# SOME STUDIES ON THE COMPETITIVE COLONISATION OF CELLULOSIC SUBSTRATES BY MICROORGANISMS

by

DAVID JOHN PENN, B.Sc.

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## SUMMARY

In an investigation of aspects of the competitive colonisation of straw by microorganisms, fungi were first isolated from samples of straw to determine the predominant species present. Straw treated with ammonia solutions was also isolated from; under these conditions, Coprinus cinereus became the dominant fungus.

After screening the more commonly occurring cellulolytic fungi for high linear growth rate and cellulolytic ability, eight fungi were selected for further studies.

An assessment of the optimum pHs and temperatures for linear growth showed how particular fungi may be encouraged to dominate the colonisation of straw by control of one or both of these parameters.

The eight fungi varied considerably in their ability to break down straw in pure culture, C. cinereus and Fusarium culmorum being rather better than the others, and tests indicated that C. cinereus and two other fungi may be able to cause some breakdown of lignin.

Most of the more commonly isolated fungi were found to be capable of inhibiting the growth of others in interaction studies. This ability varied widely, Trichoderma spp. possessing by far the greatest capacity, followed by C. cinereus and then Chaetomium globosum. C. cinereus was generally more successful at higher pHs.

Tests on C. cinereus indicated that no powerful stable volatile or non-volatile toxins effective against the assay fungi used are produced.

Several fungi showed an aggressive ability towards C. cinereus in microscopic studies of hyphal interaction. C. cinereus appeared to possess little or no aggressive ability.

The study suggests that domination of straw by C. cinereus is favoured by alkaline pHs and high temperatures, and points to the way these and other factors may affect competition by other fungi.

Key words: Straw, cellulolytic, fungi, competition, Azotobacter spp.

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CONTENTS

# CHAPTER 1

# INTRODUCTION

## 1.1 THE STUDY

THIS THESIS describes a programme of research into aspects of the competitive colonisation by microorganisms of cropped barley straw. The work was carried out as part of an investigation aimed at developing a process for the fungal upgrading of straw to produce an animal feedstuff. Physiological studies and observations of interactions between fungi previously isolated from straw were used to provide an insight into some of the factors affecting domination and maintenance of domination of the substrate by particular fungi.

## 1.2 STRAW AS A WASTE MATERIAL

About fifteen million tons of grain are produced in the United Kingdom each year (Annual Abstract of Statistics, 1975) and along with that grain, around ten million tons of that much less nutritionally valuable material, straw (N.F.U., 1973). About two-

thirds of this is barley straw, about a quarter wheat straw and most of the rest oat straw (N.F.U., 1973). Its main use is as a bedding material for livestock and as a component in feed rations.

As a result of changes in agricultural practice and increases in production over the past few decades, more than a third of the straw now produced is surplus to requirements and must somehow be disposed (ACAH, 1973). The problems created by the need to dispose of this straw have been the subject of three reports published recently (ACAH, 1973; N.F.U., 1973; McLean, 1973) and research has been stimulated which is directed towards its better utilisation. A major cause of this surplus lies in the recent trend towards specialisation in arable farming in the drier eastern counties and stock farming in the wetter western and northern counties, with the effect of separating to a large extent the supply of and the demand for straw (Fulbrook, et al, 1973). Partly as a consequence of this, but also partly due to increased intensification of livestock, avoiding the use of straw as bedding, on-farm utilisation of straw has declined considerably.

### 1.2.1 The use and disposal of straw

Table 1 below shows the methods of use and disposal of straw currently practiced in the United Kingdom. About one third is used for bedding, about a third is burnt and a further fifteen percent is used for feeding. It will also be seen that 38.2% of straw produced is disposed of; most is burnt in the field and it is primarily this method of disposal, concentrated in the eastern half of the country (80% of all straw burned) that has given rise to the present concern over straw disposal. Damage is often caused to hedgerows, trees and even buildings, and may destroy much of the beneficial natural wildlife and insect populations (ACAH, 1973).

Table 1. Methods of use and disposal of straw in the United Kingdom (NFU, 1973).

| Method of use or disposal | Millions of tons | Percentage of total |
|---|---|---|
| Feeding | 1.40 | 15.0 |
| Bedding and crop storage | 3.40 | 36.4 |
| Inter-farm sales | 0.86 | 9.3 |
| Burned | 3.42 | 36.6 |
| Ploughed in | 0.15 | 1.6 |
| Non-agricultural uses | 0.10 | 1.1 |
| Total | 9.23 | 100.0 |

In addition, a large quantity of organic matter and energy is lost to the atmosphere. Burning, however, is considered to be the cheapest method of disposal (McLean, 1973). Also, pathogens that can survive saprophytically on straw may be lessened and wild oats are probably also reduced, though the apparent cheapness of the method is probably the main reason for its widespread use (ACAH, 1973).

### 1.2.2 The recycling of straw

The enormous tonnage of straw produced each year represents a valuable source of energy, locked primarily in the reduced carbon present. Ploughing in straw transfers this energy to the soil, where it is made use of by the soil microflora. This may create temporary problems with regard to the availibility to plants of nitrogen in the soil, but the added organic matter contributes to the maintenance of good soil structure (McLean, 1973). A long-term experiment undertaken to determine the effects of chopping and ploughing in of straw on soil

structure and fertility showed that the method of disposal has little or no effect on the productive capacity of the soil, provided that adequate supplies of fertiliser are added (Rutherford, 1975). Ploughing in may even contribute to the biological control of plant pathogens in the soil (O'Callaghan, 1975). However, the method of disposal is often not appropriate for economic and technical reasons; the Advisory Council for Agriculture and Horticulture has stated that at present there is no feasible economic alternative to burning for most farmers who have a straw surplus problem (ACAH, 1973).

### 1.2.3 Other uses for straw

Most saved straw is used in animal bedding and crop storage. Its use as bedding is likely to continue to decline as intensive livestock systems, requiring little or no bedding, increase in number.

Straw fed to ruminants is used primarily to provide roughage in maintenance rations. Its structural characteristics, discussed later, are such that the energy-rich cellulose present is not readily available for breakdown by rumen microflora, so the material has a low digestibility. In addition, it also has a low protein equivalent.

Clearly, it would be desirable if an economic method could be found to improve the feed value of straw, by increasing the accessibility of some of the nutritionally valuable components within.

## 1.3 THE UPGRADING OF STRAW

The poor digestibility of straw, owing primarily to the crystallinity of the cellulose, the presence of lignin and possibly also hemicelluloses and in certain parts, silica, has led many people to investigate possible ways of modifying the material to improve its quality as a feedstuff. Chemical, physical and biological treatments have all been studied. A review of the subject has been

written by Hart, et al (1975).

1.3.1 Chemical treatments

The study of the use of sodium hydroxide as a means of improving the digestibility of straw has a long history. In 1900, Kellner and Köhler (O'Callaghan, 1975) boiled straw in NaOH and raised its organic matter digestibility (OMD) from around 40% to 88%. Later, Beckmann (1921) introduced a similar process that roughly doubled digestibility; eight parts of a 1.5% NaOH solution were added to one part of straw for four hours or more. The process became widely adopted in Norway for a little time, but suffered the major disadvantage of requiring vast quantities of wash water to remove the NaOH, thus leaching out much of the valuable solubilised material. The pollution problem thereby created eventually led to the process being discontinued. Interest in the method has recently revived, however, with a number of workers seeking ways of bettering it; smaller quantities of more concentrated alkali, removing the need to wash it out afterwards (Wilson and Pigden, 1964; Singh and Jackson, 1971; Hartley et al, 1974; Phoenix, et al, 1974) and neutralisation of the excess alkali with organic acids (Wilson and Pigden, 1964; Donefer, et al, 1969; Carmona and Greenhalgh, 1972) are both being tried. Plants are now being set up in Great Britain using a sodium hydroxide based process, but it is too early at present to evaluate its viability (Farmer's Weekly, 30/4/76).

Other possible chemical treatments, giving lower increases in digestibility than NaOH treatment, include the use of ammonia (Loosli and McDonald, 1968; Guggolz et al, 1971; Han and Callihan, 1974) and urea (Donefer, et al, 1969) solutions, or liquid ammonia; all have the advantage of providing additional nitrogen in the ruminant diet. Dilute sulphuric acid (Porteus, 1976), sodium hypochlorite (Chandra and Jackson, 1971) and hydrogen peroxide (Chandra and Jackson, 1971;

Han and Anderson, 1974) also appear to show some promise.

### 1.3.2 Physical treatments

Physical treatments applied to straw include pressure-cooking (Han and Callihan, 1974), grinding and chopping (Carmona and Greenhalgh,1972; Hartley, et al, 1974), none of which appear to increase significantly its digestibility, and steam treatment, which is reported to give rise to increases in digestibility for rice straw from 29% to 61% (Hart, et al, 1975).

### 1.3.3 Biological treatments

Treatments designed to improve the digestibility of straw to ruminants must ultimately have the effect of facilitating the breakdown and utilisation of energy-rich polysacharides, primarily cellulose and probably also hemicelluloses, in straw, by the bacteria active in the rumen of ruminants. The rumen is essentially an anaerobic fermentation vessel. It is primarily because the rumen bacteria have only a limited ability to attack the "ligno-cellulose complex" in straw and utilise the energy that straw has a low digestibility. Several workers have therefore been investigating the value of a primary fermentation outside the rumen aimed at increasing the availability of this energy, thus increasing its digestibility, prior to its use as a ruminant feed.

The use of microorganisms in the preparation of food is, of course, a very well established practice; obvious examples are the production of yoghurt, cheese, bread and beer. Furthermore, the farmer is already familiar with the principle of fermentation, in the production of silage and composting; straw fermentation is not so very different.

Biologically based methods of upgrading cellulosic wastes

have been studied primarily with the aim of creating protein-rich feeds (Han et al, 1971; Rogers et al, 1972; Eriksson, 1974). Both fungi and bacteria have been used as possible agents of straw upgrading. Using an acid hydrolysis pretreatment on rye straw to release simple sugars from the hemicelluloses, Lekprayoon (1972) has created a protein-rich liquor, the yeast Candida utilis being the fermenting organism. Han (1975) has used the cellulolytic bacterium Cellulomonas in symbiosis with Alcaligenes sp. to produce protein from rice straw pretreated with dilute alkali. There are doubts about the economic viability of these processes at present (Han and Anderson, 1974).

Very little work appears to have been undertaken on the fungal attack of straw to improve its digestibility, without some kind of chemical or physical pretreatment. Hartley et al (1974) have carried out trials involving the use of fungi to improve the feed value of straw and also wood wastes. They used several Basidiomycetes which can utilise the cellulose and lignin in wood, causing white-rot. The fungi were grown on finely ground barley straw and also on beech, oak and poplar sawdust, some samples receiving an alkali treatment after the fermentation. The digestibility of straw after receiving fungal and alkali treatments ranged from 70 to 82%, depending on the fungus and the sodium hydroxide concentration used, compared with 46% for the untreated control. Most of this increase is reported to be due to the action of the alkali; it was considered that only a small contribution was made by the fungus.

Eriksson (1974) has mutated fungi so that they can no longer attack cellulose and hemicelluloses, but can still colonise and attack the lignin in wood chips. This worker is also studying the chemical delignification of straw to improve its feed value. The use of enzyme extracts to hydrolyse the cellulose in straw has also been suggested (Brown, 1976); such systems must be aseptic and it

seems likely that some form of pretreatment of the straw would be necessary.

The small amount of work that has been carried out on the fungal upgrading of straw suggests that the approach has promise (Worgan, 1976) and this study is oriented towards such upgrading methods. Fungi are favoured over bacteria primarily because, owing to their mycelial habit, they may be capable of colonising a substrate rapidly in the low moisture conditions of a semi-solid fermentation system and, as fungi are already present in straw, a sterilisation step may not be necessary.

## 1.4 THE BIOCHEMICAL COMPOSITION AND STRUCTURE OF STRAW

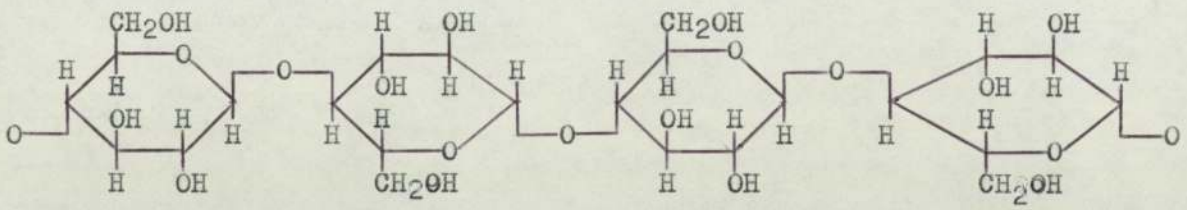
For the purposes of this study, straw may be regarded as the entire aerial part of mature cereals except the grain. Most of this is hollow stem with occasional nodes and nearly all the remainder is leaf material. At the time of harvesting, these components consist almost entirely of dry cell wall material with an outer layer of cuticle and middle lamella between the cells. This wall is composed of a primary cell wall, formed during the growth of the cell and, primarily in epidermal, fibre and xylem cells, a secondary wall is laid down inside the primary wall after growth has finished. This is composed of a thin outer ($S_1$), thick middle ($S_2$) and thin inner ($S_3$) layers.

### 1.4.1 Biochemical composition

The constituents of straw are cellulose, hemicelluloses, miscellaneous pentosans, structural proteins and lignin, together with other minor constituents (Rege, 1927).

1.4.1.1 Cellulose

Cellulose is the most abundant compound found in plants and contributes about 45-50% of the dry weight of straw(Rege, 1927). It is a polymer of D-glucose, the units being linked between the 1-carbon atom of one and the 4-carbon atom of the next, in the β-configuration. The structural formula of a small section of the molecule is conventionally represented as:



Lengths of the molecule average around 10,000 anhydro-glucose units (Jane, 1970). Cellulose is highly suseptible to attack by a number of microorganisms.

1.4.1.2 Hemicelluloses

Hemicelluloses are metabolisable by a wide range of microorganisms and constitute about 35% of the dry weight of straw (Chang, 1967). It is the name given to a mixed group of hetero-polysaccharides, and consists primarily of xylan, a mixed polymer with a polyxylose backbone and many side chains consisting mainly of uronic acids (Albersheim, 1965). Hemicelluloses also include mannan, arabinoxylan and arabinogalactan.

1.4.1.3 Lignin

Lignin comprises about 10-14% of the dry weight of straw (Chang, 1967). It is a three-dimensional polymer of three substituted phenylpropane units, cinnamyl alcohols, comparable amounts of each being present in straw (Kirk, 1971). It is somewhat resistant to

microbial attack.

1.4.2 Organisation of polymers in the cell wall

The linear polymeric molecules of cellulose are aligned together to form "elementary fibrils" with a diameter of around 35Å aggregated together to form "microfibrils" with a diameter of around 250Å (Talmadge et al, 1973). In primary cell walls the elementary fibrils are probably surrounded by loosely deposited cellulose which, together with glyco-proteins, bind the fibrils together to form microfibrils (Talmadge et al, 1973). These microfibrils have highly crystalline regions called "micelles" interrupted at regular intervals of around 500Å by amorphous regions (Muhlethaler, 1967). In secondary cell walls they are arranged in ordered layers (Jane, 1970).

The hemicelluloses form an amorphous matrix binding the microfibrils together (Jane, 1970) probably by means of hydrogen bonds(Valent and Albersheim, 1974). Lignin is laid down after cell growth has finished, mainly in the middle lamella and primary cell wall (Neish, 1965). Relatively small amounts are present in the secondary cell wall (Jane, 1970; Cowling, 1963). It holds the microfibrils, and probably also hemicelluloses (Talmadge et al, 1973), in fixed positions, probably by means of covalent bonds, to form the "ligno-cellulose complex" (Jane, 1970).

Pectins are found as a major constituent of the middle lamella and to a lesser extent the primary cell wall (Jane, 1970). Glycoproteins are also present in small quantities, probably forming crosslinks between microfibrils and thus contributing to the structural strength of the cell wall (Muhlethaler, 1967). Silica is a further component, constituting about 2% of barley straw (Lloyd, 1921), concentrated mainly in the dwarf cells but also present in the cuticle (Van Soest and Jones, 1968; Lloyd, 1921).

The crystallinity of cellulose and the presence of the lignin matrix interspersed among parts of the cellulose and hemicellulose are believed to be the main causes of the resistance to microbial attack of the otherwise suseptible components. (Pigden and Bender, 1972). The presence of silica may also be important (Van Soest and Jones, 1968).

## 1.5 THE MICROBIOLOGICAL COLONISATION OF STRAW

To colonise and break down straw, a fungus must be able to attack and utilise substances within it. These will be energy-rich substances, mainly sugars and polysaccharides, and nitrogen-containing compounds, mainly protein and nucleic acids, together with a few materials present in small quantities. There is a low level of simple sugars present in straw (Chang, 1967), readily utilisable by fungi, and much of the polysaccharide present may be utilised by fungi possessing the necessary polysaccharidases, though, as mentioned earlier, much of this is rendered resistant to attack.

Polymeric substances utilisable by fungi must first be broken down outside the cell by means of extra-cellular enzymes, primarily cellulases, hemicellulases and polyphenyl-oxidases.

The exact nature and mode of action of cellulase is still a matter of debate (Nisizawa, 1973) but the current predominantly accepted model, due primarily to Reese *et al.* (1950), is that a complex of enzymes, collectively called cellulase, act synergistically to degrade native cellulose, the extent of the ability varying widely between species and the particular substrate (Nisizawa, 1973). A "$C_1$" component is supposed to separate the cellulose fibrils and microfibrils, thus destroying the crystallinity of the substrate, and a "$C_x$" component is believed to hydrolyse the amorphous cellulose, either at the ends of the molecules (exo-1-4 $\beta$ glucosidases) to produce mono- and di-saccharides, or randomly at the internal glycoside bonds (endo-1-4 $\beta$ glucosidases) to produce shortened molecules. The $C_1$ and

$C_x$ components between them produce glucose, cellobiose and cellotriose. Cellobiose is absorbed through the cell wall of the fungus, where it is hydrolysed to glucose by 1-4 $\beta$ glucosidase ("cellobiase").

Some fungi, for example Trichoderma viride and Aspergillus fumigatus , are known to possess all the above components but are not able to effect significant breakdown of cellulose when combined with other polymers in a natural substrate and it has been hypothesised (Cowling, 1958) that an additional "X" factor, absent in such fungi, must also be present, functioning to break the ligno-cellulose bond.

### 1.5.1 Breakbown of lignin

The ability to break down lignin may be confined to Basidiomycetes, though Aytoun (1953) has suggested that Trichoderma viride is capable of limited ligninolytic activity and Kirk (1971) refers to certain Ascomycetes showing some ligninolytic activity.

The mechanism for the breakdown of lignin has not been fully elucidated. Because of the variety of different monomer units, bond types and functional groups present in lignin, it is probably only partially degraded by most fungi able to attack it; they may possess only a proportion of the total range of enzymes necessary for total breakdown. It is likely that degradation proceeds via the splitting of the aromatic ring (Kirk, 1971). Polyphenol-oxidases are widely implicated as important agents in the breakdown of lignin, but may only play a minor role, along with other enzymes (Kirk, 1971).

## 1.6 THE ECOLOGY OF STRAW COLONISATION

The ecology of plant debris colonisation has been extensively studied (Waksman, 1945; Garrett, 1951) and important determinants have been elucidated. These are discussed in the following sections.

1.6.1 Biochemical and physical determinants

At the time of harvest, propagules of many different microorganisms will be present on the surface of straw, but will not necessarily be able to colonise and exploit it after harvest. Physical conditions must first be appropriate, but also of great importance is the nature and organisation of the biochemical components of the substrate. The ecological groups of fungi successively colonising plant debris are primarily characterised by their capacity to gain access to and utilise components of the substrate (Garrett, 1951). Garrett (1963) observed that primary colonisers are normally unable to utilise cellulose and lignin, but possess high growth rates and rapid spore germination and are generally Phycomycetes. These he called primary sugar fungi. Cellulose and hemicellulose decomposing fungi follow, with slower-growing ligninolytic and cellulolytic fungi, usually Basidiomycetes, coming at the end of the succession. Also found late in the succession are "secondary sugar fungi" (Garrett, 1963), unable to utilise cellulose themselves but able to quickly assimilate sugars produced by the cellulases of other fungi present.

Studies on the fungal ecology of amassed plant debris—composts—have concentrated on the effects of spontaneous self-heating during colonisation (Miehe, 1907; Rege, 1927; Gregory and Lacey, 1963; Chang and Hudson, 1967; Fergus, 1964). Self-heating has a profound effect on the populations of microorganisms present; during the thermophilic phase, temperatures around 50°C or higher occur and only a few thermophilic microorganisms are able to grow (Cooney and Emerson, 1964). Later, when the temperature falls, mesophilic organisms recolonise from the outer, cooler parts of the compost heap.

The ecological and biochemical changes occurring during the self-heating of wheat straw amended with ammonium nitrate have been followed by Chang and Hudson (1967) and Chang (1967) respectively.

Enriching the substrate with additional nitrogen has a stimulatory effect on microbial activity, and it is known (Levi and Cowling, 1968) that fungal activity can correlate well with the carbon to nitrogen ratio of the substrate, though fungal activity will, of course, depend on the availibility of the carbon and nitrogen present. Where C:N ratios are high, for example in wood (around 500:1) and straw (130:1) (Gray and Biddlestone, 1973), microbial activity is limited by the level of nitrogen, whereas low C:N ratios, found, for example, in animal wastes (5-10:1 ; Gray and Biddlestone, 1973), can give rise to the release of excess nitrogen.

A second important determinant of the ecology of litter decomposition is the pH of the material. It affects ion availability, cell membrane permeability, enzyme activity and thus the growth rate of fungi. As fungi can have widely differing pH optima for linear growth and antagonistic ability (Weindling, 1938), pH can exert a powerful influence in determining which fungi dominate a substrate.

Other important physical determinants are temperature and moisture content. The optima and range of each tolerated by fungi vary widely and changes in either can completely alter the pattern of colonisation of a substrate (Moore-Landecker, 1972).

### 1.6.2 Microbial interactions

In addition to the biochemistry and physical conditions of a substrate affecting the microbial ecology of colonisation, the microorganisms present themselves play an important part; i.e., microorganisms will interact with each other, both directly and indirectly, through their effects on biochemical and physical conditions. These interactions exert a profound influence on microbial ecology. Reviews of the subject include those of Waksman (1937) and d'Aeth (1939).

The interactions concerned with in this study are principally

those in which at least one of the interacting species is harmed, a description Park (1960) used to define the term "antagonism" and which is adopted here. Antagonism may be mediated by the action of antibiotics, it may involve mycoparasitism, or it may simply involve competition for nutrients or some other feature of the environment. Antibiosis may often be involved in mycoparasitism. Antagonism that does not include competition for features of the environment I have called "aggression".

The competitive ability of a fungus is likely to depend upon all the above-mentioned factors, and may involve others in addition.

### 1.6.3 Antibiotics and antibiosis

One of the most important factors in many antagonistic relationships between fungi is the ability to produce antibiotics (Whittaker and Feeney, 1971). Since Fleming's discovery of penicillin (Fleming, 1929) an enormous amount of work has been conducted on the production of antibiotics and it is now known that many fungi are able to produce them (Broadbent, 1966). The term antibiotic has been defined by Gottlieb and Shaw (1970) as organic substances produced by microbes, which are deleterious at low concentrations to the growth or metabolic activities of other microorganisms. Certain common fungal metabolic products, such as carbon dioxide, alcohols and organic acids can also have an inhibitory effect, but at relatively high concentrations. Together with the wide variety of true antibiotics produced in small quantities, these metabolites may exert a profound and complicated effect on the microbial ecology of soil and other complex organic substrates (Hutchinson, 1971).

Much of the investigatory work on antibiotics and microbial ecology has been done in the context of soil microbiology. It is known that fungistatic and growth factors of microbial origin-and probably also of plant origin (Balis and Kouyeas, 1968)—are present in

almost all soils, influencing spore germination and the growth and development of microorganisms (Park, 1960; Balis and Kouyeas, 1968; Fries, 1973), though there is little direct evidence for the presence of these factors in natural substrates (Brian, 1960). Fungistatic metabolites may also be self-inhibitory (Balis and Kouyeas, 1968) and are associated with the commonly observed "staling" of fungal cultures *in vitro* (Burnett, 1976) and the widespread fungistasis found in soils (Dobbs and Hinson, 1953). It is still a matter of debate as to how important microbial fungistatic metabolites are in contributing to soil fungistasis. Park considers it to be of prime importance (Park, 1960), though nutrient levels in soils will also be important (Ko and Lockwood, 1967). Stimulators, including nutrients, and biotic and abiotic inhibitors may all be involved in a dynamic balance determining the level of fungistasis (Watson and Ford, 1972).

Antibiotics have a wide variety of structures, including dipeptides, polyoxins, aromatics and heterocyclic compounds. Some are unstable to light (eg., gliotoxin), heat (eg., cephalosporin N) or oxygen (eg., cephalosporin $P_3$). A number of inhibitory substances are volatile, able to exert their influence at a distance, through the gas phase (Hutchinson, 1973). $CO_2$, ethanol, acetaldehyde and hydrogen cyanide are examples; such volatiles are probably quite important in their effects on soil microbial populations (Hutchinson, 1973).

Antibiotics vary widely in their potency, both in terms of the quantities required to produce an effect on other organisms and in terms of the range of organisms affected. Sensitivity to antibiotics also varies widely from fungus to fungus (Brian, 1960). Temperature and pH are known to greatly affect antibiotic production, or even to prevent production altogether (Burnett, 1976).

Mechanisms of action include the inhibition of cell wall formation, the interference in several ways with respiration (Gottlieb

and Shaw, 1970) or the inhibition of protein synthesis (Gottlieb and Shaw, 1970). Mechanisms of resistance to antibiotics include the possession of a cell wall barrier to antibiotic activity (Brian, 1960), production of antibiotic-destroying enzymes, the possession of biosynthetic pathways that are not affected by the antibiotic (Moore-Landecker, 1972) and chemical antagonism by metabolites (Brian, 1960).

### 1.6.4 Mycoparasitism

Parasitism between fungi—"mycoparasitism"—occurs when one fungus, the parasite, attacks and derives nourishment from another fungus (the host) of a different species (Odum, 1971). General reviews of the subject are given by Barnett (1963,1964) and Barnett and Binder (1973).

Gäumann (1946) recognised two broad overlapping types of mycoparasitism; when death of the host is not caused, the relationship is called "biotrophic" and when the host is killed, the parasitism is "necrotrophic".

Biotrophic parasites are generally highly host specific. They occur mainly among Chytrids and Mucorales, the host usually also being a Phycomycete; among the Mucorales, specialised haustoria are produced which penetrate the host to effect attack (Karling, 1960). A few members of Fungi Imperfecti are also biotrophic parasites, but none produce haustoria; instead they produce antibiotics or possibly enzymes that alter the permeability of the host cell membrane, causing a release of nutrients which benefit the parasite.

Necrotrophic parasites operate by releasing enzymes or toxic substances which kill the host with subsequent absorption of nutrients by the parasite. Several Fungi Imperfecti and Basidiomycetes are necrotrophic parasites, Trichoderma viride and Gliocladium roseum among the former and several Polyporales among the latter (Keyes, 1968).

In a study of antagonism between coprophilous fungi from rabbit dung, Ikeduigwu and Webster (1970a,b) found that Coprinus heptemerus and, to a much lesser extent, several other Coprinus species, were able to antagonise and kill competitors when the antagonist's hyphae were very close to or in contact with hyphae of the competitor. This they referred to as "hyphal interference". The mechanism probably involves the action of one or more unstable antibiotics or enzymes. Evidence was not obtained that nutrients from the victim are utilised by the antagonist, so the interaction may well not be parasitic.

### 1.6.5 Competitive ability

The term "competitive saprophytic ability" (CSA) has been used by Garrett (1950,1963) to refer to the physiological ability of a fungus to compete successfully for a substrate. Garrett (1956) has suggested that the competitive success of a fungus depends on its CSA and its inoculum potential—i.e., the level of active propagules present. The possession by a fungus of any one of the following qualities can confer on it a high CSA (Garrett, 1956):

1) A high growth rate and rapid germination of spores

2) A high level of metabolic efficiency in enzyme production and substrate utilisation

3) An ability to produce substances that are toxic to other soil microorganisms

4) An ability to tolerate the toxic substances produced by other soil microorganisms.

Other important considerations are the tolerance of a fungus to physical factors and their variation, such as temperature, pH and moisture content. Several studies have been made to assess the CSA of fungi (Butler, 1953a,b; Rao, 1958, 1959; Wastie,1961; Mitchell

and Dix, 1975).

The competitive ability of a fungus is of greatest importance when the substrate is limiting so that demand for nutrients exceeds supply and when the nature of the substrate and the physical conditions present allow a number of different fungi to colonise, as is the case with fresh, damp straw.

Growth rate and physical presence are important factors in successful colonisation of new substrates (Wastie, 1961). Prior colonisation has been shown, in certain instances, to be a factor of prime importance in determining competitive success (Bruehl and Lai, 1966, 1968). It is common in the biological control of disease and decay to initially establish in the substrate a fungus able to prevent colonisation by another, undesired fungus. More than one mechanism may play a part in determining which fungus dominates, though removal of simple nutrients by the primary coloniser is likely to be important (Hulme and Shields, 1972; Fokkema, 1973). It should also be mentioned that plant pathogens that are able to survive saprophytically play a significant role in the decay of plant litter (Garrett, 1963, 1970); their presence in the host before its death can facilitate their survival (Forbes, 1974). A number of pathogenic saprophytes have been found surviving in straw debris (Bruehl and Lai, 1966, 1968; Garrett, 1966, 1972).

In the colonisation of substrates already occupied, factors other than growth rate may be of greater value, such as tolerance to inhibitory metabolites (Park, 1960; Butler, 1953b), or parasitic ability. Thus, the competitive ability of a particular fungus is likely to depend upon whether or the substrate being competed for has been colonised and, if so, upon which other fungi are present.

## 1.7 STRUCTURE AND OBJECTIVES OF THE RESEARCH PROGRAMME

The aim of this research programme is to provide some understanding of aspects of the competitive colonisation of barley straw by fungi, within the context of a larger research programme aimed at developing a process for the fungal upgrading of straw to produce an animal feedstuff.

Fungi were isolated from both old stored straw and fresh incubated straw, in order to obtain both potential upgraders of straw and their antagonists. Linear growth rates and the cellulolytic ability of the more commonly found isolates were determined and those with high growth rates or cellulolytic ability, or both were studied to determine their pH and temperature optima and to assess their ability to break down straw and lignin. After a preliminary study of interactions between fungal colonies, several macroscopic and a microscopic study of aspects of the interactions between *Coprinus cinereus* and a selection of other fungi were made. A preliminary study of the potential for establishing an association between fungi and nitrogen-fixing *Azotobacter* species was also carried out.

# CHAPTER 2

# FUNGI PRESENT IN STRAW

## 2.1 INTRODUCTION

THE PURPOSE of this part of the study is two-fold. First, a preliminary assessment of the kind of fungi likely to be found in barley straw after a period of storage is made. This may be of importance if the straw is to be subsequently fermented without prior sterilization, in the production of an animal feedstuff. It is useful to know which fungi are present from the point of view of their possible toxicity to animals and it may also be of value to know whether there are present large populations of fungi which may compete with the intended agent of breakdown during the fermentation of the straw. Secondly, an isolation programme is conducted to determine as far as possible the kind of cellulolytic fungi present and colonising fermenting fresh straw. This is of value because among these fungi may be one that would be worth encouraging the growth of, and also because it is important to know which fungi may be present as possible competitors to the intended agent of straw breakdown.

