ECOLOGICAL STUDIES ON THE MICROBIAL UPGRADING OF STRAW

by

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Being a thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy

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Summary

The work reported here was part of a larger research programme designed to obtain a method of increasing the digestibility of waste straw using a selected microorganism. The experiments which are the subject of the thesis were carried out to investigate the ecology of the environmental niche thus created and to examine the possibilities of controlling this environment to allow a selected fungus to become the dominant coloniser.

Coprinus cinereus (Schaeff ex Fr.) S F Gray sensu Konr., an edible, endemic coloniser of straw was the selected microorganism and it was found to be widespread on material from the bale and the field and also in soils which had been under cereals. A technique was developed to isolate this fungus using aqueous ammonia and this being so, it was suggested that this species may warrant being placed in the 'chemoecological' grouping of 'ammonia fungi'.

It was shown that the presence of free ammonia can produce a unique selective pressure in favour of *C cinereus* which was not observed using other nitrogen sources at an elevated pH. The optimum levels of ammonia and temperature required to bring this about were investigated, together with the nature of this selective pressure.

The qualitative and quantitative effects of ammonia on the microbial populations of straw over a two week period were studied and the implications of the results for the proposed upgrading technique were considered.

The utilisation of lignin and cellulose by a number of isolates of *C cinereus* were compared under conditions of varying pH and temperature and capabilities were found to differ with the isolates.

The feasibility of the straw upgrading process was then discussed taking into consideration the results and conclusions obtained from this research programme.

Straw, Ammonia, Microorganisms, Wastes, Biodegradation.

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CHAPTER ONE

INTRODUCTION

1.1 THE PROBLEM OF SURPLUS STRAW

1.1 (i) The size of the problem

The study described in this thesis was carried out as part of a larger work programme aimed at developing a process for the fungal upgrading of straw. Before detailing this work it is necessary to outline the agricultural practices which have instigated renewed interest in straw upgrading over the last 10-15 years.

The two main waste products of agriculture which are likely to cause pollution problems are the manures from livestock units and straw from cereal enterprises. Both problems are relatively recent having been brought about as a result of specialisation in farming to obtain economies of scale. In earlier times the main use for straw was as bedding for farm animals where it soaked up faeces and urine. The bedding was removed from the livestock areas, allowed to decompose and returned to the soil as farmyard manure. However during the 1960's many farms 'rationalised' their farming systems (O'Callaghan, 1975). This led to the present situation where the drier eastern counties now support mainly arable farming, while stock farming is found mainly in the wetter and hillier western and northern counties, thus, in effect separating the supply of and traditional demand for, straw (Fulbrook *et al*, 1973), whilst more intensive crop management and a national increase in the acreage under cereals has given rise to the current situation where about 50% of the straw produced in the UK is surplus to requirements (Farmers Weekly April 7th 1978).

The problem was highlighted in the early 1970's when increases in oil, fertiliser and consequently animal feed prices together with increased concern from environmental pressure groups encouraged interest in profitable agricultural waste management. The problems created by the need to dispose of excess straw became the subject of a number of reports (ACAH, 1973; McLean, 1973; National Farmers Union, 1973).

The total area of cereals grown in England and Wales during 1977 was approximately 3.1 million ha, comprising 1.9 million ha. barley, 1 million ha wheat and 0.15 million ha oats. Although the yield of straw depends upon species, variety, season and length of cut, it can be estimated that from this about 4 tonne/ha for wheat straw and 2.5 tonne/ha for barley and oat straw will be harvested. Consequently the total annual resource is about 4.8 million tonnes of barley straw, 4.0 million tonnes of wheat straw and 0.4 million tonnes of oat straw (ADAS Advisory leaflet MAFF 1977). In 1977 the actual total production was 10.25 million tonnes. These figures vary from year to year as do the number of tonnes surplus to farm needs. ADAS calculations (Farmers Weekly, April 7th 1978) indicate that in 1976 there were 3.3 million tonnes of wheat and barley straw for ex-farm use, and that this figure increased to 5.2 million tonnes in 1977. In these years 2.19 million tonnes and 4.23 million tonnes respectively were burnt.

These figures are compared to those from the NFU report of 1973 in table 1.1, as can be seen, by 1977 surplus straw production had increased significantly as had the percentage burnt. The year 1976 was somewhat anomalous in that Britain suffered extremes of weather conditions which made it economical to transport excess straw from regions of high production to those of lower production for animal bedding and fodder.

Table 1.1 Straw production and surpluses

	1973	1976	1977
Total production m tonnes	9.45	10.55	10.25
% Surplus to requirements	38.2	31.3	50.7
% Burnt	36.6	20.8	41.3
% Ploughed in	1.6	6.1	1.9

1.1(ii) Current use and Disposal of Straw

The main on farm uses of straw are as components of feed rations and as bedding material for livestock. Barley and oat straws have the higher nutritional content, so that most of the straw excess to farm needs comes from wheat. The bulk of this is burnt in the field without baling.

The uses of straw outside agriculture are limited, these include packaging, building boards, paper production and to fire grain drying furnaces; the methods in use in 1973 were catalogued by the NFU.

The variability in straw quality, its seasonal nature and its bulk are difficulties which have to be overcome before considering uses of straw which involve its removal from the site of production. Consequently burning has become the most economical method of disposing of waste straw for the farmer (Mclean, 1973). Stubble burning has been part of accepted farming for centuries while only recently has burning the straw as well come to be standard practice. Stubble burning alone was not so widespread nor on such a scale that it gave rise to more than local comment or problems. However the changes in farming technique previously outlined have meant that straw burning has become an integral part of post-harvest operations in many parts of England, the problems of smoke and damage to hedges and wildlife have increased correspondingly, causing "official and public concern" (NFU, 1973).

Opinions vary as to the desirability of burning waste straw. The case for (other than financial considerations) is mostly based on the feeling of farmers that burning reduces many crop parasites and pests. The efficacy of this in some cases cannot be denied (Hughes, 1978; Howell, 1969; Fromm and Feltz, 1976). However this effect is mainly restricted to organisms which survive on plant debris ie saprophytes and does not apply to those which require living tissue for growth. There is no evidence that burning reduces levels of weeds (except in some situations wild oats) nor levels of insect pests (ACAH, 1973; NFU, 1973).

There are two real alternatives to burning for the disposal of straw in the field,one is to plough it in after chopping (NFU, 1973). This technique is also the subject of controversy and its safe use depends mainly on the type of soil (Schröder and Grewehr, 1970). The costs and difficulties involved are reflected in the small proportion of straw production dealt with in this way. Long term trials carried out by the Ministry of Agriculture, Fisheries and Food have shown that ploughing straw in has little or no long term effect on the productive capacity of the soil, provided that adequate NPK dressings were provided (McLean, 1973). Other workers have shown that there is a possibility of increase in the release of Mn and Fe ions into the soil where large amounts of plant residues are incorporated into normally well drained soil, thus creating the potential for manganese toxicity (Elliott and Blaylock, 1975). Cochran *et al* (1977) have shown that phytotoxic products are produced from wheat and barley straw when ploughed in, but only after the debris has been colonised by microorganisms. Iswaran and Harris (1968) showed that phytotoxicity was brought about when straw incorporated into soil was colonised by an early flush of facultative anaerobic bacteria, many of which were toxigenic to plants causing

inhibition of root growth. The second possibility is that of direct drilling. The field is not ploughed and crop residues remain. The cereal is then sown directly into the soil, so reducing the time and expense normally incurred by ploughing and preparation. Over the past few years much work has been carried out to develop other outlets for excess straw, for example the production of pulp for paper mills (Wilton, 1975; Atchison, 1976), the use of wheat straw to produce pulp for the fabrication of a corrugated medium for use in packaging (Madan and Jain, 1976) and the burning of straw directly to produce heat for drying or in engines to provide mechanical or electrical power (Leach, 1976). It has been suggested that waste straw could be converted by pyrolysis into a fuel oil (McCann and Sadler, 1976), equipment has been developed which can 'plant' straw in fields to produce protective hedges for newly sown crops (Anon. Farmers' Weekly, January 21st 1977) and it is possible to produce compressed straw briquettes which may be used to replace low grade coals in industry and on the farm (Anon. Farmers' Weekly, April 11th 1975). This is a small sample of recent non-feedstuff research, the amount of which has led to the suggestion that straw may be grown for industry and the by product become a crop in its own right (Stacey, 1976). These trends are encouraging, however as can be seen from the figures these alternatives have not reduced the amount of waste straw which is burnt to date.

1.2 THE UPGRADING OF STRAW

When investigating outlets for excess straw a further consideration must be whether using waste straw outside agriculture as a non-feedstuff resource is really desirable. Straw is a potentially useful source of energy for the ruminant. One of the anomalies of cereal production is that energy is fixed in both the seed and the straw. There is about half as much energy in the straw which is usually wasted, as in the seed, which is used. However the availability of energy in straw is low,

about 70% of it is in the form of cellulose and hemicellulose, but this is bound tightly with lignin which renders it inaccessible to the gut flora of the ruminant. This will be discussed in greater detail in a later section. If the lignin bonds could be loosened or removed straw could be a useful energy source for the ruminant. Straw is a product of agriculture and as such, is already within the farm environment. The cereals which it supported were grown for consumption and hence had to be free from pesticide contamination and so consequently is the straw. It could be suggested therefore that to remove straws for industrial use is a waste of a resource especially when concern is growing about the amount of foodstuffs grown purely for animal use which could be used for human consumption (Wilson and Brigstock, 1977). In response to this feeling a number of workers have been investigating the possibilities of upgrading straw from the low quality filler which is its main feed value at present, to a much improved source of energy and/or protein for animals. This has involved chemical, physical and biological techniques, the development of new systems and also the modernising of methods which have been known for many years. Methods of improving the nutritive value of cereal straws have tried to overcome the two major feeding constraints of low intake and low digestibility. A brief review of current techniques and research follows.

1.2(i) Chemical Methods

The concept of chemically treating straw to improve its feeding value has been known for more than half a century. In 1900 Kellner and Kohler prepared fodder cellulose by boiling rye straw in a solution of sodium hydroxide (O'Callaghan, 1975). In 1922 Beckmann soaked chopped straw in 1.5% w/v NaOH for 24 hrs. This was then drained and washed in running water. The limitations of this process were in the

use of large quantities of wash water which caused losses by leaching of up to 25% of the solubilised material and produced large quantities of polluted wash water. This method is still much used in Norway, even though reports have shown that the discharge of polluted waters to streams is highly injurious to fish populations (Srekvik, Bergheim and Selmer-Olsen, 1976). In 1972 about 70,000 tonnes of feedstuff were produced using this process (Rexen *et al*, 1976). Recently research has been carried out in a number of laboratories into methods of simplifying this technique by using smaller quantities of more concentrated alkali. Temperature and pressure increases have been employed removing the need to wash the alkali out after treatment and decreasing the time involved (Wilson and Pigden, 1964; Singh and Jackson, 1971; Greenalgh, 1972). The development of this process has followed two lines, the on farm treatment of straw, and its collection for processing at a central factory. A number of firms now produce equipment to chop and spray straw in the field (Farmers' Weekly October 10th and 15th, 1976), while the large animal feed firm BOCM Silcock has a number of process plants operating in this country. However there are still conflicting reports being produced as to the economic viability of these processes for the farmer (Farmers' Weekly May 13th, July 22nd, July 29th, 1977; Taylor *et al*, 1977).

Other possible alkali treatments to increase straw digestibility have been investigated. These include the use of KOH, $Ca(OH)_2$ and NH_4OH, but their efficiency proved to be less than that of NaOH (Anderson and Ralston, 1971; Guggolz *et al*, 1971). The use of ammonia has also been studied by Loosli and McDonald (1968) and by Han and Anderson (1974). The possibility of increasing straw digestibility with ammonia has also been investigated in Russia by Laguta (1961) and Bondarev and Gurhilch (1962). The use of ammonia has the added advantage of incorporating some nitrogen into the straw. Chandra and Jackson (1971) compared the

effects of sodium sulphite, sodium sulphide, sodium carbonate, hydrogen peroxide and sodium hypochlorite with sodium hydroxide and found that sodium hypochlorite alone produced equivalent increase in digestibility, but had the disadvantage of leaving residual chlorine which inhibited the rumen microflora.

1.2(ii) Physical Methods

There are a number of physical treatments applied to straw at the present time. These are chopping, milling and occasionally grinding techniques. Such methods do not increase the digestibility of the straw and may decrease the amount which the ruminant will consume. Further research is required in this direction (Palmer, 1976). Experimental work has been carried out by Han and Callihan (1974) into the possibilities of pressure cooking straw to improve digestibility, with little success. Hart *et al* (1975) reported that steam treating rice straw will give an increase in straw digestibility of from 29-61%.

1.2(iii) Microbiological Methods

The development of biological methods of upgrading cellulosic wastes, has formed the basis of a considerable number of research projects in recent years. Most of these techniques utilise the waste product as an energy source to produce biomass and increase the overall protein content. Bacteria have been used by some workers in these processes. Han (1975) used the cellulolytic bacterium *Cellulomonas* in symbiosis with an *Alcaligenes sp* to produce protein from a rice straw fermentation. A wide range of fungi have also been investigated by a number of workers. Lekprayoon (1972) used the yeast *Candida utilis* while Han *et al* (1976) grew *Aureobasidium pullulans* on acid hydrolysates of ryegrass straw to produce a single cell protein. The fungus *Sporotrichum pulverulentium* has been used to give a product with a high protein content from the wastes of a newsprint mill (Eriksson and Larrson, 1975).

All these techniques involve the use of 'high' technology, the substrates may require considerable pretreatment and the actual degradation is often carried out in batch or continuous flow fermentors. Consequently the possibility of such processes being economically feasible in the near future is questionable (Han and Anderson, 1974). In an effort to overcome this problem research has been carried out into the use of 'low' technology whereby protein content of cellulosic wastes could be increased at the place of production (Poole and Smith, 1976). This work involves the use of two fungi growing on loose barley straw heaps after chemical adjustment of the environment. One fungus utilises the cellulose while another 'scavenges' excess carbohydrates. A similar process to this has been developed by Heltay and Petöfi (1965). In this case a compost was produced from rice straw supplemented with a nitrogen source. The whole was pasteurised and inoculated with *Agaricus bisporous* in a similar way to commercial mushroom production. The resultant compost was termed "Mycofutter" and it has been reported to be a suitable protein and carbohydrate source for "all meat, milk, egg and wool producing domestic animals".

Little work appears to have been undertaken on the microbiological colonisation of straws and other cellulosic substrates to improve their digestibility, rather than to improve the protein content. Zadražil (1974, 1975) suggested the use of spent compost on which *Pleurotus sp* had been grown as a ballast feed for domestic animals.

The potential of using isolated extra-cellular fungal cellulases to hydrolyse waste substrates has been investigated by a number of workers (Dunlap 1975) but these are usually designed with the intention of retrieving the carbohydrates so produced for use as chemical feedstock in other processes. Gupta *et al* (1973) used *Trichoderma viride* extra-cellular cellulase to degrade rice straw and newsprint in this way.

These techniques often involve manipulation of the genetic material of the fungi to produce mutants yielding more active enzymes in larger quantities (Eriksson, 1974). Here again complex equipment is required.

It has been established that little work has been carried out on the fungal upgrading of waste straw to increase digestibility, though that which has, has shown promise (Worgan, 1976). This then was the basis of the study being carried out at the Biodeterioration Information Centre; to develop a 'low' technology process by which the digestibility of waste straw could be increased using fungi.

1.3 THE FEEDING VALUES OF STRAWS

The straws most usually encountered in Britain are those of wheat, barley and oats. The value of these as feedstuffs to ruminants varies considerably, depending upon the method of harvesting, on the season and also on the biochemical composition and structure of the straws (Table 1.2). Because straws are harvested at maturity when the cereal stems are greatly thickened to support the ripe ears, the lignin content is high and this correspondingly reduces their digestibility to the ruminant.

Table 1.2 The Feeding Value of Straw (mean values from a number of varieties)

	DM %	SE (Per 100 lb)	PE (Per 100 lb)	CRUDE FIBRE %
Wheat Straw	86.0	13	0.1	36.6
Barley Straw	86.0	23	0.7	33.9
Oat Straw	86.0	20	0.9	33.9
Medium Meadow Hay	85.0	32	3.6	28.1
Poor Meadow Hay	85.0	27	2.9	30.4

These thickened supportive tissues differ between varieties of the same cereal, giving different digestibility values. This could be significant in the energy contribution to diets (Palmer, 1976). Yet few workers report the variety of straw used in feeding trials. Various methods of feeding straw have been employed, as a forage alone or as a supplement to other forages in the diet. Recent work has shown that its use as a replacement for up to 30% of the metabolisable energy, primarily for beef cattle is possible after grinding and milling through a 4 mm screen (Palmer, 1976). However its primary value is as a fibrous filler, it has little value for pigs and none for poultry. It has been estimated on 1974 prices that at 15% of straw in the diet, total costs would need to be 33% cheaper than the equivalent concentrate without straw because of lowering of performance (NFU 1973). There is therefore considerable scope for a straw product with improved digestibility levels to ruminants.

1.4 STRAW, AMMONIA AND THE RUMINANT DIGESTIVE SYSTEM

Before considering the production of a ruminant feedstuff it is necessary to understand at least the basic functioning of its digestive system.

There exists an intimate functional mutualism between the ruminant and its gut microbiota. The micro-organisms depend on the ruminant for the intake of food, its mixing and propulsion, secretion of saliva and removal and supply of substances through the rumen wall. The ruminant gains the ability to utilise food sources which its own enzymes would not be capable of dealing with. One of the most important host adaptations is the subdivision of the stomach into four compartments; the rumen, the reticulum, the omasum and the abomasum. The first two of these compartments form a large functionally integrated sac, the rumen-reticulum which is filled with digesta and micro-organisms. The favourable conditions in the rumen permit the growth of many species of

bacteria and protozoa. Because the rumen-reticulum is in front of the compartments where the animal's own digestive activities occur the ruminant can utilise the bodies of the micro-organisms and some of their 'waste' products as a source of nutrients.

The main constituents of straw are cellulose, hemicelluloses, miscellaneous pentosans, structural proteins and lignin (Rege, 1927). Micro-organisms are found in the rumen which can break down and utilise all of these except lignin.

Counts of cellulose digesting bacteria in the rumen are in the order of 10^6 or 10^7/ml (Hungate 1950, 1957; Kistner, 1960; Kistner *et al*, 1962). There are also protozoa present which are capable of utilising cellulose, although it has been postulated that symbiotic cellulolytic bacteria are present in the protozoa and are the active organisms (Hungate, 1966).

Hemicelluloses will undergo digestion in the rumen to about the same extent as cellulose (Heald, 1953). McAnally (1942) showed that pure hemicellulose is almost completely digested.

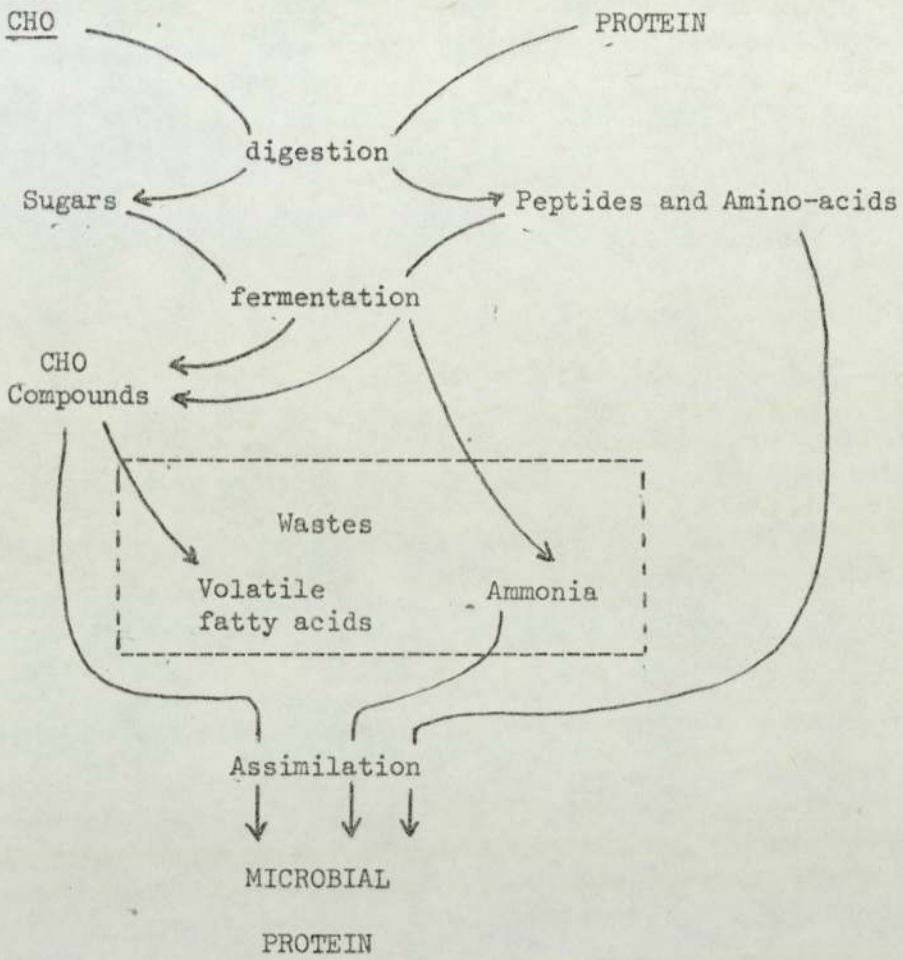
The nutritional requirements of the rumen bacteria are of considerable practical interest since much of the ruminant feed is used by the rumen microbes and is only indirectly destined for the host. The carbohydrate and nitrogen nutrition of the ruminant and its rumen microflora are closely interrelated (Fig 1.1). This must be taken into account when considering a ruminant feedstuff. Proteins are the most common nitrogenous materials in forages and in nature they provide the major part of the nitrogen used by the rumen microflora and the host. The most obvious method of dealing with proteins would seem to be for the rumen microflora to digest and assimilate them directly into microbial protein using the high energy phosphate available from fermentation. This does occur, but amino acids may also be used in other ways. Some anaerobic

microorganisms use them to yield high-energy phosphates eg arginine can yield these in the process of splitting off nitrogen. (Hungate, 1966). When protein is fermented in this manner to provide energy, ammonia is formed. This may be assimilated again into amino-acids, but the reaction is only possible when carbohydrates are available. The relative importance of proteins as energy sources or as monomers for cell synthesis depends on the ratio of carbohydrate:nitrogen in the rumen. Ammonia is essential for some rumen bacteria and stimulatory for others (Hungate, 1966) and is found at levels of 0-130 mg/100 ml in the rumen (Johns, 1955).

