of randomised blocks within controlled environment rooms at Stirling University.

Results were recorded on a daily basis as numbers and weight of healthy mushrooms produced, (at veil break), weight of infected material produced at cessation of tissue expansion and assessment of numbers of initials in healthy and infected beds.

3. Characterisation of bacterial isolates

Isolates exhibiting the ability to antagonise M.perniciosa were subjected to a range of tests outlined in Bergey's manual, (Eighth edition), (Buchanan and Gibbons, 1974), as well as those suggested by Lelliott, Billing and Hayward (1966), Thornley (1960), Bradbury (1970), Conn, Jennison and Weeks(1957) and by Wong and Preece (1979). Isolates were also examined using the API 2CE pre-packaged diagnostic system, (API System S.A. Montalieu Vercieu (France)).

The range of tests employed is shown in Table M 10. Specific procedures for tests were as outlined below.

(1) The ability to pit A.bisporus tissue

Antagonism towards A.bisporus was tested using the tissue block technique previously described (Section V1C).

Table M 10

Tests employed for the characterisation of bacterial isolates

Ability to pit A.bisporus tissue
Antagonism towards M.perniciosa   Utilisation of:-   Citrate
The ability to rot potato tissue   Glucose
White line test   Manitol
Fluorescein production   Inositol
Carotenoid production   Sorbitol
Number of flagella   Rhamnose
Growth at 41°C   Sucrose
Gram's stain   Melobiose
Oxidase reaction   Amygdaline
Nitrate reduction   Arabinose
Catalase reaction   Trehalose
Hydrolysis of starch   Geraniol
Hydrolysis of gelatin   L - Valine
Levan formation from sucrose   D L - Arginine
β galactosidase production
Arginine hydrolase production
Lysine decarboxylase production
Ornithine decarboxylase production
H_2S production

Urease production
Deamination reaction
Indole (Kovac's reaction)
Acetoin production

(2) Antagonism towards M.perniciosa

The ability of bacterial isolates to antagonise M.perniciosa
was tested by the methods previously described (Sections V1A, V2).

(3) Soft rot of potato tissue

Sections of firm potato tissue (c. 7 mm thick), prepared under
aseptic conditions, were placed in sterile Petri dishes containing SDW
(c. 3 mm deep). A heavy inoculum of the test bacterium was then
streaked across the potato tissue prior to incubation at 25°C for 48 h.
The tissue was examined for soft rotting by pressing with a loop and
compared to uninoculated controls, (Bradbury, 1970).

(4) White line production in agar

Test isolates were inoculated on to Pseudomonas agar F (PAF)
(Difco), (Table M.11).

Isolates were streaked in close proximity to known 'reacting'
isolates of Pseudomonas sp., (NCPPB 387 or NCPPB 1311). Plates were
incubated at 25°C $\pm$ 1°C for 24 h prior to examination. The production
of a white line within the agar between the test and 'reacting' isolates
is indicative of the test bacterium being Ps. tolaasii.
(Wong and Preece, 1979).

(5) Pigment production

The production of diffusable pigments by test isolates was
determined on Kings' B media for the excretion of yellow-green or blue
pigments that fluoresce under ultraviolet light (λ = 365 nm) and on King's
Medium A for the excretion of orange-red carotenoid pigments, (King,
Ward and Raney, 1954), (Table M.11).

Table M 11

Special media for the identification of bacterial isolates

| Constituent | $g\ell^{-1}$ |

Pseudomonas Agar F. (Wong and Preece, 1979)

Bacto-tryptone	10
Bacto-proteose peptone No. 3	10
dipotassium phosphate	1.5
magnesium sulphate	1.5
Bacto-agar	15
Bacto-glycerol	10

Kings' A medium (King, Ward and Raney, 1954)

Bacto-peptone	20
Bacto-agar	15
glycelol	10
dipotassium sulphate, (anhydrous)	10
magnesium chloride, (anhydrous)	1.4

pH 7.2

Kings' B medium (King et al. 1954)

Proteose-peptone No. 3	20
Bacto-agar	15
glycelol	10
dipotassium hydrogen phosphate, (anhydrous)	1.5
magnesium sulphate; septa hydrate	1.5

Nutrient gelatin

Bacto yeast extract	3
peptone (Oxoid L37)	5
gelatin	120

Table M 11 (continued)

Constituent	$g\ell^{-1}$

Palleroni and Doudoroff's (1972) media

ammonium chloride	1
magnesium sulphate septahydrate	5
ferric ammonium citrate	.5
calcium chloride	.05
Ionagar	10

:- in Na / K phosphate buffer, (pH 6.8)

(6) Number of flagella

The number of flagella arising from each bacterium was determined by examining pure culture suspensions directly. Droplets of a bacterial suspension were gently dried on to coated copper grids, (80 mesh), and stained with a drop of lead citrate stain prepared as previously described, (Section III 5Ci). Grids were rinsed in SDW prior to examination using the transmission electron microscope.

(7) Growth at 41°C

The ability of test isolates to grow at 41°C was determined by inoculating isolates to Nutrient broth and incubating cultures for 48 h. Where growth did not occur, the media remained clear.

(8) Gram's stain

Bacterial isolates were each smeared on to clean glass microscope slides and air dried. Smears were heat fixed and covered with a drop of crystal violet solution (0.5%) for 3 min. Samples were rinsed with 1% iodine in 2% aqueous potassium iodide and covered with a drop of iodine. The slides were subsequently rinsed with SDW and EtOH. Slides were again rinsed with SDW and counter-stained with 0.5% safranin. Gram +ve cells stained purple, Gram -ve cells stained red. (Buchanan and Gibbons, 1974)

(9) Oxidase reaction

A small amount of a 24 h old bacterial culture was smeared on to clean filter paper which had been dampened with a little freshly prepared 1% aqueous tetramethyl-p-phenylene diamine dihydrochloride using a sterile loop. A dark bluish-violet colour formed at the smear within 10s. is a positive result, (Kovacs, 1956).

(10) Catalase reaction

     Droplets of 3% aqueous hydrogen peroxide were dropped on to single colonies of test bacteria. The production of oxygen as bubbles at the colony surface indicates the presence of the catalase enzyme.

(11) Hydrolysis of starch

     Bacterial isolates were each streaked on to water agar plates containing 1% wheat starch. Plates were incubated for 24 h at 20°C $\pm$ 1°C prior to flooding of the culture with 1% iodine solution. Hydrolysis of the starch was indicated by the presence of a clear zone visualised within the medium.

(12) Hydrolysis of gelatin

     Gelatin hydrolysis was tested on nutrient gelatin, (Table M 11). Tubes inoculated with test bacteria were incubated for 72 h at 20°C $\pm$ 1°C. Prior to examination, tubes were cooled to 4°C for 30 min. Liquifaction was scored as positive where the medium flowed freely, (Lelliott, Billing and Hayward, 1966).

(13) Levan formation

     Plates of Nutrient agar (Oxoid CM3) containing 3% sucrose were streaked with test bacteria. The development of large white mucoid colonies were assumed to indicate the production of levan, (Lelliott et al, 1966).

(14) Carbon utilisation

     For carbon utilisation tests the basal medium of Palleroni and Doudoroff (1972) was utilised, (Table M 11). Carbon sources, (Table M 10) were included in the media at the concentration of 1%. Sugars were sterilised by filtration prior to incorporation in the cooled media.

EXPERIMENTAL WORK AND RESULTS

CHAPTER I

GROWTH, SURVIVAL AND ECOLOGY OF M.PERNICIOSA IN VIVO AND IN VITRO

The aim of the work described in this chapter was to investigate factors influencing symptom development and disease spread, and the effect of host and non-host organisms on growth and survival of the pathogen.

I. OPTIMA FOR GROWTH OF M.PERNICIOSA IN VITRO

1. Temperature

The temperature at which liquid cultures of M.perniciosa were grown significantly affected growth of the fungus.

Optimum temperature for vegetative growth, determined on the basis of dry weight of mycelium produced in OD media was determined as 25°C, (Figure 1.1). Temperature range for at least 50% of maximum growth was determined as between 17°C and 32.5°C. Growth at 16°C, that temperature recommended as the maximum cropping temperature for A.bisporus, (Brady and Gibson 1976b), was determined as 34% growth rate of maximum.

2. Substrate pH

M.perniciosa liquid cultures grown in OD media assessed on a dry weight basis showed optimum substrate pH to be 4.1. Growth declined markedly at pH values above this figure to 33% of maximum at pH 5.0. Changes in substrate pH above pH 5.0 produced no significant further decrease of the dry weight of mycelium produced, (Figure 1.2).
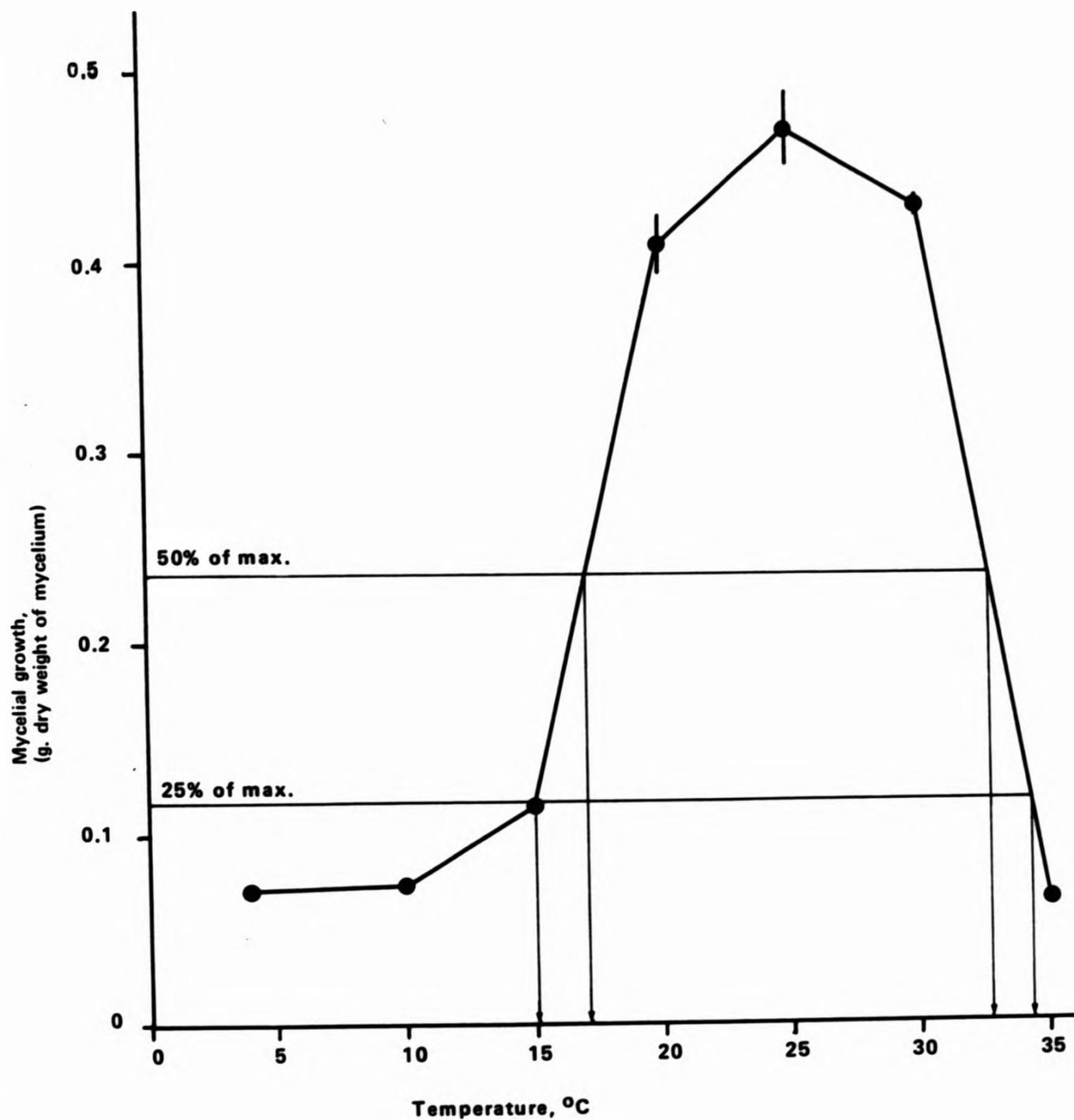
FIGURE 1.1 The effect of changes in temperature on vegetative growth of M.perniciosa (mean dry weight $\pm$ SEM) in liquid OD media at pH 5.4 after 10 days in the light.

Mycelial growth
(g. Dry weight of mycelium)
± SEM
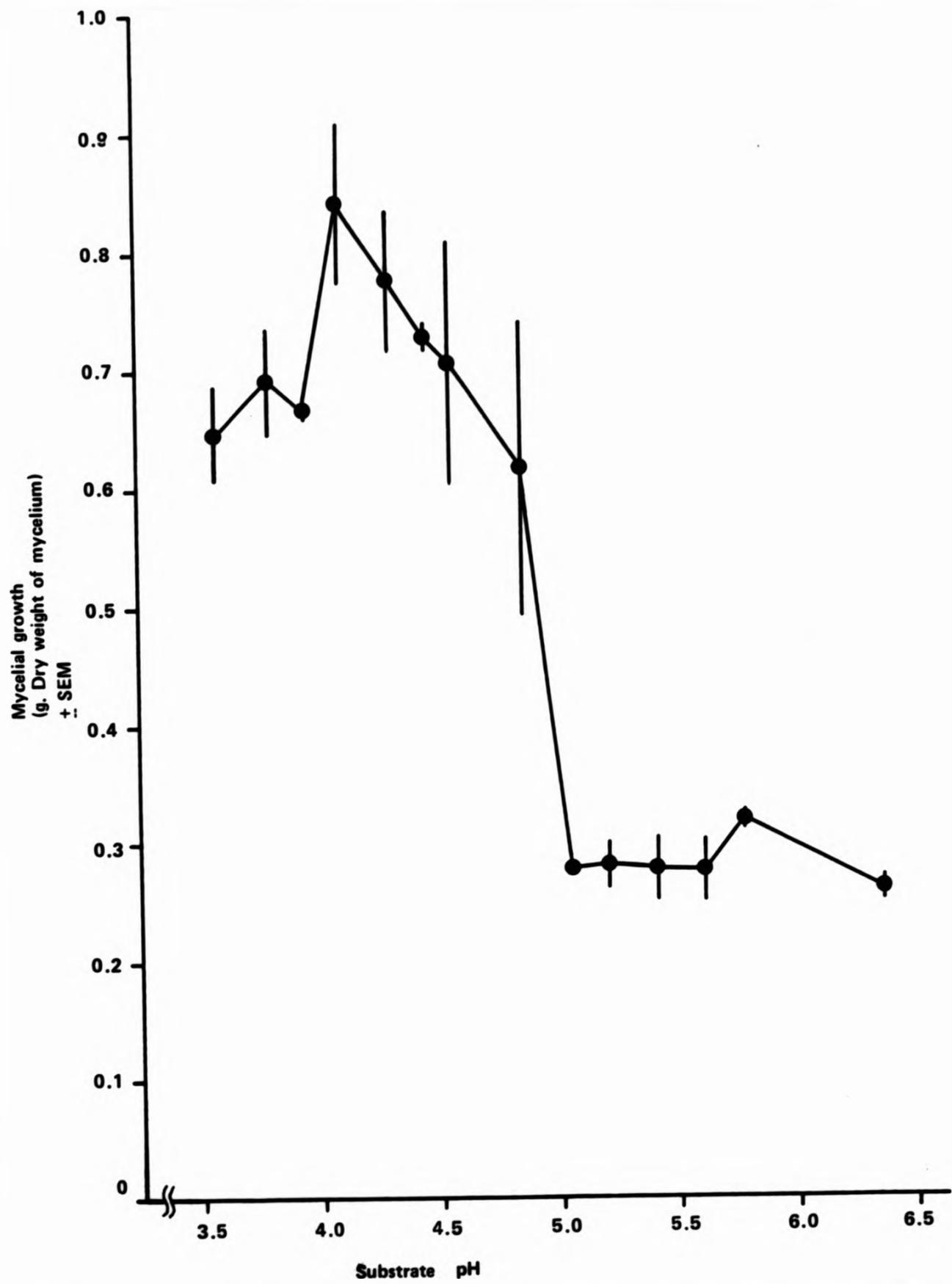
Substrate   pH

FIGURE 1.2   The effect of changes in substrate pH on vegetative growth of M.perniciosa in liquid OD media after 10 days at 25°C. (mean dry weight ± SEM)

3. The influence of Carbon:Nitrogen (C:N) ratio

Optimum C:N ratio for vegetative growth was determined in OD
media with sucrose as the sole carbon source and calcium nitrate as the
sole nitrogen source.   Variation in C:N ratio was achieved by
altering the amount of available nitrogen in order to minimise any
osmotic effects due to sucrose.   Optimum C:N ratio was determined as
55:1 with a C:N ratio range for at least 75% of maximum growth
between 10:1 and 77:1.   Growth was 97% of maximum at C:N ratio of
35:1, typical of mushroom casing soil, (Vedder 1978), compared to
growth of between 78% and 94% of maximum over the C:N range 20:1 to
11:1, as found in commercial composts at the start and the end of
the growing cycle, (Vedder 1978), (Figure 1.3).

II. SUBSTRATE UTILISATION AND ENXYMIC ACTIVITY

The ability of M.perniciosa in vitro to utilise a range of
artificial substrates containing a range of carbon and nitrogen
sources was investigated.

Carbon source utilisation was compared on defined media
containing specific carbon sources.   Growth of M.perniciosa was
measured as radial growth of the fungus on agar plates after 10 days'
incubation at $20^{o}C \pm 1^{o}C$ on media containing 10 g available
carbon litre^{-1}.

M.perniciosa utilised chitin (Crab shell poly-N-acetylglucosamine),
(6.96 $\pm$ 1.12 cm), galactomannan polysaccharide (6.59 $\pm$ 0.40 cm),
sucrose (6.0 $\pm$ 0.46 cm), and Na-carboxymethyl cellulose
(1500 $\pm$ 400 cP) (5.88 $\pm$ 0.11 cm) as sole carbon sources about equally.
Differences were not significant.   M.perniciosa was seen not to
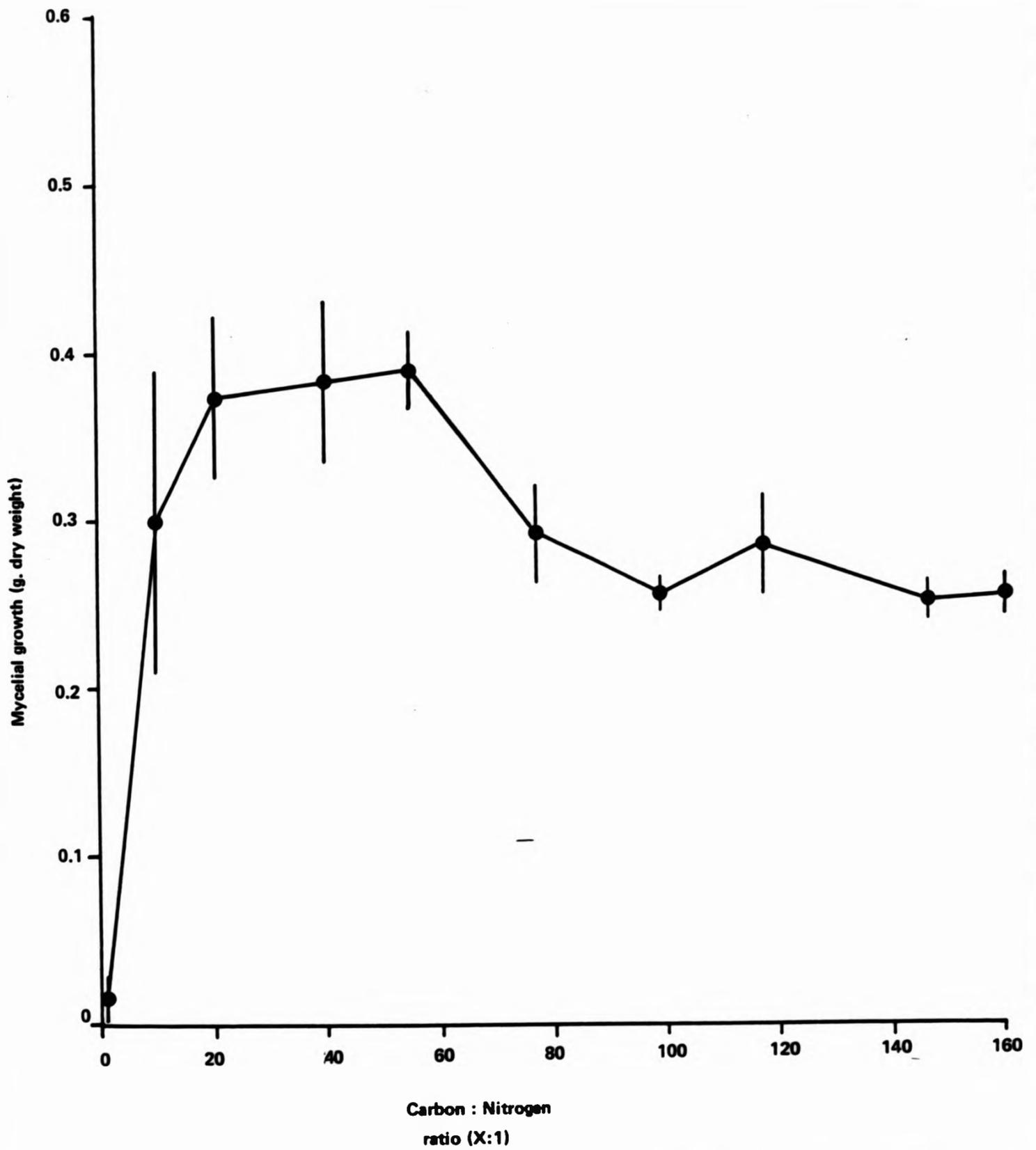
FIGURE 1.3   The effect of changes in substrate Carbon:Nitrogen (C:N) ratio on vegetative growth of *M.pernicious* in OD media after 10 days at 25°C. (mean dry weight ± SEM)

Table 1.1

The ability of M.perniciosa to colonise media containing specific
carbon sources, after 10 days incubation at 20°C.

Carbon source	Colonisation by M.perniciosa
Na-carboxymethyl cellulose (high viscosity, 1500 $\pm$ 400 cP)	+
Native cellulose (Whatman No. 1)	-
Xylan (Larchwood)	-
D-xylose	-
Sucrose	+
Chitin (Crabshell poly-N-acetylglucosamine)	+
Pectin (Apple, 250 grade)	-
Galactomannan polysaccharide (Gum locust bean)	+

+ = Substrate utilised

- = Substrate not utilised.

Table 1.2

Enzyme production by M.perniciosa in vitro

Enzyme	Production by M.perniciosa		
	(a)	(b)	(c)
Alkaline phosphatase	+	+	+
Esterase C4	-	+	+
Esterase lipase C8	+	+	+
Lipase C14	-	-	-
Leucine arylamidase	+	+	+
Valine arylamidase	+	+	+
Cystine arylamidase	-	-	-
Trypsin	+	+	-
Chymotrypsin	+	+	-
Phosphatase acid	+	+	+
Galactosidase α	-	-	-
Galactosidase β	-	+	-
Glucuronidase	-	+	+
Glucosidase α	-	-	-
Glucosidase β	+	+	+
N Acetyl B glucosaminidase	+	+	+
Mannosidase	+	+	-
Fucosidase	-	+	-
Amylase	+	+	+
CMCase	+	+	+

(a) - Mycelial sample from Czapek Dox liquid cultures

(b) - Mycelial sample from Czapek Dox + mushroom liquid cultures

(c) - Sterile filtered supernatant from (b).

+ = Enzyme produced

- = Enzyme not produced

utilise substrates containing native cellulose, (Whatman No. 1
paper), xylan (Larchwood), D-xylose or pectin (Apple, 250 grade),
as sole carbon sources, (Table 1.1).

Growth of the fungus was not significantly affected by the
variation of the type of nitrogen source present in OD media with
sucrose as the sole carbon source, with utilisation of urea, nitrate
and ammonium ion nitrogen sources being equally good.

Further to this, investigations into the enzymic activity of
M.perniciosa were carried out by assaying culture filtrate samples for
activity using the API ZYM pre-packaged diagnostic system.

Samples of mycelium from M.perniciosa cultures grown in Czapek Dox
liquid medium and from those grown in Czapek Dox/mushroom media
consisting of Czapek Dox liquid medium with the addition of sterile
filtered juice from pressed healthy 'button stage' A.bisporus sporophores
(0.2 μl cm^{-3}) were withdrawn after incubation for 10 days at 25°C $\pm$ 1°C.
In order to differentiate between wall bound and free enzymes samples of
sterile filtered supernatant from M.perniciosa Czapek Dox/mushroom media
cultures were also taken. Controls were of uninoculated Czapek Dox/
mushroom media.

Samples were assayed for the presence of a range of enzymes using
65 μl of sample suspension or filtrate.

M.perniciosa grown in Czapek Dox liquid medium was seen to produce
a smaller range of enzymes than when grown in Czapek Dox liquid medium
with the addition of sterile filtrates of juice from pressed healthy
A.bisporus sporophores, (Table 1.2). Notably, production of β galacto-
sidase, β glucuronidase, esterase C4 and α fucosidase were detected in
the presence of sporophore filtrates but not in the simple defined media.

The role of these enzymes is likely to be not only in pathogenesis against the host but they may have a role in increasing the general saprophytic activity of M.perniciosa within the compost and casing layers of mushroom beds.

In addition, amylase activity, determined by the ability of mycelial suspensions and filtrate droplets to hydrolyse starch in agar medium, visualised by flooding the plate surface with 1% iodine solution after 4 h incubation at 20°C, was noted in M.perniciosa cultures grown in media both with and without the addition of host sporophore filtrates.

Cellulase activity was determined as the ability of sterile filtrates from M.perniciosa liquid cultures to alter the viscosity of 1% carboxymethyl cellulose, (CMC), buffered at pH 5.2. Cellulase activity was identified as a reduction in substrate viscosity with a corresponding reduction in flow time through a capillary viscometer.

Supernatant (2 cm^3) from M.perniciosa OD media cultures with CMC as the sole carbon source, incubated at 20°C $\pm$ 2°C, was added to 8 cm^3 of 1% CMC and tested for cellulase activity viscometrically.

Viscometers were calibrated with a maximum viscosity (100%) taken as the time for 8 cm^3 of 1% CMC + 2 cm^3 SDW at 30°C to pass through the capillary section of the viscometer, the time taken for the same volume of SDW being taken as minimum viscosity, (0%).

Viscosity of buffered CMC substrate decreased rapidly following the addition of sample filtrates from both 4 days old and 35 days old M.perniciosa cultures, (49.2% and 78.5% reduction respectively after 1 min.). Subsequent reduction in viscosity was then less rapid (79.8%

and 97.2% reduction relative to time 0 after 20 min. respectively).
Overall, data of substrate viscosity in the presence of M.perniciosa
culture filtrates when plotted against time gave a curved graph
indicating endo-enzymic cleavage of the long chain molecular substrate
in media colonised by M.perniciosa, (Figure 1.4).

III GERMINATION OF M.PERNICIOSA CONIDIA

Conidia produced by M.perniciosa are primarily of two types.

The phialidic or verticillium-like spores are thin walled and
uniseptate. It was noted that these spores, in some cases, split at
the centrally positioned septum resulting in the occurrence of two
unicellular propagules, (Plate 1.1). Germination was observed to be
directly by one or more germ tubes from either or both of the conidial
cells.

The chlamydospore form, produced in older cultures is
initially bicellular having a lower hyaline cell and an upper cell
which becomes melanised and warty on maturation. Young chlamydospores
contain numerous fat droplets and are multinucleate, with the upper
cell remaining thin walled until fully expanded prior to melanisation,
(Plate 1.2).

When detached from the mycelium, the lower cell, while capable of
germination, has a tendency to die and split open resulting in what is
in reality a single celled spore with the remnants of the lower cell
attached.

The remaining warty cell has no germ pores and with the only
apparent weak spot at the point of the septum between the upper and
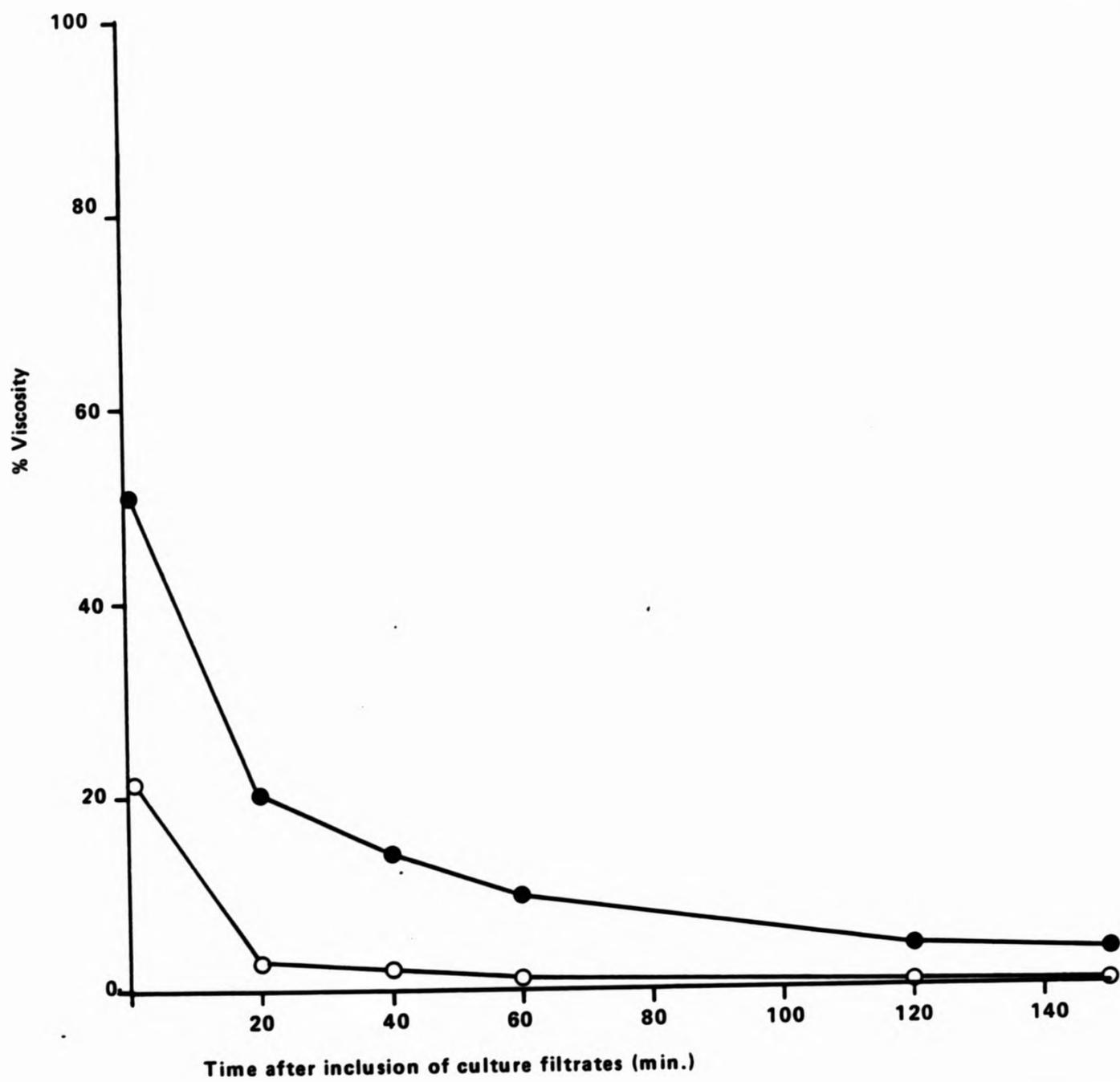
FIGURE 1.4 Changes in viscosity at 30°C of 1% Na-carboxymethyl cellulose in the presence of filtrates from 4 day old (●—●) and 35 day old (O—O) M.perniciosa liquid OD media cultures incubated at 20°C.
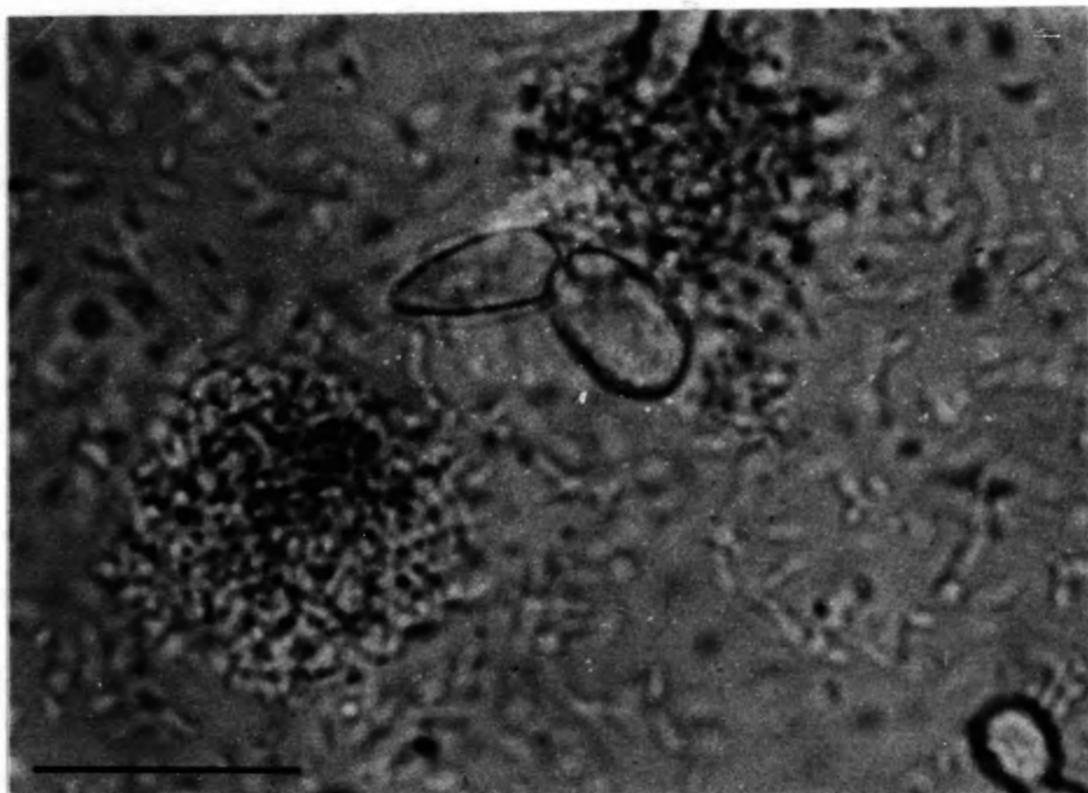
PLATE 1.1.   Light micrographs of <u>M.perniciosa</u> verticillate conidia
              isolated from 10 day old MM agar cultures incubated
              at 20°C.
              Staining was in cotton blue in lactophenol.

    i) Uniseptate conidium

    ii) Verticillate conidium split to produce two unicellular
        propagules.

Bar represents 10 µm

PLATE 1.1.   Light micrographs of M.perniciosa verticillate conidia
              isolated from 10 day old MM agar cultures incubated
              at 20°C.
              Staining was in cotton blue in lactophenol.

     i)   Uniseptate conidium

    ii)   Verticillate conidium split to produce two unicellular
          propagules.

Bar represents 10 μm

PLATE 1.2.   Young (10 day old culture) <u>M.perniciosa</u>
chlamydospores stained in 0.06% Neutral red.
Note the presence of fat droplets and multiple
nuclei, in both the upper (u) and lower (1)
cells.

Bar represents 10 μm

PLATE 1.2.   Young (10 day old culture) **M.perniciosa**
chlamydospores stained in 0.06% Neutral red.
Note the presence of fat droplets and multiple
nuclei, in both the upper (u) and lower (l)
cells.

Bar represents 10 μm

lower cells of the original bicellular spore, (Plate 1.3). Germination at this point is therefore likely, as suggested by Smith (1924), but later discounted by Vincent-Davies, (1972). Germination was observed both at this point and as a result of splitting of the chlamydospore wall or by production of a secondary vescicle prior to germ tube growth.

1. Spore germination in various substrates

Germination of M.perniciosa chlamydospores and verticillate conidia was investigated in order to determine the factors influencing this aspect of growth of the fungus.

Germination of spores was seen to be dependent on the nature of the substrate in which they were incubated. Germination of both chlamydospores and verticillate conidia was best in sterile filtered juice from pressed healthy A.bisporus sporophores at 66% and 100% respectively, (Figure 1.5a). Chlamydospores had a low level of germination in other treatments, 0-3.7%, in SDW, sterile de-ionised water (SDeW), ½ strength Czapek Dox liquid medium, methanol and chloroform extracts of healthy sporophores. In SDW containing ion-chelating agents (di-hydroxybenzoic acid (DHBA) or ethylene-diamine tetra acetic acid (EDTA)) germination improved slightly to 5% and 4.2% respectively such that a possible role for chelating soil microorganisms in the germination of M.perniciosa chlamydospores by affecting general fungal metabolism as suggested by Weinberg (1970) cannot be entirely discounted.

Differences in germination of verticillate conidia and chlamydo-spores indicate differences in the response to the nature of the substrate with chlamydospores requiring the presence of host material.

Germination levels for verticillate conidia were higher than in all corresponding chlamydospore treatments, (Figure 1.5b).

PLATE 1.3   Mature 42 day old, heavily melanised,
M.perniciosa chlamydospores showing the warty cell
structure with the remains of the lower hyaline cell
still attached (X).
Note the presence of a possible germ pore at the
septum between the upper and lower cells of the
developing spore, (arrowed).

Bar represents 5µm.

PLATE 1.3   Mature 42 day old, heavily melanised,
M.perniciosa chlamydospores showing the warty cell
structure with the remains of the lower hyaline cell
still attached (X).
Note the presence of a possible germ pore at the
septum between the upper and lower cells of the
developing spore, (arrowed).

Bar represents 5μm.

(a)

% Germination
of chlamydospores
after 72 h

(b)

% Germination
of verticillate
conidia after 48 h

SUBSTRATE
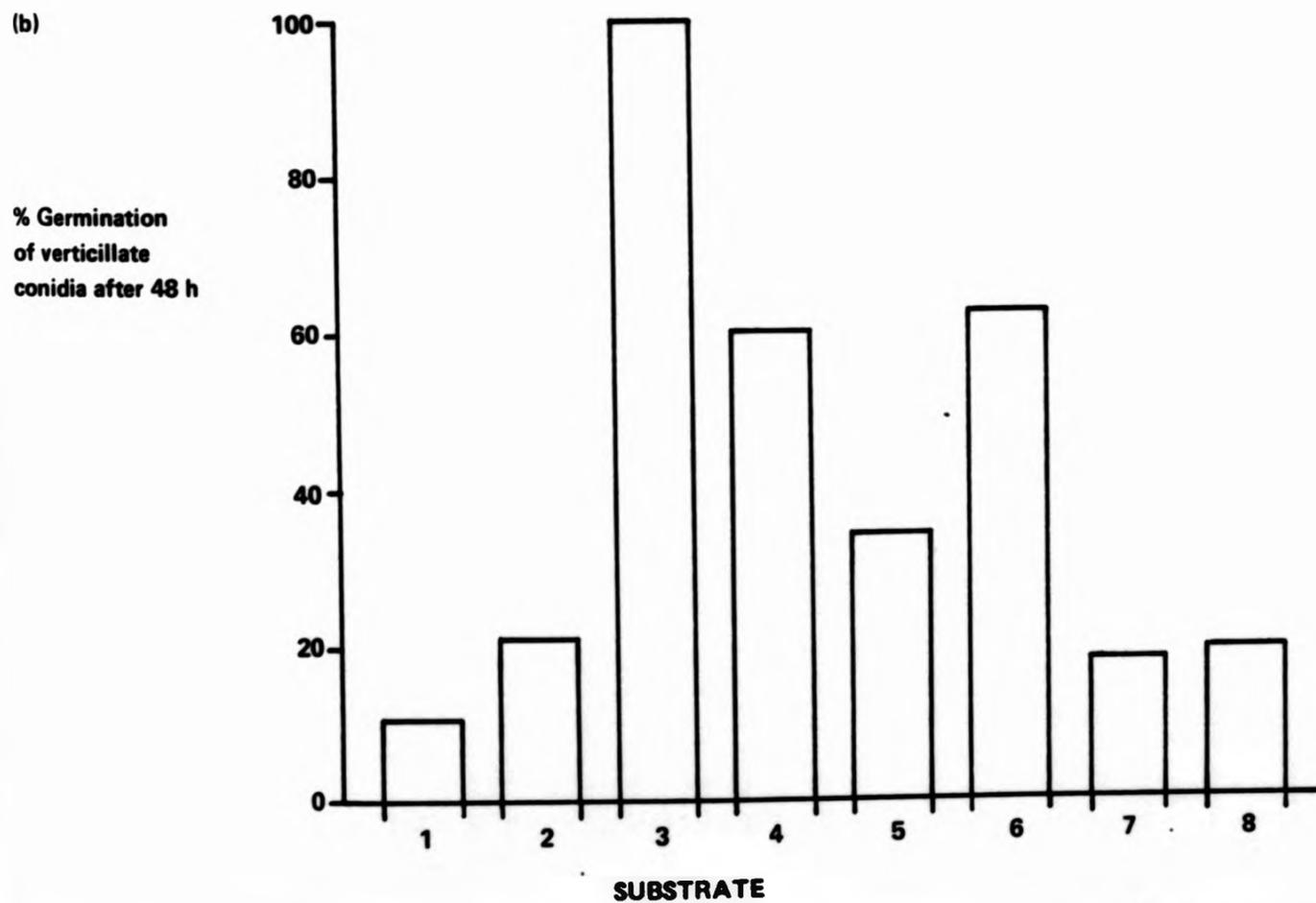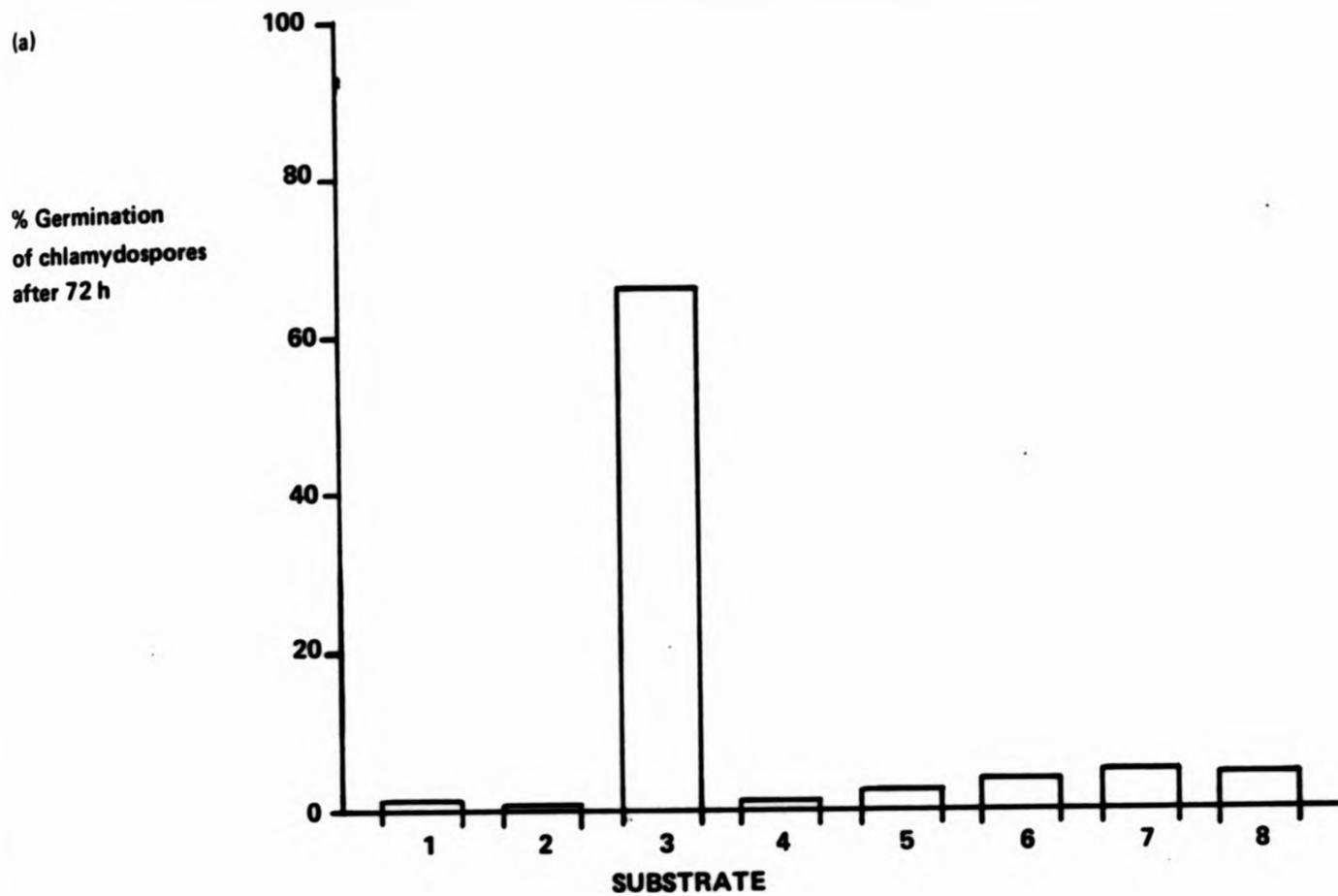
FIGURE 1.5   Percentage germination of M.perniciosa conidia from 10 day old MM agar cultures, incubated at
25°C ± 2°C in :-
1) SDeW
2) SDW, pH 6.7
3) Sterile filtered juice from pressed A.bisporus sporophores
4) Chloroform extract of (3)
5) Methanol extract of (3) , 6) Half strength Czapek Dox liquid medium, 7) 0.4 M DHBA,
8) 0.4 M EDTA

Low levels of germination of chlamydospores in methanol and chloroform

extracts of A.bisporus sporophores indicates the polar nature of the

stimulating components in extracts of host tissue. That this is not

simply a nutritional effect is indicated by the low level of

germination in ½ strength Czapek Dox liquid medium, suggesting the

involvement of specific vitamins or amino acids.

Germination of verticillate conidia was similarly best in juice

from pressed A.bisporus sporophores, although relatively high levels of

germination in other substrates compared to the level of germination of

chlamydospores indicates that chlamydospores are heterotrophic for a

wider range of nutritional components than verticillate conidia.

It is apparent therefore, that verticillate conidia are nutritionally

less demanding than chlamydospores and that germination is influenced

largely by availability of nutrients in the substrate.

Ion chelators have no significant effect on germination of

verticillate conidia relative to SDW treatments.

2. The effect of substrate on germ tube growth

Growth of germ tubes of verticillate conidia was significantly

reduced after 18 h incubation at 20°C $\pm$ 2°C in the presence of sterilised

juice from pressed healthy A.bisporus sporophores compared to controls in

SDW and to those spores harvested dry and incubated in moist chambers. (figure 1.6)

This result is in apparent contradiction to those described above

whereby germination of conidia was reported to be greatest in juice from

pressed A.bisporus sporophores, indicating a difference in the factors influencing spore germination and subsequent germ tube growth.

There was no significant difference in germ tube growth in juice from pressed healthy A.bisporus sporophores where sterilisation was by filtration or by autoclaving, (15 min. at 1 kg cm^{-2}), indicating that factors in host tissue capable of inhibiting extension growth of M.perniciosa hyphae are fully heat stable, (Figure 1.6).

3. The effect of temperature

Germination of 10 day old chlamydospores and verticillate conidia in ¼ strength sterile filtered juice from pressed healthy A.bisporus sporophores was examined at various temperatures on clean glass slides in the dark. Percentage germination was determined after 24 h for verticillate conidia and after 72 h for chlamydospores.

Maximum germination of verticillate conidia, (86.5%), was at 18°C and at 25°C for chlamydospores, (70% germination), with at least 75% of maximum germination recorded over a range of 13.6°C - 29.0°C and 15.8°C-26.3°C respectively, (Figure 1.7). Germination of verticillate conidia took place over a wider temperature range than was the case for chlamydospores. Germination in both cases was seen to occur over the complete temperature range adopted in commercial mushroom cropping houses.

4. The effect of light

Spores harvested from 14 day old MM agar cultures incubated at 20°C $\pm$ 2°C were placed in 20 µl droplets of ¼ strength sterile filtered juice from pressed healthy A.bisporus sporophores on clean glass slides

**Germn Tube
length
(Arbitrary units)**
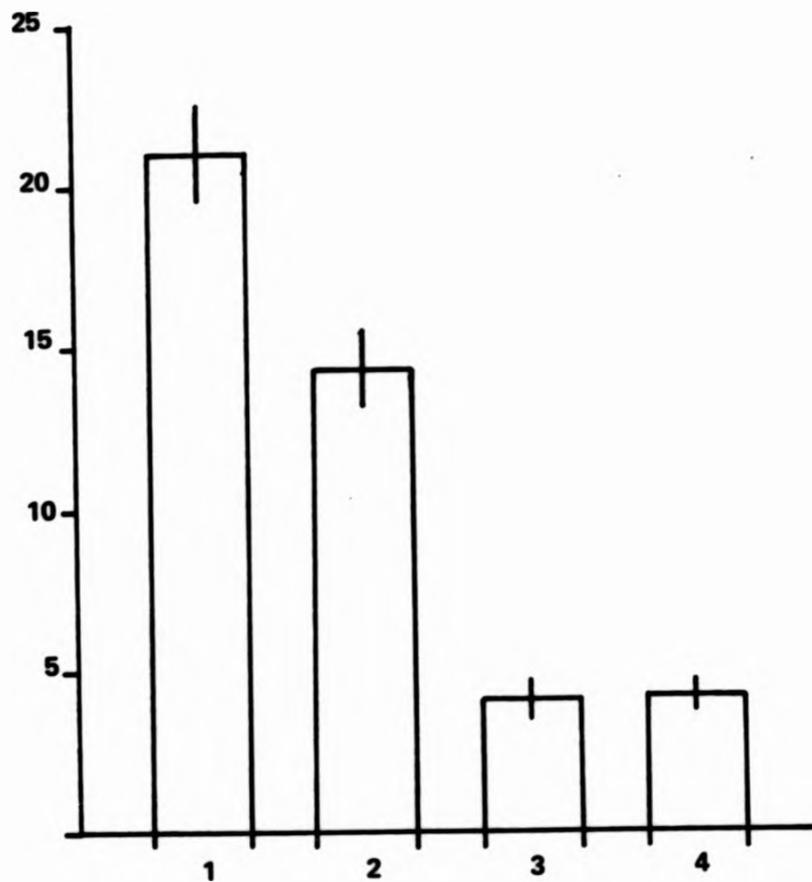
FIGURE 1.6   Germ tube length of germinated M.perniciosa verticillate conidia harvested from the margin of 10 day old MM agar cultures incubated at 25°C $\pm$ 1°C in :-

1) SDW
2) 100% humidity
3) Sterile filtered ½ strength juice from healthy A.bisporus 'cup stage' sporophores
4) Autoclaved ½ strength juice from healthy A.bisporus 'cup stage' sporophores
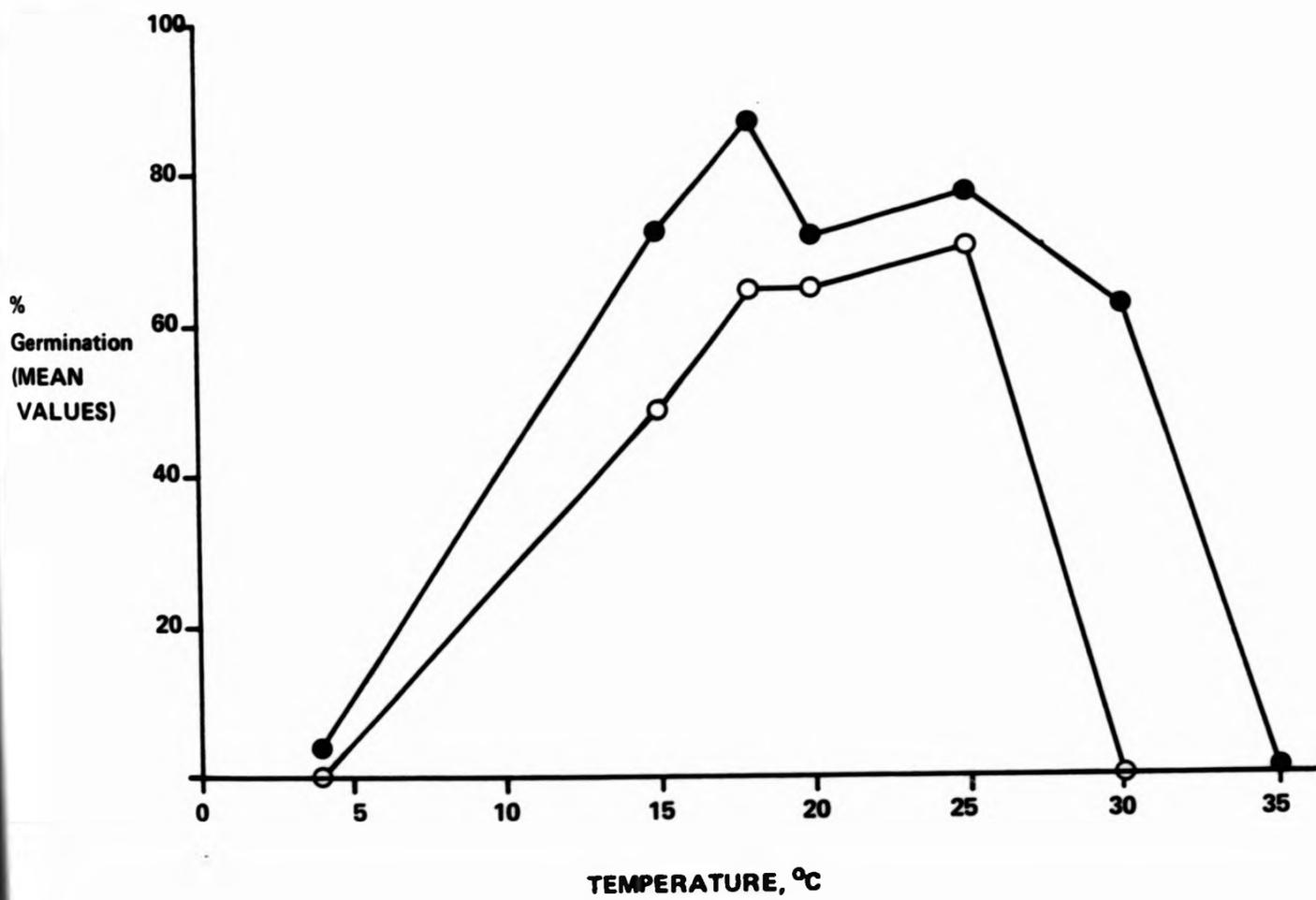
FIGURE 1.7   The effect of temperature on the percentage germination of M.perniciosa verticillate conidia (●—●) and chlamydospores (○—○) harvested from 10 day old MM agar cultures incubated at 25°C after 24 h and 72 h respectively in ½ strength sterile filtered juice from pressed A.bisporus sporophores.

and incubated in moist chambers at 20°C $\pm$ 1°C under U.V. light,
(366 nm), Tungsten light or in the dark. Germination levels were
assessed after 24 h for verticillate conidia, and after 72 h for
chlamydospores.

Germination of verticillate conidia was not significantly
affected by variation of the light regime.

Germination of chlamydospores was greatest in the dark compared
to Tungsten or U.V. light, but differences were not significant,
(Figure 1.8).

5. The effect of age on spore germination

Spores harvested from 14 day old IM agar cultures, incubated at
20°C $\pm$ 2°C were stored at ambient humidity for varying lengths of time
at 18°C $\pm$ 1°C. Subsequent germination of verticillate conidia and
chlamydospores was investigated at 18°C and 25°C respectively in 10 μl
droplets of ½ strength sterile filtered juice from pressed healthy
A.bisporus sporophores on clean glass slides in moist chambers.

Verticillate conidia were seen to have a generally high germination
potential of between 58% and 100% for spores up to 69 days old,
(Figure 1.9). Germination levels were found to vary, with low levels
occurring after 12 days and 61 days storage. Such variation may have
been due to unidentified factors other than the age of the spore.

Chlamydospores were seen to germinate readily 7 days after
harvesting (66% germination). Germination potential decreased rapidly
to 3% after 14 days with a minimum of 1% after 21 days storage.
Germination potential subsequently increased to a secondary maximum of

% Germination
of conidia

100
80
60
40
20
0

Tungsten light

U.V. light

Dark
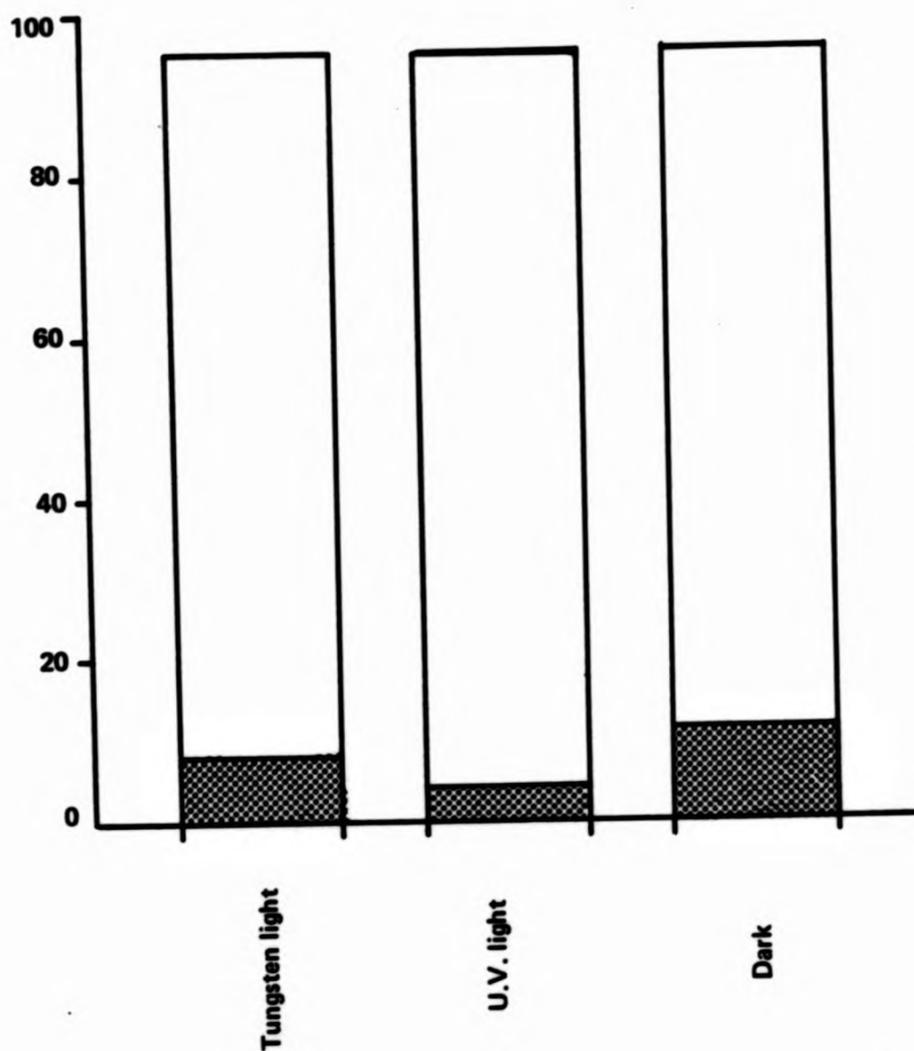
FIGURE 1.8   The effect of light on germination of M.perniciosa verticillate conidia (☐)
and chlamydospores (▨) after 24h and 72h respectively at 20°C in sterile
filtered juice from pressed A.bisporus 'button stage' sporophores.

11% after 42 d storage, with a further decrease in germination levels for chlamydospores stored for longer periods (Figure 1.9). It is likely that chlamydospores less than 14 days old have thinner cell walls with a lower degree of melanisation than older spores, young spores having a correspondingly higher sensitivity to nutritional or environmental factors such as U.V. light. Chlamydospores less than 14 days old may be more akin to verticillate conidia than to older chlamydospores which apparently go through a maturation phase prior to a secondary peak germination potential.

6. Environmental influence on sporulation

The effect of altering light and temperature on sporulation of M.perniciosa was investigated. Spores from M.perniciosa Czapek Dox agar cultures in 250 cm^3 conical flasks of uniform diameter were harvested when the agar was completely colonised. SDW (100 cm^3) + 10 μl of Tween 80 was added to each flask and the surface of cultures scraped with a sterile glass rod and flasks shaken on a wrist action shaker for 1 h. The concentration of the resulting spore suspension was subsequently measured using a haemocytometer.

Chlamydospore production was seen to be greatest at 18°C, falling to 36% of maximum at 20°C, and 8% of maximum at 30°C. Optimum temperature for the production of verticillate conidia was determined as between 20°C and 25°C, (Figure 1.10).