## 2.2 ISOLATION OF FUNGI FROM OLD STRAW

A stack of barley straw bales which had been exposed to varying weather conditions in the open air over a period of nine months, from January to September, was chosen as the material for this isolation progamme. Old stored straw was chosen because it is quite possible that, in addition to the accumulation of air spora on its surface over the months, a significant amount of active colonisation of the material by certain fungi may also have occurred and this could alter significantly the fungal populations present immediately prior to the intended fermentation process, compared with that of straw fresh from the combine harvester. The straw used was collected from a farm near Clent, Worcestershire, England. It was not supposed to be representative of barley straw stored throughout the country, from the point of view of its fungal flora, but it was felt to be beyond the scope of this study to examine samples of straw from other areas in addition. The fungi isolated in this work will therefore not necessarily yield an accurate picture of fungi in barley straw throughout the country. Furthermore, while an attempt was made to isolate a wide range of fungi present, it is accepted that the techniques used will be very unlikely to yield a complete picture of the existing fungal flora.

It is not known what biocides, if any, the straw had been exposed to while in the field.

A number of techniques have been developed for the isolation of fungi, often for application in soil microbiology. The dilution plate method, to be described later, is often used, though heavily sporing fungi tend to be over-represented and slower-growing or germinating fungi under-represented by this technique (Garrett, 1951) and it does not discriminate between active and dormant fungi (Warcup, 1960). The soil plate technique (Warcup, 1950) has a broadly similar

pattern of selectivity (Warcup, 1960). Separation of growing mycelium from soil particles by removal of hyphal tips (Waksman, 1916) or washing the material under investigation with sterile water (Harley and Waid, 1955) both reduce the number of isolates originating from spores (Warcup, 1960). The hyphal isolation method of Warcup (1955) yields many fungi not isolated by the dilution plate method, especially slower-growing fungi. A greater proportion of isolates by this method, though not all, will be active in the substrate, compared with the dilution plate method (Warcup, 1957).

Enrichment culture techniques, in which a specific energy substrate such as cellulose is added to the soil to encourage the growth of fungi able to utilise the added substrate are widely used in the selective isolation of fungi (Garrett,1963). In addition, a number of techniques have been developed in which a substrate is implanted in soil, allowed to become colonised and is then removed for isolation of fungi. Agar (Chesters, 1940; Thornton, 1952; La Touche, 1948), cellophane (Tribe, 1960; Went and de Jong, 1966), filter paper (Stapp and Borstels, 1934) and straw (Garrett, 1963) are among the substrates that have been used. These techniques have most often been used to selectively isolate cellulolytic fungi; a screen (Thornton, 1952; Eggins and Lloyd, 1968), designed to selectively isolate only actively growing fungi able to utilise the screened substrate, has also been used.

For this experiment, the isolation of a broad range of fungi present in the chosen straw sample, both as resting propagules and as active colonisers, was desired. The dilution plate technique and direct plating of pieces of straw onto agar were the methods chosen for this study. These techniques will be to some degree selective, as has been pointed out above, and so this isolation programme will not yield all the fungi present in the straw. In particular, it is

difficult to isolate sterile fungi and many Basidiomycetes using these methods; some of the slower-growing fungi are likely to be under-represented or missed.

All artificial media used will, of course, be selective, but many have been created to select out specific chosen types of fungi. Important among these are cellulose agars, designed to favour the isolation of cellulolytic fungi. Such an agar is used in this study, along with a glucose-starch agar and malt agar. Details of the composition of these media are given in Appendix 1.

### 2.2.1 EXPERIMENTAL PROCEDURE

Samples of straw were collected aseptically from both the inside and the surface of a representative bale of straw in the stack, so that any active colonisation of the moist inside of the bale and any superficial colonisation, perhaps arising from air spora, could both be represented in the isolations.

#### 2.2.1.1 The dilution Plate Method

In this method, approximately 10g of straw and a few glass beads to aid separation of fungal thalli from the substrate, were shaken together in 100ml of sterile distilled water for five minutes. Dilutions of $10^{-1}$, $10^{-3}$ and $10^{-5}$ml of this suspension per ml of sterile distilled water were then prepared. One ml of freshly shaken suspension from each dilution level was pipetted into each of sixteen Petri dishes for each of the three agars to be used (malt agar, glucose starch agar and Eggins and Pugh cellulose agar). The molten media were cooled to 45°C, poured into the Petri dishes and thoroughly mixed with the dilution sample. 30mg/l of rose bengal was incorporated into each medium to suppress bacterial growth (Ottow, 1972); its mildly inhibitory effect on the linear extension

of the colonies is also of value (Martin, 1950), since the better the separation of colonies on the surface of the agar, the easier it is to subculture and identify individual colonies.

The fungi inhabiting barley straw of primary interest in this study are mesophiles. However, it is quite possible that at some time during storage the temperature inside the straw stack rose sufficiently to allow the development of a significant population of thermophilic fungi (Cooney and Emerson, 1964). Half of the prepared plates were therefore incubated at 45°C and the other half at 25°C, giving a replication level of plates in this part of the experiment of eight.

2.2.1.2 Direct Inoculation

Pieces of straw collected aseptically from both the surface and the inside of the straw stack were placed directly onto the surface of plates of agar media of the same composition as used in the dilution plate technique, again with rose bengal incorporated into each at 30mg/l. Three pieces of straw, each about 5cm long, were placed in each plate. Ten replicates were made at each of two temperatures, 25°C and 45°C.

The plates were periodically examined and, whenever possible, the growing fungi identified *in situ*. When necessary, fungi were subcultured onto the same medium without rose bengal and identifications were made from these pure cultures. Because of the selective bias of the techniques used, in particular, the bias of the dilution plate method towards heavily sporulating fungi, no attempt was made to determine percentage frequencies of isolation of species appearing on the plates, although a note was made of those fungi occurring most frequently.

2.2.2 RESULTS

The following fungi were isolated from straw using the dilution plate technique: Chaetomium globosum, C. aureum, Aspergillus fumigatus, A. versicolor, A. glaucus ser., Penicillium expansum, P. brevicompactum, 15 other Penicillium spp., Fusarium sp., Alternaria sp., Gliocladium roseum and Botrytis cinerea.

Fungi isolated from straw using the direct inoculation technique were: Chaetomium globosum, Chaetomium sp., Trichoderma viride, Graphium sp., Rhizopus nigricans, Mucor sp. and Streptomyces sp.

Although no quantitative studies were made, it was clear that Penicillium species were very common. Several species, including Penicillium expansum, were isolated with a high frequency. Chaetomium globosum and, to a lesser extent, Aspergillus fumigatus were other frequently occurring isolates.

There is a contrast between the two techniques used in the fungi isolated. Two Phycomycetes were isolated by direct inoculation and very few Fungi Imperfecti, whereas the dilution plate technique yielded almost entirely Fungi Imperfecti; fungi belonging to this class often spore heavily and thus their isolation is often facilitated by this method, though Phycomycetes also sporulate heavily.

Isolations of fungi at 45°C were rare. Malbranchea pulchella var. sulphurea was isolated twice and the thermotolerant fungus Aspergillus fumigatus, often found active at mesophilic temperatures (Cooney and Emerson, 1964), was isolated on a number of occasions. Thermophilic Actinomycetes and bacteria were also found. Chang and Hudson (1967), investigating the self-heating of amended wheat straw, found large populations of thermophilic fungi. It appears likely that the straw bales studied here at no time developed a self-heating phase.

Most of the fungi isolated in this experiment are known to be cellulolytic (Siu, 1951) and have often been isolated from

cellulosic materials. The results of this experiment do not show which fungi isolated have been active on the straw, however, rather than arising from dormant propagules.

There appear to be no strictly comparable studies of fungi isolated from straw reported in the literature; the studies of Chang and Hudson (1967) and Eastwood (1952) concentrate on the fungal ecology of self-heating composts of straw amended with ammoniun nitrate. Chang and Hudson (1967) reported the presence of Alternaria tenuis, Cladosporium herbarum, Aureobasidium pullulans and several Aspergillus and Penicillium species at the beginning of their isolation programme, before the temperature of the compost reached its maximum. Eastwood (1952) reported the presence of Chaetomium globosum, Trichoderma viride, Mucor spinescens, Aspergillus fumigatus, Monatospora sp., Aspergillus terreus and Basidiomycete mycelium at the edge of her straw compost.

## 2.3 CELLULOLYTIC FUNGI ACTIVE IN FRESH INCUBATED STRAW

The aim of this work was to isolate as many as possible of the more active cellulolytic fungi indigenous to and actively colonising fresh incubated barley straw, in order to provide an assessment of its fungal ecology during colonisation and also to yield fungi potentially valuable as upgraders of straw or potential competitors to such fungi during the upgrading fermentation. It is likely that there exist fungi of value as upgraders of straw that are not indigenous to it. It is outside the scope of this study to seek such fungi. This study is also confined to mesophilic fungi. Since the fungi being sought are cellulolytic, techniques designed to favour their isolation were included. Eggins and Pugh cellulose agar, selective for the isolation of cellulolytic fungi, was used in all techniques (Eggins and Pugh, 1962). The medium contains 1.0% cellulose powder which has previously been ball-milled for 72 hours to reduce the particle size. Enzymatic

dissolution of the cellulose by fungi is reflected in a reduction of of the opacity of the medium. It thus performs a demonstrative function of cellulolytic ability in addition to its selective function.

## 2.3.1 EXPERIMENTAL PROCEDURE

Two sectional wads of straw, each about nine inches thick, were removed from a fresh bale of barley straw from the same source as that used in the isolation programme described in Section 2.2. These "pads" were placed in separate large polythene bags and sufficient distilled water added to each to give a moisture content of 100% on a dry weight basis, found in preliminary work to provide adequate moisture for good fungal growth. Nitrogen as ammonium chloride was added to the water of one of the pads to give a nitrogen level of 0.001g/g straw. Both pads were incubated in a large cupboard at 25°C; the bags were left open so that air was freely available to the straw. The relative humidity of the cupboard was maintained at close to 100%.

The following methods of isolation were used:

1. Direct inoculation onto agar (with four variations).
2. The dilution plate technique.
3. "Screened substrate" tube baiting.
4. "Straw strip" tube baiting.

Isolations were made on days 0, 7, 14, and 21, except where otherwise stated. Incubation of all plates was at 25°C.

### 2.3.1.1 Direct inoculation

Small pieces of straw were collected aseptically from several parts of the pads and placed in sterile containers. From these samples, 100 strips approximately 2 cm long were prepared. Four different procedures were then followed, each using Eggins and Pugh cellulose agar with 30mg/l rose bengal incorporated to suppress bacterial growth.

Fifty of the strips were half embedded in an inclined position directly in the agar of ten plates, five per plate.

The other fifty strips were subjected to a serial washing procedure, following the method of Harley and Waid (1955), to remove a proportion of the fungal spores lying on the surface of the strips. Five washings were made; Harley and Waid found that this removed more than 90% of detachable fungal fragments capable of developing into a mycelium and greatly increased the number of actively growing fungi isolated. The washed pieces of straw were then plated on agar in the same way as the first fifty strips.

Half of the pieces of straw from both sets of fifty were removed after 24hrs incubation. It was thought that this modification might favour the isolation of actively growing fungi, especially those with high growth rates, and discriminate against the isolation of fungi arising from spores, by not allowing them sufficient time to germinate and colonise the agar.

2.3.1.2 The dilution plate technique

Dilution plates were prepared by aseptically collecting samples of straw from the pads and drying them at 30°C for 48hrs in an incubator, to obtain a material sufficiently low in moisture to enable it to be ground up while minimising the reduction in viable propagules present and with as little alteration in populations as was feasible. It is likely, however, that, due to drying out, some reduction in viable propagule numbers occurred (Warcup, 1960). The material was then ground in a small sterilized coffee-grinder and approximately 1g suspended in 100ml sterile distilled water. Warcup (1960) has found, in dealing with soil, that the sediment settling out from this suspension and discarded during the preparation of dilution plates may contain fungi not present in the bulk of the suspension, so,

in this experiment, the suspension was repeatedly shaken up to maintain homogeneity during the preparation of the dilutions. One ml of a dilution of $10^{-5}$ ml of this suspension per ml of sterile distilled water was pipetted into Petri dishes, followed by molten Eggins and Pugh cellulose agar at around 45°C. Preliminary work showed that this dilution gave an optimum dispersion of colonies on the plates. Emerging colonies were subcultured onto fresh cellulose agar plates containing no rose bengal.

2.3.1.3 "Screened substrate" tube baiting

A screened substrate technique was employed in this experiment as an aid to the selective isolation of actively growing cellulolytic fungi. The technique has been used with some success by several workers (Malik, 1970; Seal, 1973; Barnes, 1974). The screened substrate assemblies were of the same pattern as that developed and used by Eggins and Lloyd (1968), except that two of the substrate strips incorporated were Whatman's plastic-backed chromatography paper and the other two small lengths of straw. A broad glass fibre band was used as the screen and the substrates were mounted on the side of a boiling tube. Five tubes were buried in each pad and each was removed and replaced with a new assembly every week for three weeks. After removal of a tube, the substrates were plated out onto Eggins and Pugh cellulose agar so that ten plates were prepared each week for each pad. Day 0 isolations were not made using this technique.

2.3.1.4 "Straw strip" tube baiting

This technique is designed to selectively isolate fast-growing cellulolytic fungi and may also favour those fungi with a high level of competitive ability. Figure 1 below shows the assembled unit, referred to as a "straw strip tube". After assembly and steril-

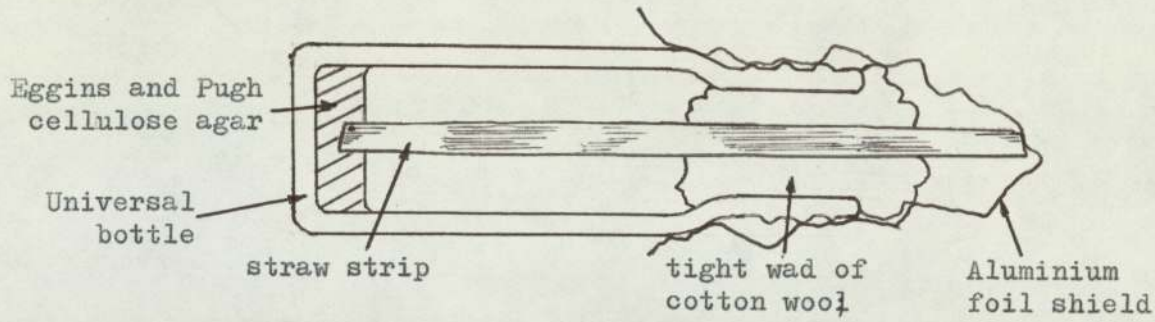



Figure 1. Section through a straw strip tube.

ization the protective aluminium foil is removed and the tube placed in a pad with the emerging strip in contact with it. Insofar as competition between fungi for possession of the straw strip is likely to occur, the technique may tend to select out fungi with good competitive abilities. A complete, intact piece of internodal stalk was always used. Fungi were found to penetrate the cotton wool plug at a much lower rate than they colonised the straw strip.

Each tube placed in the pads was examined at frequent intervals for the presence of mycelium colonising the strip. When mycelium was seen inside the tube, sections of the strip were plated out onto Eggins and Pugh cellulose agar. Loops of agar from the base of the tube were also plated out. Replication was arranged so that at all times there were five tubes in each pad. Whenever one was removed for isolations, another replaced it. Overall, fourteen tubes from the unamended pad and twelve from the amended pad were used.

2.3.1.5 Direct observation

Garrett (1963) has observed that much valuable additional information about the presence of fungi colonising substrates can be

gained by direct examination of samples under the microscope. Samples were therefore taken at regular intervals for examination, no attempt being made to isolate the fungi observed. Fungi not fruiting could not be identified.

## 2.3.2 RESULTS

### 2.3.2.1 Plating on agar

The fungi isolated by plating pieces of straw onto Eggins and Pugh cellulose agar are given in Table 2.

The broadest range of species arose from the technique in which the straw pieces were plated onto the agar without any modification to increase selectivity; by contrast, when the pretreatment was to both wash the straw pieces and remove them from the agar after twenty-four hours, one fungus only was isolated, namely *Fusarium* sp. This was the most commonly isolated fungus, often also being the most common isolate on any one occasion. *Alternaria tenuis* was only isolated on days 0 and 7, while *Gliocladium roseum* emerged in the later part of the fungal succession, being isolated only on day 14. *Trichoderma viride* was only isolated from unamended straw washed but not removed from the agar; it occurred quite frequently on these occasions. *Cephalosporium* sp. was isolated only from amended straw and *Graphium* sp. only from unamended straw, on day 14 only. Only one isolation of *Coprinus cinereus* was made, from unamended straw.

These plating techniques have yielded mainly fast-growing fungi; Waksman (1944) has stated that direct plating tends to discriminate against slower-growing fungi, so it is probable that other cellulolytically active fungi with low growth rates were present in the straw but were not isolated.

Table 2. Fungi isolated from fresh straw at 25°C by plating on agar

| Technique* | Day | Fungi isolated | |
|---|---|---|---|
| | | Unamended pad | Amended pad |
| 1. Direct plating of five pieces of straw per plate. | 0 | Chaetomium globosum<br>Fusarium sp. | Chaetomium sp.<br>Fusarium sp. A. tenuis<br>Cephalosporium sp.<br>Penicillium sp. |
| | 7 | Alternaria tenuis<br>Graphium sp.<br>Fusarium sp. | Chaetomium globosum<br>Alternaria tenuis<br>Fusarium sp. |
| | 14 | Graphium sp.<br>Gliocladium roseum<br>Fusarium sp.<br>Coprinus cinereus | Chaetomium globosum<br>Gliocladium roseum<br>Graphium sp.<br>Doratomyces sp. |
| 2. As in 1. above, but with removal of straw pieces after 24hrs incubation. | 0 | Fusarium sp.<br>Aspergillus fumigatus | Fusarium sp.<br>Cephalosporium sp. |
| | 7 | Fusarium sp.<br>Alternaria tenuis | Fusarium sp.<br>Alternaria tenuis |
| | 14 | Fusarium sp.<br>Graphium sp.<br>Aspergillus fumigatus | Fusarium sp.<br>Cephalosporium sp.<br>Aspergillus fumigatus |
| 3. Plating of five pieces of washed straw per plate. | 0 | Fusarium sp.<br>Trichoderma viride | Aspergillus fumigatus |
| | 7 | Fusarium sp.<br>Alternaria tenuis<br>Trichoderma viride | Fusarium sp.<br>Alternaria tenuis<br>Cephalosporium sp.<br>Aspergillus fumigatus |
| | 14 | Fusarium sp.<br>Trichoderma viride<br>Graphium sp. | Gliocladium roseum<br>Streptomyces sp. |
| 4. As in 3. above, but with removal of straw pieces after 24hrs incubation. | 0 | Not recorded | Not recorded |
| | 7 | Fusarium sp. | Fusarium sp. |
| | 14 | Fusarium sp. | Fusarium sp. |

*See text for details

2.3.2.2 The dilution plate technique

Fungi isolated by the dilution plate technique are shown in Tables 3 and 4. Chaetomium globosum was the most commonly isolated fungus on all occasions, though it was somewhat more frequently isolated from the amended pad than the unamended pad. Several other Chaetomium species were also isolated. Fusarium sp., Trichoderma viride and Aspergillus fumigatus were all commonly isolated throughout the incubation period, though T. viride was rare in the unamended pad and A. fumigatus rare in the amended pad. Alternaria tenuis was only found in early isolations, about equally in both pads, and Gliocladium roseum was isolated only on day 21, from the amended pad.

2.3.2.3 Screened substrate tube baiting

Tables 3 and 4 also show the fungi isolated by means of the screened substrate tube baiting technique. Chaetomium globosum, absent from day 7 isolations, became by far the most frequently isolated fungus from both pads on days 14 and 21. Fusarium sp. again occurred commonly at all times. Aspergillus fumigatus only appeared once, and Alternaria tenuis was again not isolated late in the succession.

2.3.2.4 Straw strip tube baiting

As can be seen from Tables 3 and 4 the straw strip tube baiting technique yielded predominantly Fusarium sp., at all times and in both pads, although other fungi occurred on occasions.

2.3.2.5 Direct observation

The identifiable fungi observed by examining samples of straw from the pads under the microscope, no attempt being made to isolate them, are shown in Table 5. Curvularia sp., Bipolaris sp. and Humicola sp. were not isolated by any of the techniques used and

Table 3. Fungi isolated by the dilution plate technique, the screened substrate tube baiting technique and the straw strip tube baiting technique, from unamended fresh barley straw at 25°C.

| Technique: | Dilution plates* | | | | Screened substrate tubes | | | Straw strip tubes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day: | 0 | 7 | 14 | 21 | 7 | 14 | 21 | 9 | 10 | 11 | 16 | 17 | 18 | 21 | 23 |
| Chaetomium globosum | 22 | 20 | 29 | 23 | | X | X | | | | | | | | |
| C. cochlioides | 7 | 1 | | | | | | | | | | | | | |
| C. elatum | 7 | | | | | | | | | | | | | | |
| Chaetomium spp. | | 3 | 7 | | | X | | | | | | | | | |
| Fusarium sp. | 11 | 14 | 24 | 16 | X | X | X | X | X | X | X | | X | X | X |
| Alternaria tenuis | 15 | 2 | | | | X | | | | X | | | | X | |
| Cephalo-sporium sp. | | | | | | | | | | | | | | | |
| Graphium sp. | | | | | | | | | | | | | | | |
| Trichoderma viride | 2 | 4 | 1 | | X | | | | | | | | | | |
| Trichoderma sp. | 1 | | | | | | | | | | | | | | |
| Aspergillus fumigatus | 20 | 21 | 30 | 26 | | | X | | | | X | | | | |
| Aspergillus sp. | | | | | | | | | | | | | | | |
| Penicillium sp. | | | | | | | | | | | X | | | | |
| Coprinus cinereus | | | | | | | | | | | | | | | |
| Gliocladium roseum | | | | | | | | | X | | X | | | | |
| Botrytis sp. | | | | | | X | | | | | | | | | |
| Streptomyces sp. | | X | X | X | | X | | | | | | | | | |
| Arthrobotrys sp. | | | | | X | | | | | | | | X | X | |
| Unidentified | 13 | 4 | 2 | | | | | | | | | | | | |

*Numbers refer to the number of times the fungus was observed on the dilution plates.

Table 4. Fungi isolated by the dilution plate technique, the screened substrate tube baiting technique and the straw strip tube baiting technique, from amended fresh barley straw at 25°C.

| Technique: | Dilution plates* | | | | Screened substrate tubes | | | Straw strip tubes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day: | 0 | 7 | 14 | 21 | 7 | 14 | 21 | 9 | 10 | 11 | 16 | 17 | 18 | 21 | 23 |
| Chaetomium globosum | 39 | 21 | 35 | 23 | | X | X | | X | | | | | | |
| C. cochlioides | | | | | | | | | | | | | | | |
| C. elatum | | | | | | | | | | | | | | | |
| Chaetomium spp. | 12 | | 6 | 4 | | | | | | | | | | | |
| Fusarium sp. | 18 | 17 | 25 | 6 | X | X | X | X | | | X | X | | X | X |
| Alternaria tenuis | 12 | 2 | | | X | | | | | | | | | | |
| Cephalosporium sp. | | | | | | | | | | | | | | | |
| Graphium sp. | | 1 | | | | | | | | | | | | | |
| Trichoderma viride | 20 | 12 | 9 | 11 | | | | | | | | | | | |
| Trichoderma sp. | | | | | | | | | | | | | | | |
| Aspergillus fumigatus | 6 | 7 | 6 | 11 | | | | | | | | | | | |
| Penicillium sp. | | | | 1 | | | | | | | | | | | |
| Coprinus cinereus | | | | | | | | | | | | | | | |
| Gliocladium roseum | | | | 3 | | | | | | | | | | | |
| Botrytis sp. | | | | | | | | | | | | | | | |
| Arthrobotrys sp. | | | | | X | | | | | | | | | | |
| Streptomyces sp. | | | X | X | | | | | | | | | | | |
| Unidentified | 13 | 3 | 3 | | | | | | | | | | | | |

*Numbers refer to the number of times the fungus was observed on the dilution plates.

Table 5. Fungi observed colonising fresh straw incubated at 25°C.

| Fungi observed | Unamended pad | | | | Amended pad | | | |
|---|---|---|---|---|---|---|---|---|
| Day: | 3 | 7 | 14 | 21 | 3 | 7 | 14 | 21 |
| Chaetomium globosum | | | | X | | X | X | X |
| Fusarium sp. | | | | X | | | X | X |
| Alternaria tenuis | X | X | | | X | | | |
| Cephalosporium sp. | | | | X | | X | | X |
| Graphium sp. | | X | X | | | X | | |
| Trichoderma viride | | X | X | | | | X | |
| Trichoderma sp. | | | | X | | | | |
| Aspergillus fumigatus | | X | X | X | | | X | |
| Penicillium sp. | | X | X | X | | X | | |
| Coprinus cinereus | | | | X | | | | |
| Gliocladium roseum | | X | X | | | X | X | X |
| Botrytis sp. | | | X | X | | X | X | X |
| Arthrobotrys sp. | | | | | | X | | |
| Humicola sp. | | X | X | X | | | | |
| Bipolaris sp. | | | | | | | X | X |
| Curvularia sp. | | | | | | | | X |
| Streptomyces sp. | | | X | | | | X | X |

Botrytis sp., isolated only twice, was seen to be a common inhabitant. Fusarium sp. did not appear as dominant as its frequency of isolation would suggest. Chaetomium globosum perithecia began to appear by the beginning of the second week in the amended pad and by the third week were present in masses on the surface of the straw, but did not appear until later in the unamended pad.

Throughout the incubation period, the pH of both pads was monitored by taking two samples from each pad each week and immersing them in distilled water for ½hr. The pH of each was then determined using Whatman-BDH indicator papers. Figure 2 shows the change in pH

of each pad over the incubation period.

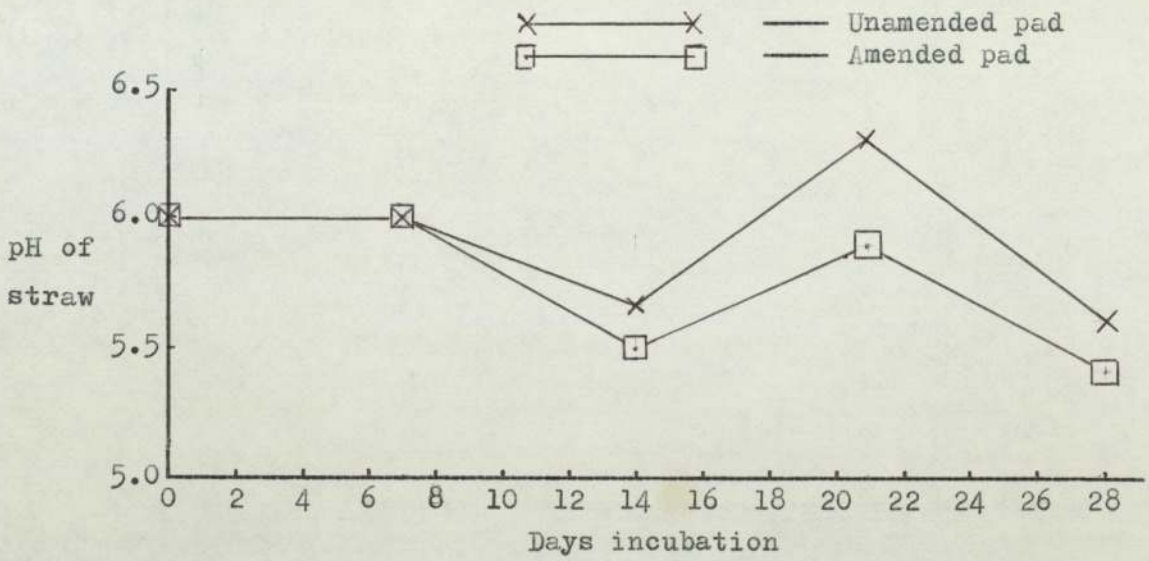



Figure 2. Change in pH of straw pads during incubation.

The C:N ratio of each pad was determined in triplicate at the beginning and end of the incubation period. Carbon was determined by the method described by Mills (1973) and nitrogen by the micro-Kjeldahl method (Humphries, 1956). The results are shown in Table 6. The low C:N ratio of the amended pad compared to the unamended pad may well have led to the higher fungal metabolic activity in the former (Levi and Cowling, 1968).

Table 6. Percentage nitrogen and carbon to nitrogen ratios for unamended and amended pads at the beginning and end of and end of incubation at 25°C.

All figures are averages of three replicate analyses.

| | Unamended pad, Day 0 | Amended pad, Day 0 | Unamended pad, Day 28 | Amended pad, Day 28 |
|---|---|---|---|---|
| % Nitrogen* | 0.404 | 0.523 | 0.480 | 0.694 |
| C:N ratio | 131:1 | 101:1 | 110:1 | 77:1 |

*Total Kjeldahl nitrogen.

2.3.3 Discussion

The isolation techniques used differ greatly in the fungi revealed. The dilution plate technique gave the widest range of fungi and probably included those originating from dormant propagules (Warcup, 1960). The highly sporulating fungi Aspergillus fumigatus, Fusarium sp., Trichoderma viride and Chaetomium globosum were all frequently isolated.

Direct plating of pieces of straw onto agar plates yielded a narrower range of fungi. Where the pieces of straw were removed from plates after 24hrs the number of fungi was reduced to four. This modification is likely to favour the isolation of fast-growing fungi already active in the substrate. Serial washing of the straw pieces further increased the degree of selectivity, the modification yielding mainly Trichoderma viride and Fusarium sp. The fungi isolated with this method were probably embedded in the straw to the greatest extent, and were likely to be actively colonising the substrate. Serial washing combined with removal of the straw pieces from the agar after 24hrs led to the isolation of Fusarium sp only.

Fusarium sp. was also the main isolate using the screened substrate technique, designed to selectively isolate actively growing fungi. It is unlikely that ingress of fungal propagules occurred from the entry of microfauna, since the screen was seen to possess a very fine mesh ($<0.5\mu$).

The straw strip tube baiting technique was highly selective, the dominant isolate again being Fusarium sp.

Direct observation revealed the presence of all the genera of fungi isolated , except Doratomyces sp. In addition, three others not isolated were observed.

It is difficult to assess C. cinereus populations at the end of the incubation period; although fruiting bodies and initials

of this fungus were occasionally seen in the unamended pad, the selective isolation techniques employed yielded only a single isolate.

Taking the results from the different techniques together, the indications are that only a small number of cellulolytic fungi were active in the incubated straw. In the unamended pad, these were initially mainly Alternaria tenuis and Fusarium sp., the former later declining and the latter increasing, to be joined by others, including Chaetomium globosum, Graphium sp., Gliocladium roseum, Aspergillus fumigatus and Penicillium spp. The amended pad gave a generally narrower range of fungi, but encouraged the growth of C. globosum, Cephalosporium sp. and Trichoderma viride and discouraged A. fumigatus. Fusarium sp. appeared to be a common inhabitant of the straw throughout the incubation period of both pads, but was not often seen in the direct observation of the straw. Streptomyces sp. was also common late in the successions of both pads.