Urea is hydrolysed in the rumen to ammonia and carbon dioxide (Huhtanen and Gall, 1955). The ammonia thus formed is assimilated as outlined above. Nitrogenous constituents of the feed undergo microbial conversions in this way before they are assimilated by the host. This has led to the practice of feeding ruminants simple forms of fixed nitrogen such as urea. The feeding of good quality proteins would be wasted, these being broken down in the rumen and reassembled as microbial protein. Huber, Lichtenwalner and Henderson (1974) fed corn silage to lactating cattle to which 0.43% ammonia solution had been added immediately prior to feeding. They also treated the silage with ammonia solutions at ensiling and found that higher milk yields were obtained than with urea treated silage. The activities of the rumen microflora must be taken into account when considering a straw upgrading process. Increasing protein content is often worthless if the feed is aimed at ruminants, as the amino acids will only reach the animal indirectly after having been metabolised by its gut microorganisms.

Fig 1.1

Carbohydrate and Nitrogen Interrelationships in the rumen (from Hungate, 1966)



1.5 THE CHEMISTRY AND ANATOMY OF STRAW

Straw can be considered as the aerial part of a cereal plant which remains after harvesting of the ears. This consists mainly of hollow stems and the remainder of the leaf material. Most of the material remaining at harvest is from dry cell walls with an outer cuticle layer and a middle lamella between the cells. This wall is composed of a primary cell wall formed during growth and mainly in epidermal, fibre and xylem cells a secondary wall is laid down within the primary, after growth has ceased. This is composed of three layers, the S_1 layer (thin outer) S_2 (thick middle) and S_3 (thin inner).

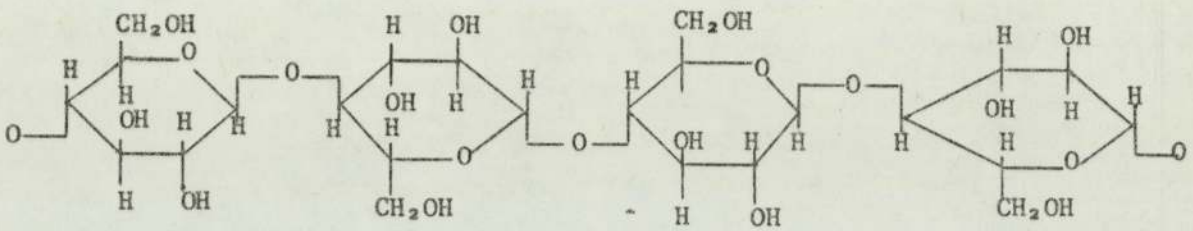
The main constituents of straw are as follows:-

1.5(i) Cellulose

Cellulose is one of the major constituents of straw amounting to 45-50% of its total weight (Rege, 1927). Chemically it is a linear polymer of D-glucose. The glucose residues are joined together through β-glycoside linkages as in cellobiose, and cellulose may be thought of as consisting of repeated cellobiose units. (Fig 1.2).

Fig 1.2

Structure of the Cellulose Molecule



Cellulose is highly susceptible to attack by a number of micro-organisms. The mechanism of cellulose breakdown has been closely investigated over a number of years. The degradation of cellulose is brought about by a complex of enzymes which are collectively termed cellulases. Reese *et al* (1950, 1952) postulated that a non hydrolytic enzyme C_1 was responsible for the initial attack on native cellulose, followed by a hydrolase C_x which completed the degradation to soluble sugars. This was modified to a "multiple Cx" theory (Reese, 1959). More recent work by Selby and Maitland (1965, 1967) has shown that cellulases can be fractionated into three components, a C_1 component which may or may not be an enzyme which "activates" the cellulose, a Cx hydrolytic enzyme which breaks down cellulose to cellobiose and monosaccharides, (this acts at the ends of the molecule and is an exo-1-4 β glucanase)and a second Cx enzyme which hydrolyses the amorphous cellulose randomly at the internal glycoside bonds to produce

shortened molecules, this is an endo-1-4 β (glucanase). So that it is now felt that for the degradation of cellulose there is a cellulase system containing 'sub-cellulases' which will attack cellulosic chains of a certain length to produce between them glucose, cellobiose and cellotriose.

Some fungi, for example *T 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 postulated that an additional 'X' factor, absent in such fungi, must also be present functioning to break the ligno-cellulose bond.

1.5(ii) Hemicelluloses

Hemicelluloses are heteropolysaccharides (Pigman and Goepp, 1948) that is, they yield two or more monosaccharides or related compounds on hydrolysis. They are metabolisable by a wide range of micro-organisms and constitute about 35% of the dry weight of straw (Chang, 1967). The hemicelluloses found in straws are mainly pentosans, that is, they yield pentose sugars on hydrolysis by fungal enzymes, the most common product being D xylose. There are no pure polymers of xylose, the existing ones are termed xylans which comprise a polyxylose backbone with side chains of uronic acids and some L-arabinose. Xylan is about 20% of wheat straw by weight (Sørensen, 1957).

1.5(iii) Lignin

Lignin comprises about 10-14% of the dry weight of straw (Chang 1967). According to Nord and Schubert (1967) the term Lignin does not imply a single and chemically defined compound but refers to a group of highly polymerised amorphous substances possessing a similar composition. The lignin found in monocotyledons is defined as "a constituent which when oxidised with nitrobenzene yields hydroxybenzaldehyde, vanillin and

syringaldehyde" (Brauns, 1960). It is a three-dimensional polymer which is somewhat resistant to microbial attack. The main exoenzymes which are produced by fungi capable of degrading lignin appear to be laccase and peroxidase (Trojanowski, 1969). These according to Trojanowski are active in the process of demethoxylation of vanillic acid and lignin. The demethoxylation process constitutes an important step in the primary process of lignin breakdown (Leonowicz and Trojanowski, 1965). However, the role of fungal laccase (polyphenol oxidase) in lignin depolymerisation is still a matter for dispute and interpretation.

1.5 (iv) The relationship between the basic polymers in straw

The linear polymeric molecules of cellulose are aligned together to form 'fibrils' with a diameter of about 35Å which come together in bundles to form 'microfibrils' with a diameter of about 250Å (Talmadge *et al*, 1973). Within the microfibrils are crystalline regions called 'micelles' which are interrupted at regular intervals by amorphous regions.

Hemicelluloses form an amorphous matrix binding the microfibrils together (Jane, 1970). Siner *et al* (1976) showed that decomposition of beechwood cellulose was much increased after pre-treatment with xylanase. They conclude that in this case the cellulose could only be attacked after the xylan was at least partially removed and this supports the theory that xylan is deposited between and encrusts the cellulose fibrils.

Lignin is laid down after cell growth has finished, mainly in the middle lamella and primary cell wall. It would appear to hold the microfibrils and therefore probably also the hemicelluloses into fixed positions (Talmadge *et al*, 1973) to form the "ligno-cellulose complex" (Jane, 1970).

Other components found in the cell wall are pectins (Jane, 1970), some glycoproteins (Muhlethaler, 1967) and silica (Lloyd, 1921). The presence of the latter may be of importance in reducing the susceptibility of the cell wall to microbial attack (Van Soest and Jones, 1968) together with the effects of the 'ligno-cellulose' complex.

1.6 THE PROPOSED TECHNIQUE

When investigating the prospects of upgrading wastes biologically, many authors have turned to organisms whose characteristics may be well understood and documented, but which occupy an ecological niche unrelated to the waste product in question. This usually means that the waste must undergo often expensive pre-treatments and sterilization before optimum conditions for the growth of the chosen organism can be obtained. There is an argument however for choosing an organism with desired characteristics from the indigenous microbial flora of the waste product, one which dominates during natural decomposition under conditions which can easily be controlled and reproduced in the laboratory or in industrial and agricultural situations. Such an organism may also be adapted to the efficient utilization of the proposed substrate, and may therefore have an ecological and physiological advantage over the non-indigenous one. Whether or not this is the case, by providing suitable selective conditions, it is possible to encourage the dominance of a selected organism in non-sterile substrates. Beer and wine production and the process of cheese maturation are examples of this. It was decided to look at the problem of waste straw upgrading from this angle and to investigate techniques which would rely on low technology and could if possible be carried out on the site of straw production. The aim was to isolate an organism from waste straw itself which would respond to environmental control such that it becomes the dominant

coloniser of the straw. The product of this controlled compost would have increased digestibility to the ruminant and possibly also increased protein content, although this is of secondary importance.

Earlier workers (Eggins and Seal personal communications) have isolated *Coprinus cinereus* (Schaeff ex Fr) fairly consistently from composting pig slurry/straw manures. Rege (1927) isolated *C cinereus* from rice straw and also investigated its ability to decompose cellulosic materials. Eastwood (1952) isolated what was identified as *C lagopus* from composting barley straw, this however was probably *C cinereus* as *C lagopus* is usually confined to leaf litter (Chang and Hudson 1967). Chang and Hudson also isolated *C cinereus* from nitrogen amended straw composts after the peak heating periods. The pH of most of these composts was high, usually around 8.5 which was probably due to the presence of ammonia produced by the bacterial breakdown of urea or urine in the composts. However, those composts where ammonium salts were added as the nitrogen sources (Eastwood and Chang and Hudson) usually maintained a lower average pH although still above pH 7. Preliminary work showed that *C cinereus* could be selectively isolated at alkaline pH using ammonia as a nitrogen source (Penn 1977). Other work carried out in this laboratory (McShane 1976; Penn 1977) has suggested that *C cinereus* has physiological characteristics which may be useful in a straw biodegradation process, so that the starting point of this project was to investigate further the effect of ammonia on the fungal flora of straws.

There were two main reasons for choosing ammonia as the selective agent in this work. Previously the use of urea had promoted *C cinereus* growth, probably because of the release of ammonia, but ammonia solution appeared to be much more selective. The second factor which was considered was the possibility of linking this upgrading process to an animal slurry treatment technique which was under investigation (Seal,1973). This

involved increasing the pH of slurry to above 7 at which point ammonia gas was released (Culp and Culp, 1972). The ammonia was then blown off and collected as the slurry passed through a specially constructed tower. This reduced the nitrogen content of the slurry and produced ammonia which could possibly be used in the straw upgrading process. The basis of the proposed technique, was to encourage the growth of an endemic fungus on waste straw using freely available ammonia. This would adjust the environmental conditions to select for its growth.

1.7 *C CINEREUS*, THE ORGANISM AND ITS CONFUSED NOMENCLATURE

C cinereus was selected for use in the proposed technique. This organism is a mesophilic basidiomycete often associated with manure piles, compost heaps and other masses of self heating plant material. Fries (1955) published an extensive investigation into the physiology of the genus *Coprinus* where she studied the growth substance, nitrogen and carbon requirements of twenty-six different strains representing nineteen species of *Coprini*. In other papers (1953, 1956) she investigated temperature and pH requirements of a similarly large number of strains.

The physiology and genetics of *C cinereus* in the laboratory has been investigated by many workers (Day and Anderson, 1961; Moore, 1967; Madelin, 1959). Their work will be discussed more fully in later chapters in conjunction with the results obtained from this piece of work. Buller (1924) remarked that "there is a good deal of confusion in the literature of mycology in respect to the nomenclature of *Coprinus lagopus* and its allies" and this confusion has persisted to the present day. *Coprinus cinereus* (Schaeff ex Fr) S F Gray sensu Konr has been studied as *C lagopus* (Anderson, 1959; Casselton, 1966; Cowan and Lewis, 1966; Day, 1963; Day and Anderson, 1961; Lewis, 1961; Madelin, 1956; Moore, 1967; Morgan, 1966), as *C stercorarius* (Brunswick, 1924; Mounce, 1921 and 1922), as *C macrorhizus* (Buller, 1924; Dickson, 1936), as

C fimetarius (Fries, 1955) and as *C fimetarius var cinereus* (Buller, 1931). The major confusion has arisen between the identities of *C lagopus* and *C cinereus*, very similar species which arise on soil and litter and straw and dung respectively. Orton (1957) maintained that the *C lagopus* described by Buller (1924) was in fact *C cinereus*, as it was described from dung composts. Many workers return to this description of Buller's so that a vast amount of confusion has arisen. The problem was the subject of a long and detailed paper by Pinto-Lopes and Almeida (1970). They used previous literature and experimentation in an effort to unravel synomyms, relationships and incorrect nomenclature.

Many workers have used *C cinereus* as a tool in genetic experiments because of its ease of culture in the laboratory. However most of these have named their fungus *C lagopus*, so that the confusion has been perpetuated. It is often difficult to compare results of work carried out in different laboratories because the identity of the fungus under examination is often disputed and cultures are rarely lodged with relevant national collections to allow examination.

The isolates used in this piece of work have all been positively identified as *C cinereus*. The initial isolate was identified by Roy Watling at the Royal Botanic Gardens Edinburgh and subsequent isolates were identified by key (Orton, unpublished data) and by crossing monokaryons with those of a known *C cinereus* culture (Appendix 2). *C cinereus* is the only *Coprinus* species which will grow well at 30-35°C and also produces highly characteristic microsclerotia (Watling personal communication).

1.8 AMMONIA AND MICRO-ORGANISMS - HEALTH CONSIDERATIONS

Preliminary work indicated that ammonia may possibly select for and encourage the growth of *C cinereus*. It should be possible for the animal's gut microflora to assimilate any residual ammonia which may be consumed. However there are dangers from the ingestion of excessive

amounts of ammonia. This is recognised by the farmer so that the quantities of urea fed are limited to a relatively low controlled level. Part of the ammonia produced in the rumen is absorbed via the portal system and passes to the liver (Lewis, 1960). Normally the bulk of this is removed there and converted to urea. Under some circumstances the quantity of ammonia absorbed by this route exceeds the level which the liver can detoxify and signs of toxicity appear. (Lewis, Hill and Annison, 1957). Carbohydrate starvation can contribute to ammonia toxicity because in their absence proteins are often fermented and this process yields ammonia (Juhasz, 1962). An increase in rumen pH will also increase the possibility of toxicity. Bearing this in mind levels of ammonia which remain in the product after upgrading must be investigated. However it is unlikely that these will be dangerously high because as previously stated, silage with relatively large amounts of ammonia has been fed to cattle very successfully.

A second health factor which must be considered is the use of micro-organisms in the proposed technique. Since the initial identification of 'turkey X disease' in 1960's as the result of the production of mycotoxins in feedstuffs, much research has been undertaken into the production of toxins, the fungi involved and the conditions required for the occurrence of mycotoxins. Fungi which are known to be toxin producers are frequently found on straw eg *Fusarium spp* and species of *Aspergillus*, so that the organism which is used in the process must grow to the exclusion of other fungi. However, ammonia has proved to be useful in inactivating aflotoxin in corn (Brekke, Sinnhuber *et al*, 1977).

Fungi can be dangerous to both man and animals in other ways. A. *fumigatus* can cause *Aspergillosis* in the lungs of both and has been implicated in causing mycotic abortions in cattle. Actinomycetes are known to initiate allergic reactions in man eg Farmers' Lung disease.

Consequently these factors must be checked and controlled. This is investigated further in later chapters.

1.9 OBJECTIVES OF THE RESEARCH PROGRAMME

The objectives of the project as a whole have been discussed in a previous section. The scope of the work detailed in this thesis is restricted to a study of the ecology of the fungi, and to a lesser extent other microorganisms, which are associated with straw under artificially produced and controlled 'composting' conditions.

It has been postulated that ammonia can create an environment which will bring about the selective growth of *C cinereus*. The work set out here was undertaken to confirm this. The major objectives of this research programme can be listed as follows:-

1. To investigate *C cinereus* and its appearance on straws from the field. This involved the devising of a selective technique for its isolation from soils and vegetable matter, and the utilisation of this method to study the distribution of *C cinereus* in agricultural environments.
2. To confirm the selective nature of ammonia towards *C cinereus* and to assess the extent of selectivity at different levels.
3. To study the effect of ammonia solutions on microbial populations and relationships, enabling the means by which ammonia brings about *C cinereus* selection to be elucidated.
4. To lay down preliminary parameters which will bring about the selective growth of *C cinereus* on straw, and to ensure that a controlled compost can consistently be produced.

CHAPTER TWO

ISOLATION OF *COPRINUS CINEREUS* FROM STRAWS

2.1 INTRODUCTION

The aim of the work detailed in this chapter was to study the effect that amending straw with various sources of the ammonium ion had on the mycoflora of that straw, and particularly to investigate the effects on the growth of *Coprinus cinereus* in relation to the rest of the fungal population. It was in no way intended to be a complete isolation programme as selective features were introduced at all stages of the work, it being the intention that only actively growing fungi were studied which could be isolated successfully on Eggins and Pugh cellulose agar (Eggins and Pugh, 1962) at an incubation temperature of 30°C. It was felt that these conditions would isolate the majority of fungi which may compete with *C cinereus* and also that the use of cellulose agar would not preclude the isolation of non-cellulolytic organisms although it may mean that they may be under represented (Dickenson and Kent, 1972).

2.2 GENERAL ISOLATIONS

2.2(i) Materials and Methods

Three sources of ammonia were studied along with five sources of straw, the necessity or otherwise of adding mineral salts to the straw to encourage *C cinereus* growth was also investigated.

The sources of straw were as follows:-

A. Shrewley, Warwickshire - Barley straw from the bale.

B. Northampton - Barley straw from the field.

C. Sarn Bach, Gwynedd - Barley straw from the field.

D. Worthing, Sussex - Wheat straw from the field.

E. Spalding Lincs. - Barley straw from the field.

Five grammes of air dry straw were placed aseptically into glass jars of 454 g capacity, three jars per treatment, these being then amended as shown in Table 2.1. The percent ammonia was calculated as the actual weight of ammonia in AR solution per gramme of air dry straw and other ammonia sources were calculated to have the same amount of nitrogen as contained in Eggins and Pugh cellulose agar, excluding yeast extract (appendix 1). The nitrogen sources were made up to 20 ml with sterile distilled water and added to the jars which were capped with aluminium foil, the jars were then rotated to ensure even distribution of the solutions over the straw. These were incubated at 30°C for 10 days after which five 5 cm strips of straw were removed aseptically from each jar, washed with five changes of sterile distilled water (Harley and Waid, 1955) chopped, and plated on to Eggins and Pugh cellulose agar with the addition of rose bengal to suppress bacterial growth. The details of this technique and results of preliminary experiments carried out are given in appendix 2. Fifteen plates per treatment were incubated at 30°C ± 1°C and observed after 4 and 7 days; the isolates were identified and the percent of *C cinereus* and other isolates noted. Isolations were also carried out on straws from three of the above sources before incubation or amendment, these being washed and plated as previously described.

The initial and final pH of the straws in the jars were taken by soaking 1 g of straw in 20 mls of distilled water for 1 hour and the pH taken using a glass electrode with a Pye model 78 pH meter.

The pHs were noted as they may have been important in determining the isolations obtained and pH changes brought about by microbial activity were also noted.

Table 2.1 Nitrogen Sources Added to Straws

Treatment Number	Treatment
1	Mineral Salts Medium(1) + 10% ammonia(2)
2	Mineral Salts Medium + 1% ammonia
3	Mineral Salts Medium + 0.1% ammonia
4	Mineral Salts Medium + 0.01% ammonia
5	Distilled water + 10% ammonia
6	Distilled water + 1% ammonia
7	Distilled water + 0.1% ammonia
8	Distilled water + 0.01 ammonia
9	Distilled water.
10	Mineral Salts Medium + Urea(3)
11	Mineral Salts Medium + ammonium sulphate(3)
12	Mineral Salts Medium + ammonium sulphate and fumaric acid(4)

(1) Mineral salts as in Eggins and Pugh medium (appendix 1)

(2) Ammonia in proprietary AR ammonia as percent of weight of air dry straw

(3) Added at levels equivalent to the total inorganic nitrogen in Eggins and Pugh medium

(4) Added as a buffer at a level of 2g/l of solution (Fries 1955).