Relative abundance of chlamydospores and verticillate conidia was also affected, with chlamydospore : verticillate conidia ratio greatest at 18°C, (2.4:1), the lowest at 25°C, (0.45:1), (Figure 1.11).
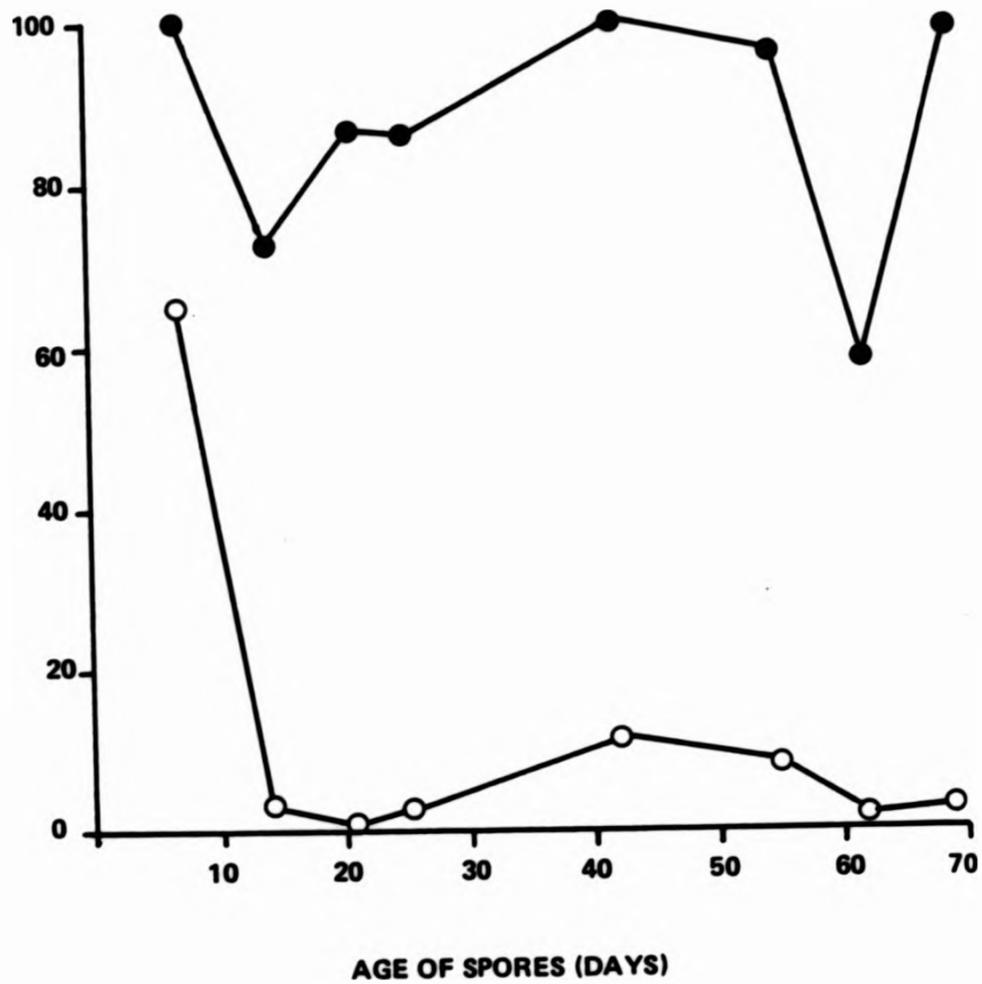
FIGURE 1.9   Germination potential of M.perniciosa conidia in 20 ul droplets of sterile filtered juice from A.bisporus sporophores after storage at 20°C. Verticillate conidia (●—●) after 24 h at 18°C, (c. 1 x 10⁵ spores cm⁻³), Chlamydospores (O—O) after 72 h at 25°C, (c. 1 x 10⁵ spores cm⁻³).

MEAN
No. Spores
$(x\ 10^5/cm^3)$
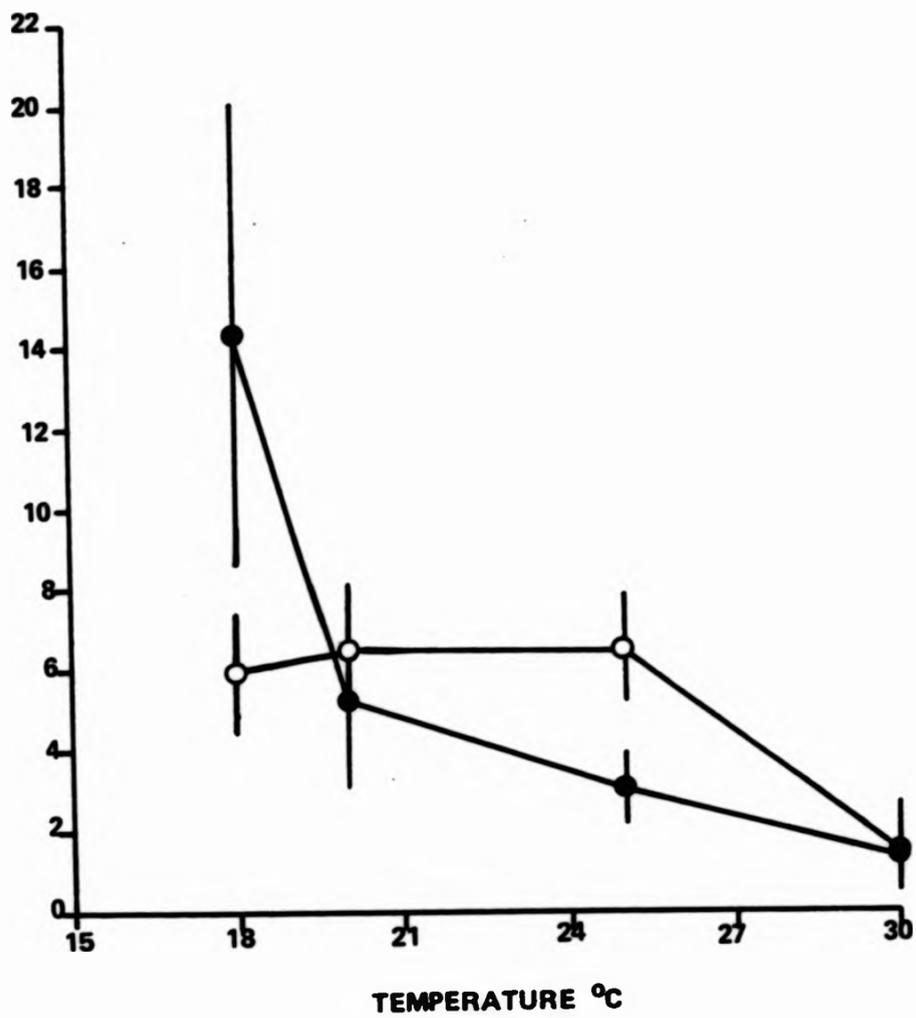
TEMPERATURE °C

FIGURE 1.10   The effect of changes in temperature on sporulation of
M.perniciosa, chlamydospores, (O—O) and
verticillate conidia, (●—●) after 28 days incubation
on Czapek Dox agar in the light. (mean values ± SEM)

Ratio of
chlamydospores:
verticillate
conidia (x:1)
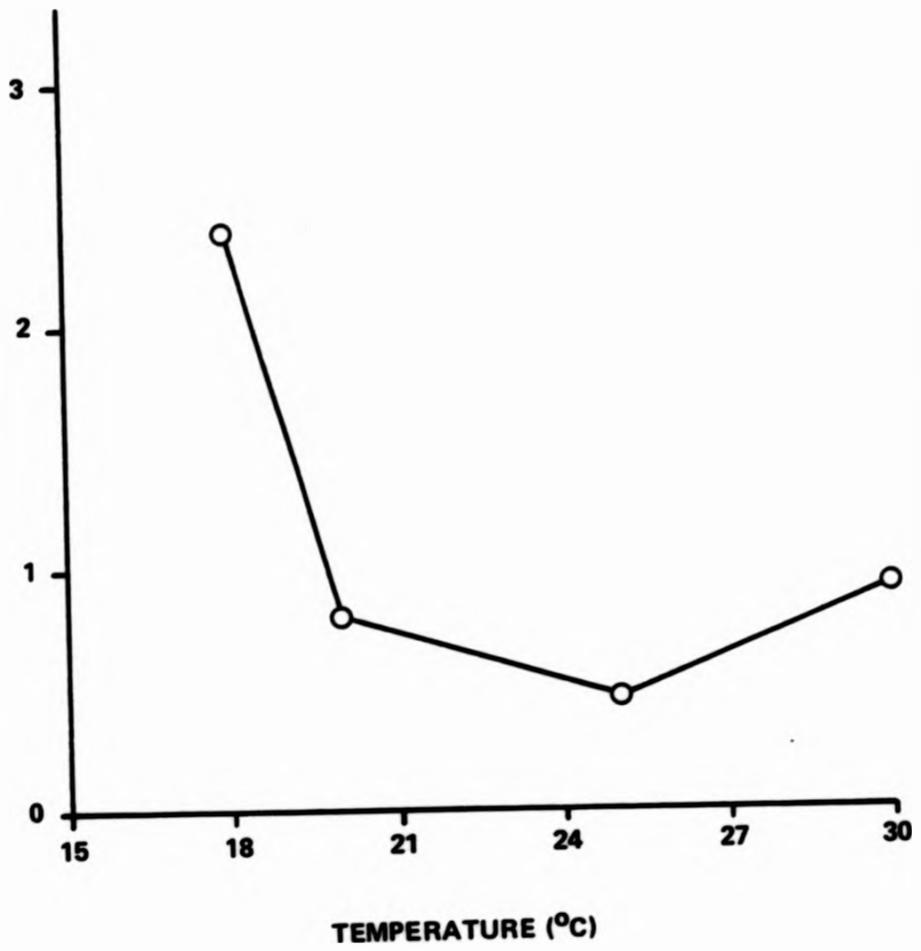
TEMPERATURE (°C)

FIGURE 1.11   The effect of changes in temperature on the relative abundance of chlamydospores
and verticillate conidia produced by M.perniciosa on liquid Czapek Dox media
incubated for 28 days in the light.

Sporulation was also affected by the light regime under which cultures were grown.

Total spore production was greatest in full light with sporulation significantly reduced in U.V. light and under dark conditions, (Figure 1.12). The relative abundance of chlamydospores compared to verticillate conidia was increased in U.V. light and under dark conditions, (Figure 1.13). This is likely to be due to the increased resistance to U.V. light of melanised structures, in addition to a possible direct effect of light on spore morphogenesis.

7. Aerial dispersal of conidia

In view of the uncertainty with which the ability of M.perniciosa conidia to be dispersed in air currents has been reported, (Zoberi 1961, Fletcher and Ganney 1968, Cross and Jacobs, 1968), dispersal of conidia within cropping houses infected with M.perniciosa was investigated.

Initial assessment of the suitability of the Cascade Impactor for collection of conidia confirmed that spores released from MM agar cultures into an air flow of 0.1 m sec.$^{-1}$ at 55% R.H., could be trapped using this apparatus. Both chlamydospores and verticillate conidia were seen to be aerially dispersed under such laboratory conditions. While chlamydospores were readily distinguished on impaction discs stained with cotton blue in lactophenol identification of verticillate conidia was more difficult due to the tendency for them to split at the central septum in some cases, resulting in two single celled propagules rather than the bi-cellular spore commonly associated with the fungus.

Use of the trap within a cropping house (A.bisporus strain D621)

MEAN
Number of spores
x 10^5 cm^{-3}

3.0

2.0

1.0

0

(i)

(ii)

LIGHT   U.V.   DARK
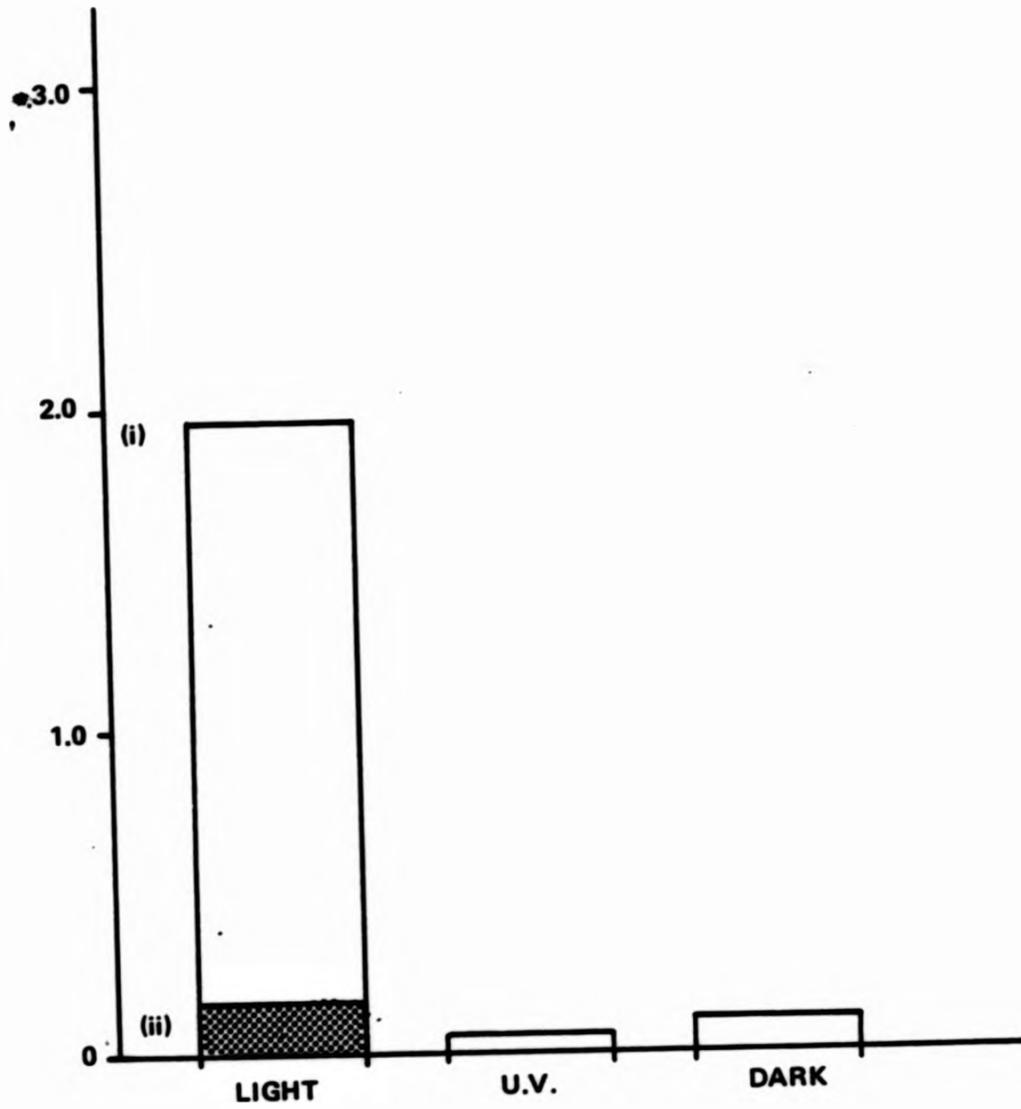
FIGURE 1.12 The effect of Tungsten light (13.5 Watts m^{-2}), Ultraviolet light (Phillips 'Black light' fluorescent tubes) and dark on the production of M.perniciosa chlamydospores (☐) and verticillate conidia (▨) on malt agar incubated at 20°C for 42 days.

Ratio of
chlamydospore:
verticillate
conidia (x:1)
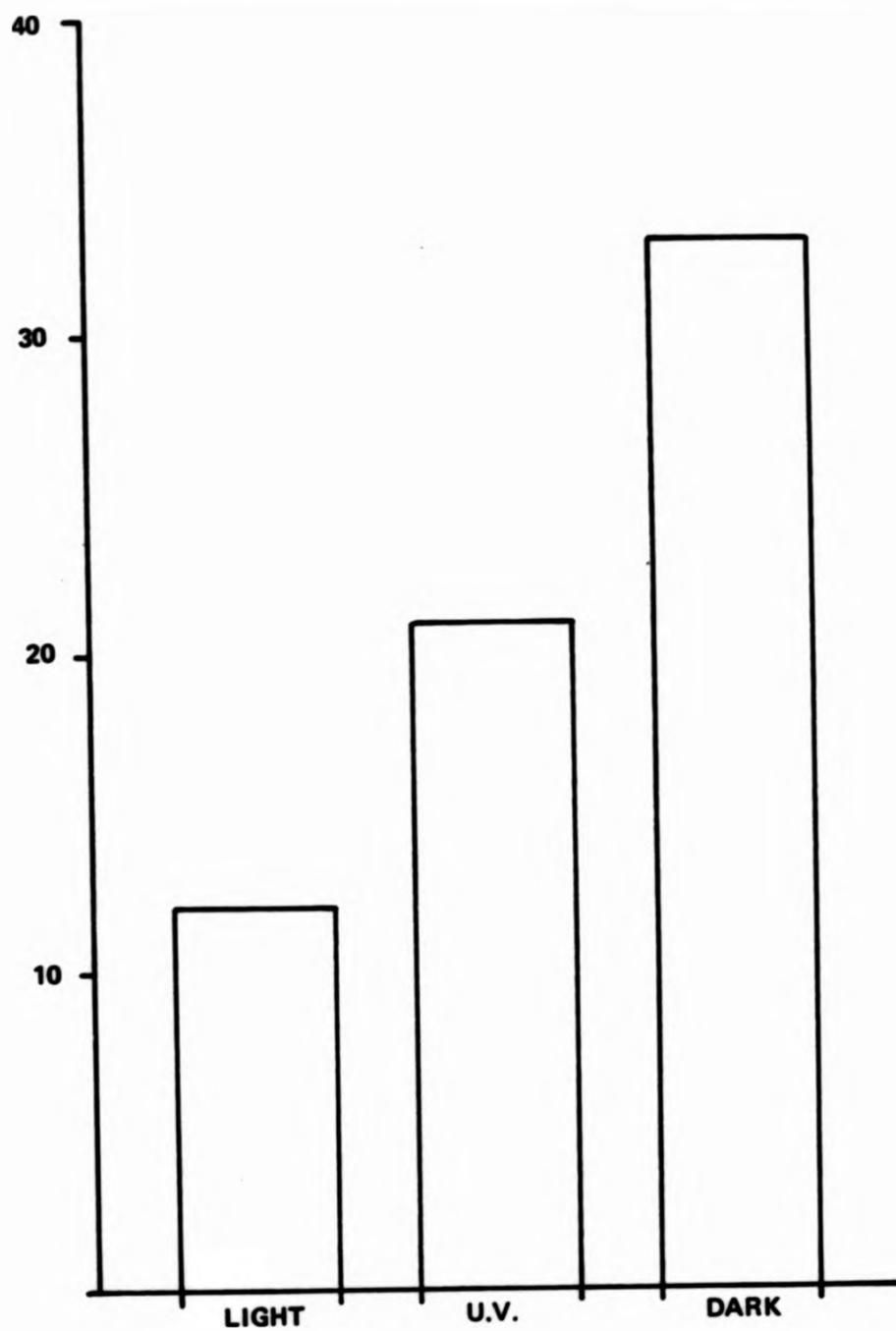
FIGURE 1.13   The effect of light (135 Watts m^{-2} Tungsten lamp), ultraviolet light
(Phillips 'Black light' fluorescent tubes), and dark on the relative
numbers of chlamydospores and verticillate conidia produced on
malt agar incubated for 42 days at 20°C.

artificially inoculated with M.perniciosa revealed that both chlamydospores and verticillate conidia were aerially dispersed. The maximum spore load measured was 110 propagules m^{-3} of air, where 37.7% of sporophores were infected.

Monitoring of temperature and relative humidity within the cropping house indicated that release of both chlamydospores and verticillate conidia could not be directly related to either of these factors. Spore release was most closely related to the age of the crop itself, $(r = -0.89, p < 0.01)$, (Figure 1.14).

The use of spore trapping apparatus was necessarily restricted to the period prior to 'veil break' of healthy sporophores before the release of spores by A.bisporus which resulted in all slides in the Cascade Impactor being covered with Agaricus spores, such that identification of M.perniciosa conidia was impossible.

IV COLONISATION OF NATURAL SUBSTRATES BY M.PERNICIOSA

1. Casing soil

Growth in casing soil inoculated with an M.perniciosa conidial suspension, (c. 1×10^4 spores g^{-1} of soil), in the absence of A.bisporus sporophore tissue, was not detected by direct microscopic observation under non-sterile conditions.

Routine observation of symptom development in infected beds revealed that in some cases M.perniciosa may grow in casing soil in the immediate vicinity of infected sporophore and sclerodermoid tissue,

In order to determine whether the ability of M.perniciosa to grow
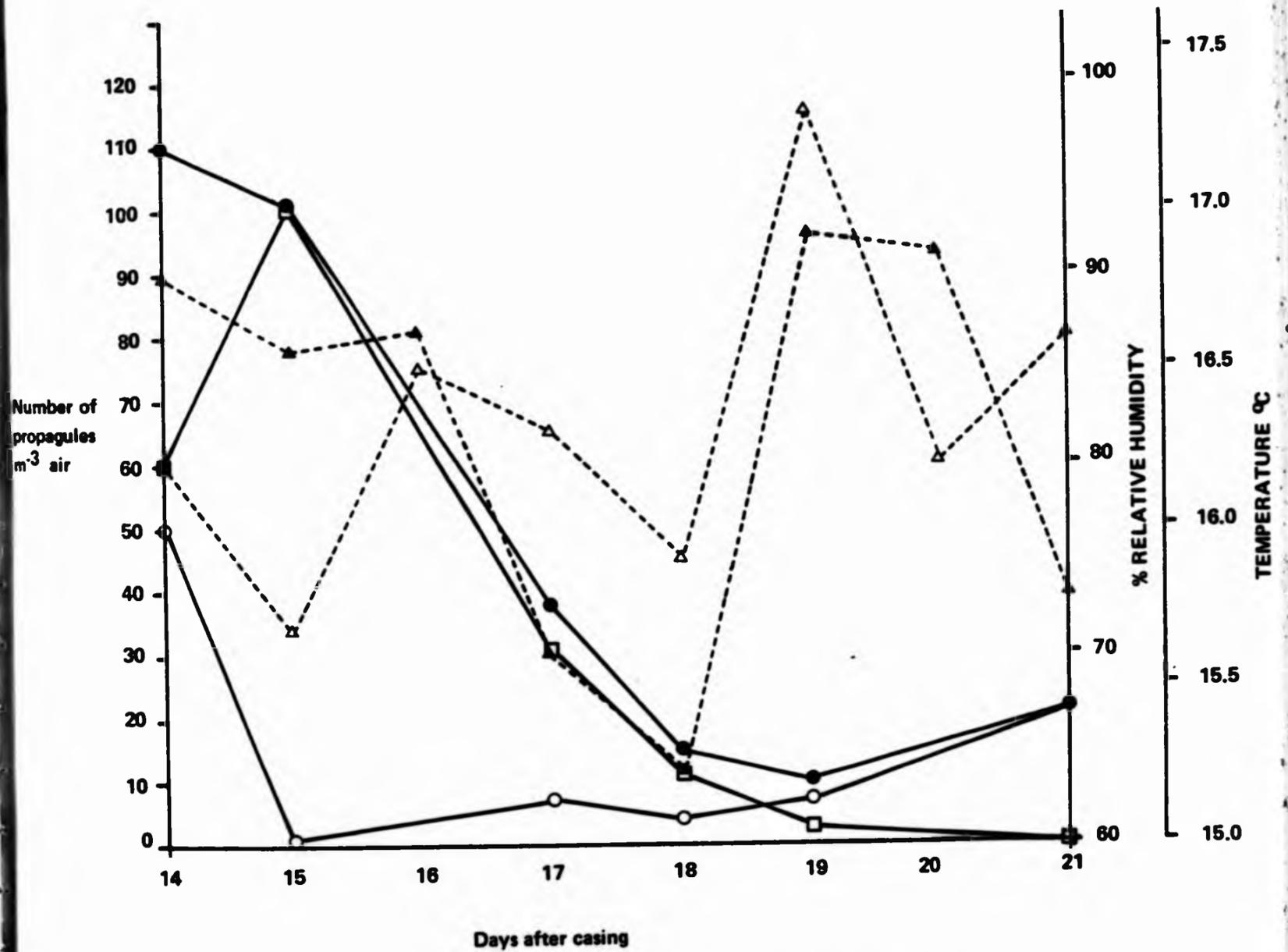
FIGURE 1.14   The relationship between aerial populations of M.perniciosa conidia and environmental factors over heavily infected commercial beds, prior to 'veil break' of A.bisporus sporophores.

●—●   Total M.perniciosa propagules
○—○   M.perniciosa chlamydospores
□—□   M.perniciosa verticillate conidia
▲—▲   % Relative humidity (wet and dry bulb hygrometer)
△—△   Temperature, °C

from infected tissue into the surrounding casing soil was influenced by the microbial population numbers of bacteria and actinomycetes were determined using the soil dilution technique.

It was found that the ability of M.perniciosa to grow into the casing soil could not be directly related to the total microbial population of the substrate. (Table 1.3).

2. Theoretical colonisation index (TCI) of M.perniciosa in natural substrates

As a comparative measure of the ability of M.perniciosa to colonise a range of natural substrates Theoretical Colonisation Index (TCI) values were determined using the method of Mitchell and Dix, (1975a).

Verticillate conidia, (10 day old) and chlamydospores (35 day old) were applied to strips of water agar placed on the surface of a range of substrates in closed plastic boxes, and incubated at 25°C $\pm$ 1°C for 24 h. Agar strips were subsequently removed from the substrate surface and placed on clean glass slides prior to staining with cotton blue in lactophenol and microscopic examination.

The TCI value is defined as the product of percentage germination and the mean germ tube length after 24 h incubation, with a low TCI value indicating a poor ability to colonise the substrate examined.

Results indicate a TCI value of zero for chlamydospores in fresh used mushroom compost and casing soil and for clay soil substrates with no germination after 24 h. Samples on mixed casing/compost weathered for 9 months at ambient temperatures, (between -10°C and +10°C) after

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Table 1.3

The relationship between the ability of M.perniciosa to colonise casing soil and soil microbial populations

(a) = Total population of bacteria and actinomycetes gram of soil $^{-1}$

(b) = Growth of <u>M.perniciosa</u> in casing soil from infected tissue

+ = < 1 cm

++ = > 1 cm

Microbial Population x 10^7 gram^{-1} (a)	Growth of M.perniciosa (b)	Microbial Population x 10^7 gram^{-1} (a)	Growth of M.perniciosa (b)
0.843	+	1.923	-
0.916	+	1.968	-
1.051	-	1.970	+
1.070	++	1.981	+
1.123	+	1.988	++
1.188	+	1.989	+
1.264	+	2.043	-
1.277	-	2.176	+
1.298	-	2.242	+
1.314	+	2.310	-
1.327	-	2.326	++
1.389	-	2.356	-
1.393	-	2.364	+
1.533	++	2.408	++
1.553	-	2.570	+
1.556	-	2.679	-
1.577	-	2.731	-
1.634	+	2.843	-
1.647	+	2.850	+
1.676	-	3.007	-
1.677	-	3.021	-
1.694	+	3.305	-
1.725	-	3.313	-
1.791	-	3.626	++
1.811	++	3.676	+
1.846	-	20.220	-
1.898	-		

cropping of A.bisporus was low at 1.38 compared with values for woodland soil and unused casing soil, (21.06 and 23.42 respectively).

Verticillate conidia similarly showed lowest TCI values in fresh used compost and casing, (316.39 and 372.36 respectively) while TCI values for weathered casing / compost mix and unused casing soil were higher at 377.11 and 874.0 respectively. TCI values for verticillate conidia were greatest in clay soil samples (1351.16) and woodland soil samples (2031.74), (Table 1.4). The low TCI values for both types of spore in fresh used mushroom substrates suggests that such material contains undefined factors capable of inhibiting growth of M.perniciosa. Increased TCI values for both types of conidia on weathered casing / compost mix indicates that such factors are degradable or that low TCI values in fresh substrates are due to the presence of a specific microflora antagonistic towards M.perniciosa that is superseded by other micro-organisms on older material.

3. Longevity of M.perniciosa mycelium in spent compost and casing soil

An attempt was made
In order to investigate the ability of M.perniciosa to survive as viable mycelium in casing and compost material in the absence of actively growing A.bisporus mycelium. After cropping, Barley seeds were inoculated with a conidial suspension of M.perniciosa, (after soaking in water for 48 h and autoclaving, (30 min. at 1 kg cm^{-2})) and incubated for 21 days at 18°C ± 2°C. The Fully colonised seeds were incorporated into spent mushroom compost and casing soil (c. 200 seeds l^{-1} of soil), and stored at 4°C and 18°C. Samples were withdrawn, (10 seeds) at 7 day

Table 1.4

Theoretical colonisation indices (TCI) for M.perniciosa 10 day old verticillate conidia and 35 day old chlamydospores on a range of substrates after 24 h at $25^{o}C \pm 1^{o}C$.

Substrate	Verticillate conidia			Chlamydospores		
	% Germination	Hyphal length arbitary units	TCI	% Germination	Hyphal length arbitary units	TCI
CONTROL	98.66	21.15	2086.66	6.33	4.09	25.89
WOODLAND SOIL	98.82	20.56	2031.74	7.14	2.95	21.06
CLAY SOIL	99.35	13.6	1351.16	0.0	0.0	0.0
UNUSED CASING	100.0	8.74	874.0	5.96	3.93	23.42
CASING/ COMPOST (9 month post cropping)	88.94	4.24	377.11	0.51	2.70	1.38
FRESH CASING (post cropping)	90.82	4.10	372.36	0.0	0.0	0.0
FRESH COMPOST (post cropping)	78.51	4.03	316.39	0.0	0.0	0.0

intervals, surface sterilised with mercuric chloride, and rinsed in three changes of SDW prior to plating on to malt (+ streptomycin) agar and incubated at 20°C $\pm$ 1°C. In the absence of A.bisporus tissue viability of M.perniciosa mycelium was seen to decrease rapidly, (Table 1.5). Mycelium was eliminated from seeds within 7 days in casing at 4°C, within 7 days in casing and compost at 18°C and within 14 days in compost at 4°C.

4. The effect of compost diffusates on germination and growth of conidia

In order to further investigate the effect of compost on fungal growth aqueous diffusates from compost fully colonised by A.bisporus were assayed for the ability to reduce germination and germ tube growth of Cladosporium herbarum and M.perniciosa conidia. Compost diffusates were prepared by steeping colonised compost in SDW for 18 h at 4°C. The resultant diffusates were centrifuged and sterilised by filtration. Separation into high molecular weight (HMW) and low molecular weight (LMW) fractions was by dialysis through cellophane into SDW for 48 h at 4°C. Resulting fractions were concentrated by freeze drying and subsequently resuspended in SDW. In order to eliminate any possible effects on germination caused by differences in osmotic potential between treatments all samples were adjusted to an osmotic potential of 1200 milliosmols Kg^{-1} by the addition of poly-ethylene glycol 6000.

Dialysis fractions and 'complete' (undialysed) diffusate samples were assayed against C.herbarum conidia and M.perniciosa verticillate conidia on glass slides, controls were of conidia in SDW.

Table 1.5

Viability of M.perniciosa mycelium incubated on sterile barley seed at 18°C for 21 days prior to burial in spent mushroom compost and casing soil with time, at 4°C and 18°C.

Time after burial	% of seeds containing viable mycelium			
(Days)	4°C		18°C	
	Casing	Compost	Casing	Compost
0	100	100	100	100
7	0	30	0	0
14	0	0	0	0

Germination of M.perniciosa verticillate conidia and C.herbarum conidia was not significantly affected by 'complete' or HMW diffusate samples relative to SDW controls. Germination of the conidia of both types of fungi was however significantly reduced in the presence of the LMW fraction, ($p < 0.05$), (Figure 1.15). The absence of any fungistatic or fungitoxic effect of the complete sample suggests the presence of undefined factors, possibly nutritional, which may negate any adverse effect on germination due to LMW components.

Germ tube growth of C.herbarum increased in all diffusate samples relative to SDW controls, while growth of M.perniciosa germ tubes was seen to be reduced by both HMW and LMW fractions relative to controls (89% and 85% reduction respectively), although this was not reflected in 'complete' samples, (Figure 1.16). The factors influencing the processes of spore germination and subsequent growth are therefore different, with an apparent specificity against M.perniciosa in the host substrate.

Membrane integrity of M.perniciosa germ tubes in compost diffusates was determined using the Trypan Blue histochemical stain as a measure of germ tube survival, with uptake of the high molecular weight stain indicating membrane disruption. Microscopic observation of germ tube terminal cells revealed a significant reduction in cellular integrity, relative to SDW controls and to non-dialysed 'complete' diffusates, ($p < 0.05$) in both HMW and LMW fractions, (65.5% and 52.0% reduction respectively), (Figure 1.17). This mirrors results for germ tube growth discussed above and indicates the fungi-toxic rather than fungistatic nature of LMW components.

FIGURE 1.15a

The effect of compost diffusates on germination of 14 day old M.perniciosa verticillate conidia incubated at 20°C for 48 hours

S.D.W. = Sterile distilled water
i   = Non dialysed diffusates from sterile compost colonised by A.bisporus.
ii   = High molecular weight fraction of (i)
iii   = Low molecular weight of (ii)
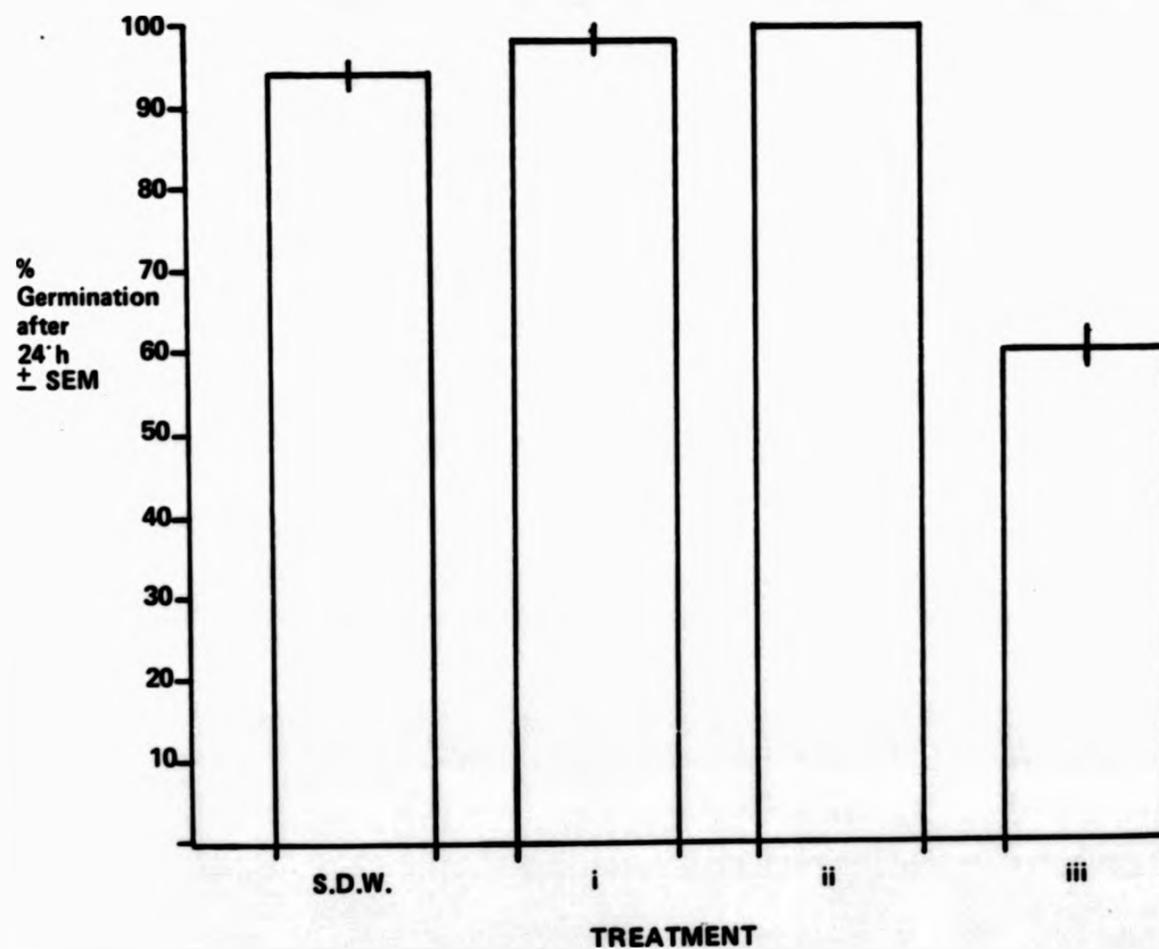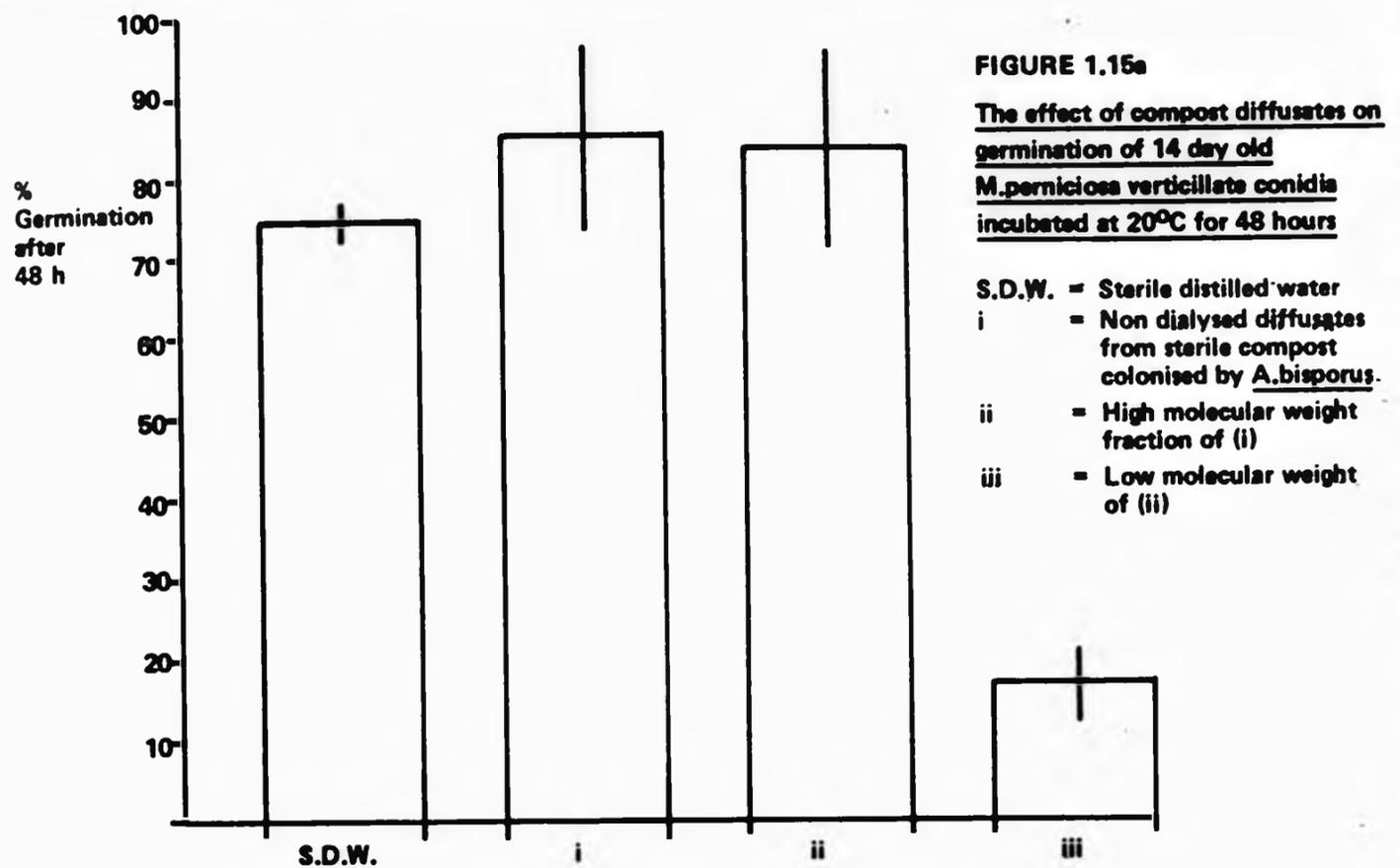
FIGURE 1.15b   The effect of compost components on germination of 8 day old conidia of Cladosporium herbarum incubated at 20°C for 24 hours

S.D.W. = Sterile distilled water
i   = Non dialysed diffusates from sterile compost colonised by A.bisporus
ii   = High molecular weight fraction of (i)
iii   = Low molecular weight fraction of (ii)

FIGURE 1.16a

The effect of compost components on growth of
M.perniciosa verticillate conidia germ tubes after
48 h incubation

S.D.W. = Sterile distilled water

i   = Non dialysed diffusates from sterile compost
        colonised by A.bisporus

ii   = High molecular weight fraction of (i)

iii   = Low molecular weight fraction of (ii)

Germ tube length
(µm)

TREATMENT

Germ tube length
(µm)

FIGURE 1.16b

The effect of compost components on
growth of Cladosporium herbarum germ
tubes after 24 h incubation.

S.D.W. = Sterile distilled water

i   = Non dialysed diffusates from
        sterile compost colonised by
        A.bisporus

ii   = High molecular weight
        fraction of (i)

iii   = Low molecular weight
        fraction of (ii)
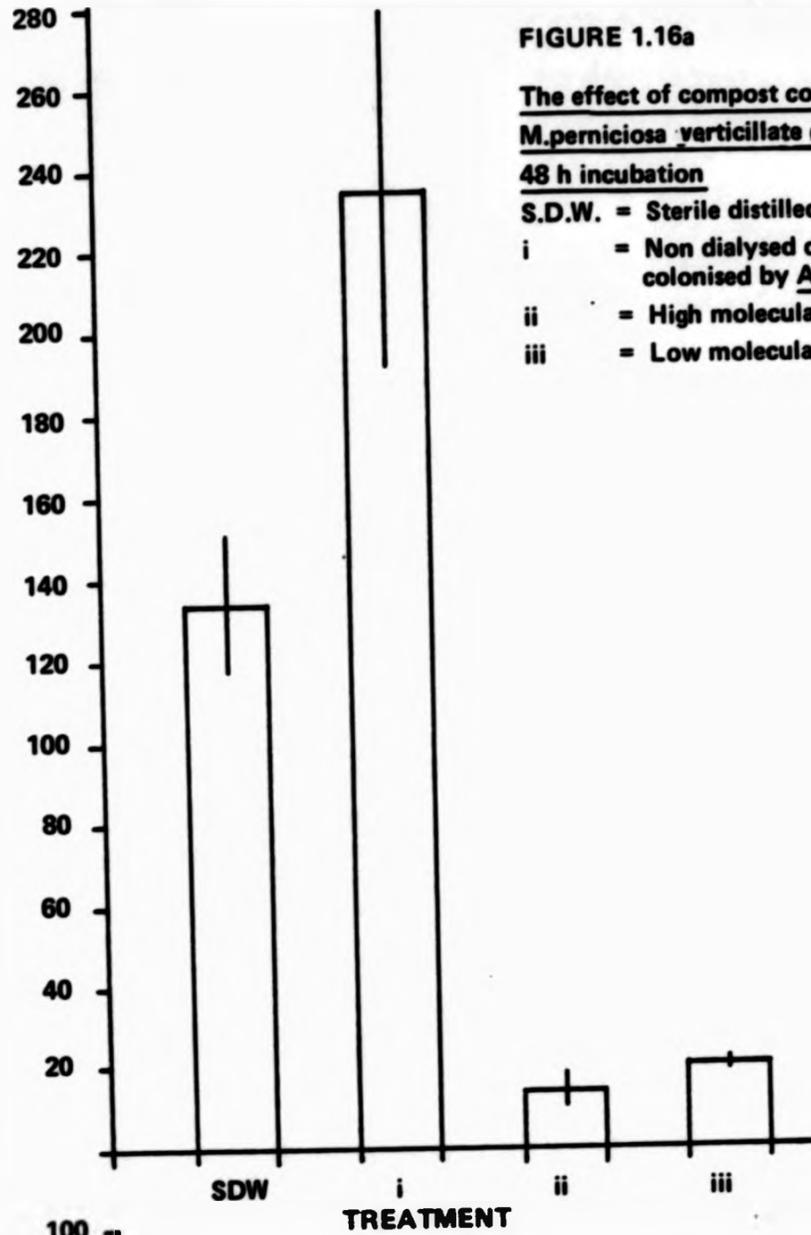
% Germ tubes
surviving
after 48 h
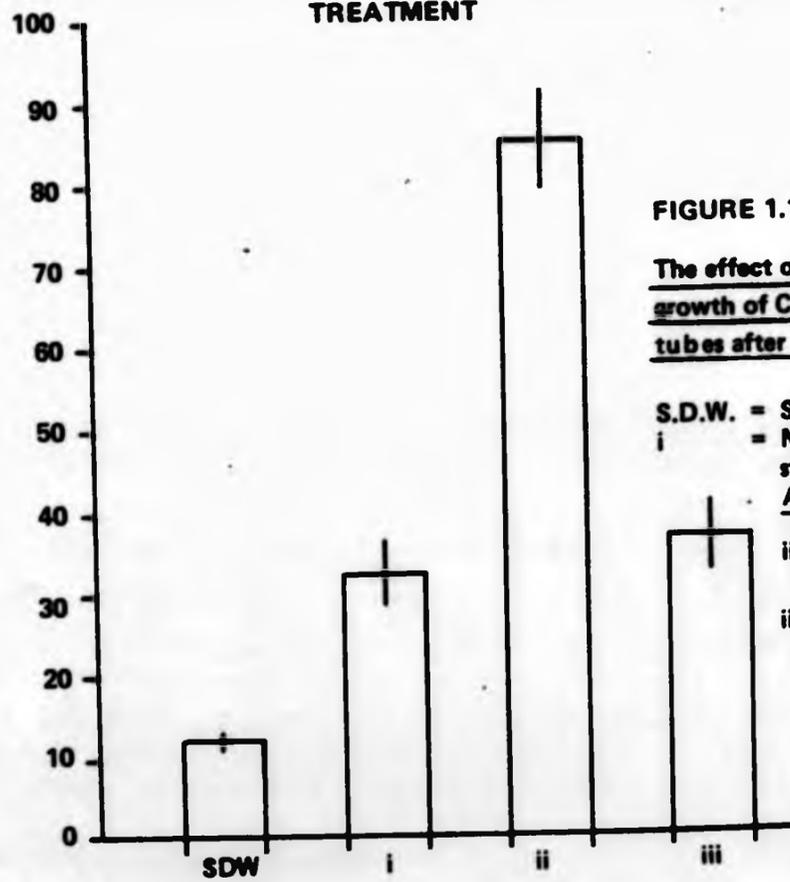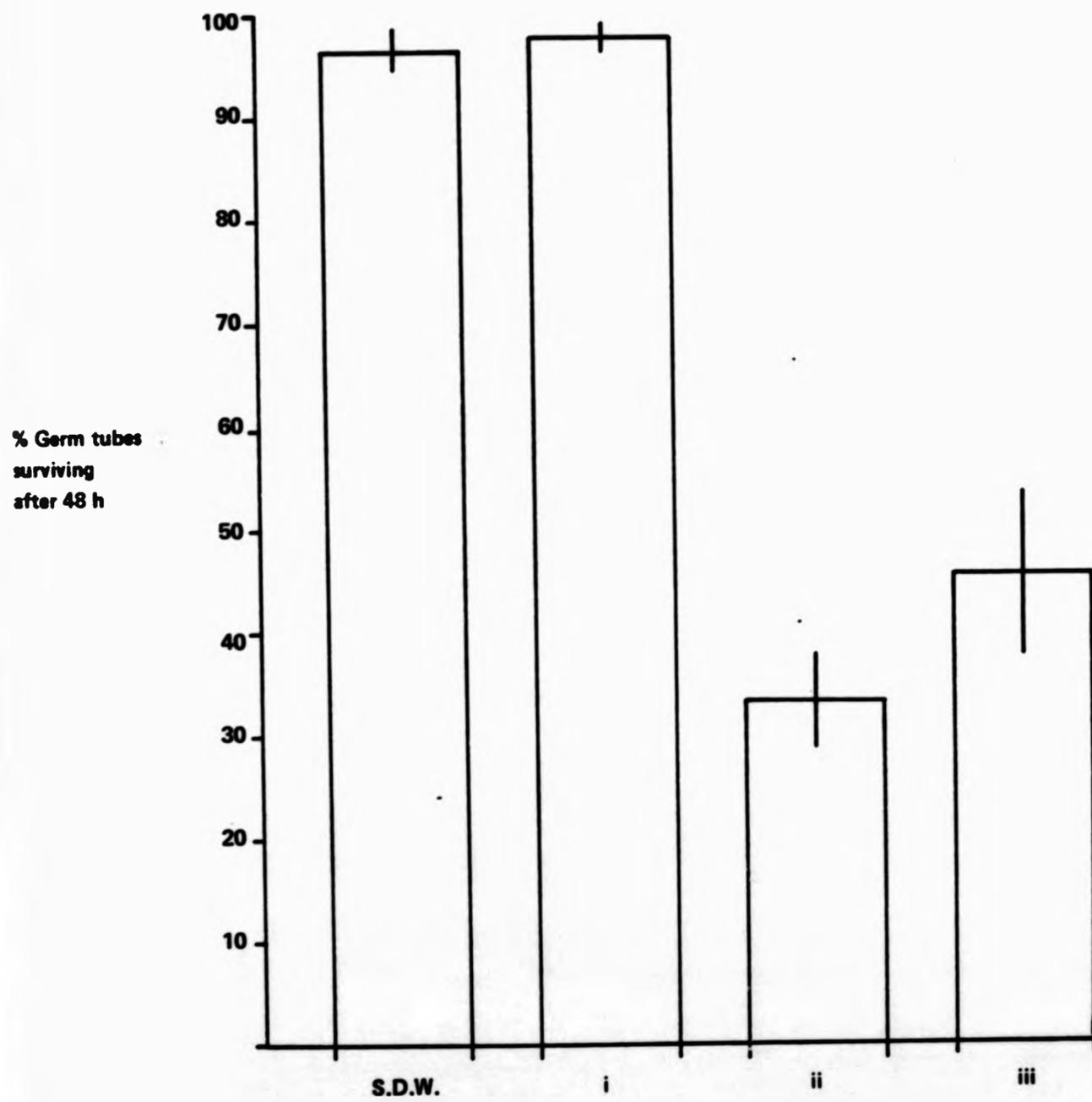
FIGURE 1.17   The effect of compost diffusates on survival of 14 day old M.perniciosa verticillate conidia
                incubated at 18°C for 48 hours

S.D.W.   =   Sterile distilled water
i   =   Non dialysed diffusates from sterile compost colonised by A.bisporus
ii   =   High molecular weight fraction of (i)
iii   =   Low molecular weight fraction of (ii)

Viability of ungerminated M.perniciosa conidia in diffusates,
and dialysis fractions of diffusates, from sterile compost previously
colonised by M.perniciosa, at uniform osmotic potential, prepared as
previously described, was examined in order to determine whether
spore viability was affected by components of the host substrate.
Spores were maintained at 4°C, in order to prevent germination, in
20 µl droplets of sample diffusates on clean glass slides in moist
chambers. In view of the problem associated with germination of
chlamydospores, spore viability was determined histochemically using
the Trypan Blue vital staining technique. Uptake of the stain -
indicating disruption of the cell membrane with resultant cell death,
(Rossall, Mansfield and Hutson, 1980).

Viability of chlamydospores was reduced in the presence of both
LMW (14.5% viable) and SDW (8.6% viable) compared to 'complete' (66.3%
viable) and HMW (61.0% viable) fractions, over an eight day period,
(p <0.05%), (Figure 1.18). This may reflect a leaching rather than a
toxic effect in the former two cases.

Viability of verticillate conidia was significantly reduced in
LMW fractions (72.1% viable) relative to SDW controls (95.8% viable)
and to both 'complete' and HMW fractions, (97.5% and 96.3% viable
respectively), measured over a 48 h period, (p <0.05), (Figure 1.19).
The effect of diffusates on verticillate conidia differs from that
noted for chlamydospores, particularly in that response to the presence
of diffusates is more rapid in the former case and appears to reflect
a susceptibility of verticillate conidia to fungitoxic LMW components
present in compost colonised by the host.

It is apparent that compost colonised by A.bisporus contains low

% Dead
spores
(chlamydospores)

TIME AFTER TREATMENT (DAYS)
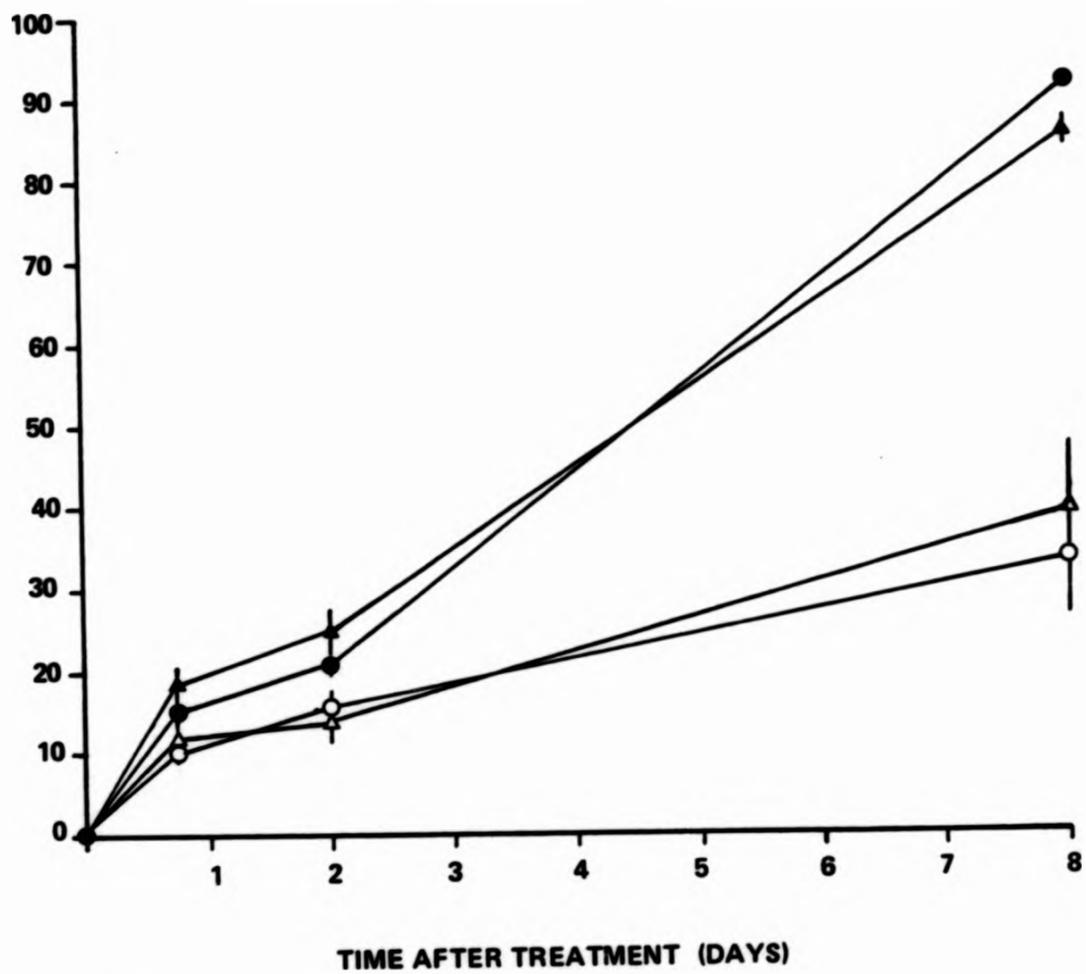
FIGURE 1.18   The effect of A.bisporus compost components on survival of 14 day old M.perniciosa chlamydospores .
Assay was by staining with Trypan Blue after incubation at 4°C.

i)   ●─●   S.D.W.
ii) O─O   Non dialysed diffusate from sterile compost colonised by A.bisporus
iii) △─△   High molecular weight dialysis fraction of (ii)
iv) ▲─▲   Low molecular weight dialysis fraction of (ii)

% DEAD SPORES

TIME AFTER TREATMENT (HOURS)
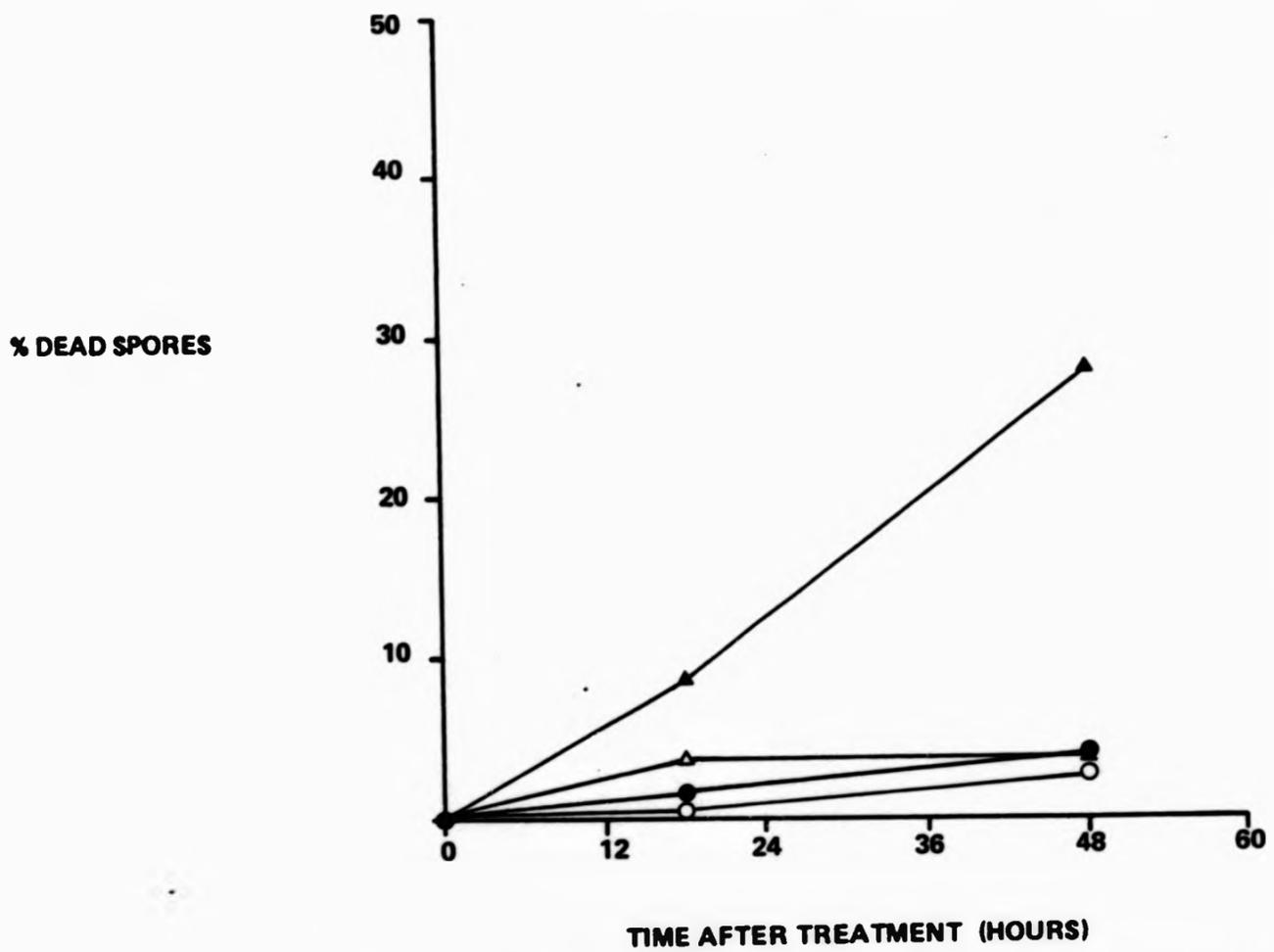
FIGURE 1.19   The effect of A.bisporus compost components on survival of 8 day old M.perniciosa
                   verticillate conidia. Assay was by staining with Trypan Blue after incubation at 4°C

i)   ●─●   S.D.W.
ii) ○─○   Non dialysed diffusates from sterile compost colonised by A.bisporus
iii) ▲─▲   High molecular weight fraction of (ii)
iv) △─△   Low molecular weight fraction of (ii)

molecular weight components reducing the growth and germination of
M.perniciosa conidia. This is apparently due to the presence of more
than one factor, however, in view of the differing response of
germination and germ tube growth of both C.herbarum and M.perniciosa
conidia. That such effects are not apparent in 'complete' samples
suggests that inhibition is due to fungitoxic activity in the low
molecular weight sample which can be overridden by factors, possibly
nutritional, present in the high molecular weight phase.

5. Spectrophotometric determination of growth of M.perniciosa in compost

The ability of M.perniciosa to colonise mushroom compost has
previously been the subject of contradictory reports, (Smith 1924,
Vincent-Davies 1972). Therefore, the ability of M.perniciosa to grow
in such material was determined quantitatively using a spectrophoto-
metric assessment of esterase activity in colonised substrates as a
measure of fungal mass, (Swisher and Carroll, 1980).

In preliminary experiments M.perniciosa esterase activity was
related to total dry weight of fungus by incubating samples of mycelium
with 25 cm^3 of SDW containing 8 μl cm^{-3} of fluorescein diacetate for
1.5 h at 20°C $\pm$ 1°C in an orbital incubator (100 r.p.m.). Incubation
of the mycelium resulted in production of a green colouration in the
substrate due to hydrolysis of fluorescein diacetate by fungal esterase.
The sample was filtered through tared membrane filters, (0.22 μ pore
size) and absorbance of the supernatant at 490 nm determined. Dry
weight of mycelium was also determined. A calibration line relating
esterase activity to dry weight of fungus was constructed (Materials
and Methods, II 7C), enabling quantitative determination of M.perniciosa

mycelium present in complex substrates.

Sterile commercial composts were then examined in order to quantitatively determine the ability of the fungus to colonise such substrates.