The pH of the pads is likely to have played an important part in determining the isolations from the two pads and the differences between them. Figure 2 shows that the pH of both pads remained acid throughout the incubation period, with the amended pad becoming slightly more acid than the unamended pad. This is probably partly due to the acidity of the ammonium chloride added to the former and partly due to the additional nitrogen stimulating microbial metabolic activity, thus increasing the organic acid metabolites produced. While the acid conditions prevailing in both pads is conducive to the growth of most fungi, it is known that some prefer alkaline conditions (Fries, 1956) and it is shown later that Coprinus cinereus prefers alkaline pHs for optimum linear growth. This may be why it was so rarely isolated in the unamended pad and not at all in the amended pad.

## 2.4 FUNGI ISOLATED FROM AMMONIA-TREATED STRAW

### 2.4.1 Introduction

Preliminary experiments at the Biodeterioration Information Centre have shown that when either urea or ammonia solutions were added to fresh, unsterilised straw, its pH rose and colonisation by Coprinus cinereus during fermentation was very much encouraged. Since this fungus has a high linear growth rate and appeared to have the potential to dominate the substrate under alkaline conditions, an isolation programme to identify its important competitors under these conditions was carried out.

### 2.4.2 Experimental procedure

The vessels used in this experiment were standard 1 lb jam jars. Each was filled with 10g fresh straw followed by 20ml of ammonia solution. Three different concentrations of ammonia solution were used; 2.0%, 1.0% and 0.1%, expressed as percentages of the dry weight of the straw. Controls were set up containing distilled water in place of ammonia solution. Three replicate jars were prepared for each nutrient regime.

Two of the techniques used in the isolations from the straw pads were used again in this experiment, the direct plating of straw pieces onto Eggins and Pugh cellulose agar and the screened substrate baiting technique, a microscope slide being used in place of a boiling tube and the substrate being plated onto Eggins and Pugh cellulose agar. Two slides, each mounted with a piece of cellulose paper and a strip of straw as screened substrates, were placed in each jar each week. 30mg rose bengal was incorporated in the media to suppress bacterial growth. Direct observation of samples of straw from the jars was used as an aid to gaining knowledge of the fungi present. All jars were incubated at 30°C. in the dark. Isolations were made at weekly intervals, fresh slides replacing each pair removed.

## 2.4.3 Results

The fungi observed or isolated in this experiment are shown in Table 7.

Table 7. Fungi observed in or isolated from ammonia-treated straw incubated at 30°C. A = isolated by direct plating on agar, B = isolated by the screened substrate technique, C = observed.

| | | 2.0% $NH_3$ | | | 1.0% $NH_3$ | | | 0.1% $NH_3$ | | | Control* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day: | | 7 | 14 | 21 | 7 | 14 | 21 | 7 | 14 | 21 | 7 | 14 | 21 |
| Coprinus cinereus | A | | | 4 | 4 | 4 | 4 | 4 | 3 | 3 | | | 1 |
| | B | | | 2 | 4 | 4 | 4 | | 2 | 2 | | | |
| | C | | | X | X | X | X | X | X | X | | | |
| Chaetomium globosum | A | | | | | 1 | 1 | 4 | 4 | 2 | 4 | 4 | 3 |
| | B | | 1 | 2 | | | | 2 | 2 | 2 | 4 | 4 | 4 |
| | C | | | X | | X | X | X | X | X | X | X | X |
| Aspergillus fumigatus | A | | 2 | | | 2 | 1 | | | | | | 1 |
| | B | 1 | 4 | 4 | | 1 | 2 | | | | | | |
| | C | | | X | | | | | | | | | |
| Fusarium sp. | A | | | | | | | | | 1 | 1 | | 2 |
| | B | | | | | | | | | | 1 | 1 | 1 |
| | C | | | | | | | | | X | X | X | |
| Alternaria tenuis | A | | | | | | | | | | | 1 | 1 |
| | B | | | | | | | | | | X | | X |
| | C | | | | | | | | | | X | | X |
| Gliocladium roseum | A | | | | | | | | | | | 1 | |
| | B | | | | | | | | | | | | |
| | C | | | | | | | | | | | X | X |
| Cephalosporium sp. | A | | | | | | | | | | | | |
| | B | | | | | | | | | | | | |
| | C | | | | | | | | | | | X | |
| Penicillium sp. | A | | | | | | | | | | | | |
| | B | | | 2 | | | | | | | | | |
| | C | | | | | | | | | | | | |
| Trichoderma viride | A | | | | | | | | | 1 | | | 1 |
| | B | | | | | | | | | 1 | | | |
| | C | | | | | | | | | | | | |
| Graphium sp. | A | | | | 1 | | | | | | | | |
| | B | | | | | | | | | | | | |
| | C | | | | | | | | | | | | |

Figures refer to the number of the four replicate plates from which the fungus was isolated.

*Sterile distilled water used in place of ammonia solution.

It can be seen that Coprinus cinereus was the predominant fungus found in the straw treated with 1.0% $NH_3$ and was the only species found on day 7 at that concentration. At 0.1% $NH_3$, it was found slightly less often than Chaetomium globosum, the dominant fungus of the control jars. Aspergillus fumigatus was isolated mainly from the straw treated with the two highest concentrations of ammonia, most commonly on days 14 and 21. 2.0% $NH_3$ in the straw appeared to prevent the growth of most fungi, at least in the early part of the incubation period. It is possible that loss of gaseous ammonia from the jars occurred during incubation and this would be likely to influence the species emerging over time.

A second experiment was carried out to determine more precisely the optimum concentration of ammonia for the isolation of Coprinus cinereus.

## 2.5 THE OPTIMUM AMMONIA CONCENTRATION FOR THE ISOLATION OF COPRINUS CINEREUS.

### 2.5.1 Experimental procedure

Jars of straw were prepared and fungi identified from in exactly the same way as for the last described experiment, this time five ammonia concentrations being used (1.5, 1.2,1.0, 0,8 and 0.5% $NH_3$) at a replication level of three, and fungi being isolated and observed only on day 14. The pH of the straw samples was also determined on day 14, by standing the straw in a small quantity of distilled water for $\frac{1}{2}$hr, decanting it and measuring the pH using Whatman- BDH indicator papers.

### 2.5.2 Results

The fungi isolated or observed on day 14 in this experiment are shown in Table 8. Coprinus cinereus grew best at 0.5%$NH_3$; the jars appeared to contain almost pure cultures of it, there being just

Table 8. Fungi observed and isolated on day 14 from ammonia-treated straw incubated at 30°C.

| % $NH_3$* | Fungi isolated and observed on day 14 | pH on day 14 |
|---|---|---|
| 1.5 | Very little *Chaetomium globosum*; *Coprinus cinereus*, not fruiting | 7.6 |
| 1.2 | *C. globosum*; *C. cinereus*, not fruiting | 7.6 |
| 1.0 | *C. globosum*; *C. cinereus*, very slightly fruiting; *Paecilomyces* sp. | 7.5 |
| 0.8 | Very little *C. globosum*; *C. cinereus*, fruiting; *Paecilomyces* sp. | 7.7 |
| 0.5 | Very little *C. globosum*; *C. cinereus*, profuse fruiting; *Paecilomyces* sp. | 8.9 |

*Expressed as a percentage of the oven-dry weight of the straw.

a small amount of growth of *Chaetomium globosum* and, rarely, *Paecilomyces* sp. At higher ammonia concentrations, *C. cinereus* became less dominant and populations of *C. globosum* were higher.

As the Table shows, the average pH of the samples at day 14 was around 7.6, except in the case of the samples containing 0.5% $NH_3$, where the average pH was 8.9 on day 14.

It appears that *Coprinus cinereus* achieves the highest degree of dominance over fungal competitors when the concentration of ammonia is around 0.5%.

One of the isolates of *C. cinereus* obtained from this experiment was used in all subsequent experiments. It corresponds with *C. cinereus* (Shaeff ex Fr.) S.F.Gray *sensu* Konr. (Pinto-Lopes and Almeida, 1971; Kuhner and Romagnesi, 1953; Orton, 1957). Spores are 10-11 X 6-7$\mu$, small brown sclerotia are produced and the isolate

grows above 40°C. The species is the same as C. fimetarius L. ex Fr. (Pinto-Lopes and Almeida, 1971) and appears morphologically and physiologically very similar to the C. fimetarius (L. ex Fr.) Fr. strain II studied by Fries (1955), though Pinto-Lopes and Almeida (1971) believe this to be C. radiatus (Bolt. ex Fr.) S.F.Gray sensu Lange.

# CHAPTER 3

# PHYSIOLOGICAL STUDIES

## 3.1 INTRODUCTION

CONSIDERATION OF the various factors involved in determining the ecological pattern of substrate colonisation by fungi was given in chapter 1. In this chapter, experiments are described which are designed to yield information about some of these factors in an assessment of the potential of fungi to break down cellulosic substrates or of others to compete with such fungi. The fungi chosen for this study were those isolated and observed most frequently from fresh straw, as described in chapter 2. *Coprinus cinereus*, which achieved very high population levels in straw under alkaline conditions, was included in these fungi. Linear growth rate, cellulolytic ability, optimum pH and optimum temperature for linear growth were all studied and, in addition, preliminary studies were made on the ligninolytic ability of fungi and their ability to bring about weight losses in straw. All these factors are likely to be important in influencing either competitive or degrading ability.

## 3.2 ASSESSMENT OF LINEAR GROWTH RATE OF SELECTED FUNGI

Linear growth rate is a factor of great importance in determining the ability of a fungus to succeed in the competitive primary colonisation of a substrate (Wastie, 1961). Primary colonisers of substrates often possess high growth rates; an important example of this is the common occurrence of fast-growing Phycomycetes in the early stages in the decay of organic debris (Garrett, 1951), enabling them to capture readily available nutrients in advance of other fungi.

Linear growth rates were determined on artificial media in Petri dishes in this study.

### 3.2.1 Experimental procedure

The twenty-one most commonly isolated fungi were inoculated onto the edge of plates of Eggins and Pugh cellulose agar. (For composition, see Appendix 1.) Inocula were taken from the edge of colonies actively growing on the same medium. Three replicates of each fungus were prepared and the plates incubated at 25°C. ± 0.5°C. in the dark. Linear growth rates were determined by measuring the average radius of the colonies using a rule, on days 2, 4, 7, 9, 11, 14, and 19 and on additional days where necessary. When condensation on the underside of the lid obscured the view of the colony, the lid was temporarily replaced with a fresh one.

### 3.2.2 Results

Rates of growth of the fungi are given in Table 9 and plots of growth against time are shown in Figure 3. It can be seen that there is a wide range of growth rates among the fungi selected. At one extreme, there is the common soil fungus *Trichoderma viride* with a very high growth rate (18.0 mm/day) and near the other extreme is *Gliocladium roseum* (2.9mm/day), also commonly found in soil.

Table 9. Linear growth rates of selected cellulolytic fungi from straw, on Eggins and Pugh cellulose agar at 25°C.†
Figures are average growth rates between days 4 and 11 unless asterisked, and all are averages of three replicates.

| Fungus | Linear growth rates (mm/day) | Fungus | Linear growth rates (mm/day) |
|---|---|---|---|
| Coprinus cinereus | 8.0* | Cephalosporium sp. 1 | 2.4 |
| Chaetomium globosum | 7.1 | Cephalosporium sp. 2 | 8.7* |
| Chaetomium cochlioides | 8.4* | Cephalosporium sp. 3 | 8.6* |
| Chaetomium sp. 1 | 6.2 | Trichoderma viride | 18.0* |
| Chaetomium sp. 2 | 6.3 | Trichoderma sp. 1 | 19.3* |
| Chaetomium sp. 3 | 6.5 | Trichoderma sp. 2 | 17.7* |
| Chaetomium sp. 4 | 5.0 | Trichoderma sp. 3 | 2.9 |
| Chaetomium sp. 5 | 4.1 | Alternaria tenuis | 5.1 |
| Chaetomium sp. 6 | 4.9 | Gliocladium roseum | 3.0 |
| Chaetomium sp. 7 | 6.2 | Aspergillus fumigatus | 4.4 |
| Fusarium culmorum | 12.2* | | |

*Average growth rate between day 4 and day when plate became covered.
†See Appendix 2 concerning numbered species.

## 3.3 ASSESSMENT OF CELLOLOLYTIC ABILITY OF SELECTED FUNGI

The measure of cellulolytic ability provides a general indication of the potential of a fungus to colonise and break down a cellulosic substrate quickly. It may be a factor of some importance in determining the competitive ability of a fungus (Garrett, 1966).

The cellulase complex employed by a fungus may enable it to break down the cellulose in one type of substrate but not another, due either to inherent limitations of the cellulase complex (Halliwell, 1963) or to the presence of interfering components, for example, lignin. The specificity of the cellulase complex may thus give rise to difficulties in the comparative assessment of cellulolytic abilities (Halliwell, 1963).

Many workers have been concerned with the assessment of

the cellulolytic ability of fungi (Gascoigne and Gascoigne, 1960; Halliwell, 1963) and a variety of techniques have been developed. Most of these employ some artificially prepared form of cellulose. Loss of dry weight of filter paper has been used by Reese (1946), Garrett (1962), Chang (1967) and others. Hazra et al (1958) acid-treated and ball-milled the paper prior to exposure in a rapid assay technique. Perfused chromatography paper was used by Malik and Eggins (1970) and Mills (1973), loss of tensile strength being the measure of cellulolytic activity, and cotton textile has been assessed for attack by the same method (Abrams, 1950; Siu and Sinden, 1951; Allsopp, 1973). Preparations of cell-free cellulase extracts have also been used (Walseth, 1952; Thomas, 1956), the substrate sometimes being synthetic soluble forms of cellulose such as carboxy-methyl cellulose (Agarwal et al, 1963; Domsch and Gams, 1969); the reduction in viscosity of the solution can be used as an index of cellulolytic activity (Agarwal et al, 1963), or alternatively, the production of reducing sugars (Nisizawa, 1963).

Monitoring the dissolution of cellulose incroporated into agar is a well established approach. McBeth (1916), Scales (1916), Aschan and Norkrans (1953) and others have used the diameter of cellulose clearing in agar plates as a measure of cellulolytic ability. One of the limitations of this technique is that some fungi tend to obscure the zone of clearing. Aschan and Norkrans (1953) and Savory et al (1967) have incorporated chemicals in the medium to suppress growth and then measured the degree of clearing caused by cellulases diffusing through the medium. Rautela and Cowling (1966) used anaerobiosis to check fungal growth and measured the depth of enzymatically produced clearing in a cellulose medium contained in a boiling tube. These techniques exclude the effects of fungal growth and the measure of cellulolytic activity will depend upon the rate of diffusion of the

enzyme complex and its stability. Walsh and Stewart (1969) used a modified technique in which the fungus was not suppressed; they measured the intensity of clearing close to the fungus by counting the number of undissolved cellulose particles remaining in a specific area of the medium in a Petri dish, after fungal growth had taken place. The method appears to be reliable in comparative measurements of cellulolytic activities (Walsh and Stewart, 1969).

The technique chosen for this study involves the assessment of four levels of intensity of clearing of ball-milled cellulose powder (Whatman's CF11) in agar regularly over a period of time. It is hoped that the method method might provide more information on the nature of the cellulolytic activity occurring. The factors that will contribute to intense cellulolytic activity and thus intense clearing (Walsh and Stewart, 1969) are likely to include a high rate of production of the cellulase complex per unit time and space, a high number of substrates each unit of cellulase complex can convert per unit time, good stability of the enzyme and a high rate of diffusion. Both stability and diffusibility must be good if a high intensity of clearing is achieved.

### 3.3.1 Experimental procedure

All the fungi selected for the linear growth rate study except *Trichoderma* sp. 1 and 2 were inoculated from the margin of actively growing colonies onto the centre of 20ml of Eggins and Pugh cellulose agar poured into Petri dishes, in triplicate. It was clear from the linear growth rate study that, as far as their ability to clear cellulose agar is concerned, there was very little difference between *T. viride* and *Trichoderma* sp. 1 or 2. Incubation was at 25°C. in the dark. The extent of each clearing intensity level in the plates was determined by comparing the transmission of daylight through the

inoculated plates with a series of sterile plates containing standard thicknesses of Eggins and Pugh cellulose agar. Each level of thickness was identified with a scale number from 0 to 4; the number of mls of agar in the Petri dishes for each scale number are shown in Table 10.

Table 10. Volume of agar in standard plates for estimation of cellulolytic ability.

| Scale Number | No. of mls of Eggins and Pugh cellulose agar in Petri dish | Label |
|---|---|---|
| 0 | 20 | no clearing |
| 1 | 15 | slight cleaing |
| 2 | 10 | moderate clearing |
| 3 | 5 | good clearing |
| 4 | - | excellent clearing |

A satisfactory standard for scale number 4 could not easily be made in this manner; instead, the criterion used was that typescript should be easily read through the agar.

On days 2, 4, 7, 9, 11, 14, and 19 the diameter of each level of clearing in the incubated plates was assessed by comparison with the standard plates using a daylight background and recorded to the nearest 0.5 cm.

### 3.3.2 Results

Figure 4 shows for each fungus the extent of each level of clearing of cellulose plotted against time, using averages of the three replicates. *Cephalosporium* spp. 1 and 3, *Chaetomium cochlioides* and *Chaetomium* spp. 4 and 5 all tended to obscure the medium, making accurate measurements difficult. In these cases, accuracy may be

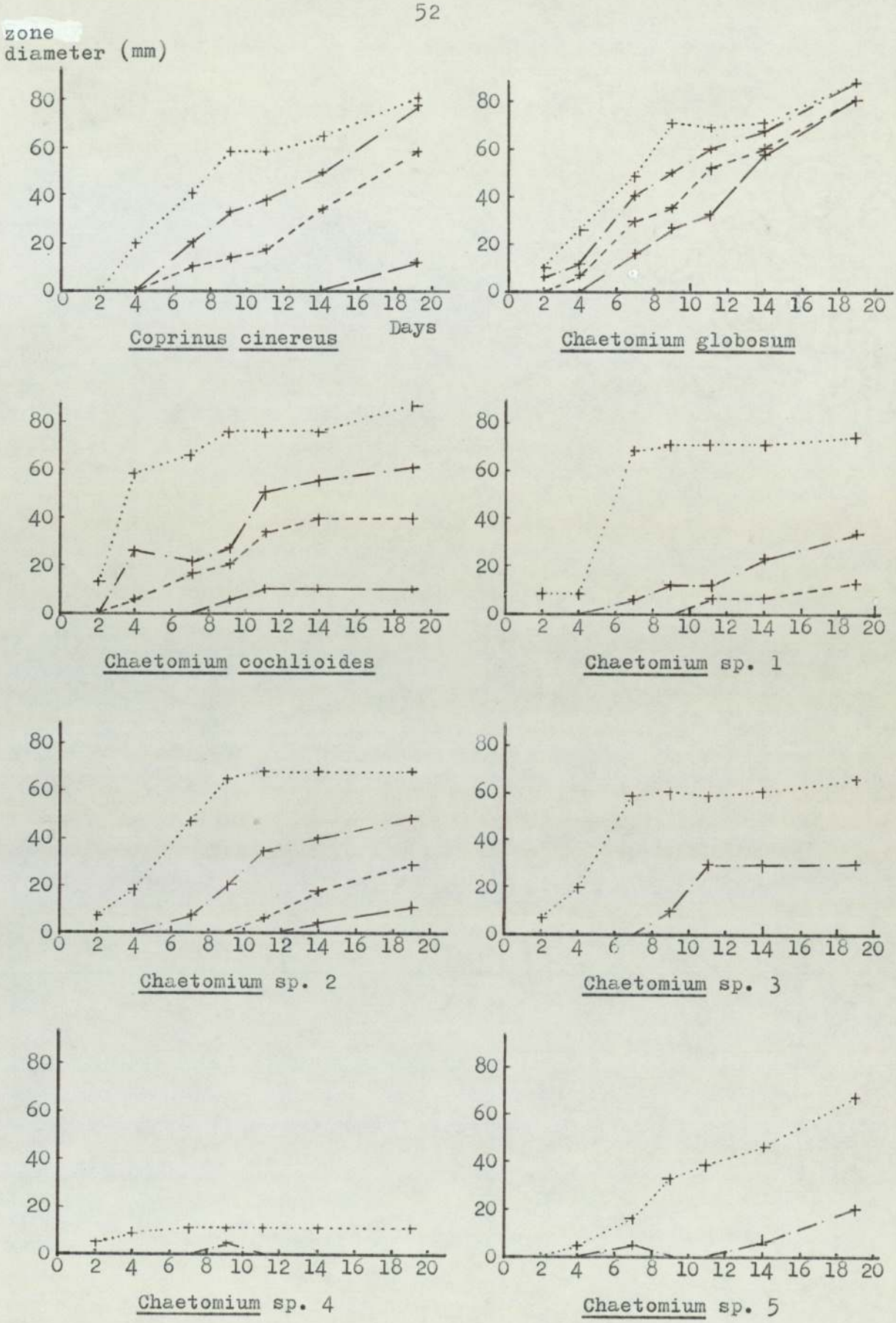



Figure 4. Extent and intensity of clearing of cellulose agar by fungi, at 25°C. (..... =slight clearing, - - - - =moderate clearing, -------- =good clearing, ——— =excellent clearing. For definition of these terms, see text.)

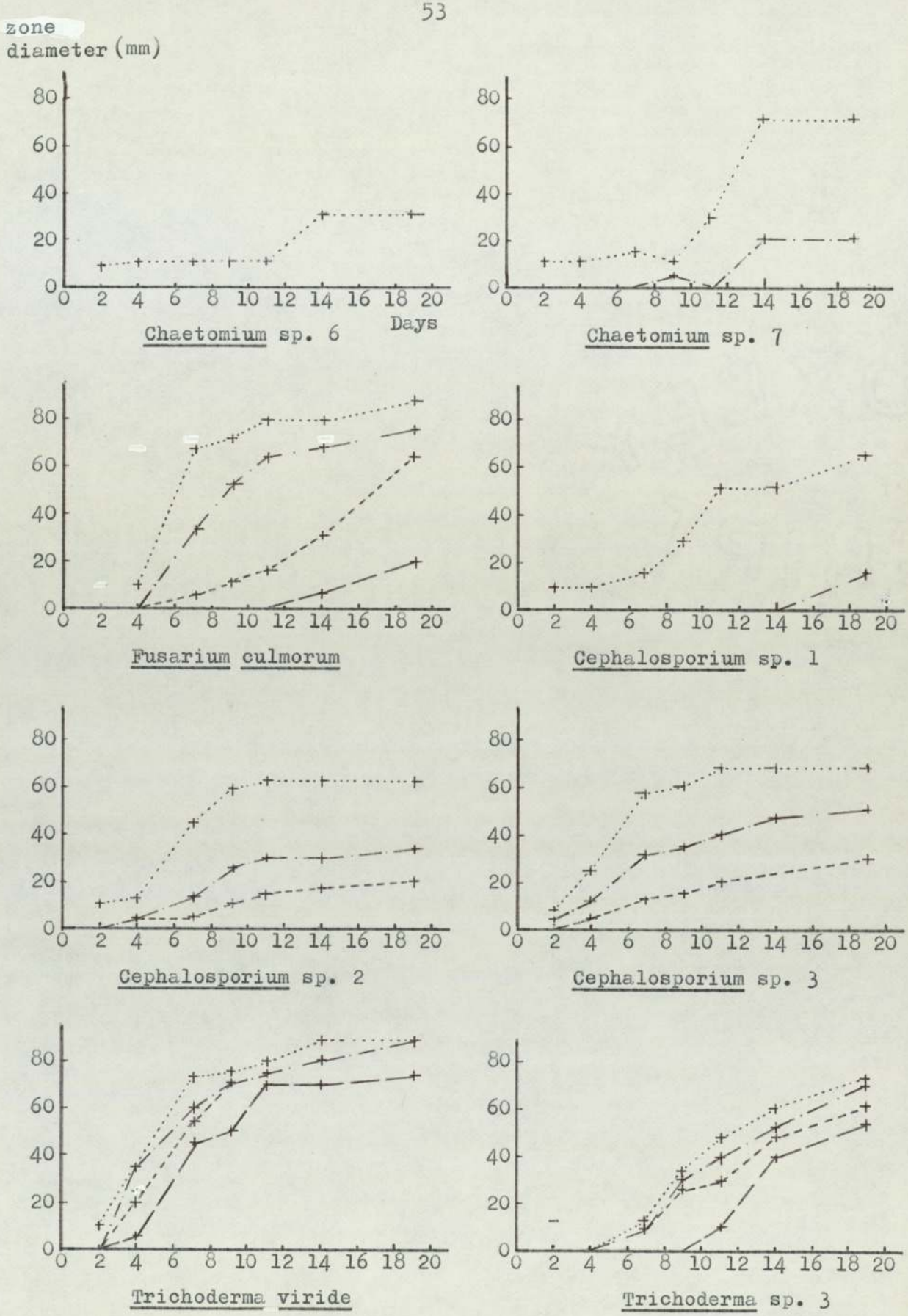



Figure 4 (cont.)

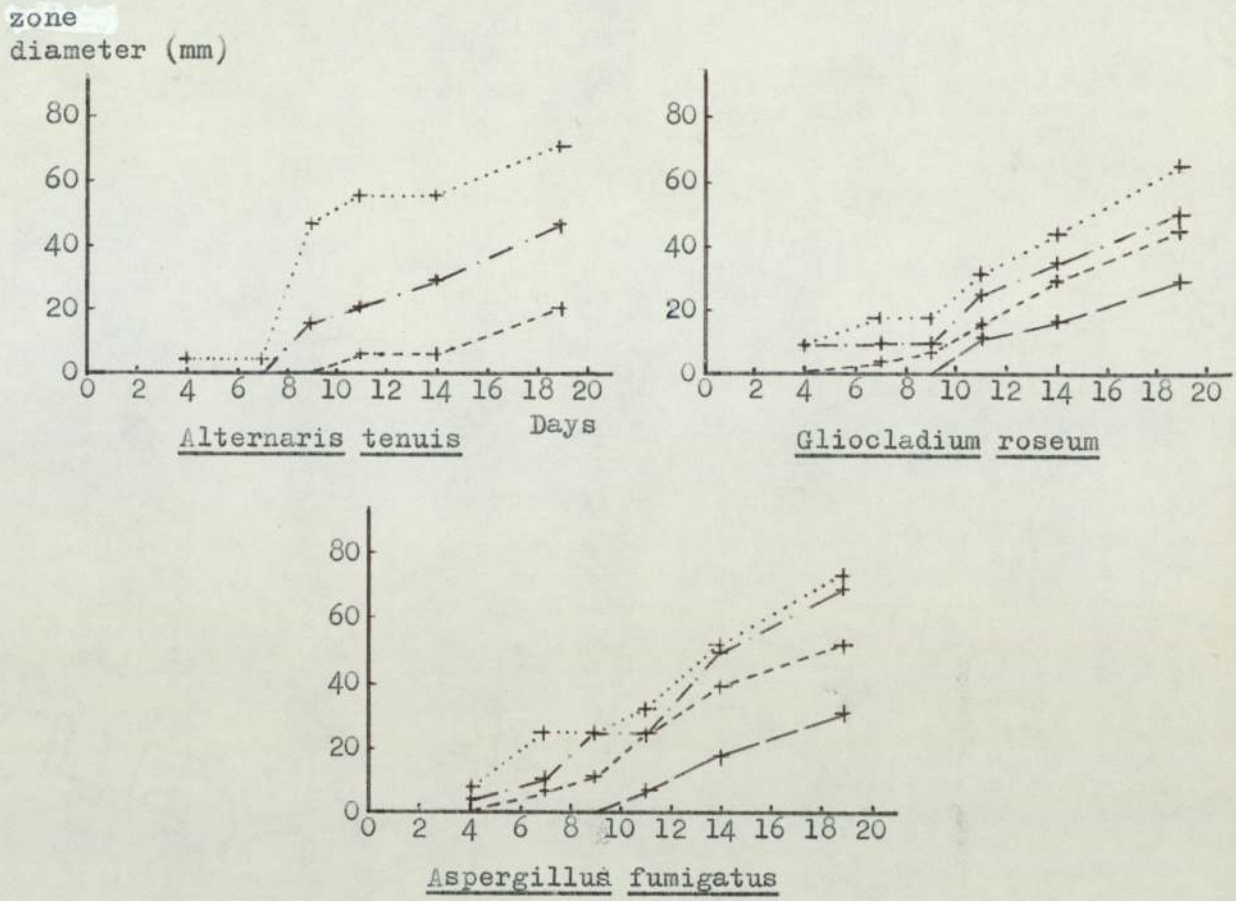



Figure 4 (cont.)

rather lower than ±0.5cm otherwise achieved.

It can be seen that the fungi vary widely in the rate and intensity of clearing produced. The rate of clearing is clearly limited by growth rate, important in the cases of Aspergillus fumigatus, Gliocladium roseum and Trichoderma sp. 3. Several Chaetomium spp. produced a low intensity slowly and Chaetomium spp. 1 and 3 a low intensity, but rather more quickly. Fungi able to produce intense clearing quickly were Chaetomium globosum, Trichoderma viride and to a lesser extent, Fusarium culmorum, Coprinus cinereus and Chaetomium cochlioides.

The experiment provides only an indication of the potential of the fungi to break down the cellulose in straw; it is not necessarily available to fungi capable of clearing cellulose agar. It must also be added that the carbon in asparagine and yeast extract, components of Eggins and Pugh cellulose agar, may be preferentially utilised by some fungi before they begin to exploit the cellulose (Bravery, 1968) and, since this was not checked, it is possible that some cellulolytic abilities are under-estimated.

## 3.4 OPTIMUM pH FOR LINEAR GROWTH OF SELECTED FUNGI IN VITRO

Six fungi with a high growth rate and good cellulolytic ability, and two—Aspergillus fumigatus and Gliocladium roseum—with known potential as good competitors, were studied to determine their optimum pH for linear growth. A. fumigatus is commonly found in fermenting straw (Chang and Hudson, 1967) and G. roseum is a strong mycoparasite (Barnett and Lilly, 1962). These eight fungi are used in nearly all the remaining studies of this thesis.

### 3.4.1 Experimental procedure

As a preliminary step in this study, investigations were made into the best means of buffering Eggins and Pugh cellulose agar

throughout the range of pHs 4 to 9. McIllvaine's buffer (McIllvaine, 1921) was tried, but did not allow pHs above around 7.5 to be maintained. A trial using Sorensen phosphate buffer was found to be satisfactory for this experiment, though Trichoderma viride was still able to cause a change of 2.0 pH units at an initial pH of 5.3

The buffer solutions were prepared after the method of Fries (1956) in which the required medium was made by mixing 400mls of buffer solution with 600mls of concentrated medium solution. Mixing was carried out after autoclaving separately, immediately prior to pouring the plates. Table 11 shows the composition of the six buffer solutions used and the pH each produced in the final autoclaved medium. Three replicates were prepared for each fungus,

Table 11. Composition of buffer solutions used in pH study.

| No. of mls of solutions* | Final pH |
|---|---|
| 346mls $KH_2PO_4$ + 54mls HCl | 4.0 |
| 387mls $KH_2PO_4$ + 13mls HCl | 5.3 |
| 21mls $Na_2HPO_4$ + 379mls $KH_2PO_4$ | 5.9 |
| 174mls $Na_2HPO_4$ + 226mls $KH_2PO_4$ | 6.5 |
| 400mls $Na_2HPO_4$ | 7.7 |
| 107mls $Na_2HPO_4$ + 293mls $K_3PO_4$ | 8.7 |

*0.1N HCl solutions, 1/15 M phosphate solutions.

tested at six different pHs. The fungi were inoculated from the margin of actively growing colonies onto the edge of the plates and incubated at 25°C. in the dark. Measurements of colony radii were made with a rule on days 1, 2, 3, 6 and 8. Linear growth rates were calculated from the data of days 2 and 8, unless the fungus had covered the plate before day 8, in which case the last day on which

a measurement could be made was used. The pH of the agars was measured on day 0 using a stab electrode and again on day 6, at a point on the agar which the fungus had colonised.