2.2 (ii) Results and Discussion

The results are detailed in table 2.2 and from these it can be seen that at 10% ammonia levels no isolates were obtained while at 0.1% and 0.01%, other species of fungi were observed as well as *C cinereus*. However at the 1% ammonia level, on all but one occasion, *C cinereus* was isolated as the only actively growing fungus on the straw.

It can be seen that the use of mineral salts was unnecessary for the successful selection of *C cinereus* and that urea maintained alkaline conditions and encouraged *C cinereus* growth but not to the exclusion of other fungi.

Ammonia in the form of ammonium sulphate did not select for *C cinereus*, possibly because of the drop in pH which was not successfully controlled by the addition of fumaric acid as used by Fries (1955). Table 2.3 and table 3.1 in appendix 3 show the percentage appearance of isolates on the straws from different areas after the twelve treatments, and figures 2.1-2 illustrate various points of these results graphically. Figure 2.1 shows the species and number of fungi isolated from three non-amended straws. The isolations were carried out within two days of the straws' arrival at the laboratories and therefore should have given an indication of the fungi which may have been actively growing on the straws in the field or during storage.

Aspergillus fumigatus was found on all three straws to varying degrees. The large amount found in straw B could be explained if the sample, prior to collection, had been lying in the field for some time under damp conditions, although if this were so, one would expect to find elevated levels of all other isolates, which did not occur.

Alternaria tenuis was isolated from all samples, and in large amounts from samples B and C. This organism is widespread and has been isolated from rhizospheres of wheat (Jooste, 1966; Mangan, 1967) oats and barley (Kirilenko, 1968), it is also a frequent coloniser of other

Table 2.2 Isolation of *C cinereus* from nitrogen amended straws

Treatment No.	Mean pH of 3 jars		Straw Collection locations				
	Initial pH	Final pH	A	B	C	D	E
1	9.8	9.0	(1) -	-	-	-	-
2	9.0	7.6	++	+	++	++	++
3	7.8	7.2	+	+	+	(+)	+
4	6.9	6.8	(+)	+	(+)	+	(+)
5	10.0	8.5	-	-	-	-	-
6	9.2	8.0	++	++	++	++	++
7	7.4	7.3	++	+	+	(+)	++
8	7.2	7.2	+	(+)	+	(-)	(+)
9	6.5	6.6	(+)	(+)	(+)	(+)	(+)
10	7.3	7.8	+	+	(+)	+	(+)
11	6.7	5.6	(-)	(+)	(+)	(+)	(+)
12	6.4	5.8	(-)	(+)	(+)	(+)	(+)

(1) - = *C cinereus* was not isolated

(-) = *C cinereus* was not isolated but other fungi were

++ = *C cinereus* alone was isolated

\+ = *C cinereus* + *Chaetomium globosum* were isolated

(+) *C cinereus* + a number of other species were isolated

Table 2.3 Isolations From Unamended Straws

Source of Straw	Percent Appearance (15 plates)						
	Aspergillus fumigatus	*Coprinus cinereus*	*Chaetomium globosum*	*Alternaria tenuis*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*
Shrewley	26.67	6.67	100	6.67	60	6.67	6.67
Northampton	10.0	0	6.67	100	66.67	6.67	0
Sarn Bach	20	6.67	33.33	100	66.67	0	0

vegetative parts of plants and has been isolated from the culm base of rye, wheat and barley (Lal and Yadav, 1964) and is also frequently isolated from air spora. *A tenuis* has an optimum temperature for linear growth on agar at 25-26°C but will grow up to a maximum temperature of 31-32°C (Pestinskaya, 1956) and has a capacity for cellulose decomposition on varied substrates, hence its isolation in this piece of work could be fully expected.

Chaetomium globosum was also isolated from all three straws; 100% isolation was obtained from straw A and lesser amounts from straws B and C. This is the commonest of the *Chaetomium* species, especially on plant remains and other cellulosic substrates and it has often been isolated from straw and straw composts (Eastwood, 1952; Chang and Hundson, 1967).

C cinereus appeared on a single plate from straw A and similarly from straw C and was not isolated at all from straw B.

A species of *Fusarium* was found to be colonising all straws in about equal amounts. The genus *Fusarium* has an extraordinarily wide range of host plants and is known throughout the world principally as a pathogen of cereal roots and ears. There are reports (Bruehl and Lai, 1966; Lai and Bruehl, 1968) that straw colonised by *Fusarium sp* is only slightly colonised by other organisms and that dead straw is, under some conditions, less intensively colonised then live (Cook and Bruehl, 1968).

Sordaria fimicola was isolated on one plate from one straw sample. This organism has previously been shown to respond to selective techniques for cellulolytic fungi (Pugh *et al*, 1963) and has been isolated from arable land, (Miller, Giddens and Foster, 1957) from permanent wheat fields, on mouldy plant remains (Frankland, 1966; Yadav, 1966) and on seed material of plants, including wheat (Machácek *et al*, 1951) oats and barley (Malone and Muskett, 1964). It is possible that this isolate was from a spore contamination from the soil, although it does have a fast rate of growth and can be isolated with screened

immersion tubes (Chesters, 1948) so that it may possibly have colonised the straw whilst it lay in the field.

Trichoderma viride is used here as a species aggregate to include all *Trichoderma* isolates with green conidia (Webster, 1964; Rifai, 1969) and was isolated on a single plate from straw A and similarly from straw B. This is one of the most widely distributed soil fungi and it occurs as an early coloniser on numerous plant roots. It has an optimum temperature for linear growth and for mycelium production between 20 and 28°C, but good growth has been recorded up to 32°C (Ward and Henry, 1961).

These initial isolates compare quite closely to the results obtained by Penn (1977) in his more comprehensive isolation programme.

Having established a 'baseline' of the mycoflora from fresh straw which was isolated under the detailed conditions, the results from treatments 1-12 can be compared with these and each other and hence the changes in the mycoflora studied. Table A.3.1 shows the fungi which were isolated and the percent appearance of each one for each of the straw samples A-E and for treatments 1-12. In all cases of raised pH and ammonia concentrations *C cinereus* was the dominant fungus, being joined by *Ch globosum* as the amount of added ammonia was reduced; further species were isolated as the amount of ammonia added was lowered to 0.01% of the straw.

In treatments 3 and 7 (0.1% ammonia) *Scopulariopsis brevicaulis* was isolated from sample D, this was at an initial pH of between 7.4 and 7.8, being about the optimal for the growth of *S brevicaulis* which has been quoted as between pH 7 and 8 in vitro (Krempl-Lamprecht, 1961). This species is also a mesophile, tolerating a temperature maximum of 38°C (Apinis, 1963) and there are many reports that this organism will decompose cellulose (Marsh *et al*, 1949; Waksman, 1916). Therefore the conditions provided by treatments 3 and 7 were ideal for the selective growth of *S brevicaulis*.

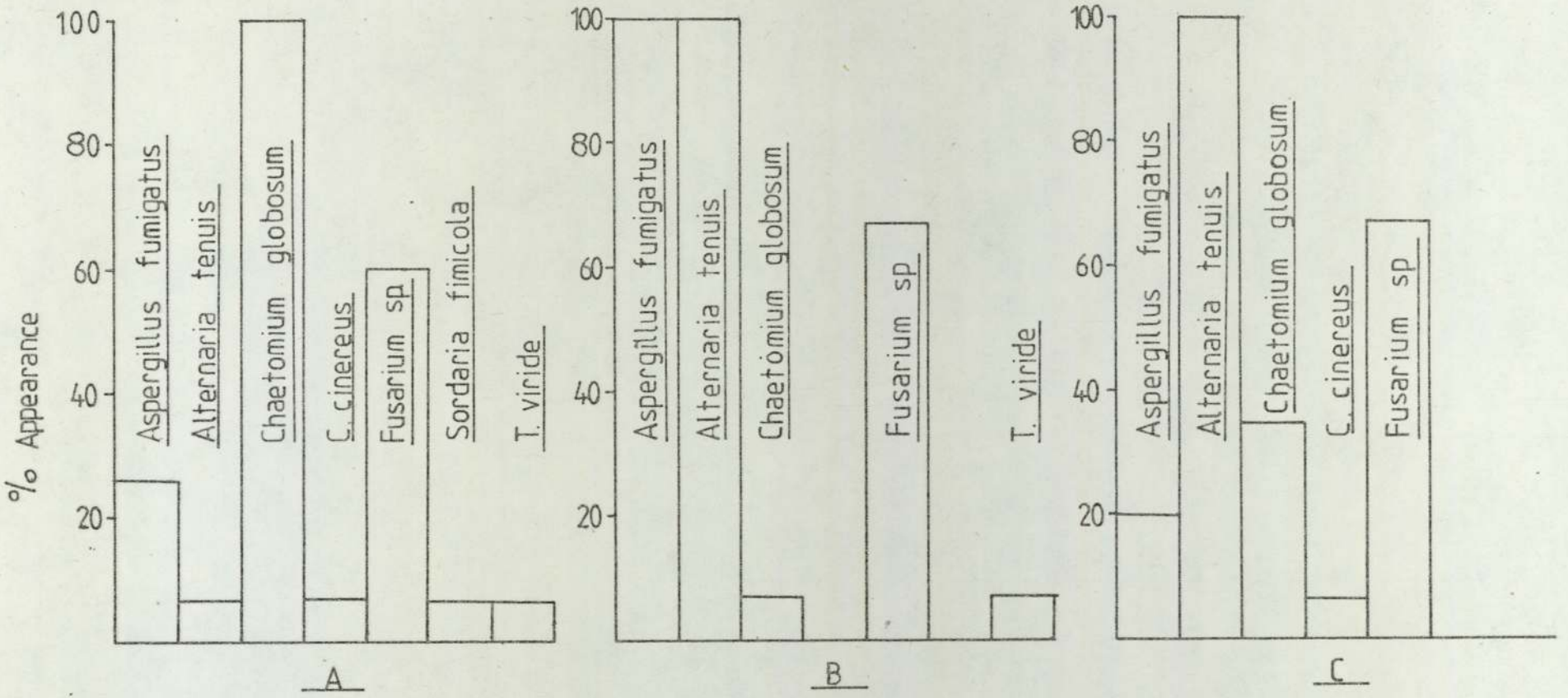


Fig 2·1 Initial isolations from unamended straws

Aspergillus versicolor was also isolated from straw D after treatment 7. This species has previously been isolated from mouldy hay (Gregory and Lacey, 1963)and appears very frequently on stored grain. Its optimum temperature for growth on agar is 25-30°C (Panasenko, 1944) and it will tolerate a very wide range of pH (Warcup, 1951). Therefore the appearance of *A versicolor* here is not unexpected.

In treatments 4 and 8 (0.01% ammonia) the above were again isolated along with a single appearance of *T viride*. *A fumigatus* was isolated from treatment 4 jars but not treatment 8 and this may indicate that the addition of mineral salts favours the growth of this fungus.

Botrytis cinerea was isolated on a single plate from straw E. This fungus is often found on rotting plant material, has a maximum temperature tolerance of 33°C and grows well at pH values between 2 and 8 (Panasenko, 1944).

The control jars (treatment 9) gave a larger number of isolates than the ammonia treated jars, but the incubation period produced a reduction in the *Fusarium sp* and *A tenuis* populations. *Fusarium sp* did not appear at all and isolation of *A tenuis* was reduced to 60% appearance in one straw sample. The inhibition of *Fusarium sp* would seem to correlate with the results of Cook and Bruehl as to the relative colonising ability of this genus on live and dead cereal straws. The reason for *A tenuis* population depletions is not quite so clear, especially as it was isolated on 60% of plates taken from straw C. It does not seem to have been brought about by antagonistic reactions with other fungi as the same isolates appear in all three straws. However, it has been stated that *A tenuis* survives better in dry habitats (Orpurt and Curtis, 1957) and the increase in RH after treatment may be the cause of its depletion.

In the control jars *C cinereus* was isolated from all straws though at much lower levels than most of the other treatments - similarly with *Ch globosum*. *S brevicaulis* was again isolated from straw D. *T viride* was isolated from two of the samples and its appearance here, but not at higher pHs, can be explained as acid conditions have been shown to favour *T viride* growth (Brown, 1958; Cowley, 1963; Taylor and Parkinson, 1964) although it has been found at pH values between 3.1 and 8. Hence at this lower pH *T viride* can compete more successfully. *Aspergillus fumigatus* was isolated here but at lower levels than in the isolations from fresh straws and this may indicate that some of the initial isolations were in fact obtained from spores rather than actively growing hyphae. *A versicolor* was also isolated.

Treatment 10 (urea) in general provided good conditions for the growth of *C cinereus* and *Ch globosum* and also for some growth of *A fumigatus* although at lower levels than in the control. The lack of any *T viride* isolations may be explained by the fact that urea is not a very suitable source of nitrogen for *T viride* (Loub, 1956).

Treatments 11 and 12 did not select especially for the growth of *C cinereus*. Treatment 11 gave a much lower percent isolation of *C cinereus* while maintaining high levels of *Ch globosum* along with single isolations of *B cinerea* and *S brevicaulis*. *A versicolor* appeared on every plate from two straws, D and E and *A fumigatus* at reasonably high levels from all but one straw.

Treatment 12 included fumaric acid in an effort to counteract the tendency of sulphate salts to turn the medium acidic as they are broken down. However, this was not successful and the isolation pattern was much the same as that of treatment 11 with *C cinereus* not being isolated at all on two occasions. There were single isolations of *Sordaria fimicola*, *Fusarium sp* and *Alternaria* and again the appearance of much *A fumigatus*. The addition of fumaric acid also seemed to favour the isolation of *T. viride*.

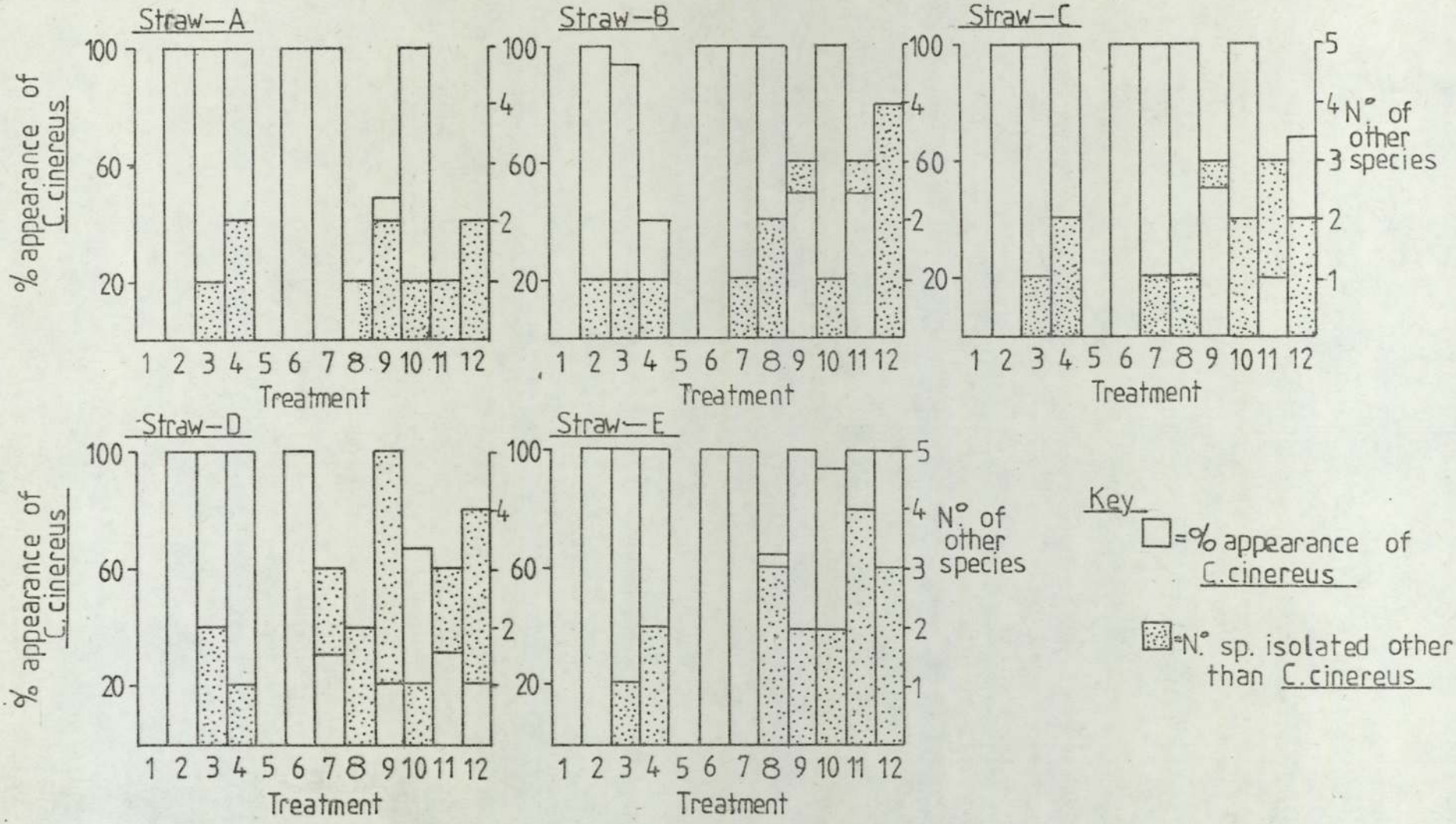


Fig 2·2 C.cinereus isolates and other species numbers compared

Figure 2.2 compares the numbers of other species isolated with the percent appearance of *C cinereus* and it can be seen that the largest variety of species on average were produced by treatments 11 and 12, followed by control treatment 9 and then the various ammonia treatments up to 10% where no isolations were obtained at all.

The total number of isolations other than *C cinereus* obtained from treatments 1-4 were compared statistically with those obtained from 5-8 (appendix 4). The results show that there was a significant difference between the number of species isolated so that treatments 5-8 produced, overall, fewer species.

2.2 (iii) Conclusions

C cinereus was isolated from straw consistently by using ammonia solutions - a figure of about 1% ammonia seemed to be the most selective for *C cinereus* isolation and this was standard over the five straws which were used.

The other treatments investigated provided useful information as to how they modified the straw mycoflora but did not produce the results required for further work.

Statistical analysis showed that ammonia used alone without any mineral salts aided in the selection process and that *A fumigatus*, a potential human and livestock pathogen was discouraged when mineral salts were not included in the medium. However, another pathogen, *S brevicaulis*, was isolated from one straw which had been treated with lower ammonia concentrations and these factors have to be considered when investigating a potential process for producing an animal feed-stuff.

2.3 STRAW SURVEY - ENGLAND & WALES

2.3 (i) Introduction

Two points emerging from the results of the previous section suggested further investigation into the abundance of *C cinereus* on straw. One was that ammonia could, it seemed, very easily be used as a selective

agent in a form of enrichment culture, making it fairly easy to isolate *C cinereus* from straw, and the other point was that all of the five straws studied at random had proved to be supporting an indigenous population of *C cinereus* either as actively growing hyphae or as spores. Also, it was felt at this time that a natural inoculum of *C cinereus* might make seeding of a compost with spores unnecessary, or on the other hand the indigenous population might possibly interfere with the growth of any spore inoculum that may be introduced. Consequently it was decided to enlarge the study and to investigate the occurrence of *C cinereus* on straws obtained from various areas of England and Wales.

2.3 (ii) Materials and Methods

During the harvest of 1975, straw samples were collected from bales and from the field (table 2.4) at 44 sites over England and Wales (table 2.5 and Fig 2.3). These were collected in sterile polyethylene containers and transported to the laboratories where they were dealt with within two days. The samples were subjected to the same conditions described in the previous section excepting that only 1% ammonia solution with no added mineral salts was used as the selective agent. Two of these samples, Sarn Bach and Worthing, were never introduced into the laboratory and the work carried out in the main university building some distance away. This was to ensure that the *C cinereus* isolations which seemed so consistent were not coming from laboratory contamination where large quantities of straw were stored and where *C cinereus* was used in pure culture. After ten days, serial washing was employed as previously described, the straws were plated onto Eggins and Pugh cellulose agar and were incubated at 30°C.

Table 2.4 Description of samples collected from sites in England and Wales

Straw	Number of Samples
Baled barley	7
Baled Wheat	5
Barley from the field	20
Wheat from the field	9
Oats from the field	3
Total	44

2.3 (iii) Results

Coprinus cinereus was isolated from every sample investigated including the two samples which had been studied away from the laboratory (Table 2.5). Plating out of the straws was not strictly necessary on a simple appearance/non-appearance survey because, after a suitable period and exposure to light, (about 7 days onward), the fungus began to fruit in the jars and could be harvested for identification from there.