In order to differentiate between the effects of compost and A.bisporus mycelium on growth of M.perniciosa, a range of substrates were examined. Sterile unused compost, sterile autoclaved compost supplemented with diffusates from A.bisporus compost cultures and ethylene oxide sterilised compost previously colonised by A.bisporus were each inoculated with an M.perniciosa conidial suspension from 14 day old MM agar cultures, $(4.0 \times 10^4$ spores $g^{-1})$. Cultures were subsequently incubated at 20°C $\pm$ 1°C. Compost diffusates, ('complete', low molecular weight, and high molecular weight fractions) prepared as previously described and added separately, were included at the rate of $0.25 \ cm^3 \ g^{-1}$ of compost.

Growth of M.perniciosa in treated and untreated compost was determined by withdrawing 1.0 g samples of compost and incubating them each with fluorescein diacetate in SDW as previously outlined. Blanks were of uninoculated sterile compost incubated in the same manner. The amount of M.perniciosa mycelium present in the compost was then calculated from the calibration line.

Growth of M.perniciosa in unsterile compost was examined by direct observation of inoculated compost only, the fluorescein diacetate hydrolysis technique being calibrated specifically for M.perniciosa in the absence of any other fungi or bacteria.

Growth of M.perniciosa was seen to be significantly greater in

compost with the addition of sterile filtered diffusates from compost
colonised by A.bisporus, and in that supplemented with HMW dialysis
fractions of compost diffusates compared to all other treatments,
(p <0.05). Growth in sterile compost previously colonised by A.bisporus
prior to sterilisation was reduced however.

The ability of M.perniciosa to colonise mushroom compost was
clearly demonstrated. Growth of M.perniciosa affected by the presence
of the host with a 16.8% reduction in growth of M.perniciosa in compost
colonised by A.bisporus compared to uncolonised material. 'Complete'
and HMW diffusates increased growth of M.perniciosa in compost by 74.9%
with LMW fractions showing no significant effect. (Table 1.6).

These results suggest the presence of anti-fungal components in
A.bisporus mycelium which are not extracted using the diffusion
technique, with an increase in growth of M.perniciosa in the presence
of compost diffusates possibly reflecting the presence of additional
nutrients leached from compost colonised by A.bisporus.

The balance between nutrition and fungistasis of composted
material in relation to growth of M.perniciosa previously observed in
spore tests was reflected in the ability of the fungus to colonise
natural substrates. That fungistasis was observed in sterile samples
previously colonised by A.bisporus suggests that inhibitory activity
may be due to the host rather than any naturally occurring members of
the microflora typically associated with A.bisporus in vivo.

Table 1.6

The effect of A.bisporus mycelium (killed) and diffusates from compost
colonised by A.bisporus on the ability of M.perniciosa to colonise
mushroom compost assessed by spectrophotometric determination of
M.perniciosa esterase activity.

Substrate	mg dry weight of M.perniciosa mycelium g^{-1} of substrate, (a)
Compost colonised by A.bisporus and sterilised with ethylene oxide	19.60 ± 2.90
Sterile compost with the addition of SDW (osmotic potential 1200 milliosmols Kg^{-1})	23.55 ± 4.43
Sterile compost with the addition of sterile filtered diffusates from non-sterile compost colonised by A.bisporus, (osmotic potential 1200 milliosmols Kg^{-1})	41.20 ± 3.32
Sterile compost with the addition of HMW dialysis fraction of diffusates from non-sterile compost colonised by A.bisporus (osmotic potential 1200 milliosmols Kg^{-1})	41.20 ± 4.95
Sterile compost with the addition of LMW dialysis fraction of diffusates from non-sterile compost colonised by A.bisporus (osmotic potential 1200 milliosmols Kg^{-1})	28.92 ± 1.19
Non sterile compost	Growth observed but not quantified

V.   INTERACTIONS OF <u>M.PERNICIOSA</u> WITH HOST AND NON-HOST FUNGI

1.   <u>Germination of chlamydospores in the presence of A.bisporus initials</u>
     <u>at various pH values</u>

In order to determine whether the stage of development of
<u>A.bisporus</u> initials affects germination of <u>M.perniciosa</u> chlamydospores,
30 sporophore initials, (<u>A.bisporus</u> Strain D621), were each placed on
sterile cavity slides with 20 μl droplets of a conidial suspension of
the pathogen, (c. 1 x 10^5 spores cm^{-3}) in citrate:phosphate buffer at$^{(Hale, 1965)}$
different pH values, and incubated at 23°C $\pm$ 1°C for 5 days in moist
chambers.

Germination was determined microscopically after killing sporelings
with one drop of cotton blue in lactophenol. The level of germination
of chlamydospores was uniformly low, (<20%). Germination levels were
affected both by pH of the substrate and by the size of the accompanying
<u>A.bisporus</u> initial, (Figure 1.20).

The percentage germination of chlamydospores increased with an
increase in the size of <u>A.bisporus</u> initial present.

Germination levels at the pH values investigated, were highest
overall at pH 7.2, that closest to the pH values of the casing layer of
a commercial crop; however, this treatment also gave a higher degree
of scatter in the relationship between initial size and percentage
germination than at pH 7.6, (Table 1.7).

In order to determine the minimum size of <u>A.bisporus</u> initial having
an effect on germination of <u>M.perniciosa</u> chlamydospores plots of
percentage germination against initial size were extrapolated to give
values for the minimum size of initial capable of increasing germination

% GERMINATION
OF M.PERNICIOSA
CHLAMYDOSPORES

SIZE OF A.BISPORUS INITIALS (mm)
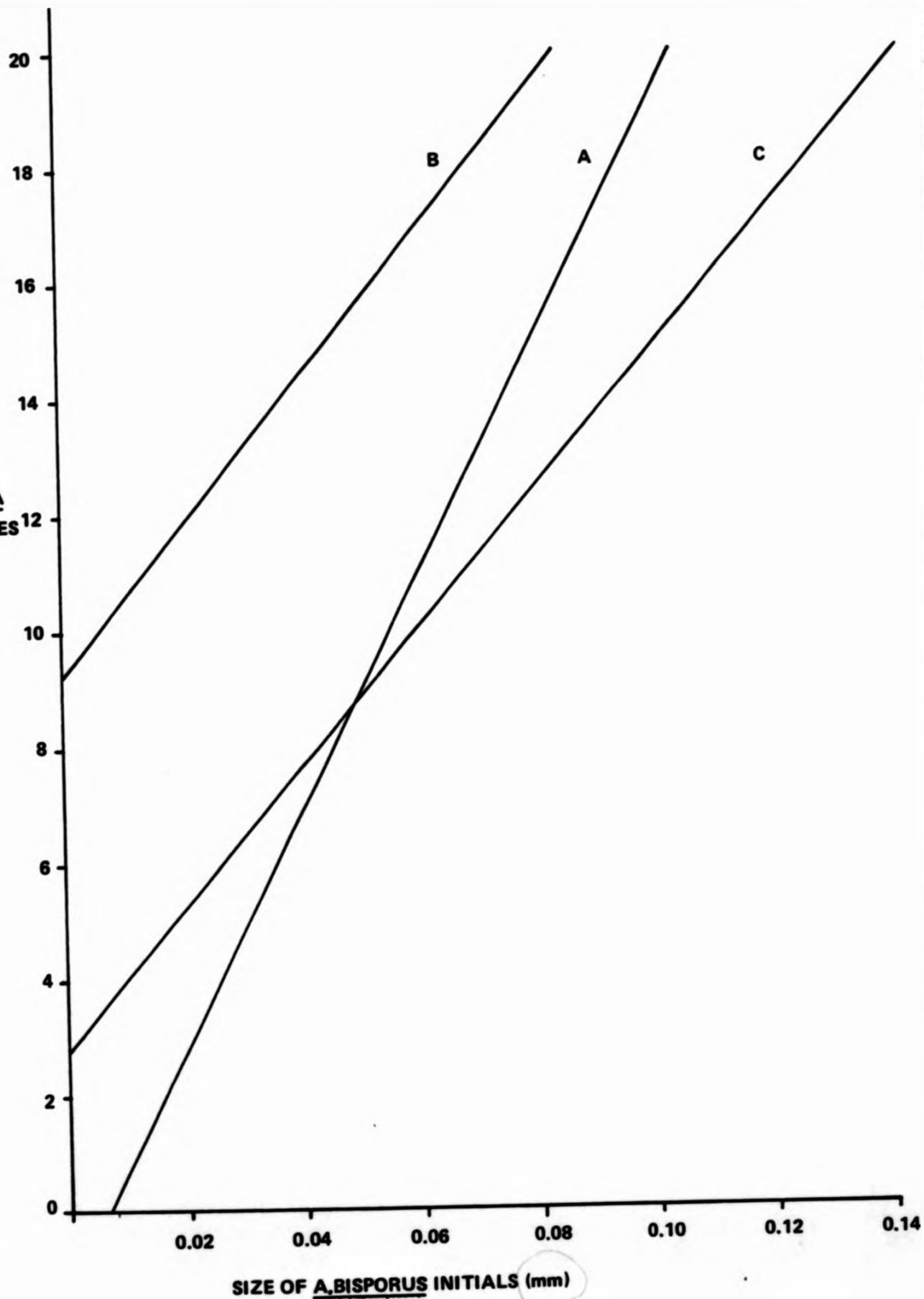
FIGURE 1.20 Germination of 45 day old M.perniciosa chlamydospores in the presence of A.bisporus (D621) initials produced under axenic conditions at A) pH 6.3, B) 7.2, C) pH 7.6. Incubation was for 5 days at 23°C prior to staining with cotton blue in lactophenol and microscopic examination.

Table 1.7

Relationship between initial size (A.bisporus Strain D621)in axenic culture
and germination of M.perniciosa chlamydospores at various pH values.
The tabulated values were determined by extrapolation of recorded data
to predict the minimum size of A.bisporus sporophore initial to increase
germination of 45 day old M.perniciosa chlamydospores.

	pH value for substrate		
	6.3	7.2	7.6
Minimum initial size to increase germination of chlamydospores relative to SDW controls (mm).	0.062	0.0	0.0
Scatter of data (ϱ)	0.37	0.58	0.67

relative to controls of chlamydospores in the absence of A.bisporus material, (Figure 1.20). ·

At pH 6.3, A.bisporus initials 62 µm in diameter were determined as being the minimum able to increase germination of chlamydospores while at pH 7.2 and 7.6, there appears to be no minimum size of A.bisporus material capable of increasing germination of M.perniciosa chlamydospores compared to control samples in the absence of A.bisporus tissue or mycelium, (Table 1.7).

2. Numerical threshold for infection (NTI) of mature A.bisporus sporophores

The numerical threshold for infection (NTI) of A.bisporus sporophore by M.perniciosa chlamydospores was determined for button stage sporophores, (Strain D621) by the application of 10 µl droplets of 10 day old chlamydospore suspensions in SDW of known concentration to the cap surface of 10 healthy freshly picked (< 1 h) sporophores at each spore concentration.

Inoculated sporophore caps were incubated at $6°C \pm 1°C$ for 10 days in moist chambers and subsequently examined for evidence of spore germination and subsequent infection of the host at the point of inoculation.

Infection was seen to occur at all spore concentrations with a minimum NTI value for 100% infection of 2 spores/inoculation site, (Table 1.8).

3. Growth of M.perniciosa in the presence of host and non-host fungi

Growth of M.perniciosa in the presence of a range of fungi (commonly found in soils and composts) was determined in vitro using

Table 1.8

Infection of healthy A.bisporus sporophores (button stage) at differing M.perniciosa spore loads. 10 day old chlamydospores were applied to cap surfaces prior to incubation for 10 days at 6°C prior to microscopic examination of inoculation sites.

Spores / inoculation site (10 μl droplet)	% infection of healthy sporophore caps
1	50
2	100
5	100
10	100
50	100
100	100
250	100

dual cultures on agar plates in order to determine which fungi are capable of antagonising M.perniciosa. In addition, the growth of M.perniciosa in the presence of different strains of host (A.bisporus) mycelium was also investigated.

The patterns of growth of mycoparasites in the presence of host and non-host fungi in vitro was investigated by Gray and Morgan-Jones, (1981). They found that the growth of mycoparasites in the presence of vegetative mycelium of other fungi could be placed in one of four categories, (Table 1.9).

In order to examine the pattern of growth of M.perniciosa in the presence of host mycelium, commercial strains of A.bisporus (D621 and A6) and an experimental strain (G.C.R.I. 431) were inoculated on to separate Czapek Dox agar plates in 9 cm Petri dishes. Strain 431 commonly forms initials in sterile culture while the commercial strains do not. After establishment of growth of A.bisporus, (c. 2-3 mm) plates were inoculated with M.perniciosa and linear growth of the pathogen from the point of inoculation recorded at regular intervals.

Growth rate of M.perniciosa in the presence of all A.bisporus strains tested was seen to fall off from 6.4 mm $\pm$ 0.53 mm day^{-1}, before contact between the two fungi, to 0.2 mm $\pm$ 0.09 mm day^{-1} on contact, (Growth pattern D, with no apparent difference between strains of A.bisporus), (Figure 1.21).

Growth of M.perniciosa in the presence of A.bisporus was further investigated using glass Petri dishes, (15 cm diameter), filled with sterile compost, (c. 50 g) and inoculated with a single 5 mm disc cut from the margin of a 10 day old M.perniciosa Malt agar culture at the

Table 1.9

Patterns of growth of mycoparasites in the presence of host and non-host

fungi in vitro

(After Gray and Morgan-Jones, 1981)

Category A

    Growth of a mycoparasite in the presence of non-host fungi is
unaffected with a uniform linear growth rate both before and after
contact between the two fungi.

Category B

    Where a mycoparasite is in the presence of mycelium of a potential
host, the growth rate of the pathogen decreases gradually, both before
and after contact between the two fungi, although growth of the
mycoparasite does not stop altogether.

Category C

    Where the fungi are mutually antagonistic, growth of the mycoparasite
is initially uniform, with a sudden cessation of growth on contact
between the two fungi.

Category D

    In the presence of vegetative mycelium of natural host fungi myco-
parasites exhibit an initial reduction in growth rate, ultimately
leading to a cessation of growth of the pathogen following contact
between the two fungi.

LINEAR
GROWTH
OF
M.PERNICIOSA
(mm)

TIME AFTER INOCULATION (DAYS)
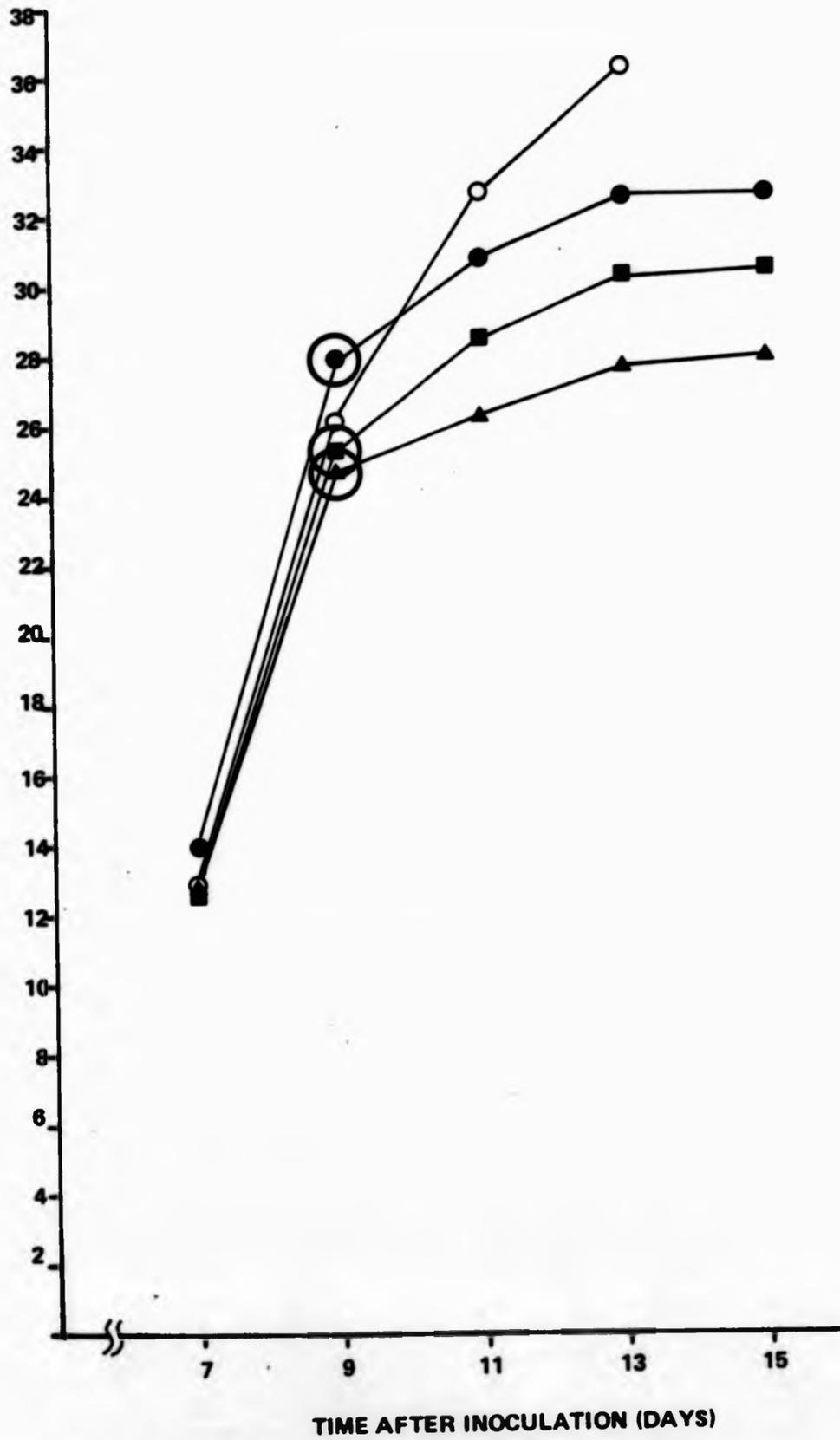
FIGURE 1.21   Growth reaction of M.perniciosa grown in the presence of different strains of A.bisporus on Czapek Dox agar at 16°C. (Point of colony contact indicated by a circle).

●—●   A.bisporus (A6)
■—■   A.bisporus (D621)
▲—▲   A.bisporus (G.C.R.I. 431)
○—○   Control · M.perniciosa only

same time inoculation with similar discs cut from the margin of 14 day old A.bisporus cultures, placed 6 cm apart.

Controls were of the two fungi inoculated separately to dishes containing compost and incubated under identical conditions.

Growth of M.perniciosa was examined after 66 days incubation at 16°C $\pm$ 4°C, the extent to which the pathogen had colonised the substrate being determined microscopically. In the presence of A.bisporus mycelium, growth of M.perniciosa was significantly less than in controls, (Strain D621 p <0.05, Strain 431 p <0.01), (Table 1.10).

That A.bisporus (Strain 431) produces initials in sterile culture has no significant effect on growth reaction of M.perniciosa to host mycelium suggesting that the onset of pathogenesis is related to factors such as substrate and environment rather than simply the availability of susceptible tissue.

The growth reaction of M.perniciosa towards vegetative mycelium of a number of other Agaricus species, was examined in paired cultures. as previously described.

It was noted that growth rate of M.perniciosa gradually declined as the other fungus was approached in all cases examined, with an eventual cessation of growth of the pathogen following contact between the two colonies, a pattern of growth considered typical of a myco-parasite in the presence of a host fungus, (Gray and Morgan-Jones, 1981). (Figure 1.22).   This has indicated that M.perniciosa is likely to infect Agaricus macrosporus, A.bitorquis and A.silvicola in addition to the natural host, A.bisporus.

The growth reaction of M.perniciosa towards basidiomycete fungi

Table 1.10

Growth of M.perniciosa in sterilised compost with and without A.bisporus
at 16°C after 66 days incubation.

(a) = Mean $\pm$ SEM

** = p <0.01

Compost/A.bisporus strain	Linear growth of M.perniciosa (mm) (a)
Sterile compost	68.5 ± 7.2
Sterile + A.bisporus (D621)	10.4 ± 3.9
Sterile + A.bisporus (431)	9.2 ± 3.2

LINEAR
GROWTH
OF
M.PERNICIOSA
(mm)

TIME AFTER INOCULATION (DAYS)
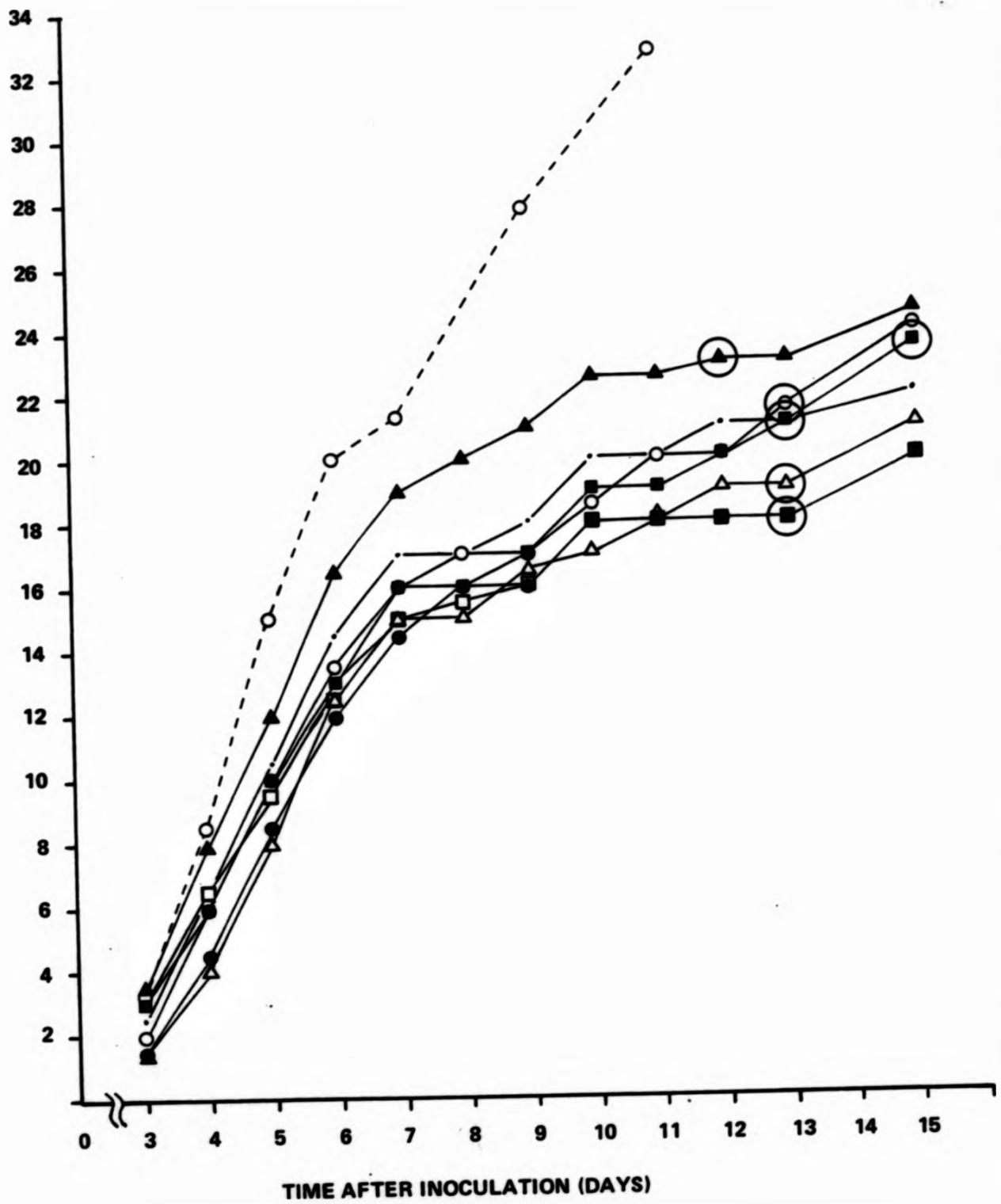
FIGURE 1.22   Growth reaction of M.perniciosa when grown with a range of Agaricus species in paired culture
on Czapek Dox agar at 20°C. (Point of colony contact denoted by a circle)

●—● A.bisporus (D621)
■—■ A.bisporus (522)
○—○ A.macrosporus (B)
□—□ A.bitorquis   W2
△—△ A.bitorquis   W32
—·—· A.bitorquis   W4
▲—▲ A.silvicola
○--○ Controls:- M.perniciosa in the absence of other fungi

other than <u>Agaricus</u> species in paired culture was examined using the dual culture technique previously described.

Growth of <u>M.perniciosa</u> was seen to decrease steadily in the presence of <u>Flammulina velutipes</u>, <u>Stropharia merdaria</u> and <u>Schizophyllum commune</u>, with cessation of growth of <u>M.perniciosa</u> following contact between the two fungi in each of the cultures, typical of growth pattern D as outlined above, indicating an ability of <u>M.perniciosa</u> to parasitise these fungi.

In the presence of <u>Coprinus sp.</u> and <u>Volvariella volvacea</u>, <u>M.perniciosa</u> exhibited a steady decline of growth rate, both before and after the point of colony contact in each case, with the pattern of growth of <u>M.perniciosa</u> resembling that previously defined as Category B, indicating a possible role for these two fungi as hosts for <u>M.perniciosa</u> under some conditions.

The pattern of growth of <u>M.perniciosa</u> in the presence of <u>Pleurotus ostreatus</u> could not be placed in any of the four categories previously described.

The extent of growth of <u>M.perniciosa</u> in the presence of <u>P.ostreatus</u>, following an initial tailing off of growth rate, was seen to be reduced after contact between the two fungi indicating extreme antagonism of <u>P.ostreatus</u> towards <u>M.perniciosa</u>. Such reduction in the extent of colonisation was considered to be a separate growth reaction to those described above and was termed Category E.   (Figure 1.23).

Growth patterns of <u>M.perniciosa</u> in the presence of a range of fungi other than basidiomycetes was also investigated.

In the presence of <u>Chaetomium globosum</u>, <u>C.olivaceum</u>, <u>Geotrichum</u>
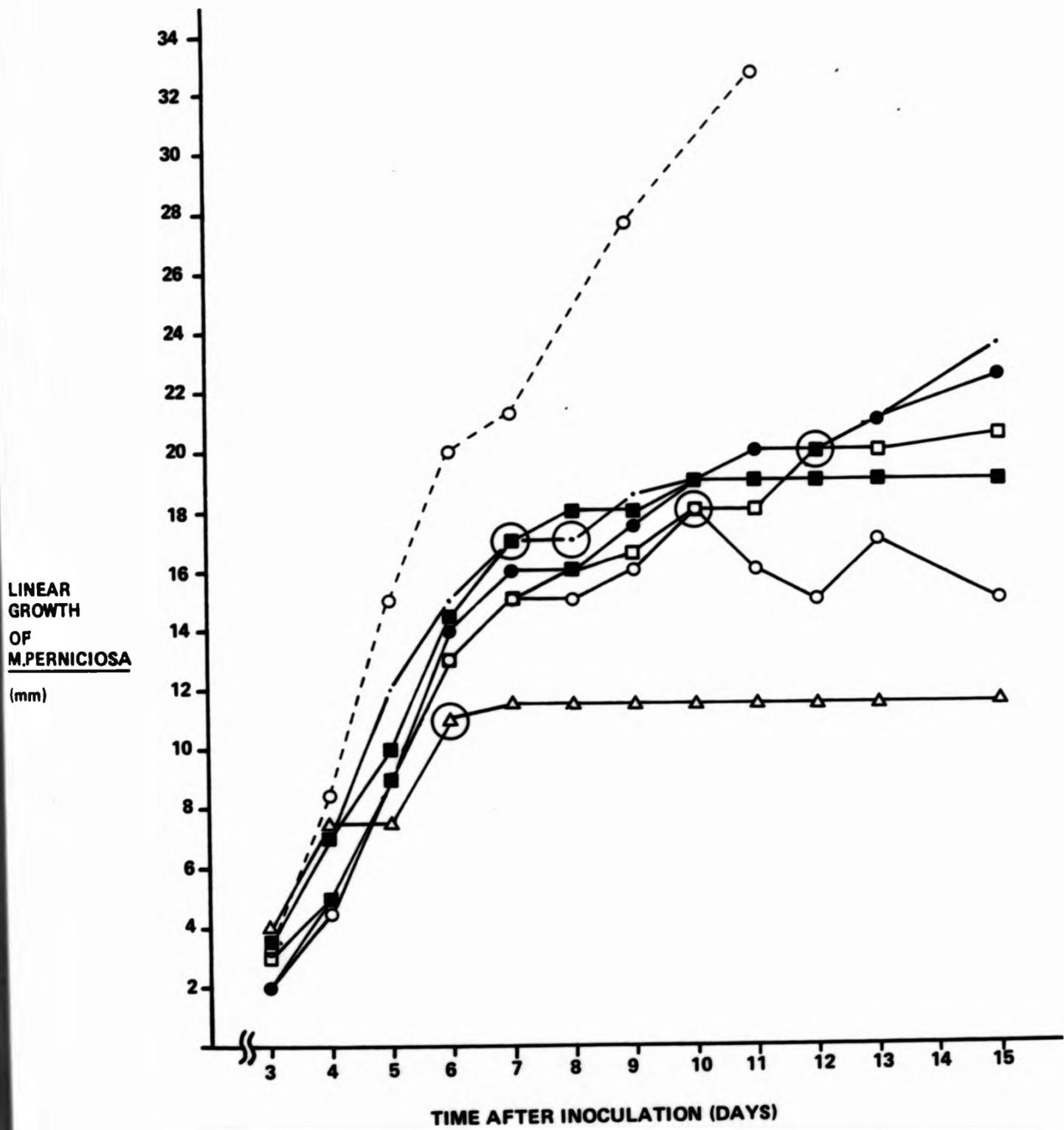
FIGURE 1.23 Growth reaction of M.perniciosa when grown in paired culture with a range of basidiomycete fungi on Czapek Dox agar at 20°C. (Point of colony contact denoted by a circle)

● —● Coprinus (H_2H_5)
■ —■ Flammulina velutipes (Curt. ex Fr.) Karst
○ —○ Pleurotus ostreatus (P11) (Jacq. ex Fr.) Kummer
□ —□ Psilocybe merdaria Stropharia merdaria. (fr.) Quel Syn. Psilocybe
△ —△ Schizophyllum commune Fr.
. —. Volvariella volvacea (Bull ex Fr.) Singer
○ --○ Control:- M.perniciosa in the absence of other fungi

candidum, Gliocladium roseum, Penicillium expansum, P.frequentens and
Verticillium fungicola the growth rate of M.perniciosa was uniform with
a sudden cessation of growth, indicating mutual antagonism between the
fungi, (Figures 1.24, 1.25). In addition, M.perniciosa was seen to be
self-inhibitory.

M.perniciosa was inhibited to an extreme degree by Trichoderma
viride, Mucor hiemalis, Rhizopus tritici and Botrytis cinerea.
Inhibition may be due wholly or in part to the rapid growth rate of
these fungi, which rapidly colonised the entire plate surface.

Differing relationships between M.perniciosa and a range of fungi
were therefore observed (Table 1,11).

Antagonism towards M.perniciosa was frequently expressed as a
reduction in the extent of substrate colonisation by M.perniciosa,
(Category E),(Figures 1.23 to 1.25). Zones between M.perniciosa and
challenge fungi were noted as being clear with little or no contact
between the two fungi in some cases, (Plate 1.4).

4. Infection of fruit body tissues of basidiomycete fungi

In order to investigate the ability of M.perniciosa to infect, and
sporulate on, fleshy fruit bodies of fungi other than the natural host,
A.bisporus, tissue samples and complete sporophores were artificially
inoculated in the laboratory and in the field with M.perniciosa.

Tissue blocks, c. 1.5 x 1.5 cm, from mature fruit bodies collected
primarily in the grounds of Stirling University, were excised under
sterile conditions and inoculated with a single 20 µl droplet of an
M.perniciosa conidial suspension, (c. 1.0×10^5 spores cm^{-3}). Blocks

LINEAR
GROWTH
OF
M.PERNICIOSA
(mm)

TIME AFTER INOCULATION (DAYS)
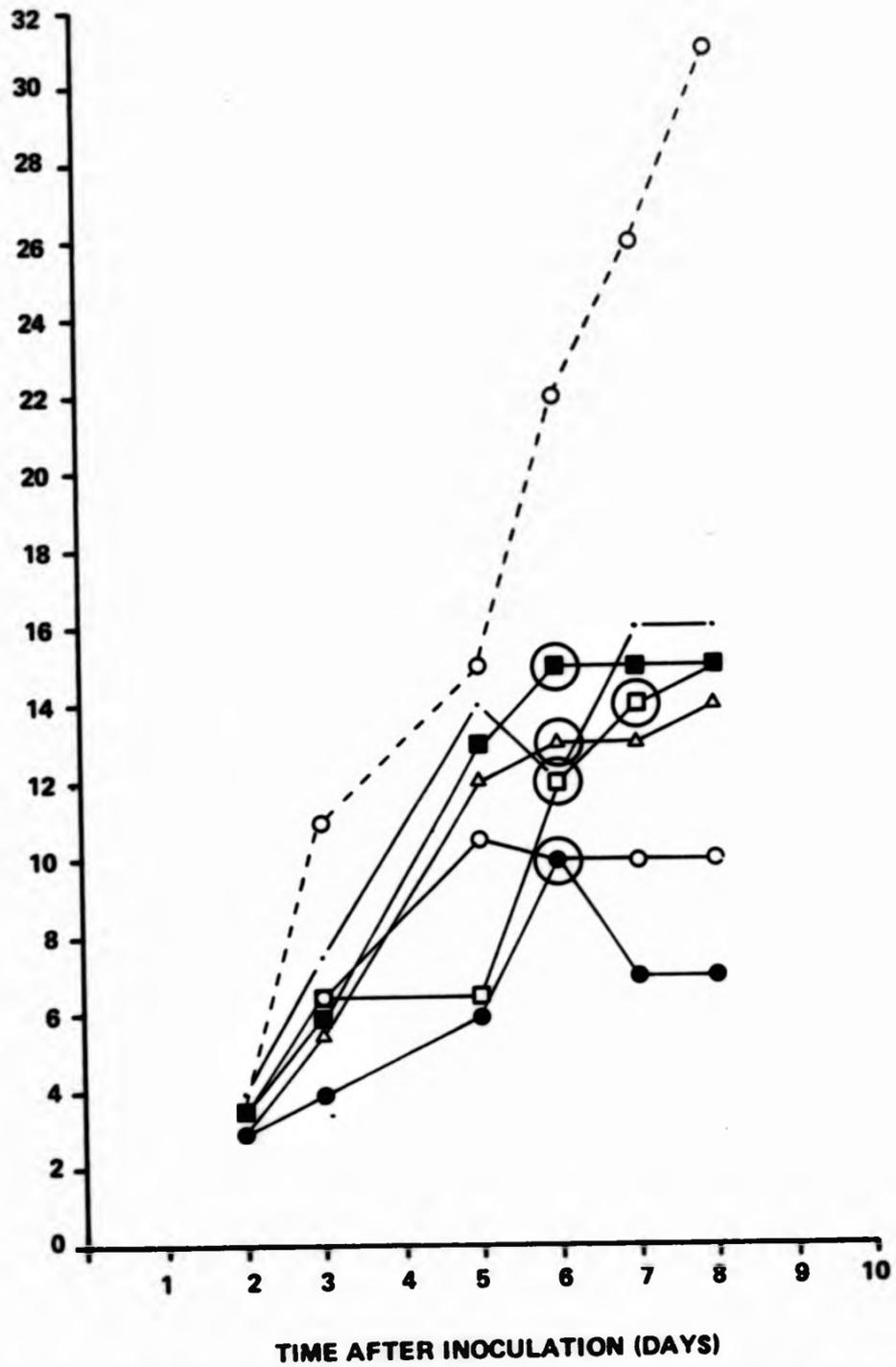
FIGURE 1.24   Growth reaction of M.perniciosa in the presence of a range of saprophytic fungi in dual
culture on Czapek Dox agar at 20°C. (Point of colony contact denoted by a circle)

●—●   Botrytis cinerea   Pers. ex Pers.
■—■   Chaetomium globosum   Kunze. ex Fries
○—○   Chaetomium olivaceum   Cooke & Ellis
□—□   Cladosporium herbarum   (Pers.) Link ex S.F. Gray
△—△   Geotrichum candidum   Link ex Pers.
—·—   Gliocladium roseum   Bainier
○-·-○   Control:-   M.perniciosa in the absence of other fungi

LINEAR
GROWTH
OF
M.PERNICIOSA
(mm)

TIME AFTER INOCULATION (DAYS)
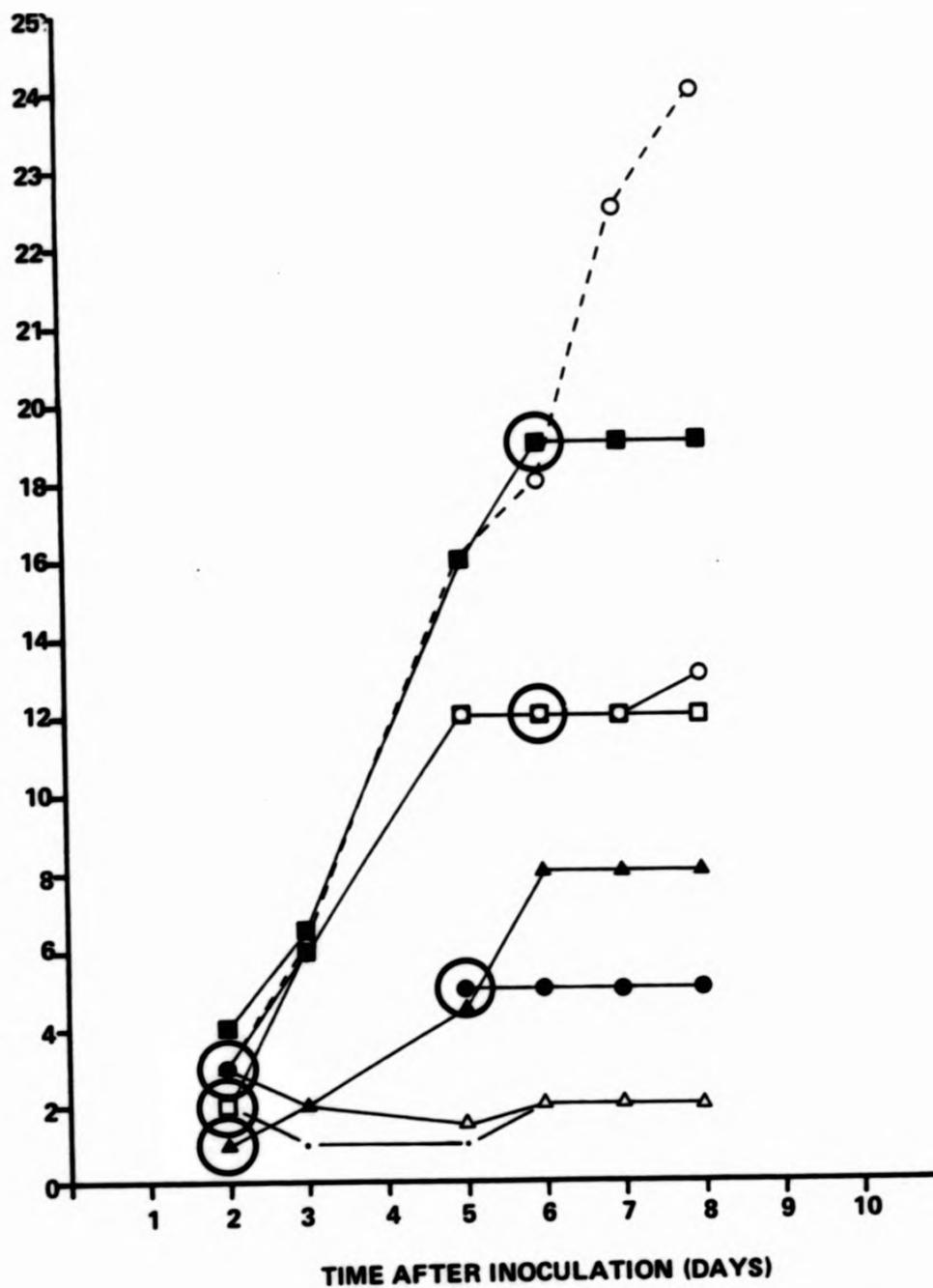
FIGURE 1.25   Growth reaction of M.perniciosa in the presence of a range of saprophytic fungi in dual
culture. on Czapek Dox agar at 20°C. (Point of colony contact denoted by a circle)

●—● Mucor hiemalis
■—■ Mycogone perniciosa
○—○ Penicillium expansum
□—□ Penicillium frequentans
△—△ Rhizopus tritici
—.— Trichoderma viride
▲—▲ Verticillium fungicola

○--○ Control:- M.perniciosa in the absence of other fungi

Table 1.11

The relationship between M.perniciosa and a range of fungi in vitro

Species	Relationship towards M.perniciosa
A.bisporus (Lange.)Sing. (A6)	host
A.bisporus (D621)	host
A.bisporus (S22)	host
A.bisporus (G.C.R.I. 431)	host
A.macrosporus (Møller & Schaeff.) Pilat (B)	host
A.bitorquis (Quel) Sacc. (W2)	host
A.bitorquis (W4)	host
A.bitorquis (K32)	host
A.silvicola (Vitt.) Peck	host
Coprinus sp.	potential host
Flammulina velutipes (Curt. ex Fr.). Karst.	host
Pleurotus ostreatus (Jacq. ex Fr.)Kummer (P11)	antagonist
Stropharia merdaria (Fr.) Quel	host
Schizophyllum commune Fr.	host
Volvariella volvacea (Bull. ex Fr.)Sing.	potential host
Botrytis cinerea Pers. ex Pers.	mutual antagonist
Chaetomium globosum Kunze. ex Fries.	mutual antagonist
Chaetomium olivaceum Cooke & Ellis	mutual antagonist
Cladosporium herbarum (Pers.)Link ex S.F. Gray	mutual antagonist
Geotrichum candidum Link ex Pers.	mutual antagonist
Gliocladium roseum Bainier	mutual antagonist
Mucor hiemalis Wehmer S.L.	antagonist
Penicillium expansum Link ex Thom.	mutual antagonist
Penicillium frequentens Westling	mutual antagonist
Rhizopus tritici Saito	antagonist
Trichoderma viride Pers. ex Fr.	antagonist
Verticillium fungicola	mutual antagonist
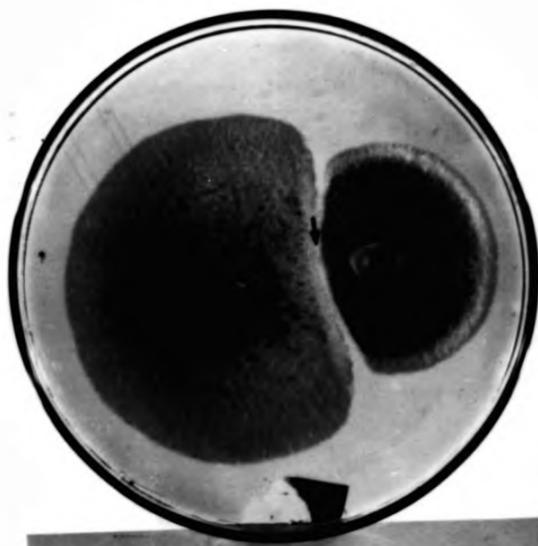
Chaetomium olivaceum vs M. perniciosa

PLATE 1.4.   Antagonism of M.perniciosa. In dual plate
cultures, zones between M.perniciosa and antagonistic
fungi were generally clear, (arrowed in this example),
with little or no contact between the two fungi.
Incubation was at 20^{0} for 9 days on Czapek Dox agar.

Mp = M.perniciosa

Co = Chaetomium olivaceum

Chaetomium olivaceum vs M. perniciosa

PLATE 1.4.   Antagonism of M.perniciosa. In dual plate
cultures, zones between M.perniciosa and antagonistic
fungi were generally clear, (arrowed in this example),
with little or no contact between the two fungi.
Incubation was at 20^{0} for 9 days on Czapek Dox agar.

Mp = M.perniciosa

Co = Chaetomium olivaceum

were subsequently incubated on clean glass slides in moist chambers at 18°C $\pm$ 2°C and examined periodically for growth of M.perniciosa.

In addition, fruit bodies of a range of fungi were inoculated in the field, subject to availability, with 20 µl droplets of an M.perniciosa conidial suspension, (c. 1.0 x 10^5 spores cm^{-3}) applied directly to the cap surface and observed periodically for evidence of growth and sporulation of the pathogen.

Members of the Agaricales, Boletales and Russulales (classified according to Hawksworth, Sutton and Ainsworth, (1983)), were seen to be potential hosts for the pathogen in the field. Infection of Agaricus campestris (L.ex Fr.), Agaricus macrosporus (Møller & Schaeff.) Pilat, Lepista nuda (Bull. ex Fr.) Cooke, Paxillus involutus (Fr.) Fr., Lactarius pubescens (Fr. ex Krombh.) Fr., Russula atropurpurea (Krombh.) Britz. and R.ochroleuca (Pers. ex Secr.) Fr. was noted with growth and sporulation of M.perniciosa occurring both in tissue blocks in the laboratory and on sporophores in the field, although Wet Bubble disease symptoms of cap distortion, typical of M.perniciosa infection of A.bisporus sporophores, were not observed. (Table 1.12).

Table 1.12

Alternative host species of fleshy fungi for M.perniciosa under laboratory
and field conditions.   M.Perniciosa conidial suspensions from a 1½ day old
malt agar culture were applied to sporophore tissues and examined at intervals
after incubation in (a) closed plastic boxes or (b) under ambient conditions in
the wild.

Classification, (Hawksworth, Sutton and Ainsworth, 1983). ORDER Family Species	(a) Growth of M.perniciosa on excised tissue blocks	(b) Infection of mature fruit bodies
AGARICALES		
Agaricaceae		
Agaricus campestris (L. ex Fr.)	+	+
A.macrosporus (Møller & Schaeff.) Pilat	+	+
Amanitaceae		
Amanita rubescens ((Pers.)Fr.)S.F.Gray	-	-
Coprinaceae		
Coprinus plicatus (Fr.)Fr.	-	-
Coprinus micaceus (Bull. ex Fr.) Fr.	-	-
Psathyrella lacrymabunda Syn.	-	-
Psathyrella candalleana (Fr.)Maire	+	+
Strophariaceae		
Hypholoma fasciculare (Hud S. ex Fr.) Kummer	-	-
Pholiota squarrosa (Muller ex Fr.) Kummer	-	-
Tricholomataceae		
Armillaria mellea (Vahl. ex Fr.) Kummer	-	-
Collybia peronata (Bolt. ex Fr.) Kummer	-	-
Mycena galericulata (Scop. ex Fr.) S.F.Gray	-	-
Mycena pura (Pers. ex Fr.) Kummer	-	-

Table 1.12 (Continued)

Classification, (Hawksworth, Sutton and Ainsworth, 1983).
 	
ORDER
 	
  Family
 	
    Species
	(a)
	
	Growth of M.perniciosa on excised tissue blocks
		(b)
		
		Infection of mature fruit bodies

Tricholomataceae (cont.)		
Lepista nuda (Bull. ex Fr.) Cooke	+	+
BOLETALES		
Boletaceae		
Boletus badius (Fr.) Kühn	−	−
B.edulis (Bull. ex Fr.)	−	−
B.subtomentosus L.ex Fr.	−	−
Paxillaceae		
Paxillus involutus (Fr.) Fr.	+	+
RUSSULALES		
Russulaceae		
Lactarius pubescens (Fr. ex Krombh)Fr.	+	+
Russula atropurpurea (Krombh.)Britz	+	+
R.mairei Sing.	−	−
R.nigricans (Bull.ex Merat)Fr.	−	−
R.ochroleuca (Pers. ex Secr.) Fr.	+	+
APHYLLORALES		
Polyporaceae		
Meripilus giganteus (Pers. ex Fr.)Karst	−	−
Pleurotus ostreatus (Jacq. ex Fr.)Kummer S.F. Gray	−	−

Table 1.12 (Continued)

Classification, (Hawksworth, Sutton and Ainsworth, 1983)
 	
ORDER
 	
  Family
 	
    Species
	(a)
	
	Growth of M.perniciosa on excised tissue blocks
		(b)
		
		Infection of mature fruit bodies

APHYLLORALES (cont.)
 	
  Thelephoraceae
 	
    Stereum hirsutum (Wide ex Fr.)
                   S.F. Gray
	-	-

GASTEROMYCETALES Lycoperdon perlatum Pers.	-	-

CHAPTER 2

I   STRUCTURAL FEATURES OF INFECTED TISSUE

This chapter describes work aimed at determining the nature of diseased tissue in infected beds under controlled conditions. The morphology of infected sporophores and of sclerodermoid tissue was examined, and host/parasite interactions investigated using light and electron microscopy.

The exact nature of antagonism at the host/parasite interface was examined.

1.   Gross morphology of sclerodermoid tissue

In the presence of M.perniciosa development of A.bisporus sporophores can be extensively disrupted, with infection of tissue resulting in the production of malformed sporophores and the appearance of sclerodermoid masses, (Smith, 1924, Vincent-Davies, 1972). In some cases apparently healthy sporophores may arise from sites closely surrounded by sclerodermoid tissue, (Plate 2.1). Some sclerodermoid tissue may possess features reminiscent of healthy sporophore tissue, such as cap, gill or stipe. In some cases complete but infected sporophores may arise from the surface of sclerodermoid tissue at a point clear of the casing surface, (Plate 2.2), indicating that each sclerodermoid mass is not the result of unchecked growth of a single undifferentiated initial but is the result of the combining of a number of such initials. That this is the case is further suggested by the fact that undifferentiated sclerodermoid tissue appears to be made up of numerous joined tissue spheres'.

PLATE 2.1. A normal <u>A.bisporus</u> sporophore (strain 649) arising from cropping beds heavily infected with <u>M.perniciosa</u> in the close vicinity of sclerodermoid tissue, at 16°C, 38 days after casing.

PLATE 2.1.   A normal A.bisporus sporophore (strain 649)
arising from cropping beds heavily infected with M.perniciosa
in the close vicinity of sclerodermoid tissue, at 16°C,
38 days after casing.

PLATE 2.2.   Development of A.bisporus tissue (strain A6)
in healthy (h) and infected (i) cropping 'beds'. Note
the development of complete but infected sporophore tissue
clear of the casing layer in some instances, (arrowed).
Cultivation was using standard commercial compost and
casing at commercial temperatures (Vedder 1978).
Pots were infected with M.perniciosa (2.4 x 10^2 spores cm^{-1})
at the time of casing.

PLATE 2.2.   Development of A.bisporus tissue (strain A6)
in healthy (h) and infected (i) cropping 'beds'.   Note
the development of complete but infected sporophore tissue
clear of the casing layer in some instances, (arrowed).
Cultivation was using standard commercial compost and
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Pots were infected with M.perniciosa (2.4 x 10^2 spores cm^{-1})
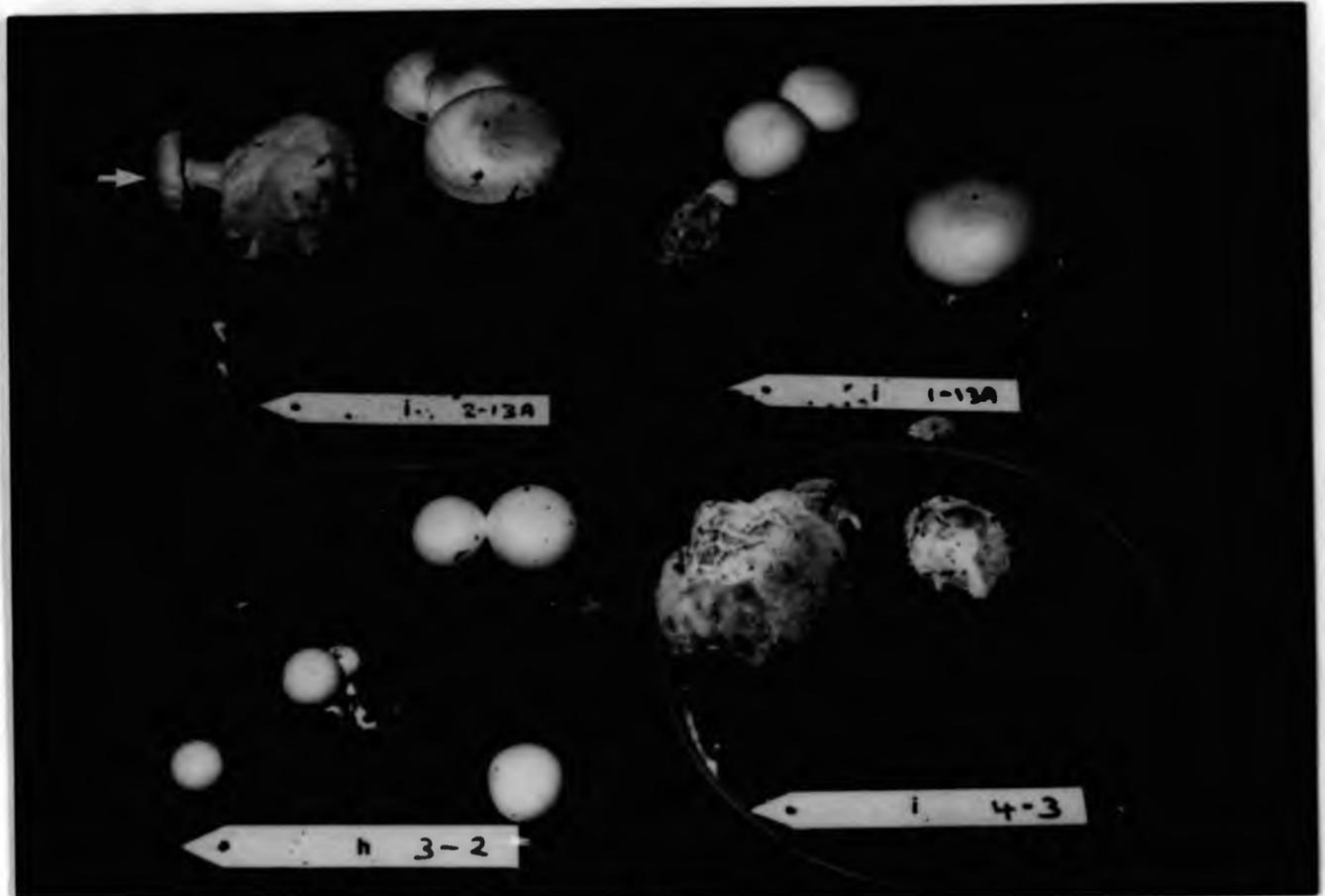at the time of casing.

PLATE 2.2. Development of <u>A.bisporus</u> tissue (strain A6)
in healthy (h) and infected (i) cropping 'beds'. Note
the development of complete but infected sporophore tissue
clear of the casing layer in some instances, (arrowed).
Cultivation was using standard commercial compost and
casing at commercial temperatures (Vedder 1978).
Pots were infected with <u>M.perniciosa</u> (2.4×10^2 spores cm^{-1})
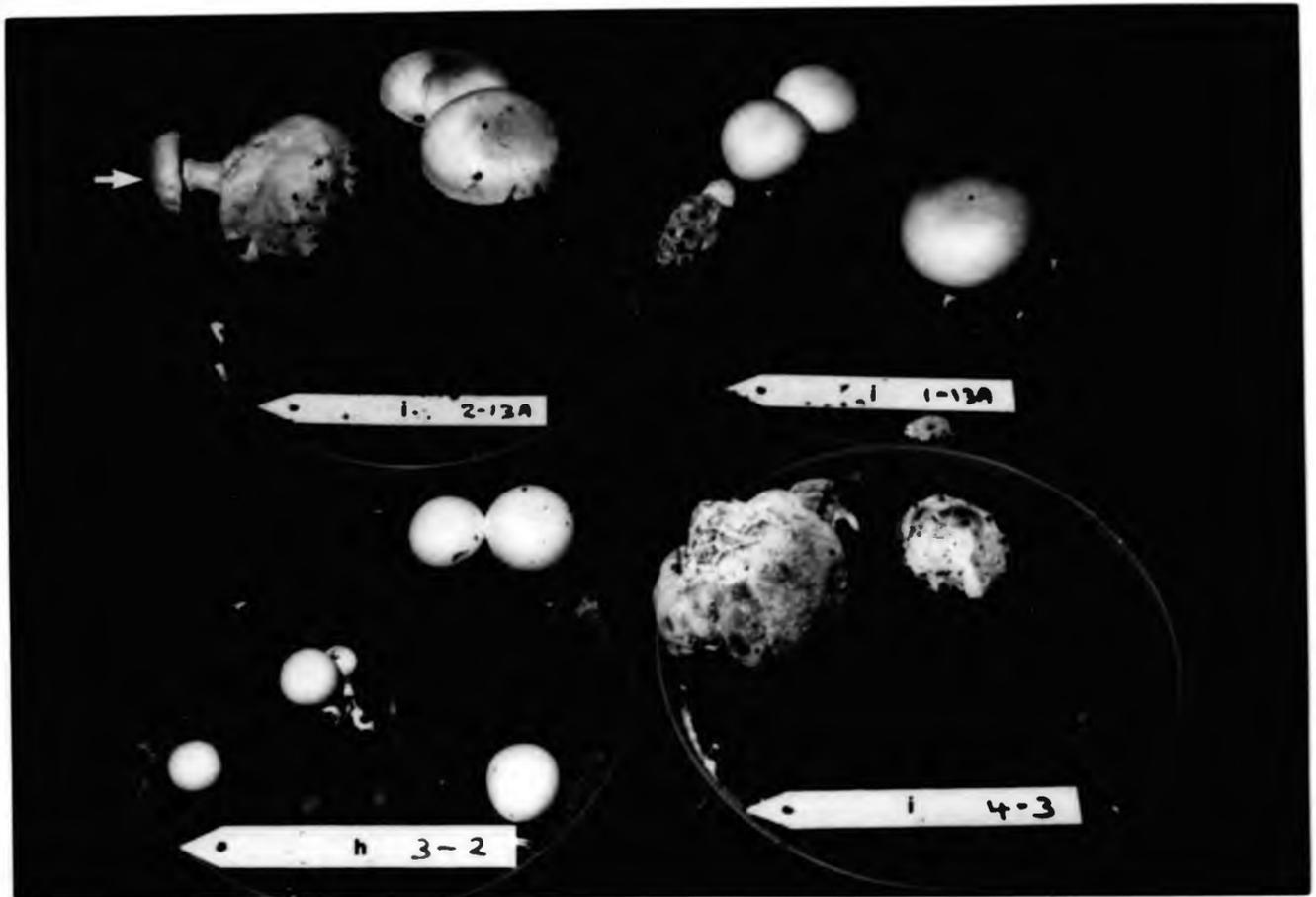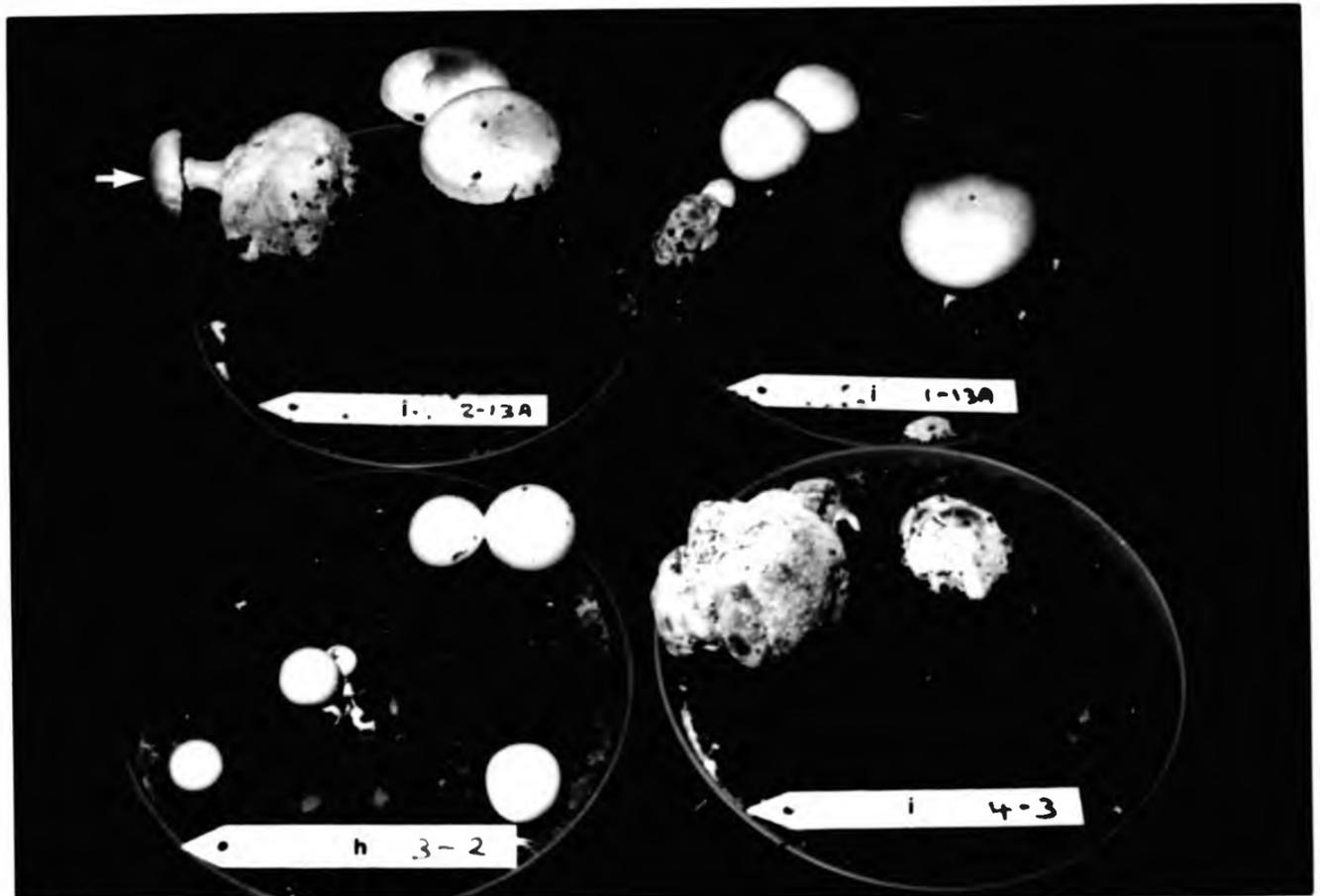at the time of casing.