### 3.4.2 Results

The average growth rates of the fungi at the six pHs are set out in Table 12 and the effects of the fungi on the pH of the agars

Table 12. Effect of pH on the linear growth rate of eight fungi cultured on Eggins and Pugh cellulose agar at 25°C.

| | Growth rate (mm/day)* | | | | | |
|---|---|---|---|---|---|---|
| pH at day 0: | 4.0 | 5.3 | 5.9 | 6.5 | 7.7 | 8.7 |
| Coprinus cinereus | 3.6 | 6.8 | 7.7 | 11.3 | 13.0 | 10.0 |
| Chaetomium globosum | 2.0 | 7.3 | 7.5 | 8.7 | 12.8 | 6.8 |
| Chaetomium cochlioides | 2.0 | 5.9 | 8.6 | 10.3 | 10.2 | 9.0 |
| Cephalosporium sp.† | 4.2 | 10.9 | 11.3 | 12.2 | 10.1 | 8.3 |
| Fusarium culmorum | 7.3 | 15.0 | 15.5 | 20.0 | 15.1 | 9.0 |
| Trichoderma viride | 13.0 | 15.5 | 21.0 | 14.7 | 2.6 | 1.5 |
| Gliocladium roseum | 1.8 | 2.8 | 2.9 | 2.9 | 3.3 | 2.3 |
| Aspergillus fumigatus | 2.5 | 3.5 | 4.4 | 2.2 | 1.5 | 0.2 |

*Figures are averages of three replicates and are averages between days 2 and 8 or the last day on which a measurement could be made.
† Cephalosporium sp. 2.

at day 6 are shown in Table 13. In Figure 5, the growth rates of the fungi are plotted against pH, both the initial pH (full line) and the pH after colonisation (broken line).

Availability of elements, membrane permeability and enzyme activity are all influenced by pH and contribute to determining the pattern of growth with pH. In addition, these parameters are known to be affected by the temperature prevailing. Fries (1953) has found that the shift in pH caused by fungal growth is also influenced by

Table 13. The effect of fungal growth on the pH of buffered Eggins and Pugh cellulose agar.

| | pH of agar after 6 days growth | | | | | |
|---|---|---|---|---|---|---|
| pH at day 0: | 4.0 | 5.3 | 5.9 | 6.5 | 7.7 | 8.7 |
| Coprinus cinereus | 4.5 | 5.8 | 5.9 | 6.5 | 7.2 | 8.3 |
| Chaetomium globosum | 5.0 | 5.1 | 5.6 | 6.4 | 7.5 | 8.7 |
| Chaetomium cochlioides | 5.3 | 5.8 | 6.0 | 6.5 | 7.2 | 8.4 |
| Cephalosporium sp. | 5.1 | 5.2 | 5.2 | 6.3 | 7.2 | 8.3 |
| Fusarium culmorum | 4.3 | 4.7 | 5.3 | 6.1 | 6.9 | 8.2 |
| Trichoderma viride | 3.0 | 3.3 | 4.4 | 5.8 | 6.9 | 8.7 |
| Gliocladium roseum | 4.5 | 5.6 | 5.8 | 6.5 | 7.5 | 8.7 |
| Aspergillus fumigatus | 4.0 | 4.3 | 5.0 | 6.1 | 7.8 | 8.7 |

temperature; Coprinus fimetarius changed the pH of media in an alkaline direction at high temperatures but in an acid direction at lower temperatures.

The fungi tested were found to possess widely differing pH optima. While most were on the acid side of neutrality, as is the case with the majority of fungi in general (Lilly and Barnett, 1951), Coprinus cinereus, Chaetomium globosum and Gliocladium roseum all had alkaline pH optima. Sharp and Eggins (1970) isolated C. globosum less from soil at acid pHs than at alkaline pHs and Fries has shown that several Coprinus spp. possess alkaline pH optima (Fries, 1956). Some of her isolates were found to possess two pH optima with a minimum between at around pH7.4-7.8 and it was suggested that this may be due to changes in the availability of essential elements occurring with changes in pH. A similar double peak was observed by McShane (1976) in the growth of Coprinus cinereus, but a single peak only was observed in this experiment.

Trichoderma viride and Aspergillus fumigatus, both with

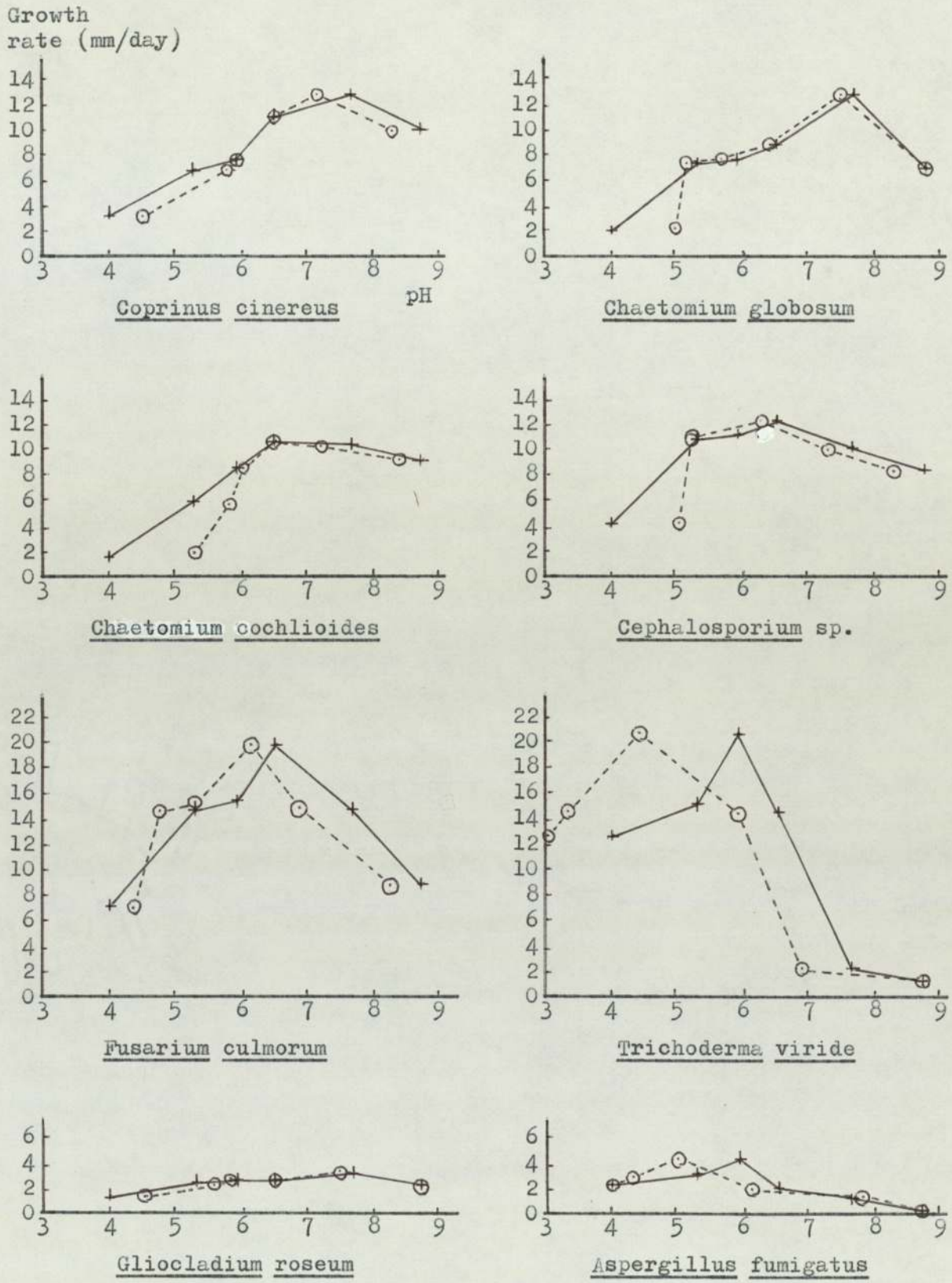



Figure 5. Effect of pH on the linear growth rate of selected fungi, and their influence on the pH of the medium (Eggins and Pugh cellulose agar) at 25°C. (⟵+ = growth rate v. initial pH, ⊙---⊖ = growth rate v. pH after colonisation.)

acid pH optima, were rather intolerant of alkaline pHs.

Fungal cellulases are, in general, stable and active over a wide range of pHs, usually with optimum activity around pH4-5.5 (Mandels and Reese, 1965). The cellulase complex of T. viride has been shown to possess maximum activity at pH 4.1 (Hanstein, 1960), while Gliocladium roseum cellulase possesses optimum activity between pHs 6.4 and 8 (Isaac, 1954). The optimum pH for Chaetomium globosum cellulase is reported to be 4.8-5.2 (Agarwal et al, 1963). Sharp (1970) has studied the cellulolytic activity of C. globosum, T. viride and and G. roseum, as measured by the depth of clearing of cellulose agar in Cowling tubes, and found optima for depth of clearing around pH 4.5-5.5 for each fungus. Mills (1973) found that Aspergillus fumigatus possessed two optima for cellulolytic activity, both at acid pHs; this fungus and a number of others, however, were found to possess significant cellulolytic activity at alkaline as well as acid pHs and definite clearing of cellulose agar by all eight fungi was seen at pH 8.7 in this experiment.

It is clear from the experiment carried out in this study that the relative linear growth rates of the fungi tested are very much dependent on the pH of the substrate. In any attempt to favour the growth or competitive advantage of a selected fungus or fungi, control of the pH of the substrate may therefore play an important part. If, for example, it is desired to encourage the growth of Coprinus cinereus and impede the growth of the other fungi being studied here, it may be useful to maintain the pH of the substrate above pH 8.

## 3.5 EFFECT OF TEMPERATURE ON LINEAR GROWTH RATE OF SELECTED FUNGI

Since temperature is known to play a vital part in determining which fungi in the mixed mycoflora of a complex substrate will dominate, an *in vitro* study was carried out to examine the effect of temperature on the linear growth rates of selected fungi. The fungi studied were the same eight that were chosen for the pH study.

### 3.5.1 Experimental procedure

The fungi were inoculated from actively growing colonies onto the edge of plates of Eggins and Pugh cellulose agar (pH 5.5) and incubated in the dark at four different temperatures; 15.0°C, 20.0°C, 25.0°C and 30.0°C, ±0.5°C. Three replicates at each temperature were prepared. Linear growth rates were calculated from measurements of the radii of fungal colonies on their first day of active growth and then on day 9 or on the last day on which measurements could be made.

### 3.5.2 Results

The linear growth rates of the fungi are plotted against temperature in Figure 6. It is clear from these results that relative growth rates between the fungi are very much dependent upon temperature. For example, *Aspergillus fumigatus*, by far the slowest-growing fungus at 15°C., is faster-growing than *Cephalosporium* sp. and *Gliocladium roseum* at 30°C., and, while at 20°C. only two of the seven other fungi had lower growth rates than *Coprinus cinereus*, at 30°C. only *Trichoderma viride* grew faster. Since *C. cinereus* still has a high growth rate at 40°C. (McShane, 1976) and *Trichoderma viride* has been shown to possess a very low growth rate above 30°C. (Lai and Bruehl, 1968), it is likely that at some point above this temperature, the growth rate of *C. cinereus* will exceed that of all the fungi tested, with the possible exception of *Aspergillus fumigatus*. This fungus possesses

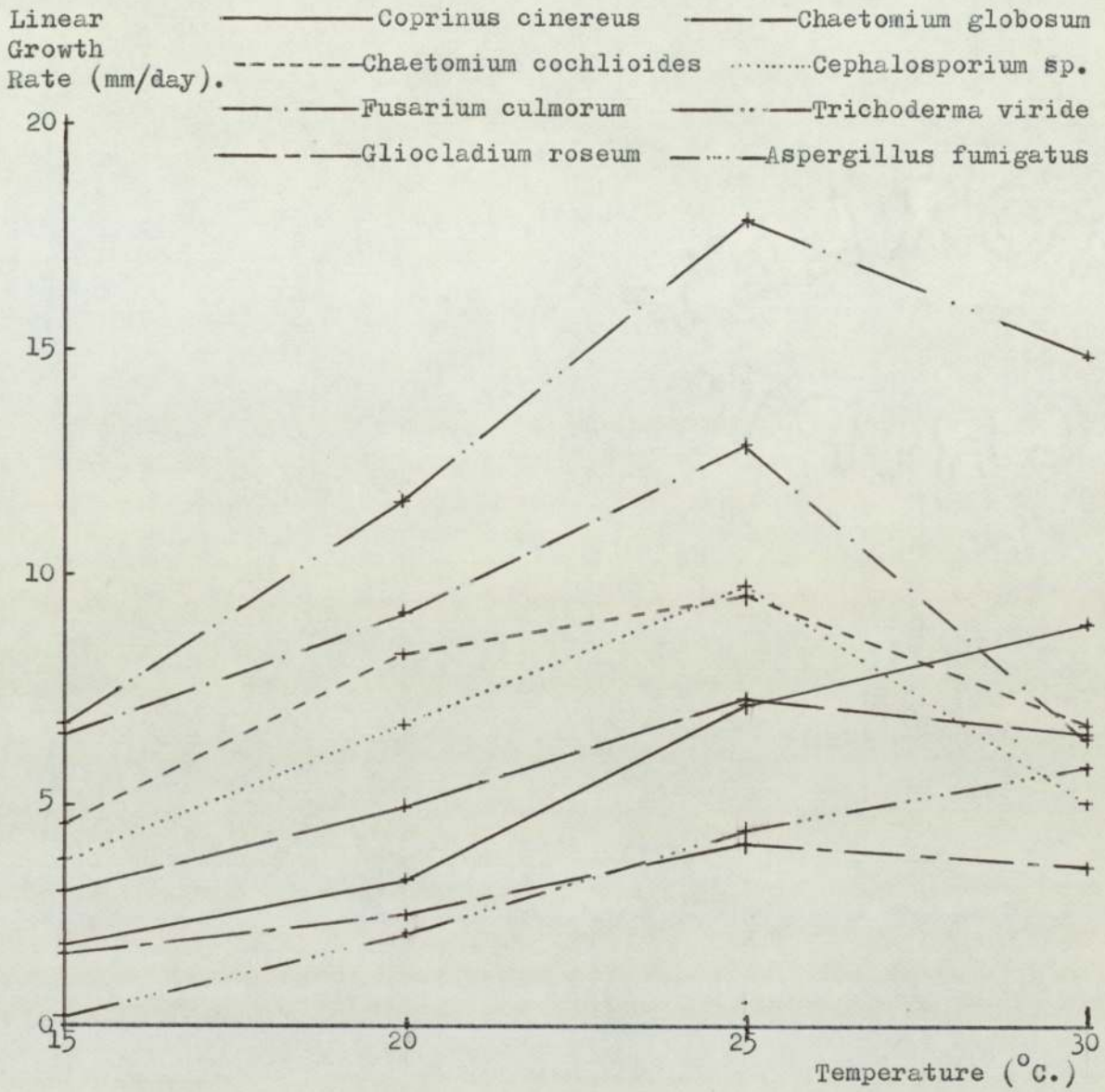



Figure 6. Effect of temperature on the linear growth rate of fungi growing on Eggins and Pugh cellulose agar.

an optimum linear growth rate of around 14 mm/day at 35°C. (Rege, 1927) also the optimum temperature for growth of C.cinereus (Chang, 1967). Reduction of growth rate above optimum temperatures operates mainly through partial or complete enzyme inhibition. Probably as a result of this, nutrients which are not essential requirements at one temperature can become so at a higher temperature. It is known that the growth rate of Coprinus cinereus at 44°C., near to its maximum temperature for growth, is much enhanced by the presence of methionine in the medium (Fries, 1953). It may be possible to exploit such occurrences

to facilitate domination of a substrate by a desired fungus.

This experiment, demonstrating the importance of temperature in determining relative growth rates, gives some information as to how temperature control might be used to help create and maintain dominance by a particular fungus.

## 3.6 LIGNINOLYTIC ABILITY OF SELECTED FUNGI

It is recognised that an important factor in limiting the digestibility of straw to ruminants is the presence of lignin, enmeshed with the cellulose, hemicelluloses and other polysaccharides present (Chandra and Jackson, 1971; Pigden and Bender, 1972; Eriksson, 1974). Breakdown, removal or at least separation of the lignin from the carbohydrate will therefore be likely to increase the digestibility of straw (Tomlin et al, 1965). Reference has already been made of the possibility of using fungi as agents of lignin breakdown and it was considered of value to carry out a preliminary assessment of the potential of the eight fungi under study to break down lignin.

Several methods have been used by researchers in the determination of ligninolytic ability. Fungi may be grown on a lignin-containing natural substrate and the levels of lignin present before and after colonisation determined (Waite et al, 1964). Alternatively, qualitative assessment can be made on artificial media. Clearing of agar with ball-milled beech-wood incorporated in it has been used (Eggins, 1965); the method was not used here since the lignin is of wood origin. The methods of Sundman and Näse (1971) and Bavendamm (1928) are both good at differentiating between white-rot and brown-rot fungi, though there are occasional anomalies (Sundman and Nase, 1971). The widely used Bavendamm's test was used used for this experiment. The presence of polyphenol oxidases excreted by fungi is detected by the production in the medium of deep-coloured zones around the mycelium,

due to the degradation of tannic or gallic acid incorporated in the agar.

### 3.6.1 Experimental procedure

Eggins and Pugh cellulose agar was buffered to six pHs (4.4, 5.8, 6.3, 6.9,8.8 and 9.5) by the procedure described in Section 3.4.1; gallic acid was added to each buffer before autoclaving so that the final medium contained 0.5% gallic acid. The prepared plates were inoculated centrally with 4 $mm^2$ agar plugs taken from the edge of actively growing colonies and incubated at 25°C. in the dark. Three plates per fungus were prepared. Uninoculated controls were prepared at each pH for comparison purposes. Colony diameters were measured at suitable intervals and notes taken of changes in the colour of the media.

### 3.6.2 Results

The observations, and the pH at which each was made, are set out in Table 14. pH figures refer only to the initial pH of the

Table 14. Assessment of the ligninolytic ability of selected fungi using the Bavendamm test.

| Fungus | pH | Observations |
|---|---|---|
| Coprinus cinereus | 5.8 | Moderate darkening of medium } by day 5 |
| | 6.3 | Good darkening of medium } by day 5 |
| | 6.9 | Very slight darkening of medium } by day 5 |
| Chaetomium globosum | 5.8 | Moderate darkening of medium } By day 7 |
| | 6.3 | " " " " } By day 7 |
| Chaetomium cochlioides | 4.4-9.5 | No reaction |
| Cephalosporium sp. | 4.4-9.5 | No reaction |
| Fusarium culmorum | 4.4 | Very slight darkening of medium |
| Gliocladium roseum | 4.4-9.5 | No reaction |
| Trichoderma viride | 6.3 | Slight, but definite, darkening of medium |
| Aspergillus fumigatus | 4.4-9.5 | No reaction |

media.

The darkening caused by Fusarium culmorum was so slight that it may not be caused by the production of polyphenol oxidases, but that caused by Trichoderma viride, however, was quite definite, and indeed, this fungus has been reported by Aytoun (1953) to be capable of limited lignin decomposition. Cephalosporium chrysogenum has been reported to be ligninolytic (Pisano, 1970) but the species studied here produced no reaction at all. Chaetomium globosum produced definite darkening at pHs 5.8 and 6.3; a positive response by this fungus has also been reported by Sundman and Näse (1971) and Haider and Domsch (1969) found that C. piluliferum appeared to be highly ligninolytic. There has been some debate and doubt, however, over the ligninolytic ability of C. globosum (Savory and Pinion, 1958).

Coprinus cinereus produced a much deeper reaction than C. globosum, at around the same pH range; Fries (1955) has reported that Coprinus fimetarius and several other Coprinus spp. are capable of decomposing lignin.

This experiment indicates that Coprinus cinereus and Chaetomium globosum are probably both capable of producing producing polyphenol oxidases and perhaps also T. viride, but all at acid pHs only. These fungi may therefore be capable of some lignin decomposition under suitable physical conditions.

## 3.7 WEIGHT LOSS OF STRAW INDUCED BY SELECTED FUNGI

The ability of each of the eight fungi to break down straw was assessed by growing each in pure culture on straw and determining the weight loss—due primarily to respiration of reduced carbon—brought about. The ability to do this may be influenced by the presence of other colonising fungi; such influences will, of course, not be detected in this experiment.

### 3.7.1 Experimental procedure

Approximately 10g samples were dried for 18hrs at 80°C. and weighed. Sufficient nutrient solution, containing 1.0g/l $KH_2PO_4$, 0.5g/l KCl, 0.2g/l $MgSO_4$, 0.1g/l $CaCl_2$ and 15.7g/l $(NH_4)_2SO_4$, was added to each sample to provide a moisture content of 300% of the dry weight of the straw and 1.0% nitrogen in addition to that already present. The vessels were loosely capped, autoclaved at 10 lbs/in$^2$ for 20 minutes and each inoculated with five infected 1cm$^2$ plugs of Eggins and Pugh cellulose agar. It is recognised that autoclaving is likely to cause some chemical change in the straw, affecting its suseptibility to attack. The level of replication was three. All the vessels, including three sterile controls, were incubated at 25°C. in the dark for 28 days and dried for 48hrs at 80°C. before being reweighed.

### 3.7.2 Results

Percentage weight losses of the colonised straw, corrected for the weight loss of the controls, are presented in Table 15. All figures are averages of the three replicates.

The experiment shows that all the fungi are capable of

Table 15. Percentage weight losses of autoclaved straw colonised by pure cultures of selected fungi after 28 days incubation at 25°C.

| Fungus | Percentage weight loss*† | Fungus | Percentage weight loss*† |
|---|---|---|---|
| Coprinus cinereus | 18.4 | Fusarium culmorum | 17.9 |
| Chaetomium globosum | 14.4 | Trichoderma viride | 9.9 |
| Chaetomium cochlioides | 9.8 | Gliocladium roseum | 12.8 |
| Cephalosporium sp. | 12.3 | Aspergillus fumigatus | 11.2 |

*Corrected for weight loss of sterile controls.

†Average of three replicates.

breaking down straw, Coprinus cinereus and Fusarium culmorum being rather better than the others. How much effect autoclaving the straw had on its resistance to attack is not known. It has little effect on the digestibility of straw (Hartley et al, 1974), a parameter related to the availability of straw to microorganisms in the rumen of ruminants and so it is possible that it has little effect on resistance to attack by fungi.

# CHAPTER 4

# COLONY INTERACTION STUDIES

## 4.1 INTRODUCTION

IT WAS pointed out in chapter 1 that among the factors which contribute to the competitive success of a fungus colonising a substrate, its antagonistic ability in the conditions prevailing may be of great importance when other invaders are present or when the substrate has already been colonised by other microorganisms. As part of the programme of work investigating competition between fungi colonising straw, studies were carried out to see whether some of the more prevalent fungi found in straw possess antagonistic abilities, by observing interactions between pairs of fungi growing *in vitro*. Competitive success of fungi paired against each other and the ability of *Coprinus cinereus* to produce volatile and non-volatile antibiotic factors were investigated.

Interactions between microorganisms have been studied by a number of workers over the past few decades and techniques developed by some of them have been used in these studies.

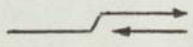
## 4.2 PAIRING OF SELECTED FUNGI ON AGAR. I. PRELIMINARY STUDY.

In this study, twelve of the most commonly occurring fungi isolated from straw were paired against each other on agar. Although such an *in vitro* study has limitations as a model for observing occurrences between fungi in a habitat such as straw, since it is nutritionally, physically and ecologically different, it has been widely used in the study of antagonistic ability (Zeller and Schmitz, 1919; Porter, 1924; Lai and Bruehl, 1968; Eckstein and Liese, 1970) and has been a very useful means of detecting antagonistic potential.

There are several types of interactions which may occur between fungi sharing an agar plate. Before colonies meet, volatile or diffusing non-volatile metabolites of one may affect the other; after meeting, competition for nutrients and space begins, when fungi able to quickly absorb and utilise nutrients may be favoured. Synergistic as well as antagonistic factors may be present in these interactions. During mingling of the colonies, antibiotic effects over short distances, parasitism and possibly "hyphal interference" (Ikeduigwu and Webster, 1970a) may also play a part in determining the relative position of colonies on the plates.

Porter (1924) has created the following categories and corresponding symbols to represent the various types of colony interaction he observed:


Mutually intermingling


Overgrowth of one organism by another

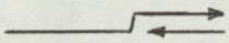
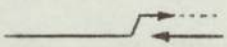

Mutual inhibition at a short distance


Mutual inhibition at a considerable distance

Skidmore and Dickinson (1976) used a partly quantified system based on Porter's categories. Other types of representation have also been used (Zeller and Schmitz, 1919; Eckstein and Liese, 1970; Fokkema, 1973).

The categories and symbols used in this experiment, based

Table 16. Categories and symbols of types of interaction between fungi, as used in this study

| | Symbol | Type of interaction |
|---|---|---|
| 1 |  | Mutual intermingling. Position of arrows indicates extent. |
| 2 |  | Overgrowth of one fungus by another. Position of arrows indicates position and degree of overlap. |
| 3 |  | Overgrowth of one fungus by another; dotted line indicates very sparse growth. |
| 4 |  | Complete inhibition of growth of both colonies. Position of arrows indicates position and distance between colonies. |

on Porter's with some modifications, are shown in Table 16. Interactions on the borders of two categories were common. The overlap of category 1 with categories 2 and 3 made it difficult to accurately present occasional interactions observed; unless one colony was quite clearly overgrowing another, the interaction was represented as mutual intermingling.

### 4.2.1 Experimental procedure

The twelve fungi used in this study, all isolated from the straw pads studied earlier, are shown in Figure 7. These fungi were paired against each other by inoculating pairs together with agar plugs, taken from actively growing colonies, placed onto opposite edges of Eggins and Pugh cellulose agar plates. The plates were prepared in duplicate and incubated at 25°C. in the dark. Observations on the progress of the fungi were made at frequent intervals and colony front positions and any noteworthy occurrences recorded.

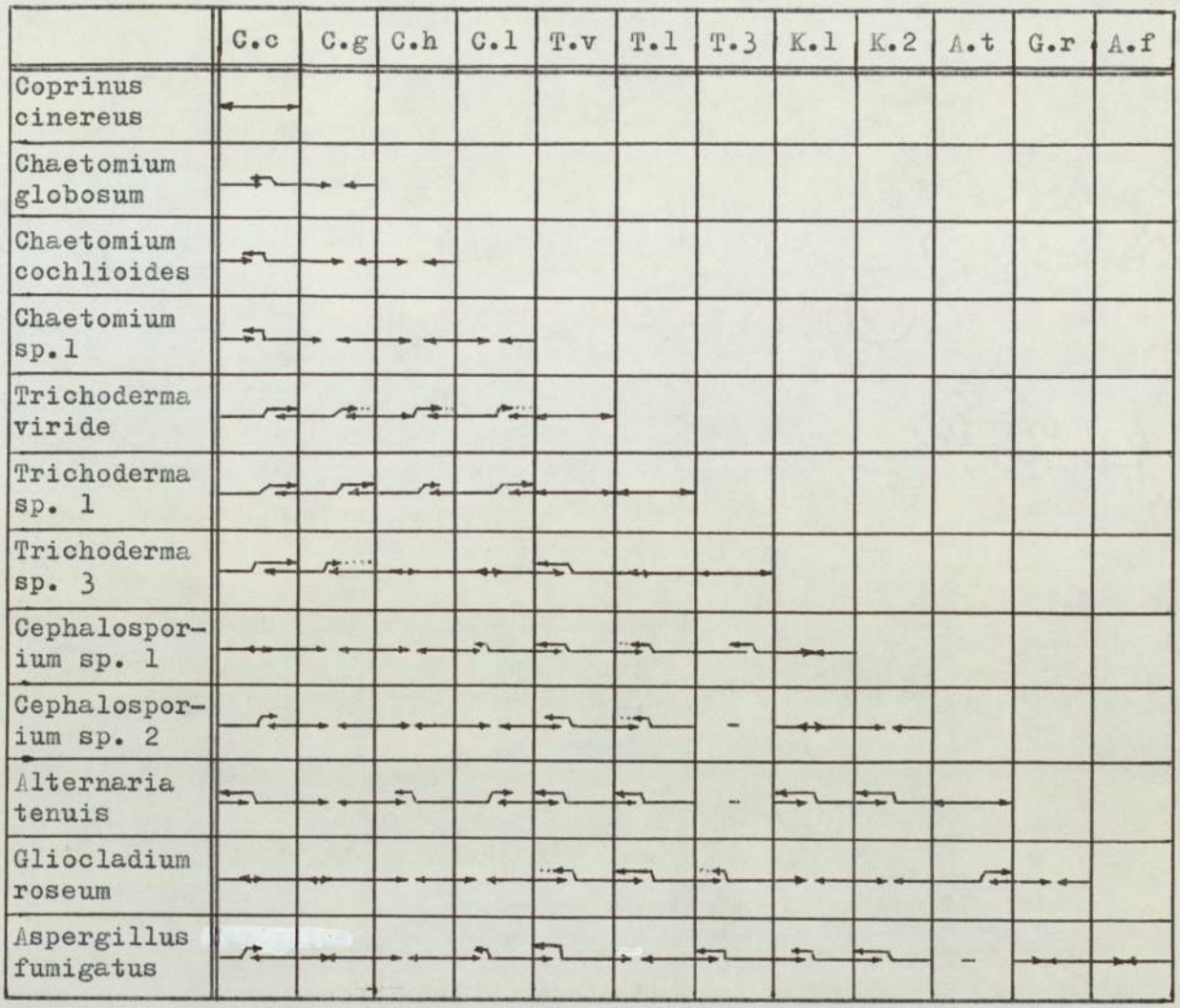



Figure 7. Interactions between fungi paired on cellulose agar at 25°C. Arrows originating from left refer to fungi listed vertically. See text for explanation of symbols.

4.2.2 Results

Figure 7 shows the observed patterns of colony interaction. Each box represents a vertical cross-section through the agar cut at the inoculation points, taken when the growth of both fungi had ceased. The arrows represent the direction and extent of growth of a fungus across the plate.

It can be seen that in the vast majority of cases, both of the paired fungi were halted, either before, at or after contact between the colonies had occurred. In several instances, however, complete overgrowth of one fungus by another was observed. Mutual inter-

mingling of colonies, with no clear domination of one by another, was also occasionally observed; this was usually accompanied by some inhibition of growth.

The Trichoderma spp. were by far the most successful at overgrowing the fungi paired with them, though the Chaetomium spp. gave some resistance. It is thus clear that the Trichoderma spp. are highly competitively successful under the conditions of this experiment. Coprinus cinereus was able to overgrow, either partially or completely, four other fungi, Cephalosporium sp. 2 three others, Cephalosporium sp. 1 two others and the rest one other or none at all.

Regarding indications of poor competitive ability, Alternaria tenuis was either partially or completely overgrown by five fungi, Aspergillus fumigatus three and the others two or less, in all cases excluding the Trichoderma spp. Cephalosporium sp. 2 and Gliocladium roseum were overgrown only by Trichoderma spp.