2.3 (iv) Discussion

It appeared that *Coprinus cinereus* was very wide-spread on barley, wheat and oat straws and that it had been on the straws when they were harvested before baling. This could explain the numerous accounts of the isolation of *C cinereus* from farmyard/composting situations where straw and ammonia derived from urine from livestock are constantly coming into close contact. What had been done in this laboratory technique was to duplicate the conditions which occurred so readily in the natural ecological niche which *C cinereus* inhabits, thus providing an ideal situation for selective isolation.

Fig 2·3 Sampling sites in England and Wales

Table 2.5 Straw Survey - Sampling Areas and Isolation of *C cinereus*

Sample Number	Area	Straw type	Source ie Field or Bale	Presence of *C cinereus*
	Norfolk/Lincs			
1	Guist	Barley	Field	+
2	Wells-next-the-Sea	"	"	+
3	Blakeney	"	"	+
4	Lodling	"	"	+
5	Holme	"	"	+
6	Neverham	Wheat	"	+
42	Spalding	Barley	"	+
	West Midlands			
7	Sutton Coldfield	Barley	"	+
8	Wishaw	Wheat	"	+
32	Coleshill	Wheat	"	+
33	Tamworth	Barley	"	+
38	Shrewley	Barley	Baled	+
39	Northampton	Barley	Field	+
	Cheshire			
9	Twemlow	Barley	Field	+
10	Holmes Chapel	"	"	+
	Cotswolds			
11	Chipping Campden	Oats	Field	+
12	Snowhill	Barley	"	+
13	South Guiting Power	"	"	+
14	North Guiting Power	"	"	+
15	Blackwell	Wheat	"	+
16	Dorridge	Barley	"	+
17	Newbold-on-Stour	Wheat	"	+
18	Winchcombe	"	"	+
19	Stretton-on-Fosse	Barley	"	+
20	Lower Slaughter	"	"	+
21	Lower Swell	"	"	+
22	Condicote	"	"	+
23	Broadway	Oats	"	+
	Hereford and Worcester			
24	How Capel	Wheat	Baled	+
25	How Capel	Barley	Baled	+
26	Brampton Abbotts	Wheat	"	+
27	Brampton Abbotts	Barley	"	+
44	Worcester	"	"	+
28	Church Broughton	Wheat	Field	+
29	Muchover	Barley	"	+
30	Ashby	Wheat	"	+
31	Osmaston	Barley	"	+
34	Yoxhall	Oats	"	+

Sample Number	Area	Straw type	Source ie Field or Bale	Presence of *C cinereus*
	Cornwall			
35	Bodmin	Barley	Baled	+
	North East			
36	Durham	Wheat	Field	+
37	Durham	Barley	"	+
43	Hartlepool	"	"	+
	Wales			
40	Sarn Bach	Barley	Field	+
	South East			
41	Worthing	Wheat	Field	+

The use of ammonia as a selective agent for isolating from natural substrates and its possible use in a straw upgrading process will be expanded in the following chapters.

CHAPTER THREE

THE ISOLATION OF *COPRINUS CINEREUS* FROM SOILS

3.1 INTRODUCTION

Having shown *Coprinus cinereus* to be widespread on straws it was decided to extend the isolation programme to include a study of this organism in the soil; to look for its presence and hence to possibly discover the origins of the inoculum found on straws. Traditional plate techniques were first investigated followed by the designing of a method which utilised ammonia to select for *C cinereus* as in the straw isolations. This technique was then used to compare the presence of *C cinereus* in a number of farm and non-farmland soils.

3.2 PLATE ISOLATIONS

3.2(i) Techniques

The techniques used to isolate fungi from the soil are numerous and varied, as are reviews of the subject, all are in one way or another selective and will detect microbes with particular growth forms, biochemical capabilities or other properties. This, combined with the heterogeneous nature of soil which provides suitable microhabitats for a great variety of microorganisms means that it is impossible to devise a single procedure which will isolate all forms present in a soil sample. Consequently when planning a general isolation programme a number of techniques must be used in conjunction. However, the selective nature of isolation methods can be put to positive use when the study requires the isolation of a particular physiological, biochemical or taxonomic group of microorganisms.

One of the major divisions of fungal isolation techniques can be said to be into those which will isolate actively growing

hyphae and those which simply isolate all propagules, be they hyphal fragments or spores. The importance of this distinction was pointed out by Waksman as early as 1916. Bearing this in mind, it was decided that at this stage of the project the occurrence of *C cinereus* in soils would be studied alone and that no attempt would be made to discriminate between actively growing hyphae and spores.

The dilution plate technique (Waksman, 1927; Garrett, 1951; Warcup, 1960) is a tool frequently used by microbial ecologists. This involves plating out serial dilutions of the soil suspensions under investigation, but this selects for the heavily sporing fungi and the spores themselves, so that spores and hyphae which are more firmly attached to the substrate are under-represented, if at all. Warcup (1959) failed to isolate any basidiomycetes from wheatfield and pastureland soils using this technique although he did isolate a number of species by more direct hyphal plating methods from the same plots; neither did he isolate any basidiomycetes using the soil plate method (Warcup 1950). The study of basidiomycete ecology in the past has often been restricted to the analysis of fruit body appearances and their occurrence in rhizospheres, with the investigation of vegetative hyphae and spore occurrence in the soil neglected to such an extent that Chesters (1948) called basidiomycetes "the missing link in soil mycology". Warcup (1959) began to rectify this by isolating a total of nineteen species from wheatfield soils and sixteen from pastureland soils by direct hyphal plating, by isolating from vegetable detritus and roots and by picking out sclerotia, none of the wheatfield species were observed fruiting in the field. One of the fungi isolated in this way was an unidentified *Coprinus* species, T.198, this organism was fruited in culture but not identified.

The techniques used in this work (Warcup 1955) involve the removal of fungal hyphae directly from soil sediments using fine instruments and a stereo-dissecting microscope, it is tedious to apply and requires considerable technical skill in its operation. The soil plate method on the other hand allows isolation of fungi from a large number of separate soil samples quickly and conveniently and is consequently often used when making initial surveys of soil mycofloras. However, as previously stated this technique does not often produce basidiomycete isolations, possibly because it does to some extent select for spores, but also because the slower growing basidiomycetes are often swamped out on the plates by the faster growing microfungi; even when such colonies are removed on appearance they may have already inhibited the development of basidiomycetes. It was decided therefore, to investigate the possibilities of using this very straightforward technique with selective media to isolate *C cinereus* from soils in preference to microfungi. Warcup (1957) concluded that soil plates tend to favour faster growing species present in relatively low numbers, therefore if microfungi could be suppressed *C cinereus* which has a relatively speedy hyphal extension rate compared to other basidiomycetes may possibly be selectively isolated.

3.2(ii) Selective media

In general there are fewer useful selective mycological media than bacteriological ones and until recently the inhibition of non-basidiomycete fungi has not been very successful. Earlier workers (Russel, 1956) have described the use of ortho-phenyl phenol in media to select for white rot fungi, while more recently (Hunt and Cobb, 1971; Raabe and Hurliman, 1971; Taylor, 1971) have incorporated benomyl[1] into media and succeeded in isolating

many basidiomycetes, as have other workers (Maloy, 1974; Coggins and Jennings, 1975). A very useful review of selective agar media for the isolation of basidiomycetes has been produced by Hale and Savory (1976).

Initially the use of a selective media without suppressants was studied without success. A soil known to contain *C cinereus* was plated onto Warcup plates using three media known to be favoured by species of *Coprinus*. These were Potato Maltose Agar (Appendix 1), Lange's horse extract agar (Lange, 1952) and Eggins and Pugh cellulose agar (Eggins and Pugh, 1962). The pHs of these media were adjusted to 7.9-8.0 as this has been shown to be optimal for *C cinereus* growth on agar (McShane, 1976). The plates were then incubated at 30°C and 35°C and assessed qualitatively over a period of sixteen days without the occurrence of *C cinereus* being noted on any of them.

It was then decided to investigate the possibilities of using benomyl to suppress the growth of microfungi. Preliminary experiments were carried out to assess the effect of benomyl levels on *C cinereus* growth (Appendix 2), and it was noted that levels of above 25 ppm included in Eggins and Pugh cellulose agar began to seriously reduce growth rates. On this evidence cellulose media were produced incorporating 0.5, 5, 10, 15, 20, 25 and 30 ppm benomyl and the *C cinereus* containing soil was plated out on these as above. The results of this preliminary work showed no reduction in microfungal numbers until 5 ppm, at 10 ppm similar numbers of colonies appeared but growth was much sparser. The effect was then studied more closely.

FOOTNOTE

(1) Benomyl is a 1-butyl carbonyl-2-benzimadazole carbamic acid methyl ester and is marketed as 'Benlate' by Du Pont. This is a heat stable wettable powder: 2 parts Benlate = 1 part benomyl.

3.2(iii) Experimental methods

Eggins and Pugh cellulose agar was made up with 5 µg/l rose bengal (Martin, 1950). This compound inhibits many bacteria and actinomycete species, reduces fungal colony size which helps to prevent overgrowth of plates and in this case helps to define white hyphaed colonies against the otherwise also white cellulose agar. The media was split into four, one lot remaining unamended whilst to the other three were added 10, 15, and 20 ppm benomyl. A seeded soil sample was produced by adding 1 ml of a 13 x 10^4 spore/ml suspension of *C cinereus* spores to 10g of soil and mixing thoroughly.

Warcup plates were then made using each amended medium, 0.1g and 0.01g of the seeded soil were placed in ten replicate plates of each giving twenty plates per medium. Two sets of controls A and B were also set up using seeded soils produced with previously sterilised soil (A) (forty minutes autoclaving on three consecutive days) and non-sterile soil (B) both being plated with unamended Eggins and Pugh cellulose agar. The plates were incubated for four days, assessed visually and then incubation was continued for a further three days when the total colony count and basidiomycete counts were taken on each plate. The *C cinereus* colonies having been produced from single spore inocula were monokaryotic but were still easily recognised by the production of oidia. (plate 3.1)

3.2(iv) Results

Table 3.1 shows the percentage of colonies isolated which were *C cinereus* (mean of ten replicates). The medium which contained 20 ppm benomyl gave the largest percent appearance of 86.6%, however at this level the total number of colonies began to drop showing some inhibition of *C cinereus* also

Plate 3.1 Oidia on monokaryotic hyphae of *C cinereus* X100 lens magnification

quantities of actinomycetes were isolated (Plates 3.2 and 3.3). The media which contained 15 ppm benomyl gave the second highest percent *C cinereus* appearance of 84.3% and also the highest total recovery and was therefore adopted for future work. Statistical analysis (Appendix 4) of the colony counts showed significant differences between treatments and supported the above conclusions.

The light, cleared areas on the plates containing 20,25, 30 and 40 ppm in the photographs are caused by actinomycetes.

3.2(v) Further work

It would seem that Eggins and Pugh cellulose medium with an amendment of 15 ppm benomyl may prove useful in the isolating of *C cinereus* from soils. However, success in isolating from heavily seeded soils is not comparable to success in isolating from the field. Therefore ten soil cores were taken aseptically from a soil previously shown to contain *C cinereus*. These cores were bulked together and mixed thoroughly, from this soil fifty samples of 0.01g of soil were taken and plated onto Warcup plates using Eggins and Pugh cellulose agar amended with 15 ppm benomyl. They were then incubated at 30°C and examined after four and seven days.

Results

Although good suppression of microfungi was observed on all plates *C cinereus* was isolated from only two of them.

3.2(vi) Discussion

This low level of *C cinereus* occurrence could possibly be accounted for in two ways. Firstly, that the method devised above is inefficient for use in the field and possibly isolation of living hyphae does not respond to the same factors as spore

isolation with regard to benomyl sensitivity. Secondly that this figure is a true reflection of the numbers found in that particular sample. Warcup in his studies of soil basidiomycetes examined 566 hyphal isolation plates from wheatfields to recover only 62 colonies. Should this be so, plate isolating of *C cinereus* from soils would be a very laborious task. This section of the project would have been investigated further had time permitted. The recovery experiment could have been repeated using fragmented hyphae as seed instead of spores and a much enlarged field isolation programme should have been undertaken. However at this point, experiments running parallel to those outlined above were giving useful results. This was the devising of a technique to isolate *C cinereus* from soils using ammonia as the selective agent. This method promised to be very useful as a comparative technique and so it was decided not to pursue the use of benomyl as a selective agent any further. However, the results of the work are presented here because it was felt that the observations on the effects of benomyl on *C cinereus* may be of interest and use in their own right, and may be worth further investigations.

Table 3.1 Recovery of *C cinereus* from seeded soils using benomyl

Eggins & Pugh Medium plus:-	Soil(g)	% isolates which were *C cinereus*
10 ppm (1)	0.1	C (2)
	0.01	80.5
15 ppm	0.1	C
	0.01	84.3
20 ppm	0.1	C
	0.01	86.6
Control A	0.1	C
	0.01	100
Control B	0.1	C
	0.01	C *C cinereus* Indistinguishable

(1) Benomyl as ppm of medium:

(2) C = All plates with 0.1g soil gave confluent colonies which could not be counted.

Plate 3.2 Soil plates showing inhibition of microfungi by 0.5-20.0 ppm benomyl.

Plate 3.3 Soil plates showing inhibition of microfungi by 25.0-40.0 ppm benomyl.

3.3 ISOLATIONS USING AMMONIA

3.3(i) Introduction

Enrichment techniques have been used frequently to isolate organisms present only in small numbers in the soil. These usually involve pre-treating or 'enriching' samples with chemicals so that the organism to be isolated grows at the expense of other types. The use of ammonia to isolate *C cinereus* from straw could be classed as a technique of this type. The success of this method on straw suggested that it may also work with soil samples and so it was decided to investigate these possibilities further.

Soil baiting techniques have been employed by many workers and can be looked upon as extended enrichment techniques. These usually involve the addition of sterile 'baits' to the soil which will be colonised by fungi, retrieved and isolated from; pythium and other water moulds are frequently isolated from hemp seeds floating in water covering the soil sample (Sparrow, 1957). Other baits used range from Buckwheat for the isolation of *Rhizoctonia solani* (Papavizas and Davey, 1959) to pieces of carrot to isolate *Thielaviopsis brasicola* (Yarwood, 1946). Since these early workers the techniques have been used frequently and much expanded. Straw has been one of the organic substrates frequently used in such methods since the early work of Sadasivan (1939) to isolate fungi from soil. It has also been used by Butler (1953) and Garret(1963) in the assessment of competitive saprophytic fungal ability. Warcup (1959) plated soil suspensions onto sterilized wheat straw to isolate basidiomycetes and free the cultures from bacteria, since he found many basidiomycetes grew well on straw whereas bacterial growth was slight. Straw pieces are also often included in media to encourage basidiomycete fructification (Watling, 1971). It is clear that there is a long precedent for using straw as a bait, frequently for basidiomycete isolation.

The two techniques outlined here, enrichment and baiting were combined to produce a method which successfully and consistently led to the isolation of *C cinereus* from the soils under examination.

3.3(ii) Materials and Methods - preliminary experiments

Initially the work was carried out on a fairly large scale using jars of 454g nominal capacity to contain the soil sample, with about 20 g of soil to ensure a reasonably heavy inoculum for these preliminary experiments. The soil was placed aseptically into sterile jars and mixed with about 1 g of hammer milled straw which had been previously sterilised by autoclaving at 15 lb per sq in for 40 mins, after first moistening the milled straw. Into this was pushed a small sheaf of about 10 straws (sterilised as above) 10 cm in length. Twenty jars were prepared in this manner and to half of these were added sterile universal bottles to receive ammonia solutions, duplicate jars were then treated as follows:-

1. Ammonia solution containing 0.1g ammonia made up to 5 mls with sterile distilled H_2O added:-
   (1) to the soil - Series A
   (2) to the universal - Series B
2. Ammonia solution containing 0.01g ammonia as above to series A & B.
3. Ammonia solution containing 0.001g ammonia as above to series A & B.
4. Control a. Sterile soil inoculated with *C cinereus* spores + 0.01g ammonia to series A & B.
5. Control b. Sterile soil inoculated with *C cinereus* with 5 mls H_2O alone to series A & B.

These were capped with aluminium foil and incubated for 9 days at 30°C after which the jars were removed and observed for fruit body production for identification, and the amount of hyphal growth after this period assessed visually.

3.3(iii) Results and discussion

The results of the visual assessment are shown in table 3.2 and it can be seen that the basic concept behind the technique was successful. The ammonia stimulated *C cinereus* growth which in turn colonised the milled straw and eventually the hyphae extended along the straw pieces. For isolation and identification purposes the pieces could be removed, the bottom 2 cm snipped off to remove contaminating soil and the straw plated out to give a pure culture of *C cinereus*. The best growth was shown in the jar where ammonia had been added at the 0.1g level and to the universal bottles, whereas this higher concentration added to the soil directly caused inhibition of growth.

This preliminary work gave the basis for a useful comparative isolation technique which was not in itself quantitative. The unwieldy jars were replaced by universal bottles with Durham tubes to contain the ammonia solutions (Plate 3.4). In this form the isolation bottles could be set up and sterilised complete with Durham tubes in large batches ready for incoming soil samples. On arrival 5g of the sample were placed aseptically into the bottles, mixed with milled straw as above and 5 cm pieces of straw placed into the soil, 0.13 g of ammonia were placed in the Durham tube; a dilute ammonia solution was made up with sterile distilled H_2O and distributed between the tubes to give this amount. The 0.013g was arrived at by dividing the amount added to the larger jars (0.1g) by the ratio of surface area of the soil exposed in

Plate 3.4 Arrangement of apparatus for isolating *C cinereus* from soil.

the jar and the universal bottle. This technique was then used to carry out a more detailed study of the soils from three farm sites.

Table 3.2 Preliminary isolations from soil

	0.1g	0.01g	0.001g	Control a	Control b
Series A	+	++	+	+++	-
	+	++	+	+++	-
Series B	+++	+	+	+++	-
	+++	++	+	+++	-

Key

+ Some sparse growth along the straw strips.

\- No visible growth

++ Good growth

+++ Copious amounts of hyphae

3.3(iv) Farmland Survey

Introduction

The purpose of this section of the project was to use the devised *C cinereus* soil isolation method to compare the occurrence of this fungus in cereal soils, pastureland and non-agricultural soils, to assess whether its appearance was linked to any physical or chemical characteristic of the soils or if it could be directly related to cereal growing. Soil biotic and environmental factors which it was considered may have a potential bearing on the mycoflora of the soils, particularly *C cinereus*, were noted.

When sampling from the field thought must be given to the factors which may bring about an alteration in the mycoflora during the sampling procedure. The most obvious change that can take place is that of temperature. Two approaches have been made to solve this problem, some workers have refrigerated samples to slow down multiplication of cells while others have attempted to keep them at the temperature at which they were sampled by storing in thermos flasks (Parkinson et al. 1971). This latter is probably the most satisfactory, however, it was felt that as in this case only the presence or absence of an organism was being studied the time, effort and expense required to transport and store samples in thermos flasks was not warranted. A change in aeration and water content may also alter the sample mycoflora; therefore plastic bags were used to help maintain moisture content in transit. These were of polythene which allows the passage of air but not water vapour, hence helping to maintain aerobic conditions in the soil sample (Stotzky et al., 1962).

When sampling soils it is necessary to devise a plan of sampling which will allow statistical analysis of the results obtained. This is because the small size and lack of numbers of the samples taken constitute only a small part of the whole, but are assumed to be representative of it. Therefore the parameters and populations are not measurements of the actual situation, but are only estimates, consequently, statistics are necessary to assess the reliability of this estimate.

3.3(v) Materials and Methods

The three farms chosen for this study were all on different soil types, two were situated in Herefordshire and one in Worcestershire. These farms were chosen as they had grown both wheat and barley over the previous twelve months and had areas of

very long standing pastureland which had not been cultivated for many years.

At each farm soil samples were taken from a field which had been under barley for at least two seasons, one which had similarly been under wheat and an area of pastureland as previously mentioned. Samples were also taken from non-agricultural land in the same area. At each sample site the soil temperature was taken at 5 cm depth using a "Comark" electronic portable thermometer. The aspects, covering crop and recent vegetation and fertilizer histories were also noted. Ten samples per field were taken at random using a 10 × 4 cm soil corer which was flamed between each core using 70% alcohol and a gas blowlamp. The top 0.5 cm of the cores was removed with sterile implements to prevent contamination from the surface. The cores were placed into polythene bags and on return to the laboratory were stored at 4°C until required which was normally within 24 hours of return.

Each sample was thoroughly mixed and from this was taken material to find:-

1. The pH - 1g of soil was mixed with 1 ml of distilled water (Tansey and Jack 1976), whirlimixed and the pH of the suspension taken using a glass probe and pH meter.
2. The percent organic material by weight loss after ashing.
3. The percent water content by weight loss on drying to constant weight.
4. Presence of *C cinereus* - 5g of each sample were placed into sterile isolation tubes as outlined previously. These tubes were incubated at 30°C for 8 days after which the straw strips were removed and plated onto Eggins and Pugh cellulose medium and the presence or absence of *C cinereus* noted.