Sclerodermoid tissue, harvested by cutting level with the casing layer when the tissue was seen to have ceased expanding, coinciding with browning of the tissue due to the presence of M.perniciosa chlamydospores, was assessed for the number of 'tissue spheres' assumed to represent one A.bisporus initial each. The validity of this assumption was tested by the determination of dry weights of such tissue and calculating dry weight values per assumed initial. Data values of dry weight per initial for sclerodermoid tissue showed a lower statistical variability (SEM = 17.6%) than for healthy 'cup stage' sporophores, (SEM = 18.6%) indicating that the surface features of sclerodermoid tissue give a consistent representation of the number of initials developing on infected beds.

On the basis of visual assessment of the number of initials developing in heavily infected and healthy beds, it was determined that the number of A.bisporus initials that develop is not significantly affected by either the presence or absence of M.perniciosa.

The ratio of fresh weight : dry weight was determined for healthy and infected 'cup stage' sporophores and for mature sclerodermoid tissue. Ratios were found not to be significantly different, (15.0:1, 18.0:1 and 16.9:1 respectively). Conversion of fresh weights to dry weights on the basis of these ratios indicated that the total dry weight biomass produced in either healthy or infected beds is not significantly different.

Infected tissue was structurally variable, with rheological

strength, determined using a hand-held penetrometer, of between 0.0
and 53.8 g mm^{-2}, (mean 16.7 $\pm$ 2.4 g mm^{-2}). Internally, tissue was
mainly cream in colour with a spongy texture, interspersed with areas
of dark brown or rust colour which were often wet in appearance,
(Plate 2.3).

2. The occurrence of bacteria in sclerodermoid tissue

Routine isolations from sclerodermoid tissue under aseptic
conditions on to Malt agar, Nutrient agar and King's B agar, commonly
yielded not only M.perniciosa but also a number of species of bacteria.
The occurrence of bacteria within apparently entire tissue was
unexpected and isolations from healthy sporophore caps under identical
conditions did not yield bacteria.

A number of possible roles of bacteria within infected tissue were
considered.

i   Bacteria may be antagonistic towards A.bisporus in their own right
but play no part in the development of Wet Bubble disease symptoms per
se.

ii   Bacteria may be antagonistic towards M.perniciosa, playing no
direct part in the development of disease symptoms.

iii   Bacteria may be antagonistic towards A.bisporus and may act in
association with M.perniciosa in the development of disease symptoms,
implying that Wet Bubble disease may in fact be of complex etiology.

iv   Bacteria may be saprophytic and may take no direct part in
disease development.

In order to determine the nature of the relationship between
bacteria and constitutive fungi in sclerodermoid tissue, a number of

PLATE 2.3.   Internal cross section of typical mature
sclerodermoid tissue produced on commercial mushroom beds.
Note the spongy appearance of the tissue which is interspersed
with areas which are dark brown or rust in colour.   These
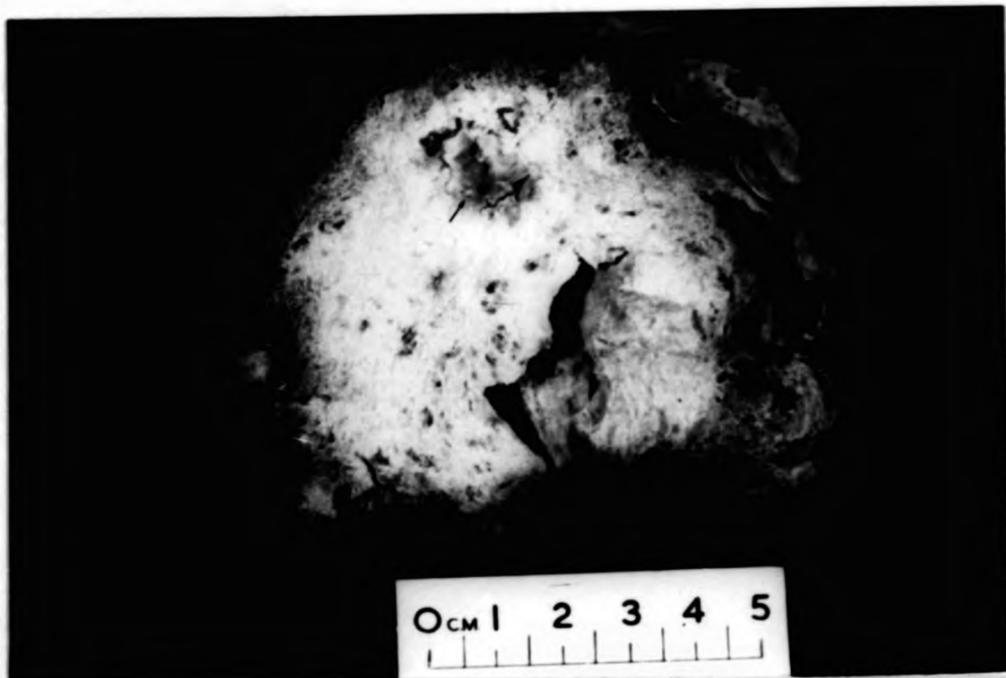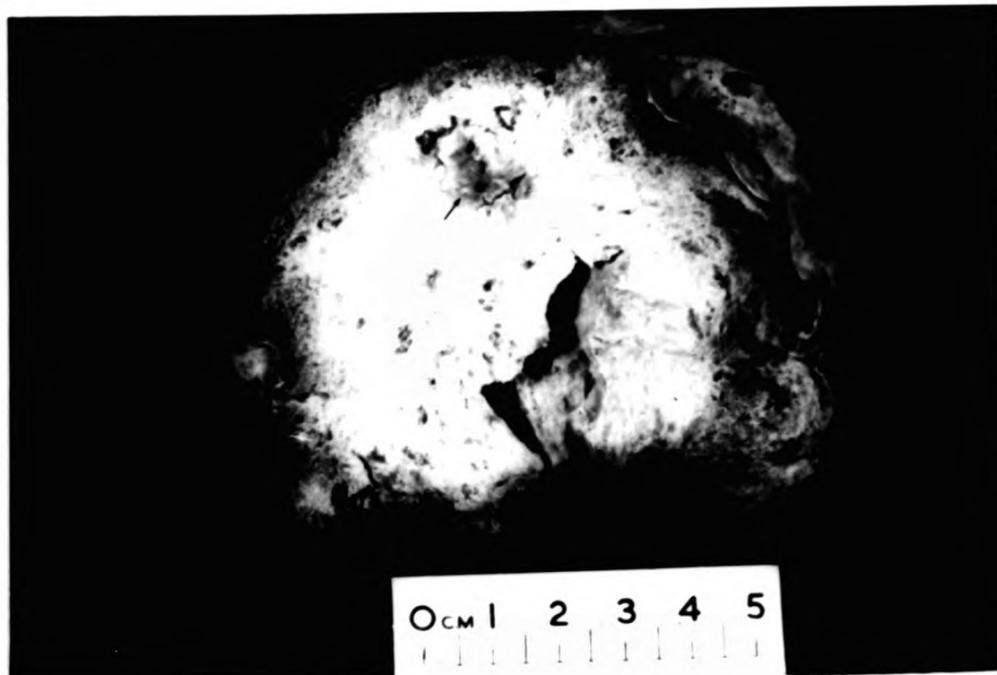areas may be wet in appearance.(→).

PLATE 2.3.   Internal cross section of typical mature
sclerodermoid tissue produced on commercial mushroom beds.
Note the spongy appearance of the tissue which is interspersed
with areas which are dark brown or rust in colour.   These
areas may be wet in appearance,(→).

characteristics of the bacterial isolates and the tissue from which
they were isolated were considered.

The percentage of sclerodermoid masses infected with bacteria was
less than 100 (71%), with 18% having more than one type of bacteria
present. This indicates that bacterial infection is not a pre-
requisite for the development of the disease, but the possibility that
the full range of symptoms previously described are expressed only in
the presence of bacteria, cannot be discounted at this stage.

The strength of sclerodermoid tissue was measured using a hand-
held penetrometer and the occurrence of bacteria determined by plating
out tissue samples on to Nutrient agar under sterile conditions.

It was observed that bacteria were typically isolated from 'weak'
tissue (mean strength 15.8 g mm^{-2}) compared to that from which bacteria
were not isolated, (mean strength 30.8 g mm^{-2}).

Strength of tissue and the presence or absence of bacteria
appeared to be inter-dependent, (x^2 = 39.9, p <0.01), with more
bacteria occurring in 'weak' tissue than predicted using an assumption
of independence between tissue strength and the occurrence of bacteria,
(Figure 2.1).

Bacterial isolates were tested for antagonism towards A.bisporus
using the tissue block technique. Bacterial suspensions in SDW from
24 h old Nutrient agar cultures were applied to tissue blocks of
healthy sporophore cap tissue, (Strain Sinden A6), (c. 1 cm x 1 cm x
0.5 cm), excised under sterile conditions and incubated in moist chambers
for 3 days at 20°C $\pm$ 2°C. Some bacterial isolates from sclerodermoid

Percentage of sclerodermoid masses

Rheological strength of sclerodermoid tissue (grams mm^{-2})

The percentage of sclerodermoid tissues containing bacteria □ and those with none in different rheological strength classes
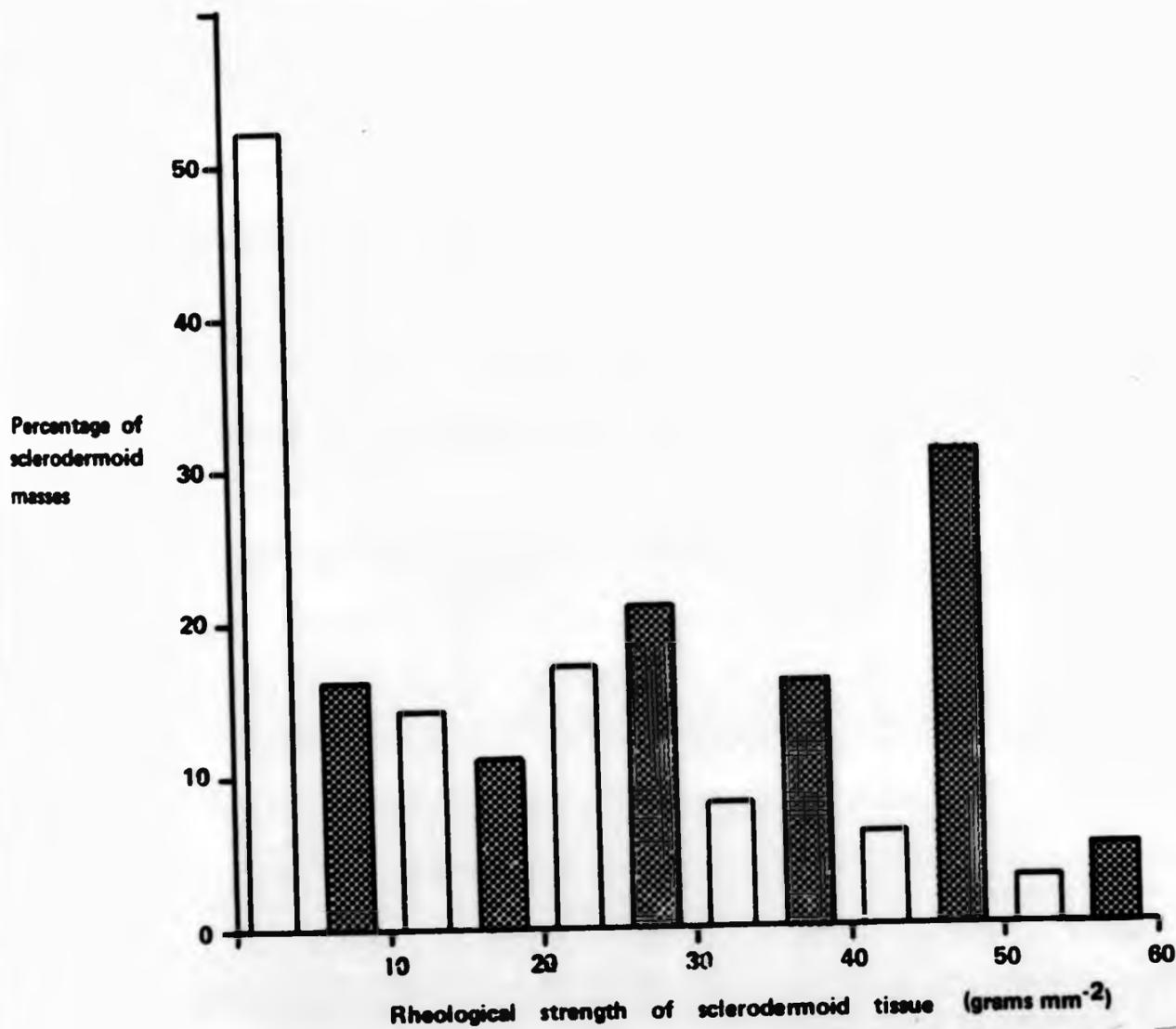
FIGURE 2.1 Histogram showing the frequency of occurrence of sclerodermoid tissue, containing bacteria (□) and those without (▨) of different rheological strengths. Samples (c. 2mm^3) were excised under axenic conditions and streaked on to nutrient agar and incubated at 20°C for 5 days in order to determine the presence or absence of bacteria.

tissue were capable of causing browning of healthy A.bisporus tissue, (37.5%), although the distribution of such bacteria could not be related to the strength of the tissue from which they were isolated. This suggests therefore that the role of bacteria is related to factors other than the ability to antagonise A.bisporus within sclerodermoid tissue.

In order to determine whether bacteria occurring in sclerodermoid tissue were antagonistic towards M.perniciosa bacterial isolates were each streaked on to Czapek Dox agar and the plates immediately reinoculated with M.perniciosa prior to incubation at 23°C $\pm$ 1°C for 10 days. Some isolates reduced growth of the fungus, relative to controls grown under identical conditions in the absence of bacteria. It wasobserved that those isolates antagonising M.perniciosa in vitro were predominantly isolated from weaker tissue, (<35 grams mm^{-2}). Bacterial isolates not antagonistic towards M.perniciosa were evenly distributed over the entire strength range of sclerodermoid tissue. (between 0 and 60 grams mm^{-2}), (Figure 2.2).

Within sclerodermoid tissue specific areas of wet/dark tissue occur in many instances. Specific isolations from such areas commonly yielded bacteria antagonistic towards M.perniciosa and not to A.bisporus, (71.4%).

Overall, it is apparent that some bacteria may be associated with disease symptoms, although the relationship between bacteria and host and pathogen fungi remains unclear.

The influence of the mushroom bed microflora on disease development in vivo has not previously been considered.

FIGURE 2.2 Strength — Frequency histograms of M.perniciosa sclerodermoid masses containing bacteria isolated from a range of commercial cropping beds 21-60 days after casing

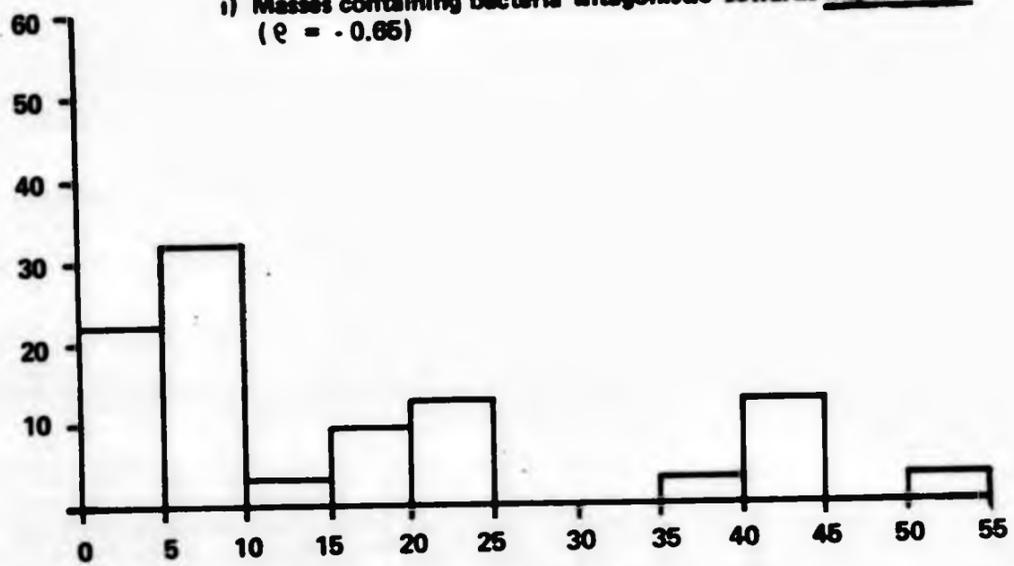Routine isolations both from commercial casing material and from infected tissue yielded bacteria which in some instances were antagonistic towards M.perniciosa in vitro. The effect of such bacteria, and of those from similar sources that did not affect growth of the pathogen, on disease development was investigated.

Axenic cultures of A.bisporus, produced in 3.5ℓ flasks using a modification of the method of Long and Jacobs, (1974), (Materials and Methods I7) enabled investigation to be carried out on the ability of M.perniciosa to infect sporophore tissue in the absence of the microflora commonly found in commercial casing soil and the role of bacteria in the development of Wet Bubble disease.

Following colonisation of sterile compost by A.bisporus cultures were cased with sterile activated charcoal and infected with a conidial suspension of M.perniciosa (c. 1×10^5 propagules g^{-1} of casing), at the time of casing. Cultures were then inoculated with an SDW suspension of bacteria (c. 1×10^6 g^{-1} of casing) from a 24 h old nutrient broth culture. Baterial isolates were either those antagonistic towards M.perniciosa in vitro, or those showing no effect in dual culture tests. Control flasks were either inoculated with M.perniciosa alone or uninoculated healthy axenic A.bisporus cultures. All control and inoculated flasks received the same volume of SDW.

In axenic A.bisporus cultures infected with M.perniciosa in the presence of bacteria antagonistic towards the pathogen, the time between casing and development of initials was significantly greater than that for cultures infected with M.perniciosa in the presence of non-antagonistic bacteria, (62.2 days after casing $\pm$ 17.3 days and

30.0 days $\pm$ 1.8 days respectively, p <0.05).

The time between casing and development of initials in cultures infected with M.perniciosa only was 24.4 days $\pm$ 2.5 days, (Table 2.1).

Cultures infected with M.perniciosa alone resulted in the development of featureless sclerodermoid masses which exuded liquid, both brown and clear, and produced the smell commonly associated with Wet Bubble disease in commercial beds.

Flasks infected with M.perniciosa in the presence of antagonistic bacteria produced no healthy sporophores, but 4.5% of sclerodermoid initials exhibited some features of normal sporophore tissue, (i.e. cap stipe or gill).

It is apparent from this work, therefore, that bacteria antagonistic towards M.perniciosa may delay the development of disease symptoms on infected beds, but that the role of bacteria in disease development per se is secondary. M.perniciosa can produce the extreme symptoms of Wet bubble disease of A.bisporus under axenic conditions, in the absence of naturally occurring micro-organisms, and as such the disease is unquestionably of simple etiology. The presence of bacteria antagonistic towards M.perniciosa in the casing layer may modify the development of symptoms by direct antagonism of the pathogen prior to infection. Any resultant slowing of the growth rate of M.perniciosa, as exhibited in vitro, may enable the host to differentiate cap, stipe or gill tissue.

The possibility that bacteria may antagonise M.perniciosa to such a

Table 2.1

The effect of infection of axenic A.bisporus cultures with M.perniciosa and

bacteria on the days from casing to emergence of initials

* indicates significant differences (p <0.05)

Treatment	Days from casing to initial emergence					Mean $\pm$ SEM
	Replicate number					
	1	2	3	4	5	
Healthy culture	34	40	66	128	128	79.2 $\pm$ 20.6
Infected with M.perniciosa	21	34	21	25	21	24.4 $\pm$ 2.5
Infected with M.perniciosa and antagonistic bacteria	26	39	39	93	114	62.2 $\pm$ 17.3
Infected with M.perniciosa and non antagonistic bacteria	26	26	30	34	34	30 $\pm$ 1.8
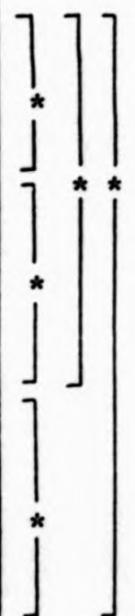
degree that disease development could be significantly reduced on a commercial scale is discussed elsewhere, (Chapter 4).

II THE FINE STRUCTURE OF INFECTED SPOROPHORE AND SCLERODERMOID TISSUE

The structure of infected tissue was examined in order to determine the relationship between M.perniciosa and A.bisporus in vivo. Previous descriptions have been restricted to light microscope studies of tissue, (Smith, 1924) and electron microscope studies of enzymic degradation of host and pathogen cell walls, (Vincent-Davies, 1972).

Smith, (1924), described the infection of A.bisporus sporophores occurring with penetration of the tissue by M.perniciosa between the host cells. Subsequent growth was seen to be both inter and intra-cellular. Most of the cellular disruption was considered to be caused by enzymic degradation, although this was not defined.

Vincent-Davies, (1972), investigated the role of enzymes in the development of symptoms. He demonstrated the production of a range of enzymes by M.perniciosa capable of degrading A.bisporus cell walls. The role of enzymes in disease development was not fully determined and it was suggested that enzymic action could not satisfactorily account for the full range of 'Wet Bubble disease' symptoms observed.

1. Selection of methods

The relationship between M.perniciosa and A.bisporus has been examined here both with light and electron microscopy.

Initially, experiments were carried out to select fixation and

staining methods most suitable for the determination of spatial arrangements of the two fungi and of their ultrastructure.

The use of fresh sectioned and 'squash preparation' material was found to be unsatisfactory. The use of such techniques did not clearly differentiate between the host fungus and the pathogen. Of those tested, (Materials and Methods III 3A), 0.06% Neutral red gave the best differentiation, the comparatively fine pathogen hyphae taking up the stain to a greater extent than the host.

Accumulation of the stain by the pathogen indicates that such cells are living, (Ikeduigwu and Webster, 1970a). Reduced uptake of the stain by A.bisporus is indicative of cell death although it is likely that the difference in cytoplasmic density between host and pathogen hyphae would commonly result in the fine dense pathogen hyphae taking up stain to an apparently greater extent than the host which tends to have more diffuse and extensively vacuolated hyphae, (Plate 2.4).

In order more closely to examine the relationship, by light microscopy, between the two fungi, tissue was embedded in methacrylate resin following fixation in 2% glutaraldehyde. Sections were either stained in 1% toluidine blue in 5% boric acid or with periodic acid - Schiff's reagent; both techniques gave good staining. Periodic acid-Schiff's reagent clearly showed the cell walls of the two fungi, although interactions between the two fungi were not as clear as in samples examined using the scanning and transmission electron microscopes.

Material was prepared for scanning electron microscopy, (SEM), by

i)

ii)

PLATE 2.4.   Light micrographs of A.bisporus (Ab) gill
tissue at veil break, 7 days after infection with
M.perniciosa (Mp), and incubation at 16°C in moist chambers.

i) Staining is of fresh material with 0.06% neutral red.
Accumulation of stain is in the pathogen hyphae.

ii) Staining of embedded transverse sections is with 1%
toluidine blue in 5% boric acid.

Bar represents 50μm.

i)

ii)

PLATE 2.4.   Light micrographs of A.bisporus (Ab) gill
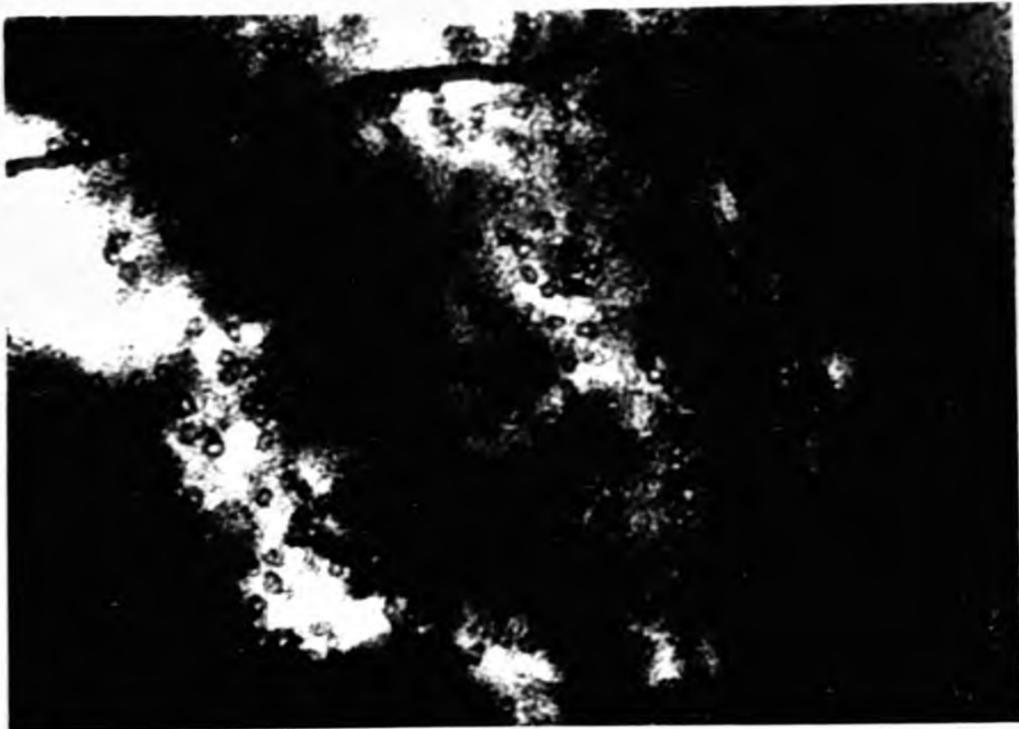tissue at veil break, 7 days after infection with
M.perniciosa (Mp), and incubation at 16°C in moist chambers.

i) Staining is of fresh material with 0.06% neutral red.
Accumulation of stain is in the pathogen hyphae.

ii) Staining of embedded transverse sections is with 1%
toluidine blue in 5% boric acid.

Bar represents 50µm.

fixing tissue in either 2% glutaraldehyde with post-fixing in 1% osmium tetroxide, osmium tetroxide vapour or osmium tetroxide vapour with post-fixing in 0.05% thiocarbohydrazide. In addition, unfixed material was examined by rapid freezing of tissue in liquid nitrogen immediately prior to examination. Fixed material was dehydrated through a graded ethanol series and substituted with amyl acetate. Samples were critical point dried prior to coating with gold and subsequent examination.

While all preparation regimes gave good tissue preservation, minimum distortion was caused by fixing material with osmium tetroxide vapour and post-fixing with thiocarbohydrazide. (Hayat, 1978).

High resolution investigations of healthy and infected tissue were made using the transmission electron microscope, (T.E.M.). Tissue was prepared by fixation in 1% potassium permanganate (Manocha, 1965) or in 2% glutaraldehyde with post-fixing in 1% osmium tetroxide (Sabatini, et al 1963). Samples were dehydrated in a graded ethanol series and substituted with propylene oxide and resin.

Samples fixed using potassium permanganate were granular in appearance when examined using the T.E.M. Samples fixed with glutaraldehyde / osmium tetroxide and stained with uranyl acetate / lead citrate showed improved resolution and this technique was adopted for all T.E.M. work.

2. Host cell morphology in infected tissue

Host cells were affected by M.perniciosa in two distinct phases of sporophore development. Infection may occur at an early stage in

development, in which case, cellular disruption was extensive. Where
infection occurred late in sporophore development, the effect of the
pathogen was less marked. Where cellular differentiation was complete,
as was the case with fully developed sporophores, the effects of
infection were largely that of altering individual host cells either by
disrupting cytoplasmic organisation or by rupturing the cells. Where
infection of developing fruit bodies occurred, the effect was primarily
in altering host cytoplasm and differentiation. Total cellular
disruption in such cases was rare.

A. A.bisporus initials

Observation of infected and healthy initials using the T.E.M.
showed an alteration in host cells in the presence of the pathogen.
While all cells in healthy tissue appeared intact, a small proportion
in infected tissue appeared dead due to the total lack of cytoplasm or
to the breakdown in the plasmalemma. Cell wall degradation was not
observed in host tissue, (Table 2.2). Host cells were extensively
vacuolated in the presence of the pathogen, (52.7% of cells showing
greater than 50% vacuolation across sections measured under the T.E.M.).
In contrast, healthy A.bisporus cells in initials of a comparative size
showed only 18.0% of cells with in excess of 50% vacuolation. Numbers
of mitochondria, fat droplets and Woronin bodies within host cells were
not significantly different in healthy and infected A.bisporus cells
and as such, the overall metabolic activity appears similar in healthy
and infected initials. The primary effect of infections is, therefore,
on the vacuolation of host cells within developing sporophore initials.

Table 2.2

Ultrastructure of intact A.bisporus cells in healthy tissue and tissue artifically inoculated with M.perniciosa at various stages of development.
(Mean values ± SEM of 200 cell sections from each of four samples).

Tissue type	% cells showing 50%+ vacuolation across T.E.M. sections	% cells containing Woronin bodies / fat droplets	Mitochondria per cell section.	% cells showing cytoplasmic abnormality (granulation and increased membrane development).
Healthy sporophore:				
Stipe	13.3 ± 3.0	2.7 ± 1.7	1.0 ± 0.0	2.0 ± 1.2
Cap	2.3 ± 0.3	61.0 ± 2.7	1.0 ± 0.0	1.0 ± 1.0
Gill (trama)	50.7 ± 4.3	55.0 ± 4.0	5.0 ± 0.0	5.3 ± 1.2
Initial (2-5 mm)	18.0 ± 9.8	7.7 ± 5.0	1.3 ± 0.3	1.7 ± 0.7
Infected sporophore:				
Stipe	16.7 ± 7.7	5.7 ± 2.8	3.7 ± 0.3	4.3 ± 1.8
Cap	37.7 ± 1.5	51.7 ± 0.9	5.0 ± 0.0	8.7 ± 0.3
Gill (trama)	15.0 ± 1.7	6.0 ± 0.0	1.0 ± 0.0	5.2 ± 0.7
Sclerodermoid mass:				
Mature (interior)	98.3 ± 0.3	0.0 ± 0.0	1.0 ± 0.0	44.7 ± 2.0
Mature (surface)	100.0 ± 0.0	10.3 ± 5.4	1.0 ± 0.0	52.3 ± 3.0
Initial (2-5 mm)	52.7 ± 16.4	3.0 ± 0.6	3.0 ± 1.0	28.3 ± 9.5

Table 2.2 (continued)

Tissue type	% cells showing wall degradation	% cells with discontinuous plasmalemma	% cells without cytoplasm
Healthy sporophore:			
Stipe	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cap	0.0 ± 0.0	0.0 ± 0.0	10.2 ± 2.5
Gill (trama)	0.0 ± 0.0	5.3 ± 0.9	7.9 ± 2.3
Initial (2-5 mm)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Infected sporophore:			
Stipe	0.0 ± 0.0	0.0 ± 0.0	54.8 ± 1.1
Cap	7.1 ± 3.6	5.7 ± 0.3	21.1 ± 4.2
Gill (trama)	12.0 ± 1.9	5.3 ± 0.9	32.1 ± 6.9
Sclerodermoid mass:			
Mature (interior)	39.6 ± 1.1	9.7 ± 0.3	48.7 ± 2.8
Mature (surface)	61.8 ± 8.4	43.3 ± 2.6	46.3 ± 3.2
Initial (2-5 mm)	0.0 ± 0.0	0.3 ± 0.3	1.3 ± 0.3

in agreement with the previously noted rapid rate of expansion of
infected tissue on cropping beds. In addition infected initials
show a high incidence of cytoplasmic abnormality, particularly in
granulation and membrane development (Table 2.2). Early infection
of developing initials results in the development of undifferentiated
cells, where cell types cannot be identified.

B. Mature sclerodermoid tissue

In order to define clearly the physical nature of sclerodermoid
tissue, embedded material was sectioned (5 μmsections) and observed
under the light microscope following staining in 1% toluidine blue.
One area of sclerodermoid tissue was selected and drawn on to acetate
sheets using a camera lucida attachment, (Leitz). Diagrams were
compiled to give a three dimensional image of the types of cells
present in infected tissue and the spatial relationship between host
and pathogen, (Figure 2.3).

A.bisporus cells within mature sclerodermoid tissue do not appear
directly to resemble any tissue type found in normal sporophores.
Sclerodermoid tissue was found to be made up of predominantly
A.bisporus cells, (Table 2.3), (A.bisporus : M.perniciosa = 4.05:1 by
volume). Air or water space accounting for 46.9% of the volume of
fully expanded tissue, in comparison to healthy A.bisporus cap tissue
consisting exclusively of A.bisporus cells with c. 10% of air or water
space. A.bisporus hyphae had no apparent orientation within scleroder-
moid tissue and were made up of a range of cell types, from apparently
normal cells through to rounded hypertrophic cells, (range 2.6μ - 22.0μ,

FIGURE 2.3

Juxtaposition of A.bisporus and M.perniciosa hyphae in mature sclerodermoid tissue.

Note the presence of dolipore septa (X) in A.bisporus hyphae (A), M.perniciosa (M)

having no septal pore apparatus visible under the light microscope.

M.perniciosa hyphae were typically constricted at points of penetration or emergence

through A.bisporus cells, (Y).

M.perniciosa chlamydospores were also present within infected tissue, (C).

Table 2.3

Relative distribution of fungal components in fully expanded sclerodermoid tissue

Sclerodermoid component	Mean % volume ± SEM
A.bisporus	39.99 ± 3.76
M.perniciosa	13.10 ± 0.51
Air / Water	46.91 ± 2.79

mean diameter 7.9 $\pm$ 1.0µ); (Plate 2.5). The extensive hypertrophy of individual cells together with the high percentage of air / water spaces resulting in the spongy nature of the tissue.

Examination of host cells in sclerodermoid tissue revealed an increase in cell death compared to immature tissue, (Table 2.2). In some instances the plasmalemma appeared broken or poorly defined under the T.E.M. More commonly, cells appeared to lack cytoplasm altogether, possibly reflecting a breakdown of the cell wall or plasmalemma at a specific point remote from individual cross sections, (Table 2.2). In contrast to infected and healthy initials, where the level of metabolism, indicated by the numbers of mitochondria per cell profile, appears similar, examination of host cells in mature sclerodermoid tissue revealed an alteration in host metabolism compared to healthy cells. Cells exhibited an increase in granulation, vacuolation and inclusion of membranes within the cytoplasm when compared to those healthy 'button stage' sporophores, (Plate 2.6). Of those occurring at the surface of sclerodermoid tissue, 52.3% were affected, with 44.7% showing cytoplasmic abnormality within the tissue.

C. Infected sporophore tissue

It was noted that where sporophore development and differentiation has occurred, the effect of infection by M.perniciosa on host cells is less marked than in developing initials.

Healthy sporophores, (button stage), were inoculated with a conidial suspension in SDW either to the stipe base or directly to the cap surface. Samples of stipe, cap and gill tissue were prepared for examination using the TEM after 3 days incubation in moist chambers.

PLATE 2.5.   Scanning electronmicrograph of an internal
area of mature sclerodermoid tissue.   A.bisporus (Ab)
hyphae appear to consist of apparently normal cells and
hypertrophic cells, (X).   Also present are M.perniciosa
hyphae shown here with developing conidiophores, (arrowed).
Note the high proportion of air/water space which appears
to contribute to the spongy nature of the tissue.

Fixation was in osmium tetroxide vapour with post fixing
in thiocarbohydrazide.

Bar represents 10 μm.

PLATE 2.5.   Scanning electronmicrograph of an internal
area of mature sclerodermoid tissue.   A.bisporus (Ab)
hyphae appear to consist of apparently normal cells and
hypertrophic cells, (X).   Also present are M.perniciosa
hyphae shown here with developing conidiophores, (arrowed).
Note the high proportion of air/water space which appears
to contribute to the spongy nature of the tissue.

Fixation was in osmium tetroxide vapour with post fixing
in thiocarbohydrazide.

Bar represents 10 μm.

PLATE 2.6.   Transmission electronmicrographs of <u>A.bisporus</u> cells in
healthy and infected tissue at various stages of
development.   Cultivation was at 16°C post initiation.

i)   Healthy 'button stage' cap tissue showing normal
cell development.
Staining was with uranyl acetate/lead citrate.

ii)   Sclerodermoid tissue cells showing increased production
of membranous material, (m), 4 days after emergence
under commercial conditions.   (Vedder, 1978).
Staining was with uranyl acetate/lead citrate.

iii)   Sclerodermoid tissue cells showing extensive
vacuolation, (V), 10 days after emergence under
commercial conditions, (Vedder, 1978).
Staining was in silver proteinate. ?

Bars = 1μm

i)

ii)

PLATE 2.6.   Transmission electronmicrographs of <u>A.bisporus</u> cells in
healthy and infected tissue at various stages of
development.   Cultivation was at 16°C post initiation.

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Staining was with uranyl acetate/lead citrate.

iii) Sclerodermoid tissue cells showing extensive
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commercial conditions, (Vedder, 1978).
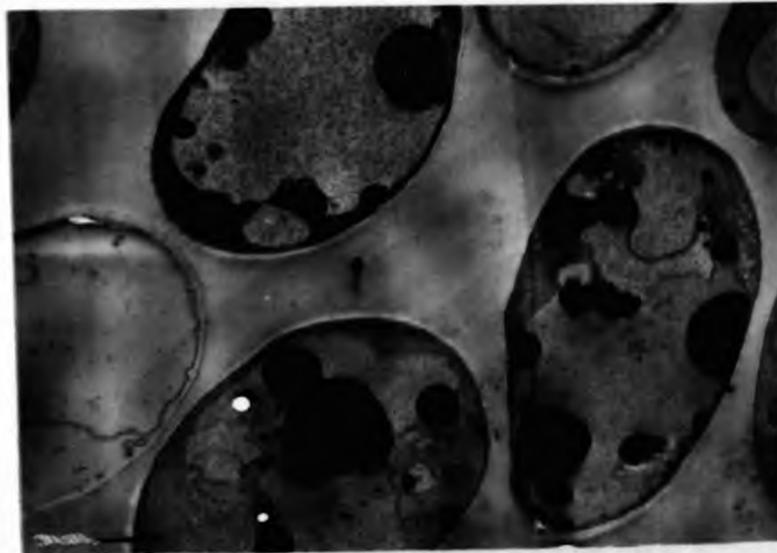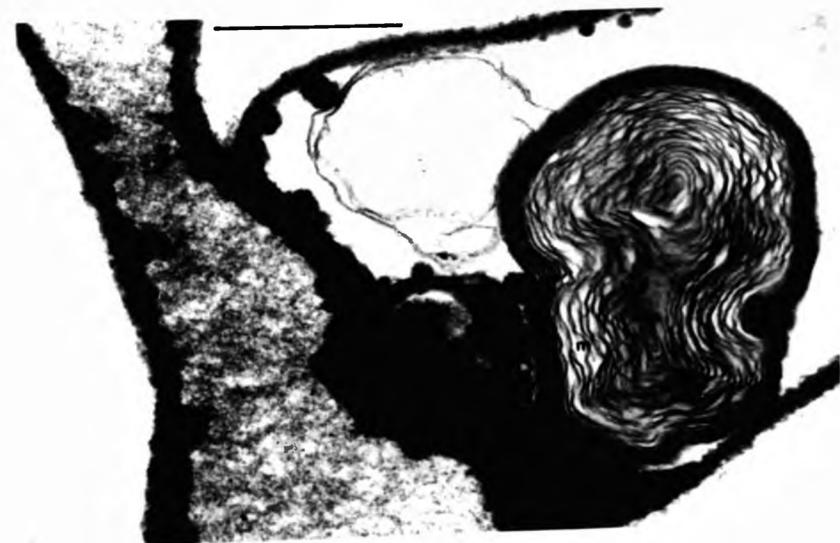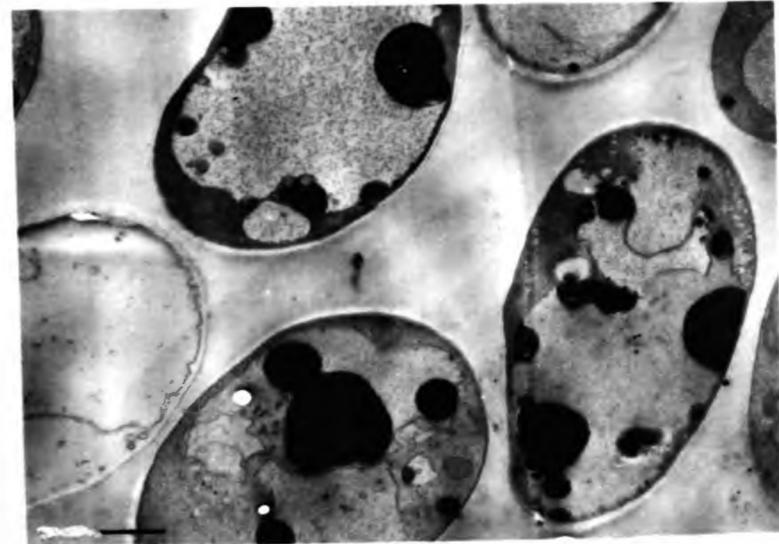Staining was in silver proteinate.

Bars = 1μm

i)

ii)

iii)

iii)

Uninoculated sporophore tissue was also prepared in an identical manner.

Where infection was at the stipe base, the host cells remained largely intact with no evidence of cell wall breakdown, (Table 2.2). There was a significant increase in the number of cells without visible cytoplasm at the plane of the sections examined, (54.8% increase, p < 0.01). This may indicate wall breakdown, with resultant loss of cytoplasm, at a point other than that visible in the sample.

Within live cells vacuolation, the occurrence of fat droplets and Woronin bodies was unaffected although there was a slight increase in the occurrence of mitochondria, (p < 0.01). There was a slight but non-significant increase in the number of cells showing cytoplasmic abnormality.

Where M.perniciosa was directly inoculated on the cap surface infection of both cap and gill tissue occurred. In both cases, cell wall degradation was noted with an increase in the extent of cytoplasmic and plasmalemma breakdown, (Table 2.2). The occurrence of cells apparently lacking cytoplasm in healthy tissue, indicates that both cap and gill tissue may have been senescent at the time of examination. Host cells within cap tissue showed a marked increase in vacuolation in the presence of M.perniciosa, (35.4% increase, p <0.01). There was a marked increase in the number of mitochondria per cell profile in the presence of the pathogen indicating an increase in the rate of cell metabolism, although this was associated with an increase in cytoplasmic abnormality in terms of granulation and membrane development.

In contrast gill tissue, which in healthy tissue has a high apparent metabolic rate at sporulation with a relatively large number of

mitochondria per cell compared to other types of tissue within healthy
sporophores, showed a decrease in the number of organelles per cell,
(Table 2.2). The number of mitochondria per cell profile and the
percentage of cells containing Woronin bodies and fat droplets was
significantly reduced, (p $<$0.01).

3. M.perniciosa hyphae in infected tissue

Examination of M.perniciosa hyphae within infected tissue
revealed that hyphae are made up of a number of different forms of
cells.

Using the T.E.M. actively growing apices were seen to contain a
prominent apical body or 'spitzenkorper' (Hawksworth, Sutton and
Ainsworth, 1983) found in association with wall vescicles,
(Plate 2.7). In observations of more mature hyphae, M.perniciosa
septa were seen to be perforated but with no complex pore apparatus,
commonly having Woronin bodies in close association, (Plate 2.8).
The majority of hyphal cells were seen to be electron dense and
highly granular in nature. Some M.perniciosa cells were present
which were less dense. Such cells contained little granulation and
had an apparent high metabolic activity, with numerous mitochondria
and multiple vescicles, (lomasomes (Bracker, 1967)), (Plate 2.9i).
Lomasomes were observed to be closely associated with the plasmalemma,
apprently discharging their contents to the environment outside the cell,
(Plate 2.9ii). These cells may, therefore, have a role in enzyme transport
across the cell wall. The rare occurrence of such cells, (18.1%) agrees
closely with the observed frequency of host cell wall breakdown at
specific A.bisporus/M.perniciosa contact points, (12.6%). These cells

PLATE 2.7.   Transmission electronmicrograph of _M.perniciosa_
hyphae in infected _A.bisporus_ 'cup stage' sporophore cap
tissue 6 days after inoculation with the pathogen and
incubation at 16°C.   An accumulation of vescicles (arrowed)
is associated with the developing hyphal tip.

Note the variation in granulation between cells of the
pathogen within the same hyphal strand.

Staining was in uranyl acetate/lead citrate.

Bar represents 1 µm.

PLATE 2.7.   Transmission electronmicrograph of <u>M.perniciosa</u> hyphae in infected <u>A.bisporus</u> 'cup stage' sporophore cap tissue 6 days after inoculation with the pathogen and incubation at 16°C.   An accumulation of vescicles (arrowed) is associated with the developing hyphal tip.

Note the variation in granulation between cells of the pathogen within the same hyphal strand.

Staining was in uranyl acetate/lead citrate.

Bar represents 1 μm.

PLATE 2.8.   Transmission electronmicrograph of a septum
within M.perniciosa in 'cup stage'   A.bisporus cap tissue
isolated from commercial beds.   No complex pore apparatus
is apparent.   The septum is perforate with Woronin bodies
(w) and mitochondria (m) in close association.

Staining was in uranyl acetate/lead citrate.

Bar represents 0.5 μm.

PLATE 2.8.   Transmission electronmicrograph of a septum
within M.perniciosa in 'cup stage'   A.bisporus cap tissue
isolated from commercial beds.   No complex pore apparatus
is apparent.   The septum is perforate with Woronin bodies
(w) and mitochondria (m) in close association.

Staining was in uranyl acetate/lead citrate.

Bar represents 0.5 μm.

i)

ii)

PLATE 2.9. Transmission electronmicrograph of M.perniciosa (Mp) in infected 'button stage' A.bisporus (Ab) sporophore tissue, 8 days after inoculation to the cap surface and incubation at 16 C.

i)   M.perniciosa cell showing little granuolation with an apparent high metabolic rate and containing numerous mitochondria (m) and lomasomes (l). Staining was in uranyl acetate/lead citrate/ Ruthenium Red. Bar represents 3µm.

ii)   Lomasome discharging contents to the extracellular matrix between M.perniciosa and neighbouring host cells.   At this point the host cell wall is unclear with possible separation of component layers, (arrowed).   Staining was in uranyl acetate/lead citrate/ Ruthenium Red. Bar represents 0.1 µm.

i)

ii)

PLATE 2.9.   Transmission electronmicrograph of <u>M.perniciosa</u> (Mp)
in infected 'button stage' <u>A.bisporus</u> (Ab) sporophore tissue, 8 days
after inoculation to the cap surface and incubation at 16°C.

i)   <u>M.perniciosa</u> cell showing little granuolation with an apparent
high metabolic rate and containing numerous mitochondria (m) and
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ii) Lomasome discharging contents to the extracellular matrix
between <u>M.perniciosa</u> and neighbouring host cells.   At this point
the host cell wall is unclear with possible separation of component
layers, (arrowed). Staining was in uranyl acetate/lead citrate/
Ruthenium Red.
Bar represents 0.1 μm.

may represent a specific site of the production of enzymes utilised in pathogenesis.

4. Course of infection of A.bisporus sporophore tissue

Sporophore tissue artificially inoculated with M.perniciosa was incubated in moist chambers at 20°C ± 2°C. Examination of the tissue using the S.E.M., revealed that initially following infection, growth of the pathogen was intercellular within the tissue and not directly on the sporophore surface. This was followed by the emergence of verticillate conidiophores through the tissue surface. Colonisation of the host surface subsequently occurred, together with the production of chlamydospores,

5. Morphology of the host/parasite interface within sclerodermoid tissue

The exact nature of any cell/cell interactions at the host/parasite interface was investigated using the T.E.M. and S.E.M. Examination by S.E.M. of internal areas of infected sporophores tissue prepared by freeze fracture (Materials and Methods III 5D), enabled the observation of M.perniciosa hyphae within the sporophore tissue. Pathogen hyphae were seen to ramify intercellularly with penetration of host hyphae occurring apparently at random, (22.7% of cells penetrated), (Plate 2.10). Penetration of host hyphae was by fine M.perniciosa hyphal strands, (Plate 2.11i). S.E.M. examination of internal freeze fractured and surface samples of sclerodermoid tissue revealed that host cells were hypertrophic in the presence of the pathogen. Host cells were generally smooth walled, (Plate 2.12). In some instances there was localised host cell wall degradation at specific host / pathogen contact

PLATE 2.10. Scanning electronmicrograph of unfixed freeze fractured A.bisporus (Ab) 'cup stage' sporophore cap tissue 4 days after inoculation with an M.perniciosa (Mp) conidial suspension to the cap surface and incubation at 14°C. The pathogen hyphae ramify intercellularly with penetration of host hyphae occurring apparently at random. Cambridge MkI, 15 kV accelerating voltage.
Bar represents 20 μm.

PLATE 2.10.   Scanning electronmicrograph of unfixed freeze
fractured A.bisporus (Ab) 'cup stage' sporophore cap tissue
4 days after inoculation with an M.perniciosa (Mp) conidial
suspension to the cap surface and incubation at 14°C.   The
pathogen hyphae ramify intercellularly with penetration of
host hyphae occurring apparently at random.   Cambridge MkI,
15 kV accelerating voltage.
Bar represents 20 μm.

i)

ii)
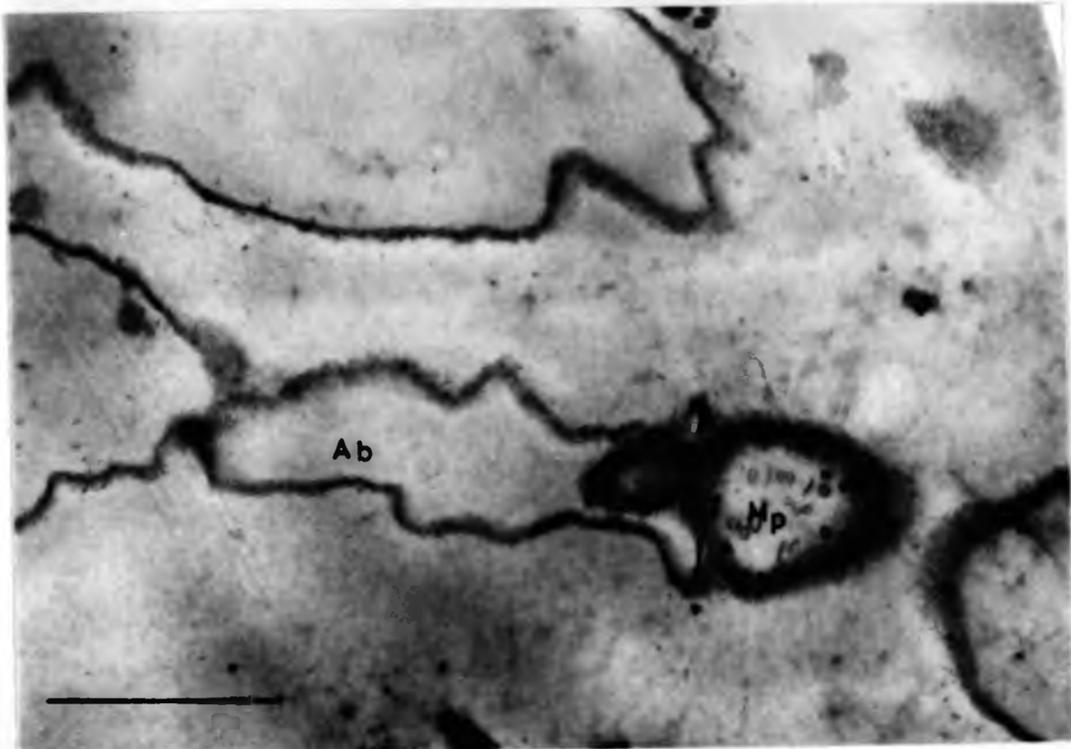
PLATE 2.11.   Transmission electronmicrographs of penetration of
A.bisporus (Ab) hyphae by M.perniciosa (Mp) in sporophore (button
stage) cap tissue 4 days after inoculation of the pathogen to the
cap surface and incubation at 14°C.   Pathogen cells are typically
more granular than those of the host and enter A.bisporus cells by
means of fine hyphal strands.
Staining was in 1% potassium permanganate.

i)   Penetration point of M.perniciosa hyphal strand entering an
A.bisporus hypha, in infected sporophore cap tissue (button stage).
Bar represents 3 µm;

ii) M.perniciosa hypha growing within an A.bisporus cell in
infected sporophore cap tissue, (button stage).
Bar represents 1 µm.

i)

ii)

PLATE 2.11.   Transmission electronmicrographs of penetration of
A.bisporus (Ab) hyphae by M.perniciosa (Mp) in sporophore (button
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Bar represents 3 μm;

ii) M.perniciosa hypha growing within an A.bisporus cell in
infected sporophore cap tissue, (button stage).
Bar represents 1 μm.

PLATE 2.12.   Scanning electronmicrograph of the surface
of sclerodermoid tissue produced on commercial cropping beds
35 days after casing, showing M.perniciosa (Mp) hyphae in
close association with A.bisporus (Ab) hyphae.   A.bisporus
hyphae show no sign of extensive cell wall breakdown.
Note the present of bacteria (b) associated both with the
host and with the pathogen.

Fixation was in osmium tetroxide vapour, Cambridge MkI SEM
15 kV accelerating voltage.

Bar represents 10 μm.

PLATE 2.12.   Scanning electronmicrograph of the surface
of sclerodermoid tissue produced on commercial cropping beds
35 days after casing, showing M.perniciosa (Mp) hyphae in
close association with A.bisporus (Ab) hyphae.   A.bisporus
hyphae show no sign of extensive cell wall breakdown.
Note the present of bacteria (b) associated both with the
host and with the pathogen.

Fixation was in osmium tetroxide vapour, Cambridge MkI SEM
15 kV accelerating voltage.

Bar represents 10 μm. .

points where M.perniciosa was seen to be sunken into A.bisporus hyphae, (Plate 2.13). Ultrathin sections were stained with silver proteinate, or at the time of fixation with ruthenium red, (Materials and Methods 5IIIB,C). While healthy sporophore hyphae showed no evidence of cell wall breakdown in the sections examined, cells from gill tissue, (7%), and stipe tissue, (12%), from sporophores of the same age but infected with M.perniciosa showed cell wall breakdown.

Wall breakdown was highly localised, affecting discreet sections of individual cell walls. Initially, small areas of wall material were seen to be swollen, (Plate 2.14i). Following this separation of the component layers was noted, (Plate 2.14ii). Subsequent layer breakdown appeared to be by perforation of the chitin / glucan wall layer, followed by splitting across the cell wall, (Plate 2.14iii).

Where the pathogen was sunken into host hyphae wall breakdown was extremely localised, (Plate 2.11ii).

In sclerodermoid mass initials cell walls appeared to be intact compared to healthy controls. In mature sclerodermoid tissue 39.6% of cells in the tissue interior showed some evidence of cell wall breakdown. At the surface of mature sclerodermoid tissue 61.8% of host cell walls were degraded to some extent.

The specific nature of this process in individual cells was apparent by the occurrence of wall fragments within infected tissue, (Plate 2.15). Such fragments were degraded only at the point of breakage and not over the entire section. The wall breakdown process occurs at precise M.perniciosa / A.bisporus contact points and not
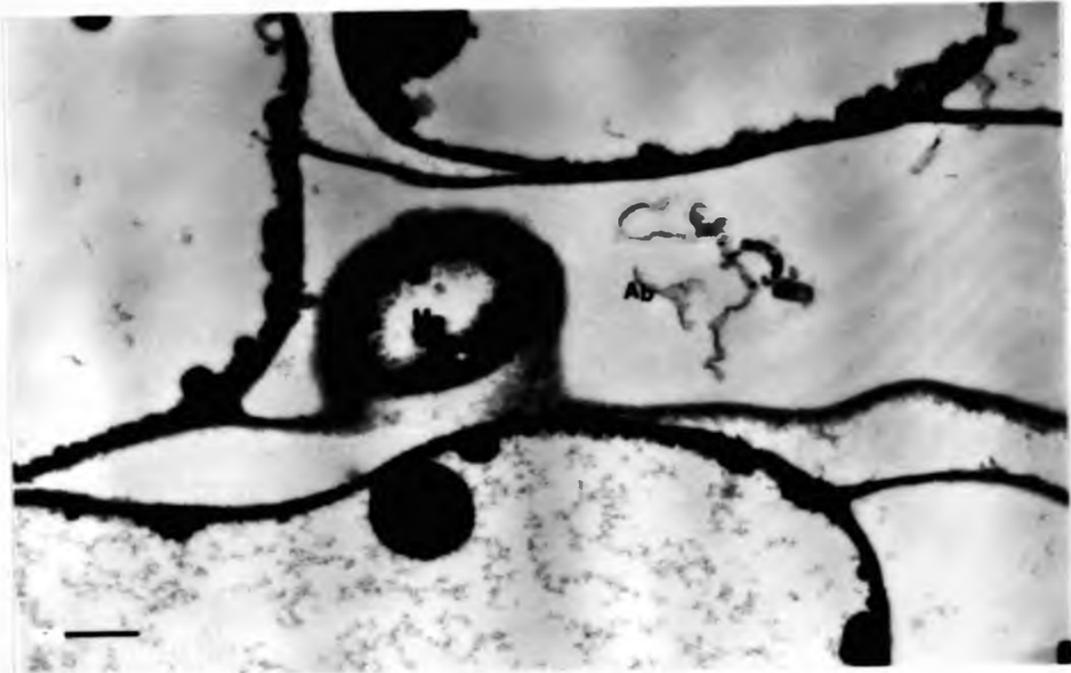
PLATE 2.13.   Transmission electronmicrograph of a transverse
section of A.bisporus (Ab) hymenial cells in gill tissue in-
fected with M.perniciosa (Mp).   Host tissue was inoculated
with an M.perniciosa conidial suspension at 'veil break' and
incubated for 6 days at 16°C prior to fixation and sectioning.
Note the localised cell wall degradation at the specific host/
pathogen contact point, (arrowed).

Staining was in uranyl acetate/lead citrate.

Bar represents 1 μm.

PLATE 2.13.   Transmission electronmicrograph of a transverse
section of A.bisporus (Ab) hymenial cells in gill tissue in-
fected with M.perniciosa (Mp).   Host tissue was inoculated
with an M.perniciosa conidial suspension at 'veil break' and
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Note the localised cell wall degradation at the specific host/
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Staining was in uranyl acetate/lead citrate.

Bar represents 1 µm.

PLATE 2.14. Transmission electron micrographs of sections across A.bisporus cell walls in cap tissue (button stage) inoculated with an M.perniciosa conidial suspension to the cap surface. Incubation was at 14°C in moist chambers. Samples were of cap pseudoparenchyma 0.5 cm from the inoculation site. (Ab = A.bisporus)

Staining was in Uranyl acetate/lead citrate.

Bars represent 0.1 μm.

i) Small areas of cell wall material (w) were seen to be swollen over a limited area. (x).

ii) This was followed by separation (arrowed) of the wall (w) component layers.

iii) Larger breakdown within the wall (w) was by perforation (arrowed) of the inner chitin/glucan layer prior to splitting of the cell wall.

PLATE 2.14.   Transmission electron micrographs of sections across
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Bars represent 0.1 μm.

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       component layers.

iii)   Larger breakdown within the wall (w) was by perforation
       (arrowed) of the inner chitin/glucan   layer prior to
       splitting of the cell wall.

PLATE 2.14.   Transmission electron micrographs of sections across
A.bisporus cell walls in cap tissue (button stage) inoculated with
an M.perniciosa conidial suspension to the cap surface.   Incubation
was at 14°C in moist chambers.   Samples were of cap pseudoparenchyma
0.5 cm from the inoculation site.   (Ab = A.bisporus)

Staining was in Uranyl acetate/lead citrate.

Bars represent 0.1 µm.

i)   Small areas of cell wall material (w) were seen to be
      swollen over a limited area. (x).

ii)   This was followed by separation (arrowed) of the wall (w)
      component layers.

iii) Larger breakdown within the wall (w) was by perforation
      (arrowed) of the inner chitin/glucan   layer prior to
      splitting of the cell wall.

iii)

Ab

W

iii)

Ab

W

───

iii)

Ab

w

PLATE 2.15.   Transmission electronmicrograph of sclerodermoid
tissue from commercial cropping beds 28 days after casing.
Inoculation with M.perniciosa was at the time of casing.
M.perniciosa (Mp) hyphae with dense granular cytoplasm ramify
throughout the tissue, predominantly inter-cellularly between
less dense A.bisporus (Ab) hyphae.   The specific nature of
host cell wall degradation (arrowed) is demonstrated by the
presence of discrete host cell wall sections (X) within the
tissue.

Staining was in uranyl acetate/lead citrate.

Bar represents 2 μm.

PLATE 2.15. Transmission electronmicrograph of sclerodermoid
tissue from commercial cropping beds 28 days after casing.
Inoculation with M.perniciosa was at the time of casing.
M.perniciosa (Mp) hyphae with dense granular cytoplasm ramify
throughout the tissue, predominantly inter-cellularly between
less dense A.bisporus (Ab) hyphae.   The specific nature of
host cell wall degradation (arrowed) is demonstrated by the
presence of discrete host cell wall sections (X) within the
tissue.