It is difficult to say from the observations of this much
experiment how/competitive successes are due to antibiosis or parasitism and how much due to competition for residual nutrients in colonised agar and tolerance to excreted metabolites.

Aggressive abilities are possibly operating in the occurrence of mutual inhibition before or at contact between colonies. The Chaetomium spp., especially C. globosum, and G. roseum frequently showed mutual inhibition of this type and also on occasions Cephalosporium spp.

Both temperature and pH were excluded as variables in this experiment, but it is probable that the observed antagonism is very much dependent upon both (DeVay, 1956). The results only indicate competitive success in the conditions of this experiment, which differ in several ways from a complex habitat such as straw, and also indicates competitive success against the other chosen fungi, not against other

fungi in general.

## 4.3 PAIRING OF SELECTED FUNGI ON AGAR. II. A pH STUDY.

As was stated in the previous section, the antagonistic ability of a fungus may very much depend upon the pH of its substrate. In this experiment, Coprinus cinereus, which shows promise in being able to maintain dominance in non-sterile alkaline straw (see section 2.4), was paired against the seven other selected fungi on agar at six different pHs. Several of these other fungi were shown to possess some antagonistic potential in the last described experiment.

### 4.3.1 Experimental procedure

The fungi were inoculated in pairs onto opposite edges of Eggins and Pugh cellulose agar so that C. cinereus was paired with each of the other seven fungi, at a replication level of three. The agar was buffered to six pHs (4.0, 5.3, 5.9, 6.5, 7.7 and 8.7), using Sorensen's buffer in the manner described in section 3.4.1. Inoculation times were adjusted to take into account relative growth rates of the fungi, in order to allow both fungi of a pair to become well established before they met. The plates were incubated at 25°C in the dark. Observations were made at frequent intervals and colony front positions and any noteworthy occurrences recorded.

### 4.3.2 Results

The types of interaction observed are shown in Figure 8, using the same system of representation employed previously. Photographs of the interactions eight days after inoculation with C. cinereus are also given (Figure 9).

There was a general trend towards increasing antagonistic ability on the part of C. cinereus the higher the pH, except against

| Fungus \ pH | 4.0 | 5.3 | 5.9 | 6.5 | 7.7 | 8.7 |
|---|---|---|---|---|---|---|
| Chaetomium globosum | | | | | | |
| Chaetomium cochlioides | | | | | | |
| Cephalosporium sp. | | | | | | |
| Fusarium culmorum | | | | | | |
| Trichoderma viride | | | | | | |
| Gliocladium roseum | | | | | | |
| Aspergillus fumigatus | | | | | | |

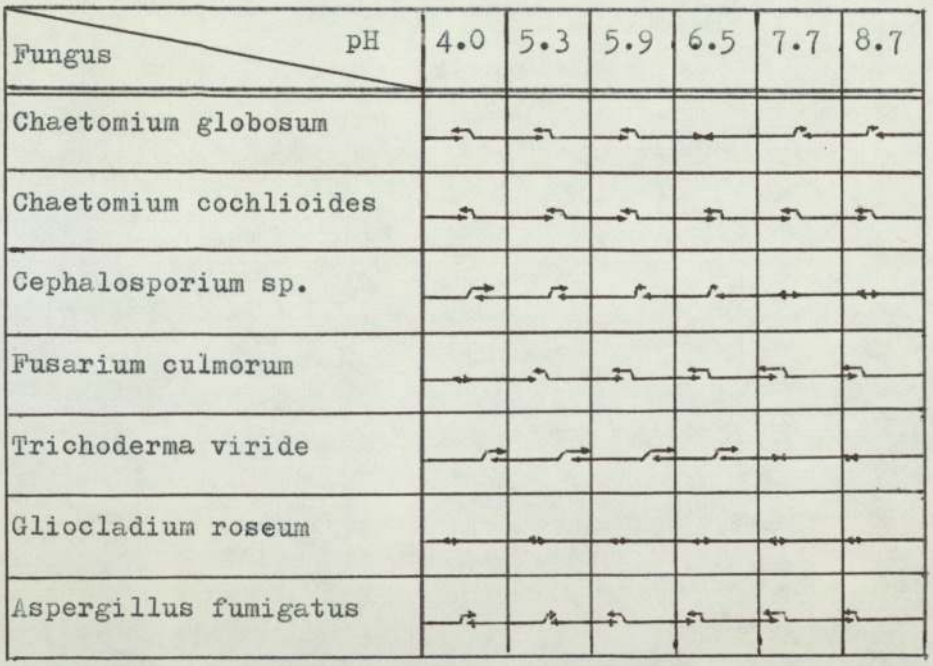


Figure 8. Interactions between Coprinus cinereus and seven fungi paired against it on cellulose agar at 25°C., buffered at six pHs. For key to symbols, see Table 16. Arrows originating from left refer to fungi listed vertically.

Chaetomium cochlioides and Gliocladium roseum, where interaction was largely independent of pH, and Chaetomium globosum, where the antagonistic ability of C. cinereus was greatest at pH 6.5; at pHs 7.7 and 8.7 there was slight overgrowth by C. globosum. Against Cephalosporium sp., C. cinereus was overgrown at acid pHs, but not at alkaline pHs. Fusarium culmorum was completely overgrown at alkaline pHs and partially so at acid pHs. C. cinereus inhibited Trichoderma viride at alkaline pHs, when the latter was very much retarded in its growth rate by the pH of the medium, though even then, it still completely inhibited the growth of C. cinereus. Aspergillus fumigatus slightly overgrew C. cinereus at pHs 4.0 and 5.3; otherwise, it was overgrown by C. cinereus.

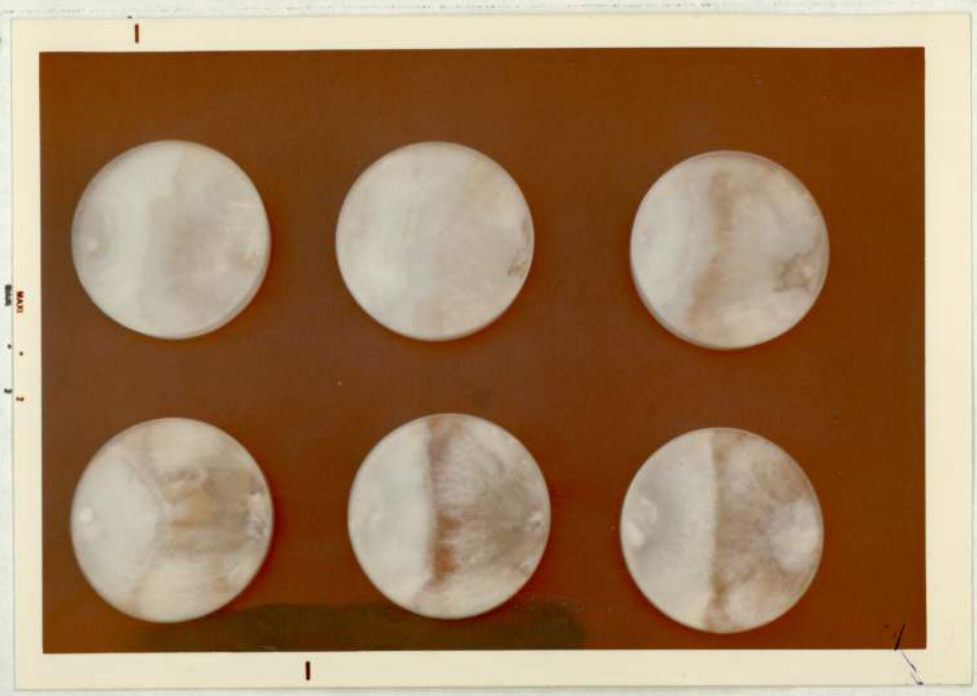
The efficiency of fungi at assimilating and utilising nutrients in the agar will alter with pH and of course the speed of assimilation

will be a function of hyphal extension, itself dependent upon pH. Since the growth rate and competitive success of C. cinereus increased together with pH, nutrient competition appears likely to be the mechanism operating. This view is strengthened by the observation that the competitive success of C. cinereus was not improved at higher pHs when paired against those fungi that also increased their growth rate as pH rose.

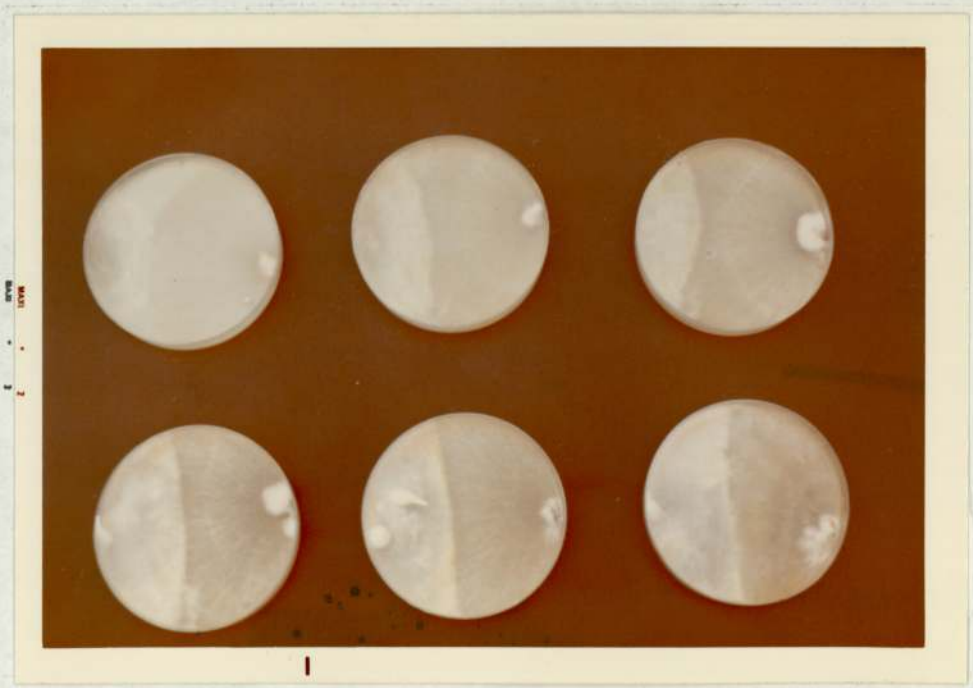
Other factors which may play a part in the change in interactions with pH are the changes in type, quantity and stability of metabolites excreted by the fungi. Gliotoxin, for example, is produced only at acid pHs and becomes inactivated at alkaline pHs (Bilai, 1963).

C. cinereus v. Chaetomium globosum

Figure 9. Fungi interacting on cellulose agar at six pHs. Initial pHs of the agars are: top row, left to right, 4.0, 5.3, 5.9; bottom row, left to right, 6.5, 7.7, 8.7. In all cases, C. cinereus is inoculated on the left of the plates. Photographs were taken 8 days after inoculation with C. cinereus.



C. cinereus v. Chaetomium cochlioides



C. cinereus v. Cephalosporium sp.

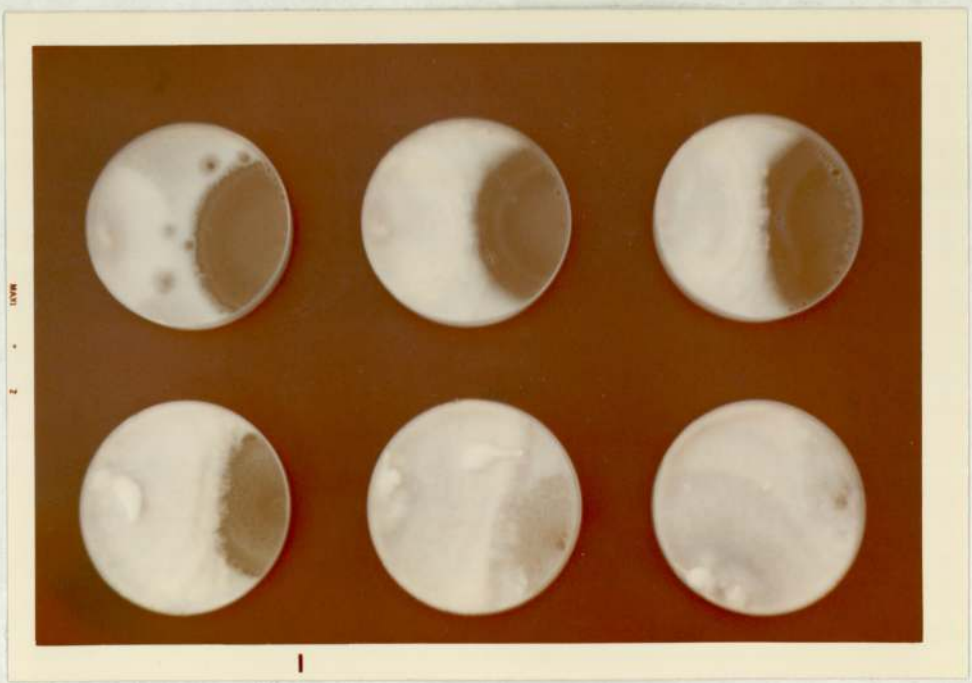
Figure 9. (cont.)

C.cinereus v. Fusarium culmorum

C. cinereus v. Trichoderma viride

Figure 9. (cont.)

C. cinereus v. Gliocladium roseum



C. cinereus v. Aspergillus fumigatus

Figure 9. (cont.)

## 4.4 AN INVESTIGATION INTO THE PRODUCTION BY COPRINUS CINEREUS OF NON-VOLATILE INHIBITORY METABOLITES

The work described earlier in this chapter indicates that Coprinus cinereus possesses some antagonistic ability, though it does not show whether this is due to simple success in competing for nutrients in the agar, to the excretion of inhibitory metabolites or to some mechanism involving direct hyphal contact.

In this section and the next, experiments attempting to determine whether C. cinereus produces toxic metabolites are described and chapter 6 is devoted to an investigation of its hyphal interaction with other fungi.

The experiment described here is designed to detect the production by C. cinereus of non-volatile inhibitors effective against the seven other selected fungi. A technique developed by Gibbs (1967 ), in which Cellophane is used to separate metabolites deposited in the agar from the fungus producing them, was used in this study.

### 4.4.1 Experimental procedure

Plates of Eggins and Pugh cellulose agar, each containing 20mls of medium, were prepared and left for three days to allow excess moisture to evaporate. The surface of the agar in each plate was then covered with a disc of Cellophane (PT300 grade), previously boiled for half an hour to remove plasticizers, and the plates then left for 24hrs, again to allow surface moisture to evaporate. Half of the plates thus prepared were inoculated centrally with 10mm diameter plugs of C. cinereus cultures taken from the margin of actively growing colonies, the other half being left uninoculated as controls. Preliminary trials with the technique showed that after a period of around 40hr, the fungus, having penetrated the Cellophane,

began to colonise the underlying agar, so after 40hrs had elapsed, the Cellophane sheets on all plates were removed, together with the cultures of C. cinereus. By this time, the average diameter of the colonies was 46mm. The plates were incubated at 25°C. for twenty-four hours and examined. Those plates which were seen to have become colonised were discarded and sufficient of the remainder were inoculated with the eight fungi (one being C. cinereus) used in previous experiments, five of each onto plates pretreated with C. cinereus and five of each onto untreated plates. All plates were incubated at 25°C. in the dark and measurements of colony diameter made daily.

4.4.2 Results

Plots of the growth of the colonies versus time, averaged from the five replicates, are shown in Figure 10. In most cases the growth rates of the controls were fairly consistently slightly higher

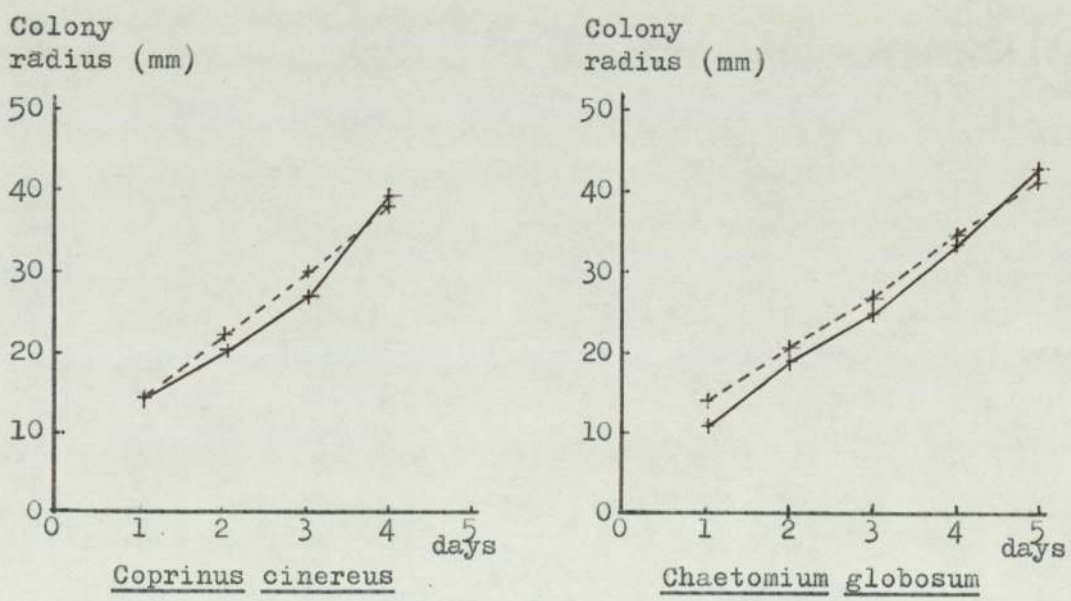



Figure 10. Growth of fungi on cellulose agar in the presence of non-volatile metabolites of C. cinereus, at pH 5.5 and 25°C. (--------=growth of control colonies, ————=growth of test colonies.)

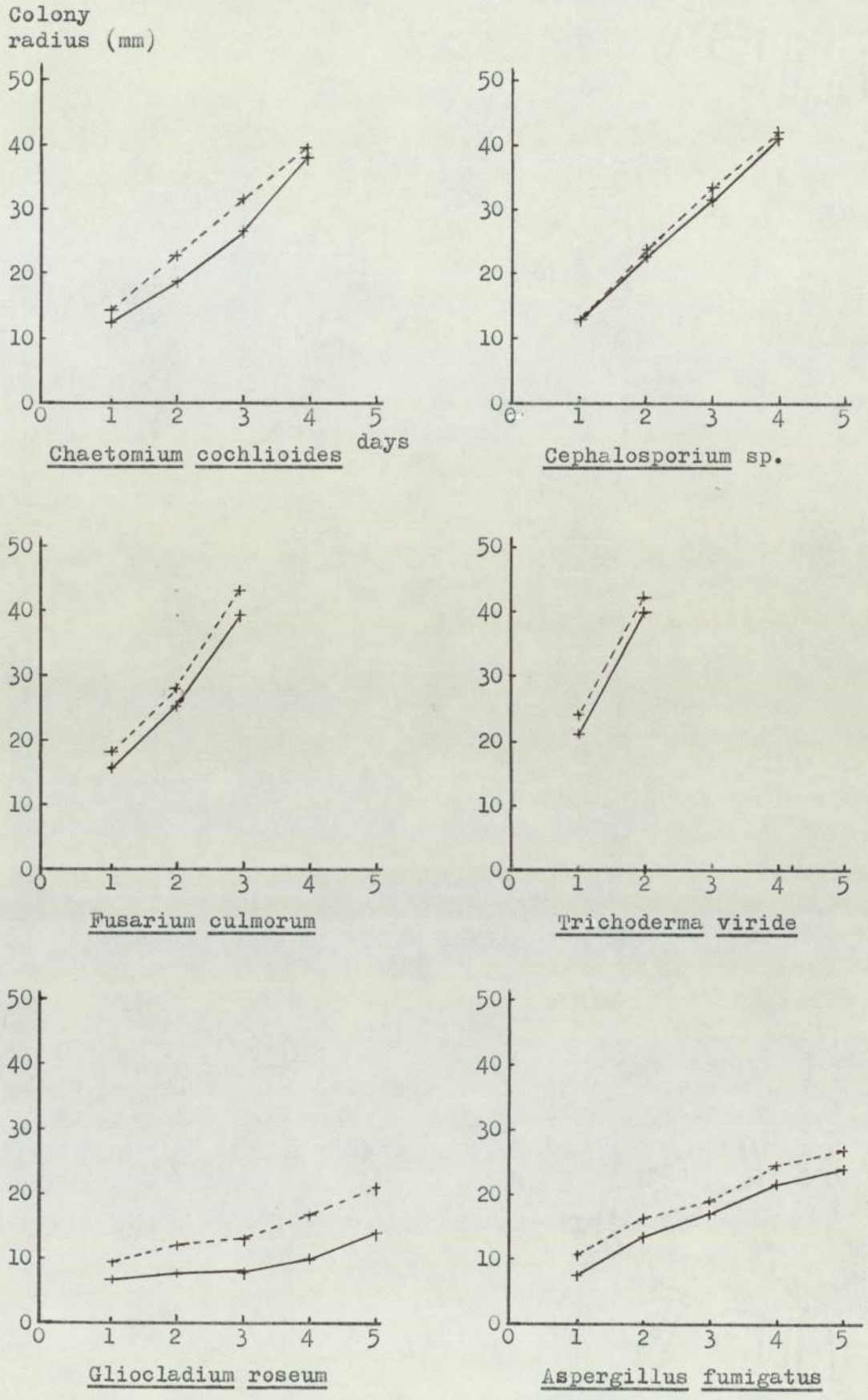



Figure 10. (cont.)

than growth rates of the test colonies growing on agar previously exposed to C. cinereus, but apparently only until the colony diameter had reached the same as that of the C. cinereus colony before it, after which rates tended to increase, to give overall rates about the same as the controls. This depression of growth rates may be due to reduction by C. cinereus of nutrient levels in the medium or to the deposition of one or more metabolites with mildly inhibitory qualities. Presumably the marked effest on Gliocladium roseum is due to its particular sensitivity to one of these mechanisms. Certainly no powerful, stable non-volatile antibiotic is being deposited in the agar, though of course it is possible that a non-volatile toxin is produced by C. cinereus at some pH other than 5.5 or at a different temperature to 25°C. These were major limitations of this experiment; it would certainly be of value to repeat it, with pH and temperature as variables.

## 4.5 AN INVESTIGATION INTO THE PRODUCTION BY COPRINUS CINEREUS OF VOLATILE INHIBITORY METABOLITES

It was mentioned in chapter 1 that many fungi are able to produce volatile toxic metabolites; the experiment described in this section is designed to determine whether any such substances are produced by Coprinus cinereus that are effective against the fungi under study. The technique used is based on that devised by Dick and Hutchinson (1966). The lid of a plate of an actively growing colony (the "assay" fungus) is replaced with an upturned plate of another fungal colony (the "test" fungus) and the linear growth rate of the former is compared with that of a control.

### 4.5.1 Experimental procedure

Plates of Eggins and Pugh cellulose agar were inoculated

centrally with 10mm diameter plugs of C. cinereus cultures taken from the margin of colonies actively growing on the same agar. After incubation of these for three days, more cellulose agar plates were inoculated centrally with the eight test fungi (the same fungi as used in previous experiments) and the lids of half replaced with the upturned three-day old cultures of C. cinereus. the lids of the other half were replaced with upturned plates of sterile cellulose agar, as controls. Each assembly was held together with Sellotape (Figure 11). Five replicates were prepared. All incubations were at 25°C.

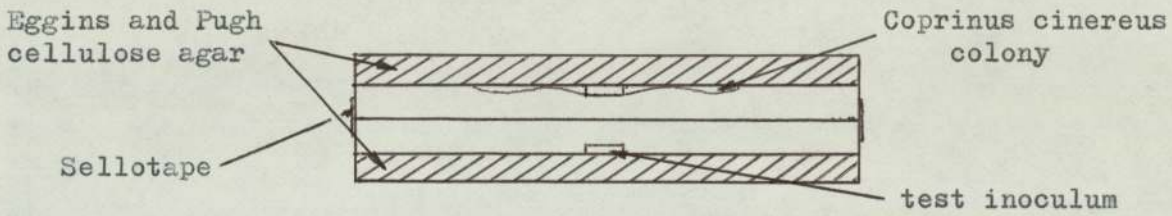



Figure 11. A section through a volatile test assembly.

in the dark. When the assay fungus had colonised most of the agar the assembly was dismantled and colony diameters measured.

4.5.2 Results

The diameter of the test and control colonies at the time of dismantling are given in Table 17. It can be seen that in most cases there is no significant difference between test and control colonies. There was quite a definite difference, however, with Trichoderma viride and Fusarium culmorum and possibly also Aspergillus fumigatus. Some morphological differences were also apparent. Growth and sporulation of T. viride and F. culmorum were much sparser and there was more hyphal branching in the presence of C. cinereus than in its absence. This effect may be due to the

Table 17. Diameter of fungal colonies with and without exposure, at a distance, to a colony of Coprinus cinereus.

Figures are averages of five replicates.

| Fungus | Test | Control | Fungus | Test | Control |
|---|---|---|---|---|---|
| Coprinus cinereus | 60.6 | 61.0 | Fusarium culmorum | 57.6 | 63.2 |
| Chaetomium globosum | 68.6 | 69.4 | Trichoderma viride | 61.4 | 73.0 |
| Chaetomium cochlioides | 68.0 | 68.2 | Gliocladium roseum | 53.8 | 54.6 |
| Cephalo-sporium sp. | 71.0 | 72.2 | Aspergillus fumigatus | 59.4 | 62.8 |

All measurements are ±1.0 mm.

production by C. cinereus of either a very mild inhibitor or an antibiotic produced in very small quantities.

# CHAPTER 5

## INTERACTIONS BETWEEN FUNGI AND AZOTOBACTER spp.

### 5.1 INTRODUCTION

IN THIS chapter, experiments are described which were designed to assess the feasibility of growing a cellulolytic fungus in association with a free-living nitrogen-fixing bacterium. If such an association could be established in the commercial fermentation of straw to produce an animal feedstuff, it might be found unnecessary to add expensive industrially produced nitrogenous materials to encourage fungal growth. It is possible that nitrogen-fixing by bacteria occurs in wood at ground level (Sharp and Millbank, 1973), encouraging fungal colonisation of this other nitrogen-poor substrate. The selective effect of limited nitrogen availability may still operate in such an ecology, and this may help to control the colonisation of a non-sterile substrate by unwanted fungi.

## 5.2 GROWTH OF AZOTOBACTER spp. WITH SELECTED FUNGI ON AGAR

Preliminary experiments were first carried out to see whether fungi and Azotobacter spp. could be grown together on agar and whether the fungus could benefit from the presence of the bacterium on a nitrogen-poor medium.

### 5.2.1 Experimental details

The following bacteria and fungi were used: Azotobacter chroococcum, A. vinelandii, A. beijerinckii, A. paspali, Coprinus cinereus, Chaetomium globosum, Trichoderma viride, Gliocladium roseum, Paecilomyces varioti and Aspergillus niger. The Azotobacter spp. and the last two named fungi were obtained from the Torrey Research Station, Aberdeen and the Commonwealth Mycological Institute, respectively; the remaining fungi were isolates from barley straw and have been used in previous experiments. All the fungi are known to be cellulolytic.

The bacteria were first grown on Norris's medium (see Appendix 1), which contains no nitrogen, and Azotobacter chroococcum was then subcultured with each of the fungi, in pairs, onto plates of a modified Norris's medium in which the glucose was replaced with 1% ball-milled cellulose, as used in Eggins and Pugh cellulose agar. The plates were prepared in triplicate. It was hoped that enzymatic hydrolysis of the cellulose by fungal cellulases would release glucose for assimilation by A. chroococcum and the A. chroococcum would fix atmospheric nitrogen for utilisation by the fungus, thus allowing both microorganisms to grow, with mutual dependence on each other for nutrients. All incubations were at 27°C., the optimum temperature for growth of Azotobacter spp. (Norris, 1959). For comparison purposes, plates inoculated with only A. chroococcum and only a fungus were also prepared. Assessment of growth was by visual inspection only.

The following methods of inoculation were tried:

1) Streaks of A. chroococcum and fungal spores, meeting at one point.

2) A saline suspension of A. chroococcum cells added to the medium at 45°C., immediately prior to pouring and then, after solidification, inoculation with 5mm diam. plugs of fungal mycelium.

3) Inoculation with fungi as in 2, followed by incubation for a week and then the pouring of a thin layer of a saline solution of A. chroococcum cells onto the surface of the agar.

This last method gave the best results but, even so, both fungal and bacterial growth were very poor and sparse. It appeared that the low levels of nitrogen and available carbon prevented sufficient growth of either microorganism for them to begin adequate nutrient production themselves.

When 0.5g/l of cellulase (BDH) was incorporated in the agar at 45°C. immediately prior to pouring, inoculation after two day's incubation at 25°C. with a thin layer of a saline suspension of A. chroococcum cells led to good growth. No fungus was used in this experiment, but the result shows that the presence together of cellulose and cellulase allows good growth of A. chroococcum.

To enable both organisms to grow a little without mutual interdependence on nutrients, trials were carried out with small amounts of filter-sterilized glucose or ammonium sulphate added to the N-free cellulose agar. When 0.01g/l ammonium sulphate and 0.1g/l glucose were added together ("low-N cellulose agar") in several cases fungal growth was significantly better with Azotobacter spp. present than in its absence. In this experiment, all four Azotobacter spp. were separately paired with Coprinus cinereus, Chaetomium globosum and Trichoderma viride, the plates being inoculated as in Figure 12. The results are summarised in Table 18; the dots indicate the amount

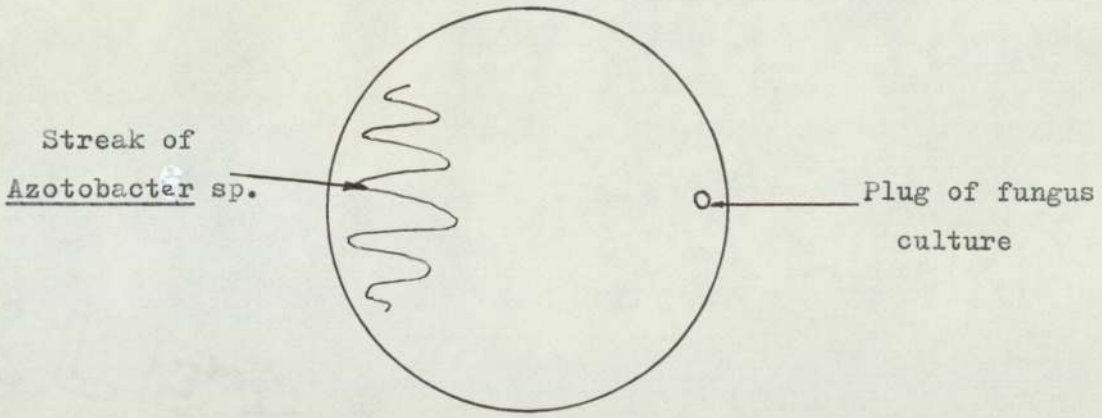



Figure 12. Method of inoculation of a modified Norris's medium containing 0.01g/l $(NH_4)_2SO_4$, 0.1g/l D-glucose and 1% ball-milled cellulose.

of mycelium produced on the surface of the agar (averaged from three replicates), as assessed visually; one dot represents very sparse growth and four dots growth similar to that produced on a complete medium. It can be seen that Coprinus cinereus faired better with all the Azotobacter spp. than on its own and in the presence of A. beijerinckii was particularly good; fruiting initials were also

Table 18. Fungal growth on a modified Norris's medium* in the presence of Azotobacter spp.

| | Coprinus cinereus | Chaetomium globosum | Trichoderma viride |
|---|---|---|---|
| A. chroococcum | • • • | • | • |
| A. beijerinckii | • • • • | • • | • |
| A. vinelandii | • • • | • • | • |
| A. paspali | • • | • | • |
| Control (no Azotobacter) | • | • | • |

*"low-N cellulose agar". See Appendix 1. for composition.

produced. Chaetomium globosum only grew better with A. beijerinckii and A. vinelandii than on its own, the main response being increased perithecial production. Trichoderma viride did not benefit from the presence of any Azotobacter spp.