Controls were also set up using sterilized soil produced by 40 minutes autoclaving at 15 lb per sq in for 3 consecutive days, this was then dried. The water content was then returned to pre-autoclave levels with sterile distilled water.

The air temperature, weather conditions and signs of animal activity were also noted at each site.

The pH taken in this manner bears little relation to that found in the soil environment, however it does give a comparative view of the field soil pH.

3.3(vi) Results

Tables 3.3 - 3.5 summarise the results from the three farm sites. It can be seen that a higher number of *C cinereus* isolates were obtained from soils which had been under cereals than from all other soils. From four of these soils 100% *C cinereus* isolation was obtained and high levels (70 and 80%) were obtained from the others; whereas only 20 - 40% *C cinereus* isolations were obtained from the soils which had not been under cereals. Statistical analysis (appendix 4) of this data shows that there was no correlation between the number of isolates and the moisture content, organic content, soil temperatures or soil pH. However there was a positive correlation between the number of isolates and the covering crop or vegetation as outlined above. The soil pH varied between pH 3 - pH 7 and was dependent on soil type and treatment as could be expected. Soil temperatures found were mainly between 5°C and 8°C except for a pastureland soil at How Caple which was only 3.6°C. The percent moisture content depended very much on the soil type, drainage and cultivating techniques employed. The organic content measured as weight loss after ashing varied greatly even within fields of the same soil type. This could be expected because of the vastly different

Table 3.3

CHARACTERISATION OF SOILS AND *C.CINEREUS* ISOLATIONS

Farm & Soil Type	Crop or Vegetation	Recent Fertiliser History	Animal Associations	Air Temp and Conditions	% Moisture Content *	% Organic Content *	Soil Temp °C *	Soil pH *	Number *C cinereus* Isolations
White House Farm How Caple	Wheat	Unknown	-	6°C Dry Overcast Windy	14.65 ± 1.314	3.064 ± 2.608	6.2 ± 0.6708	7.00 ± 0.232	10
Herefordshire OS 677353 Light Sandy Ross Soil	Barley	Unknown		6°C Dry Overcast Windy	16.43 ± 0.810	1.956 ± 0.601	6.7 ± 0.2739	6.13 ± 0.324	10
	Pasture	110 units N Compound fertilizer	Horses Evidence of past sheep grazing	4°C Heavy Rain	23.30 ± 3.245	5.606 ± 0.986	3.6 ± 0.5477	6.59 ± 0.525	2
	Grassland (Roadside bank)	-	-	11°C Dry Overcast	22.91 ± 2.119	4.633 ± 1.486	6.4 ± 0.670	7.61 ± 0.245	4

Table 3.4

Farm & Soil Type	Crop or Vegetation	Recent Fertiliser History	Animal Associations	Air Temp and Conditions	% Moisture Content *	% Organic Content *	Soil Temp °C *	Soil pH *	Number *C cinereus* Isolations
Longlands Farm	Wheat	2.5 cwt Compound Fertilizer	-	11°C Fair and Dry	18.43 ± 2.380	3.582 ± 1.291	8.3 ± 0.913	6.79 ± 0.375	10
Whitbourne Hall	Barley	2.5 cwt Compound Fertilizer	-	10°C Fair and Dry	23.28 ± 0.948	4.030 ± 0.735	7.0 ± 0.283	7.06 ± 0.2911	8
Near Worcester	Pasture	Top dressing of Nitrogen	None during sampling but cattle and sheep at previous dates	10.5°C Fair and Dry	24.63 ± 3.965	6.049 ± 1.623	8.3 ± 0.761	6.52 ± 0.471	4
Sandstone/ Clay	Woodland (Much Birch)	-	-	9°C Fair and Dry	22.86 ± 5.291	4.442 ± 1.067	7.7 ± 0.992	4.99 ± 0.509	3

* ± Standard Deviation

Table 3.5

Farm & Soil Type	Crop or Vegetation	Recent Fertiliser History	Animal Associations	Air Temp and Conditions	% Moisture Content *	% Organic Content *	Soil Temp °C *	Soil pH *	Number *C cinereus* Isolations
Park Farm Farm	Wheat	K 50 units	-	6.8°C Heavy Rain High Winds	27.59 1.596	8.6153 1.423	5.6 ± 0.422	6.76 ± 0.551	7
Brampton Abbot Ross-on-Wye	Barley	50 units N 50 units P 50 units K Lime 1 kg/Acre	Many Rabbit Pellets	7°C Heavy Rain High Winds	19.07 ± 3.806	2.3814± 1.079	7.1 ± 0.291	6.98 ± 0.423	10
OS 618274	Pasture	150 units N Top dressing annually	Cattle	8.5°C Showery Overcast	15.65 ± 0.760	2.119 ± 0.4605	5.1 ± 0.711	6.52 ± 0.829	4
	Grassland (Village Green)	-	-	9°C Showery Overcast	24.23 ± 1.173	7.371 ± 0.451	6.5 ± 0.333	7.47 ± 0.901	4
	Woodland	-	-	9°C Showery Overcast	20.89 ± 2.010	9.045 ± 2.850	5.0 ± 0.910	7.19 ± 0.351	4

* ± Standard Deviation

histories, treatments, access by animals etc which were found in different fields.

3.3(vii) Discussion and Conclusions

The effects on soil mycofloras of adding various chemicals directly or in the laboratory has been investigated by a number of workers. Hora (1958,1959) described the changes in the fungus flora after treatment of the forest floor in a pine plantation with lime and various fertilisers. Petersen (1970) used potassium, calcium and sodium carbonate alone and in combination to bring about the appearance of 'fireplace fungi' on non burnt ground. Other workers in addition to Hora have used amendment with nitrogen sources to select out and encourage the growth of certain groups of microorganisms (Lehmann and Hudson,1977; Sagara,1973). While a combination of increase in pH and addition of nitrogen has been achieved by treating the soil with urea or urine (Lehmann,1976; Lehmann and Hudson,1977; Sagara and Hanarda,1965; Sagara,1973 and 1974) and by the addition of aqueous ammonia or agents producing ammonia on breakdown (Sagara,1974 and 1975). By treating soils with urea or ammonia solutions Sagara has isolated, amongst others, fungi usually associated with the coprophilous fungus successions and from the results of this work he proposed to place the species thus isolated in a chemoecological group which he called "ammonia fungi" and defined as "a group of fungi which sequentially develop reproductive structures exclusively or relatively luxuriantly on the soil after a sudden addition of ammonia, some other nitrogenous materials which react as bases by themselves or on decomposition, or alkalis". It would be premature at this stage to say that *C cinereus* falls into this category as its appearance in the field after such treatment has not been reported and field treatments have not been carried out by this laboratory. However its appear-

ance in the laboratory under similar treatment conditions would seem to indicate that it is possible that *C cinereus* along with some other coprophiles could fall into this new grouping. Both Lehmann and Sagara have isolated *Coprini* which are more usually associated with burnt ground after these treatments, for example, *C echinosporous*. Sagara also isolated *C lagopus* and a new species *C neolagopus* from soils treated with urea and urea or ammonia respectively.

The natural habitats of *C cinereus* equate with the experimental conditions outlined in Sagara's definition. It is often isolated in farm situations, from straw where the opportunity for the bales to come into contact with animal faeces or urine is present or from straw/dung composts where there is obviously a high concentration of ammonia present. Sagara found that the occurrence of NH_4-N together with an alkaline condition were the essential factors required to bring about these fungal occurrences and that an alkaline condition alone was much less successful. To explain the occasional appearance of 'ammonia fungi' after alkaline treatment alone, Sagara postulated that the treatment was bringing about a small release of ammonia from organic material in the soil. This 'unique' effect of ammonia was also noted in this project with relation to *C cinereus* growth and selection and will be discussed further in a later chapter. It is interesting to note that Sagara classes *Chaetomium globosum* in this group and in the previous chapter this organism was found to be a frequent competitor of *C cinereus* on straw amended with ammonia.

Coprinus cinereus has been shown to tolerate and in fact favour alkaline pH under laboratory conditions (McShane, 1976). Other *Coprini* have been isolated from alkaline environments, for

example alkaline lakes (pH 8.8) (Anastasiou 1967) and from soil (pH 8.4) (Fries 1956) and have also been shown in the laboratory to be able to tolerate alkaline pHs (Fries 1953). The growth of *Coprinus cinereus* on straw under alkaline conditions is compared in a later chapter to its growth when amended with ammonia as mentioned above.

The role of ammonia in this work and that of Sagara is not clear. As mentioned previously *C cinereus* growth is actively favoured by the presence of ammonia, but ammonia is also known to inhibit some microbial growth. Hora and Baker (1972) found that volatiles produced by *Streptomyces sp* were able to suppress conidial growth of other genera and later work (Hora and Baker,1975) showed that this inhibition was due to ammonia suppressing germination of fungal spores. Schippers and Palm (1973) showed that ammonia may act as a fungistatic agent in some soils. It may be that it is this dual role which brings about the selective isolation of *C cinereus* from soils and straws. It is known that *C cinereus* excretes ammonia from its sporophores on maturation and this has been found to play a regulating role in the production of sclerotia (Moore and Jirjis, 1976). This may be an important factor in controlling the relationships and successions within dung or straw composts, and the appearance of other coprophiles on urea and urine treated soils seems to lend weight to this suggestion.

The origin of the *C cinereus* propagules isolated from the soils under investigation is of interest. The isolation technique used does not differentiate between actively growing hyphae and spores or sclerotia. The hyphae and sclerotia of an unidentified *Coprinus species* were isolated from Australian wheat field soils where their fruitbodies had never been recorded, by Warcup, but searches for the hyphae of *C cinereus* in the soils investigated

here were not successful, although a full isolation programme with the numerous replicates required to make such data significant was not carried out.

The correlation between fields under cereal crops and the isolation of *C cinereus* has already been pointed out. The possibility of the effect of fertilisers being important here seems unlikely as nitrogen was also added to the pastureland soils which showed no increased occurrence of *C cinereus* over the non agricultural soils. This is not incompatible with the suggestion that *C cinereus* may be an "ammonia fungus" as compound fertilisers usually contain the ammonium ion in a form which does not become alkaline in the soil thus not fulfilling the requirements of Sagara's definition. Compound fertilisers had been used on the agricultural soils investigated.

It is difficult to pinpoint the source of the *C cinereus* inoculum in the cereal field soils, although suggestions can be made. Discarded straw will lie around the unploughed outer edge of a field, possibly for a whole season, colonisation of this by *C cinereus* could occur under conditions which allow development to maturity, when spores would be dispersed over the field. Ammoniacal nitrogen would be available from animal excrement and from fertilisation by animal wastes should this occur. Stubble and unwanted straw often lies in the field for some days after harvesting prior to burning, usually, the wetter the weather, the longer they will remain and the better the conditions for fungal attack. It may be that these wastes are colonised by *C cinereus* and although mature sporophores may not be produced the thick walled chlamydospores and sclerotia would be and hence act as an inoculum in the field. In this way the soil, if not actually supporting hyphal growth would be acting as a sink for

the *C cinereus* propagules. Allowing cattle to graze in stubble fields would enhance the possibilities of the above happening by adding plentiful supplies of ammoniacal nitrogen. In situations where the stubble is not burnt, but chopped and ploughed in, the chances of inoculating the soil are even greater. Later, spores may be transferred to cereal stems during growth in rain splashes or in soil particles on the wind, these may be spread through combine harvesters and hence transferred to the bale, thus explaining the presence of *C cinereus* on baled and non-baled straws alike as noted in the previous chapter.

To summarise this section, *Coprinus cinereus* can be isolated from soils by using aqueous ammonia and this being so, may warrant being placed in the chemoecological group of "ammonia fungi" as defined by Sagara. The increased frequency of *C cinereus* isolations from cereal fields suggests a connection with straw at this early stage in the field, but further work to establish the nature of the propagules in the soil is necessary.

3.3(viii) Suggestions for further work

It was felt that the time required to devise and put into practice a technique for differentiating spore and active hyphal growth in the soil under these conditions could not be justified in this project. The ecology of *C cinereus* in the field although of great interest was not of direct relevance to the study of *C cinereus* as it may be employed in a waste straw biodegradation process. However the subject is undergoing further investigation by other personnel from these laboratories.

The occurrence of *C cinereus* on cereal stems during growth would be of interest as would an investigation into its presence

on straw litter remaining from season to season.

It may also be possible to extend the isolation technique devised into a quantitative method by using the tubes as "most probable number" tubes and diluting the soil inocula with sterile soil or sand.

CHAPTER FOUR

ASSESSMENT OF COMPARATIVE CELLULOSE AND LIGNIN DECOMPOSING ABILITIES OF SOME *COPRINUS CINEREUS* ISOLATES

4.1 INTRODUCTION

The role of the cellulose-lignin complex in the plant has been likened to "a sort of biological reinforced concrete" (Walker, 1975), where the cellulose in the cell walls of higher plants is encrusted and penetrated by the complex, three dimensional polymer of phenyl propaniod (C_6-C_3) units which comprises lignin. Together lignin and cellulose contribute much towards the mechanical rigidity and strength of the higher plants and the 'encrusting' and 'penetrating' described above produce a very strong bond between the two. Because of this, much of the cellulose present in a highly lignified plant is not available for breakdown by the cellulolytic gut microflora of the ruminant. Straw which is harvested at maturity when the cereal stems are fully strengthened to support heavy ears of grain, has much of its potentially useful cellulose locked in this non-utilisable form with lignin. The problem has been discussed in greater depth in Chapter One. Bearing this in mind therefore, the ability of *C cinereus* to utilise cellulose and lignin under various conditions, and the time spans required for significant depletion of either would be of interest if this organism is to be used to upgrade waste straw. The removal of lignin would release more cellulose for breakdown by both fungus and animal, whereas an efficient, rapid utilisation of this available cellulose by *C cinereus* may result in an actual decrease in the digestibility of the straw to the ruminant. The experiments described in this chapter are designed to give an indication of the likely activities of *C cinereus* with regard to the breakdown of

cellulose and lignin, also, of equal importance, to compare the activities and variabilities of *C cinereus* isolates from different areas to assess strain differences for future work.

4.2 ASSESSMENT OF CELLULOLYTIC ABILITIES

4.2(i) Review of techniques

There are many difficulties to be considered when investigating the breakdown of cellulosic substrates by micro-organisms. When studying cellulolytic abilities some form of homogenous, easily characterised substrate is usually used and this often bears little resemblance to the state of the cellulose as it would be encountered in nature; neither does it take account of the structural relationships between cellulose, lignin and hemicelluloses as mentioned previously. Also, as shown by Mandels (1965) fungi will often utilise different substrates at different phases of spore germination and growth. Hence, it is often difficult to relate laboratory assessments of cellulolytic abilities to the situations in which the fungus would be encountered in its 'natural' environment. The situation is further confused by the fact that cellulase is not a single enzyme acting alone but a complex of enzymes and these, present in the system of any micro-organism may enable it to break down the cellulose in one type of substrate, but not another, so that the specificity of the cellulase complex may also give rise to difficulties in the comparative assessment of cellulolytic abilities (Halliwell,1963).

However, many workers have been interested in the abiliites of fungi to break down cellulose, and consequently a variety of techniques has emerged. Most studies on cellulose decomposition have involved the use of modified celluloses prepared from 'native' cellulose, and it is questionable whether the results can be related to the in'vivo' situation. Some workers have utilised

Cellulose papers; for example, the loss of weight of filter papers (Reese, 1946; Garrett, 1962; Chang, 1967) and loss of tensile strength of strips of chromatography paper after fungal growth (Malik and Eggins, 1970; Mills, 1973).

Enzyme techniques have been used where the change in viscosity of soluble cellulose derivatives, brought about by cell-free cellulase extracts is used as a measure of cellulolytic ability (Levinson and Reese, 1950; Norkrans and Ranby, 1956). Measuring the amounts of reducing sugars produced after treating cellulose with enzymes obtained from microorganisms has also been used (Levinson et al 1951).

Other cellulose derivatives which have been employed are acid swollen cellulose (Rautela and Cowling, 1966; Tansey, 1971), Cellophane (Tribe, 1957) and carboxymethyl cellulose (Reese, Siu and Levinson, 1950). Smith (1977) used dyed cellulose powder incorporated into a growth medium to assess comparative cellulolytic abilities; as the cellulose is broken down the dye is released into the medium in amounts proportional to the cellulose breakdown.

Scales (1916) and McBeth (1916) first measured cellulolytic activity by determining the magnitude of a clear zone formed in an opaque cellulosic medium. Aschan and Norkrans (1953) and Savory et al (1967) incorporated chemical inhibitors into similar media to prevent hyphal growth while cellulase secretion continued to clear the medium. This removed the problem of the mycelium obscuring cleared zones and excluded the effects of hyphal growth, so that clearing depended solely on production and diffusion of the cellulases. Walsh and Stewart (1969) counted microscopically the numbers of remaining cellulose particles in a measured area of the petri plate after the growth of organisms on a cellulosic medium to obtain a measure of cellulolytic ability.

Rautela and Cowling (1966) used tubes of cellulose containing media, the anaerobic nature of the lower depths of the media columns prevented hyphal growth so that cellulases continued to diffuse and a clearing front could be measured as it advanced down the tube. This technique along with a plate clearance method was utilised in the following experiments.

4.2(ii) Materials and methods

Two solid media techniques were used to assess and compare the cellulolytic abilities of five *C cinereus* isolates obtained from straws from various parts of the country (Table 4.1).

Table 4.1 Origins of *C cinereus* isolates

Isolate No.	Area
AS-1	Isolated by C McShane (1976)
AS-2	Shrewley, Warwickshire
AS-3	Northamptonshire
AS-4	Sam Bach, Gwynedd
AS-6.	Spalding, Lincs

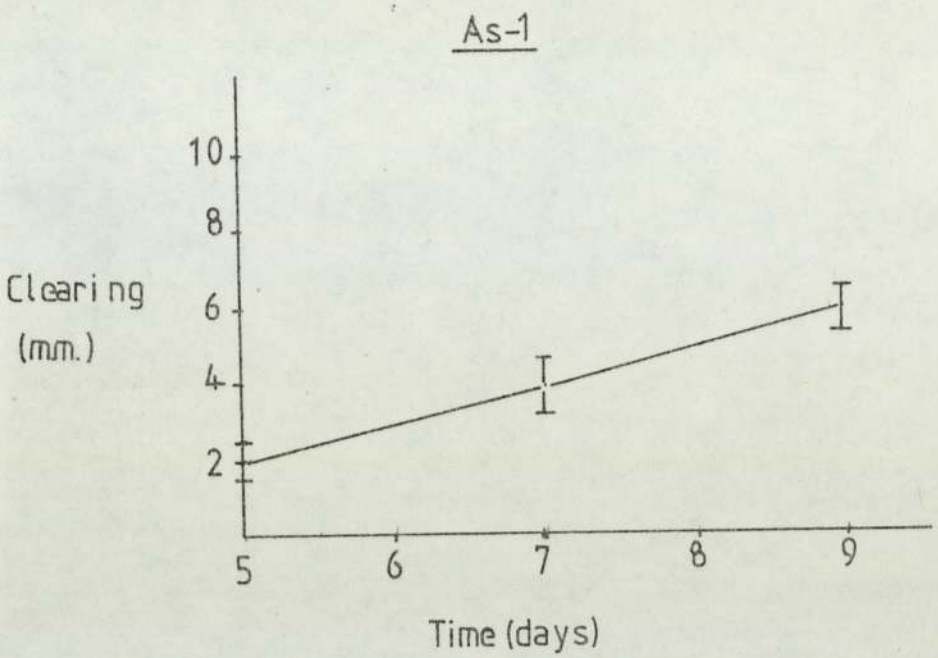
a. The first technique used was that described by Rautela and Cowling (1966). Test tubes (18mm) were filled to a level of 60 mm with Eggins and Pugh cellulose agar, these were closed with oxoid caps and autoclaved at 15 lb/square inch for 20 minutes then, to prevent settling out of the cellulose the method of King (1972) was used. The tubes were spun on a 'whirlimix' and plunged into crushed ice until the media solidified. The tubes were then inoculated with 8 mm plugs of *C cinereus* mycelium taken from the growing edges of four day cultures on Eggins and Pugh cellulose medium. A total of five replicate tubes were prepared per isolate. The tubes were incubated at 30°C in a non-illuminated incubator and inspected daily

for signs of clearing of the media. A standard was produced for comparative purposes consisting of a tube containing Eggins and Pugh basic medium without the addition of ball-milled cellulose. Measurements were not taken until this standard had been reached, so that virtually total removal of cellulose was being measured. When this standard of clearing had been reached in the tubes the advancing front was measured every 2 days. Three measurements were taken on each tube and the mean used for assessment, this was because often the clearing did not occur in a uniformly straight line.

b. The second technique used to study relative cellulolytic activity was the comparison of the clearing of cellulose agar in plates. Again five replicates per isolate were prepared. Plates of Eggins and Pugh medium without cellulose were poured and allowed to solidify, these were then given a 3 mm overlay of the same medium but this time containing ball-milled cellulose. This thinner layer made the cleared zone more easily distinguishable than on plates containing solely cellulose medium. The plates were then inoculated and incubated as previously described. Hyphal extensions and cleared zones were measured daily from above and below the plates respectively. It was not possible to measure hyphal extensions accurately from below as the growing edge of fine, white hyphae could not be distinguished through the opaque medium. The results are the means of two diameter measurements taken at right angles to each other.