Staining was in uranyl acetate/lead citrate.

Bar represents 2 μm.

PLATE 2.15. Transmission electronmicrograph of sclerodermoid tissue from commercial cropping beds 28 days after casing. Inoculation with M.perniciosa was at the time of casing. M.perniciosa (Mp) hyphae with dense granular cytoplasm ramify throughout the tissue, predominantly inter-cellularly between less dense A.bisporus (Ab) hyphae. The specific nature of host cell wall degradation (arrowed) is demonstrated by the presence of discrete host cell wall sections (X) within the tissue.

Staining was in uranyl acetate/lead citrate.

Bar represents 2 μm.

uniformly throughout infected tissue.

The majority of cell / cell contact points showed no evidence of host cell wall breakdown however (87.4%). Most contact points where A.bisporus cells were closely aligned to M.perniciosa hyphae consisted of apparently unaffected host cell walls closely abutted to pathogen hyphae, (Plate 2.16).

This observation is in apparent contradiction to previous theories relating to the role of cell wall degrading enzymes in development of Wet Bubble disease. Smith, (1924), considered that the action of cell wall degrading enzymes was the primary cause of disease symptom development. Observations reported here indicate that, while cell wall degradation does occur, not all host/parasite contact points are affected. It is unlikely, therefore, that pathogenesis is solely related to the occurrence of such cell wall degrading enzymes.

PLATE 2.16. Transmission electronmicrographs of host/pathogen contact points.
In the majority of cases in which M.perniciosa (Mp) cells were closely aligned to A.bisporus (Ab) cell walls, host cell wall degradation was not observed.

Staining was in uranyl acetate/lead citrate.

i)   M.perniciosa/A.bisporus contact point in sclerodermoid tissue (mature), isolated from commercial cropping beds 21 days after casing.   Infection of beds with M.perniciosa was at the time of casing.   Bar represents 1.5 μm

ii)   Close up of (i).   Circled area of above showing closely aligned host and pathogen cell walls with no evidence of host cell wall degradation at the interface, (arrowed).
      p = plasmalemma

      c = microfobrillar chitin

      g = β1.3, β1.6 glucan. (After Vincent-Davies 1972).

Bar represents 0.1 μm.

i)

PLATE 2.16. Transmission electronmicrographs of host/pathogen contact
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    casing.   Bar represents 1.5 μm

ii) Close up of (i).   Circled area of above showing closely
    aligned host and pathogen cell walls with no evidence of host
    cell wall degradation at the interface, (arrowed).
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PLATE 2.16. Transmission electronmicrographs of host/pathogen contact points.
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i) M.perniciosa/A.bisporus contact point in sclerodermoid tissue (mature), isolated from commercial cropping beds 21 days after casing. Infection of beds with M.perniciosa was at the time of casing. Bar represents 1.5 μm

ii) Close up of (i). Circled area of above showing closely aligned host and pathogen cell walls with no evidence of host cell wall degradation at the interface, (arrowed).

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Bar represents 0.1 μm.

ii)

CHAPTER 3

I. THE PRODUCTION OF ANTIFUNGAL AND GROWTH PROMOTING SUBSTANCES BY
    M.PERNICIOSA AND A.BISPORUS

This chapter describes experiments designed to investigate the
mechanisms of pathogenicity in M.perniciosa and to examine the possible
means by which A.bisporus may maintain a level of defence towards
potential pathogens. In addition, the possible production of compounds
capable of increasing fungal growth by both the host and the pathogen
was investigated. The ability of some of these compounds to inhibit
bacterial growth was also investigated.

The initial approach was to demonstrate the antifungal or growth
promoting activity of A.bisporus sporophore tissues and of M.perniciosa
culture extracts. A.bisporus tissue infected by M.perniciosa was also
examined and the origin of active compounds consequently deduced by
comparisons with extracts from the individual fungi.

1. M.perniciosa

The production of biologically active inhibitory compounds by
M.perniciosa was investigated initially by bioassaying crude culture
filtrates against Cladosporium herbarum. Sterile filtrates from liquid
malt cultures, incubated for 23 days at 18°C $\pm$ 2°C, were concentrated
by freeze drying and resuspended in c. 5 cm^3 of SDW. Droplets of
filtrate (20 μl) were placed on clean glass slides with C.herbarum
conidia from an 8 day old Czapek Dox agar culture. Germination of
conidia in the presence of sample filtrates from M.perniciosa cultures
after incubation in moist chambers for 18 h at 20°C $\pm$ 2°C was

significantly reduced relative to controls in uninoculated freeze dried liquid malt media, (76.5% $\pm$ 0.5% reduction, p <0.001), indicating that antifungal compounds produced by M.perniciosa were present in the culture medium.

In order to investigate whether the presence of antifungal substances in M.perniciosa cultures was influenced by the age of the culture, conical flasks (250 cm^3), each containing 100 cm^3 of liquid malt media were inoculated with three 3 mm diameter discs cut from the margin of 14 day old M.perniciosa MM agar cultures and incubated at 20°C $\pm$ 1°C. At regular intervals, cultures were filtered and the supernatant freeze dried, resuspended in 20 cm^3 of SDW and sterilised by filtration. Sample filtrate droplets (20 µl) were placed on clean glass slides and assayed for antifungal activity by the addition of 20 µl droplets of a conidial suspension of either Botrytis cinerea or Cladosporium herbarum. Samples were incubated for 18 h in moist chambers at 20°C $\pm$ 2°C, prior to microscopic examination. The antifungal activity of M.perniciosa culture filtrates increased with the age of the culture. C.herbarum showed a higher degree of sensitivity to M.perniciosa culture filtrates than did B.cinerea. Germination of C.herbarum conidia, relative to controls in SDW, was not significantly reduced in filtrates from 2 day old cultures, but germination was significantly reduced in those from 4.5 day old cultures, (32.7% $\pm$ 1.0% reduction) and in filtrates from 6 day old cultures, (100% reduction, p < 0.001). Germination of B.cinerea conidia was not affected however in the presence of filtrates from 4.5 day old M.perniciosa cultures, but was reduced in the presence of those from 6 day old cultures, (100% reduction, p <0.001), (Table 3.1).

Table 3.1

The effect of filtrates of M.perniciosa liquid malt cultures incubated at 20°C on germination and germ tube growth of C.herbarum and B.cinerea conidia on glass slides after incubation at 18°C for 18 h in moist chambers.

(A) = Germ tube length as a percentage relative to controls.

Age of M.perniciosa culture (days)	Cladosporium herbarum		Botrytis cinerea	
	% germination	Germ tube length (A)	% germination	Germ tube length (A)
Uninoculated liquid malt controls	97.3 ± 0.4	100 ± 0.0	100 ± 0.0	100 ± 0.0
0	98.6 ± 0.9	244.3 ± 25.3	100 ± 0.0	112.6 ± 12.5
2	95.3 ± 1.5	320.0 ± 3.5	100 ± 0.0	105.0 ± 2.6
4.5	65.5 ± 1.0	48.0 ± 4.8	100 ± 0.0	91.6 ± 7.0
6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Germ tube length of B.cinerea conidia was slightly increased
relative to controls, in the presence of filtrates from M.perniciosa
cultures, (0 and 2 day old, 12.6% and 5.0% increase respectively).
In older samples, germ tube length was reduced, (8.4% reduction in
filtrates from 4.5 day old cultures), with no germination of conidia
in filtrates from 6 day old cultures reflected in an absence of germ
tubes, (100% reduction).

The effects of M.perniciosa culture filtrates on germ tube length
was emphasised using C.herbarum. Germ tube length was markedly
increased relative to controls, of uninoculated media, in the presence
of filtrates from 0 day old and 2 day old M.perniciosa cultures,
(144.3% and 220.0% respectively). In filtrates from older cultures
germ tube length was, however, reduced with a 52.0% reduction, relative
to controls, in filtrates from 4.5 day old M.perniciosa cultures and
100% reduction in filtrates from 5 day old cultures reflecting a
complete lack of germination, (p <0.001).

This indicates the presence of stimulants of an undefined nature
in young (4.5 day old) cultures. Such stimulatory effects, additional
to the effect of unused nutrients from M.perniciosa cultures, are
however, subsequently overcome by the production of fungistatic or
fungitoxic substances in older cultures, (Table 3.1).

The manner in which M.perniciosa is able to antagonise A.bisporus
hyphae was consequently investigated. Four factors were examined, the
ability of M.perniciosa culture filtrates to kill host cells, the
alteration of the level of host cell vacuolation and cytoplasmic
granulation, changes in host cell width, and the extent of electrolyte
leakage from host tissue.

Germ tube length of <u>B.cinerea</u> conidia was slightly increased relative to controls, in the presence of filtrates from <u>M.perniciosa</u> cultures, (0 and 2 day old, 12.6% and 5.0% increase respectively). In older samples, germ tube length was reduced, (8.4% reduction in filtrates from 4.5 day old cultures), with no germination of conidia in filtrates from 6 day old cultures reflected in an absence of germ tubes, (100% reduction).

The effects of <u>M.perniciosa</u> culture filtrates on germ tube length was emphasised using <u>C.herbarum</u>. Germ tube length was markedly increased relative to controls, of uninoculated media, in the presence of filtrates from 0 day old and 2 day old <u>M.perniciosa</u> cultures, (144.3% and 220.0% respectively). In filtrates from older cultures germ tube length was, however, reduced with a 52.0% reduction, relative to controls, in filtrates from 4.5 day old <u>M.perniciosa</u> cultures and 100% reduction in filtrates from 5 day old cultures reflecting a complete lack of germination, (p <0.001).

This indicates the presence of stimulants of an undefined nature in young (4.5 day old) cultures. Such stimulatory effects, additional to the effect of unused nutrients from <u>M.perniciosa</u> cultures, are however, subsequently overcome by the production of fungistatic or fungitoxic substances in older cultures, (Table 3.1).

The manner in which <u>M.perniciosa</u> is able to antagonise <u>A.bisporus</u> hyphae was consequently investigated. Four factors were examined, the ability of <u>M.perniciosa</u> culture filtrates to kill host cells, the alteration of the level of host cell vacuolation and cytoplasmic granulation, changes in host cell width, and the extent of electrolyte leakage from host tissue.

Tissue strips, c. 0.5 cm x 1.0 cm x 1.0 cm, were excised from healthy 'cup stage' sporophore caps, under sterile conditions, and incubated for 5 days in Czapek Dox / yeast extract media, (c. 0.5 g tissue, 50 cm^{-3} media) at 22°C $\pm$ 1°C in an orbital incubator, (100 r.p.m.). Growth of A.bisporus hyphae resulted in spheroidal flocular growth from the inoculum. This was taken as being biologically similar to mature sporophore cap tissue within the limits of the requirement for undamaged A.bisporus hyphae.

In order to investigate the possibility that production of toxins by M.perniciosa may vary in the presence or the absence of the host, the effect of filtrates from two types of M.perniciosa cultures on A.bisporus was investigated, those grown on Czapek Dox liquid medium and those grown on Czapek Dox liquid medium supplemented with 0.3 µl cm^{-3} of sterile filtered juice from pressed healthy A.bisporus 'button stage' sporophores (Czapek Dox + mushroom liquid medium). M.perniciosa cultures were of 50 cm^3 media inoculated with two 3 mm diameter discs cut from the margin of 14 day old Malt agar cultures, and incubated for 8 days at 20°C $\pm$ 1°C. Extracts were collected by filtration and the supernatant sterilised by filtration 0.2 µm pore size prior to assay against A.bisporus. Controls were sterile uninoculated Czapek Dox media with and without filtrates from pressed healthy sporophores.

The effects of filtrates from M.perniciosa cultures on A.bisporus were tested by the incorporation of sterile filtered medium from the two types of M.perniciosa cultures, and from control samples, into separate A.bisporus test flasks, (1.0 µl cm^{-3}) prepared as described above, and the host mycelium examined periodically for changes relative to controls.

At intervals following the inclusion of sample filtrates into

growth media, samples of A.bisporus mycelium were withdrawn from the culture flasks and mounted on clean glass slides and examined micro- scopically. Cell death was determined using the Trypan blue vital staining technique, with uptake of the large molecular weight stain indicating membrane disruption in killed cells.

After 4.5 h incubation, c. 30% cell death had occurred in flasks containing filtrates from M.perniciosa cultures grown both with and without the addition of host filtrates in the culture medium, relative to controls, (p <0.01). After 24 h cell death had increased to 78.0% $\pm$ 0.9% in the presence of filtrates from M.perniciosa cultures containing host filtrates, while in those flasks containing filtrates from M.perniciosa Czapek Dox cultures without the addition of host filtrates, cell death was increased to 73.0% $\pm$ 5.0%, relative to controls, (p <0.01). This indicates that the presence of host components is not a prerequisite for the production of toxic substances capable of antagonising A.bisporus by M.perniciosa, (Figure 3.1i).

Live cells of A.bisporus were examined microscopically following the inclusion of antagonistic M.perniciosa culture filtrates for evidence of cytoplasmic granulation and vacuolation. While controls, with the addition of uninoculated media, as used for the culture of M.perniciosa, showed a maximum level of 21.8% $\pm$ 6.2% of cells showing extensive vacuolation after 24 h, test samples with the inclusion of M.perniciosa culture filtrates showed a marked increase to 97.9% $\pm$ 1.2% cells vacuolated after 9.5 h in the presence of filtrates from M.perniciosa cultures containing host filtrates in the growth media. Samples with the addition of filtrates from unsupplemented M.perniciosa Czapek Dox cultures similarly showed an increase in the level of

FIGURE 3.1 i

% Dead A.bisporus cells (a)

INCUBATION TIME (hours after sample inclusion)

FIGURE 3.1 ii

% Live A.bisporus cells vacuolated (a)

INCUBATION TIME (hours after sample inclusion)
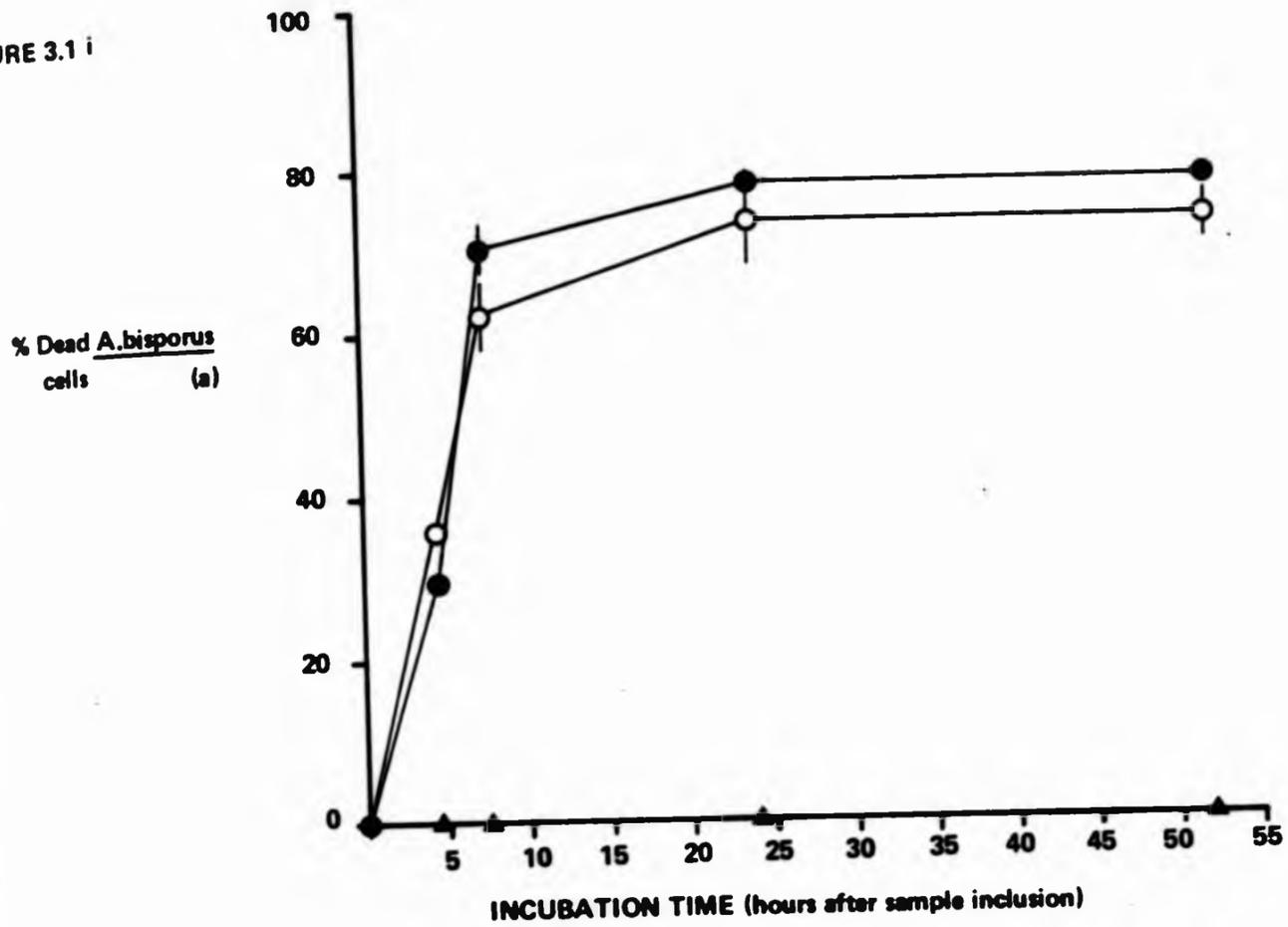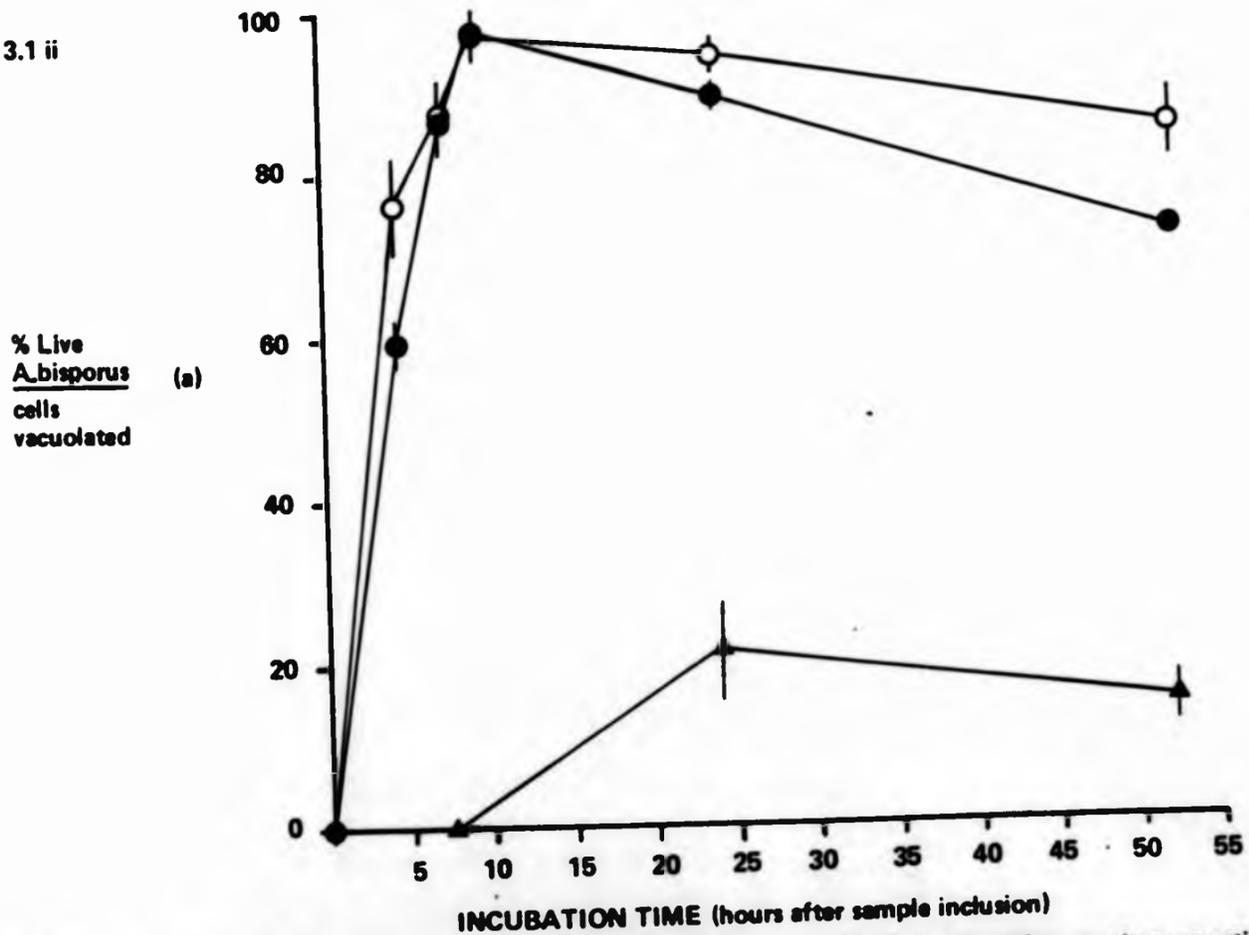
Alteration of A.bisporus hyphae produced in 5 day old shake cultures incubated at 22°C after incorporation of filtrates (0.2 μm pore size) from M.perniciosa cultures incubated for 8 days at 20°C.

O—O M.perniciosa sample — Czapek Dox liquid medium (0.3 μl cm^{-3}) culture filtrate

●—● M.perniciosa sample — Czapek Dox + mushroom liquid medium (0.3 μl cm^{-3}) culture filtrate.

▲—▲ Control — 0.3 μl cm^{-3} uninoculated Czapek Dox + mushroom liquid medium.

(a) = Mean value ± SEM.

vacuolation, rising to 97.7% $\pm$ 3.2% of cells vacuolated after 9.5 h,
(p <0.01), (Plate 3.1, Figure 3.1ii).

The width of host hyphae was seen to increase significantly in the
presence of M.perniciosa culture filtrates. In the presence of filtrates
from M.perniciosa cultures grown on Czapek Dox medium supplemented with
host filtrates A.bisporus hyphal width was increased, relative to
controls, by 65.5% $\pm$ 13.3%, while for those samples in the presence of
filtrates from M.perniciosa cultures grown on unsupplemented Czapek Dox
liquid medium, hyphal width increased by 124.0% $\pm$ 15.3% relative to
controls.

For the assessment of electrolyte leakage from antagonised
A.bisporus tissue, cap tissue samples were prepared as previously
described and rinsed in sterile deionised water, (SDeW), twice and placed
in 50 cm^3 SDeW in chromic acid cleaned flasks prior to the addition of
sample filtrates prepared as described above. Sterile filtrates from
M.perniciosa cultures grown on Czapek Dox liquid media supplemented with
sterile filtrates of juice from pressed healthy sporophores and those
from cultures grown on Czapek Dox liquid medium without host filtrates
were added to A.bisporus tissue in SDeW and solution conductivity
determined at regular intervals. Flasks were maintained at 20°C $\pm$ 1°C
in an orbital incubator, (25 r.p.m.). Sample blanks were of correspond-
ing M.perniciosa culture filtrates with or without host filtrates in the
culture medium added to SDeW without A.bisporus tissue, with controls of
A.bisporus tissue in SDeW with the addition of uninoculated Czapek Dox
medium containing host filtrates, and of unsupplemented Czapek Dox
medium.

i)

ii)

PLATE 3.1. Light micrographs of A.bisporus hyphae from shake cultures incubated at 22°C for 5 days, 9.5 hours after the inclusion of:

i) Filtrates (0.2 μm pore size) from M.perniciosa Czapek Dox cultures incubated at 20°C for 8 days, causing extensive vacuolation in the host.

ii) Uninoculated Czapek Dox liquid medium as control for (i).

Bar represents 10 μm.

i)

ii)

PLATE 3.1.   Light micrographs of A.bisporus hyphae from
shake cultures incubated at 22°C for 5 days, 9.5 hours
after the inclusion of:

i) Filtrates (0.2 μm pore size) from M.perniciosa Czapek
Dox cultures incubated at 20°C for 8 days, causing extensive
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Bar represents 10 μm.

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shake cultures incubated at 22°C for 5 days, 9.5 hours
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Dox cultures incubated at 20°C for 8 days, causing extensive
vacuolation in the host.

ii) Uninoculated Czapek Dox liquid medium as control for (i).

Bar represents 10 μm.

Solution conductivity for flasks containing A.bisporus tissue in SDeW following the inclusion of filtrates from M.perniciosa cultures increased rapidly. Conductivity increased by 34.6% $\pm$ 4.7% min. after the inclusion of filtrates from M.perniciosa Czapek Dox / sporophore filtrate cultures, while those flasks containing A.bipsorus in SDeW with the addition of M.perniciosa unsupplemented Czapek Dox culture filtrates showed an increase of solution conductivity after 30 min. of 23.8% $\pm$ 4.9%, (Figure 3.2, Table 3.2).

2. A.bisporus

Activity of A.bisporus sporophore tissue was investigated initially by assaying freeze dried sterile filtrates from pressed sporophores, resuspended in methanol, against Cladosporium herbarum. Samples were spotted on to TLC plates and sprayed with a conidial suspension of C.herbarum conidia in ½ strength Czapek Dox liquid medium. Plates were then incubated in moist chambers for 48 h at 20° $\pm$ 2°C. Antifungal activity appeared as white areas on the plate where C.herbarum had failed to grow. Antifungal activity was noted both in healthy A.bisporus sporophore samples and in samples from sporophore tissue infected with M.perniciosa, prepared in a similar manner, (Plate 3.2). In addition, both of these samples exhibited antibacterial activity when assayed against Bacillus megaterium using the aesculin spray technique by spraying samples on TLC plates with a nutrient broth suspension of B.megaterium from a 24 h old culture and incubating plates for 24 h at 23°C in moist chambers. Plates were then resprayed with 0.2% aesculin in 0.1% ferric citrate containing 0.5% yeast extract. After a further

FIGURE 3.2 Changes in conductivity of SDeW containing A.bisporus hyphae from shake cultures incubated at 22°C for 5 days following the inclusion of filtrates (0.2 μm pore size) from M.perniciosa cultures (a) or of sample blanks and incubated at 20°C in an orbital incubator (25 rpm).
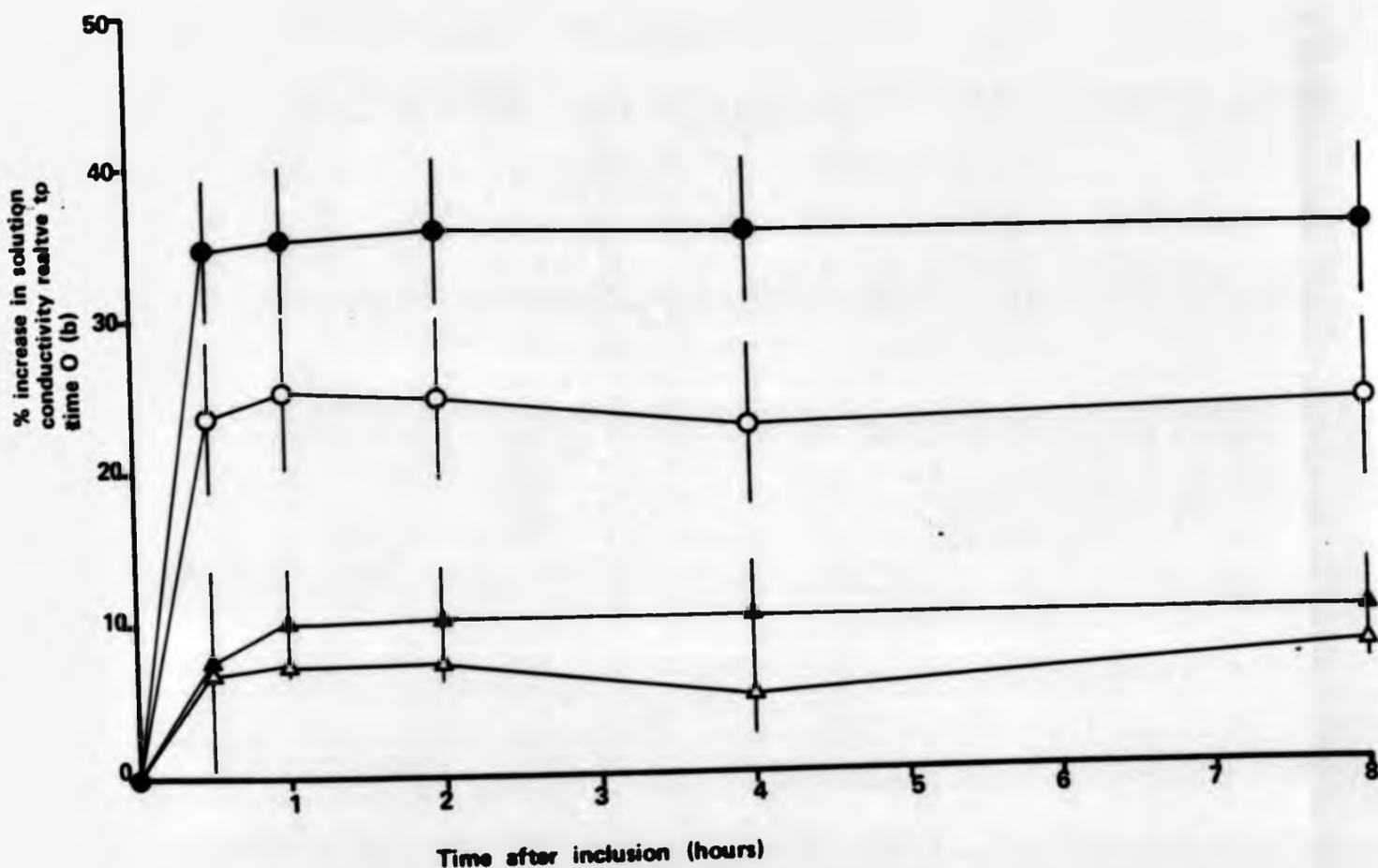
Time after inclusion (hours)

% increase in solution conductivity realtve to time O (b)

●—● A.bisporus substrate conductivity with the addition of sterile filtered M.perniciosa culture medium, Czapek Dox + mushroom liquid medium, (0.3 μl cm⁻³)

O—O A.bisporus substrate conductivity with the addition of sterile filtered M.perniciosa culture medium, Czapek Dox liquid medium, (0.3 μl cm⁻³)

▲—▲ Control — A.bisporus substrate conductivity with the addition of uninoculated Czapek Dox + mushroom liquid medium, (0.3 μl cm⁻³).

△—△ Control — A.bisporus substrate conductivity with the addition of uninoculated Czapek Dox liquid medium, (0.3 μl cm⁻³)

(a) = M.perniciosa cultures — 10 days ● 23° ± 1°C.

(b) = Mean values ± SEM with sample values adjusted relative to blanks.

Table 3.2

Changes in conductivity of SDeW containing <u>A.bisporus</u> hyphae from shake cultures incubated at 22°C for 5 days following the inclusion of filtrates (0.2 μm pore size) from <u>M.perniciosa</u> cultures incubated for 10 days at 23°C or of sample blanks and incubated at 20°C in an orbital incubator (25 r.p.m.)

Mean values (mmho cm^{-1}) ± SEM

Time from sample inclusion	Blank 1 - M.perniciosa Czapek Dox filtrate in SDeW	Blank 2 - M.perniciosa Czapek Dox + mushroom filtrate in SDeW	Control 1 - A.bisporus in SDeW with Czapek Dox medium	Control 2 - A.bisporus in SDeW with Czapek Dox + mushroom medium	Sample 1 - A.bisporus in SDeW with M.perniciosa Czapek Dox filtrate	Samples 2 - A.bisporus in SDeW with M.perniciosa Czapek Dox + mushroom filtrate
0 min.	4.82 ± 0.08	4.75 ± 0.03	5.60 ± 0.55	5.53 ± 0.03	4.82 ± 0.21	3.51 ± 0.13
30 min.	4.92 ± 0.05	4.91 ± 0.03	5.99 ± 0.38	5.91 ± 0.01	6.04 ± 0.03	4.82 ± 0.03
1 h	4.97 ± 0.03	4.92 ± 0.00	6.13 ± 0.41	5.93 ± 0.02	6.16 ± 0.03	4.86 ± 0.03
2 h	5.01 ± 0.05	4.96 ± 0.02	6.13 ± 0.41	5.94 ± 0.03	6.17 ± 0.02	4.91 ± 0.03
4 h	5.03 ± 0.07	4.99 ± 0.02	6.13 ± 0.41	5.94 ± 0.03	6.10 ± 0.06	4.91 ± 0.03
8 h	5.02 ± 0.07	4.98 ± 0.03	6.12 ± 0.41	5.96 ± 0.03	6.12 ± 0.03	4.89 ± 0.04
24 h	5.01 ± 0.06	4.97 ± 0.02	6.09 ± 0.42	5.94 ± 0.04	6.05 ± 0.03	4.88 ± 0.03
48 h	5.03 ± 0.06	4.98 ± 0.03	6.13 ± 0.43	5.97 ± 0.03	6.02 ± 0.03	4.89 ± 0.03

PLATE 3.2.   Plate bioassay of solvent extracts of healthy
             and infected tissue.

Extracts equivalent to 0.5 gram fresh weight of tissue were spotted
onto a TLC plate and air dried prior to bioassay against _Cladosporium
herbarum_.   (Extent of antifungal zone arrowed).

Sample No.
1.   Methanol extract of healthy A.bisporus 'button stage' sporophores.

2.   Methanol extract of A.bisporus 'button stage' sporophores
      infected with M.perniciosa

3.   Methanol phase of partitioned extract of healthy A.bisporus
      'button stage' sporophores.

4.   Chloroform phase of partitioned extract of healthy A.bisporus
      'button stage' sporophores.

5.   Chloroform phase of partitioned extract of sclerodermoid tissue.

6.   Methanol phase of partitioned extract of sclerodermoid tissue.

PLATE 3.2.   Plate bioassay of solvent extracts of healthy
              and infected tissue.

Extracts equivalent to 0.5 gram fresh weight of tissue were spotted
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Sample No.
1.   Methanol extract of healthy A.bisporus 'button stage' sporophores.

2.   Methanol extract of A.bisporus 'button stage' sporophores
      infected with M.perniciosa

3.   Methanol phase of partitioned extract of healthy A.bisporus
      'button stage' sporophores.

4.   Chloroform phase of partitioned extract of healthy A.bisporus
      'button stage' sporophores.

5.   Chloroform phase of partitioned extract of sclerodermoid tissue.

6.   Methanol phase of partitioned extract of sclerodermoid tissue.

PLATE 3.2.   Plate bioassay of solvent extracts of healthy
              and infected tissue.

Extracts equivalent to 0.5 gram fresh weight of tissue were spotted
onto a TLC plate and air dried prior to bioassay against Cladosporium
herbarum.   (Extent of antifungal zone arrowed).

Sample No.
1.   Methanol extract of healthy A.bisporus 'button stage' sporophores.

2.   Methanol extract of A.bisporus 'button stage' sporophores
      infected with M.perniciosa

3.   Methanol phase of partitioned extract of healthy A.bisporus
      'button stage' sporophores.

4.   Chloroform phase of partitioned extract of healthy A.bisporus
      'button stage' sporophores.

5.   Chloroform phase of partitioned extract of sclerodermoid tissue.

6.   Methanol phase of partitioned extract of sclerodermoid tissue.

period of incubation, (24 h at 23°C), hydrolysis of the aesculin by actively growing bacteria, resulted in a brown colouration with zones of inhibition appearing as colourless areas on the plate.

A.bisporus antifungal components were further investigated by extracting from tissues with methanol:chloroform (1:1), (Materials and Methods IV 5). Solvent fractions were partitioned prior to concentration on a rotary evaporator. Samples were then spotted on to TLC plates and assayed for activity using the Cladosporium spray technique. Antifungal activity was confined to the non-polar chloroform fraction, with no apparent activity in the methanol phase, (Plate 3.2). Antifungal activity of the non-polar chloroform fraction from extracts of healthy A.bisporus sporophores, A.bisporus cap tissue infected with M.perniciosa and from A.bisporus vegetative mycelium were tested on agar plates against M.perniciosa, A.bisporus and Cladosporium herbarum by the application of extracts to Whatman AA discs, (13 mm diameter) at the rate of extract from ½ g fresh weight of tissue per disc. Malt agar plates were inoculated with the fungus used in the test either by seeding the plate surface with a conidial suspension in SDW, (M.perniciosa or C.herbarum) and allowing the agar surface to dry in a sterile air flow, or by a single inoculation directly on to the agar, (A.bisporus), prior to the positioning of the loaded AA disc centrally on the agar surface. Antifungal activity was greatest in healthy A.bisporus sporophore tissue. Antifungal activity was also present, at a reduced level, in tissue infected with M.perniciosa but A.bisporus vegetative mycelium showed no activity, (Table 3.3, Plate 3.3). The antifungal compounds observed in A.bisporus sporophore tissue were active against M.perniciosa, C.herbarum and, surprisingly, A.bisporus

Table 3.3

Inhibition of fungal growth on malt agar plates incubated at 18°C for 10 days by chloroform extracts of healthy 'button stage' A.bisporus sporophores, similar tissue infected with M.perniciosa and of vegetative A.bisporus mycelium.

(a) = Extract of 0.5 g fresh weight of tissue / 13 mm AA disc

(b) = Mean width of inhibition zone, (mm), $\pm$ SEM

Extract origin - Tissue type (a)	Level of inhibition of extracts against test fungus (b)		
	M.perniciosa	A.bisporus	C.herbarum
Healthy A.bisporus sporophore cap tissue	10.65 ± 0.30	7.50 ± 0.0	9.28 ± 0.29
A.bisporus cap tissue infected with M.perniciosa	8.59 ± 0.34	0.00 ± 0.0	0.00 ± 0.0
A.bisporus vegetative mycelium	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0

Extract on AA discs
from:
Healthy A.bisporus   A.bisporus tissue   A.bisporus
   sporophore.   infected with   vegetative
                         M.perniciosa   mycelium

M.perniciosa

A.bisporus

PLATE 3.3.   Malt agar plate bioassays of chloroform extracts of
healthy and infected A.bisporus sporophores and A.bisporus vegetative
mycelium incubated for 10 days at 18 C.

Extracts equivalent to 0.5 gram fresh weight of tissue were placed
onto AA discs which were positioned centrally onto malt agar plates
seeded with M.perniciosa or Cladosporium herbarum or inoculated with
A.bisporus.   Antifungal activity was greatest in healthy A.bisporus
sporophores and least in vegetative mycelium.

Bars represent 2 cm.

Healthy A.bisporus   A.bisporus tissue
   sporophore   infected with
                         M.perniciosa

C.herbarum

C.herbarum

Extract on AA discs
from:
Healthy A.bisporus   A.bisporus tissue   A.bisporus
sporophore.   infected with   vegetative
                            M.perniciosa   mycelium

M.perniciosa

A.bisporus

PLATE 3.3.   Malt agar plate bioassays of chloroform extracts of
healthy and infected A.bisporus sporophores and A.bisporus vegetative
mycelium incubated for 10 days at 18°C.

Extracts equivalent to 0.5 gram fresh weight of tissue were placed
onto AA discs which were positioned centrally onto malt agar plates
seeded with M.perniciosa or Cladosporium herbarum or inoculated with
A.bisporus.   Antifungal activity was greatest in healthy A.bisporus
sporophores and least in vegetative mycelium.

Bars represent 2 cm.

Healthy A.bisporus   A.bisporus tissue
sporophore   infected with
                        M.perniciosa

C.herbarum

C.herbarum

Extract on AA discs
from:

| Healthy A.bisporus sporophore. | A.bisporus tissue infected with M.perniciosa | A.bisporus vegetative mycelium |

M.perniciosa

A.bisporus

PLATE 3.3.   Malt agar plate bioassays of chloroform extracts of
healthy and infected A.bisporus sporophores and A.bisporus vegetative
mycelium incubated for 10 days at 18°C.

Extracts equivalent to 0.5 gram fresh weight of tissue were placed
onto AA discs which were positioned centrally onto malt agar plates
seeded with M.perniciosa or Cladosporium herbarum or inoculated with
A.bisporus.   Antifungal activity was greatest in healthy A.bisporus
sporophores and least in vegetative mycelium.

Bars represent 2 cm.

| Healthy A.bisporus sporophore | A.bisporus tissue infected with M.perniciosa |

C.herbarum

C.herbarum

vegetative mycelium, indicating a lack of specificity in their action.

The activity of diffusates from the different tissues from A.bisporus sporophores on the growth of M.perniciosa was examined in order to identify any variation in antifungal activity within different parts of sporophores. Tissue from freshly picked 'button stage' sporophores, (c. 3 g) was excised under sterile conditions and placed on sterile cellophane over Czapek Dox agar in 9 cm Petri dishes for 72 h at 4°C. The sporophore tissue and cellophane was subsequently removed and the agar inoculated with M.perniciosa on the site previously covered by the A.bisporus tissue. Cultures were then incubated at 23°C $\pm$ 2°C, and linear growth recorded. Growth of M.perniciosa was significantly reduced in the presence of diffusates from gill tissue, 30.5% $\pm$ 1.1% reduction relative to untreated controls, (p <0.01), after 360 h, while diffusates from cap and stipe tissue had no significant effects. In a modification of this experiment in order to test for active compounds which may be inactivated when exposed to air, diffusion through cellophane from cap and stipe tissue was carried out under nitrogen at 4°C prior to inoculation of the agar with M.perniciosa. This resulted in the stimulation of growth of M.perniciosa of 36.6% $\pm$ 5.0% and 33.5% $\pm$ 2.7% relative to controls from cap and stipe tissue respectively, (Figure 3.3), indicating the presence of both inhibitory and stimulatory compounds within healthy sporophore tissue.

In order to determine whether antifungal activity present in sporophore tissue is in fact due to synthesis by A.bisporus and not to the surface microbial population, healthy sporophores grown under axenic conditions in 3.5 ℓ glass containers, as previously described, (Materials and Methods, I7), were extracted directly in chloroform at 4°C and tested

Growth of M.perniciosa (% relative to controls)

140
130
120
110
100
90
80
70
60

0 2 4 6 8 10 12 14 16 18 20 22 24 26

INCUBATION TIME (Days)
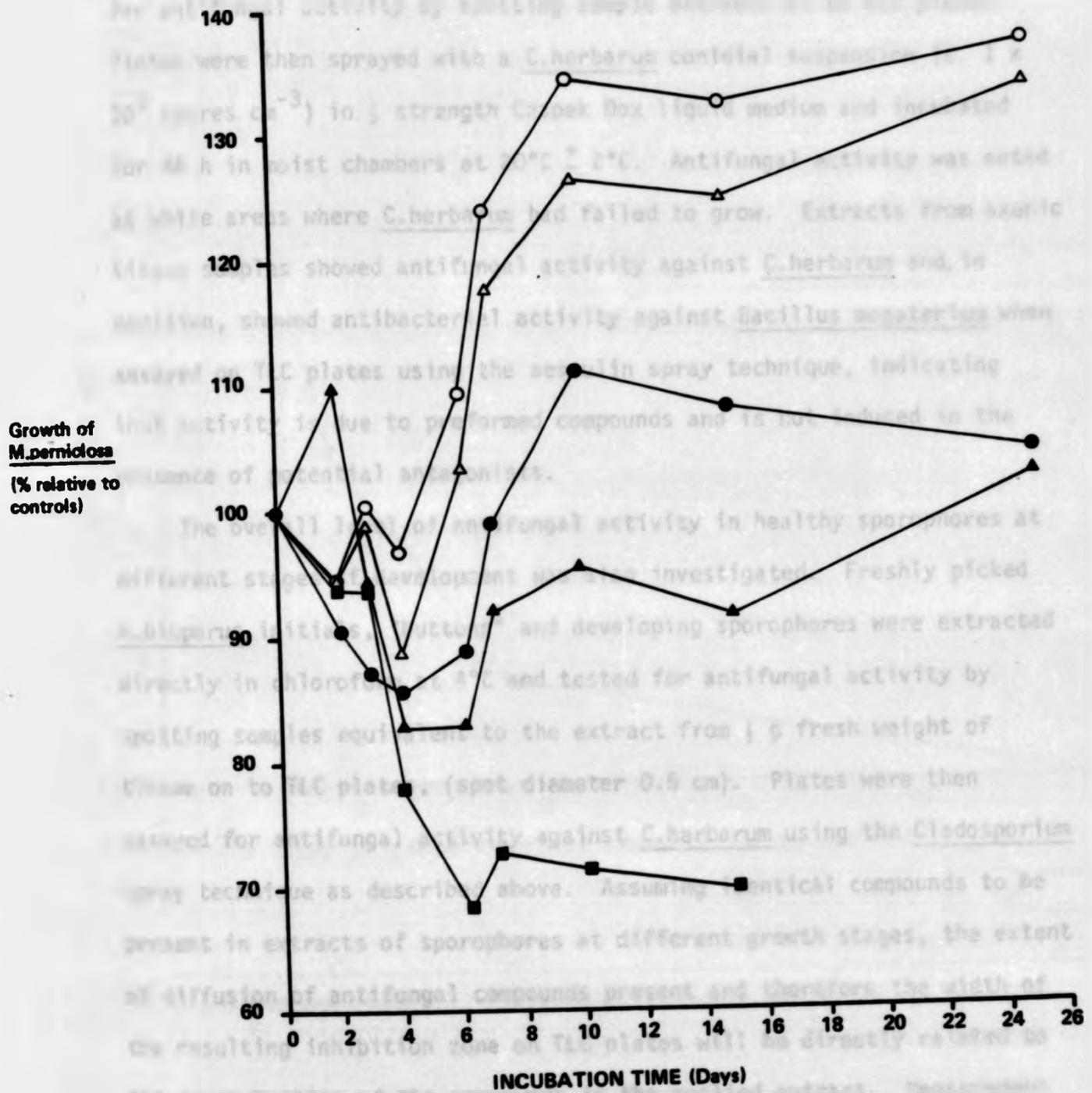
FIGURE 3.3   Mean change in linear growth of M.perniciosa relative to untreated controls on Czapek Dox agar containing diffusates through cellophane of A.bisporus sporophore tissue and incubated at 23°C:-

■—■ Gill tissue diffusates

●—● Cap tissue diffusates

▲—▲ Stipe tissue diffusates

○—○ Cap tissue diffusates (N_2)

△—△ Stipe tissue diffusates (N_2)
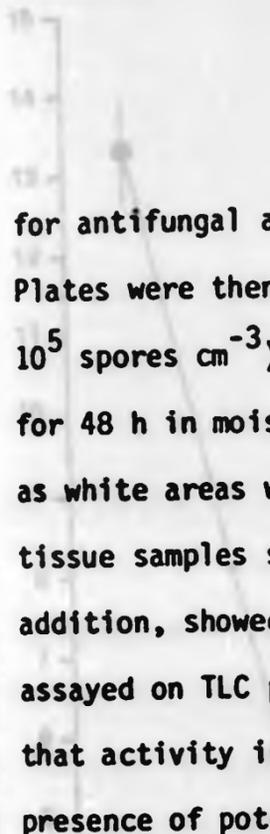
for antifungal activity by spotting sample extracts on to TLC plates. Plates were then sprayed with a C.herbarum conidial suspension (c. 1 x 10^5 spores cm^{-3}) in ⁴ strength Czapek Dox liquid medium and incubated for 48 h in moist chambers at 20°C ± 2°C. Antifungal activity was noted as white areas where C.herbarum had failed to grow. Extracts from axenic tissue samples showed antifungal activity against C.herbarum and, in addition, showed antibacterial activity against Bacillus megaterium when assayed on TLC plates using the aesculin spray technique, indicating that activity is due to preformed compounds and is not induced in the presence of potential antagonists.

The overall level of antifungal activity in healthy sporophores at different stages of development was also investigated. Freshly picked A.bisporus initials, "buttons" and developing sporophores were extracted directly in chloroform at 4°C and tested for antifungal activity by spotting samples equivalent to the extract from ⁴ g fresh weight of tissue on to TLC plates, (spot diameter 0.5 cm). Plates were then assayed for antifungal activity against C.herbarum using the Cladosporium spray technique as described above. Assuming identical compounds to be present in extracts of sporophores at different growth stages, the extent of diffusion of antifungal compounds present and therefore the width of the resulting inhibition zone on TLC plates will be directly related to the concentration of the compounds in the applied extract. Measurement of antifungal zone widths directly on TLC plates was, therefore, used as a measure of the overall antifungal activity of the sporophore extracts.

Antifungal activity was seen to decline with sporophore maturation, (Figure 3.4), with initials showing the highest level of inhibition.
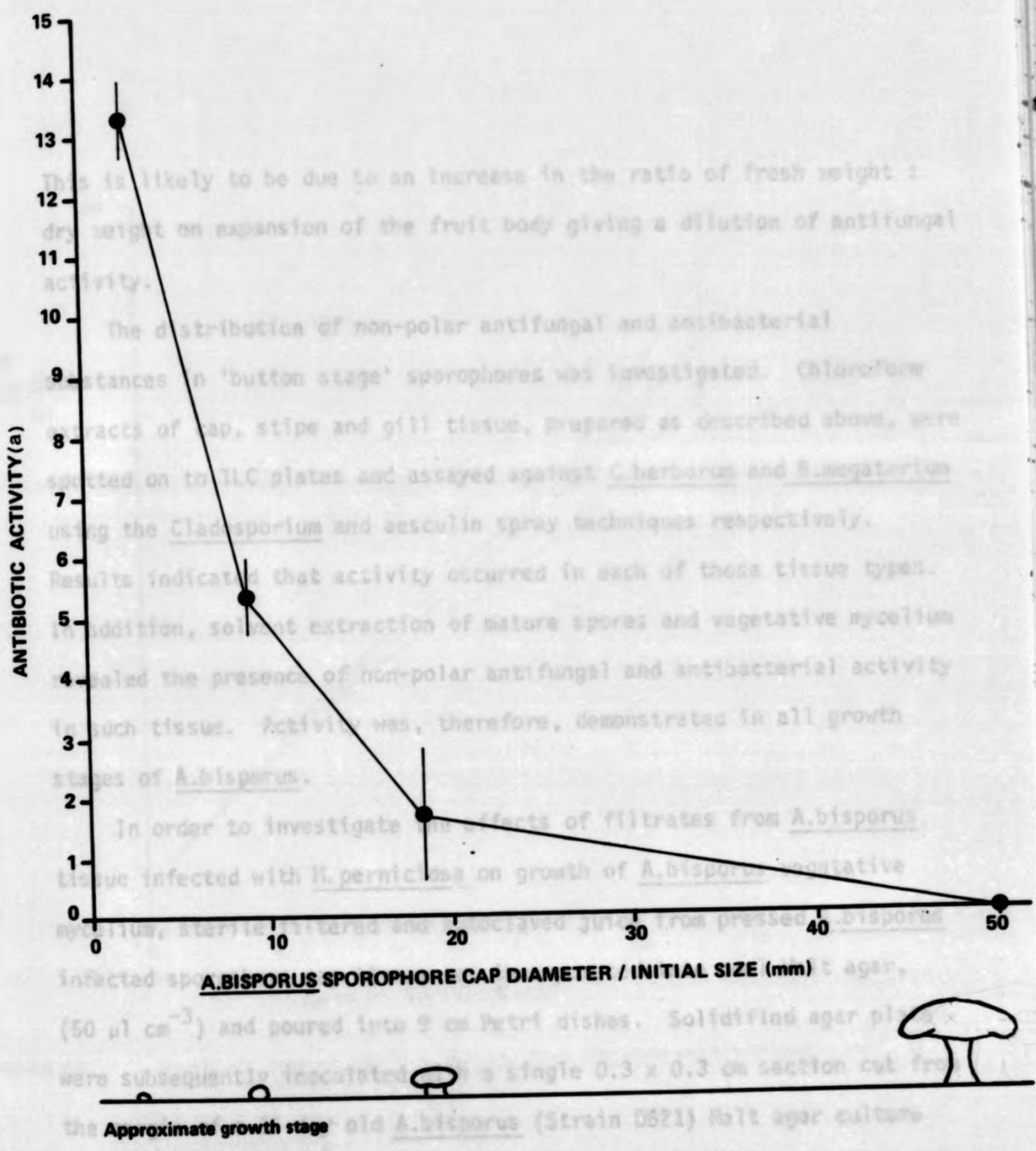
FIGURE 3.4 **Antibiotic activity of chloroform extracts of healthy A.bisporus sporophores at various stages of development. Sporophores were harvested from commercial cropping beds.**

(a) = TLC zone width (½ gram fresh tissue weight) on C.herbarum bioassay plates (mm, mean values ± SEM)

This is likely to be due to an increase in the ratio of fresh weight :
dry weight on expansion of the fruit body giving a dilution of antifungal
activity.

The distribution of non-polar antifungal and antibacterial
substances in 'button stage' sporophores was investigated. Chloroform
extracts of cap, stipe and gill tissue, prepared as described above, were
spotted on to TLC plates and assayed against C.herbarum and B.megaterium
using the Cladosporium and aesculin spray techniques respectively.
Results indicated that activity occurred in each of these tissue types.
In addition, solvent extraction of mature spores and vegetative mycelium
revealed the presence of non-polar antifungal and antibacterial activity
in such tissue. Activity was, therefore, demonstrated in all growth
stages of A.bisporus.

In order to investigate the effects of filtrates from A.bisporus
tissue infected with M.perniciosa on growth of A.bisporus vegetative
mycelium, sterile filtered and autoclaved juice from pressed A.bisporus
infected sporophore cap tissue was incorporated into cool Malt agar,
(50 μl cm^{-3}) and poured into 9 cm Petri dishes. Solidified agar plates
were subsequently inoculated with a single 0.3 x 0.3 cm section cut from
the margin of a 15 day old A.bisporus (Strain D621) Malt agar culture
and incubated at 20°C $\pm$ 1°C. Linear growth of A.bisporus was not
affected by the presence of filtrates of either healthy or infected
sporophore tissue whether sterilised by filtration or by autoclaving,
relative to controls of A.bisporus grown on Malt agar in the absence of
tissue filtrates, (Figure 3.5). The general nature of growth of
A.bisporus was, however, altered, with cultures on media containing
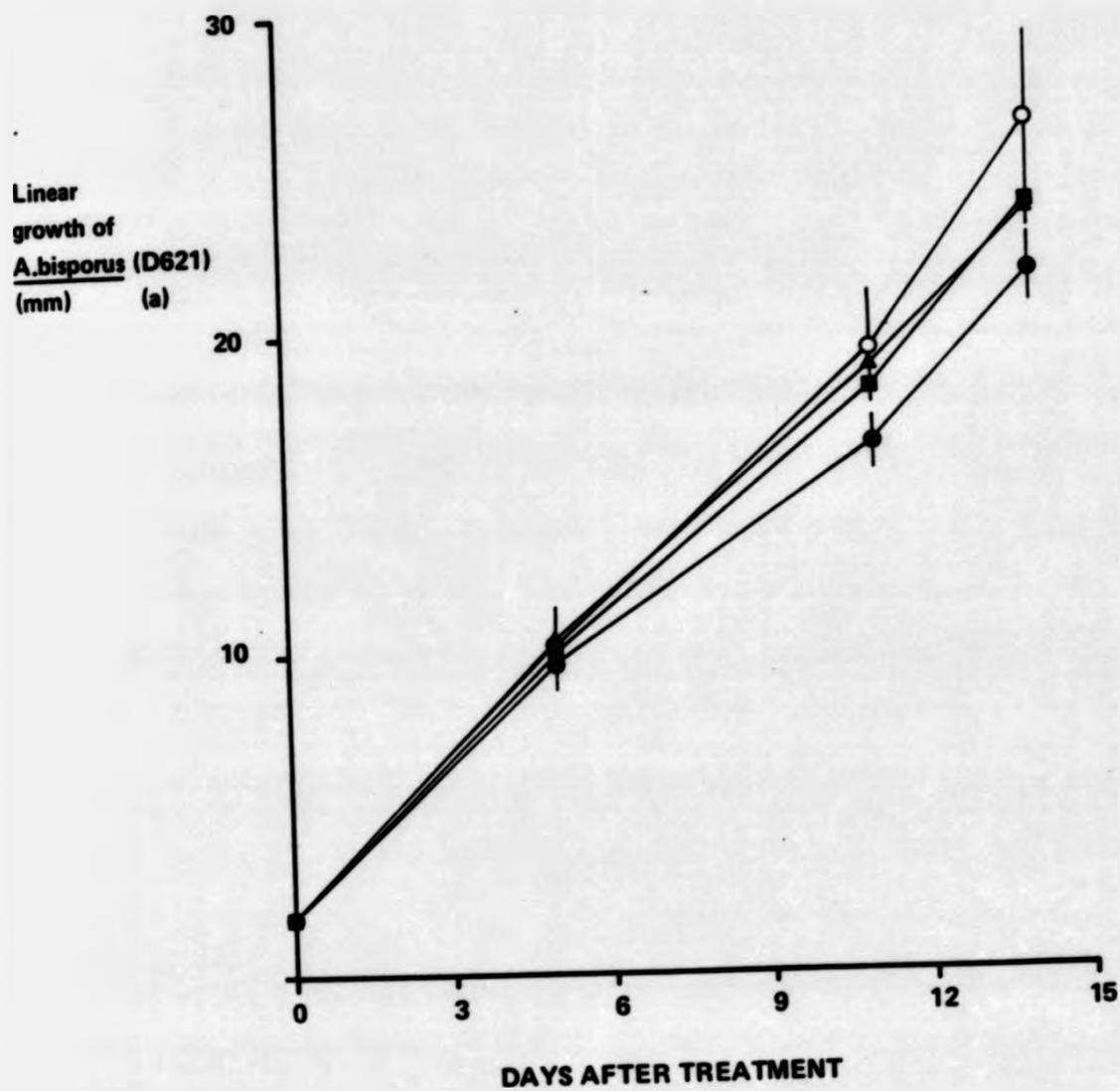
FIGURE 3.5   Growth of A.bisporus (D621) on Malt agar containing crude extracts of healthy and infected material and incubated at 20°C.

●—●   Autoclaved juice of pressed __A.bisporus__ sporophores (button stage) infected with __M.perniciosa__

■—■   Sterile filtered juice of pressed healthy __A.bisporus__ sporophore cap tissue

▲—▲   Sterile filtered juice of pressed A.bisporus sporophore tissue infected with __M.perniciosa__

O—O   Control — __A.bisporus__ on Malt agar only

(a) Mean values ± SEM

filtrates from healthy sporophores of an apparently sparser nature than those containing filtrates from A.bisporus tissue infected with M.perniciosa, (Plate 3.4).

3. Fractionation of antifungal and stimulatory substances

Samples showing antifungal and stimulatory activity were extracted from healthy A.bisporus tissue and M.perniciosa mycelium in a range of solvents prior to chromatographic separation. Fractionated compounds were subsequently subjected to a number of tests in order to determine the nature of active compounds.

A. Extraction of M.perniciosa mycelium

M.perniciosa mycelium was extracted initially using ice cold methanol, followed by fractionation of extracts, following concentration on a rotary evaporator, in Sephadex LH 20 on a 31 cm column, (flow rate 2 cm^3 min.$^{-1}$). Sample volume was 2 cm^3. Fractions were tested for antifungal activity by spotting on to TLC plates and assaying using the Cladosporium spray technique. Active fractions were subsequently separated on TLC plates, eluting in chloroform:methanol, (6:1 or 8:1), with the position of antifungal activity being determined on developed chromatograms using the Cladosporium spray technique. A number of modifications of the extraction procedure were tested and it was found that separation was equally good with a simplified procedure of extraction of M.perniciosa mycelium directly in chloroform and subsequently partitioning the solvent extract in methanol. Antifungal activity was retained in the chloroform fraction which was pale yellow

PLATE 3.4.   Plate cultures of A.bisporus (strain D621) incubated at
20°C for 15 days:-

i)   on malt agar

ii) on malt agar containing sterile filtrates from A.bisporus
sporophores infected with M.perniciosa, at the rate of (50µl cm^{-3}).
Note the abundance of aerial growth of A.bisporus in the presence of
low concentrations of extracts from infected tissue.

i)

ii)

PLATE 3.4.   Plate cultures of A.bisporus (strain D621) incubated at 20°C for 15 days:-

i)   on malt agar

ii) on malt agar containing sterile filtrates from A.bisporus sporophores infected with M.perniciosa, at the rate of (50µl cm^{-3}). Note the abundance of aerial growth of A.bisporus in the presence of low concentrations of extracts from infected tissue.

i)

ii)

PLATE 3.4.   Plate cultures of A.bisporus (strain D621) incubated at 20°C for 15 days:-

i) on malt agar

ii) on malt agar containing sterile filtrates from A.bisporus sporophores infected with M.perniciosa, at the rate of (50µl cm^{-3}). Note the abundance of aerial growth of A.bisporus in the presence of low concentrations of extracts from infected tissue.

in colour and had no distinguishing smell. Samples exhibited a
characteristic U.V. absorption spectrum over the range 239.5 nm -
367.4 nm, (Figure 3.6). Chloroform extracts were separated directly on
TLC plates. The assay of chloroform extracts of mycelium from
M.perniciosa liquid malt cultures incubated for 10 days at 20°C $\pm$ 2°C
using the Cladosporium spray technique revealed the presence of a number
of biologically active compounds. Zones inhibiting fungal growth were
observed on chromatograms of mycelial extracts, developed in chloroform :
methanol (4:1) over the band Rf. 0.13 - 0.52. Overall antifungal
activity was seen to be greater in cultures grown in media containing
sterile filtered juice from pressed healthy 'button stage' A.bisporus
sporophores than in cultures grown on defined media, (Plate 3.5). Lipid
extraction of M.perniciosa mycelium using the method of Deven and
Manocha, (1975), (Materials and Methods IV 5), retained antifungal
activity in the chloroform fraction. Development of chromatograms of
chloroform extracts in toluene : acetic acid : water, (4:1:5) resulted
in antifungal activity remaining on the base line, indicating that
activity is not due to the presence of phenolic substances. Development
of chromatograms in chloroform:methanol (6:1) showed antifungal activity
to occur in a single band at Rf. 0.24, (Plate 3.6). This active zone
exhibited peak U.V. absorption at 234.2 nm, (Figure 3.7). In addition,
this antifungal zone was seen to have antibacterial activity when assayed
against Pseudomonas tolaasi, (NCPPB 1116), using the aesculin spray
technique.