These results show clearly that a nutritional association between a cellulolytic fungus and a nitrogen-fixing bacterium can be created in vitro with each organism providing metabolites essential for the growth of the other.

## 5.3 GROWTH OF AZOTOBACTER spp. WITH FUNGI ON STRAW

In this section, experiments investigating the growth of fungi and bacteria together on straw are described. Percentage weight loss of the straw is used as an index of fungal activity.

### 5.3.1 Growth of fungi with Azotobacter chroococcum

#### 5.3.1.1 Experimental procedure

Vessels containing about 10g of barley straw were dried at 103°C., weighed and sufficient of a one-tenth normal strength buffer solution of the same type as used in the experiment described in section 3.4, adjusted to pH 7, added to give a final moisture content of 250% of the dry weight of the straw. The vessels were covered loosely and autoclaved at 15 lbs/in$^2$ for 10 minutes. One set of vessels was inoculated with A. chroococcum first and a week later one of six fungi and a second set with the fungi first and a week later the bacterium. The fungi used are shown in Table 19. Fungi were inoculated by placing 5mm$^2$ pieces of infected Eggins and Pugh cellulose agar in scattered positions in the straw; A. chroococcum was inoculated by the addition of 10ml of saline suspension of cells to each jar, which was then shaken to disperse the inoculum. Inoculations of bacterium and each of the fungi alone, and sterile controls, were also set up. Each

regime was set up in triplicate. After 25 days incubation at 27°C. in the dark (32 days with those vessels inoculated with A. chroococcum one week before the fungus) the straw was dried at 103°C. for 48 hrs and reweighed.

5.3.1.2 Results

Percentage weight losses of straw are presented in Table 19. The average weight loss brought about by A. chroococcum alone was 0.75% and the average weight loss of sterile controls was zero.

Table 19. Percentage weight loss of straw colonised by mixed cultures of Azotobacter chroococcum and each of six fungi.

| Fungus | Percentage weight losses† | | |
|---|---|---|---|
| | Fungus alone | Fungus, then A. chroococcum* | A. chroococcum,* then fungus |
| Coprinus cinereus | 16.1 | 18.5 | 2.8 |
| Chaetomium globosum | 16.4 | 18.6 | 4.1 |
| Gliocladium roseum | 14.7 | 18.3 | 3.8 |
| Trichoderma viride | 10.0 | 12.0 | 2.7 |
| Paecilomyces varioti | 17.0 | 16.7 | 1.9 |
| Aspergillus niger | 14.6 | 10.0 | 3.6 |

*One-week gap between inoculations.

†Average of three replicates.

The results clearly show that the presence of Azotobacter chroococcum when the fungus was inoculated caused fungal growth to be greatly inhibited. When the inoculation sequence was reversed, a synergistic response occurred with all the fungi except Paecilomyces varioti and Aspergillus niger, straw weight losses being greater in the presence of both microorganisms together than with either alone.

5.3.2 Growth of fungi with four Azotobacter spp.

In this experiment, the fungi used were Coprinus cinereus and Chaetomium globosum; both gave good weight losses of straw with A. chroococcum. They were paired against each of four Azotobacter spp. to see which association would lead to the greatest weight loss.

5.3.2.1 Experimental procedure

The experimental procedure was identical to that adopted in the last experiment, except that this time incubation was terminated 21 days after inoculation with the fungus, instead of 25 days. Since inoculation of A. chroococcum before the fungus has been shown to have an inhibitory effect of microbial attack of the straw, this regime was omitted here. Controls of Azotobacter sp. alone were incubated for 14 days.

5.3.2.2 Results

Percentage weight losses are presented in Table 20. The weight losses are all lower than those obtained in the previous experiment, presumably at least partly due to the shorter incubation period of this experiment. Why C. cinereus produced a lower weight loss with A. chroococcum as well as the other Azotobacter spp than on its own is not known.

Table 20 Percentage weight losses of straw colonised by mixed cultures of one of four Azotobacter spp. and either Coprinus cinereus or Chaetomium globosum.

| | A. chroococcum | A. vinelandii | A. beijerinckii | A. paspali | No bacterium |
|---|---|---|---|---|---|
| Coprinus cinereus | 11.8 | 11.9 | 10.7 | 10.1 | 14.0 |
| Chaetomium globosum | 13.3 | 13.4 | 12.6 | 12.5 | 11.0 |
| No fungus | 3.7 | -0.3 | 2.2 | 0.3 | - |

Percentage weight losses are averages of three replicates and have been corrected by subtracting the weight loss of sterile controls.

In the previous experiment, a synergistic effect between C. cinereus and A. chroococcum was produced. Inoculum levels of the Azotobacter spp. were not carefully controlled in either this or the last experiment and this may be the cause of the low weight loss due to C. cinereus and Azotobacter spp. in this experiment. It could also explain the high weight loss produced by A. chroococcum on its own (3.7%) in this experiment compared with that produced in the last (0.75%). The experiments need repeating with controlled quantities of Azotobacter spp. and possibly a longer incubation period, before definite conclusions may be drawn. However, this experiment and the last indicate that some mutually supportive association can exist between Chaetomium globosum and all four Azotobacter spp. and also between several other fungi and A. chroococcum, provided that the bacterium is not allowed to become established on the substrate before the fungus. Certainly further work, including microscopic examination of interactions between the fungi and Azotobacter spp., would be worthwhile.

# CHAPTER 6

# MICROSCOPICAL STUDIES OF HYPHAL INTERACTION

## 6.1 INTRODUCTION

MICROSCOPICAL STUDIES of interactions between fungi have been carried out by several workers. Techniques have been developed by them and contributions to an understanding of the mechanisms of antagonistic interactions have been made. In this chapter, the development and use of techniques to study microscopically the mechanisms of antagonism between selected fungi from straw, particularly in relation to Coprinus cinereus, are described. It is possible, using such studies, to determine whether antagonistic effects are exerted over a distance or only on contact with opposing hyphae, and also what type of impairment is caused to the victim's hyphae.

### 6.1.1 Experimental procedure

The experiments described here were designed to enable hyphal interaction between fungi to be monitored, using the same nutrient regime as in previous studies, ie., Eggins and Pugh cellulose

agar. Direct examination of fungi in a Petri dish was found to be impractical, primarily because contamination is difficult to avoid and the opacity of the agar used causes the illuminating beam of light to be scattered and severely reduces image clarity.

Trials were first carried out to develop a culturing system enabling continuous monitoring of hyphal interaction. Pieces of Eggins and Pugh cellulose agar cut from plates were placed on sterile slides, inoculated with two fungi and a coverslip placed on the surface of the agar. In this way, fungi were able to colonise the underside of the coverslip in places where no agar was present to scatter the light beam, using the pieces of agar as a nutrient base. The assembly was placed on a bent glass rod in a Petri dish; beneath the rest were several pieces of paper towelling moistened with sterile water. Figure 13 shows a completed assembly. The photograph shows one of several designs tried, the more successful of which are shown in Figure 14.

Figure 13. Completed slide culture assembly for microscopic examination of hyphal interaction, with lid removed.

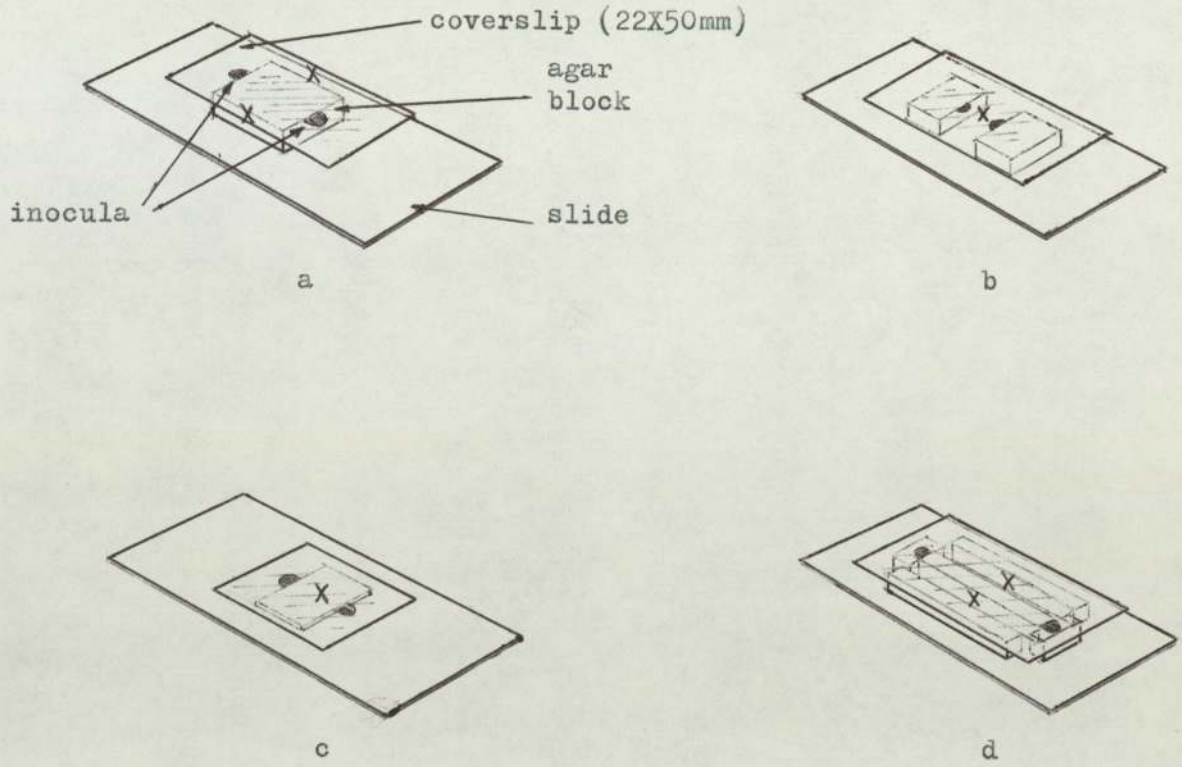



Figure 14. Slide culture assemblies used in the observation of hyphal interaction. X = area of interaction.

Growth between the agar block and the coverslip was slow and sparse, presumably due to low oxygen tension. While the assembly in Figure 14a was easy to prepare, the length of the interacting fronts was rather short and contamination was common; the arrangement of Figure 14b gave longer interacting fronts and less contamination but the fronts were very uneven, making clear observation of interactions often rather difficult. The arrangement of Figure 14c generally gave sparse, very slow growth and, although it was possible to observe hyphae using this technique, images were not really sharp enough for satisfactory photographs to be taken. Since the special conditions of low oxygen tension may severely affect antagonistic ability, the technique was not used in comparative studies. The configuration of Figure 14d overcame the the problem of contamination and yielded many interesting observations;

the area of interaction was relatively large, but condensation on the ungerside of the coverslip, a problem with all the slide culture arrangements, again often obscured vision and may also have contributed to the uneveness of the hyphal fronts.

The use of boiled and autoclaved Cellophane (grade PT300), 20 X 40mm, placed immediately beneath the coverslip, was also tried; in this way, interaction between fungi growing on a nutrient medium containing cellulose (it was assumed that soluble nutrients would diffuse from the agar along the moist Cellophane) could be observed. Colony fronts were extremely uneven, causing difficulties in observing interactions and, as the technique always gave rise to high levels of contamination, it eventually had to be abandoned.

Microscopic observations of interactions were also made using modifications of a technique devised by Tribe (1966). Pieces of boiled and autoclaved Cellophane were placed on the surface of agar in a Petri dish and the agar inoculated with two fungi so that the colonies met on the Cellophane, which was then removed, placed on a slide and examined. In preliminary trials, six pieces of Cellophane, 10 x 20mm, were placed in a circular arrangement, but it was found better to use the arrangement shown in Figure 15, the pieces of Cellophane being 20 x 40mm. It was usually possible to observe a continuum of

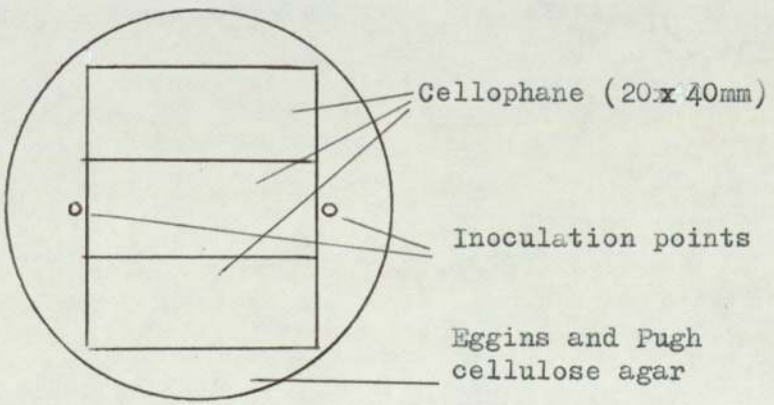



Figure 15. Arrangement of Cellophane on agar for microscopic examination of hyphal interactions.

progress in interaction by examining the length of a colony front, at one end of which the colonies were still separate, while at the other end, well interpenetrated. Again, hyphal fronts were sometimes very uneven using this technique, but it provided a very much greater area for the examination of interactions and was used for many of the studies; most of the photographs taken were of these Cellophane cultures.

All interaction cultures were incubated at 25°C. in the dark. The same eight fungi used in previous work were used here, each being paired with Coprinus cinereus, at pHs 5.3, 5.5 (unmodified Eggins and Pugh agar), 5.9, 6.5, 7.7 and 8.7. Pairings of all the fungi with C. cinereus at all pHs were made using the cellophane technique (Figure 15) and a number of pairings at several pHs were made using slide culture techniques. Here, the main arrangement used was that of Figure 14d.

### 6.1.2 Results

The types of interaction observed fall broadly into the the following categories:

a). Inhibition of linear growth at a distance. This was apparent when a fungal colony, on being approached by its partner, lost its normal circular outline, often later becoming penetrated by the opposing fungus. Changes in the direction of growth of individual hyphae, some even growing back towards the parent colony, were occasionally seen. The effects are presumably due to the production of an antibiotic substance tolerated by the producer more than by its partner,

b). Changes in mycelial morphology, mainly increased branching or swelling of hyphae and bursting of hyphal tips, all of which may be due to changes in osmotic pressure differences across cell membranes (Robertson, 1958; Robinson and Park, 1966).

This may occur through alterations in the permeability of the membrance, caused directly or indirectly by the action of antibiotics (Gottlieb and Shaw, 1970) or through changes in tonicity in the vicinity of hyphae. The loss of turgor pressure following membrane damage or bursting of the cell causes adjacent septa to curve towards the damaged cell, indicating a pressure difference across the septa (Ikeduigwu and Webster, 1970a).

c). Changes in appearance of cell contents. Hyphae were often seen to lose their opacity or to become highly refractive. Increased vacuolation and granulation of the cytoplasm were also observed. Loss of opacity is associated with loss of turgor pressure and vacuolation may be associated with leakages from hyphae (Robinson and Park, 1966).

d). Direct interaction between hyphae. On numerous occasions, hyphae were seen entwining other hyphae and possibly even penetrating the cell wall, one hypha growing inside another. The hyphae of many fungi are known to respond positively to the presence of others by growing alongside or entwining them (Burnett, 1976); if damage is not caused and a nutritional exploitation not involved, the relationship is regarded as merely"fungicolous" (Barnett and Binder, 1973). However, a number of fungi appeared not only to contact and sometimes coil around the hyphae of others, but also to kill cells and utilise nutrients derived from them, in which case, a parasitic relationship is likely to be occurring.

It should be added, finally, that mutual intermingling of hyphae, without any apparent interaction at all was also commonly seen.

In the following sections, the types of interaction observed, if any, with each of the pairings made, is described.

6.1.2.1 Coprinus cinereus and Chaetomium globosum

Chaetomium globosum showed a clear aggressive ability against Coprinus cinereus, but only at pHs 6.5 and 7.7. At these pHs, a brown stain diffusing in advance of the colony front appeared to reduce the growth rate of C. cinereus.

Loss of opacity occurred in nearly all hyphal tip cells of C. cinereus (though not the tip itself) at distances of up to around 200μ from C. globosum hyphae, mainly at pH 6.5 (Figures 16 and 17) but also occasionally at pH 7.7, mostly only after contact (Figure 18). At pH 8.7, although no definite microscopic evidence of aggression was apparent, C. cinereus colonies were markedly appressed on being penetrated by the C. globosum colony; the antagonism may be simply due to competition for nutrients.

Loss of opacity presumably arises from the loss of refractivity of hyphae that occurs when turgor pressure is lost and the cell flattens. It has been observed in the hyphal interference exhibited by Coprinus heptemerus and also in some instances other coprophilous fungi (Ikeduigwu and Webster, 1970a). These workers also observed the retention of refractivity in the hyphal tip of an affected tip cell, thought to be due to the presence of vacuoles. They associated loss of opacity with death of the affected cell. Hedger and Hudson (1974) also report loss of opacity as an effect of antagonism.

At pHs 6.5 and 7.7 occasional cells of C. cinereus up to 5mm or so away from C. globosum became to varying degrees vacuolated, though this was not observed often. Several workers have observed this effect in connection with interacting fungi ( Aytoun, 1953; Dennis and Webster, 1971a). Production of vacuoles in cytoplasm is probably associated , in this context, with changes in cell membrane permeability and osmotic pressure within the cell.

C. cinereus hyphae were often seen being entwined by those

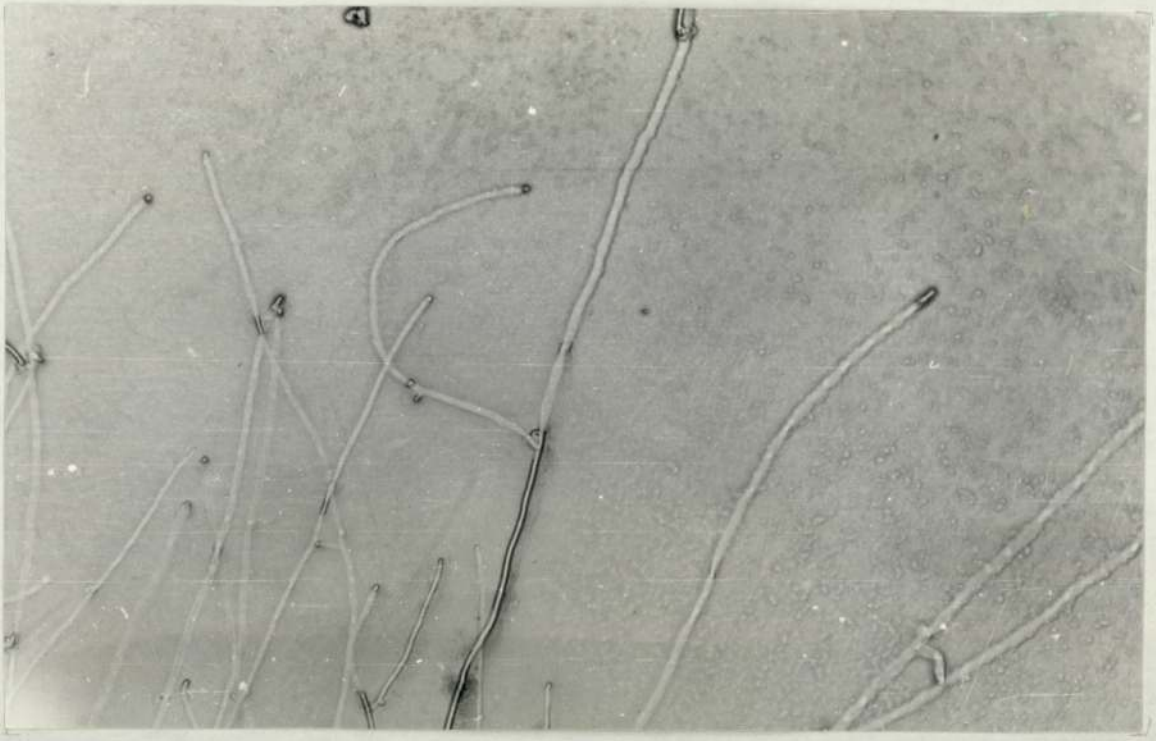


Figure 16. Loss of opacity of Coprinus cinereus (except at hyphal tips) at approach of Chaetomium globosum. pH 6.5. x200.

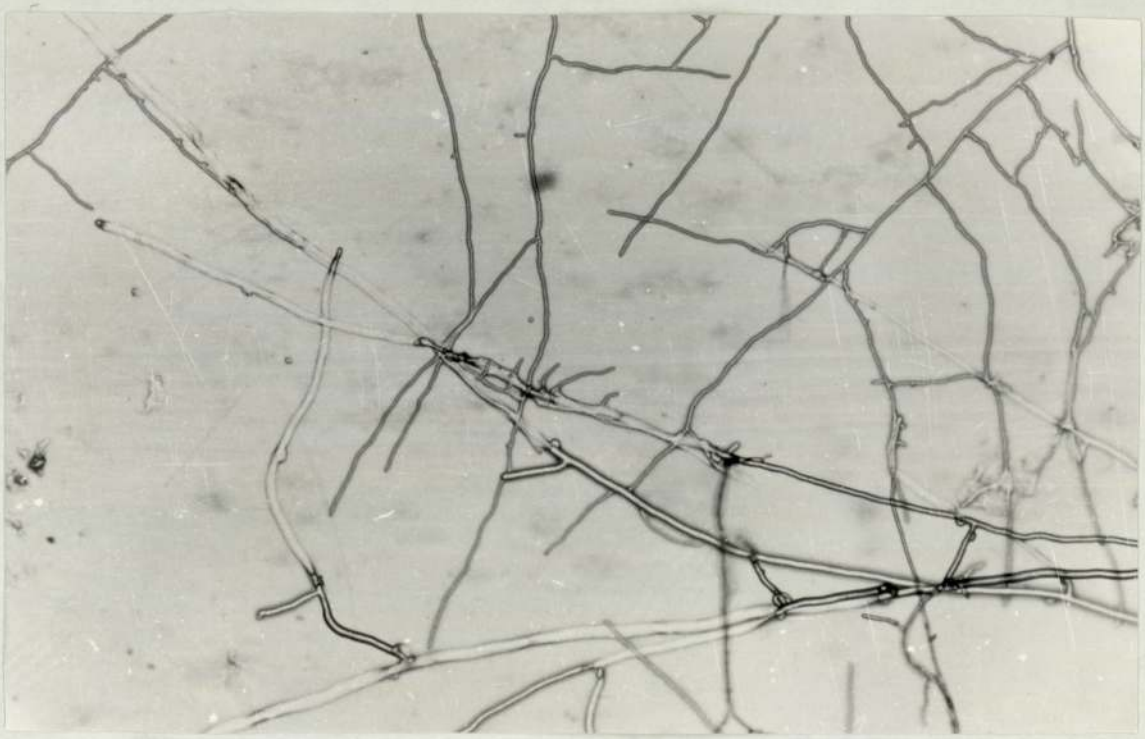


Figure 17. Loss of opacity, entwining and probably penetration of Coprinus cinereus by Chaetomium globosum. pH 6.5. x 100.

Figure 18. Loss of opacity, entwining and probably penetration of Coprinus cinereus by Chaetomium globosum (narrower hyphae). pH 7.7. x 100.

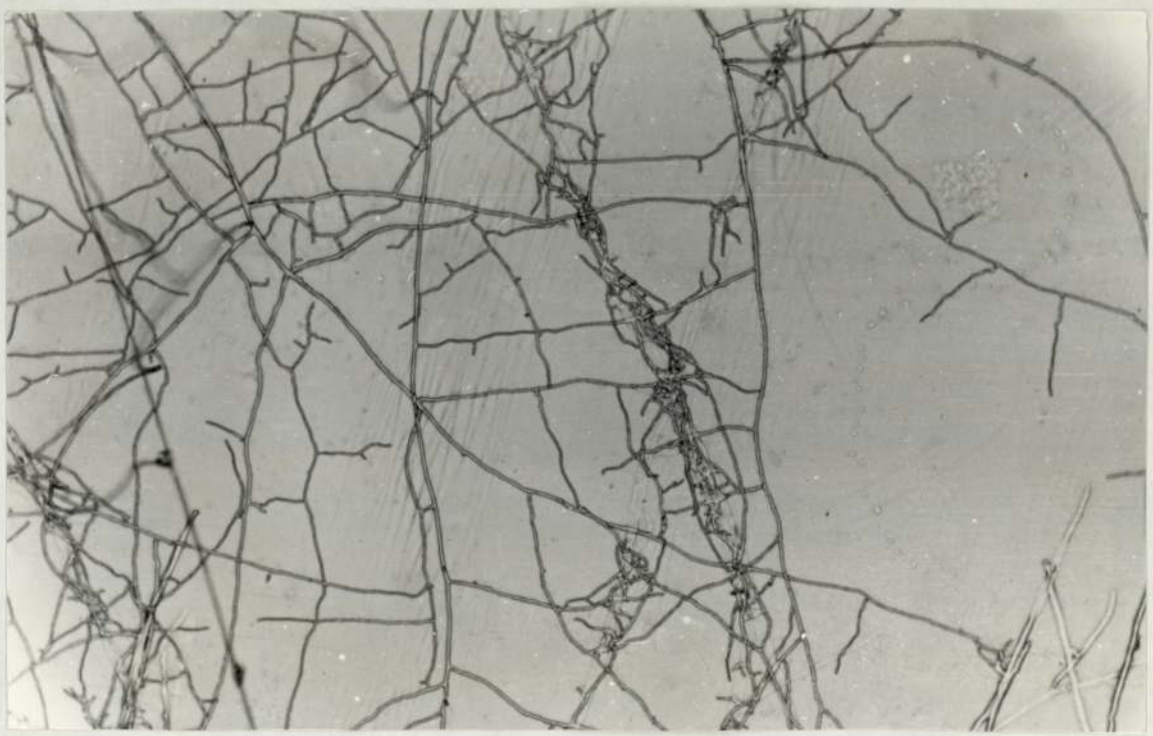


Figure 19. Chaetomium globosum hyphae entwining Coprinus cinereus hyphae. The latter have lost their opacity. pH 6.5. x 100

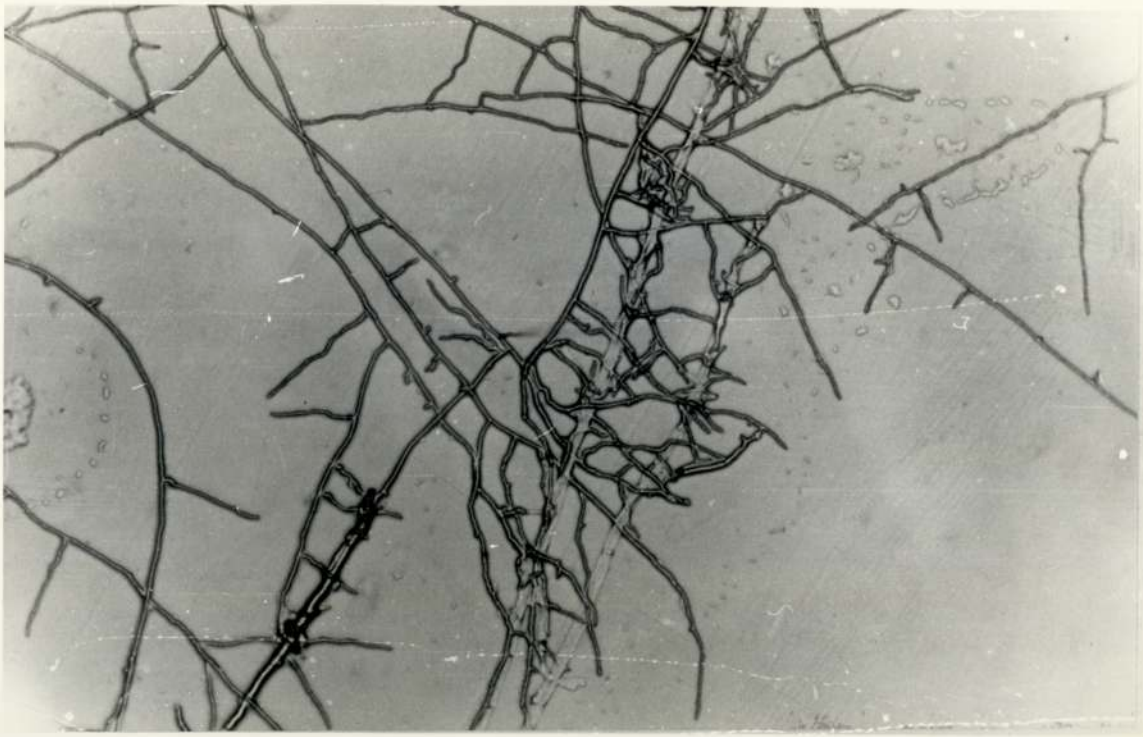


Figure 20. Chaetomium globosum hyphae aggregating around Coprinus cinereus hyphae. Two of the latter have lost their opacity and a third has become highly refractive. pH 6.5 x 100.

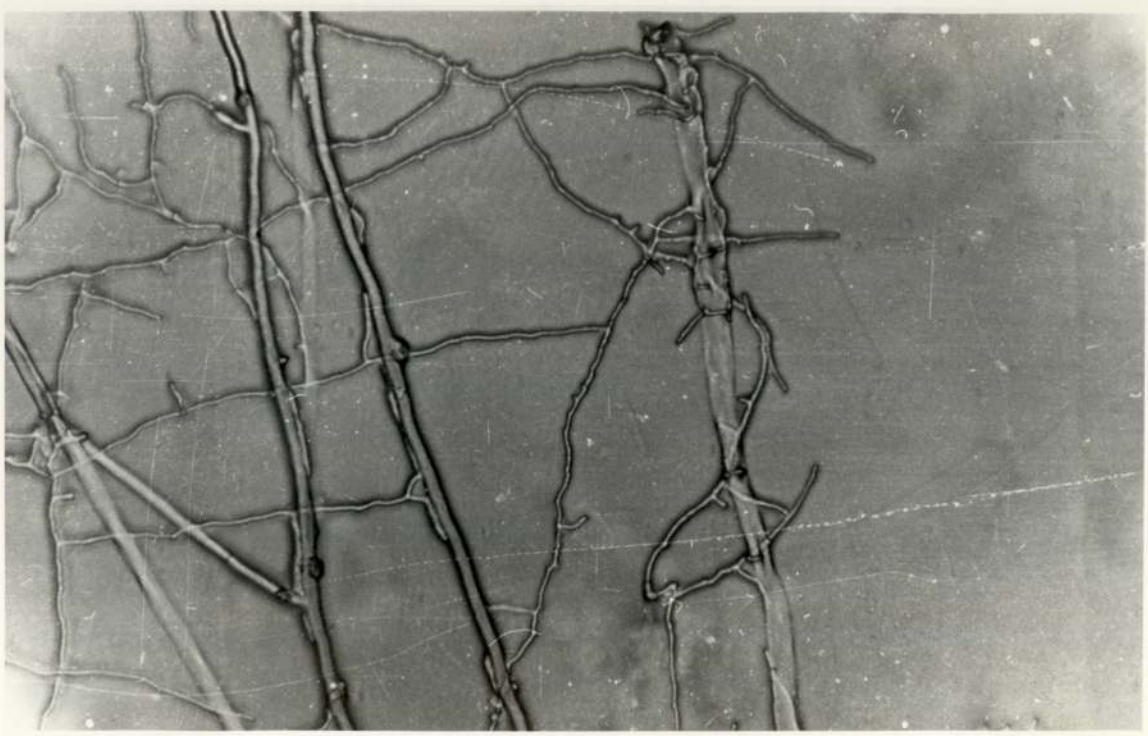


Figure 21. Chaetomium globosum hyphae growing alongside, entwining and possibly penetrating hyphae of Coprinus cinereus. pH 7.7 x 200.