4.2(iii) Results

a. The extent of clearing in the tubes is shown in figure 4.1 and table 4.2. The standard described in 4.2(ii) was not reached by any isolate until day 5 after which the front of the cleared zone advanced slowly. Analysis of variance was performed on the final



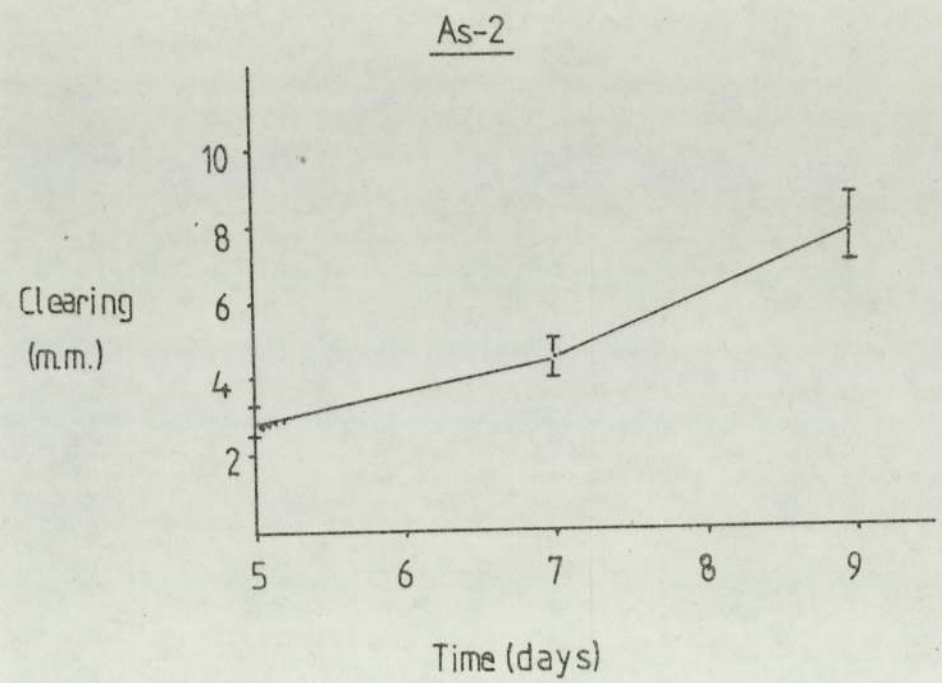
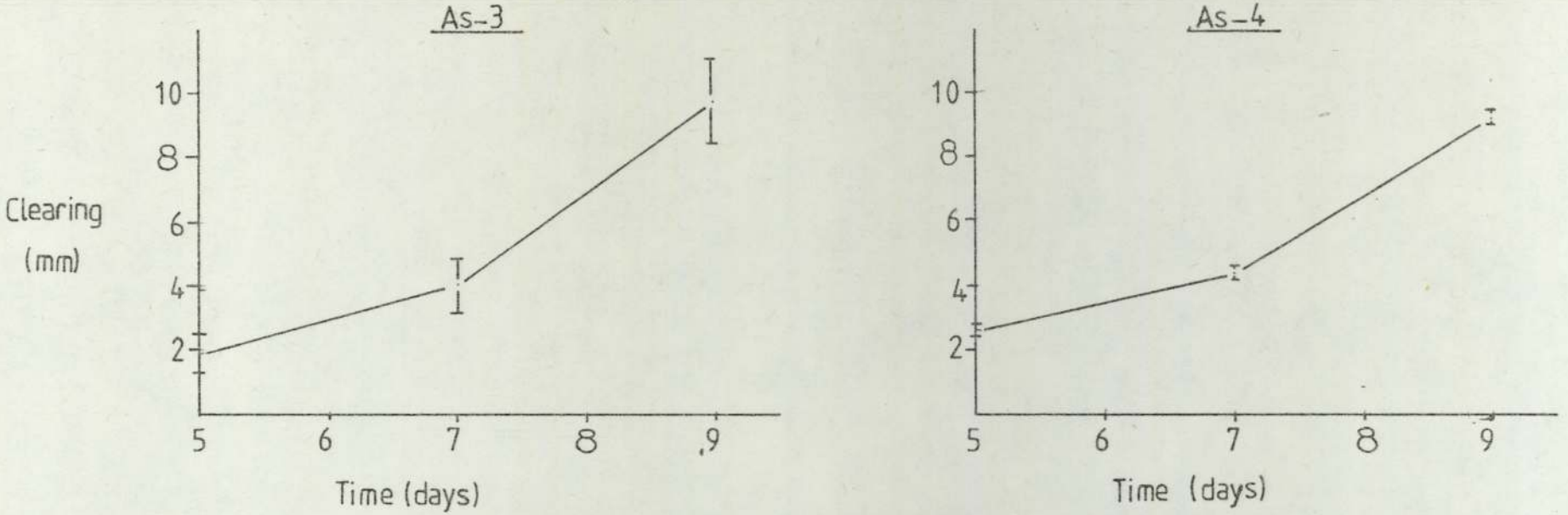


Fig. 4.1. Clearing in cellulose medium of "Cowling" tubes.


As-3
Clearing
(mm)
10
8
6
4
2
5
6
7
8
9
Time (days)

As-4
10
8
6
4
2
5
6
7
8
9
Time (days)

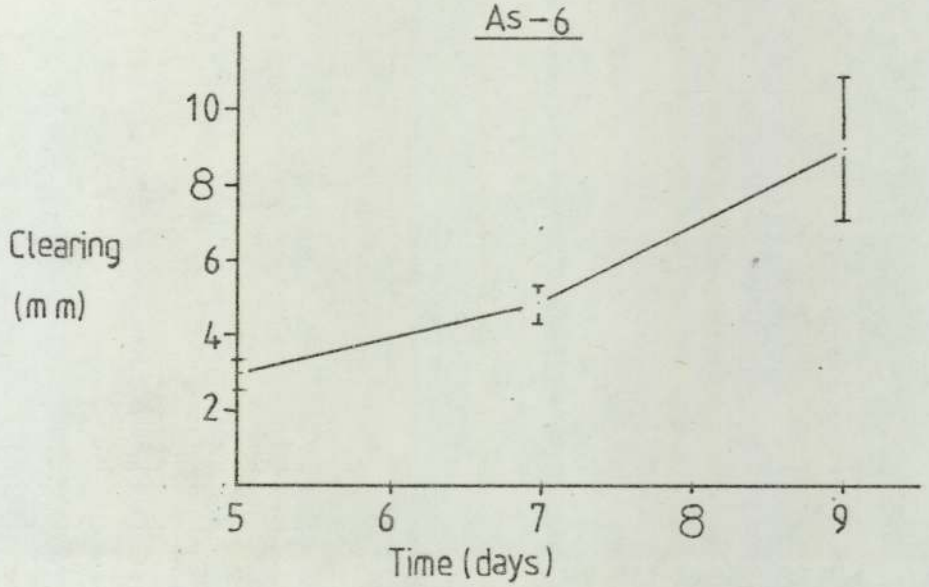

As-6
Clearing
(mm)
10
8
6
4
2
5
6
7
8
9
Time (days)

Fig 4·1 con't

measurements after 9 days (Appendix 4) and significant differences were found between the isolates as high as the 1% probability level. After 9 days problems arose in that agar shrinkage due to water loss was allowing cellulases to pass down between the agar and the glass instead of being forced to diffuse through the medium. This gave rise to areas of clearing many centimetres deep which appeared at irregular intervals round the edges of some tubes and consequently the experiment was not continued beyond day nine.

b. Hyphal extensions and cellulose clearing are shown in figure 4.2 and rates of growth and clearing in table 4.3.

Analysis of variance was carried out on mean rates of growth from 50 hours to 122 hours and on mean rates of cellulose clearing from 0 to 150 hours. Significant differences were found up to the 0.1% level between growth rates and also between cellulose clearing rates up to the same level. Detailed analysis of these differences will be discussed at the end of this chapter along with results from section 4.3.

Table 4.2

Cellulose Utilization in 'Cowling' Tubes

Day	Clearing mm ± SD				
	AS-1	AS-2	AS-3	AS-4	AS-6
5	2.17 ± 0.59	2.83 ± 0.31	1.93 ± 0.56	2.67 ± 0.67	3.03 ± 0.61
7	3.93 ± 0.68	4.53 ± 0.52	4.00 ± 0.82	4.40 ± 0.93	4.90 ± 0.81
9	5.80 ± 0.62	7.87 ± 1.06	9.50 ± 1.52	9.08 ± 0.41	9.1 ± 2.45

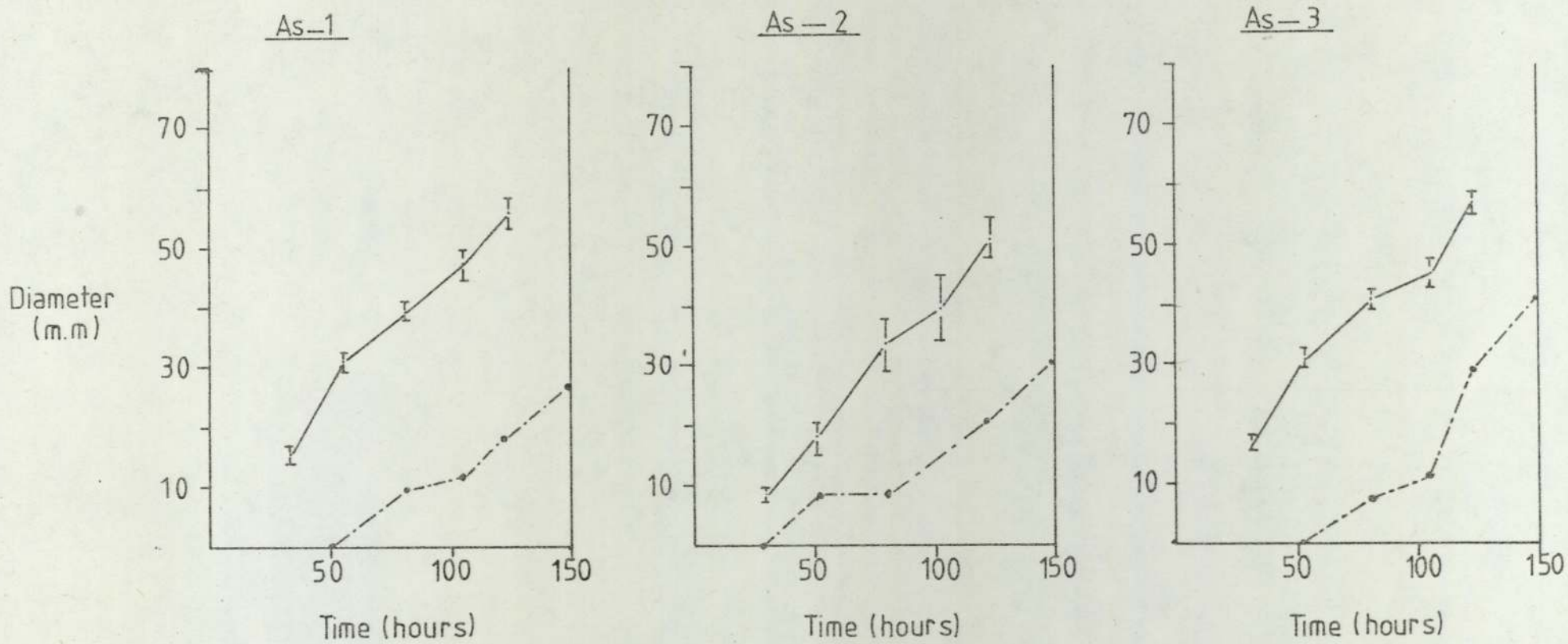


Fig 4·2 Cellulose plate clearing and growth rates

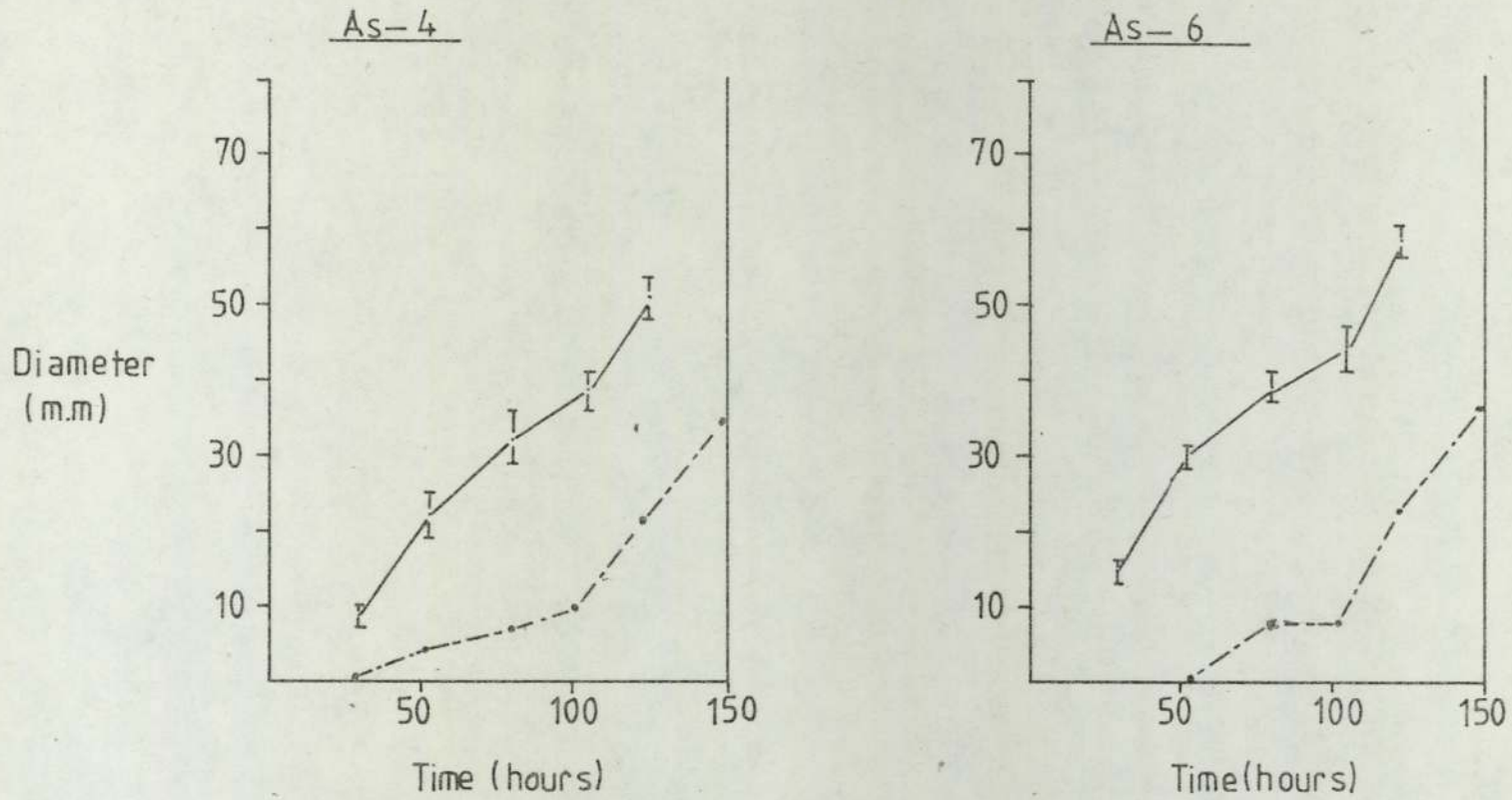

As–4
As–6
Diameter
(m.m)
70
50
30
10
50
100
150
Time (hours)
Time(hours)

Fig 4·2 cont

Table 4.3

Mean Hyphal Extension and Clearing Rates

Isolate	Mean growth rates mm/hr Between 50-122 hours ± SD	Mean clearing rates mm/hr Between 0-150 hours ± SD
AS-1	0.500 ± 0.20	0.214 ± 0.59
AS-2	0.438 ± 0.03	0.215 ± 0.70
AS-3	0.479 ± 0.02	0.422 ± 0.71
AS-4	0.449 ± 0.02	0.286 ± 0.44
AS-6	0.508 ± 0.02	0.333 ± 0.13

4.2(iv) Discussion

A number of workers have studied the cellulolytic abilities of *Coprinus cinereus* (Rege,1927; Fries,1955; Chang,1967 and 1977, Jodice,1971 and Penn 1977) and some have come to opposing conclusions, finding that *C cinereus* is either not cellulolytic, in as much as it was not seen to clear cellulose agar plates (Rege,1927) or that it shows "strongly cellulolytic activity" (Jodice,1971). Most researchers however have obtained results somewhere between those two extremes, and find that *C cinereus* is cellulolytic though not as active as such organisms as *Chaetomium thermophile, Aspergillus fumigatus* and *Trichodema viride* (Chang,1967; Penn,1977). Much of the variation in these results can be attributed to the numerous conditions under which the assessments have been carried out. Incubation temperatures have ranged from 25° - 35°C, a variety of pH and time spans have been used and substrates as diverse as carboxymethyl cellulose and 'native' cellulose in the form of raw vegetable matter have been employed. It was partly for these reasons that this work was carried out here, to assess the cellulolytic abilities of isolates available in this laboratory at a temperature that was being contemplated

for use in the waste straw upgrading process.

Comparing the two techniques used here, more useful results were obtained from the plate technique than from the tube method. Clearing was not noted until 50 hours had elapsed in all isolates and all showed a decrease in the rate of clearance at about 100 hours followed by an immediate increase. This slow initial rate of cellulose breakdown may be of use in the upgrading process as by this time the growth of hyphae is well established. More detailed studies of the carbohydrate nutrition of *C cinereus* on straw when ammonia is used for selection purposes are being carried out at the Biodeterioration Information Centre and there are indications that during this early lag period in cellulose utilization, hemicelluloses are preferentially utilized (Seal unpublished data). Hemicelluloses are known to have a shielding effect on cellulose (Alexander, 1961) in a similar manner to lignins and hence more cellulose may be made available for utilization by the ruminant.

4.3 ASSESSMENT OF LIGNINOLYTIC ABILITIES

4.3(i) Introduction

Lignin is the third most abundant constituent of plant tissues and crop residues after cellulose and hemicelluloses (Alexander, 1961) but in spite of this, until relatively recently little was known of the microbiology and decomposition of lignin, or of the environmental variables governing its breakdown. Three main factors seem to account for this; difficulties arising from the complexity of the lignin molecule, difficulties in assaying this substance and thirdly problems in isolating a purified lignin fraction which was suitable for use as a microbiological substrate.

Falck (1923, 1930) was probably the first to point out that in forest soils Basidiomycetes play an important part in the breakdown of lignin. Waksman and McGrath (1931) and Waksman (1931) showed *Agaricus campestris* and *Coprinus radians* decompose lignin as well as cellulose in dead plant material, and other early workers (Lindeberg, 1944 and Day et.al. 1949) followed, extending the lists of fungi thought to degrade lignin.

Several methods have been used by researchers in the determination of ligninolytic ability, although much of the early quantitative research is of doubtful value because conclusions were based upon unreliable methods for estimating lignin in plant residues. Often the isolated lignin used as a substrate, contained impurities or had already been partially degraded or altered by the production technique (Alexander, 1961).

Two main methods have been used to assess ligninolytic ability, one entails determining the disappearance of lignin when portions of sterile plants are inoculated with pure cultures of suspected organisms; this has been used by Lindeberg (1946) and many others. The other involves the use of isolated and purified lignin preparations usually incorporated into artificial media (Day et.al., 1949, Gottlieb et.al., 1950). Eggins (1965) incorporated ball-milled beech wood into media and observed its clearing by fungi. Sundman and Näse (1971) added isolated lignins to media which were flooded with ferricyanide solution after fungal growth. Ferricyanide reacts with lignin to give a green colouration and cleared areas were looked for where lignin had been utilized and hence removed. Bavendamm (1928) proposed a simple and rapid technique to determine the ligninolytic capacity of fungi involved in the rotting of wood. Most ligninolytic fungi have been observed to produce polyphenol oxidases these are enzymes which catalyse the oxidation of polyphenolic

compounds. The addition of such compounds to the medium in the form of gallic or tannic acids gives rise to dark coloured quinone containing products in the presence of polyphenol oxidases. Thus, the appearance of a brown diffusion zone under and around the growing fungal colonies indicates the production of these enzymes. Bavendamm found that fungi giving positive reactions to this test were usually white rot fungi, later workers (Lindeberg,1948; Sundman and Näse,1971) have however reported somewhat conflicting results. Nevertheless, generally the 'Bavendamm' test remains as a simple method of obtaining some indication as to whether or not a fungus is likely to be capable of degrading lignin, and as such was used for this investigation.