Chloroform fractions from M.perniciosa mycelium lipid extracts
prepared as described above were tested with a range of spray reagents :
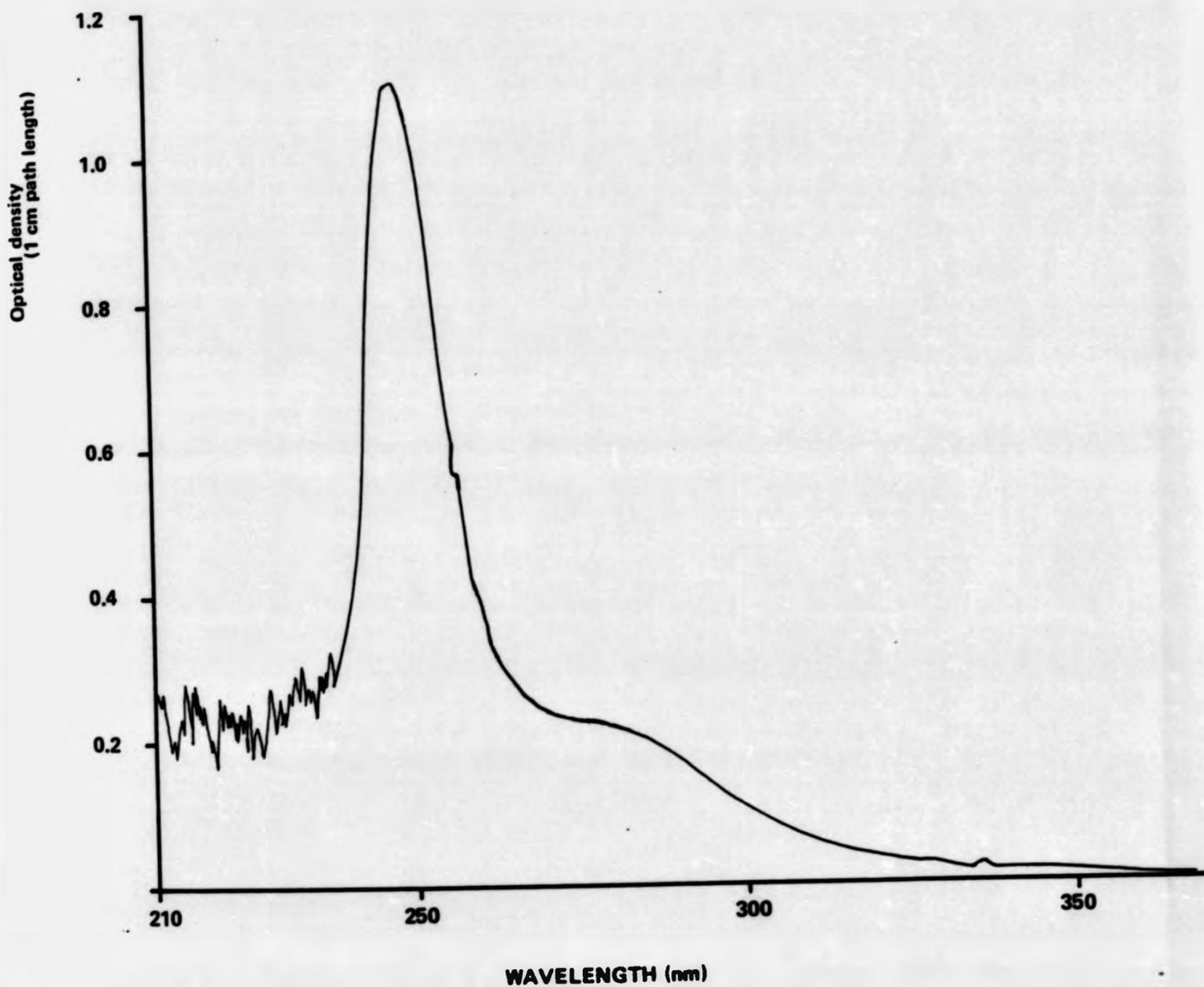
FIGURE 3.6 Absorption spectrum of M.perniciosa chloroform extract. Mycelial extracts (0.064g cm³) were partitioned in methanol:chloroform, and the chloroform phase examined using a Pye Unicam SP1800 spectrophotometer.

PLATE 3.5.   TLC Cladosporium bioassay of 10 day old M.perniciosa
              culture filtrates.

Note the presence of two antifungal bands in uninoculated Czapek Dox
liquid medium containing filtrates from healthy A.bisporus
sporophores. (3).   One of these bands appears to have been metabolised
by M.perniciosa.

Antifungal activity is greatest in M.perniciosa culture filtrates
containing sterile filtered extracts of healthy A.bisporus sporophores
(1), when compared to culture filtrates from M.perniciosa grown on
defined Czapek Dox media, (2).

Chromatogram developed in Chloroform : Methanol   4 : 1.

Sample volume:- 100 µl of extract equivalent to 0.5 gram fresh weight
of tissue was spotted onto 2.5 cm origins.

<div style="text-align:center">

1   2   3

</div>

PLATE 3.5.   TLC Cladosporium bioassay of 10 day old M.perniciosa
culture filtrates.

Note the presence of two antifungal bands in uninoculated Czapek Dox
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defined Czapek Dox media, (2).

Chromatogram developed in Chloroform : Methanol 4 : 1.

Sample volume:- 100 µl of extract equivalent to 0.5 gram fresh weight
of tissue was spotted onto 2.5 cm origins.

| 1 | 2 | 3 |

PLATE 3.5. TLC Cladosporium bioassay of 10 day old M.perniciosa
            culture filtrates.

Note the presence of two antifungal bands in uninoculated Czapek Dox
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Antifungal activity is greatest in M.perniciosa culture filtrates
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(1), when compared to culture filtrates from M.perniciosa grown on
defined Czapek Dox media, (2).

Chromatogram developed in Chloroform : Methanol 4 : 1.

Sample volume:- 100 µl of extract equivalent to 0.5 gram fresh weight
of tissue was spotted onto 2.5 cm origins.

1   2   3   4

PLATE 3.6.   TLC Cladosporium bioassay of M.perniciosa mycelium extracted in:-

1.   Water

2.   Methanol

3.   Methanol partitioned fraction.

4.   Chloroform partitioned fraction.

Chromatogram development was in chloroform : methanol 6 : 1.

A single band of antifungal activity was retained in the chloroform fraction. (arrowed).
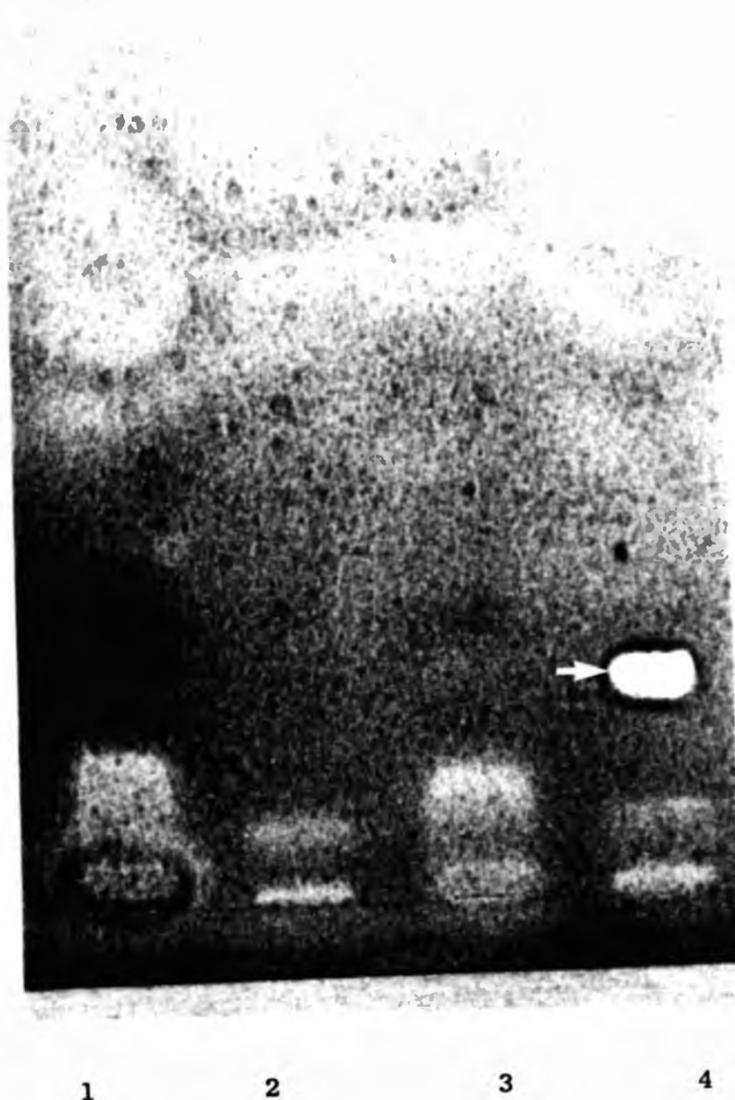
PLATE 3.6.   TLC _Cladosporium_ bioassay of <u>M.perniciosa</u> mycelium
extracted in:-

1.   Water

2.   Methanol

3.   Methanol partitioned fraction.

4.   Chloroform partitioned fraction.

Chromatogram development was in chloroform : methanol 6 : 1.

A single band of antifungal activity was retained in the
chloroform fraction. (arrowed).

1   2   3   4

PLATE 3.6.   TLC Cladosporium bioassay of M.perniciosa mycelium extracted in:-

1.   Water

2.   Methanol

3.   Methanol partitioned fraction.

4.   Chloroform partitioned fraction.

Chromatogram development was in chloroform : methanol 6 : 1.

A single band of antifungal activity was retained in the chloroform fraction. (arrowed).

Optical density (1 cm path length)

WAVELENGTH (nm)
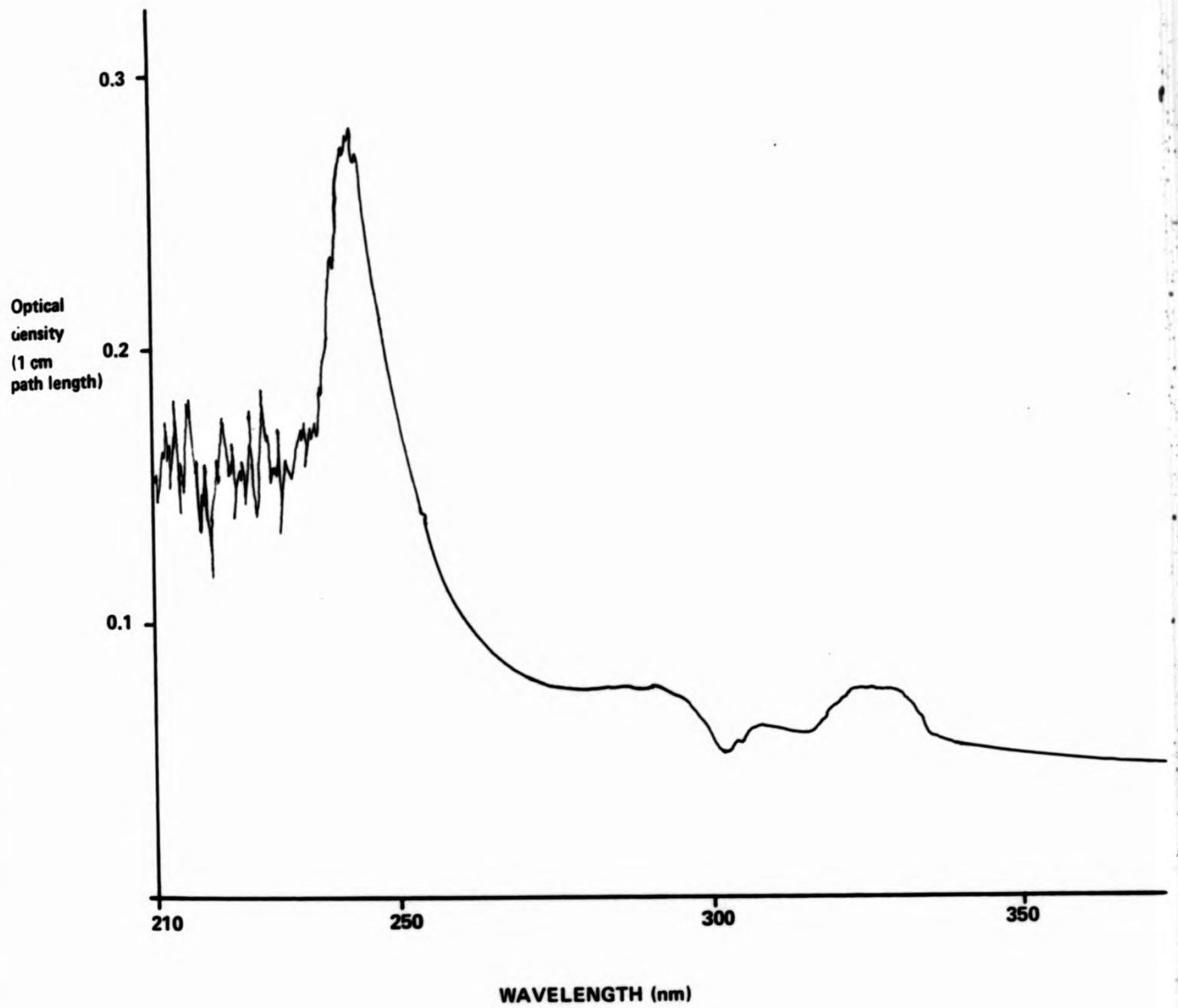
FIGURE 3.7   Absorption spectrum of M.perniciosa antifungal/antibacterial zone extracted using the method of Deven and Manocha (1975) after isolation from TLC plates (Rf. 0.24) following chromatogram development in chloroform:methanol (6:1)
Samples were examined using a Pye Unicam SP1800 spectrophotometer at a concentration of 0.064 g fresh weight cm^{-3}

directly on TLC plates in order to determine the nature of the anti-
fungal zone, (Materials and Methods IV 5). Developed chromatograms
were sprayed with 50% concentrated sulphuric acid prior to heating to
180°C for 30 min. The antifungal band at Rf. 0.24 did not coincide
with bands visualised using this method, nor with those noted on
standing developed chromatograms in a closed chromatography tank
containing iodine crystals indicating that the active zone contains a
compound of low molecular weight.

The antifungal band at Rf. 0.24, on untreated chromatograms was
noted as not coinciding with bands fluorescing under U.V. light,
(366 nm and 254 nm) indicating an absence of unsaturated bonds within
the antifungal band. In addition, TLC plates were tested with a range
of spray reagents, (Materials and Methods IV 3), in order to identify
chemically active groups within the antifungal zone. A positive
reaction was observed only on spraying the active zone with 3% vanillin
in ethanol containing 0.5% sulphuric acid followed by heating the sample
to 120°C, resulting in a blue colouration of the active zone, indicating
the presence of a higher alcohol group.

In addition to antifungal activity of M.perniciosa mycelial
extracts, it was observed that M.perniciosa may produce stimulatory
compounds, demonstrated on a single unreplicated TLC plate bioassay.
Chloroform extracts of M.perniciosa mycelium showed stimulatory activity
at Rf. 0.63 and 0.45 on chromatograms developed in chloroform : methanol,
(8:1) when assayed against C.herbarum, (Plate 3.7).

M.perniciosa was seen, therefore, to produce a number of biologically
active compounds. While no attempt was made to determine the nature of

PLATE 3.7.   TLC _Cladosporium_ bioassay of 10 day old _M.perniciosa_ culture filtrates extracted directly in chloroform at 4°C.

Chromatogram was developed in chloroform : methanol 8 : 1.

Note the presence of stimulatory zones on this unreplicated TLC plate.

Samples grown in Czapek Dox liquid medium with the addition of sterile filtrates from healthy _A.bisporus_ sporophores showed stimulatory activity, (arrowed) which was not noted in filtrates of uninoculated media.

PLATE 3.7.   TLC <u>Cladosporium</u> bioassay of 10 day old <u>M.perniciosa</u>
culture filtrates extracted directly in chloroform at 4°C.

Chromatogram was developed in chloroform : methanol 8 : 1.

Note the presence of stimulatory zones on this unreplicated
TLC plate.

Samples grown in Czapek Dox liquid medium with the addition of
sterile filtrates from healthy <u>A.bisporus</u> sporophores showed
stimulatory activity, (arrowed) which was not noted in filtrates
of uninoculated media.

PLATE 3.7.   TLC Cladosporium bioassay of 10 day old M.perniciosa
culture filtrates extracted directly in chloroform at 4°C.

Chromatogram was developed in chloroform : methanol 8 : 1.

Note the presence of stimulatory zones on this unreplicated
TLC plate.

Samples grown in Czapek Dox liquid medium with the addition of
sterile filtrates from healthy A.bisporus sporophores showed
stimulatory activity, (arrowed) which was not noted in filtrates
of uninoculated media.

stimulatory substances the antifungal activity of M.perniciosa,
increased in the presence of the host and was seen to be due to the
production of a small molecular weight alcoholic substance.

B. Extraction of antifungal substances from healthy sporophores

Activity was detected in chloroform fractions following solvent
extraction in chloroform and partitioning in methanol. In order to
reduce the level of impurities in the sample and improve partitioning
the procedure subsequently adopted was of extraction of the tissue
directly in chloroform, (4°C, X30 volume), over anhydrous sodium sulphate
(1 g g^{-1} fresh weight of tissue) with subsequent partitioning in methanol.
The retained chloroform fraction was than concentrated on a rotary
evaporator. Resultant extracts were straw coloured, and with a
distinct mushroom smell, having a characteristic U.V. absorption spectrum,
(Figure 3.8), with peak absorption at 245.6 nm.

Initially, all extraction procedures were performed in the dark, as
was subsequent development of chromatograms and bioassay of extracted
material, in order to ensure the demonstration of activity of any
compounds unstable when exposed to light. All antibiotics were, however,
fully light stable and no activity was lost in extraction under normal
laboratory conditions. Chromatograms of chloroform extracts from healthy
A.bisporus sporophores on TLC plates were initially separated in hexane :
acetone, (1:1.5), resulting in antifungal activity occurring at Rf. 0.21,
0.53 and 0.66, activity being identified using the Cladosporium spray
technique. In order to facilitate comparisons between extracts eluted in
different solvent systems, the three bands noted above were termed

FIGURE 3.8   **Absorption spectrum of chloroform extracts of A.bisporus 'button stage' sporophores. Extraction was within 1 hour of harvest from commercial beds. Samples were examined using a Pye Unicam SP1800 spectrophotometer at a concentration of 0.112 g fresh weight cm⁻³**

'Active Component' (AC) I; ACII and ACIII respectively. Separation was further improved by adopting chloroform:methanol, (6:1) as the elutant. Examination of developed chromatograms under U.V. light revealed a number of bands able to fluoresce at either 366 nm or 254 nm. Removal of these bands and of intermediary non-fluorescing bands by scraping the silica from the developed chromatogram followed by resuspending the extract components from individual bands in chloroform, with filtration of the suspension to remove any residual silica. Antifungal activity occurred at Rf. 0.29, 0.75 and 0.95, corresponding to the three bands noted above, all other bands increased germination of C.herbarum conidia relative to controls, when assayed using the glass slide bioassay technique, (Figure 3.9, Table 3.4).

On assaying developed chromatograms against M.perniciosa using the conidial suspension spray technique followed by visualisation of anti-fungal zones by browning of the M.perniciosa mycelium in the presence of iodine vapour in closed chromatography tanks, band ACIII (Rf. 0.95) only was seen to inhibit the pathogen. ACIII also showed antibacterial activity against Bacillus megaterium and Pseudomonas tolaasii when assayed using the aesculin spray technique.

Active compounds were characterised using a range of reagents. Extraction prior to testing was by the lipid extraction procedure of Deven and Manocha, (1975), (Materials and Methods IV 5), retaining antifungal activity in the chloroform fraction with the advantage of higher purity of unfractionated samples compared to previous procedures. Development of chromatograms was in chloroform:methanol, (6:1), resulting in antibiotic activity of ACI, ACII and ACIII as described above.

% Germination relative to controls (b)

160
150
140
130
120
110
100
90
80
70
60
50
40
30

0   0.1   0.2   0.3   0.4   0.5   0.6   0.7   0.8   0.9   0.10

R f value (a)
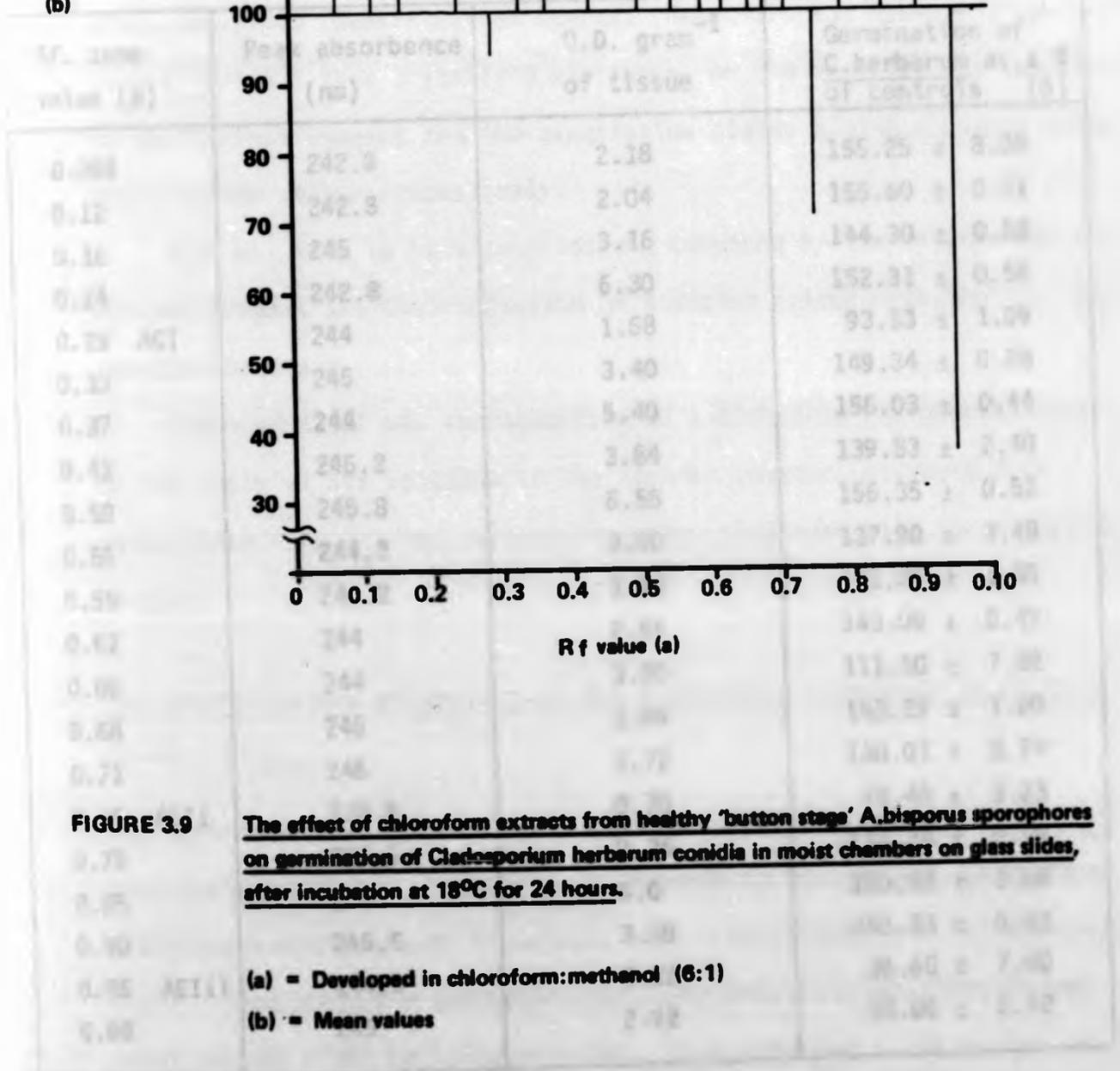
FIGURE 3.9   The effect of chloroform extracts from healthy 'button stage' A.bisporus sporophores on germination of Cladosporium herbarum conidia in moist chambers on glass slides, after incubation at 18°C for 24 hours.

(a) = Developed in chloroform:methanol (6:1)

(b) = Mean values

Table 3.4

Characteristics of fractionated components of healthy 'button stage' A.bisporus sporophores separated by TLC following extraction in chloroform

(a) = Chromatograms developed in chloroform:methanol 6 : 1.

(b) = Germination as a % of controls, (63.75% ± 1.65% germination) on glass slides in moist chambers after incubation at $18^{\circ}C$ for 24 hours in the absence of sample fractions, Mean Value ± SEM.

Rf. zone value (a)	Peak absorbence (nm)	O.D. gram^{-1} of tissue	Germination of C.herbarum as a % of controls (b)
0.069	242.8	2.18	155.25 ± 8.08
0.12	242.8	2.04	155.60 ± 0.21
0.16	245	3.16	144.30 ± 0.58
0.24	242.8	6.30	152.31 ± 0.56
0.29 ACI	244	1.68	93.53 ± 1.09
0.33	245	3.40	149.34 ± 0.28
0.37	244	5.40	156.03 ± 0.44
0.43	246.2	3.84	139.83 ± 2.40
0.50	245.8	6.56	156.35 ± 0.52
0.55	244.2	3.00	137.90 ± 3.48
0.59	246.2	3.84	152.06 ± 1.35
0.62	244	2.56	143.09 ± 0.49
0.66	244	3.00	111.50 ± 7.82
0.68	245	2.84	143.29 ± 1.00
0.71	246	3.72	130.01 ± 3.74
0.75 ACII	239.5	0.70	70.46 ± 3.23
0.79	237.5	0.36	154.28 ± 0.29
0.85	244	6.0	150.52 ± 2.66
0.90	246.5	3.40	153.33 ± 0.22
0.95 ACIII	244.5	2.82	36.60 ± 7.60
0.98	245	2.92	99.06 ± 2.42

Visualisation of lipidic substances was achieved by spraying developed chromatograms with 50% concentrated sulphuric acid and charring plates in an oven at 180°C for 30 min. ACI, ACII and ACIII were each seen to char with H_2SO_4 indicating that they are each lipid substances. Further characterisation of antifungal bands performed using the lipid identification scheme, (Materials and Methods IV 5), showed that ACI is a glycolipid substance, on the basis of its reaction to the Orcinol reagent and the ganglioside stain, giving a purple colour and a yellow colour respectively.

ACII was seen to be a gangliosidic compound by its reaction to the Orcinol reagent and the production of a violet colour with the ganglioside stain.

Compound ACIII was characterised as a Ninhydrin +ve phospholipid on the basis of its reaction to the Zinadze reagent, giving a blue colouration, and its red colouration after treatment with the Ninhydrin reagent.

4. The identification of antifungal and stimulatory compounds in sclero-dermoid tissue

Extraction of sclerodermoid tissue in chloroform:methanol as previously outlined, (Materials and Methods IV 5), yielded chloroform fractions reddish orange in colour, with a characteristic 'oily mushroom' smell. Samples had a characteristic U.V. absorption spectrum in the range 240 nm - 350 nm, (Figure 3.10). This was seen to be unique to sclerodermoid tissue and could not be mimicked by the combination of chloroform extracts from healthy 'button stage' sporophores and from

FIGURE 3.10 Absorption spectrum of chloroform extracts of mature sclerodermoid tissue. Samples were examined using a Pye Unicam SP1800 spectrophotometer at a concentration of 0.42 g fresh weight cm^{-3}.

M.perniciosa mycelium from 10 day old liquid Czapek Dox / mushroom filtrate medium cultures, (20°C $\pm$ 2°C) in either 1:1 or 4.05:1 ratios, (g g^{-1} fresh weight), (Figure 3.11), indicating novel metabolism of sclerodermoid tissue compared to the two constitutive fungi alone.

Investigation of antifungal activity by spotting samples directly on to TLC plates and testing for activity using the Cladosporium spray technique indicated that antifungal activity was retained in the chloroform fraction, (Plate 3.2). Initial separation of chloroform extracts from sclerodermoid tissue using ascending paper chromatography, (chloroform:methanol, 6:1) with assay of developed chromatograms using the Cladosporium spray technique revealed the presence of both antifungal and stimulatory bands, (Rf. 0.95 and 0.90 respectively), (Plate 3.8).

TLC separation of chloroform fractions in chloroform:methanol (6:1) with subsequent assay using the Cladosporium spray technique gave better resolution with the demonstration of two further antifungal bands at Rf. 0.64 and 0.77, in addition to antifungal activity at Rf. 0.95 which corresponded with the activity observed in healthy tissue extracts, (Plate 3.9).

All bands on developed chromatograms of chloroform extracts of sclerodermoid tissue corresponding to sectors seen to fluoresce under U.V. light, (366 nm and 254 nm) in addition to intermediary non-fluorescing bands were removed, resuspended separately in chloroform, and filtered to remove residual silica, prior to quantitative assessment of individual zones for antifungal or stimulatory activity against C.herbarum conidia, (Figure 3.12, Table 3.5). The increased sensitivity of the glass slide bioassay demonstrated a greater range of stimulatory
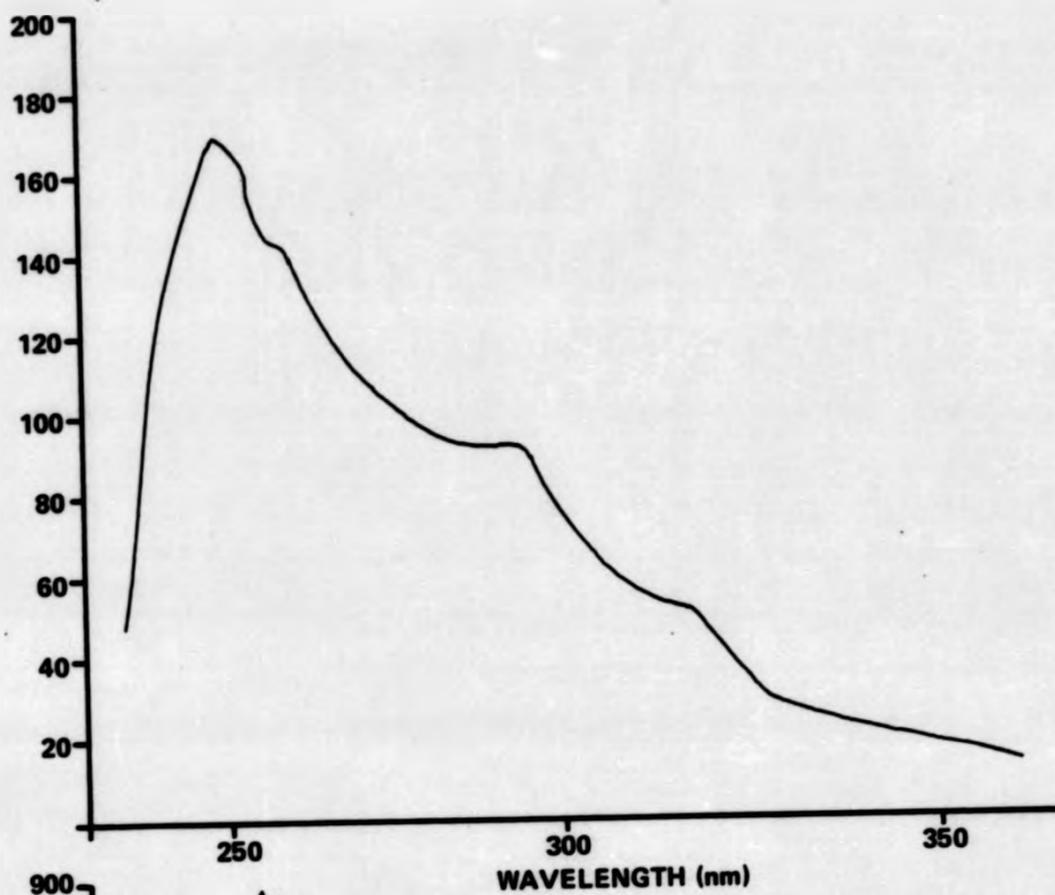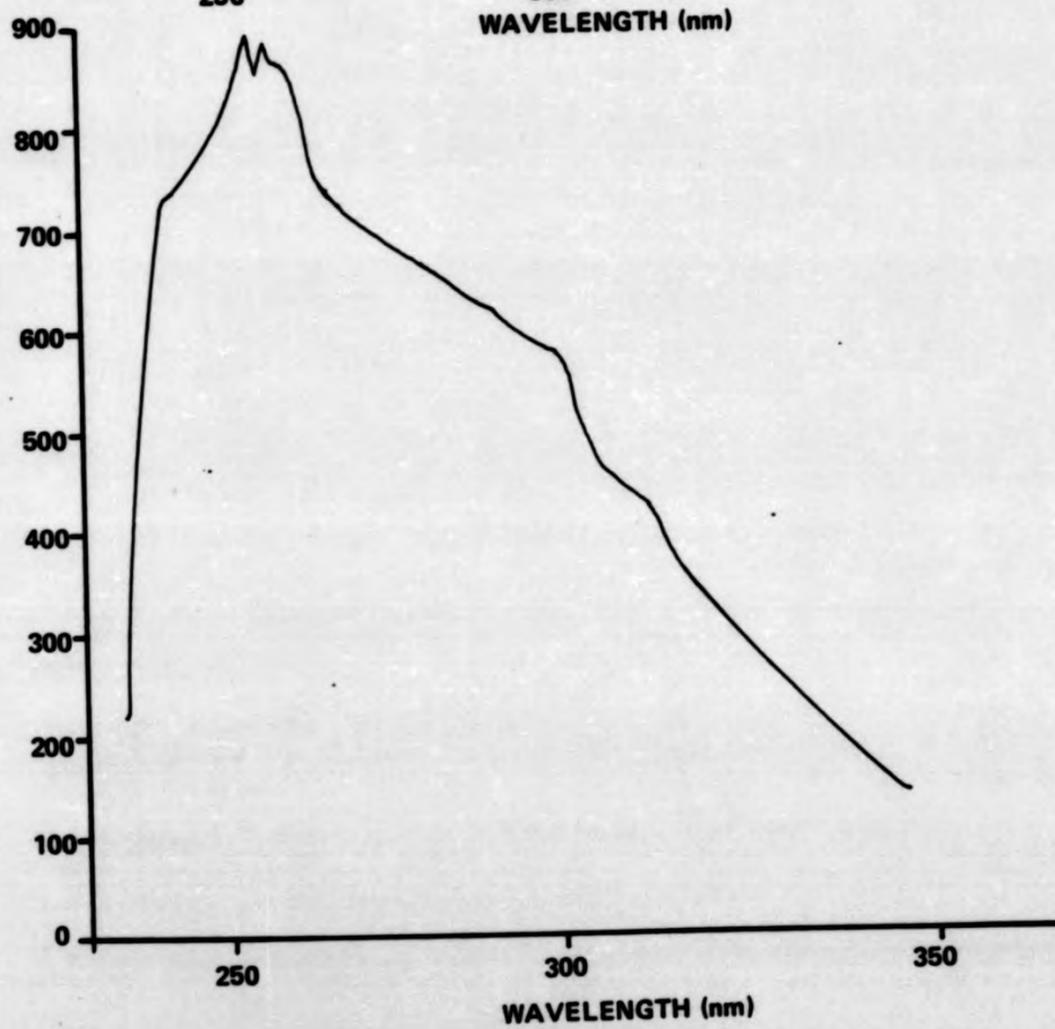
FIGURE 3.11   Absorption spectra of combined extracts from M.perniciosa mycelium from 10 day old Czapek Dox/mushroom filtrate cultures and complete A.bisporus 'button stage' sporophore chloroform extracts, (a) = 1:1, (b) = 1:4.05 g:g fresh weight.

1   2

PLATE 3.8.   Cladosporium bioassay of a paper chromatogram
of chloroform extracts of healthy A.bisporus sporophore and
sclerodermoid tissue.

Note the presence of antifungal (Af) and simulatory (s) zones
in sclerodermoid tissue, the latter zone not occurring in
healthy tissue.

Chromatogram developed in chloroform : methanol 6 : 1.

1 = extract of mature sclerodermoid tissue

2 = healthy sporophore (button stage) extract.

1   2

PLATE 3.8.   _Cladosporium_ bioassay of a paper chromatogram
of chloroform extracts of healthy _A.bisporus_ sporophore and
sclerodermoid tissue.

Note the presence of antifungal (Af) and simulatory (s) zones
in sclerodermoid tissue, the latter zone not occurring in
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healthy tissue.

Chromatogram developed in chloroform : methanol 6 : 1.

1 = extract of mature sclerodermoid tissue

2 = healthy sporophore (button stage) extract.

Chloroform extractions

Solvent - Chloroform 6 : 1 Methanol

Healthy mush'   Sclero'

1   2

PLATE 3.9.   TLC _Cladosporium_ bioassay of healthy
A.bisporus 'button stage' sporophores (1) and mature
sclerodermoid tissue (2) chloroform extracts showing
antifungal activity in both healthy and infected tissue,
(inhibitory zones arrowed).

Chromatogram developed in chloroform : methanol 6 : 1.

Chloroform extractions

Solvent :- Chloroform 6 : 1 Methanol

1   2

PLATE 3.9.   TLC <u>Cladosporium</u> bioassay of healthy
A.bisporus 'button stage' sporophores (1) and mature
sclerodermoid tissue (2) chloroform extracts showing
antifungal activity in both healthy and infected tissue,
(inhibitory zones arrowed).

Chromatogram developed in chloroform : methanol 6 : 1.

Chloroform extractions

'Solvent :- Chloroform 6 : 1 [illegible]

1   2

PLATE 3.9. TLC <u>Cladosporium</u> bioassay of healthy
<u>A.bisporus</u> 'button stage' sporophores (1) and mature
sclerodermoid tissue (2) chloroform extracts showing
antifungal activity in both healthy and infected tissue,
(inhibitory zones arrowed).

Chromatogram developed in chloroform : methanol 6 : 1.

% Germination
relative to
controls (b)

Rf value (a)

FIGURE 3.12   The effect of chloroform extracts from mature sclerodermoid tissue on germination of Cladosporium herbarum conidia in moist chambers on glass slides, after incubation at 18°C for 24 hours.

(a) = Developed in chloroform:methanol (6:1)

(b) = Mean values

Table 3.5

Characteristics of fractionated components of mature sclerodermoid tissue extracted in chloroform following separation by TLC and elution of individual zones in chloroform.

(a) = Chromatograms developed in chloroform:methanol (6:1)

(b) = Germination as a % of controls in the absence of sample fractions.

Control = 67.2% $\pm$ 8.8% germination.

Rf. zone value (a)	Peak absorbance (nm)	O.D/gram tissue	Germination of C.herbarum as a % of controls (b)
0.0	-	-	
0.11	-	-	94.05 ± 2.01
0.15	-	-	61.6 ± 5.4
0.19	-	-	14.0 ± 6.4
0.23	-	-	103.5 ± 7.8
0.25	-	-	8.5 ± 1.9
0.29	-	-	90.15 ± 4.56
0.34	-	-	109.46 ± 0.81
0.37	-	-	51.93 ± 2.23
0.39	246.5	0.22	95.37 ± 0.94
0.42	244,283	0.52,0.34	79.58 ± 3.6
0.44	247	0.27	88.86 ± 2.02
0.47	243	0.17	85.23 ± 2.54
0.51	247	0.34	58.41 ± 1.2
0.56	241	0.27	71.37 ± 1.87
0.62	247	0.29	59.57 ± 1.47
0.67	-	0.18	31.69 ± 2.33
0.73	249	0.39	113.32 ± 1.83
0.76	241	0.54	26.99 ± 0.6
0.79	-	0.21	103.65 ± 6.21
0.82	244,280.5	0.51	64.29 ± 1.54
0.85	263	1.27	81.59 ± 1.0
0.89	240,278	0.34,0.31	105.61 ± 3.14
0.94	272	0.26	26.55 ± 0.89
0.98	250.9	0.66	74.06 ± 1.06

and inhibitory substances than initially indicated using the TLC spray technique. Sclerodermoid tissue extracts contained five bands reducing germination of C.herbarum conidia by at least 50%, at Rf. 0.19, 0.25, 0.67, 0.76 and 0.94. In addition, five zones capable of increasing germination of C.herbarum conidia were demonstrated, at Rf. 0.23, 0.34 0.73, 0.79 and 0.90, although changes in germination % for these compounds was small, 3.5% - 13.5% increase, compared to those giving a reduction in germination, (Figure 3.12). The remaining fourteen bands tested each gave a reduction in germination of conidia, (4.5% - 48.5%).

The work reported here demonstrating a number of specific antifungal and stimulatory substances separately in both the host and the pathogen, enabled comparisons between the occurrence of such biologically active substances in sclerodermoid tissue and its constitutive fungi. Antifungal activity of samples at Rf. 0.25, 0.76 and 0.94 was assumed to be due to compounds of host origin, corresponding closely to bands ACI, ACII and ACIII respectively, while antifungal activity at Rf. 0.19 were seen to correspond closely to that previously noted in M.perniciosa mycelial extracts. It is likely that antifungal activity at Rf. 0.67 was similarly originated from M.perniciosa although not detected using previous methods.

Of the bands exhibiting stimulatory activity, those at Rf. 0.23 and 0.90 corresponded closely to those shown to be present in healthy sporophores, while those at Rf. 0.34, 0.73 and 0.79 were assumed to originate from M.perniciosa.

Individual zones were further examined using the lipid identification scheme, (Materials and Methods IV 5), and the test reagent schedule,

(Materials and Methods IV 3), in order to determine the nature of the active compounds. Tests confirmed that antifungal activity at Rf. 0.25, 0.76 and 0.94 was due to the presence of glycolipid, ganglioside and ninhydrin +ve phospholipid compounds indicating that these bands did indeed coincide with the presence of compounds ACI, ACII and ACIII from A.bisporus respectively. Antifungal activity at Rf. 0.19 was seen to coincide with the presence of a compound reacting only to the vanillin test for a higher alcohol group, indicating the presence of an active compound previously noted in M.perniciosa mycelial extracts. The stimulatory band at Rf. 0.23 failed to react positively to any of the test reagents, while the remaining bands tested were seen to be principally lipidic in nature, (Table 3.6).

This work has demonstrated, therefore, the occurrence of antifungal and stimulatory substances produced by both the host and the pathogen.

That these substances are present in infected (sclerodermoid) tissue indicates continued active metabolism by both the host and the pathogen in mature infected tissue and as such suggests that symptom development and the production of infected tissue may ultimately be affected by the relative proportions of such compounds.

Table 3.6

The occurrence of antifungal (AF) and stimulatory (S) compounds in
chloroform extracts from sclerodermoid tissue developed in chloroform :
methanol, (6:1) determined using the methods of Christie (1976).

(a) = Antifungal (AF) or stimulatory (S) activity assayed against C.herbarum
on glass slides after 24 hours incubation at $18^{o}C$ in moist chambers.

Rf. zone	Activity (a)	Chemical group	Likely origin - (Host or Pathogen)
0.19	AF	higher alcohol	P
0.23	S	unknown	H
0.25	AF	glycolipid	H
0.34	S	phospholipid	P
0.67	AF	phospholipid (ninhydrin +ve)	P
0.73	S	phospholipid	P
0.76	AF	ganglioside	H
0.79	S	ganglioside	H
0.9	S	phospholipid (dragendorff +ve)	H
0.94	AF	phospholipid (ninhydrin +ve)	H

CHAPTER 4

I   BIOLOGICAL CONTROL OF M.PERNICIOSA

Control of mycoparasitic diseases of A.bisporus is typically
restricted to the adoption of sanitation procedures and the application
of fungicides to the crop itself. The use of fungicides on mushroom
beds is by nature undesirable because the crop itself is a fungus, and
in view of the likelihood of mycoparasites building up resistance to
fungicides in common usage (Gandy and Spencer 1976, Samuels and Johnston,
1980, Fletcher and Yarham, 1976). Further, fungicides may fail to
control susceptible fungi in some situations, (Fletcher, Connolly,
Mountfield and Jacobs, 1980).

In an attempt to avoid the problems associated with the application
of fungicides to mushroom crops, the possibility of biological control
of mycoparasites of A.bisporus has previously been investigated using
fungi antagonistic towards Verticillium fungicola and to M.perniciosa,
(Gandy, 1975, 1979). The use of fungi antagonistic towards mushroom
pathogens as a method of disease control is, however, in contradiction
to the continued development of mushroom production which has as one of
its prime objectives, the growth of A.bisporus as a mono-culture in the
absence of competitive fungi.

At the present time, it is accepted that the microbial population
of the casing layer is an inescapable prerequisite for successful
commercial sporophore production and it seems likely therefore that
bacteria capable of surviving as part of the casing microflora are most
likely to be of practical value in controlling mycoparasitic diseases

such as Wet Bubble disease with a minimum of interference of healthy sporophore production.

Bacteria were isolated from a range of sites, principally mushroom cropping beds, either from compost or casing, or from healthy and infected tissue and screened for the ability to antagonise M.perniciosa.

Samples were initially plated out on to Nutrient agar (+ sucrose) or Kings' B agar and incubated at 25°C $\pm$ 1°C. All isolates were subsequently subcultured from single cell colonies and tested for fungitoxic activity on growth media, without the presence of A.bisporus components, as a model for the environment in the casing layer prior to the development of sporophore initials.

Bacterial isolates were tested against M.perniciosa using the dual culture technique on Czapek Dox agar in 9 cm Petri dishes by streaking plates with a pure bacterial culture at the same time as inoculating the agar surface with M.perniciosa, prior to incubation, (23°C $\pm$ 1°C). Growth of M.perniciosa was measured and expressed as a percentage of mean growth of controls of identical M.perniciosa cultures in the absence of bacteria.

Antagonism towards M.perniciosa was characterised as a reduction in growth of the fungus, and in some cases, although vegetative growth was barely affected, sporulation was reduced in the presence of antagonistic bacteria, (Plate 4.1). In all cases where fungal growth was significantly inhibited in the presence of bacteria, this occurred in the sector closest to the antagonistic bacteria, suggesting the presence of non-volatile, rather than volatile, toxins.

PLATE 4.1. 9cm dual plate cultures (Czapek Dox agar) of <u>M.perniciosa</u> (Mp) in the presence of antagonistic bacteria (b) in 9 cm petri dishes. Incubation was at 23°C for 12 days.

Note that in some instances sporulation of the fungus is reduced relative to overall fungal growth in some of the examples illustrated, (arrowed).

PLATE 4.1. 9cm dual plate cultures   (Czapek Dox agar)
of M.perniciosa (Mp) in the presence of antagonistic bacteria
(b) in 9 cm petri dishes.   Incubation was at 23°C for 12 days.

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PLATE 4.1.   9cm dual plate cultures   (Czapek Dox agar)
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Note that in some instances sporulation of the fungus is
reduced relative to overall fungal growth in some of the
examples illustrated, (arrowed).

Those isolates showing antagonism towards M.perniciosa were
subsequently screened for compatibility with A.bisporus. Isolates were
tested for their ability to brown blocks of A.bisporus (Strain Sinden
A6) cap tissue (c. 1.0 x 1.0 cm) freshly excised under sterile
conditions, initially using the rapid pitting technique adopted by
Gandy, (1968).

Bacterial isolates were grown on Nutrient agar at 25°C $\pm$ 1°C for
24 h and harvested in SDW to give a suspension of c. 1 x 10^8 cells cm^{-3}
and inoculated to the outer surface of the tissue block with the outer
layer peeled off. One 10 µl droplet of bacterial suspension was applied
to each block and samples were then placed on clean glass slides in moist
chambers and incubated at 25°C $\pm$ 1°C. The surface of each tissue block
was examined microscopically 10 min. after the application of bacteria.
The conspicuous pitting of A.bisporus cap tissue in the presence of
Pseudomonas tolaasii noted by Wong and Preece, (1979), was not
consistently observed using any of the bacterial isolates examined,
including Ps.tolaasii NCPPB 1116, 2325, 387 and 1311 included as
comparisons. Observation of tissue blocks 24 h after inoculation with
bacteria at 25°C $\pm$ 1°C as suggested by Gandy (1968), revealed a number of
isolates capable of browning cap tissue indicating a degree of
antagonism towards A.bisporus. Isolates able to cause browning of
A.bisporus tissue blocks were subsequently discarded, (Plate 4.2).

This selection procedure identified seventeen isolates capable of
significantly inhibiting growth of M.perniciosa in agar culture while
showing no effect on host tissue. The possibility of using these

PLATE 4.2. Screening of bacteria for the ability to brown mushroom tissue.

Isolates from 24 hour old nutrient agar cultures were streaked onto the upper surface of tissue blocks of 'button stage' A.bisporus sporophore cap tissue with the outer layer peeled off, prepared under aseptic conditions.

Tissue blocks were examined after 24 hours incubation at 25°C in moist chambers.   Those bacteria shown to cause browning(arrowed) of A.bisporus tissue were discarded.

In this sample of isolates Pseudomonas tolaasi (NCPPB 2325) is included alongside controls of tissue blocks inoculated with SDW.

PLATE 4.2. Screening of bacteria for the ability to brown
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In this sample of isolates Pseudomonas tolaasi (NCPPB 2325)
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with SDW.

bacteria as a means of control of Wet Bubble disease within mushroom crops as subsequently tested in small scale trials.

Experimental crops of A.bisporus (Sinden A6) were grown in controlled environment rooms at the University of Stirling as previously outlined, (Materials and Methods I2). Experimental design was of 6 randomised blocks each comprising of 18 treatments of pots inoculated with M.perniciosa and one of 17 bacterial antagonists at the time of casing. The remaining treatment acting as a control infected with M.perniciosa but without the addition of bacterial antagonists. Casing soil prior to usage was steamed to give an initial bacterial population of c. 2.3×10^5 bacteria g^{-1} (fresh weight at c. 70% moisture holding capacity, (mhc)).

Prior to use, test bacteria were cultured in nutrient broth (+ sucrose) in an orbital incubator at 23°C $\pm$ 1°C, 100 r.p.m. for 24 h. Cultures were subsequently centrifuged, (6000 r.p.m., 5500G) for 15 min., resuspended in SDW and recentifuged, twice. Isolates were finally resuspended in SDW and suspension concentration adjusted to 1.0×10^7 bacteria cm^{-3}.

Experimental crops were infected with M.perniciosa by mixing casing soil with a conidial suspension immediately prior to application to the crop. SDW conidial suspensions were prepared from 15 day old Malt agar cultures and applied at the rate of 1.32×10^3 spores g^{-1} fresh weight of casing soil, equivalent to 2.4×10^2 spores cm^{-2} of surface area. SDW bacterial suspensions were each applied to separate pots, giving an initial concentration of selected bacteria of c. 2.3×10^5 bacteria g^{-1}

of casing soil, with a total population of 4.6×10^5 bacteria g^{-1}.

Results were recorded as fresh weight of healthy sporophores, picked by cutting of the stipe, level with the casing layer, at 'veil break'. Sclerodermoid tissue was assessed as fresh weight at maximum size picked by cutting level with the casing layer. Cutting implements were sterilised in methanol, between treatments.

Results for the production of healthy sporophores in infected pots were seen to be normally distributed. Comparisons between treatments and controls grown in the absence of test bacteria on the basis of least significant differences (LSD) showed significant increases in the production of healthy sporophores in the presence of 5 of the 17 selected bacteria, (Table 4.1). Isolate I giving the maximum increase, (108.5% increase relative to controls, $p < 0.01$). Isolates B, E, G and Q also gave significant increases, ($p < 0.05$). These levels were not clearly reflected in the analysis of variance table, due to the variability between blocks, (Table 4.2i).

The level of production of sclerodermoid material in infected pots was not normally distributed, with sample variance increasing with sample mean values. Consequently, analysis was of log 10 transformed data. On the basis of LSD comparisons, significant reductions in the production of infected tissue were seen in the presence of 4 of the 17 test bacteria, (Table 4.3). Maximum reduction was with the addition of bacterial isolate J (83.1% reduction relative to controls, $p < 0.05$) isolates B, E and F also showed significant reductions in the production of infected

Table 4.1

Yield of healthy cup stage A.bisporus sporophores from infected plots in the presence of selected bacterial isolates applied at the time of casing.

Growing room air remperature:- $16^{\circ}C$ post-initiation. (g fresh weight 200 cm^{-2})

Isolate		i	ii	iii	iv	v	vi	yield / treatment
				Blocks				
A		59.11	93.22	88.44	72.48	32.27	26.37	371.89
B	*	90.41	105.46	89.62	63.79	98.89	98.48	546.75
C		74.50	60.08	73.80	98.01	129.60	91.05	527.05
D		24.48	73.00	90.57	65.38	87.07	86.49	426.35
E	*	126.07	101.12	130.16	42.28	124.22	90.03	613.88
F		117.46	83.72	76.45	37.26	87.62	40.23	442.74
G	*	117.15	45.67	75.63	123.03	76.53	102.93	540.94
H		95.66	15.30	103.44	40.61	92.58	78.93	426.52
I	**	114.78	124.73	105.25	103.77	22.77	148.85	620.15
J		48.13	94.50	95.37	66.50	44.62	143.02	492.14
K		87.11	126.17	31.16	31.71	18.35	24.71	320.21
L		0.00	46.04	49.63	91.96	31.14	60.54	279.31
M		50.12	130.10	29.06	54.78	90.32	132.27	486.65
N		52.24	81.87	56.36	22.32	138.20	25.86	376.85
O		116.60	88.87	85.41	14.69	43.04	120.37	468.98
P		74.31	76.76	103.47	110.72	51.40	69.25	485.91
Q	*	110.67	121.87	11.77	54.37	126.18	126.36	550.63
CONTROL		78.57	13.78	10.21	117.76	23.18	53.94	297.44

(* = p <0.05, ** = <p 0.01)

Table 4.2i

Analysis of variance for healthy 'cup stage' A.bisporus sporophore production
in the presence of bacteria antagonistic to M.perniciosa in infected plots
(Yield g. fresh weight)

Source of variation	d.f.	Sums of squares	Mean sums of squares
Treatments	17	28921.954	1701.291
Blocks	5	3953.971	790.994
Residual	85	100991.30	1188.133
Corrected total	107	133867.230	

Table 4.2ii

Analysis of variance of sclerodermoid mass production (log 10 transformed
data) in the presence of bacteria antagonistic towards M.perniciosa in
infected plots   (Yield g. fresh weight)

Source of variation	d.f.	Sums of squares	Mean sums of squares
Treatments	17	10.905	0.642
Blocks	5	3.279	0.656
Residual	85	37.184	0.437
Corrected total	107	51.368	

Table 4.3

<u>Yield of sclerodermoid tissue from A.bisporus plots infected with M.perniciosa</u>
<u>at the time of casing in the presence of selected bacterial isolates applied at</u>
<u>the time of casing.   (g fresh weight 200 cm^{-2}).</u>
Growing room air temperature:-   16°C post-initiation.

Isolate		i	ii	iii	iv	v	vi	Total yield / treatment
				Blocks				
A		16.22	28.84	11.49	37.52	98.12	27.96	220.15
B	*	0.00	0.15	32.36	27.79	0.00	17.22	77.52
C		24.66	24.84	0.00	18.28	9.90	0.00	77.68
D		57.26	43.83	15.67	43.10	22.38	14.54	196.78
E	*	2.98	1.81	20.61	0.00	15.11	0.00	40.51
F	*	2.27	20.55	0.00	66.22	29.58	0.00	118.62
G		0.00	10.94	28.85	5.83	41.46	8.49	95.57
H		18.99	62.91	13.34	0.00	16.06	18.80	130.10
I		0.00	16.35	12.68	0.00	59.61	5.09	93.73
J	*	0.00	7.29	19.79	15.80	0.00	0.00	42.88
K		0.00	3.74	0.00	3.02	79.08	11.10	96.94
L		107.06	64.11	21.48	21.36	18.87	0.00	232.88
M		59.05	5.00	116.73	48.06	19.31	0.00	248.15
N		40.25	0.00	47.79	75.46	1.71	49.40	214.61
O		4.65	14.61	15.98	89.80	41.75	6.08	172.87
P		0.00	17.54	0.00	12.22	11.87	51.32	92.95
Q		5.30	0.00	100.74	0.00	20.33	0.00	126.37
CONTROL		3.81	32.06	31.32	23.75	100.25	62.71	253.90

($*$ = $p <0.05$)

tissue, (p <0.05). Levels of significance were not reflected in the analysis of variance table as noted for the production of healthy tissue, (Table 4.2ii).

Isolates I and J were seen, therefore, to give the highest levels of disease control within the context of the experimental crop grown under the outlined conditions.

The manner in which control of Wet Bubble disease using bacteria was expressed, differed for bacterial isolates I and J. In the presence of isolate I the level of production of healthy A.bisporus sporophores in samples infected with M.perniciosa was increased when compared to untreated plots, such that control was expressed as an increase in yield. In contrast, the application of isolate J to infected plots, although apparently not affecting the yield of healthy sporophores when compared to infected plots without the addition of antagonistic bacteria resulted in a reduction of the amount of infected tissue developing on infected beds and may in reality be of more practical value by reducing sporulation of the pathogen, and decreasing the probability of infection to adjoining and subsequent crops.

Isolates B, E, F, G, I, J and Q were tested for compatibility on agar plates using the cross inoculum technique in order to identify pairs of bacteria that are mutually antagonistic. No such inhibition was observed, suggesting that disease control may be further increased using combinations of these bacteria, with the use of isolates I and J together most likely to be of practical value.

In addition to the recording of the quantities of healthy and

diseased tissue produced from infected experimental plots the ability of antagonistic bacteria to establish stable populations within the casing layer was examined. At regular intervals samples of casing soil (c. 1.5 g, c. 70% mhc) were removed from pots, similar to those previously described, with antagonistic bacteria incorporated into the casing layer, infected with M.perniciosa at the time of casing, and from uninfected pots, (3 samples per treatment). Replicate samples were combined and bacterial populations determined using the plate dilution technique. The determination of populations of individual test bacteria was on the basis of culture morphology. Isolates B, E, F, G, I, J and Q each attained stable populations in infected and uninfected casing soil, (Table 4.4). For all of these isolates, except isolate G, populations were higher in healthy plots than in the presence of M.perniciosa, possibly indicating a degree of mutual antagonism between M.perniciosa and test isolates. Consequently, where such bacteria are able to establish themselves in casing soil free from M.perniciosa, it may be that subsequent infection would result in a higher degree of control than in the experimental system.

Isolates I and J, those showing most potential for disease control, were seen to achieve stable populations in both healthy and infected casing soil in approximately 17 days. This closely followed the pattern of development of natural microbial populations in both healthy and infected casing soil, (Figure 4.1).

The use of bacteria antagonistic towards M.perniciosa appears to be a potentially useful method of biological control of Wet Bubble disease

Table 4.4

Populations of selected bacteria, antagonistic towards M.perniciosa, in infected and healthy A.bisporus casing soil following application to cropping beds, and incubating at $22^{\circ}C$ for 8 days and 37 days at $16^{\circ}C$.

(initial bacterial population 2.3×10^5 bacteria g^{-1})

(a) = Mean values $\pm$ SEM.