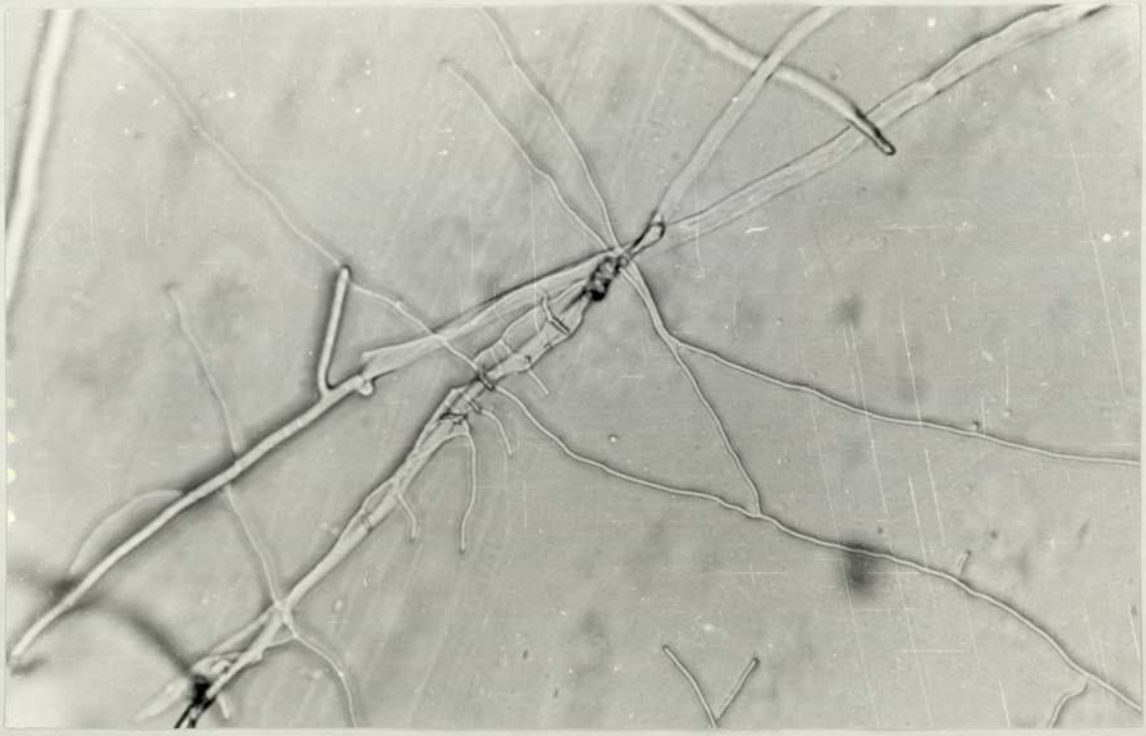


Figure 22. Chaetomium globosum growing around and possibly penetrating hyphae of Coprinus cinereus. pH 6.5 x 200.

of C. globosum (Figures 17-22), mainly at pH 6.5, occasionally at pH 7.7 and one or two equivocal observations at pH 8.7. Occasionally, evidence of possible penetration of C. cinereus hyphae by C. globosum was also observed (Figures 17, 18, 21, 22). Apparently healthy hyphae and those which had lost their opacity were both attacked (Figure 20).

Judging by the proliferation of C. globosum hyphae around an attacked length of C. cinereus hypha, it appears likely that a nutritional benefit is being derived by the former at the expense of the latter, which appears to be killed; however, no attempt was made to prove that this nutritional relationship exists. If it does, C. globosum can be regarded as a necrotrophic parasite towards C. cinereus, at least in the experimental conditions used. The question arises as to how easy it is to induce this type of behaviour in fungi by the use of artificial habitats such as that used in these experiments. It is well known that the types of nutrients present may influence both the

production of metabolites and the degree of sensitivity to them and it may be that certain changes in these factors will result in a parasitic relationship such as has been observed here, which may not occur in the field. The same point also applies, of course, to the observation of antagonism exerted before hyphal contact has occurred, a phenomenon, incidentally, which may be an integral part of the observed parasitism.

In 1924 Burgeff reported mycoparasitism by Chaetomium jonesii (Burgeff, 1924) but since then there appears to have been no further work published on mycoparasitism by Chaetomium spp. However, antagonism by C. globosum is well known. Tveit and Wood (1954, 1955) showed that several Chaetomium spp., including strains of C. globosum and C. cochlioides were able to inhibit the growth of pathogenic field fungi. Inhibition of the pathogen on agar occurred only on contact with the antagonist. Maier (1961) has also reported antagonism by these two fungi.

The antagonism observed in this study presumably operates through the action of an antibiotic; Tveit and Wood (1955) drew a similar conclusion from their observations. There appear to be no reports of the detection and isolation of any such antibiotic.

6.1.2.2 Coprinus cinereus and Chaetomium cochlioides

C. cinereus and C. cochlioides did not appear to show any aggression towards each other. Several workers,however, have reported antagonism by C. cochlioides. In addition to the work of Tveit and Wood (1955) and Maier (1961), referred to in section 6.1.2.1, there have been extensive studies into the possibility of using C. cochlioides in the biological control of cereal diseases (Kommendahl and Mew, 1975). Berry and Maier (1968) have shown that both C. cochioides and C. globosum can lose their antagonism in in vitro stdies. C. cochlioides is known to produce an antibiotic, chaetomin (Waksman and Bugie, 1944) but

it possesses no antifungal properties (Bilai, 1963).

6.1.2.3 Coprinus cinereus and Fusarium culmorum

The pairing of C. cinereus with F. culmorum yielded no evidence of any aggression. F. culmorum is not regarded to have a high degree of antagonistic ability, though it has been shown to have a good ability to maintain possession of straw (Lai and Bruehl, 1968) and to possess good competitive saprophytic ability in soil (Booth and Taylor, 1976). It also appears to have a high level of tolerance to the fungistatic products of other fungi (Wastie, 1961), and itself produces antibiotics, though none are antifungal (Bilai, 1963). The plate-pairing experiments in this study (section 4.3.2) indicated that F.culmorum possesses a low level of competitive ability against C. cinereus.

6.1.2.4 Coprinus cinereus and Cephalosporium sp.

Numerous observations of antagonism by Cephalosporium sp. towards C. cinereus were made, all at acid pHs, and C. cinereus was seen occasionally to antagonise Cephalosporium sp., the only microscopic observations made of antagonism by C. cinereus.

Loss of opacity (Figure 23), vacuolation and granulation of cytoplasm (Figure 24) were often observed at pHs 5.5-6.5, but only after colonies had begun to interpenetrate. At pH 6.5, swelling of tip cells and occasionally loss of cytoplasm from C. cinereus hyphae was also seen, at distances up to 1 mm from Cephalosporium sp. hyphae. Damaged hyphae, with granulated cytoplasm and lost opacity, were often seen to be attacked and sometimes also penetrated by Cephalosporium sp. (Figures 24-26). Overall, aggression by Cephalosporium sp. was rather spasmodic, with interacting hyphae often not leading to any damage. It appeared that only damaged hyphae of C. cinereus were entwined and sometimes penetrated; if a nutritional benefit is also derived by Cephalosporium sp., it may be regarded as being parasitic

Figure 23. Hyphae of Coprinus cinereus (growing from left to right) have lost their opacity and have become vacuolated in the presence of Cephalosporium sp. pH 5.5 x 100.

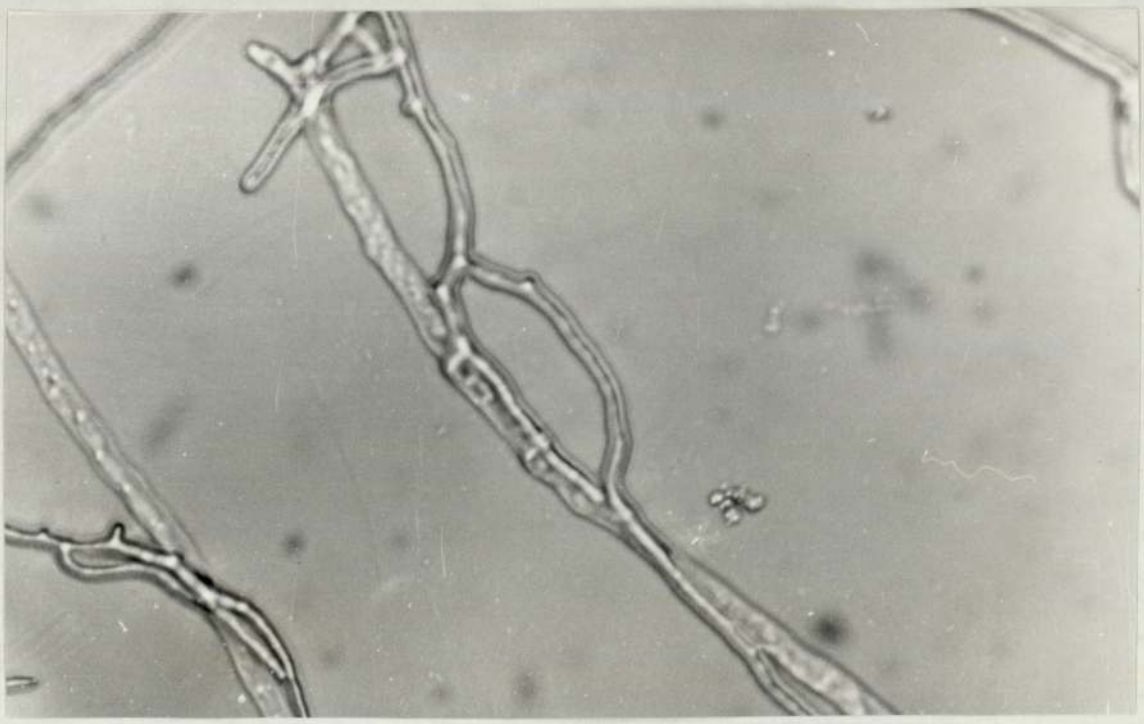


Figure 24. Two granulated hyphae of Coprinus cinereus being approached by Cephalosporium sp., in one case penetration possibly taking place. pH 5.9. x 1000.

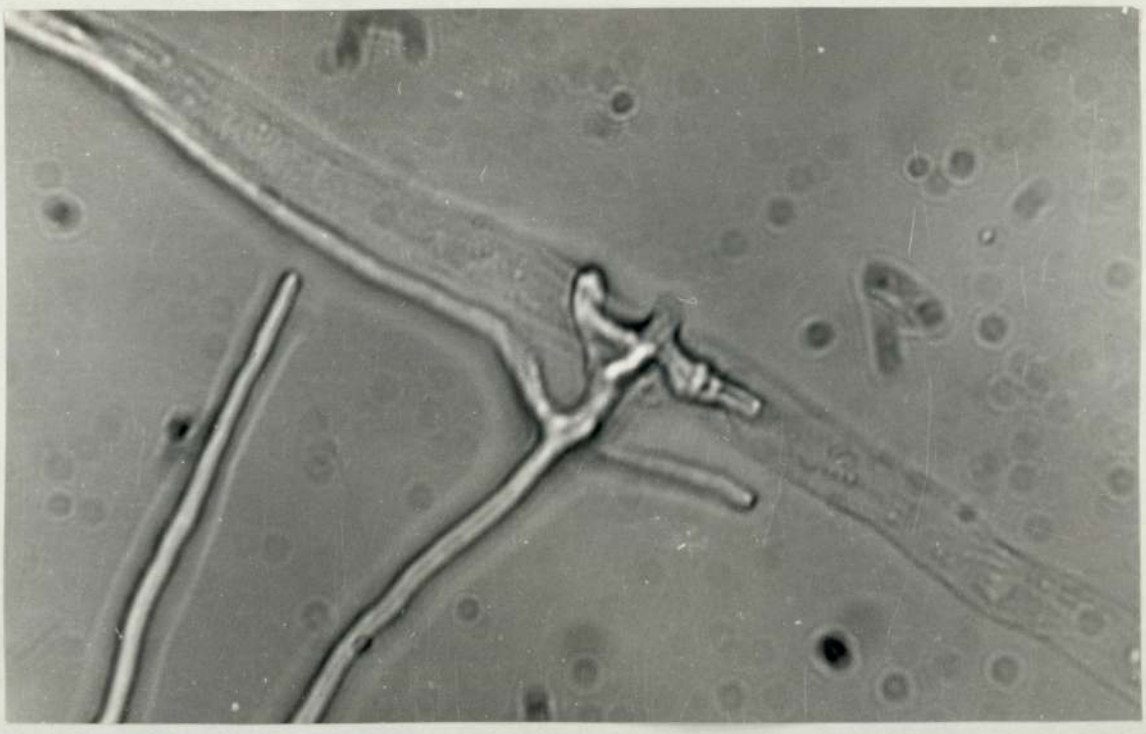


Figure 25. Cephalosporium sp. approaching and penetrating a damaged hypha of Coprinus cinereus. pH 5.5 x 1000.

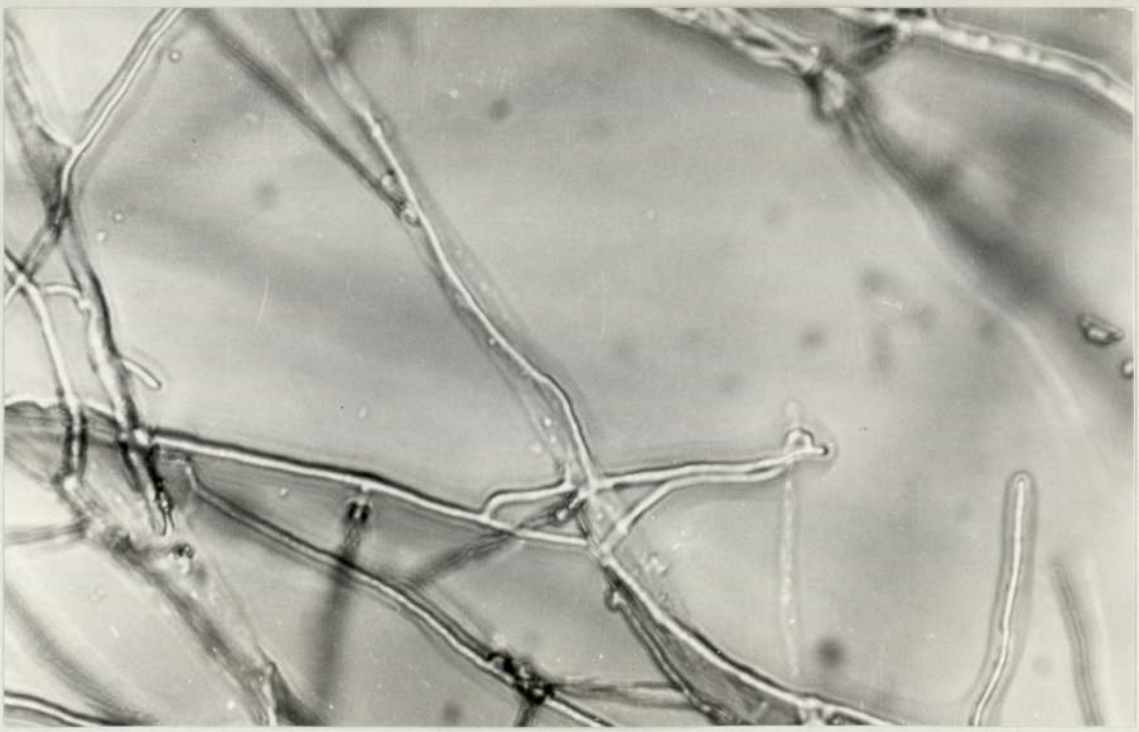


Figure 26. Growth of Cephalosporium sp. alongside and possibly within Coprinus cinereus hyphae. pH 5.5 x 400.

towards C. cinereus.

Cephalosporium spp. are known to produce a number of antibiotics, several of which are active against fungi (Broadbent, 1966). There are also reports of antagonism in Cephalosporium spp. Lai and Bruehl (1968) showed that C. gramineum has a good ability to maintain possession of straw and they also mentioned that it was able to lyse Trichoderma viride at 10°C. Kenneth and Isaac (1964) have observed three unidentified species of Cephalosporium exhibiting necrotrophic parasitism towards Helminthosporium sp.

6.1.2.5 Antagonism by Coprinus cinereus

The only observation of hyphal damage appearing to be caused by C. cinereus was made during its interaction with Cephalosporium sp., at pHs 6.5 and 7.7. The effect occurred very infrequently and was confined to small areas, always where both fungi were present. Lengths

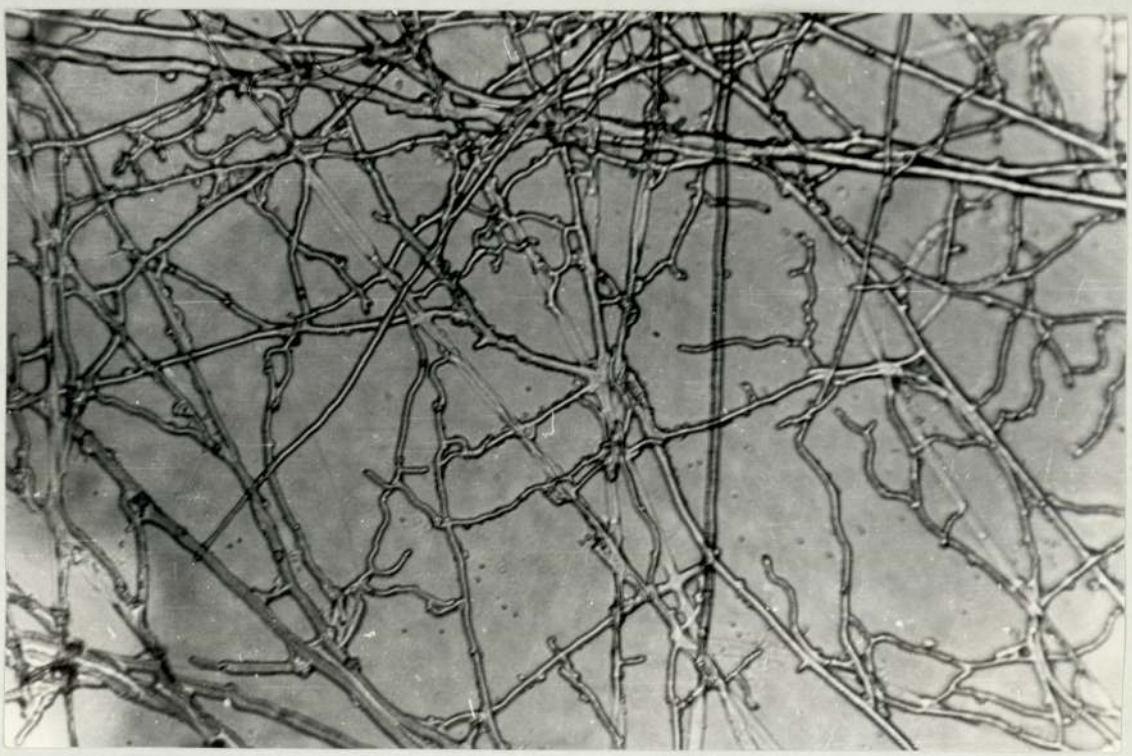


Figure 27. Loss of opacity in Cephalosporium sp. Affected cells have been approached and partially entwined by hyphae of Coprinus cinereus. pH 6.5 x 200

of Cephalosporium sp. hyphae lost their opacity and appeared to attract the growth of C. cinereus hyphae towards and alongside them (Figure 27). A causal relationship between C. cinereus and the loss of opacity was not definitely established.

Antagonism requiring contact or very close proximity (<50μ) between opposing hyphae ("hyphal interference") has been observed in C. cinereus, one of a number of Coprinus species exhibiting the phenomenon, by Ikeduigwu and Webster (1970a,b). Only one of the ten coprophilous basidiomycetes paired against it was sensitive to C. cinereus, and seven were antagonistic to it. The workers observed vacuolation and eventual loss of opacity, granulation of cytoplasm and loss of turgor pressure, along with an inability to stain with lactophenol cotton blue or to become plasmolysed. Hyphae were attracted to and entwined around affected cells, though nutritional studies failed to yield evidence that nutrients were derived from the victim.

Hedger and Hudson (1974) have also observed hyphal interference in C. cinereus in its pairings with many fungi from wheat straw compost. The fungus may produce the interference factor in only small quantities (Ikeduigwu and Webster, 1970b) and it is possible that none of the fungi chosen for pairing with it in these studies was sensitive to it.

The cell damage caused to Cephalosporium sp. does not bear much similarity with hyphal interference as described by Ikeduigwu and Webster. Possibly the fungus was exhibiting an especially high degree of sensitivity to a metabolite of C. cinereus, though observations made in the antibiotic assay studies do not support this theory. It is possible that the nutritional status of the agar used may have inhibited the expression of aggression by C. cinereus in these studies; Bassett et al (1967) found that Fomes annosus was unable to produce the antibiotic fomannosin on cellulose but was able to when using a simpler carbon source.

6.1.2.6 Coprinus cinereus and Gliocladium roseum

Gliocladium roseum was antagonistic towards Coprinus cinereus at both acid and alkaline pHs. At pHs 5.3-5.9, C. cinereus began to be affected at distances of up to around 5mm from G. roseum. The effects were quite characteristic; hyphae appeared increasingly stippled towards the older part of each cell, though not always the tip cell. Small areas, at first not extending right across the hyphae, appeared to be affected first, suggesting localised loss of turgor pressure. Vacuolation of hyphae was often observed and it is possible that vacuoles were preventing flow of cytoplasm and loss of turgor pressure from other parts of the cell (Ikeduigwu and Webster, 1970b). The damage to C. cinereus is presumably due to the production of an antibiotic by G. roseum.

At pH 5.5 only, the well-known parasitic behaviour of G. roseum was frequently observed (Figures 29-31); it coiled round hyphae of C. cinereus, growth being particularly profuse where the victim had lost opacity. Observations indicated that penetration of C. cinereus

Figure 28. Damage to hyphae of Coprinus cinereus on the approach of Gliocladium roseum. pH5.5 x 200.

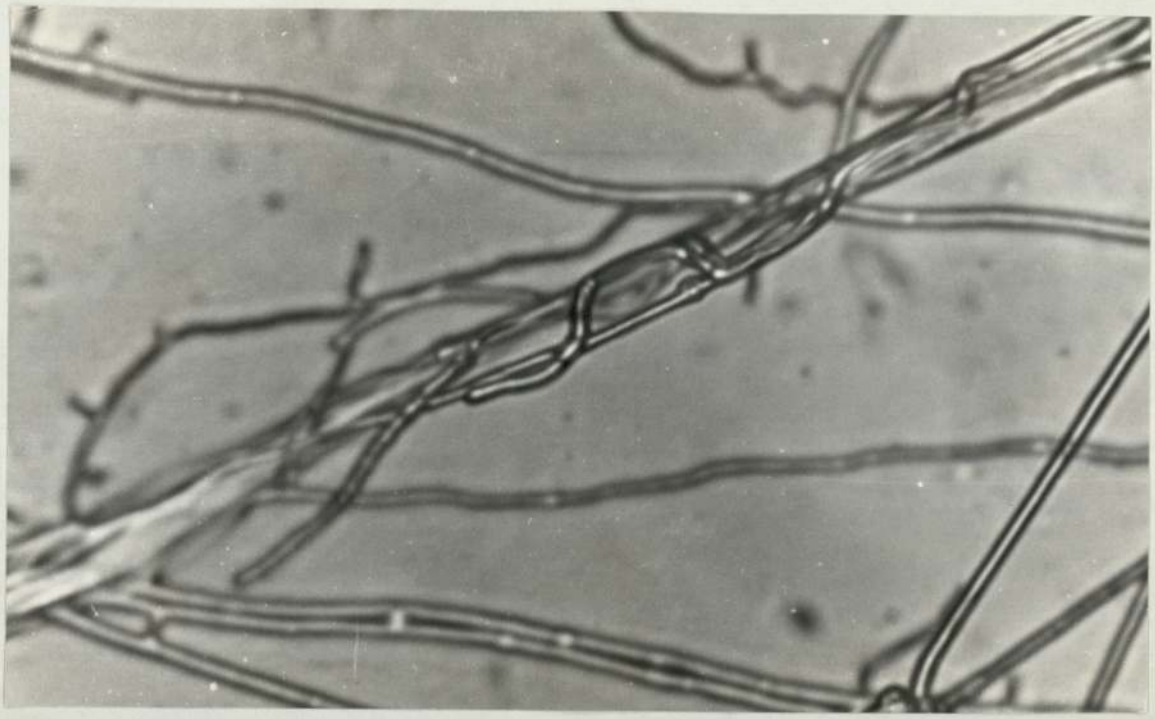


Figure 29. Gliocladium roseum entwining Coprinus cinereus hypha. pH 5.5 x 1000.

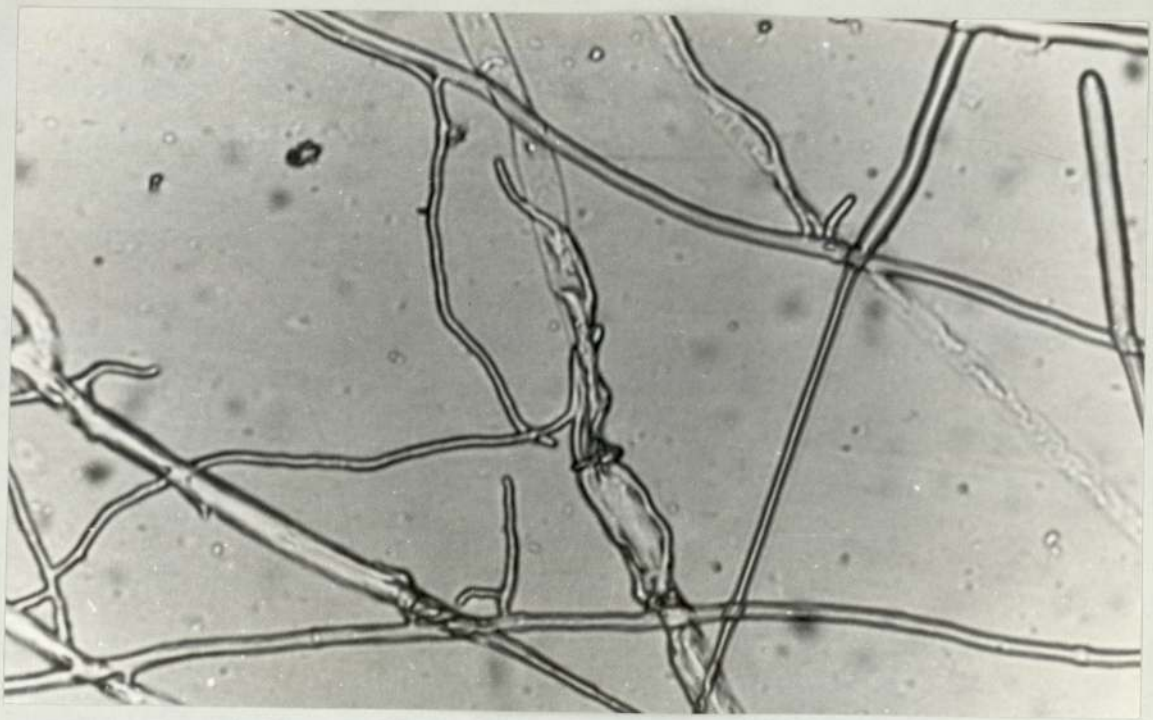


Figure 30. Gliocladium roseum entwining and possibly penetrating hyphae of Coprinus cinereus. pH 5.5 x 400.

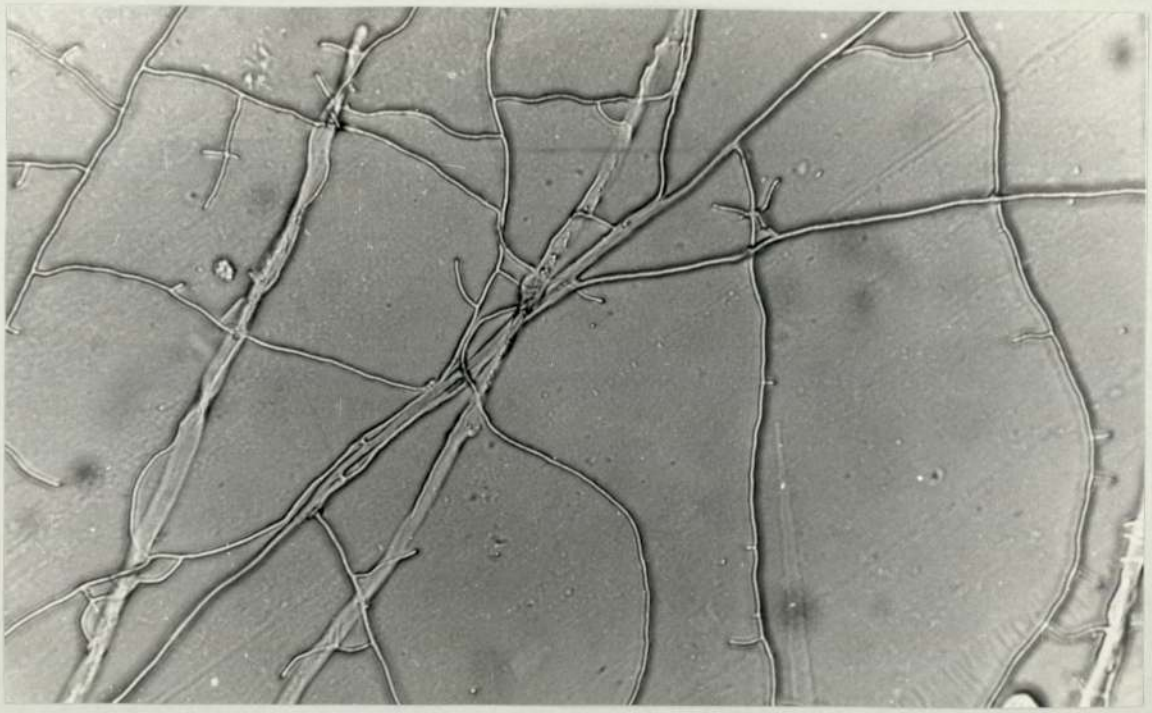


Figure 31. Loss of opacity and granulation of cytoplasm of Coprinus cinereus hyphae caused by Gliocladium roseum, which is also entwining its victim. pH 5.5 x 200.