4.3(ii) Materials and Methods

A. Effect of pH and temperature on polyphenol oxidase production

As with the previous experiment five isolates were investigated, AS-1, AS-2, AS-3, AS-4 and AS-6. The media used throughout these 'Bavendamm' tests were those containing gallic and tannic acid as described by Lindeberg (1948) (Appendix 1). Gallic and tannic acids were both used as occasionally an organism may give a positive result with one acid but not the other. Both these media were buffered at pH 6.2, 7.5 and 8.5 using Sörenson's buffers (Fries, 1956) which have been used successfully in previous similar experiments (Penn,1977). The buffer solutions and media were autoclaved separately at 15 lb/^{2}" for 20 minutes and then combined after sterilization. The plates were then inoculated with 8 mm plugs taken from the edge of actively growing colonies; three plates per set of conditions were produced. These were then incubated at three temperatures 25°, 30° and 35°C in non illuminated incubators and observed after 7 and 14 days. The results were assessed as

intensity of brown colouration appearing in the medium. A range of shades was selected for the results to be compared from the British fungus flora colour charts and were reported as very dark, dark, pale colouration or negative result. Non inoculated plates were incubated along with the experimental plates to test the effect of time and temperature on the normal colour of the media.

4.3(iii) Results

It was found that the intensity of colouration did not differ between 7 and 14 days therefore the day 7 results were reported in table 4.4.

Results were similar on gallic and tannic acid plates as far as positive and negative responses, however, the intensity of colouration did vary between the two media. All isolates showed some production of polyphenol oxidases at neutral to acid pH. At 25°C most gave positive reactions at pH 6.2 only, whereas at 30° and 35°C most showed polyphenol oxidase production at pH 7.5 and AS-3 gave a dark colouration up to pH 8.5 on tannic acid medium at 30° and 35°C.

4.3(iv) Materials and Methods

B. Further Investigations at 30°C

It was decided to extend the pHs investigated further into the acid ranges and to compare the results with those brought about by *C comatus* which has previously been reported to give a positive reaction to the 'Bavendamm' test, also to measure growth and to relate hyphal extension to polyphenol oxidase production. The procedure outlined in 4.3(ii) was repeated using tannic acid media alone and incubating at 30°C, the pH were buffered at 5.1, 6.9, 7.9, 8.2 and 8.5. Plates were inoculated with AS-1, AS-2 and *C comatus* plugs, a set of non inoculated controls were produced

Table 4.4

Production of Polyphenoloxidases by *C cinereus* under differing conditions of pH and temperature

Isolate	Temperature °C	Initial pH	Tannic Acid	Gallic Acid
AS-1	25	6.2	+	+
		7.5	-	+
		8.5	-	-
	30	6.2	+	+
		7.5	-	-
		8.5	-	-
	35	6.2	+	+
		7.5	+	+
		8.5	-	-
AS-2	25	6.2	+	+
		7.5	-	-
		8.5	-	-
	30	6.2	+	+
		7.5	+	-
		8.5	-	-
	35	6.2	+	+
		7.5	+	+
		8.5	-	-
AS-3	25	6.2	+	+
		7.5	-	-
		8.5	-	-
	30	6.2	+	+
		7.5	++	-
		8.5	++	-
	35	6.2	+	+
		7.5	++	-
		8.5	++	-
AS-4	25	6.2	+	++
		7.5	-	++
		8.5	-	-
	30	6.2	+	+
		7.5	+	-
		8.5	-	-
	35	6.2	+	+++
		7.5	+	++
		8.5	-	-
AS-6	25	6.2	+	+
		7.5	-	-
		8.5	-	-
	30	6.2	+	+
		7.5	+	-
		8.5	-	-
	35	6.2	+	+
		7.5	+	-
		8.5	-	-

as in section A. Hyphal diameters were measured on day 7.

4.3(v) Results B

A series of photographs was taken to illustrate the results (plates 4.1,2, 3, 4) and figure 4.3 shows the hyphal extensions which had been reached after seven days in relation to the strength of brown colouration observed. The two isolates of *C cinereus* grew well at alkaline pH as previously reported. The growth at pH 6.9 was not as close to that at higher pH as the histograms may at first indicate, as 9 cm was the diameter of the petri dishes and the hyphae had reached this limit at the higher pHs by day 5 or 6 whereas on lower pH plates, the mycelium had not reached the limits of the dish by day 7. The production of polyphenol oxidases at elevated pH was not conclusive as only a very light brown colouration was visible, but as in 4.3(iii) positive results were obtained at lower pHs *C comatus* grew poorly on the medium but did show a very strong positive reaction at pH 6.9.

4.3(vi) Discussion

It is often difficult to distinguish between a positive and negative reaction to this test, especially as variation in the colour and intensity of the diffusion ring is to a certain extent quite acceptable, as this indicates a variation in the product. However, it would seem that all the isolates investigated do produce polyphenol oxidases but to differing extents under various conditions of pH and temperature.

Many workers have reported *C cinereus* to give positive results to the 'Bavendamm' test (Fries, 1955; Jodice, 1971; Penn 1977) and those using 'natural' lignin containing substrates have mainly found slow but definite loss of lignin (Jodice, 1971) over a long period, although those using shorter periods have reported little

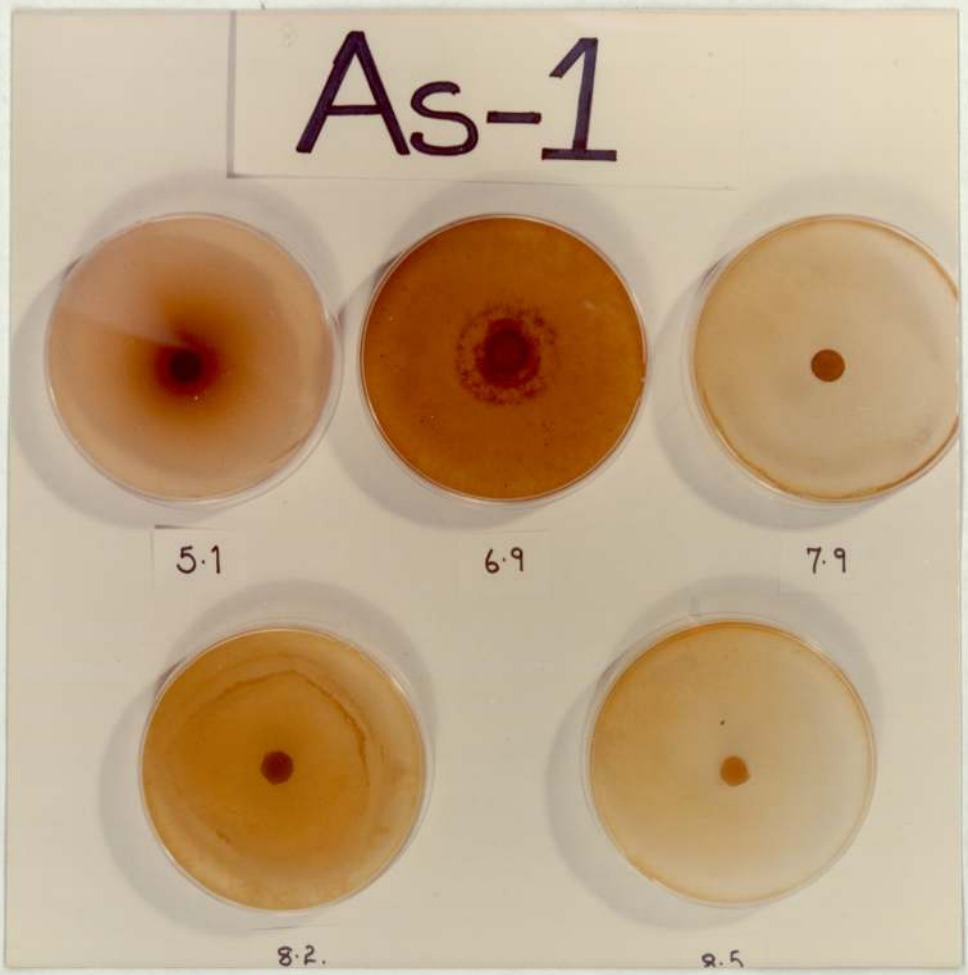


Plate 4.1 Polyphenoloxidase production by As-1 on tannic acid media at different pHs.

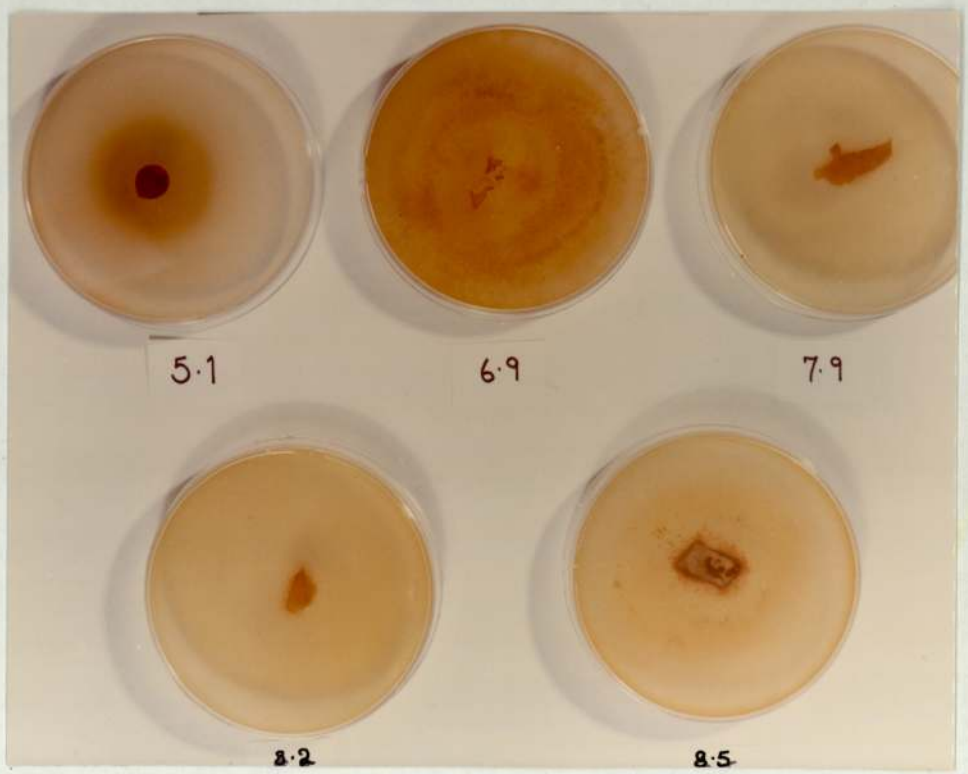


Plate 4.2 Polyphenoloxidase production by As-2 on tannic acid media at different pHs.

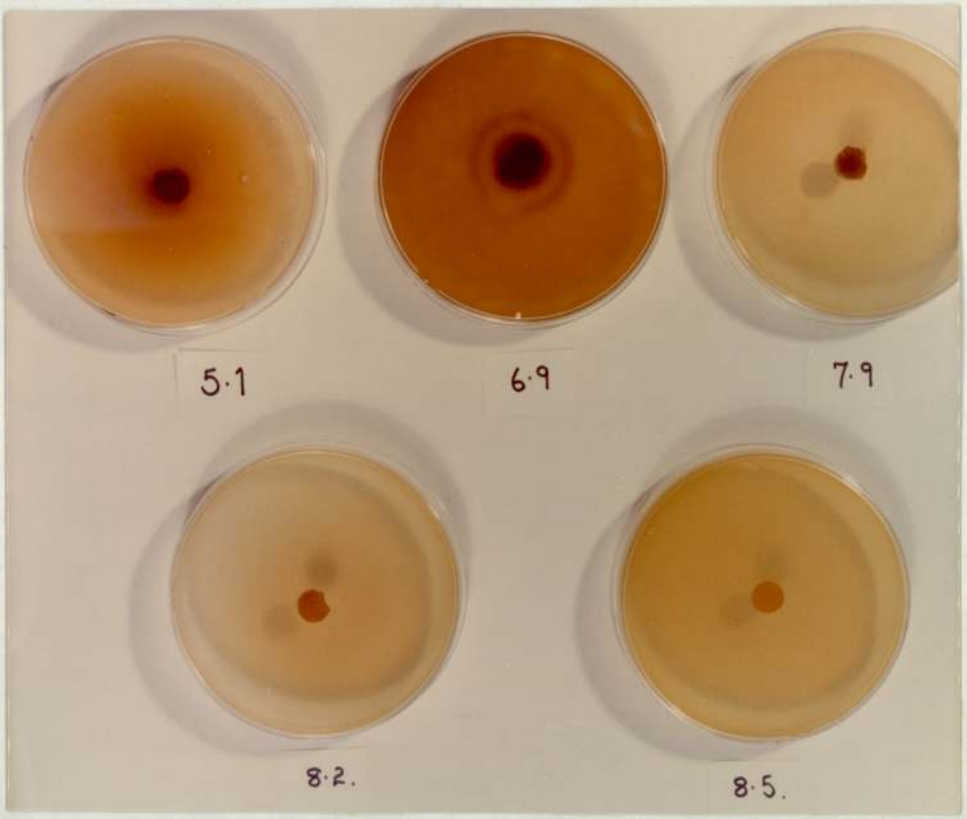
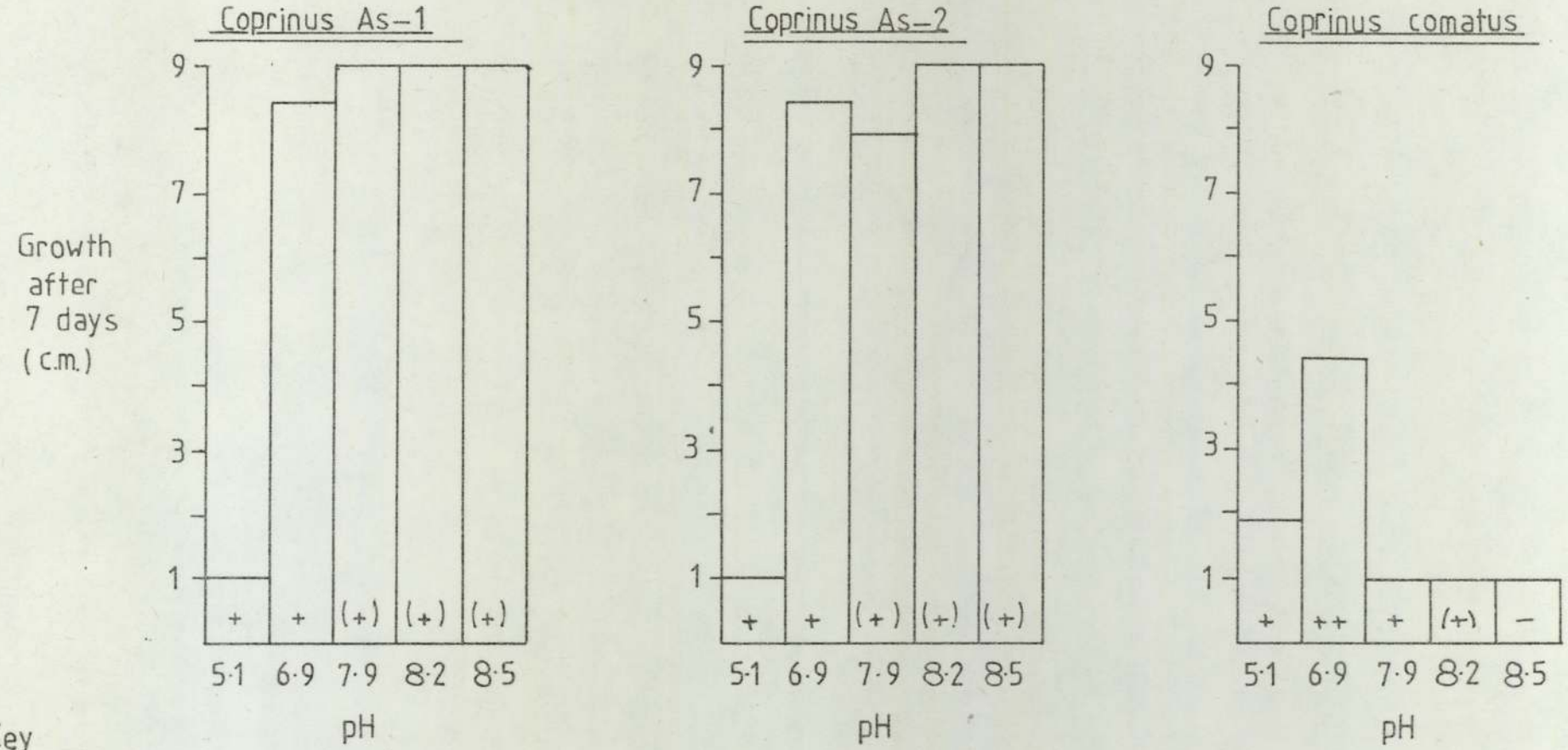


Plate 4.3 Polyphenoloxidase production by *C comatus* on tannic acid media at different pHs.

Plate 4.4 Control tannic acid plates incubated along with test plates.



Key

+ = positive to Bavendamm test
– = negative " " "
(+) = results indecisive
++ = strong positive reaction

Fig 4·3 Growth and production of polyphenol oxidases after 7 days

if any loss of lignin (Chang,1967 and 1977). Rege (1927) reported that *C cinereus* did not utilize lignin incorporated into solid media although as the source of lignin is not reported, it is difficult to comment on this result. Penn found darkening of gallic acid media up to pH 6.9 at 25°C. The results here confirm this but indicate that a positive result can be obtained up to pH 7.5 or even 8.5 in one case at higher temperatures closer to the optimum temperature for *C cinereus* growth. This phenomenon is shown more clearly on tannic acid than on gallic acid media. Figure 4.3 shows that pH conditions conducive to optimum growth do not correspond to those causing greatest production of polyphenol-oxidases, although isolates AS-3 and AS-4 (Table 4.4) in particular seem to indicate that they will respond to a combination of higher temperature and pH to give reasonable amounts of growth and polyphenol oxidase production.

4.4 CHAPTER CONCLUSIONS

There are significant differences between the *C cinereus* isolates in their growth rates, cellulolytic abilities and responses to the 'Bavendamm' test, and this may be sufficient to indicate strain differences. From these results three isolates were chosen for further work. Because of their differences or similarities AS-1, AS-3 and AS-6 were selected from the six isolates;these have similar growth rates which are significantly greater than AS-2 and AS-4. The ability to invade the substrate quickly may well be an advantage when rapid colonisation of the straw substrate is required. Isolate AS-1 was chosen as it has a significantly slower rate of cellulose breakdown, the possible benefit of this has been discussed in a previous section, AS-3 was chosen as it showed indications of having a higher rate of polyphenol oxidase production at raised pH and AS-6 was selected as the results indicated that this isolate combined fairly high polyphenol oxidase activity with

being in about the middle of the group for cellulase activity. (Detailed breakdown of significant differences can be found in appendix 4). Therefore these three isolates were used in later work.

The ligninolytic abilities of *C cinereus* may still be open to debate as mentioned in section 4.3 (vi) but it does seem that this fungus can break down and utilize lignin very slowly over a relatively long term period; this may be of little use in the proposed straw upgrading process. However, it does indicate that further work on the carbohydrate nutrition of *C cinereus* particularly under alkaline conditions is necessary before parameters for the proposed process can be laid down.

CHAPTER FIVE

OPTIMUM TEMPERATURES AND LEVELS OF AMMONIA TO FACILITATE THE SELECTIVE GROWTH OF *C CINEREUS* ON STRAW

5.1 INTRODUCTION

The work detailed in chapter two included an investigation into the possibilities of isolating *C cinereus* from straw using a range of ammonia concentrations. The results indicated that these could be used both as a selective agent and as a source of nitrogen for the growth of this fungus on straw, under non-sterile conditions. These studies however covered a wide range of ammonia levels. The work described in this chapter was designed to investigate more closely those levels which gave optimum isolation of *C cinereus* and maximum suppression of other organisms.

The aim of the second part of the work described here relates to the optimum temperature which facilitates selective colonisation of ammonia amended straw by *C cinereus*. The incubation temperature used up to this stage of the work was 30°C but this figure was based on results from other workers' experiments which were usually carried out in pure culture on artificial media, so that it was felt necessary to investigate further the effects of temperature on growth under the proposed conditions.

5.2 OPTIMUM AMMONIA LEVELS

To ensure that the cost of any future upgrading process would be kept to a minimum, it was necessary to find the lowest levels of ammonia which would give maximum *C cinereus* selection. This was carried out in laboratory scale experiments as described previously.

5.2(i) Materials and methods - ammonia investigations

The jar technique outlined in chapter two was once again utilised in this piece of work which was carried out in two stages. Firstly to obtain results over a broad spectrum of ammonia levels and secondly to investigate further the levels at which selection began and ceased, hence to clarify the useful levels of ammonia.

Triplicate jars containing 5 g of air dry straw were set up and amended with the required ammonia levels and distilled water, until a total of 20 ml of liquid had been added. The treatments were designated 1-13 and the pHs were taken as previously described before and after 12 days incubation at 30°C (table 5.1).