Isolate code	Bacteria g^{-1} of casing soil, (c.70% mhc), in plots infected with M.perniciosa at the time of casing after 45 days.(a)	Bacteria g^{-1} of casing soil, (c.70% mhc), in the absence of M.perniciosa, 45 days after casing, (a).
B	$1.56 \times 10^6 \pm 1.18 \times 10^5$	$1.14 \times 10^6 \pm 1.08 \times 10^5$
E	$3.40 \times 10^4 \pm 9.90 \times 10^3$	$8.46 \times 10^5 \pm 1.81 \times 10^5$
F	$4.20 \times 10^4 \pm 3.20 \times 10^3$	$5.20 \times 10^5 \pm 2.08 \times 10^5$
G	$9.40 \times 10^6 \pm 1.10 \times 10^5$	$8.47 \times 10^5 \pm 1.87 \times 10^5$
I	$5.90 \times 10^5 \pm 5.60 \times 10^4$	$1.20 \times 10^6 \pm 1.30 \times 10^5$
J	$8.90 \times 10^5 \pm 1.14 \times 10^5$	$1.91 \times 10^6 \pm 3.95 \times 10^5$
Q	$3.00 \times 10^5 \pm 5.30 \times 10^5$	$1.25 \times 10^6 \pm 2.38 \times 10^5$
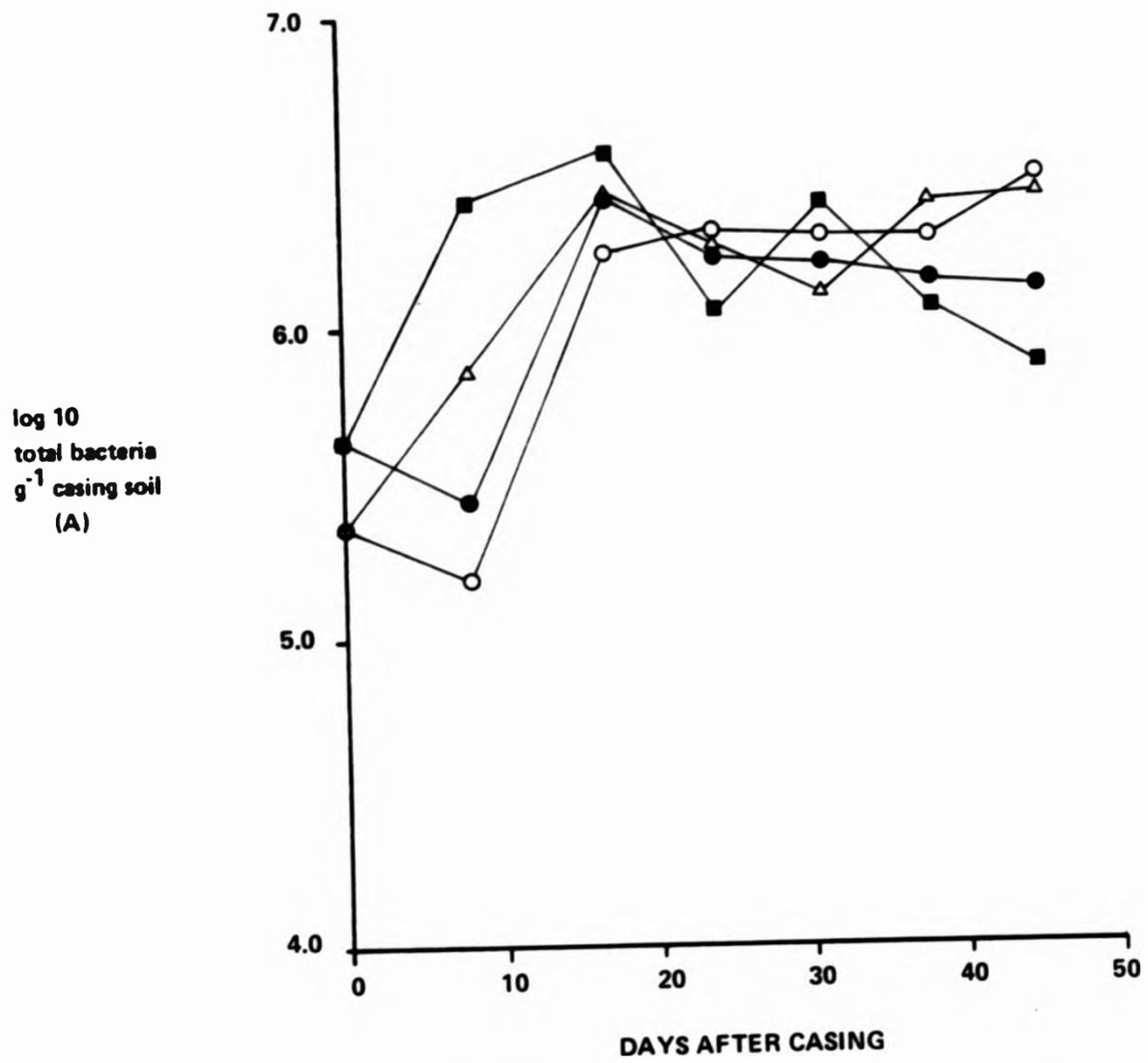
FIGURE 4.1   Populations of bacteria in A.bisporus casing soil in untreated healthy plots, (△—△), untreated plots infected with M.perniciosa, (○—○), and in infected plots treated with antagonistic bacteria (isolate I, (■—■), isolate J (●—●)) Growing room air temperature regime:-Days 0-8 = 22°C, 8-45 = 16°C.

(A) = Mean values

of <u>A.bisporus</u>. While not ensuring total control of the disease at high inoculum levels, the use of bacteria as antagonists may enable the use of fungicides as an additional means of disease control in severe cases without affecting the balance of antagonistic populations within the casing layer.

II INHIBITION OF <u>M.PERNICIOSA</u> <u>IN</u> <u>VITRO</u> AS A MEASURE OF INHIBITION <u>IN</u> <u>VIVO</u>

Inhibition of <u>M.perniciosa</u> on agar plate cultures by bacteria was necessarily used as an initial method of screening for organisms capable of antagonising the mushroom pathogen. Comparisons between results for inhibition <u>in vitro</u> with disease levels in infected plots containing antagonistic bacteria in the casing layer were made in order to assess whether inhibition of <u>M.perniciosa</u> on agar plates could in reality be used as an indication of the suitability of a particular bacterial isolate for control of Wet Bubble disease in infected cropping beds.

Correlation between inhibition <u>in vitro</u> and the effect of bacteria on the production of healthy sporophores in infected beds, was poor, (r = 0.415), (Figure 4.2), with a high degree of scatter, (S = 15.84), indicating that inhibition of <u>M.perniciosa</u> <u>in vitro</u> is a poor indication of the ability of test bacteria to influence the production of healthy sporophores in infected plots.

The correlation between inhibition of <u>M.perniciosa</u> <u>in vitro</u> and the production of infected tissue in infected plots in the presence of

Total yield
of healthy
A.bisporus
sporophores
(g fresh weight/
plot)

% INHIBITION OF M.PERNICIOSA IN VITRO RELATIVE TO CONTROLS
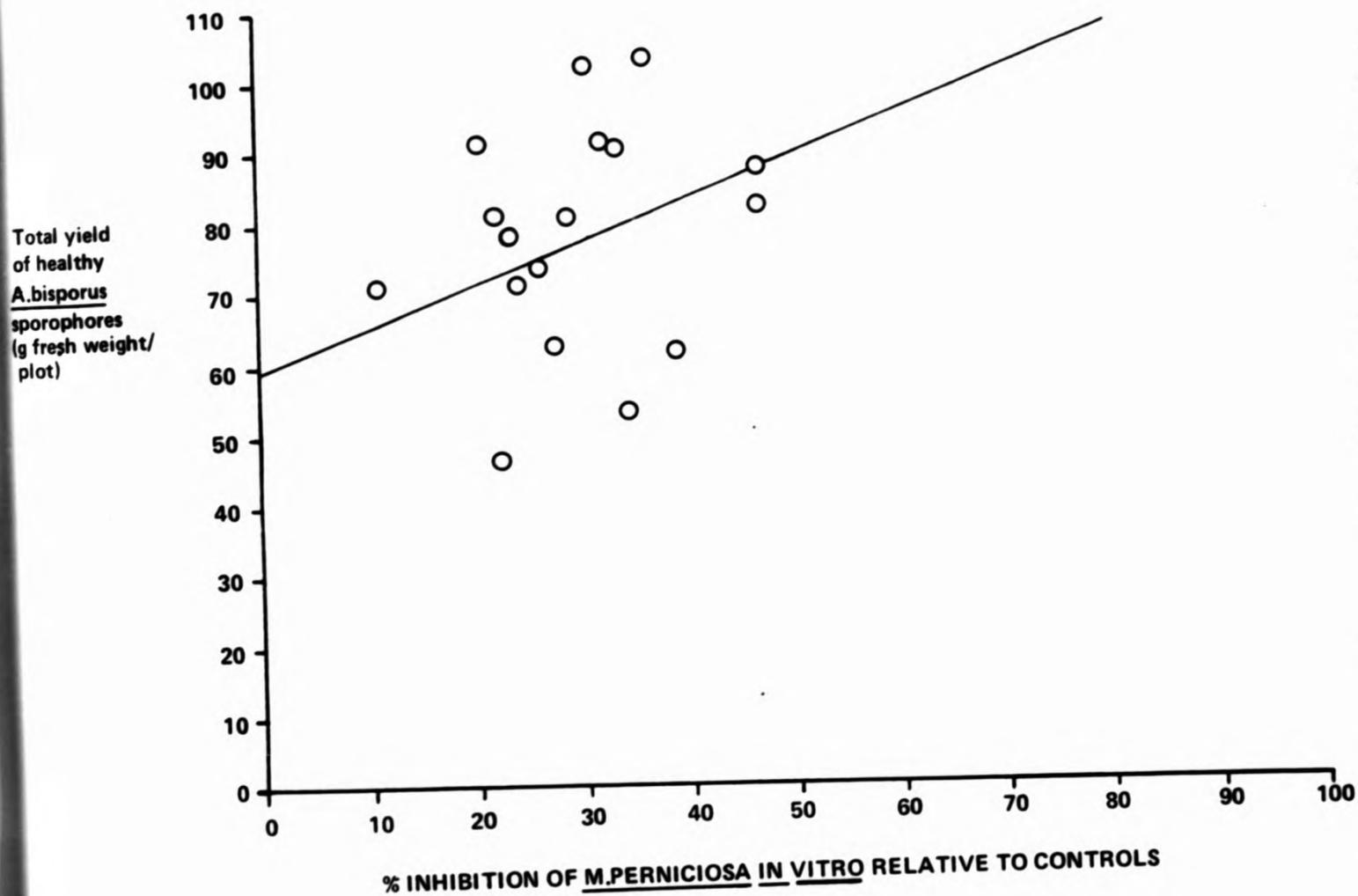
FIGURE 4.2   Relationship between yield of healthy A.bisporus sporophores in infected plots
each inoculated with bacteria antagonistic to M.perniciosa in the casing layer and
the level of that antagonism in vitro.
Sporophores were harvested at 'veil break', level with the casing layer.
Bacteria were applied to give a concentration of 2.4×10^2 spores cm^{-2} of plot
surface area at the time of casing. M.perniciosa was inoculated as a conidial
suspension at the time of casing at 1.32×10^3 spores g^{-1} fresh weight of casing
soil. Antagonism of M.perniciosa by bacteria in vitro was determined on 9 cm
Czapek Dox agar plates incubated at 23^oC for 12 days.

antagonistic bacteria incorporated into the casing layer, (log 10 transformed data) was greater, (r = 0.649), (Figure 4.3), with reduced scatter, (S = 9.045). It is apparent, therefore, that the inhibition of M.perniciosa, by bacteria, in vitro can be related to a potential for the test bacteria to influence the development of infected tissue from infected cropping plots and as such the use of agar plate tests for screening of potential antagonists appears to have been based on valid assumptions. The relationship between in vitro antagonism of bacteria towards M.perniciosa and the production of healthy sporophores appears to be more obscure however, and the screening technique adopted cannot apparently be related to the likely level of healthy sporophore production in infected beds in the presence of bacterial antagonists.

III. CHARACTERISATION OF BACTERIAL ISOLATES

Isolates exhibiting the ability to control M.perniciosa in vivo, (isolates I and J), were subjected to a range of identification tests, (Materials and Methods, V 3). Tests were those outlined in Bergey's Manual, (Buchanan and Gibbons, 1974) and those suggested by Lelliot, Billing and Hayward, (1966), and by Wong and Preece, (1979). Isolates were also examined using the API 20E pre-packaged diagnostic system, (API Systems, S.A. France).

Differences between the two isolates were apparent in agar plate cultures. Isolate I produced entire edged, flat, mucoid colonies,
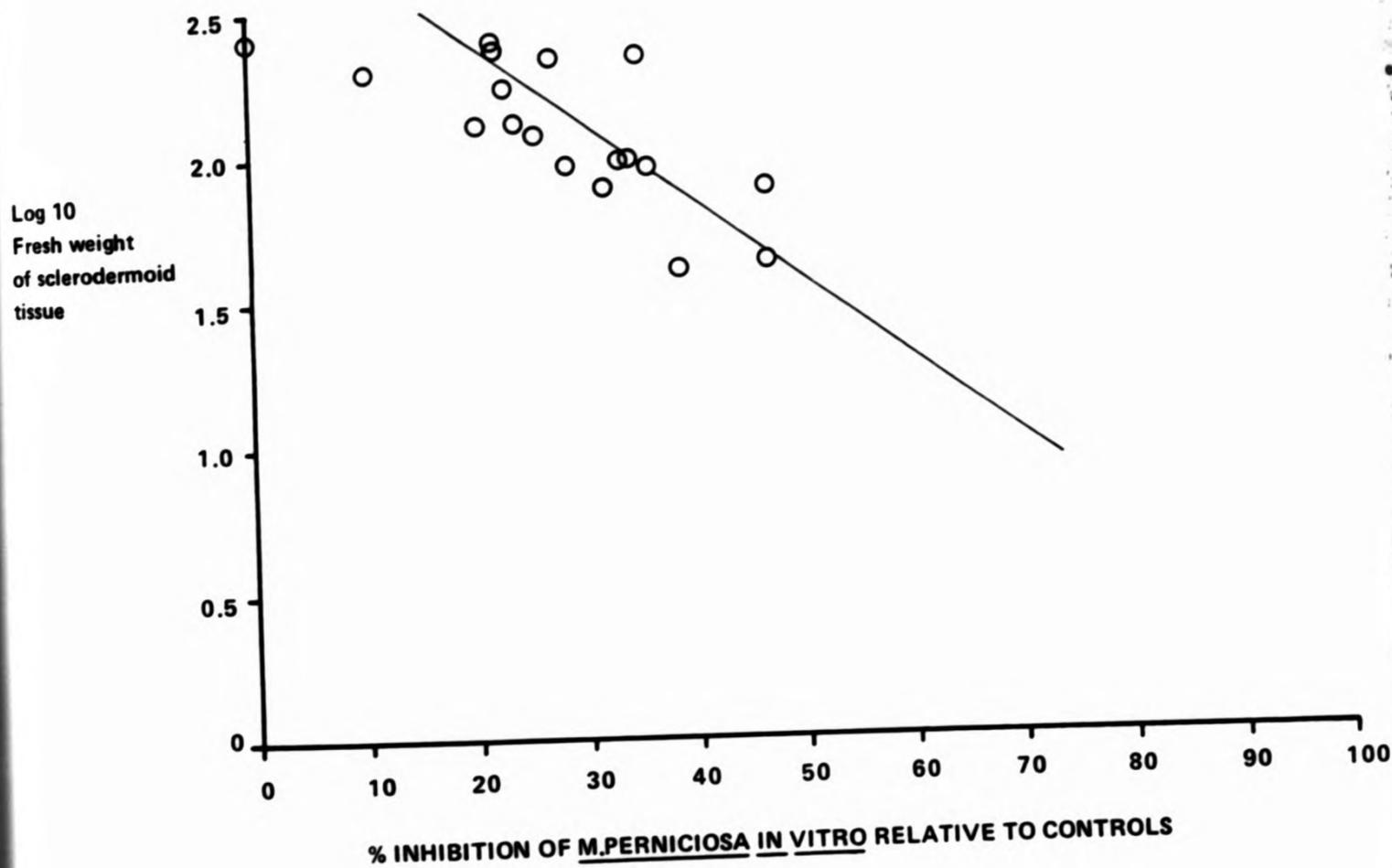
FIGURE 4.3 The relationship between yield of sclerodermoid tissue, (log 10 values) in A.bisporus plots infected with M.perniciosa and each inoculated with bacteria antagonistic towards M.perniciosa in the casing layer and the level of that antagonism in vitro. Sclerodermoid tissue was harvested when the tissue surface turned brown. Bacteria were applied to give a concentration of 2.4×10^2 spores cm^{-2} of plot surface area at the time of casing. M.perniciosa was inoculated as a conidial suspension at the time of casing, at 1.32×10^3 spores g^{-1} fresh weight of casing soil. Antagonism of M.perniciosa by bacteria in vitro was determined on 9 cm Czapek Dox agar plates incubated at $23^{\circ}C$ for 12 days.

cream / beige in colour, after 24 h incubation at 25°C $\pm$ 1°C on Nutrient agar. The colony produced a brown pigmentation in the agar after 48 h - 72 h incubation. When bacteria of this isolate were washed and concentrated by centrifugation for 'bulk' use on A.bisporus cropping beds they appeared salmon pink in colour.

Isolate J produced domed mucoid entire edged colonies after 24 h incubation at 25°C $\pm$ 1°C. Colonies were cream with a greenish tinge, having a 'ringed' appearance after 72 h incubation. Washed 'bulk' samples, prepared by centrifugation, were also salmon pink in colour. Both bacterial isolates were Gram negative, oxidase positive rods. On the basis of identification tests relating to chemical reactions and carbon source utilisation both isolates were identified as members of species 3 - 5 of the genus Pseudomonas. The only detected differences between the two isolates were in their appearance on culture plates, described above, and in the number of flagella, Isolate I being motile by either one or two flagella and Isolate J by one, (Tables 4.5, 4.6).

The two isolates may be distinguished from Ps. chlororaphis and Ps. aureofasciens by the lack of green or orange non-fluorescent pigments and are, therefore, both members of the Pseudomonas fluorescens complex.

The two isolates examined are both members of the group containing Ps. tolaasii and the 'Ginger Blotch Organism', (GBO), identified by Wong, Fletcher, Unsworth and Preece, (1982). Both of these organisms are known to cause 'blotch' of cultivated mushrooms, (Wong et. al. 1982). The isolates examined in this study can however be readily distinguished from these pathogens on the basis of colony appearance, (Wong et. al. 1982), the white line reaction (Wong and Preece, 1979) and the inability of isolates I and J to cause blotching of A.bisporus sporophore tissue.

Table 4.5

Colony charactristics and reactions of bacterial isolates I and J which, of those examined in the study were shown to have the greatest ability to reduce the severity of Wet Bubble Disease in A.bisporus beds infected with M.perniciosa by increasing production of healthy sporophores and by reducing production of sclerodermoid tissue respectively, relative to controls.

(+ = positive test reaction)

(- = negative test reaction)

Test reaction	Isolate I	Isolate J
Ability to pit A.bisporus tissue	-	-
Antagonism towards M.perniciosa	+	+
Potato rot	-	-
White line	-	-
Fluorescein production	+	+
Carotenoid production	-	-
No. of flagella	1 or 2	2
Growth at 41°C	-	-
Gram's stain	-	-
Oxidase reaction	+	+
Nitrate reduction	+	+
Catalase	+	+
Hydrolysis of starch	-	-
Hydrolysis of gelatin	+	+
Levan formation from Sucrose	+	+
β galactosidase	-	-
Arginine dihydrolase	+	+
Lysine decarboxylase	+	+
Ornithine decarboxylase	-	-
H$_2$S production	-	-
Urease	-	-
Deamination	+	-
indole (Kovac's reaction)	-	-
Acetoin	+	+

Table 4.6

Carbon source utilisation of bacterial isolates I and J exhibiting antagonism towards M.perniciosa

(+ = Carbon source utilised by test bacteria)

(- = Carbon source not utilised by test bacteria)

Test reaction	Isolate I	Isolate J
Citrate	-	-
Glucose	+	+
Manitol	-	-
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	-	-
Melobiose	-	-
Amygdaline	-	-
Arabinose	-	-
Trehalose	+	+
Geraniol	-	-
L - Valine	+	+
D L-Arginine	+	+

DISCUSSION

CHAPTER 1

GROWTH SURVIVAL AND ECOLOGY OF M.PERNICIOSA

The environment in which M.perniciosa is commonly found is that of
mushroom cropping beds. The environment in which mushrooms are
typically cultivated is unique in horticulture in the degree to which
it is controlled both in terms of substrate uniformity and the aerial
environment within the growing house. In order to parasitise the host
successfully, M.perniciosa must be able to tolerate the conditions
under which A.bisporus is cultivated.

The process of commercial mushroom production is based on empirical
development of the substrates and environments which are optimum for the
growth of A.bisporus. Commercial cultivation of A.bisporus necessitates
the use of specific composts containing a mixture of straw, horse manure,
gypsum and deep litter poultry manure as the principal constituents.
The exact nature of the mix varies from farm to farm. The processes
involved in composting and conditioning have been extensively reviewed
by Vedder, (1978) and by Gerrits, Bels-Koning and Muller (1967), who
state that the substrate should, at spawning, be of the following form:-

Total nitrogen: 1.8-2%, Ammonium nitrogen: 0.1%, C:N Ratio: 14-20:1,
pH 7.5 or below, Moisture content 70-73% and Temperature 25°C.

The conditions required within the casing layer are for an open
structured mix of, typically, peat and chalk which, for successful
initiation of A.bisporus sporophores, is otherwise nutritionally inert.
Moisture content of the casing layer should be c.70% at c. pH 7.0-7.5.
In most cases the temperature in the compost after casing is kept within
the range 25-27°C with air temperatures of 22-23°C. After a stage during

which vegetative colonisation of the casing layer takes place, sporophore initiation is induced at the time of lowering of air and compost temperatures to 15-17°C and 18-19°C respectively, with casing temperature tending to fall between these two levels.

The relationship between M.perniciosa and its environment has not previously been studied in depth. In most studies it has been considered that M.perniciosa is unable to grow successfully in compost (Smith, 1924, Vincent-Davies, 1972). The absence of infection resulting from inoculation with M.perniciosa spores of compost colonised by A.bisporus prior to casing suggests that germination may be prevented by substrate fungistasis.

Germination and growth of M.perniciosa may be prevented by biotic factors derived from existing fungal and bacterial populations, (Lloyd and Lockwood, 1966, Lockwood 1977). The ability of M.perniciosa to colonise compost is clearly important in terms of the possibility that the fungus may colonise used mushroom substrates which may then act as a source of infection for crops of A.bisporus.

This study has examined the effect of the environment on M.perniciosa at different stages of the life cycle, encompassing vegetative growth, survival and spread together with environmental influences on infection of A.bisporus, the natural host, and the relationship with fungal competitors and potential hosts in natural reservoirs of infection.

Experiments conducted in this study have demonstrated that the effect of environmental parameters varies depending on the growth stage of the pathogen. M.perniciosa reacts differently to a range of

environmental factors during vegetative growth and at the time of
sporulation and spread.

It is of interest that at different stages of mushroom culture the
environmental conditions required for healthy sporophore production are
related to the optimum conditions for development of the pathogen.

I.   GROWTH AND SURVIVAL OF M.PERNICIOSA

Theoretical optima for growth of M.perniciosa were determined in
this study by growing the fungus in plate cultures and in liquid
culture in a range of differing media and under differing environmental
conditions.

These results tie in with the apparent requirements for successful
growth of the fungus in vivo.

The three aspects considered here to be most appropriate in
determining the optimum condition for growth of M.perniciosa were
temperature, pH and C:N ratio. Optimum levels were identified in this
investigation to be 25°C, pH 4.1 and C:N of 55:1 respectively.

With respect to temperature the determined optimum value of 25°C
agrees closely with that previously determined by other workers.
Espinasse and Touze-Soulet, (1968) considered the optimum to be 23°C and
Vincent-Davies (1972) considered it to be in the range 22-25°C.   The
temperatures under which mushrooms are commercially cultivated are
likely to be between 15° and 27°C depending on the growth stage of the

crop. It is clear therefore that in order to facilitate growth a wide tolerance by M.perniciosa to variations in temperature is desirable.

Experiments conducted in this study demonstrated that M.perniciosa has a wide spread of temperatures at which it can grow vegetatively, with growth at least 25% of maximum over the range 15°C-34.5°C. This agrees closely with recommendations by Brady and Gibson, (1976b) who, on the basis of empirical observations suggested that a maximum cropping temperature of 16°C should be employed in order to minimise growth of the pathogen.

In terms of the substrate and its effect on growth of M.perniciosa determinations of the optimum pH for vegetative growth made in this study are at variance with determinations made by previous authors. Espinasse and Touze-Soulét (1969), observed that the optimum for growth was in the range pH 5.5-6.5. By contrast results from this study showed a clear optimum at pH 4.1., with a secondary peak at pH 5.775 the latter possibly coinciding with the peak observed by these previous workers. The optimum pH for vegetative growth of M.perniciosa is therefore not that of casing material, (pH 7.0-7.5), compost (pH 7.5), or of mature A.bisporus sporophores, (pH 6.4) suggesting that growth of M.perniciosa may be comparatively poor in these substrates. A similar discrepancy between the optimum C:N ratio for vegetative growth and the ratio found in substrates in which M.perniciosa typically occurs was also observed in the course of this study. While the optimum C:N ratio for growth in vitro was determined as 55:1 mushroom compost typically has a value of 15-20:1 (Hayes, 1980), casing soil a value of 35:1 (Vedder 1978), and healthy mushroom sporophores a value of 78:1 (Chang, 1980). In this study the response of M.perniciosa to

changes in substrate C:N ratio above 15:1 was slight however, and differences between the optimum for growth in vitro and substrate C:N ratios in vivo are unlikely to greatly influence growth of M.perniciosa.

Of all the parameters examined in this study pH appears to be the most significant in determining the growth rate of M.perniciosa within the ranges found in mushroom cropping environments, and as such growth is likely to be sub-optimal under conditions typically found in mushroom beds.

Under sub-optimal growth conditions some fungi have been shown to increase production of secondary metabolite toxins and antibiotics (Durbin, 1983, Smith and Berry, 1975, Weinberg, 1970) and it is likely that sub-optimal growth of M.perniciosa follows a similar pattern. Toxin production by M.perniciosa under sub-optimal conditions in the presence of the host may have the net effect of increasing the aggressiveness of the pathogen therefore. This is discussed more fully elsewhere (see Discussion Chapter 2.I).

In this study, M.perniciosa was shown to be capable of growing saprophytically in vitro on a wide range of substrates, confirming observations by previous workers, (Vincent-Davies, 1972, Barnett and Binder, 1973). Despite this the pathogen is rarely found in nature in the absence of the host.

The ability of M.perniciosa to survive and compete is likely to depend not only on the physical environment but also on the microflora of the substrate to be colonised. At any one point in the cropping cycle the chemical composition and the microflora of compost and casing tends to be fairly uniform, (Vedder 1978, Hayes, 1969,

Fergus 1978), but this tends to change to some degree between composting and cropping, (Fergus, 1964, Chanter and Thornley, 1978, Hayes, Randle and Last, 1969). The presence of antibiotics derived from the associated microflora in mushroom composts has been alluded to in reviews of composting procedures, (Sinden, 1971, Vedder, 1978), and many of the organisms present in normal composts are known antibiotic producers (Fergus, 1978, Eicker, 1980, Wilkins, 1946, Berdy, 1974). This is likely to influence the ability of M.perniciosa to colonise natural substrates therefore.

The ability of M.perniciosa to compete successfully and survive in natural substrates was examined by the determination of Theoretical Colonisation Index (TCI) values as a measure of saprophytic ability.

The concept of the TCI has typically been used to predict the ability of fungi to germinate and colonise substrates, based both on levels of germination and subsequent germ tube growth (Mitchell and Dix, 1975a, Dix and Mitchell, 1976). The inhibition of fungal spores by soil is a well known phenomenon called fungistasis. Saprophytic ability of susceptible fungi being restricted by the prevention of spore germination and germ tube growth, (Dix, 1972, Ko and Lockwood, 1967). With respect to M.perniciosa in the presence of the host the concept of competitive saprophytic ability, determined as TCI values is irrelevant, as growth is, except in exceptional circumstances, within A.bisporus tissue.

In the absence of the host there is however an ecological requirement for the fungus to shift from parasitic to saprophytic growth and TCI values can be used as an indication of the ability of M.perniciosa to survive. Verticillate conidia germinate freely in a wide range of

conditions and are normally produced before chlamydospores in culture.

Of the two types of spore produced by M.perniciosa it is verticillate conidia which are likely to be the most important in determining the ability of M.perniciosa to infect natural substrates therefore.

The saprophytic ability of a fungus is inextricably linked to a number of factors. It is considered to be directly related to the substrate which a fungus attempts to colonise (Garrett, 1970), both in terms of available nutrients and of the presence of antibiotics produced by other colonisers. Such antibiotics are likely to be secondary metabolites produced when vegetative growth is slow or has ceased in nutritionally poor substrates, (Bu'Lock, Detroy, Hostalek and Munim-Al-Shakarachi, 1974). Nutritionally poor substrates are therefore more likely to have high levels of fungistasis. In order to compete in the absence of the host M.perniciosa must have higher TCI values on nutritionally poor soils where competition for nutrients is higher than on nutritionally rich substrates such as mushroom composts.

TCI values determined in this study indicated that M.perniciosa does indeed follow this pattern with higher TCI values, in natural soils than in mushroom composts. Major changes in substrate fungistasis are also likely to occur with the presence or the absence of the host.

In this study A.bisporus was shown to produce a number of anti-fungal substances active against M.perniciosa and this is also likely to reduce the saprophytic ability of the pathogen. In mushroom substrates that have been weathered (stored for nine months post cropping), TCI values for M.perniciosa were slightly higher than in fresh mushroom compost or casing. This may reflect an increase in

saprophytic growth as host mycelium and the associated microflora are superseded by other organisms. TCI values for M.perniciosa were shown in this study to be highest in natural soils relative to mushroom compost and casing substrates and this matches the ecological requirement of the fungus, with saprophytic growth in the absence of the host.

While determination of TCI values clearly demonstrated in this study that M.perniciosa has a theoretical capability to grow saprophytically in natural substrates growth in vivo has not previously been reported per se. Experiments conducted in this study determined saprophytic growth of M.perniciosa quantitatively in natural substrates as measured by fluorescein diacetate determination of M.perniciosa esterase activity. This method indicated that the fungus is capable of considerable growth in mushroom compost under sterile conditions. Direct observation of mycelium and sporulation demonstrated that M.perniciosa is also capable of saprophytic growth in non-sterile mushroom compost. In the latter quantitative assessment was not possible because of the presence of other organisms with an associated esterase activity.

These observations contradict assumptions by previous workers (Smith, 1924, Vincent-Davies, 1972, Fletcher and Ganney, 1968) who did not observe saprophytic growth of the pathogen in natural substrates. This may be due to differences between the microbial populations of samples used in this study compared to other sources, with associated differences in the level of fungistasis influencing the extent to which M.perniciosa may grow.

The level of fungistasis in host substrates on growth of M.perniciosa was examined more closely, and in experiments to investi-

gate the effect of the host on growth of the pathogen it was noted that the response of M.perniciosa conidia to components of mushroom compost differs with respect to germination and to subsequent germ tube growth.

Germination of M.perniciosa was reduced by the presence of LMW fractions from diffusates of composts colonised by A.bisporus, although this was not observed in 'complete' (unfractionated) samples. This suggested the occurrence of fungistatic factors in LMW compost diffusates which may be overcome by the presence of factors, possibly nutritional, in HMW fractions.

Germ tube growth follows a different pattern from germination, with growth reduced both in LMW and HMW fractions compared to controls, with a corresponding increase in the number of germ tubes killed in these treatments.

A range of toxins appears to be present in A.bisporus substrates which are active against M.perniciosa. They appear to have a degree of selectivity against M.perniciosa in that C.herbarum, a saprophyte frequently used in studies of antifungal toxins because of its high sensitivity (Klarman and Sanford, 1968) was not affected to the same degree as M.perniciosa. It is likely that activity is primarily fungistatic rather than fungitoxic, with activity which was not detected in unfractionated compost diffusates being overcome by high availability of nutrients, the fractionation of HMW and LMW samples possibly separating nutrients from fungistatic substances.

Screening trials to investigate the effect of microbial populations on saprophytic growth of M.perniciosa indicated that growth of the fungus is not simply related to total population levels.

M.perniciosa was shown in plate cultures to be susceptible to antagonism by a number of soil fungi and conversely is antagonistic against others. This indicates that the susceptibility of M.perniciosa to soil fungistasis is likely to vary dependent on the make up of soil populations.

M.perniciosa is clearly capable of saprophytic growth but is susceptible to soil fungistasis which varies depending on the substrate to be colonised.

Growth of the pathogen as mycelium, and by inference survival, is better in the absence than in the presence of the host. This may explain to some extent the high incidence of Wet Bubble disease in past years where growers used loam and soil for crop casing (Gandy, 1983).

In some instances growers incorporate old casing and compost, which has been weathered, into new casing material, (Vedder, 1978). It has been demonstrated in this study that M.perniciosa may under such conditions, survive as mycelium and may infect A.bisporus crops as a result. The storing of used material for considerable periods of time prior to reuse will not reduce the risk of infection as the levels of M.perniciosa will not decrease.

Weathering and long term storage of once used material is clearly an inadequate means of ensuring freedom from M.perniciosa and other means of disinfection, such as the use of steam, are clearly essential.

In addition there is a risk of infection of fresh compost or casing material with M.perniciosa prior to colonisation by A.bisporus with saprophytic growth of the pathogen following chance infection.

In order to minimise the risk of colonisation by M.perniciosa fresh

casing or compost materials should not be stored for long periods
prior to use therefore.

II. RESERVOIRS OF INFECTION

In the course of this study the ability of M.perniciosa to grow
saprophytically has been demonstrated. This may lead to the build up
of natural reservoirs of infection in soils and in used compost and
casing substrates in the vicinity of mushroom growing sheds.

Another possible source of infection which has not previously been
considered in depth is the ability of M.perniciosa to infect basidio-
mycetes other than the natural host which may facilitate both spread
and survival of the pathogen in the wild. M.perniciosa has been classed
as a necrotrophic mycoparasite (Barnett and Binder, 1973) and as such is
likely to have a wide host range. In previous studies the ability of
M.perniciosa to infect fungi other than A.bisporus was limited to the
observation of infection of A.arvensis,(Moore,1959), Pluteus sp., Coprinus
sp. Panaeolus campanulatus and P.campestris,(Brady and Gibson, 1976b)and of
Rhopalomyces elegans, (Barron and Fletcher, 1972).

In the course of this study the ability of M.perniciosa to infect
fungi other than A.bisporus has been investigated using antagonism
between fungi in plate culture, inoculation of tissue blocks from
sporophores of potential hosts and inoculation of developing sporophores
of a range of fungi, in the wild, as a measure of the likelihood of
fungi to act as hosts of M.perniciosa under natural conditions.

Examination of antagonism and growth patterns of M.perniciosa and a range of fungi in plate cultures indicated that Agaricus macrosporus, A.bitorquis and A.silvicola together with Flammulina velutipes, Stropharia merdaria and Schizophyllum commune are likely to be susceptible to infection by M.perniciosa. In addition Coprinus sp. and Volvaria volvacae may be host fungi under certain conditions.

Investigations into the ability of M.perniciosa to infect tissue blocks and sporophores of fleshy fungi in the wild resulted in the observation of infection of a number of species by M.perniciosa for the first time. In addition to A.bisporus, A.campestris, A.macrosporus, Lepista nuda, Paxillus involutus, Lactarius pubescens, Russula atropurpurea and R.ochroleuca were all infected by M.perniciosa under field conditions. With the exception of A.bisporus this is the first report of infection of any of these species.

Some of the potential hosts, A.macrosporus, A.bitorquis and Flammulina velutipes, are fungi which are grown commercially (Vedder, 1978, Fermor, 1982) while the remainder are fungi commonly occurring in the wild in the U.K.

With respect to A.macrosporus, A.bitorquis and F.velutipes, no measure could be made of any likely crop losses resulting from infection by M.perniciosa in the present study but this may prove to be significant commercially.

Dispersal of the pathogen by sequential sporulation from infected sporophores in the wild as well as from those in cultivation would help to ensure the survival of the pathogen.

In some instances mushroom crops other than A.bisporus may be grown as 'break crops' to assist in eradicating epidemic disease,

particularly virus. The use of the <u>Agaricus</u> species, in particular, those which have here been shown to be susceptible to <u>M.perniciosa</u> may be considered in the U.K. for this purpose (Vedder, 1978, Gaze, 1985), While this may reduce or eliminate viral infection the level of Wet Bubble disease may be unaffected.

In this study <u>M.perniciosa</u> has not been shown to parasitise mycelium of any fungal species tested on plate cultures, although various fungi were seen to be mutually antagonistic. In the event of <u>M.perniciosa</u> growing in natural soils it is unlikely to parasitise non-fleshy fungi with the ability of <u>M.perniciosa</u> to grow being governed by the presence of antagonists within the substrate.

It has been the commonly held view that <u>M.perniciosa</u> survives, in the main, as resting chlamydospores, (Vincent-Davies, 1972, Fletcher and Ganney, 1968). This study has clearly shown that the fungus has a number of possible alternatives. Survival may be by saprophytic colonisation of natural substrates, with growth being comparatively good in natural soils when compared to commercial composts. Survival may alternatively be by colonisation of sporophores of wild fleshy fungi or in some cases by infecting cultivated mushroom other than the natural host. <u>M.perniciosa</u> is better equipped for spread and survival than is generally accepted in the literature and this is likely, in part, to explain the rapid spread and wide distribution of the fungus throughout the world, (Forer, Wuest and Wagner, 1974, Liao, 1981, You, Byun, Park and Shin, 1981).

III. INFECTION OF A.BISPORUS

For disease to develop to the fullest extent within a commercial crop early infection of host sporophore initials immediately after casing is necessary, (Fletcher and Ganney, 1968). The primary means by which M.perniciosa infects crops of A.bisporus has generally been considered to be by the germination of chlamydospores in the immediate proximity of A.bisporus sporophore tissue, (Vincent-Davies, 1972, Fletcher and Ganney, 1968), with little or no infection of the host occurring under any other circumstances.

Secondary infection was assumed to result from germination of verticillate conidia produced on neighbouring sporophores infected with M.perniciosa, (Vincent-Davies, 1972). This assumption centres on the presumed short life of verticillate conidia in contrast to thick walled chlamydospores which have been considered to be the principal resting spores, (Fletcher and Ganney, 1968, Vincent-Davies, 1972). Experiments conducted in this study have however demonstrated that this is not entirely the case with verticillate spores remaining viable for at least 69 days with a germination potential of between 58% and 100%. While chlamydospores germinate readily when young, (less than 10 days old), germination levels decrease as they mature ,suggesting that these spores become unsuitable for rapid build up of disease.

At the early stage of A.bisporus sporophore initiation during which infection with M.perniciosa chlamydospores is likely to lead to the maximum level of disease, the temperature within the cropping house is typically in the range 22-27°C in the air and in the casing layer.

Results of experiments carried out in this study indicate that this agrees closely with the optimum temperature for germination of chlamydospores which was determined as 25°C.

Verticillate conidia are typically produced on young conidiophores, in advance of chlamydospores. In addition the former germinate rapidly and are more likely to contribute to a rapid build up of disease during the later stages of cropping of A.bisporus than are chlamydospores, confirming the opinion of Smith, (1924). During the cropping phase of A.bisporus temperatures within cropping houses are generally lower, at 16°C-18°C, than during sporophore initiation and it is of interest that the optimum temperature for germination of verticillate conidia was observed in this study to be correspondingly lower than that for chlamydospores, at 18°C. Thus the optimum conditions for the two types of spore are closely aligned to the conditions under which A.bisporus is typically cultivated, and with the conditions likely to be prevalent at the time of the cropping cycle when the two types of spore are each most important in spread of M.perniciosa.

By contrast, Verticillium fungicola also myco-parasitic on A.bisporus, produces a single spore type which has an optimum temperature for germination of 20°C, in contrast to the two optimum temperatures for germination of the two more specialised M.perniciosa spores, (Brady and Gibson, 1976a).

In this study M.perniciosa was not observed to parasitise commercial strains of A.bisporus in plate cultures. This confirms previous observations of the relationship between the two fungi in

culture, (Vincent-Davies 1972, Espinasse and Touzé-Soulet, 1969, Gray and Morgan-Jones, 1981). Growth of M.perniciosa in plate cultures was seen in this study to follow a consistent pattern in the presence of A.bisporus, irrespective of the A.bisporus strain. Most A.bisporus strains remain vegetative in plate culture, (e.g. A6, S22, D649 used in this study), while some experimental strains may produce sporophore initials (e.g. Strain 431). The latter type, considered to be comparable to early stages of sporophore development in vivo (Elliott and Wood, 1978), similarly failed to elicit a parasitic response from M.perniciosa in culture.

The presence of susceptible tissue is not in itself sufficient to ensure pathogenesis and other factors in the biological and physical environment and in substrates are likely to be important.

Vincent-Davies (1972), considered that successful infection of A.bisporus could only be achieved where M.perniciosa spores were in direct contact with A.bisporus sporophore tissue and that chlamydospore germination was initiated by specific 'factors' present in such tissue. In experiments conducted in this study to determine the effect of A.bisporus initials of varying sizes on germination of M.perniciosa chlamydospores it was shown that there is no minimum size of A.bisporus initial which can increase germination, relative to controls. This indicates that any such 'factor' that may be involved could be present both in initials and in vegetative mycelium.

In addition to the influence of the presence of host tissue the ability of M.perniciosa chlamydospores to infect A.bisporus is

influenced both by the viability of spores and by any possible inter-
actions between spores. The maximum level of germination of
M.perniciosa chlamydospores in this study was 65% in vitro for young
spores and 14% for spores greater than 14 days old. Similar germina-
tion levels for mature chlamydospores were determined by previous
workers, (Vincent-Davies, 1972, Liao, 1981). The actual infection
potential of young spores, (10 days old) was lower in vivo than the
germination potential in vitro, with 50%of sporophores inoculated with
single chlamydospores being colonised by M.perniciosa.

Where chlamydospores are aggregated infection may not be
significantly greater than where single spores occur,as spores appear
to be mutually inhibited (Vincent-Davies, 1972). This possibly
reflects mutual inhibition of M.perniciosa in plate cultures as
observed in this study.

IV. SPREAD OF M.PERNICIOSA

While M.perniciosa is able to survive in the wild as mycelium,
the production of spores is of considerable importance in facilitating
spread of the pathogen.

The pattern of sporulation of M.perniciosa is typically with the
production of verticillate conidia preceding the production of
chlamydospores on older culture sections (Smith, 1924).

Verticillate conidia have been considered to represent propagules suited to an early build up of infection of the host, (Vincent-Davies, 1972). Experiments conducted in this study demonstrated that verticillate conidia have a generally higher germination potential than chlamydospores and germinate freely on a wide range of substrates. This supports the view that these spores are suited to a rapid build up of disease.

The factors influencing the pattern of sporulation of M.perniciosa have not previously been considered in depth and may have implications in the manner in which infection of A.bisporus crops with M.perniciosa should be prevented.

Results from this study indicate that sporulation of M.perniciosa is influenced by the environment and relates to the situation found within mushroom cropping houses. Production of verticillate spores is greatest at 25°C agreeing closely with the temperatures employed within cropping houses in the early stages of production immediately following sporophore initiation. This is in agreement with the assumed role of verticillate conidia in an early rapid build up of disease. At lower temperatures a higher proportion of chlamydospores are produced. Ecologically this fits in with a requirement during the later stages of cropping, when lower temperatures of 16°C are employed.

In trials designed to determine the effect of light on sporulation of M.perniciosa the relative abundance of chlamydospores compared to verticillate conidia was influenced by the light regime under which cultures were grown. Chlamydospores are preferentially

produced under U.V. light and under dark conditions.

The overall level of production of both chlamydospores and verticillate conidia is however greater in light than in dark conditions, although germination is unaffected by the light regime. It is clearly of benefit to mushroom growers to ensure that crops are grown in the dark in order to minimise sporulation and by inference disease spread.

The means by which M.perniciosa spores may be spread has been the subject of a number of studies. Spread of the fungus between crops has generally been attributed to dispersal of chlamydospores, (Fletcher and Ganney, 1968, Vincent-Davies, 1972, Van Zaayen and Rutjens, 1981). The means by which this occurs was investigated by Fletcher and Ganney (1968), who considered the primary means of dispersal to be by water spread. The possibility that spores may be carried by insects was also considered by these workers but no evidence that this is the case was found.

Monitoring of cropping environments in the course of this study demonstrated that both chlamydospores and verticillate conidia can be aerially dispersed. This complements previous observations by Zoberi (1961) who observed that spore release from M.perniciosa cultures may occur, although he failed to distinguish between the two types of spore. Cross, (1971), concluded that M.perniciosa is not aerially distributed, although Fletcher and Ganney (1968), had earlier succeeded in obtaining cultures of M.perniciosa on agar plates exposed in a wind tunnel to an air flow of 500 ft min.$^{-1}$ passed over infected

sporophore tissue. This work was not successfully replicated by these workers however. Efforts by these workers to identify M.perniciosa spores on slides exposed on nine consecutive days, using a Hirst spore trap, in a heavily infected crop were hampered by the presence of large numbers of A.bisporus spores on exposed slides. The high spore load of air in mushroom cropping environments is well recognised (Kleyn, Johnson and Wetzler, 1981) and the success of this study in trapping M.perniciosa spores is attributable in part to the sampling of air within cropping houses in the period immediately prior to 'veil break' and sporulation of A.bisporus.

The present study has demonstrated that aerial dispersal may significantly contribute to disease spread in vivo, with both verticillate conidia and chlamydospores being detected using the Cascade Impactor Spore Trap. The most important factor influencing spore release appeared to be the age of infected tissue on infected cropping beds, although this is not typical of spore release in some other fungi. In some instances spore release may be influenced by a number of factors, including humidity and temperature (Ingold 1967, Zoberi, 1961), and diurnal cycles (Ayres, 1983). Environmental changes within mushroom crops are generally slight however when compared to field conditions, and the changes in environment encountered in the course of this study are unlikely to be significant.

CHAPTER 2

INTERACTIONS BETWEEN <u>A.BISPORUS</u> AND <u>M.PERNICIOSA</u>

The relationships between fungal parasites and their hosts has been the subject of a number of extensive studies.

In particular, host/parasite relationships with plant pathogenic fungi, have been a large field of study comprehensively reviewed by various authors, (Wheeler, 1975, Callow, 1983).

The complexity of plant/pathogen relationships has been considered to hinder the understanding of the mechanisms of parasitism and host reaction.

However, Manocha and Lee, (1971) considered that relationships between mycoparasites and their hosts are less complex than those between plants and their pathogens. This view was supported by Barnett and Binder, (1973), who proposed that mycoparasites could be studied to determine the general principles of fungal parasitism. Generally, studies of mycoparasites have examined hyphal / hyphal interactions and not interactions between filamentous fungi and fungal tissue such as <u>A.bisporus</u> sporophores, (Manocha and Golesorkhi, 1979, Manocha and Letourneau, 1978, Evans and Cooke, 1982, Dennis and Webster, 1971 a,b,c, Pachenari and Dix, 1980, Ikeduigwu and Webster, 1970 a, b, Ikeduigwu, Dennis and Webster, 1970).

The complexities of interactions between filamentous fungi and large fleshy sporophore tissue of members of the Agaricales have not been considered in depth in most cases. Hawksworth, (1981) observed that although there are many reports of parasitisation of sporophores of Agarics only some of those of economic importance have been studied extensively. Because of the limited information available interactions

between mycoparasites such as M.perniciosa and host fungi such as
A.bisporus cannot be automatically assumed to follow the patterns
found in typical hyphal / hyphal mycoparasitic relationships. Despite
this uncertainty M.perniciosa has been classed as a necrotrophic
mycoparasite (Barnett and Binder, 1973). Such organisms typically
have no special nutrient requirements and are capable of colonising a
wide range of substrates. In most cases necrotrophs are capable of
indefinite saprophytic growth in addition to having an ability to
parasitise and grow on host fungi. Necrotrophs are facultative or
opportunistic parasites and in many cases have a broad host range.
This may be a reflection of the commonly noted production of enzymes
and toxins by such fungi. (Barnett and Binder,1973), which have been
considered to serve two functions in mycoparasites.

On the one hand, enzymes or toxins may antagonise competitors in
order to prevent access to common substrates. Compounds such as anti-
biotics may contribute to soil fungistasis, (Lockwood, 1977, Lloyd and
Lockwood, 1966). Alternatively, mycoparasites may produce enzymes and
toxins in order to antagonise fungi directly prior to parasitisation
and utilisation of the target fungus as a source of nutrition, (Dennis
and Webster, 1971,a,b, Manocha and Lee, 1971).

Such a view of the dual role of enzymes and toxins was considered
by Lewis (1973, 1974), as evidence that necrotrophic mycoparasitism may
be part of an evolutionary progression from saprotrophy to parasitism
representing an intermediary stage between saprotrophy and biotrophism
where, unlike biotrophs the presence of the host is not essential for
successful growth and survival of the pathogen.

Interactions between necrotrophic mycoparasites and their hosts

may be in advance of physical contact via the production of toxins or enzymes by the pathogen. Subsequent physical interaction may take various forms depending on the fungi involved. In some cases physical interaction may result in coiling of pathogen hyphae around the host. This type of response to the presence of host hyphae was demonstrated in a number of Trichoderma species in response to a range of fungi by Dennis and Webster, (1971c) and appears to be common in mycoparasites, (Arora and Dwivedi, 1980, Rai, Singh and Singh, 1980, Upadhyay, Rai and Gupta, 1981) although the extent of coiling may be slight in some cases, (Pachenari and Dix 1980). In necrotrophic mycoparasites infection of host hyphae generally leads to the death of the host fungus.

Dennis and Webster, (1971c) interpreted the coiling of myco-parasites around host fungi as being indicative of a high degree of aggressiveness in the pathogen. Deacon, (1976) by contrast suggested that coiling may be in response to host resistance, with coiling of pathogen hyphae enabling attack of host hyphae at a greater number of loci than would otherwise be the case.

Microscopic examination of A.bisporus tissue infected with M.perniciosa in this study showed that the development of pathogen hyphae appears to be at random, with no obvious coiling of the pathogen around the host. M.perniciosa appears to be largely unaffected by the presence of individual host hyphae and grows either around or penetrates through host cells. This may be due to the nature of the tissue in that no single host hyphae is more closely aligned to particular pathogen hyphae than its immediate neighbours. M.perniciosa is therefore unable to recognise single hyphal strands around which it

might otherwise coil. Physical interaction may involve penetration of the host.

Penetration of host fungi by biotrophic mycoparasites may be by the production of complex appressoria and haustoria prior to penetration of the host, (Tsuneda and Hiratsuka, 1980, Manocha and Golesorkhi, 1979, Manocha and Letourneau, 1978). With, in some instances, the formation of complex haustoria at the interface of host and pathogen cells in biotrophs, (Manocha and Golesorkhi, 1981). By contrast penetration of A.bisporus hyphae by M.perniciosa was observed to be by undifferentiated hyphal strands, in some instances with a slight constriction at the point of penetration. In this respect, M.perniciosa follows the pattern typical of necrotrophic mycoparasites, (Dennis and Webster, 1971c, Hawksworth, 1981).

The relationship between M.perniciosa and the natural host A.bisporus is somewhat unusual in that while M.perniciosa is clearly capable of growing saprophytically and has a wide potential host range it is seldom found in the absence of the host. Luttrell, (1974), considered that biotrophs are organisms, which, regardless of the ease with which they can be cultured, in nature obtain food from living tissue on which they complete their life cycles. It is conceivable therefore that M.perniciosa could be considered a necrotrophic mycoparasite with biotrophic tendencies. A tendency towards variable modes of parasitism has been noted for mycoparasitic hyphomycetes in general. (Gray and Morgan-Jones, 1981, Rudakov, 1978, Hawksworth, 1981).

I. TOXIN PRODUCTION BY M.PERNICIOSA AND A.BISPORUS

Action of toxins on hosts has been described in a number of instances, (Rudakov, 1978).

In some cases toxin activity may be highly specific, causing for instance, extensive host cell granuolation (Arora and Dwivedi, 1980) or vacuolation (Bu'Lock, Detry, Hostalek and Munim-Al-Shakarachi, 1974, Skidmore and Dickinson, 1976).

In some cases, growth may simply be stopped by toxins produced by mycoparasites, but in extreme cases host cells may actually burst when exposed to such toxins. This was seen to occur in hyphae of Fomes annosus and Rhizoctonia solani in the presence of Trichoderma isolates by Dennis and Webster (1971c). In this study attempts to examine interference phenomena between M.perniciosa and A.bisporus showed that in plate cultures mycelium of the two fungi do not generally make contact, with the zone between colonies of the two fungi sparsley colonised. This confirmed previous observations that the two fungi are mutually inhibitory, (Vincent-Davies, 1972, Espinasse and Touzé-Soulet, 1969). Espinasse and Touzé-Soulet, (1969) considered that mutual inhibition may be due to the presence of toxins, although no compounds were isolated. In addition there was an indication from their work that growth promoting substances capable of stimulating growth of young cultures of A.bisporus were produced by the pathogen.

In this study, time course investigations of the effect of M.perniciosa culture filtrates on A.bisporus demonstrated that antagonism of host cells may be severe under some circumstances. Where A.bisporus was grown as shake cultures, producing mycelial clumps resembling sporophore tissue, antagonism of A.bisporus by M.perniciosa

culture filtrates caused host cells to become highly vacuolated when compared to non-antagonised cells. Associated electrolyte leakage from antagonised host cells indicated disruption of cell membranes, followed by cell death. A similar response to the presence of pathogens, due to toxins, has been reported in other mycoparasitic relationships (Manocha and Maharaj, 1981) as well as in plants, as a consequence of attack by pathogens (Smith and Mansfield, 1981).

With infected tissue microscopic examination of antagonised A.bisporus cells revealed that in some instances the plasmalemma appeared broken or poorly defined and tended to be contracted from the cell wall. Cytoplasm was extensively granuolated with a high percentage of cells containing excessive membranous material. Cells were generally highly vacuolated in the presence of M.perniciosa. Sych symptoms of infection are similar to antagonism attributed, in other examples, to toxin activity. Ikeduigwu, (1976), observed that antagonism of the coprophilous fungus Ascobolus crenulatus by Coprinus heptemerus resulted in vacuolation of hyphal tips of antagonised fungi and accumulation of lipid droplets. Mitochondria, perinuclear space, and cisternae of the endoplasmic reticulum were extensively swollen and were seen to shrink from the hyphal wall as a result of toxin activity, (Ikeduigwu, 1976). Similar evidence of the effect of toxins on host fungi was noted in studies of Stereum hirsutum and Coriolus versicolor antagonised by Hypomyces aurantius (Kellock and Dix, 1984a). They observed that ultimately many membrane bound vesicles were formed within antagonised hyphae, probably as a result of solubilisation of the contents of mitochondria.

There is also evidence that Mycogone species other than

M.perniciosa produce toxins. M. Japii a mycoparasite of Inocybe rimosa
can produce the toxin Bikaverin (syn. Mycogonin) (Terashima, Ishida,
Hamasaki and Hatsuda, 1972). Bikaverin may be active as a toxin by
increasing vacuolation in target cells (Bu'lock, Detroy, Hostalek and
Munim-Al-Shakarachi, 1974, Osman and Valadon, 1984), and has a broad
spectrum of activity, having both anti-fungal and anti-protozoal
activity. Although Bikaverin was not detected in M.perniciosa cultures,
either in the presence or the absence of the host, the production of
toxins affecting host cell vacuolation may represent a means of
pathogenesis typical of Mycogone species in general.

Essentially, all toxins have been considered to be secondary
metabolites (Durbin, 1983). In many cases production of secondary
metabolites is greater where growth of the fungus is restricted, either
by nutrient depletion or by inhibition (Weinberg, 1970, Bu'lock et.al.
1974, Cornforth, Ryback, Robinson and Park, 1971).

Experiments conducted in this study indicated that, relative to
controls, A.bisporus cells exposed to M.perniciosa culture filtrates
containing juice extracted from the host in the medium showed a greater
electrolyte leakage and ultimately a higher percentage cell death than
A.bisporus cells exposed to filtrates of M.perniciosa cultures grown
without host extracts. This indicates that in the case of M.perniciosa
the production of toxins is greater in the presence than in the
absence of the host.

Mutual inhibition between M.perniciosa and A.bisporus observed in
plate cultures implies that antagonism of the pathogen by the host may
have the net effect in vivo of increasing the pathogenic ability of
M.perniciosa by causing the production of toxic secondary metabolites..

Switching on of toxin production may be due to the presence of toxins produced by the host (see later) and leading to an increase in the production of toxins active against the host by M.perniciosa.

Production of toxins by M.perniciosa is likely to be greatest in situations where the host, A.bisporus, is present and on nutritionally poor substrates, a situation occurring in the casing layer of mushroom cropping beds, (Vedder, 1978). This combination of poor nutrition and the presence of the host may account for the ability of M.perniciosa to parasitise A.bisporus when it is inoculated to the casing layer but not as a result of inoculation to compost colonised by A.bisporus with a greater availability of nutrients (Fletcher and Ganney, 1968, Vedder, 1978).

The complete characterisation of toxins produced by M.perniciosa was outside the scope of this study. Further work to determine the exact nature of M.perniciosa toxin would be desirable and could lead to a greater understanding of the biochemical relationship between host and pathogen metabolism.

In the case of the host, inhibitory properties of A.bisporus cultures have been attributed to toxins (Garibova, 1968a) and this was considered to be a possible means of disease resistance, (Garibova, 1968b). This view was also held by Vedder, (1978), who suggested that A.bisporus may contribute to the level of fungistasis in colonised compost by the production of toxins. Experiments conducted in the course of this study have demonstrated that A.bisporus may produce toxins in commercial composts and that toxins are present in sporophore tissue as well as in mycelium. Toxins demonstrated in this investigation have not previously been isolated from A.bisporus.

Toxin production has been reported for Psalliota species (syn. Agaricus)
(Wilkins, 1946, Brian, 1951). In particular, P.xanthoderma (syn.
A.xanthoderma) was shown to produce the toxin Psalliotin by Atkinson,
(1954). Psalliotin, a polar compound which is light sensitive, was not
isolated from A.bisporus in this study however. The toxins produced by
A.bisporus, all lipidic in nature, are unusual in that few known fungal
toxins belong to this group.

The mode of action of such toxins is unclear but it is probable
that lipidic substances have toxic activity by directly affecting cell
membrane integrity and permeability.

In interactions involving plant pathogens and their hosts,
permeability of host cell membranes may alter as a result of disruption
of membrane lipids, (Hoppe and Heitefuss, 1974) resulting in electrolyte
leakage and cell death. In bacteria toxic activity has been attributed to
lipidic substances in a number of instances, (Kabir,Rosenstreich and
Mergenhagen, 1978,
Jeljaszewicz and Wadström, 1978). Activity being related principally
to an ability to integrate with host cell membranes, disrupting the
lipid component of membranes and altering permeability. Such a mode of
action has been suggested for mycotoxins produced by the biotrophic
mycoparasite Piptocephalus virginiana as a means of parasitising
Choaneophora cucurbitarum, (Deven and Manocha, 1975, Manocha, 1980).
It may be that this is the case with toxins derived from A.bisporus.

The ability of A.bisporus to antagonise other organisms is possibly
as a form of resistance to infection. Because it is not a known
parasite, the production of toxins by A.bisporus is likely to be purely
defensive, or as a means of antagonising potential colonisers of
substrates.

II. TOXINS IN RELATION TO DISEASE DEVELOPMENT

Vincent-Davies, (1972) suggested that development of Wet Bubble disease could be influenced by the interaction of two unknown 'factors' derived separately from the host and from the pathogen. A balance between such 'factors' possibly influencing the relative growth of the two fungi and in turn influencing the development of diseased tissue.

The toxins produced by M.perniciosa and A.bisporus were each demonstrated in this study to be active against the other fungus in plate culture and on TLC bioassays, in addition to having activity against a number of other fungi and bacteria. These toxins may represent the 'factors' postulated by Vincent-Davies, (1972).

Where M.perniciosa overcomes the antibiosis of A.bisporus, for instance, symptoms are likely to be severe. Where the situation is reversed, A.bisporus is likely to resist infection to some extent, and symptoms will be less severe.

The role of toxins in general fungal ecology relates to a number of aspects. Both A.bisporus and M.perniciosa are capable of saprophytic growth and as such their toxin production is likely to be important in their ability to colonise substrates in the presence of other organisms. Typically natural substrates in which organisms are competing for nutrients have a level of antibiosis or fungistasis, (Lockwood, 1977), which prevents or reduces growth and germination of would be colonisers. In order to overcome fungistasis the production of toxins is important as a means of antagonising competing organisms. This study has demonstrated that both A.bisporus and M.perniciosa are capable of producing toxins which are active against each other and

against other fungi and bacteria and this is likely to assist them in colonising a wider range of substrates than would otherwise be possible.

III. GROWTH PROMOTING SUBSTANCES

Microscopic examination of intact host cells in infected tissue revealed that metabolic activity of A.bisporus, measured as the number of mitochondria per cell section, was actually greater in the presence of M.perniciosa than in the absence of the pathogen. This suggests that the host responds to the presence of the pathogen by increasing respiration. This paradox of an increase in respiration of A.bisporus in the presence of M.perniciosa, possibly as a result of the production of growth promoting substances, and the demonstration of the production of toxins by M.perniciosa which are active against A.bisporus implies that activity of these substances within infected tissue in vivo is highly localised. This is in contrast to other studies of mycoparasites (Kellock and Dix, 1984a,b) in which antagonism of Coriolus versicolor by Hypomyces aurantius resulted in solubilisation of host mitochondria. Increased respiration of infected A.bisporus tissue relative to healthy tissue was noted by Vincent-Davies, (1972) on the basis of analysis of CO_2 levels in infected beds. No distinction between host and pathogen respiration was made however. Vincent-Davies (1972) considered that growth promoting substances may in some way be involved in disease development and increased respiration of the host although no evidence to support this view was given.

In this study, microscopic examination of A.bisporus cells in sclerodermoid tissue revealed that live host cells exhibited symptoms of hyperplasia and hypertrophy when compared to cells in equivalent healthy sporophore tissue supporting the view that growth promoting substances are involved in the rapid development of diseased tissue on cropping beds.

In this study, growth promoting substances produced by both M.perniciosa and A.bisporus were demonstrated in plate cultures and on TLC bioassays. Active compounds were not characterised however and further investigations into the stability, mode of action and identification of such compounds is required.

The occurrence of growth promoting substances in mycoparasitic relationships has not been widely reported. All the major types of plant growth regulators (auxins, gibberellins, cytokinins and ethylene) have been shown to be produced by various bacterial and fungal plant pathogens, and these have been implicated in the production of gross symptoms involving gall formation, epinasty, witches' broom or similar symptoms, in a number of plant diseases (Wheeler, 1975).

The occurrence of 'plant' growth regulators in A.bisporus was investigated by Pegg, (1973) who demonstrated the presence of cytokinin like substances in sporophore tissue. The production of cytokinins was confirmed by Dua and Jandaik, (1979) who considered that such compounds may be involved in the onset of senescence in mature sporophores. Turner, Wright, Ward, Osborne and Self, (1975) reported the production of ethylene by A.bisporus although the role of this and other growth regulators in development of sporophore tissue remains unclear.