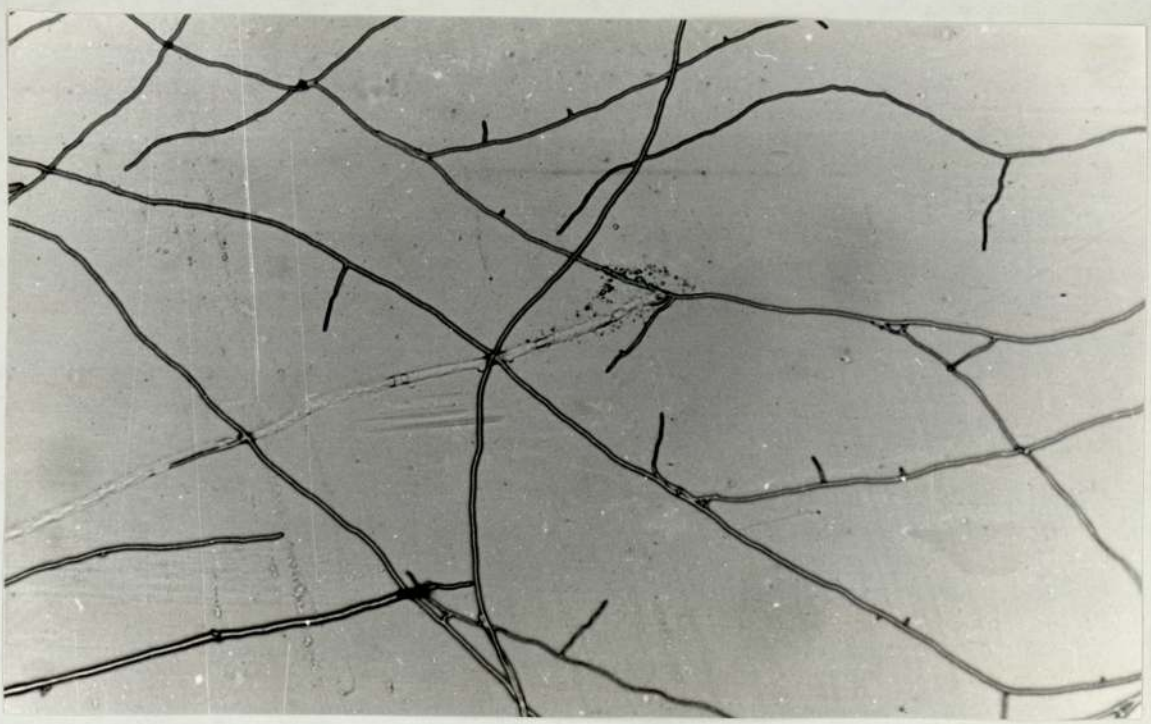


Figure 32. Hyphal tip of Coprinus cinereus bursting during contact with Gliocladium roseum. pH 7.7 x 100.

hyphae also occurred on occasions.

All studies carried out at pH 6.5 failed to yield any evidence of antagonism, but at pH 7.7 and occasionally at pH 8.7, loss of opacity in C. cinereus up to 200μ from G. roseum was observed, affecting one or two cells back from the tip cell, but not the tip cell itself. No stippling was apparent, and growth towards the affected cells by G. roseum was not seen. Bursting of hyphal tips of C. cinereus in contact with G. roseum was also occasionally seen at pH 7.7 (Fig. 32).

G. roseum is known to produce two mildly anti-fungal antibiotics, aurantiogliocladin and gliorosein (Bilai, 1963). Barnett and Lilly (1962) have shown that G. roseum is a strong necrotrophic mycoparasite with a broad host range and have characterised its behaviour during parasitism. No evidence of antagonism before colonies met was observed and it was concluded that death of the victim could not be due to a highly diffusible antibiotic. Penetration of the victim's hyphae was often seen, though not with all hosts. It normally occurred after death of the host. The mycoparasitic nature of G. roseum has also been reported by several other workers and Malik and Eggins (1970) have produced evidence indicating that it may be able to produce toxic metabolites.

The strain of Gliocladium roseum studied here appears to be antagonistic through the action of toxic metabolites and also appears to be mycoparasitic.

### 6.1.2.7 Coprinus cinereus and Trichoderma viride

The well-known antagonism of T. viride was seen at all acid pHs tried; loss of opacity and marked vacuolation in C. cinereus (Figures 33-36), giving hyphae a stippled appearence, occurred in virtually all hyphae up to around 300μ distance from T. viride. At pHs 5.3-7.7, granulation of cytoplasm was also seen. On rare occasions,

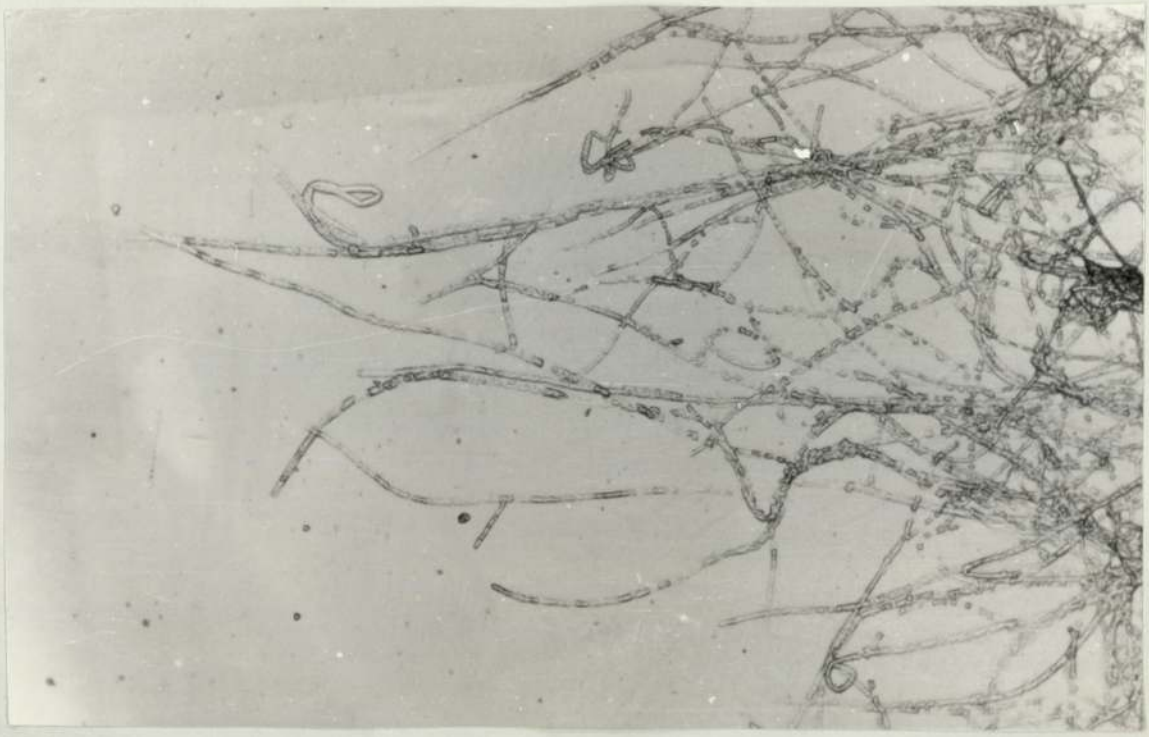


Figure 33. Damaged Coprinus cinereus hyphae, occurring at around 200μ or so from hyphal front of Trichoderma viride. pH 6.5 x 100.

Figure 34. Loss of opacity in Coprinus cinereus hyphae around 200μ from Trichoderma viride. pH 5.9 x 100.

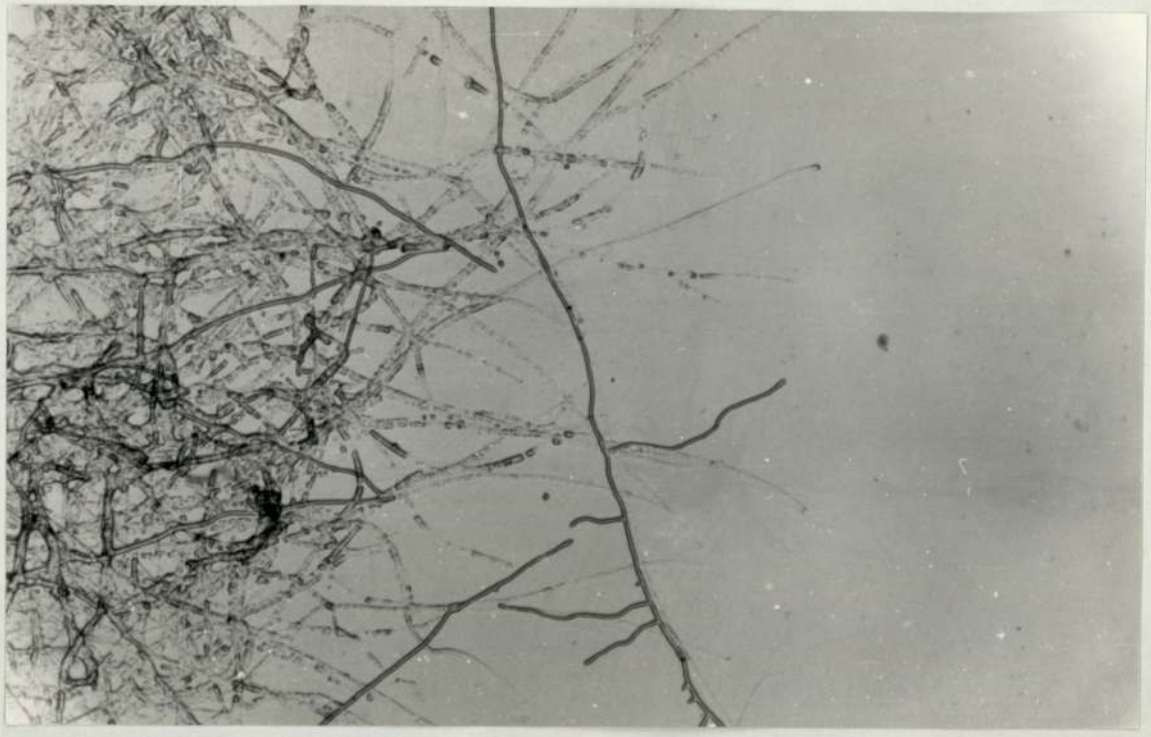


Figure 35. Damaged Coprinus cinereus hyphae being overgrown by Trichoderma viride. pH 6.5 x 100.

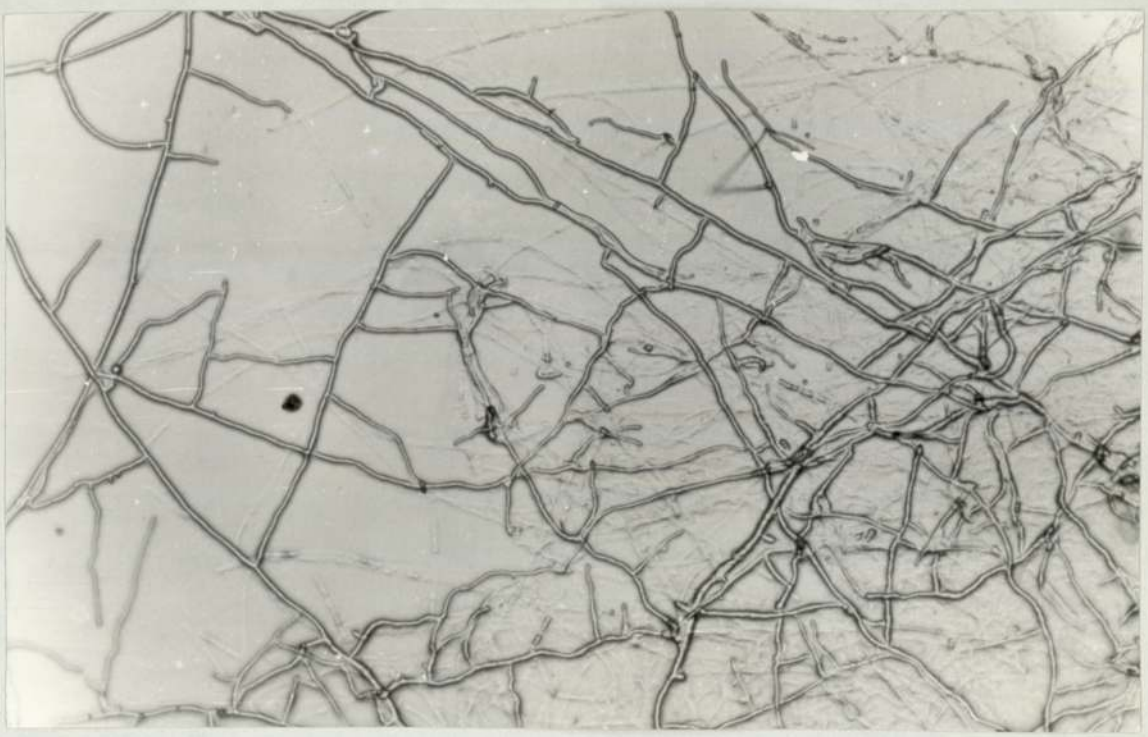


Figure 36. Necrotic colony of Coprinus cinereus being overgrown by Trichoderma viride. pH 6.5 x 100.

and only at pH 7.7, T. viride was seen to partially entwine hyphae of C. cinereus. Also at pH 7.7, it was common to see C. cinereus hyphae change their direction of growth, sometimes back towards their own colony.

Reports of antagonism by T. viride in the literature are somewhat confused by a controversy over the identity of the fungus under study. Weindling (1932) reported mycoparasitism by T. lignorum (Tode) Harz (=T. viride Pers ex Fries) and later he reported production by the same fungus, referred to now as Gliocladium virens, of an antifungal antibiotic, gliotoxin (Weindling and Emerson, 1936). Brian and co-workers, using both their own and Weindling's isolates, confirmed both the original identification of Weindling (1932) and their ability to produce gliotoxin (Brian, 1944; Brian and Hemming, 1945) and in addition, a highly active antifungal antibiotic, viridin (Brian and McGowan, 1945; Brian et al, 1946). Webster and Lomas (1964), investigating the identity of the strains used by Weindling and Brian, reported them to be Gliocladium virens; they failed to isolate either gliotoxin or viridin from Trichoderma spp. Dennis and Webster (1971a,b) have shown that several species of Trichoderma, including T. viride, can produce both volatile and non-volatile antibiotics but, while none were identified, viridin and gliotoxin were considered not to be among them. Several other antibiotics, including suzukacillin, alamethicine and trichodermin have been reported to be produced by T. viride (Dennis and Webster, 1971a).

Rishbeth (1950), Aytoun (1953) and Gibbs (1967) all found antagonism by T. viride to be greater at acid than alkaline pHs, though Dennis and Webster (1971a) report that pH has very little influence on the ability of Trichoderma spp. to inhibit fungi. Aggression was only seen at acid pHs (and occasionally at initial pH 7.7) in this study, though it was clearly antagonistic at alkaline pHs in the plate-pairing

studies (section 4.3). The dependency on pH may be due to several factors, including its effect on the stability of antibiotics (Bilai, 1963).

No clear evidence of parasitism by T. viride was obtained. Dennis and Webster (1971c) found that most of the strains they studied showed an ability to coil and occasionally to penetrate the victim and Gibbs (1967) observed necrotrophic parasitism at acid pHs. It is possible that C. cinereus is resistant to coiling by T. viride, as were some of the assay fungi used by Dennis and Webster.

6.1.2.8. Coprinus cinereus and Aspergillus fumigatus

At pHs 7.7 and 8.7 A. fumigatus inhibited the growth of C. cinereus and at pH 7.7 loss of opacity in hyphae of C. cinereus was also seen, though not always. Both effects occurred at distances of up to 300μ from A. fumigatus.

Broadbent (1966) mentions three antibiotics produced by A. fumigatus, none of which are antifungal, and a fourth, fumigatin, also not antifungal, is mentioned by Bilai (1963). The fungus has also been observed to strongly inhibit spore germination by means of a volatile inhibitor.

## 6.2 Discussion

Five of the seven fungi paired against C. cinereus showed an aggressive ability towards it. Table 21 shows the principal effects observed. Except in the case of Cephalosporium sp., the antagonistic effects were exerted before the opposing hyphae made contact, suggesting a mechanism involving antibiotics. Loss of opacity was the most commonly seen reaction to aggression. It signifies loss of turgor pressure, due to leakage of cell contents. This can arise from damage to the permeability of the cell membrane, which a number of antibiotics are

Table 21. A summary of the antagonistic effects between Coprinus cinereus and other fungi observed microscopically. Figures refer to the pH range over which the observations were made.

| Antagonistic effect | Chaetomium globosum | Cephalosporium sp. | Gliocladium roseum | Trichoderma viride | Aspergillus fumigatus |
|---|---|---|---|---|---|
| Loss of opacity | 6.5-7.7 | 5.5-6.5 | 5.3-5.9, 7.7-8.7 | 5.3-6.5 | 7.7 |
| Vacuolation | (5.9), 6.5-7.7 | 5.5-6.5 | 5.3-5.9 | 5.3-6.5 | - |
| Granulation | - | 5.5-6.5 | 5.5 | 5.5-7.7 | - |
| Effects exerted over distance | yes | no (?) | yes | yes | yes |
| Entwining | 6.5-7.7, (8.7) | 5.5-6.5 | 5.5 | (7.7) | - |
| Penetration | 6.5 (7.7) | 5.5-5.9 | 5.5 | - | - |
| Other effects | Swelling of hyphae, 6.5-7.7 | Swelling of tip cells, 6.5. Cytoplasm leakage, 6.5. | See text | See text | - |

Hyphal damage in Cephalosporium sp. was seen adjacent to hyphae of C. cinereus at pHs 6.5 and 7.7.

( ) indicates rare or equivocal observation.

known to cause (Gottlieb and Shaw, 1970).

Most observations of aggression were made at acid pHs or around neutrality. The apparent lack of aggression at pH 8.7 may be due to improved resistance by C. cinereus to attack at alkaline pHs, though toxin instability cannot be ruled out as the cause. The domination of straw by C. cinereus at highly alkaline pHs, observed in earlier studies, may be partially due to suppression of aggressive abilities in competitors at such pHs.

# CHAPTER 7

## SYNOPSIS AND GENERAL DISCUSSION

AN INVESTIGATION into the fungi present in an old stack of straw yielded a broad range of fungi, mainly Fungi Imperfecti, with some Ascomycetes and Phycomycetes; thermophilic fungi were rare. Most of the fungi found are known to be cellulolytic. The specific fungi isolated may not be representative of those in other barley straw; the populations present will depend on both the geographical location of the straw and its conditions of storage.

Pads of fresh straw, both unamended and amended with nitrogen, were incubated in the laboratory at 25°C. for one month and changes in the presence of active cellulolytic fungi followed using several isolation techniques and direct observation. Fusarium sp. and Alternaria tenuis were common in the early stages; A. tenuis later declined and Gliocladium roseum became common late in the succession. Chaetomium globosum was extremely common in both amended and unamended straw, though it, Cephalosporium sp. and Trichoderma viride were all more common in the amended pad than the unamended pad. Coprinus cinereus

was isolated on only one occasion. The pH of the pads was acid throughout the incubation period and was around 5.5 by day 28.

Isolations made from ammonia-treated straw showed that, at concentrations of ammonia around 0.5-1.5% (w/w), C. cinereus became the dominant fungus, with Chaetomium globosum present as an important competitor, less so at the higher ammonia concentrations. Paecilomyces sp. and Aspergillus fumigatus were also often present. The pH of the straw ranged from 7.5-8.9.

Several physiological studies were carried out on a selection of the fungi isolated from the pads and the ammonia-treated straw. The linear growth rates on agar of twenty-one of the more commonly isolated fungi were first determined. Cellulolytic abilities were assessed and some fungi (Coprinus cinereus, Chaetomium globosum, C. cochlioides, Fusarium culmorum and Trichoderma viride) were found to produce intense clearing of cellulose agar quickly, some a low intensity slowly and others a low intensity quickly. Those unable to produce a high intensity of clearing may cease cellulase production early in their colonisation of the agar and may also excrete an unstable cellulase complex.

Optimum pHs and temperatures for linear growth of eight fungi in vitro were assessed. It was found that T. viride and F. culmorum grew fast at acid but not alkaline pHs, while C. cinereus, Chaetomium globosum and Gliocladium roseum possessed alkaline pH optima. At low temperatures, C. cinereus possessed a low linear growth rate compared to the other fungi tested, but at 30°C., only T. viride grew faster. Higher temperatures also improved the relative linear growth rate of Aspergillus fumigatus.

Ligninolytic abilities of the eight fungi were investigated; C. cinereus and Chaetomium globosum appear to be mildly ligninolytic and probably also T. viride. All eight fungi were also found to be capable of causing significant weight losses in straw in pure culture,

C. cinereus and F. culmorum being rather better than the others. Ligninolytic ability may be a valuable attribute of a fungus being used to increase the digestibility of straw, since it may be able to increase the availability of the cellulose present by removing or separating some of the lignin from it. It may indeed be better to use ligninolytic fungi rather than cellulolytic fungi, because the latter are likely to utilise first that part of the cellulose in straw most easily available to rumen microorganisms, giving the straw a lowered digestibility (Eriksson, 1974).

Interactions between colonies of twelve fungi paired on agar plates were studied and it was found that a number of fungi isolated from straw possess significant competitive ability. Trichoderma spp. were shown to be particularly successful in competition with others. Of the remaining fungi, C. cinereus appeared to possess the highest competitive ability, followed by Chaetomium globosum.

Seven fungi were selected for pairing against C. cinereus on agar plates over a range of pHs and it was shown that, with some pairings, competitive ability varied considerably with the pH of the medium. C. cinereus was generally more successful at higher pHs, the trend being particularly marked against F. culmorum and Aspergillus fumigatus.

Tests on the ability of C. cinereus to produce volatile or non-volatile toxic metabolites showed that under the experimental conditions used, the fungus produced no powerful, stable toxins. The slight inhibition of Gliocladium roseum may be due to sensitivity to a mild toxin or to the removal of particular nutrients by C. cinereus. A volatile substance from this fungus appears to inhibit slightly the growth of T. viride and F. culmorum.

In experiments investigating the possibility of growing a fungus in association with Azotobacter spp. it was found that on a medium

containing cellulose and small amounts of glucose and nitrogen as ammonium sulphate, C. cinereus grew better with all of the four Azotobacter spp. than on its own and Chaetomium globosum better with two of the four species. When straw being colonised by either C. globosum, G. roseum or T. viride was later inoculated with Azotobacter spp. weight losses in the straw were increased by the presence of the bacterium. Results with Coprinus cinereus are equivocal.

A nutritional association between Azotobacter spp. and fungi on both agar and straw appears possible; further work needs to be done to confirm and enlarge upon these findings.

Interactions between hyphae of C. cinereus and seven other fungi were examined microscopically using several culturing systems. In contrast to the findings of other workers, C. cinereus, or at least the strain used here, did not elicit signs of any ability to cause hyphal interference, though it seemed occasionally to cause some damage to Cephalosporium sp.; it is possible that the nature of the medium used or resistance of the assay fungi to attack prevented the expression of aggressive ability.

Of the seven fungi paired with C. cinereus, five showed an aggressive ability towards it, C. cochlioides and F. culmorum being the exceptions. Aggression appeared predominantly under acid conditions. Except in the case of Cephalosporium sp., the antagonistic effects were exerted before the opposing hyphae made contact, suggesting a mechanism involving toxic metabolites. C. globosum, Cephalosporium sp., G. roseum and possibly also T. viride all were seen to entwine C. cinereus and, with the exception of T. viride, also appeared occasionally to penetrate the cell wall of the victim, displaying mycoparasitic behaviour. The domination of straw by C. cinereus at highly alkaline pHs, observed in earlier studies, may be partially due to loss of aggressive abilities by competitors at such pHs.

Garrett (1956) has stated that the factors affecting the outcome of the struggle between fungi competing for a substrate are the competitive abilities of the fungi and their inoculum potentials, competitive abilities being enhanced by efficient nutrient utilisation, rapid growth and the ability to produce and tolerate toxic metabolites.

The importance of simple competition for nutrients in determining the competitive success of the fungi studied cannot easily be assessed, though it clearly plays a significant part. The patterns of interaction with change in pH between fungi that showed no aggression towards one another, for example, C. cinereus and F. culmorum, in which penetration and overgrowth of colonies of the latter by the former progressively increased as the pH rose, strongly indicates that relative speeds of nutrient uptake are important, and that C. cinereus derives increased competitive success by increased nutrient uptake efficiency at higher pHs. Relative linear growth rate (determined partly by the level of nutrient uptake efficiency) itself will play a part, since the fungus with the highest linear growth rate is most able to gain access to nutrients in the vicinity of hyphae of two or more fungi. The fungi most frequently isolated from the straw pads tended also to be the fastest-growing, a reflection, perhaps, of the role of linear growth rate in the competition for nutrient baits used in isolation techniques.

Since temperature has also been found to influence relative growth rates, it too will be an important factor in determining the competitive success of fungi; this and pH will have operated in causing the high frequency of isolation of C. cinereus from ammonia-treated straw incubated at 30°C., compared with its low frequency of isolation from the straw pads incubated at 25°C. at acid pHs.

Evidence of aggressive abilities in interactions between fungi in vitro was common in most of the fungi tested. Inhibition of growth before the colonies met was seen in many pairings of fungi,

suggesting that diffusing toxic metabolites were exerting effects. The level of production and stability of these excretions will be influenced both by pH and temperature, as too will the degree of suseptibility to them. The effect of pH was clearly seen in the microscopic studies; only within certain pH ranges were inhibitions of hyphal growth at a distance seen. It seems likely that variations in the potency of toxins of other fungi to C. cinereus is of greater importance in its competitive success at high pHs than variations in the potency of toxins of C. cinereus to other fungi; only certain specific mild inhibitions by C. cinereus were ever observed on the one hand and, on the other, very few cases of aggression were ever seen at high pHs compared with acid pHs. Variations in potency can be due to changes in the stability or the level of production of a toxin as well as to changes in tolerance to it.

The effect of temperature on antagonistic abilities was not investigated in these studies.

It is difficult to assess the significance of the in vitro findings concerning aggression in the competitive colonisation of straw. Inhibition of C. cinereus was often seen in both the microscopic and macroscopic studies and these phenomena may occur to some degree in the natural substrate. It may be possible to incorporate chemical or physical features of the in vitro conditions in large scale semi-solid fermentations of straw, and thereby induce aggressive behaviour that would not otherwise occur, in order to encourage domination of the substrate by a particular chosen fungus.

The ability to produce toxins effective against competitors may be particularly important in the maintenance of domination of a substrate. If the toxin produced is volatile, it must be produced continuously in order to be effective; some evidence was produced in these studies that C. cinereus may be able to produce such a toxin

mildly inhibitory to T. viride and F. culmorum.

The role of inoculum potential in determining the competitive success of fungi was not investigated in this study, but it appears that the inoculum level of C. cinereus is sufficiently high to lead to rapid domination of ammonia-treated straw. It would be of value to know by how much its success in colonisation and domination of the substrate under less favourable conditions would be improved by an increase in its inoculum level and also by how much its inoculum level in cropped barley straw exceeds the lower limit required for domination of the substrate in the alkaline conditions of ammonia-treated straw. It would also be useful to know more of the part played by inoculum potential in determining population levels in the incubated straw pads.

It appears that it is possible to provide conditions in straw suitable for it to become quickly dominated by indigenous C. cinereus and for that domination to be maintained. This was clearly shown to be the case in ammonia-treated straw incubated at 30°C. and McShane (1976) has shown this also to be true at 35°C. First, the alkaline pH present confers upon it a higher linear growth rate than all the competitors selected for study, whereas at pH 5.3 several of these competitors possess much higher linear growth rates than C. cinereus. Secondly, the incubation temperature of 30°C. encourages competitive success by this fungus. While at 20°C. only two of its competitors have lower growth rates than C. cinereus, at 30°C. it grows faster than all of them except T. viride—and this fungus grows extremely slowly at the high pH of ammonia-treated straw. This argument also applies to A. fumigatus; while it possesses a high linear growth rate at 35°C. (Rege, 1927), it grows only very slowly at alkaline pHs.

The studies in which fungi were paired on plates also indicate that C. cinereus is more competitively successful at the higher pHs, where it appears to be better at competing for nutrients, probably primarily a reflection of its raised relative linear growth rate. In

addition, aggressive ability, present in five of the competitors to C. cinereus at pHs around neutrality, was absent in all but one fungus at pH 8.7.

In the acid conditions of the straw pads, C. globosum and F. culmorum appeared to be the predominant inhabitants. C. cinereus has a linear growth rate comparable with C. globosum at the pH prevailing, but rather lower than that of F. culmorum, and much lower than that of T. viride, which, however, did not appear to be particularly active. It is possible that both this fungus and A. fumigatus are not able to rapidly utilise the substrate; both caused quite low weight losses in straw in pure culture compared with most of the other fungi tested and Cowling (1958) has suggested that neither possess an 'X' factor in their cellulase complex, necessary for the attack of cellulose associated with lignin.

Control of pH at alkaline levels appears to virtually eliminate competition from F. culmorum and would also prevent competition from T. viride. A finer control of pH appears to be necessary to prevent competition from C. globosum and A. fumigatus. These two fungi and Paecilomyces sp. appear to be the only serious fungal competitors to C. cinereus in ammonia-treated straw. All three are known to be potentially hazardous to animals (Festenstein et al, 1965; Christensen et al, 1966) so it may be very important to eliminate competition from them in commercial fermentations to produce an animal feedstuff. If the use of pH and temperature as controlling factors prove to be inadequate to prevent significant populations of the fungi arising, it may be necessary to use other methods in addition. For example, it might be possible to exploit the level of moisture present, or the nutritional state of the substrate, including C:N ratios, to control the emergence of these fungi.

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## APPENDIX 1

The compositions of the media used in this study are as follows:

1). Eggins and Pugh cellulose agar.

| | |
|---|---|
| $KH_2PO_4$ | 1.0 g |
| $(NH_4)_2SO_4$ | 0.5 g |
| KCl | 0.5 g |
| L-asparagine | 0.5 g |
| Yeast extract | 0.5 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| $CaCl_2$ | 0.1 g |
| Agar | 15 g |
| Cellulose (Whatman's CF11, ball-milled for 72 hrs.) | 250 ml of 4% suspension |
| Distilled water | to 1.0 litre. |

2). Glucose starch agar.

Same as cellulose agar above, except that the cellulose is replaced with:

| | |
|---|---|
| D-glucose | 5.0 g |
| Starch | 5.0 g |

3). Malt agar.

| | |
|---|---|
| Malt extract | 20.0 g |
| Mycological peptone | 5.0 g |
| Agar | 15 g |
| Distilled Water | to 1 litre |

Appendix 1 (continued)

4). Norris's medium

| | |
|---|---|
| $K_2HPO_4$ | 1.0 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| $CaCO_3$ | 1.0 g |
| NaCl | 0.2 g |
| $FeSO_4.7H_2O$ | 0.1 g |
| $Na_2MoO_4.2H_2O$ | 0.005 g |
| D-glucose | 10.0 g |
| Agar | 15 g |
| Distilled water | to 1.0 litre |

5). Modified Norris's medium

Same as 4). above, with D-glucose replaced by 1.0% ball-milled cellulose suspension, as used in Eggins and Pugh cellulose agar.

6). Low-N cellulose agar

Same as 4). above, with only 0.1 g glucose instead of 10 g, and with 0.01 g ammonium sulphate added.

## APPENDIX 2

Fungi studied in this project which have not been conclusively identified to species level have been numbered. Tentative suggestions of the species of some of them are given below.

*Chaetomium* sp. 1 = *C. tortile*

*Chaetomium* sp. 2 = *C. crispatum*

*Chaetomium* sp. 3 = *C. dolichotrichum*

*Chaetomium* sp. 5 = *C. elatum*

*Chaetomium* sp. 6 = *C. aureum*

*Chaetomium* sp. 7 = *C. cuniculorum*

*Trichoderma* spp. 1 and 2 are probably strains of *T. viride*.