In this study, growth promoting substances were demonstrated on TLC bioassays following extraction of fungal tissue in non-polar solvents. This is not consistent with extraction methods recommended for auxins, cytokinins, or gibberellins, (Dua and Jandaik, 1979, Osborne and McCalla, 1961, Pegg, 1973, Jones, Metzger and Zeevart, 1980,) suggesting that growth promoting substances involved in the interaction between M.perniciosa and A.bisporus are not those typically occurring in plants.

Growth regulators other than auxins, gibberellins, cytokinins or ethylene have been reported to be associated with developmental changes in a number of other fungi, (Arora and Dwivedi, 1980, Thomas and Mullins, 1969, Sladky and Tichy, 1974, Park, 1963, Rimoczi and Vetter, 1975) and it is probable that this is the case in the relationship between A.bisporus and M.perniciosa.

The role of growth promoting substances in disease development remains unclear although it is possible that they are involved in the balance of 'factors' alluded to by Vincent-Davies, (1972).

The postulated 'two factor system' is likely to be, in reality, a combination of a number of factors involving host and pathogen derived anti-fungal substances (toxins) and growth promoting substances, although the origin of the latter is less clear.

IV.   THE ROLE OF BACTERIA IN DISEASE DEVELOPMENT

Another factor likely to influence development of Wet Bubble disease which had not previously been considered is the natural micro-

flora associated with A.bisporus in mushroom beds. This study has shown that under some circumstances bacteria, antagonistic towards M.perniciosa may ameliorate Wet Bubble disease symptoms. In axenic cultures of A.bisporus in the presence of M.perniciosa disease symptoms were severe with no differentiation of A.bisporus sporophore tissue. In the presence of bacteria, isolated from commercial casing soil and which were antagonistic towards M.perniciosa in plate cultures, A.bisporus differentiated sporophore tissue to varying degrees under otherwise identical conditions. It is apparent therefore that the microflora can contribute to the overall level of antibiosis effective against M.perniciosa. Many bacteria identified in mushroom composts are known antibiotic producers (Tautorus and Townsley, 1983, Fergus, 1964, Berdy, 1974, Sinden, 1971), and these may complement the production of antibiotics by A.bisporus, which were demonstrated in this study. Combined activity against M.perniciosa by A.bisporus and the associated microflora may control, to some degree, the development of infected tissue.

V. CELL WALL DEGRADING ENZYMES

Cell wall degrading enzymes have generally been implicated in the destructive activities of mycoparasites, particularly in the lysis of host hyphae (Pachenari and Dix, 1980, Elad, Chet, Boyle and Henis, 1983, Dennis and Webster, 1971c). In mycoparasitic interactions involving

fleshy fungi enzymes have similarly been considered to contribute to parasitism. Hyphae of Boletus edulis exposed to culture filtrates of the mycoparasites Hypomyces chlorinus or H.chrysospermum showed evidence of enzymic degradation of host cell walls (Touzé-Soulet, Rami, Dargent and Montant, 1978).

The primary means by which M.perniciosa parasitises A.bisporus was considered to be by enzymic degradation by Smith, (1924) and the presence of enzymes capable of degrading host cell walls was confirmed by Vincent-Davies, (1972). The production of cell wall degrading enzymes by M.perniciosa was further investigated in this study. The production of enzymes by M.perniciosa was shown to increase in the presence of the host and as such the pattern of enzyme production appears similar to that found in other mycoparasites (Pachenari and Dix, 1980, Elad, Chet, Boyle and Henis, 1983). Observations made in the course of this study of plate cultures of M.perniciosa in the presence of A.bisporus mycelium indicated that the growth rate of the pathogen is reduced in the presence of the host. In view of this, increases in production of enzymes by M.perniciosa in the presence of the host are not a fortuitous result of a stimulation of overall growth of the pathogen. Production of cell wall degrading enzymes by M.perniciosa is induced by the host, and this is similar to the response elicited from plant pathogenic fungi in the presence of susceptible host cell wall material. (English, Jurale and Albersheim, 1971).

In vivo, degradation of A.bisporus hyphae appears to be restricted to certain specific host / parasite contact points, possibly

penetration points. Microscopic examination of A.bisporus cells
antagonised by M.perniciosa revealed that disruption of host cytoplasm
was not associated with host cell wall degradation in all instances,
suggesting that enzymic attack is not a prerequisite for successful
antagonism of A.bisporus hyphae. That enzymic degradation of host
cell walls is not a prerequisite for successful mycoparasitic attack
was also demonstrated by Kellock and Dix, (1984b), who observed that
antagonism of Stereum hirsutum hyphae by Hypomyces aurantius resulted
in severe host cell disruption and death without cell wall degradation
occurring.

The highly localised breakdown of A.bisporus cell walls by
M.perniciosa suggests that while enzymes capable of such action are
generally produced by the pathogen they are restricted in vivo,
possibly to the apex of hyphae, such that at contact points between
A.bisporus and mature M.perniciosa cells host cell wall breakdown does
not occur in all instances. Alternatively, differential susceptibility
to cell wall degradation may occur between host cells, as observed
in the case of Choaneophora cucurbitarum which is differentially
susceptible to penetration by the biotrophic mycoparasite Piptocephalus
virginiana dependent on the age of host hyphae, (Manocha and Golesorkhi,
1979).

Cell wall structure in both A.bisporus and M.perniciosa is
basically similar, (Vincent-Davies, 1972), with both fungi belonging to
those whose walls are of the chitin-glucan group, (Bartnicki-Garcia,
1968). Variation in cell wall structure within the fungi of the
chitin-glucan group is not unusual, (Chet and Hutterman, 1980), and may

account for the ability of M.perniciosa to parasitise A.bisporus and resist cell wall degradation by its own enzymes. Resistance by M.perniciosa to its own enzymes was considered to be due to the presence of an outer amorphous heteropolysaccharide layer not present in A.bisporus, by Vincent-Davies, (1972).

Enzymes generally have an essential function in growth and development of the fungus from which they are derived. There typically exists a balance between lytic and synthase cell wall enzymes at the apex of growing hyphae (Farkas, 1979, Bartnicki-Garcia, 1968). Such enzymes may be directly involved in cell wall synthesis or they may be responsible for activation or modulation of other enzymes (Montgomery, Adams and Gooday, 1984, Gooday and Trinci, 1980). It is conceivable that production of cell wall degrading enzymes by M.perniciosa, which are active against A.bisporus, represents the means by which the pathogen has evolved from saprotrophy to necrotrophism as envisaged by Lewis, (1973, 1974), with production of enzymes essential for fungal growth adapted to relate to the presence or absence of susceptible host tissue. Clearly enzymic degradation of host hyphae is important in antagonism of A.bisporus by M.perniciosa but is to be considered along with the action of toxins produced by the pathogen as a means by which the host is antagonised and not as the sole means.

VI. SUMMARY OF INTERACTIONS IN WET BUBBLE DISEASE

While this study has confirmed the importance of cell wall degrading enzymes in disease development, they appear to constitute one of a

number of aspects contributing to the antagonism of the host and the development of infected tissue and are not the sole means by which M.perniciosa may attack A.bisporus.

M.perniciosa exhibits necrotrophic tendencies in many aspects of parasitism of A.bisporus although there are certain aspects which indicate a tendency towards biotrophism and as such M.perniciosa appears to be somewhat ambivalent in the mode of attack. The relationship with the host may be interactive in the first instance with the nature of host cells indicating induced hypertrophy and vacuolation, with an associated stimulation of host cell respiration.

The effect of toxins, generally implicated in necrotrophic attack of host fungi by other mycoparasites, are in this case apparently balanced by the occurrence of toxins which are produced by the host and are active against the pathogen. In vivo this is further complicated by the general occurrence of bacteria which may be antagonistic towards the pathogen. The development of Wet Bubble disease of cultivated mushrooms is apparently influenced by a combination of host and pathogen derived toxins, host and pathogen derived growth promoting substances and the level of antibiosis resulting from the organisms colonising the casing and compost layer substrates.

The demonstration of toxins produced by the host and the pathogen is of interest particularly in indicating that A.bisporus possesses a potential means of disease resistance which could perhaps be exploited in selection or breeding programmes. The identification of A.bisporus strains capable of producing greater levels of toxins than those currently available may enable the development of commercial strains with an increased resistance to pathogens.

The demonstration of the ability of M.perniciosa to produce toxins complements investigations in this study into the ecology of the fungus (see Discussion, Chapter 1) and supports the view that M.perniciosa is a more aggressive competitor than was considered to be the case by previous workers.

CHAPTER 3

BIOLOGICAL CONTROL

I. GENERAL CONCEPTS

The concept of biological control, whereby an undesirable organism may be eliminated, or the consequences of the presence of such an organism can be reduced, by the introduction of one or more antagonistic organisms (control agents) has been extensively reviewed by Baker and Cook, (1974). The development of biological control of a wide range of pests and pathogens has been examined although few appear to be commercially successful to date. Those showing most potential are organisms for the control of arthropods, (Burges and Scopes, 1971). Successful control agents of such pests may be insects as demonstrated by the control of whitefly (Trialeurodes vaporarium) by Encarsia formosa and the control of red spider (syn. two spotted) mites (Tetranychus urticae) by Phytoseilus persimilis (Hussey and Scopes, 1977). In some cases fungi may be suitable as control agents of insect pests, Verticillium lecanii giving good control of whitefly and flies of the family Sciaridae under glasshouse conditions, (Hall and Burges, 1979, Hall, 1980). These insect and fungal control agents are now commercially available, as is Bacillus thuringiensis for control of caterpillars in glasshouses (Applied Horticulture Ltd. (U.K.)).

Biological control of fungal pathogens has not been developed to the same extent, although control of fungi using biological control agents has been demonstrated in a wide range of situations, most commonly where plant pathogens either in the soil or in the phylloplane are the target organisms.

Fungi which have been successfully used as biological control agents

against fungal pathogens are typically saprophytes capable of antagonising target organisms by the production of toxins, (Marois and Mitchell, 1981, Chand and Logan, 1984, Fokemma, 1973), or are necro- trophic mycoparasites capable of saprophytic growth in the absence of the target pathogen and able to directly parasitise the target by enzymic degradation or toxin production, (Baker and Cook, 1974, Tu, 1980, Tu and Vaartaja, 1981, Spencer, 1980). The processes involved in necrotrophic attack of fungi have been extensively discussed in Discussion, Chapter 2.

Verticillium lecanii in particular has been shown to be capable of controlling fungal diseases such as carnation rust caused by Uromyces dianthi, (Spencer, 1980) and bean rust caused by Uromyces appendiculatus (Allen, 1983). Tu, (1980) considered that Gliocladium virens has potential for control of Sclerotinia sclerotiorum but this has not been demonstrated on a large scale. G.virens showed considerable potential in small scale field trials by Tu and Vaartaja (1981), as a means of reducing the incidence of root rot of white beans caused by Rhizoctonia solani although it remains unclear whether control could be achieved on a commercial scale. Only in some studies has control been achieved on a satisfactorily large scale, as is the case in the control of Fusarium oxysporum f.sp. radicis-lycopersici the causal agent of crown rot of tomato by the use of Trichoderma harzianum or Penicillium finiculosum, (Marois, Mitchell and Sonoda, 1981).

In general, the use of bacteria as a means of controlling fungal pathogens has not received the same attention as the use of fungal antagonists. The use of bacteria was considered by Newhook, (1951), who demonstrated inhibition of Botrytis cinerea using a number of

bacterial antagonists. In some instances bacteria have shown potential as control agents (Chand and Logan, 1984, Howell and Stipanovic, 1980), Pseudomonas syringae for instance, has been shown to exhibit marked antagonism towards Ceratocystis ulmi the causal agent of Dutch Elm disease, both in vitro and in vivo, (Myers and Strobel, 1983).

Similarly, a range of rhizosphere bacteria have been shown to be potential control organisms for Fusarium oxysporum f.sp. dianthi on carnation (Sneh, 1981).

Pathogenesis of A.bisporus by mycoparasites within the casing layer is similar in some respects to that of soil borne plant pathogens. A fundamental difference between natural soils and mushroom substrates occurs in that while soil microbial populations vary from site to site mushroom substrates typically have a more uniform microbial population in a standardised environment in terms of temperature and humidity (Hayes, 1980). In field soils the success of a soil borne plant pathogen appears to be influenced by the ability of the fungus to interact successfully with the abiotic and biotic factors in its immediate environment (Marois, Mitchell and Sonoda, 1981) with the influence of the microflora apparently related to the total soil population of fungi and bacteria rather than to the presence or absence of specific organisms within the microflora. Marois and Mitchell, (1981) demonstrated that there is a negative correlation between the ability of Fusarium oxysporum f.sp. radicis-lycopersici to infect host plants and total soil populations of fungi and bacteria. No correlation between infection of host plants and the level of specific micro-organisms was noted by these authors. The influence of total soil microflora populations, which vary between sites, on the ability of soil

borne plant pathogens to infect hosts may account for the lack of field scale success of biological control of soil borne diseases in some instances.

This is in contrast to results from this study that indicated that M.perniciosa is not affected by the total microbial population of casing material and may be influenced by specific types of micro-organisms.

The biological control of fungal diseases of A.bisporus has received surprisingly little attention. This may be due to the current availability of fungicides effective against many of the major fungal pathogens of A.bisporus (Van Zaayen, 1983,Van Zaayen and Van Adrichem, 1982, Fletcher,Hims and Hall,1983, Gaze,1985).Resistance to modern fungicides such as Prochloraz and Chlorthalonil may arise in fungal pathogens of A.bisporus as it has done with previously effective fungicides. Such a build up of resistance to fungicides in fungal pathogens was particularly rapid in the case of benomyl (Samuels and Johnston, 1980, Fletcher, Connolly, Mountfield and Jacobs, 1980, Fletcher and Yarham, 1976). Since it is likely that fungicide resistance will arise again, the use of biological control methods of fungal pathogens of A.bisporus may in future be of importance.

II. BIOLOGICAL CONTROL OF M.PERNICIOSA

Experiments conducted in the course of this study have demonstrated that M.perniciosa may be controlled in casing soil to some degree by the introduction of bacterial antagonists. Of those organisms tested

in this study, <u>Pseudomonas</u> bacteria belonging to species group 3, (<u>P.fluorescens</u> complex) according to the classification of Buchanan and Gibbons, (1974), were identified as having the most potential for control of <u>M.perniciosa</u> in casing soil.

Baker and Cook, (1974), and Sinden, (1971) discussed biological control in terms of the processes involved in mushroom production and concluded that the entire process is, in reality, a biological control system itself. Composting, pasteurising and conditioning of compost has been developed, albeit unwittingly, to produce a substrate which is not sterile and yet contains a specific microflora, primarily bacteria and actinomycetes, that are highly conducive to growth of <u>A.bisporus</u>, with the virtual exclusion of other fungi at the time of spawning. The application of a casing layer supporting a specific microflora conducive to the successful initiation of sporophores similarly demonstrates the use of particular types of organisms in ensuring the healthy development of the crop (Sinden, 1971, 1972). The success of this process is reflected in the relatively small number of undesirable organisms which commonly deleteriously affect <u>A.bisporus</u> in commercial production (Vedder, 1978).

The choice of control agents should depend to a considerable degree on the ability of the organism to integrate with naturally occurring populations. While necrotrophic mycoparasites have been seen to act as biological control agents against some plant pathogens (Baker and Cook, 1974, Spencer, 1980, Tu, 1980), the use of such organisms in <u>A.bisporus</u> substrates is likely to cause damage to the crop itself.

The use of necrotrophic mycoparasites to control fungal pathogens of <u>A.bisporus</u> has been examined in studies by other workers. Gandy,

(1981), failed to achieve satisfactory control of Verticillium
fungicola, the causal agent of Dry Bubble disease of A.bisporus using
Trichoderma viride and T.polysporum because of antagonism of A.bisporus
by control fungi although these had previously been shown to be
severely antagonistic towards the pathogen in vitro (De Troghoff and
Ricard, 1976). Similarly, Acremonium strictum, antagonistic towards
M.perniciosa in plate cultures, is an unsatisfactory biological control
agent because of its ability to antagonise A.bisporus, (Gandy, 1979).

The unsuitability of necrotrophic mycoparasites as control agents
of mushroom pathogens is borne out by the fact that A.bisporus is
susceptible to attack by a number of necrotrophs which cause
economically important diseases in addition to M.perniciosa such as
Verticillium fungicola, Diehliomyces microspora, Dactylium dendroides
and Trichoderma viride. A.bisporus is also susceptible to attack by
mycoparasites which only occasionally occur on commercial cropping beds.
(Sinden, 1972). As a group, necrotrophic mycoparasites are clearly
unsuitable as biological control agents of pathogens of A.bisporus.

Mushroom composts and casing materials may also be colonised by
vigorous saprophytes including Botrytis sp. Chaetomium globosum,
Geotrichum sp. Aspergillus sp. and Penicillium sp., (Vedder, 1978,
Atkins, 1974). Competition from such fungi may reduce yield from mush-
room beds and it is apparent that the presence of fungi other than
A.bisporus in mushroom substrates should not be encouraged (Eicker,
1980, Fergus, 1978).

The use of bacteria as biological control agents of mushroom
pathogens is a logical approach to disease control in that they are
less likely to cause damage to A.bisporus crops than are necrotrophic
mycoparasites.

The selection of bacteria from mushroom substrates which are capable of antagonising fungal pathogens has the further advantage that such organisms may be more likely to achieve stable populations in mushroom substrates to which they are inoculated as control agents than are organisms selected from other environments. Regular sampling of casing soil inoculated with bacteria selected for antagonism against M.perniciosa in plate cultures demonstrated that the isolates used in this study rapidly established stable casing soil populations.

In most samples examined populations of potential control agents were greater in the absence than in the presence of M.perniciosa implying that where disease control agents are introduced prior to infection with M.perniciosa disease control is likely to be greater than in the present study where control organisms were introduced at the same time as the pathogen.

These results complement work by Tautorus and Townsley, (1983, 1984) who considered that biological control of olive green mould caused by Chaetomium olivaceum on mushroom beds may be achieved using a bacterium isolated from Phase I of the composting process. Treatment of infected compost with a thermophilic Bacillus sp. (termed Bacillus AOG and resembling B.coagulans) gave an increased yield and some protection against C. olivaceum.

The losses caused by olive green mould are not high however and this specific control method is unlikely to be of economic significance. As in this example, the present study has demonstrated that organisms capable of antagonising pathogens of A.bisporus may be isolated from mushroom substrates and environments and re-introduced to control disease to some degree.

III. IN VITRO SCREENING OF POTENTIAL BIOLOGICAL CONTROL AGENTS

In order to identify organisms with potential as biological control agents rapid techniques to assess the possible antagonism against target pathogens are required.

In this study dual plate cultures of M.perniciosa and single cell cultures of bacteria were used to determine the extent of antagonism of M.perniciosa in vitro. Dowding, (1978) considered that antagonism in vitro may give a false impression of the relationships between organisms in nature. Such techniques have to be employed however in order to give a preliminary indication of the potential of organisms as control agents wherever appropriate.

Whether antagonism in plate cultures can be expressed as an ability to control pathogens in vivo may depend on the mode of antagonism. In some instances antagonism in vitro has been attributed to a change in substrate pH as a result of fungal growth (Newhook, 1951), or to nutrient impoverishment (Skidmore, 1976). The use of such antagonists as biological control agents in natural substrates is less likely to be successful than where antagonism is the result of the direct production of antifungal substances or mycoparasitic attack because soils tend to have an overall buffering effect which tends to mask the effects of pH changes or nutrient impoverishment in vivo. The occurrence of antifungal substances has been widely reported (Berdy, 1974, Betina, 1964) and the role of these in biological control examined in some cases (McGinty, McFadden, Rawlinson and Buck, 1984, Bagient and Ogawa, 1960, Rai and Singh, 1980).

Dual cultures of M.perniciosa and bacteria isolated from mushroom substrates indicated that some bacteria may significantly inhibit

growth of the pathogen. In contrast to such a single expression of antagonism in vitro the same isolates when inoculated on to casing soil infected with M.perniciosa over compost colonised by A.bisporus influenced the development of Wet Bubble disease in one of two ways. Control of M.perniciosa by bacterial antagonists was expressed as either i) a reduction in the development of infected sporophore tissue on infected beds, or ii) as an increase in the yield of healthy sporophores in the presence of the pathogen.

The correlation between inhibition of M.perniciosa in vitro and the reduction in the development of infected sporophore tissue was better than between inhibition of the pathogen in vitro and any increase in yield of A.bisporus sporophores in infected plots.

While antagonism of M.perniciosa by bacteria in plate cultures does not give a complete indication of the ability to control the pathogen in vivo it is apparent that the technique could usefully be incorporated in more extensive studies in future to assist in the selection of potential control agents.

The bacteria shown in this study to have most potential as biological control agents for M.perniciosa all showed significant levels of antagonism against the pathogen in vitro. The mode of antagonism against the fungus is unclear, although it may be by the production of toxins which have been shown to occur in other isolates from Pseudomonas bacteria belonging to species Group 3-5, (Howell and Stipanovic, 1980, Gandy, 1968). The screening techniques employed in this study to identify antagonists active against M.perniciosa that were also antagonistic against A.bisporus using a modification of the pitting and rapid pitting techniques of Gandy, (1968), and Wong and Preece, (1979),

served to eliminate potential pathogens of A.bisporus from the test programme. Some Pseudomonas bacteria belonging to the same group as those shown in this study to have potential as biological control agents of mushroom pathogens may antagonise A.bisporus as pathogens in their own right (Paine, 1919, Gandy, 1968, Wong and Preece, 1980, Wong and Preece, 1979, Preece and Wong, 1982, Wong, Fletcher, Unsworth and Preece, 1982). In contrast this group of Pseudomonas bacteria also contains those which are beneficial to A.bisporus. Hayes, Randle and Last, (1969) demonstrated that Pseudomonas putida in particular can be conducive to sporophore initiation under commercial conditions (Eger, 1972, Urayama, 1967).

Any screening programme to identify potential antagonists of fungal pathogens of mushrooms must clearly include testing for pathogenesis of A.bisporus to eliminate those which are likely to deleteriously affect the crop itself.

The introduction of biological control agents capable of antagonising M.perniciosa may act by complementing the natural antifungal action of the casing microflora, made up of predominantly Pseudomonas bacteria, (Preece and Wong, 1982, Hayes, Randle and Last, 1969, Long and Jacobs, 1974) and by complementing the production of antifungal substances by A.bisporus which were demonstrated in the course of this study, in order to reduce the incidence and severity of Wet Bubble disease.

GENERAL CONCLUSIONS AND FUTURE DEVELOPMENTS

In each of the main areas of study this work has added to the fund of knowledge concerning the relationship between M.perniciosa and its host A.bisporus. The work has also added to the understanding

of necrotrophic mycoparasitism in general.

In the area of fungal ecology the effects of the physical and biological environment on development of the pathogen have been defined more closely than was previously the case.   The consequences of altering the conditions under which A.bisporus is cultivated on the development of the pathogen can now be clearly identified.

The examination of a range of fungi as potential hosts of M.perniciosa revealed a number of species which may be infected by the pathogen.   This work could be usefully expanded to give further information on species and habitats which could act as reservoirs of infection for commercial crops of A.bisporus.

The understanding of the factors influencing chlamydospore germination remains incomplete despite the demonstration of a maturation phase of spore development.   This is an aspect of the biology of M.perniciosa which warrants further investigation.

Elucidation of the mechanism by which germination of M.perniciosa chlamydospores is stimulated by the presence of the host may assist in control of such resting spores and may further add to the understanding of host-parasite interactions in general.

Microscopic investigations into the interactions between M.perniciosa and A.bisporus revealed that in conditions under which the host is typically cultivated enzymic breakdown of host cell walls occurs, but may be of secondary importance to the action of other factors such as antifungal or growth promoting substances. Microscopically M.perniciosa is unusual in having hyphal cells which apparently have a relatively high metabolic activity and may represent the site of enzyme or toxin production used in pathogenesis.   The ultrastructure of M.perniciosa warrants further investigations.

The effect of toxins, generally implicated in necrotrophic attack

of host fungi by other mycoparasites (Barnett and Binder, 1973), are, in this case, apparently balanced by the occurrence of toxins which are produced by the host and are active against the pathogen. In vivo this is further complicated by the general occurrence of bacteria which may be antagonistic towards the pathogen. The development of Wet Bubble disease of cultivated mushrooms is apparently influenced by a combination of host and pathogen derived toxins, host and pathogen derived growth promoting substances and the level of antibiosis resulting from the organisms colonising the compost and casing layer substrates.

In each case this is the first report of such biologically active substances produced by both M.perniciosa and A.bisporus with any degree of resolution. The further identification of such compounds would assist in the understanding of host-parasite interactions in the disease system. In the case of A.bisporus the correlation between levels of antifungal substances and susceptibility to fungal infection warrants continued investigation. Identification of antifungal substances may provide a means by which disease resistance can be developed in breeding programmes.

Experiments conducted in the course of this study have demonstrated that bacteria antagonistic toward M.perniciosa may be used as control agents in infected mushroom beds. The levels of control achieved in this study were insufficient in themselves to warrant further development of the isolated strains. However, these results, and in particular those relating to the suitability of screening techniques in vitro as a measure of determining effectiveness of control agents in vivo are likely to assist further studies in this area.

Further selection of bacteria on a larger scale than possible within the context of the present study is likely

ultimately to yield isolates with the capacity to control M.perniciosa.
The use of groups of antagonists and of antagonists effective against
more than one disease, while outside the scope of this study, may
in future increase the suitability of biological control still
further.

The likely occurrence of resistance in M.perniciosa to
fungicides which are currently effective against the pathogen may
in time necessitate further developments in this area of work.

ACKNOWLEDGEMENTS

REFERENCES

ALLEN, D.J. (1983).   Verticillium lecanii on the bean rust, Uromyces
appendiculatus.   Transactions of the British Mycological Society
79 (2) 362 - 364.

ANONYMOUS (1982).   Department of Biological Science Safety Handbook
pub: University of Stirling (Stirling)

ARORA, D.K. & DWIVEDI, R.S. (1980).   Mycoparasitism of Fusarium sp. on
Rhizoctonia solani.   Kühn, Plant and Soil, 55, 43-55.

ATKEY, P.T. & BARTON, R.J. (1976).   Virus of the mushroom pathogen
Mycogone perniciosa.   Annual report of the Glasshouse Crops Research
Institute for 1975, 127.

ATKINS, F.C. (1974).   Guide to mushroom growing pub. Faber and Faber,
(London.).

ATKINSON, N. (1954).   Psalliotin, the antibiotic of Psalliota xanthoderma.
Nature, 174, 598.

AYRES, P.G. (1983).   Conidial germination and germ tube growth of
Erysiphe pisi in relation to visible light and its transmission
through pea leaves.   Transactions of the British Mycological Society,
81 (2) 269-274.

BAGIENT, N.L. & OGAWA, J.M. (1960).   Activity of the antibiotic produced
by Pullularia pullulans.   Phytopathology 50, 82

BAKER, K.F. & COOK, R.J. (1974).   Biological control of plant pathogens.
pub. Freeman press, (San Francisco)

BARNETT, H.L. & BINDER, F.L. (1973).   The fungal host-parasite
relationship.   Annual Review of Phytopathology, 11, 293-312.

BARRON G.L. & FLETCHER, J.T. (1972).   Rhopalomyces elegans Corda.   A host
of Mycogone perniciosa.   Mushroom Science, 8, 383-386.

BARTNICKI-GARCIA, S. (1968).   Cell wall chemistry, morphogenesis and
taxonomy of fungi.   Annual Review of Microbiology, 22, 87-108.

BERDY, J. (1974). Recent development of antibiotic research and

classification of antibiotics according to chemical structure.

Advances in Applied Microbiology, 18, 308-406.

BETTINA, V. (1964). A systematic analysis of antibiotics using paper

chromatography. Journal of Chromatography, 15, 379-392.

BRACKE , C.E. (1967). Ultrastructure of fungi.   Annual review

of phytopathology, 5, 343-374

BRADBURY, J.F. (1970). Isolation and preliminary studies of bacteria

from plants. Review of Plant Pathology, 49 (5), 213-218

BRADY, B.L.K. (1978).   Personal Communication, C.M.I.

BRADY, B.L.K. & GIBSON, I.A.S. (1976a).   Verticillium fungicola

CMI descriptions of pathogenic fungi and bacteria, No. 498

BRADY, B.L.K. & GIBSON, I.A.S. (1976b). Mycogone perniciosa.

CMI descriptions of pathogenic fungi and bacteria, No. 499

BRIAN, P.W. (1951).   Antibiotics produced by fungi.   Botanical

Review, 17, 357-430.

BUCHANAN, R.E. & GIBBONS, N.E. (1974).   Bergey's Manual of

Determinative Bacteriology, 8th Edition, pub. Wilkins & Wilkins

(Baltimore).

BU'LOCK, J.D., DETROY, R.W., HOSTALEK, Z. & MUNIM-AL-SHAKARACHI, A. (1974).

Regulation of secondary biosynthesis in Gibberella fujikoroi.

Transactions of the British Mycological Society, 62, (2), 377-389.

BURGES, H.D. & SCOPES, N.W. (1971). Microbial control of insects and

mites, pub. Academic Press (New York and London).

CALLOW, J.A. (1983). Biochemical Plant Pathology, pub. Wiley (Chichester,

New York, Brisbane, Toronto, Singapore).

CHAND, T. & LOGAN, C. (1984). Antagonists and parasites of Rhizoctonia

solani and their efficacy in reducing stem canker of potato under

controlled conditions.   Transactions of the British Mycological

Society, 83 (1), 107-112.

CHANG, S.T. (1980). Mushrooms as human food, Bioscience, 30 (6), 399-401.

CHANTER, D.O. & THORNLEY, J.H.M. (1978). Mycelial growth and the
initiation and growth of sporophores in the mushroom crop.
Journal of General Microbiology, 106 (1), 55-65.

CHET, I. & HUTTERMAN, A. (1980). Chemical composition of hyphal walls
of Fomes annosus. European Journal of Forest Pathology, 10, (2-3)
65-70.

CHRISTIE, W.W. (1976). Lipid analysis : isolation, separation,
identification and structural analysis of lipids. pub. Pergamon Press
(Oxford, N.York, Toronto, Sydney, Paris, Braunschweig).

CIEGLER, A. KADIS, S. & AJL, J. (1971). Microbial Toxins, Vol. 6, pub.
Academic Press (New York and London).

CONN, H.J., JENNISON, M.W. & WEEKS, P.B. (1957). Routine tests for the
identification of Bacteria. Society of American Bacteriologists'
Manual of Microbial Methods, 140-168.

CORNFORTH, J.W., RYBACK, G., ROBINSON, P.M. & PARK, D. (1971). Isolation
and characterisation of the fungal vacuolation factor Bikaverin.
Journal of the Chemical Society, C, 2786-2787.

CONSTANTIN, J.N. & DUFOUR, L. (1892). In: Three diseases of cultivated
mushrooms (Smith, F.E.V., 1924). Transactions of the British
Mycological Society, 10, 81-97.

CROSS, M.J. (1971). Studies on Verticillium malthousei. Ware.
and Mycogone perniciosa. Magnus. PhD. Thesis, University of Bath.

CROSS, M.J. & JACOBS, L. (1968). Some observations on the biology of
spores of Verticillium malthousei, Mushroom Science, 7, 239-244.

CURRIER, H.B. (1957). Callose substances in plant cells. American
Journal of Botany, 44, 478-488.

DEACON, J.W. (1976). Studies on Pythium oligandrum an aggressive
parasite of other fungi. Transactions of the British Mycological
Society, 66 (3), 383-391.

DENNIS, C. & WEBSTER, J. (1971a). Antagonistic properties of species-
groups of Trichoderma. Production of non-volatile antibiotics.
Transactions of the British Mycological Society, 57 (1), 25-39.

DENNIS, C. & WEBSTER, J. (1971b). Antagonistic properties of species-
groups of Trichoderma. ii. Production of volatile antibiotics.
Transactions of the British Mycological Society, 57 (1), 41-48.

DENNIS, C. & WEBSTER, J. (1971c). Antagonistic properties of species-
groups of Trichoderma. iii. Hyphal Interactions. Transactions
of the British Mycological Society, 57 (3), 363-369.

DE TROGHOFF, H. & RICHARD, J.L. (1976). Biological control of Verticillium
malthousei by Trichoderma viride spray on casing soil in commercial
mushroom production. Plant Disease Reporter, 60, 677-680.

DEVEN, J.M. & MANOCHA, M.S. (1975). Effect of Glutamic acid on the fatty
acid and lipid composition of Choaneophora cucurbitarium. Canadian
Journal of Microbiology, (21), 1827-1833.

DIX, N.J. (1972). Effect of soil fungistasis on spore germination and
germ tube growth of Penicillium species. Transactions of the
British Mycological Society. 48 (1), 59-66.

DIX, N.J. & MITCHELL, C.P. (1976). Soil fungistasis and the theoretical
colonisation index of some soil fungi. Transactions of the British
Mycological Society, 67 (1), 136-139.

DOMSCH, K.H. & GAMS, W. (1969). Variability and potential of a soil
fungus population to decompose pectin, xylanord carboxymethylcellulose.
Soil Biology and Biochemistry, I, 29- 6.

DOWDING, P. (1978). Methods for studying microbial interactions. Annals
of Applied Biology, 89, 166-171

EVANS, G.H. & COOKE, R.C. (1982).   Studies on mucoralean
    mycoparasites, III.   Diffusable Factors from _Mortierella_
    vinacea, Dixon-Stewart that direct germ tube growth in
    Piptocephalis fimbriata, Richardson and Leadbeater.
    New Phytologist, _91_, 245-253.

FARKAS, V. (1979).   Biosynthesis of cell walls of fungi.
    Microbiological Review, _43_ (2), 117-144.

FERGUS, C.L. (1964).   Thermophilic and thermotolerant moulds
    and actinomycetes of composts during heating.
    Mycologia, _56_, 267-284.

FERGUS, C.L. (1969).   The cellulolytic activity of
    thermophilic fungi and actinomycetes, Mycologia, _61_,
    120-135.

FERGUS, C.L. (1978).   The fungus flora of compost during mycelium
    colonisation by the cultivated mushroom, _Agaricus brunnescens_.
    Mycologia, _70_, 636-644.

FERMOR, T.R. (1982). _Agaricus macrosporus_: an edible
    fungus with commercial potential.   Scientia
    Horticulturae, _16_, 272-282.

FLEGG, P.B. (1979). Effects of competition on the development of mycelium, mycelial aggregates and sporophores of Agaricus bisporus. Scientia Horticulturae, 11, 141-149.

FLEGG, P.B. (1980). Temperature induced synchronisation of sporophore production in the mushroom Agaricus bisporus. Scientia Horticulturae, 13, 307-314.

FLETCHER, J.T., CONNOLLY, G., MOUNTFIELD, C.I. & JACOBS, L. (1980). The disappearance of Benomyl from mushroom casing. Annals of Applied Biology, 95, 73-82.

FLETCHER, J.T. & GANNEY, G.W. (1968). Experiments on the biology and control of Mycogone perniciosa Magn. Mushroom Science, 7, 221-237.

FLETCHER, J.T., HIMS, M.J. & HALL, R.J. (1983). The control of bubble diseases and cobweb diseases of mushroom with prochloraz. Plant Pathology, 32, 123-131.

FLETCHER, J.T. & YARHAM, D.J. (1976). The incidence of Benomyl tolerance in Verticillium fungicola, Mycogone perniciosa and Hypomyces rosellus in mushroom crops. Annals of Applied Biology, 84 (3), 343-353.

FOKEMMA, N.J. (1973). The role of saprophytic fungi in antagonism against Drechslera sorokiniana (Helminthosporium sativum) on agar plates and on rye leaves with pollen. Physiological Plant Pathology, 3, 195-205.

FORER, L.B., WUEST, P.J. & WAGNER, V.R. (1974). Occurrence and economic impact of fungal diseases of mushrooms in Pennsylvania. Plant Disease Reporter, 58 (11), 987-991.

GANDY, D.G. (1968). A technique for screening bacteria causing brown blotch of cultivated mushrooms. Annual Report of Glasshouse Crops Research Institute for 1967, 150-154.

GANDY, D.G. (1975). Fungicide tolerance in Verticillium fungicola (syn. malthousei). Report of the Glasshouse Crops Research Institute for 1974, 112-113.

GANDY, D.G. (1979). Inhibition of Mycogone perniciosa growth by Acremonium strictum. Transactions of the British Mycological Society, 72 (1), 151-154.

GANDY, D.G. (1981). Investigations into biological control of Verticillium fungicola. Report of the Glasshouse Crops Research Institute for 1979, 146.

GANDY, D.G. (1983), Personal communication. Glasshouse Crops Research Institute.

GANDY, D.G. & SPENCER, D.M. (1976). The use of Chlorothalonil for the control of Benzimidazole tolerant strains of Verticillium fungicola (Preuss) Hassebr. on the cultivated mushroom. Scientia Horticulturae, 5, 13-21.

GANDY, D.G. & SPENCER, D.M. (1978). Fungicides for the control of Mycogone perniciosa (Magn.). The cause of Wet Bubble on the cultivated mushroom. Scientia Horticulturae, 8, 307-313.

GARIBOVA, L.V. (1968a). Antagonistic activity of the mycelium of a cultivated mushroom - Agaricus bisporus. Mikologiya I Fitopatologiya, 2 (2), 81-87 (Abst.).

GARIBOVA, L.V. (1968b). Resistance of monospore strains of cultivated mushroom to Mycogone disease. Biol Nauk, 11 (1), 102-108 (Abst.).

GARRETT, S.D. (1970). Pathogenic root infecting fungi, pub. Cambridge University Press. (London).

GAZE, R. (1985). Personal communication. ADAS National Mushroom Consultant.

GERRITS, J.P.G., BELS-KONING, H.C. & MULLER, F.C. (1967).   Changes in
compost constituents during composting, pasteurisation and cropping.
Mushroom Science, 6, 225-243

GLAUERT, A.M. (1965).   The fixation and embedding of biological specimens.
In: Techniques for electron microscopy, 2nd edition.   Edit:
Kay, D. pub. Blackwell Scientific Publications (Oxford).

GOODAY, G.W. & TRINCI, A.P.J. (1980).   Wall structure and biosynthesis in
fungi.   Symposium of the Society of General Microbiology, 30, 207-251.

GRAY, D.J. & MORGAN-JONES, G. (1981).   Host-parasite relationships of
Agaricus brunnescens and a number of microparasitic Hyphomycetes.
Mycopathologia, 75, (1), 55-59.

GURR, E. (1960).   Encyclopaedia of microscopic stains.
pub. Leonard Hill Ltd., (London).

HALE, L.J. (1965).   Biological laboratory data. 2nd Edition.
pub. Methuen and Co. Ltd. (London).

HALL, R.A. (1980). Laboratory infection of insects by
Verticillium lecanii strains isolated from phytopathogenic fungi.
Transactions of the British Mycological Society, 74 (2),
445-446.

HALL, R.A. & BURGES, H.D. (1979). Control of aphids in glasshouses with
the fungus Verticillium lecanii.   Annals of Applied Biology,
93, 235-246.

HAMES, B.D. (1978). Methods for preparing fractionating gradients.
In: Centrifugation: a practical approach. (edit. Rickwood, D.)
pub. Information Retrieval Ltd., (London).

HARRIS, P.J. & HARTLEY, R.D. (1976).   Detection of bound ferulic acid
in cell walls of the Gramineae by ultraviolet fluorescence
microscopy.   Nature. 259, 508-510.

HAWKSWORTH, D.L. (1981). A survey of the Fungicolous conidial fungi.
In: Biology of Conidial Fungi, pub. Academic Press (New York
and London), 171-244.

HAWKSWORTH, D.L., SUTTON, B.C. & AINSWORTH, G.C. (1983). Ainsworth and
Bisby's Dictionary of the Fungi, pub. C.M.I. (London).

HAYAT, M.A. (1978).   Introduction to Biological Scanning electron
microscopy.   Pub. University Park Press. (Baltimore).

HAYES, W.A. (1969). Microbiological changes in composting
what straw/horse manure mixtures.   Mushroom Science VII
173-186.

HAYES, W.A. (1980).   Solid state fermentation and the cultivation
of edible fungi.   In: Fungal biotechnology (edit. Smith,
Berry,   and   Kristiansen), pub. Academic Press (New York
and London).

HAYES, W.A., RANDLE, P.E. & LAST, F.T. (1969).   The nature of the
microbial stimulus affecting sporophore formation in Agaricus
bisporus.   Annals of Applied Biology, 64, 177-187.

HINTON, L. (1982).   Mushrooms in Britain and Ireland.   Occasional
paper No. 25., pub. Cambridge University.

HOPPE, H.H. & HEITEFUSS, R. (1974). Permeability and membrane lipid metabolism of Phasaeolus vulgaris infected with Uromyces phaseoli II. Changes in lipid concentration & ^{32}P incorporation into phospholipids. Physiological Plant Pathology, 4, 11-32.

HOWELL, C.R. & STIPANOVIC, R.D. (1980). Suppression of Pythium ultimum induced damping off of cotton seedlings by Pseudomonas fluorescens and its antibiotic, Pyoluteorin. Phytopathology, 70, 712-715.

HUSSEY, N.W. & SCOPES, N.E.A. (1977). The introduction of natural enemies for pest control in glasshouses: Ecological considerations. In: Environmental Science Research, Vol. 11, edit. Ridgway, R.L. and Vinson, S.B., pub. Plenum Press, (New York and London).

IKEDUIGWU, F.E.O. (1976). Ultrastructure of hyphal interference between Coprinus heptemerus and Ascobolus crenulatus. Transactions of the British Mycological Society, 66, 281-290.

IKEDUIGWU, F.E.O., DENNIS, C. & WEBSTER, J. (1970). Hyphal interference by Peniophora gigantea against Fomes annosus. Transactions of the British Mycological Society, 54, 307-308.

IKEDUIGWU, F.E.O. & WEBSTER, J. (1970a). Antagonism between Coprinus heptemerus and other coprophilous fungi. Transactions of the British Mycological Society, 54, 181-204.

IKEDUIGWU, F.E.O. & WEBSTER, J. (1970b). Hyphal interference in a range of coprophilous fungi. Transactions of the British Mycological Society, 54, 205-210.

INGOLD, C.T. (1967). Liberation mechanisms of fungi. In: Airborne microbes: 17th symposium of the Society for General Microbiology, edit. Gregory, P.M. and Monteith, J.L., 102-115.

JELJASZEWICZ, J. & WASTRÖM, T. (1978). Bacterial toxins and cell membranes, pub. Academic Press (New York and London).

JENSEN, H.A. (1962). Botanical histochemistry. pub. W.H. Freeman & Co. Ltd. (London), 408.

JONES, M.G., METZGER, J.D. & ZEEVART, J.A.D. (1980). Fractionation of Gibberellins in plant extracts by reverse phase HPLC, Plant Physiology, 65 (2), 218-221.

KABIR, D., ROSENSTREICH, L. & MERGENHAGEN, S.E. (1978). Bacterial endotoxins and cell membranes, In: Bacterial toxins and cell membranes,(edit. Jeljaszewicz, J. and Wadström, T.), pub. Academic Press (New York and London), 59-87.

KELLOCK, L. & DIX, N.J. (1984a). Antagonism by Hypomyces aurantius I. Toxins and hyphal interactions. Transactions of the British Mycological Society, 82 (2), 327-334.

KELLOCK, L. & DIX, N.J. (1984b). Antagonism by Hypomyces aurantius II Ultrastructure studies of hyphal disruption. Transactions of the British Mycological Society, 82 (2), 335-338.

KING, E.O., WARD, M.K. & RANEY, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. Journal of Laboratory and Clinical Medicine, 44, 301-307.

KLARMAN, W.L. & SANFORD, J.B. (1968). Isolation and purification of an antifungal principle from infected soy beans. Life Sciences, 7, 1095-1103.

KLEYN, J.G., JOHNSON, W.M. & WETZLER, T.F. (1981). Microbial aerosols and actinomycetes in etiological considerations of mushroom workers lung. Applied and Environmental Microbiology, 41 (6), 1454-1460.

KO, W.H. & LOCKWOOD, J.L. (1967). Soil fungistasis : relation to fungal spore nutrition. Phytopathology. 57. 890-901.

KOVACS, N. (1956). Identification of Pseudomonas pyocyanea by the

   oxidase reaction.   Nature 178. (No. 4535), 703.

LAUNDON, G. (1968). In: Plant Pathologists Pocketbook compiled by:

   Commonwealth Mycological Institute, pub. Commonwealth Agricultural

   Bureaux. (Kew), 224-249.

LELLIOTT, R.A., BILLING, E. & HAYWARD, A.C. (1966). A determinative

   scheme for the fluorescent plant pathogenic pseudomonads.

   Journal of Applied Bacteriology, 29 (3), 470-489.

LEWIS, D.H. (1973). Concepts in fungal nutrition and the origin of

   biotrophy.   Biological Review, 48, 261-278.

LEWIS, D.H., (1974). In: Evolution in the microbial world (edit. Carlile,

   M.J. & Skehel, J.J.), 367-392. Cambridge University Press (London).

LIAO, Y.M. (1981). The chlamydospore germination of Mycogone perniciosa

   and its effect on the occurrence of Wet Bubble of mushroom on the bed.

   Journal of Agricultural Research of China, 30 (3), 332-339 (Abst.).

LLOYD, A.D. & LOCKWOOD, J.L. (1966). Lysis of fungal hyphae in soil and

   its possible relation to autolysis.   Phytopathology, 56, 595.

LOCKWOOD, J.L. (1977). Fungistasis in soils. Biological Reviews, 52, 1-44.

LONG, P.E. & JACOBS, L. (1974). Aseptic fruiting of the cultivated

   mushroom Agaricus bisporus. Transactions of the British Mycological

   Society, 63, 99-107.

LUFT, J.H. (1971). Ruthenium red and violet I. Chemistry, purification,

   methods for use for electron microscopy and mechanism of action.

   Anatomical Record, 171, 347-368.

LUND, B.M. & LYON, G.D. (1975). Detection of inhibitors of Erwinia

   carotovora and E.herbicola on thin layer chromatograms. Journal

   of Chromatography, 110, 193-196.

LUTRELL, E.S. (1974). Parasitism of fungi on vascular plants.

   Mycologia, 66, 1-15

McGINTY, R.M., McFADDEN, J.J.P., RAWLINSON, C.J. & BUCK, K.W. (1984).
Widespread inhibitor production in culture by isolates of
Gaeumannomyces graminis var. tritici. Transactions of the British
Mycological Society, 82 (3), 429-434.

MANOCHA, M.S. (1965). Fine structure of the Agaricus carpophore.
Canadian Journal of Botany, 43, 1329-1334.

MANOCHA, M.S. (1980). Role of lipids in host-parasite relations of a
mycoparasite. Physiological Plant Pathology, 17, 319-328.

MANOCHA, M.S. & GOLESORKHI, R. (1979). Host-parasite relations in a
mycoparasite. V. Electron microscopy of Piptocephalis virginiana
infection in compatible and incompatible hosts. Mycologia, 71,
565-576.

MANOCHA, M.S. & GOLESORKHI, R. (1981). Host-parasite relationships in
a mycoparasite. 7. Light and scanning electron microscopy of
interactions of Piptocephalis virginiana with host and non-host
species. Mycologia, 73 (5), 976-988.

MANOCHA, M.S. & LEE, K.Y. (1971). Host-parasite relationships in a
mycoparasite I. Fine structure of host-parasite and their inter-
face. Canadian Journal of Botany , 49, 1677-1681.

MANOCHA, M.S. & LETOURNEAU, D.R. (1978). Structure and composition of
host-parasite interface in a mycoparasite interface. Physiological
Plant Pathology, 12, 141-150.

MANOCHA, M.S. & MAHARAJ, R. (1981). Parasite relations in a myco-
parasite VI. Leakage of electrolytes, amino acids and sugars after
infection with Piptocephalis virginiana. Canadian Journal of
Botany , 59 (7), 1271.

MANSFIELD, J.W. & HUTSON, R.A. (1980).   Microscopical studies on
     fungal development and host responses in broad bean and tulip
     leaves inoculated with five species of Botrytis.   Physiological
     Plant Pathology, 17, 131-145

MAROIS, J.J. & MITCHELL, D.J. (1981).   Effects of fungal communities
     on the pathogenic and saprophytic activities of Fusarium oxysporum
     f.sp. radicis - lycopersici.   Phytopathology, 71 (12), 1251-1256.

MAROIS, J.J., MITCHELL, D.J. & SONODA, R.M. (1981).   Biological
     control of Fusarium crown rot of tomato under field conditions.
     Phytopathology, 71, (12), 1257-1260

MERCK, E. (1974).   Dyeing Reagents for Thin Layer and Paper
     Chromatography.   pub. Merck (Darmstadt).

MITCHELL, C.P. & DIX, N.J. (1975a).   Influence of carbon source
     in the sporulation medium on the theoretical colonisation index
     and the sensitivity of Trichoderma viride spores to soil
     fungistasis.   Transactions of the British Mycological Society
     65 (2), 259-264.

MITCHELL, C.P. & DIX, N.J. (1975b).   Growth of Trichoderma sp. under
     the influence of soil fungistasis.   Transactions of the British
     Mycological Society, 64 (2), 235-251

MONTGOMERY, G.W.G., ADAMS, D.J. & GOODAY, G.W. (1984).   Studies
     on the purification of chitin synthase from Coprinus cinereus.
     Journal of General Microbiology, 130, 291-297.

MOORE, C. (1959).   British parasitic fungi.   pub. Cambridge University
     Press (London).

MUIR, G.D. (1977). Hazards in the chemical laboratory. pub. The Chemical Society (London).

MYERS, D.F. & STROBEL, G.A. (1983). Pseudomonas syringae as a microbial antagonist of Ceratocystis ulmi in the apoplast of American elm. Transactions of the British Mycological Society, 80 (3), 389-394.

NEWHOOK, N.J. (1951). Microbiological control of Botrytis cinerea. Pers.1. The role of pH changes and bacterial antagonism. Annals of Applied Biology, 38, 169-184.

O'BRIEN, T.P., FEDER, N. & McCULLY, M.E. (1964). Polychromatic staining of plant cell walls by Toluidine blue O. Protoplasma, 59, 368-373.

O'BRIEN, T.P.& McCully, M.E. (1981). The study of plant structures. Principles and selected methods. publ. Teramocaroh. P.T.Y. Ltd., (Victoria).

O'NEIL, T.M. (1981). Narcissus Smoulder; Cause, epidemiology and host resistance. PhD thesis. University of Stirling.

OSBORNE, D.J. & McCALLA, D.R. (1961). Rapid bioassay for Kinetin and Kinins using senescing leaf tissues. Plant Physiology, 36, 219-221

OSMAN, M. & VALADON, L.R.G. (1984). Fungistatic napthoquinones of Verticillium agaricinum. Transactions of the British Mycological Society, 84 (2), 713-751

PACHENARI, A. & DIX, N.J. (1980). Production of toxins and
wall degrading enzymes by Gliocladium roseum. Transactions
of the British Mycological Society, 74 (3), 561-556.

PAINE, S.G. (1919). Studies in bacteriosis. II. A brown blotch
disease of cultivated mushrooms. Annals of Applied Biology, 5
206-219.

PALLERONI, N.J. & DOUDOROFF, M. (1972). Some properties and
taxonomic subdivisions of the genus Pseudomonas. Annual
Review of Phytopathology, 10, 73-100.

PARANJPE, M.S. & CHEN, P.K. (1979). Morphogenesis of Agaricus
bisporus. Changes in protein and enzyme activity. Mycologia, 71
(3), 469-478.

PARK, D. (1963). Evidence for a common fungal growth
regulator. Transactions of the British Mycological Society, 46
541-548.

PARK, D. (1975). A cellulolytic pythiaceous fungus. Transactions
of the British Mycological Society, 65 (2), 249-257.

PEGG, G.F. (1973). Gibberellin-like substances in the sporophores
of Agaricus bisporus (Lange) Imbach. Journal of Experimental
Botany, 24 (81), 675-688.

PREECE, T.F. & WONG, W.C. (1982). Quantitative and scanning electron
microscope observations on the attachment of Pseudomonas tolaasii
and other bacteria to the surface of Agaricus bisporus.
Physiological Plant Pathology, 21, 251-257.

RAI, B. & SINGH, D.B. (1980). Antagonistic activity of some leaf surface
microfungi against Alternaria brassicae and Drechslera graminea.
Transactions of the British Mycological Society, 75, 363-369.

RAI, B. SINGH, V.N. & SINGH, D.B. (1980). Trichoderma viride as a
mycoparasite of Aspergillus sp. Plant & Soil, 57 (1), 131-137.

REYNOLDS, E.S. (1963). The use of lead citrate at high pH as an electron
opaque stain in electron microscopy. Journal of Cell Biology, 17,208-212

RIMOCZI, I. & VETTER, J. (1975). Effect of 6-methyluracyl and
2-chloroethyl-phosphonic acid on the fructification of and crop
of various strains of champignon. Agaricus bisporus (Lange)
Singer. Acta Agronomica Academiae Scientiarum Hungaricae, 24 (Abst).

ROLAND, J.C. (1978). General preparation and staining of thin sections.
In: Electron microscopy and cytochemistry of Plant Cells. (edit.
Hall, J.L.), pub. Elsevier/North Holland Biomedical Press, 1-62.

ROSSALL, S., MANSFIELD, J.W., & HUTSON, R.A. (1980). Death of Botrytis
cinerea and B.fabae following exposure to wyerone derivatives in vitro
and during infection development in broad bean leaves. Ibid.16, 135-146.

RUDAKOV, O.L. (1978). Physiological groups in mycophilic fungi.
Mycologia, 70, 150-159.

SABATINI, D.D., BENSCH, K. & BARRNETT, R.J. (1963). Chytochemistry and
electron microscopy. The preservation of cellular ultra-structure and
enzymic activity by aldehyde fixation. Journal of Cell Biology, 17
19-58.

SAMUELS, G.J. & JOHNSTON, P.R. (1980). Benomyl and the Verticillium
diseases of cultivated mushrooms. New Zealand Journal of
Agricultural Research, 23, 155-157.

SINDEN, J.W. (1971). Ecological control of pathogens and Weedmolds
in mushroom culture. Annual Review of Phytopathology, 9, 411-432.

SINDEN, J.W. (1972). Disease problems in technologically advanced
mushroom nurseries. Mushroom Science, 8, 125-130

SIROCKIN, G. & CULLIMORE, S. (1969). Practical microbiology.
pub. McGraw Hill, (Maidenhead).

SKIDMORE, A.M. (1976).   Interactions in relation to biological control

    of plant pathogens.   In: Microbiology of Aerial Plant Surfaces,

    (edit. Dickinson, C.H. & Preece, T.F.), 507-528, Academic Press

    (New York and London).

SKIDMORE, A.M. & DICKINSON, C.H. (1976).   Colony interactions and hyphal

    interference between Septoria nodorum and phylloplane fungi.

    Transactions of the British Mycological Society, 66, 57-64.

SLADKY, Z. & TICHY, V. (1974).   Stimulation of the formation of

    fruiting bodies of the fungus Lentinus tigrinus (Bull)Fr. by

    growth regulators. Biologia Plantarum (Praha), 16 (6), 436-443.

SMITH, F.E.V. (1924). Three diseases of cultivated mushrooms.

    Transactions of the British Mycological Society, 10, 81-97.

SMITH, J.E. & BERRY, D.R. (1975).   The filamentons fungi, Vol. 1:

    Industrial mycology, pub. Edward Arnold (London).

SMITH, J.F. (1976).   Rapidly prepared composts. Report of the Glasshouse

    Crops Research Institute for 1975. 115-116.

SMITH, J.J. & MANSFIELD, J.W. (1981). Interactions between

    Pseudomonads and leaves of oats, wheat and barley.

    Physiological Plant Pathology, 18, 345-346.

SNEH, B. (1981). Use of rhizosphere chitinolytic bacteria for

    biological control of Fusarium oxysporum. F.sp. Dianthi

    in carnation.   Phytopathologische Zeitschrift, 100 (3),

    251-257.

SPENCER, D.M. (1980).   Parasitism of carnation rust

    (Uromyces dianthi) by Verticillium lecanii. Transactions

    of the British Mycological Society, 74, 191-194.

SWISHER, R. & CARROLL, G.E.C (1980). Fluorescein diacetate hydrolysis as an estimator of microbial biomass on coniferous needle surfaces. Microbial Ecology, 6, 217-226.

TAUTORUS, T.E. & TOWNSLEY, P.M.(1983). Biological control of olive green mold in Agaricus bisporus cultivation. Applied and Environmental Microbiology, 45, 511-515.

TAUTORUS, T.E. & TOWNSLEY, P.M. (1984). Analysis of an effective Antibiotic (Chaetomacin) isolated from a thermophilic Bacillus sp. against olive green mold. Appled and Environmental Microbiology, 47 (4), 775-779.

TERASHIMA, N., ISHIDA, M., HAMASAKI, T. & HATSUDA, Y. (1972). Moniliaceae: Isolation of Bikaverin from Mycogone Japii. Phytochemistry, 11, 2880.

THOMAS, D.S. & MULLINS, J.T. (1969). Role of enzymatic wall softening in plant morphogenesis : Hormonal induction in Achyla. Science, 156, 84.

THORNLEY, M.J. (1960). The differentiation of Pseudomonas from other gram negative bacteria on the basis of arginine metabolism. Journal of Applied Bacteriology, 23 (1), 37-52.

TOUZE-SOULET, J.M., RAMI, J., DARGENT, R. & MONTANT, C. (1978). Aspects particuleurdes hyphes de Boletus edulis Fr. ex. Bull. et de leur parois après actions des filtrats de culture du Hypomyces chlorinus Tul. C.R. Acad. Sc. Paris t., 286.

TSUNEDA, A. & HIRATSUKA, Y. (1980). Parasitisation of Pine Stem Rust Fungi by Monocillium nordinii. Phytopathology, 11, 1101-1103.

TU, J.C. (1980). Gliocladium virens, a destructive mycoparasite of Sclerotinia sclerotiorum. Phytopathology, 70 (7), 670-674.

TU, J.C. & VAARTAJA, O. (1981). The effect of the hyperparasite Gliocladium virens on Rhizoctonia solani and on Rhizoctonia root rot of white beans. Canadian Journal of Botany, 59 (1), 22-34.

TURNER, E.M., WRIGHT, M., WARD, T., OSBORNE, D.J. & SELF, R. (1975). Production of ethylene and other volatiles and changes in cellulase and laccase activities during the life cycle of the cultivated mushroom, Agaricus bisporus. Journal of General Microbiology, 91, 167-176.

UPADHYAY, R.S., RAI, B. & GUPTA, R.C. (1981). Fusarium udum as a mycoparasite of Mortierella subtilissima. Plant and Soil, 60 (1), 149-151.

URAYAMA, T. (1967). Initiation of pinheads in Psilocybe panaeoliformis caused by certain bacteria. Mushroom Science, 6, 141.

VAN ZAAYEN, A. (1983). Prochloraz (Sporgon) en nieuw Fungicide in de champignonteelt. Champignoncultuur, 27 (4), 163-167.

VAN ZAAYEN, A. & RUTJENS, A.J. (1981). Thermal death points for two Agaricus species and for the spores of some major pathogens. Mushroom Science, 11. Proceedings of the Eleventh International Scientific Congress on the Cultivation of Edible Fungi. Australia, 393-402.

VAN ZAAYEN, A. & VAN ADRICHEM, J.C.J. (1982). Prochloraz for control of fungal pathogens of cultivated mushrooms. Netherlands Journal of Plant Pathology, 88, 203-213.

VARIOUS, (1965). Mushroom Growers Association Bulletin, 186, 238-261.

VEDDER, P.J.C. (1978). Modern Mushroom Growing, pub. Educaboek B.V. (Culemborg).

VINCENT-DAVIES, S. (1972). Relationships between Mycogone perniciosa
magnus. and its host Agaricus bisporus (Lange). Sing. the
cultivated mushroom. PhD thesis, University of Bath.

WADSTRÖM, T. (1978). Introductory overview. In: Bacterial toxins
and cell membranes, (edit. Jeljaszewicz, J. and Wadström, T.),
pub. Academic Press (New York and London), 1-7.

WEINBERG, E.D. (1970). Biosynthesis of secondary metabolites: role
of trace metals. Advances in microbial physiology, 4, 1-44.

WHEELER, H.M.S. (1975). Plant pathogenesis, pub. Springer-Verlag
(New York, Heidelberg, Berlin).

WILKINS, W.H. (1946). Investigation into the production of bacterio-
static substances by fungi. Preliminary examination of more of
the larger Basidiomycetes and some of the larger Ascomycetes.
Annals of Applied Biology, 33, 188-190.

WONG, W.C., FLETCHER, J.T., UNSWORTH, B.A. & PREECE, T.F. (1982).
A note on ginger blotch, a new bacterial disease of the cultivated
mushroom, Agaricus bisporus. Journal of Applied Bacteriology, 52,
43-48.

WONG, W.C. & PREECE, T.F. (1979). Identification of Pseudomonas
tolaasi: The white line in agar and mushroom tissue block rapid
pitting tests. Journal of Applied Bacteriology, 47, 401-407.

WONG, W.C. & PREECE, T.F. (1980). Pseudomonas tolaasi in mushroom
crops: A note on primary and secondary sources of the bacterium
on a commercial form in England. Journal of Applied Bacteriology,
49, 305-314.

YOU, C.H., BYUN, M.O., PARK, Y.H. & SHIN, G.C. (1981). Studies on
the development of a new strain No. 705 of Agaricus bisporus
(Lange) Sing. Korean Journal of Mycology, 9 (3), 133-139 (Abst.).

ZOBERI, M.H. (1961). Take off of mould spores in relation to wind
speed and humidity. Annals of Botany, 25, 53-64.