Table 5.1 Ammonia treatments 1-13

Treatment Number	% Ammonia w/w	Initial pH	Final pH
1	10.0	10.07	9.15
2	9.0	9.83	8.75
3	8.0	9.80	8.86
4	7.0	9.83	8.85
5	6.0	9.95	8.40
6	5.0	9.65	8.85
7	4.0	9.65	9.03
8	3.0	9.47	9.00
9	2.0	9.25	8.90
10	1.0	9.25	9.10
11	0.1	8.48	9.05
12	0.01	8.48	9.00
13	distilled H_2O	7.73	8.80

As shown in chapter two the mycoflora of straw samples can vary significantly so that in order to have a constant "basic" flora for the rest of the work described in this thesis, straw from a single

source was utilised. This was obtained from the Northampton area and was baled at harvest 1975. The straw was of a malting barley variety called "Hassan". This single straw source was also used to maintain consistency with workers carrying out parallel biochemical and nutritional analyses.

The numbers of actively growing species of fungi were assessed as previously described, using the Harley-Waid technique on five single straws from each jar giving a total of fifteen plates. These were then incubated at 30°C, examined after 4 and 7 days and the percentage isolation of *C cinereus* noted along with the number of other species isolated.

Treatments 1-13 gave the results shown in figure 5.1 and from these it was decided in the second part of this work to investigate further the plateau area between 0.1% and 4% ammonia content (table 5.2). Identification of most isolates was not carried out as it was felt that this would simply be repeating previous work.

Table 5.2 Detailed investigation of ammonia levels

Treatment Number	% Ammonia w/w	Initial pH	Final pH
14	0.8	9.00	8.90
15	0.9	9.09	8.90
16	2.5	9.37	9.10
17	3.0	9.51	9.00
18	3.5	9.57	9.05
19	3.7	9.58	9.00

5.2(ii) Results

These are shown in table 5.3 and figures 5.1 and 5.2, the latter showing details of the plateau giving optimum *C cinereus* isolation. Only species isolated from jars treated with 2% ammonia and above were

identified as the single species which appeared on all these plates withstood higher ammonia levels and pH,and thus may be in direct competition with *C cinereus*. This was identified as *Scopulariopis brevicaulis*.

From figure 5.1 it can be seen that levels between 1-2% ammonia brought about 100% isolation of *C cinereus* while preventing growth of competing species. Above these levels *C cinereus* was not isolated but *S brevicaulis* was regularly obtained and at levels below 1% many other species were isolated in large numbers. Figure 5.2 shows that 100% isolation can be obtained between 0.8% and 2%. However, the lowest ammonia level which maintains suppression of other species is 0.9% and again at levels above 2% *S brevicaulis* was regularly isolated.

Table 5.3 Percentage isolations of *C cinereus* and other species after ammonia amendment

Treatment	% *C cinereus*	Other species (Sum % of each isolate)	Treatment	% *C cinereus*	Other species (Sum % of each isolate)
1	0	0	11	50	190
2	0	0	12	25	250
3	0	30	13	0	455
4	0	100	14	100	175
5	0	100	15	100	0
6	0	100	16	100	213
7	0	100	17	0	200
8	0	120	18	0	150
9	100	0	19	0	150
10	100	0			

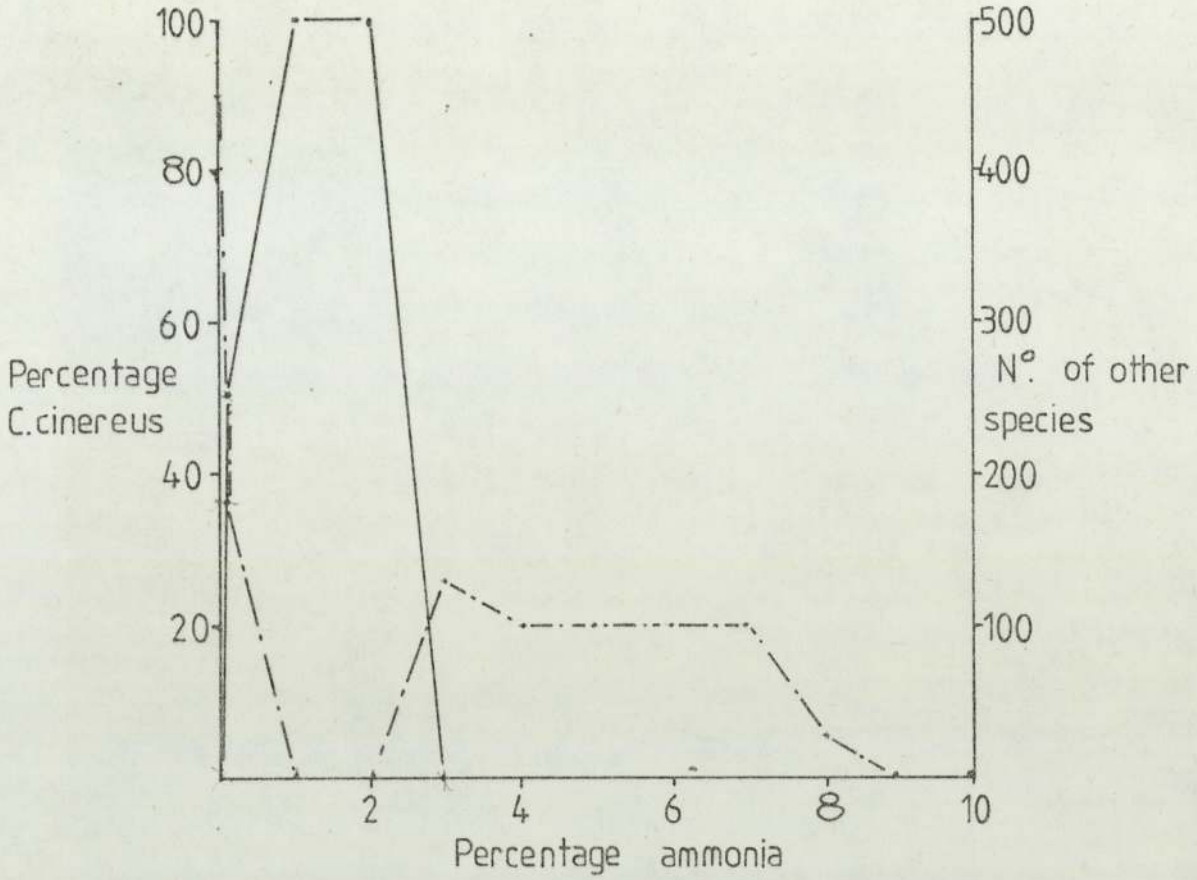


Fig 5·1 Optimum ammonia amendment for C. cinereus isolation

Key Figs 5·1, 5·2, 5·3.

—— = C. cinereus

—·— = Others

5.2(iii) Conclusions

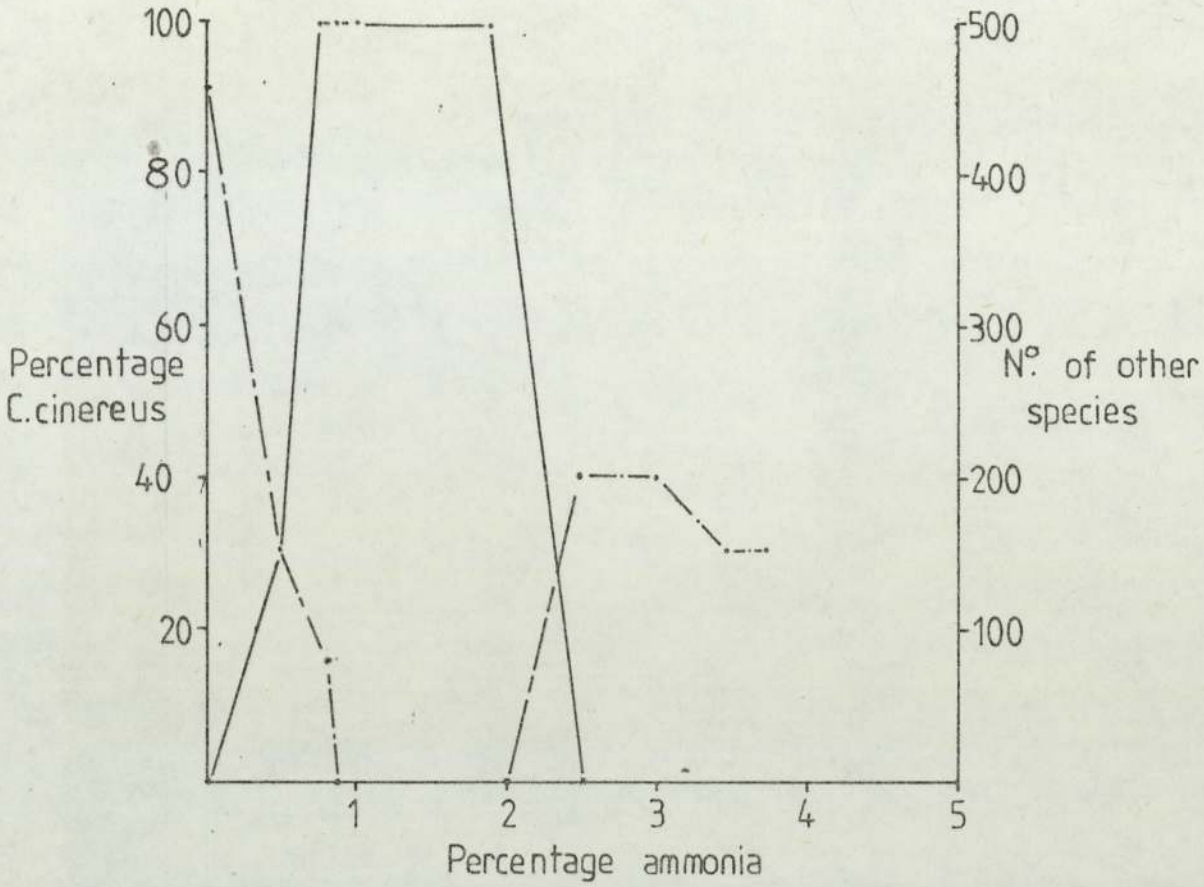
From the above results it would appear that an ammonia level of 1% was suitable for use in further work: this gave optimum isolation of *C cinereus* with maximum suppression of other species. The isolation of *S brevicaulis* in this piece of work must be given serious consideration, because as previously stated this fungus can be pathogenic to man. Levels of ammonia between 1-2% allow, it would seem, *C cinereus* to compete succe sfully with *S brevicaulis*, possibly by growth rate alone. However, as ammonia levels increase and become inhibitory to *C cinereus*, *S brevicaulis* which has a high optimum pH for growth takes over and becomes the dominant coloniser.

5.3 THE EFFECT OF TEMPERATURE ON THE COMPETITIVE ABILITY OF *C CINEREUS* ON STRAW

A number of workers have carried out investigations into the temperature tolerance ranges of *Coprinus spp*. These were usually carried out in pure culture and on artificial media (Fries, 1953; Yung Chang, 1967; McShane, 1976). Fries gave an upper limit to growth for *C fimitarius* (synonym *C cinereus*) of over 44°C with an optimum at "around" 40°C and McShane found the optimum to be about 35°C, varying slightly with strain characteristics. However, Webster (1970) states that although the temperature optimum for growth is about 35°C, fruiting does not occur at this temperature but only below 30°C. Yung Chang also found the optimum temperature for growth to be 35°C but could only obtain fertile sporophores at 25°C. This phenomenon has not been observed in this laboratory where healthy fruiting bodies are regularly obtained at 30°C.

From the combined evidence of these reports 35°C would appear to be the obvious temperature to choose to encourage rapid colonisation of straw by *C cinereus*. However in mixed culture and on natural substrates

Fig 5·2 Further investigations of optimum ammonia levels



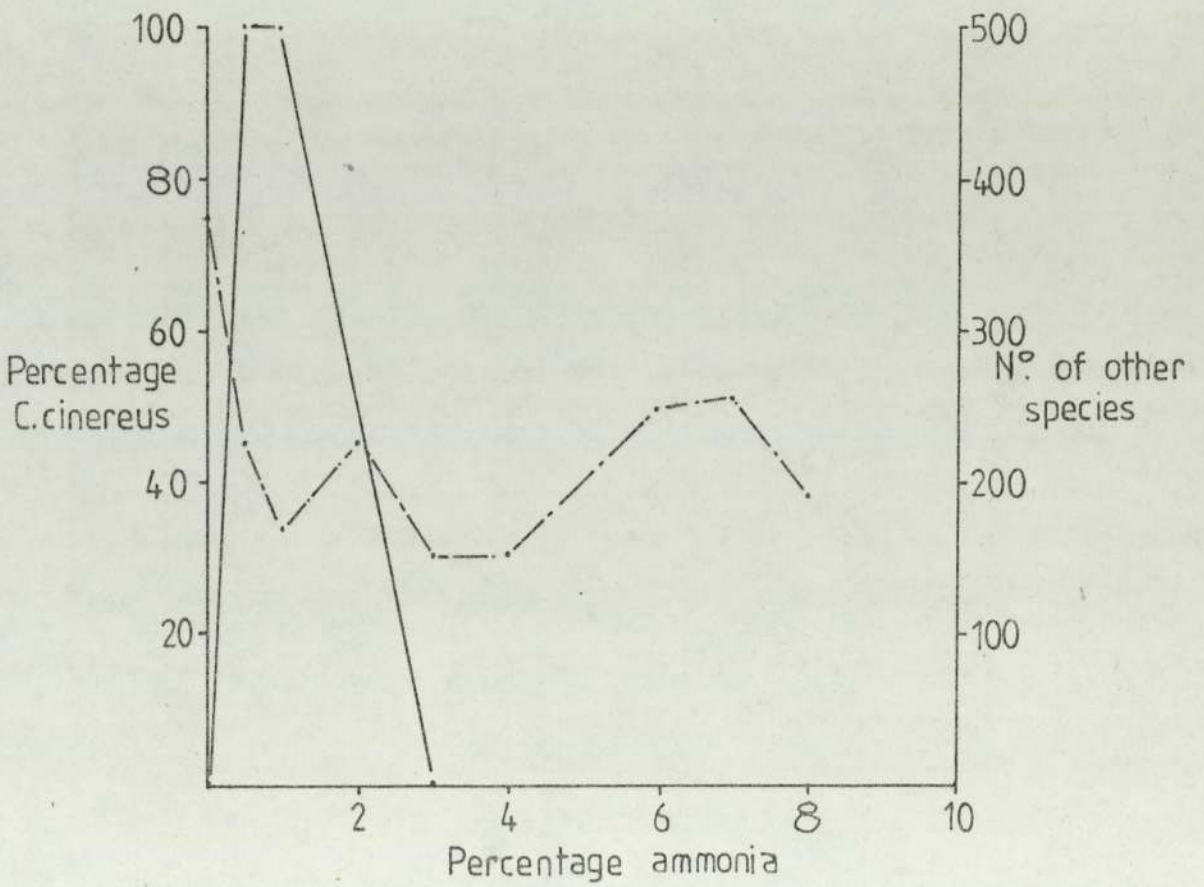


Fig 5.3 Isolations after ammonia amendments at 35°C

the choice is not so obvious as other factors are introduced, for example, *A fumigatus* is a frequent coloniser of straw and grain (Flannigan, 1969) and is a thermophilic fungus. It may therefore be undesirable to provide these higher temperature conditions where the competitive ability of *A fumigatus* may be enhanced. *A flavus*, the fungus which produces the mycotoxin, 'aflotoxin' has also been isolated from stored, moist barley grain (Mulinge and Apinis, 1969) and is a known thermophilous species.

Considering all these factors it was felt that an investigation of temperatures around 30°C, which had been utilised with some success in previous experiments, would yield useful information.

5.3(i) Materials and Methods - temperature investigations

The technique used in the ammonia studies was again repeated at 2%, 1%, 0.01% and 0 ammonia levels. Triplicate jars for each of these levels were then incubated at 20, 25, 30 and 35°C for 12 days. After this period straws were plated as described above and the presence or absence of *C cinereus* and other isolates were recorded. After considering the results thus obtained (Table 5.4) it was decided to compare 30° and 35°C more closely as these gave the better *C cinereus* isolations. The ammonia level experiment carried out at 30°C was therefore repeated at 35°C so that the curve thus obtained could be compared directly with those obtained at 30°C (fig 5.1 and 5.2).

5.3 (ii) Results

The results of the 20-35°C temperature investigations are shown in table 5.4.

Table 5.4 Appearance of *C cinereus* and other species over a range of temperatures

% Ammonia	20°C	25°C	30°C	35°C
2	++(Very Sparse)	++(Very Sparse)	++	++
1	++(Very Sparse)	+	++	++
0.1	(+)	-	(+)	(+)
Zero	-	-	-	(+)

Key:- ++ *C cinereus* alone

\+ *C cinereus* + one other species

(+) *C cinereus* + many other species

\- *C cinereus* not isolated

The best growth and selection of *C cinereus* was obtained at 30 and 35°C from the 1 and 2% ammonia levels. The results of the closer investigations are shown in table 5.5 and figure 5.3.

Table 5.5 Isolation of *C cinereus* and other species after ammonia amendment and incubation at 35°C

Treatment Number	% Ammonia	% *C cinereus* isolation	Other sp (Sum of % each isolate)
1	0	0	375
2	0.5	100	225
3	1.0	100	173
4	2.0	100	225
5	3.0	0	150
6	4.0	0	150
7	5.0	0	125
8	6.0	0	238
9	7.0	0	250
10	8.0	0	188

From figure 5.3 it can be seen that the isolation of *C cinereus* at 35°C was much the same as that obtained by incubating at 30°C. However the number and frequency of other species isolated increased substantially. Complete suppression of other species as observed at 30°C was not obtained and the fungus isolated between 1-3% ammonia was *A fumigatus* and this was combined with *S brevicaulis* at ammonia levels above 2%. It would seem that the increase of only 5°C gave the thermophilic *A fumigatus* a major advantage so that it was able to compete very successfully with *C cinereus*. The results from the wider ranging study showed that it was possible to isolate *C cinereus* alone at 35°C however it would appear from these closer observations that this was not a consistent result which could safely be relied upon.

5.3(iii) Conclusions

C cinereus was successfully grown and isolated at 35°C, however, selection did not occur as had been observed at 30°C. This would seem to indicate that although temperatures greater than 30°C have been reported as the optimum temperature in pure culture, under the circumstances of the proposed upgrading technique a temperature of 30°C would be more efficient. This temperature would also be more easily maintained by the farmer on site.

5.4 CHAPTER DISCUSSIONS

The dangers posed by the two main competitors to *C cinereus* as shown by the work described in this chapter cannot be underestimated as both are potential human pathogens. However the results indicate that it would be possible, with careful environmental control to maintain an axenic culture of *C cinereus* on straw. The maintenance of a temperature of 30°C combined with an initial ammonia amendment of 1% should remove the danger of contamination by *A fumigatus* and *S brevicaulis* respectively, although it must be borne in mind at this stage that a full

isolation programme, including the use of less selective media had not yet been undertaken. This work, combined with a study of the mycoflora over time periods, is described in later chapters. However in all later work 1% ammonia was used as the selective level for amendment.

It is interesting to note the sharp change from 100% *C cinereus* isolation to zero isolation which was observed in all cases after the 2% ammonia level. This may mean that *C cinereus* itself is unable to tolerate ammonia above these levels, and it would certainly appear that above this point *C cinereus* is not competitive. Further investigation of levels between 2 and 3% may produce intermediate percentages of *C cinereus* isolation, but it was not felt necessary to pursue this as it was proposed to use ammonia levels at the other end of the plateau ie 1% in the upgrading technique.

The results of this work do not reflect the proposed method faithfully in that the endemic *C cinereus* alone was investigated. It is envisaged that the process will include inoculation of the straw with a spore suspension so that these encouraging results should be repeatable when a much heavier inoculum of *C cinereus* is present on a larger scale.

CHAPTER SIX

THE ROLE OF AMMONIA IN THE SELECTIVE ISOLATION OF *COPRINUS CINEREUS*

6.1 INTRODUCTION

6.1(i) General

The results of experiments detailed in previous chapters have indicated that ammonia solutions can produce highly selective conditions for the isolation and growth of *C cinereus*. However the effect of ammonia on *C cinereus* growth rates has not yet been investigated, neither has its role in the observed selection process. Ammonia may simply be acting as a nitrogen source which promotes rapid growth, as an agent which raises pH and consequently favours *C cinereus* growth or a combination of these may be acting on the fungus. Alternately the possibility must be considered that it is the suppressive effect of ammonia on other species which is the selecting force. *C cinereus* may simply be capable of withstanding the adverse conditions produced by ammonia so that it is not being actively selected for, but a hostile environment is being created for its competitors. A similar situation to this is the growth of *Cladosporium resinae* on creosote treated poles. The fungal growth is not enhanced by the presence of creosote but *C resinae* can tolerate higher levels of this preservative than most other fungi and is therefore able to colonise the poles.

The work described in this chapter was designed to investigate the effect of ammonia solution on the growth rate of *C cinereus* when straw was the substrate and also to study the effect that varying nitrogen sources at an elevated pH had on these growth rates. It was hoped that from the results it would be possible to

ascertain which properties of ammonia were bringing about the selective conditions previously observed.

6.1(ii) Growth Measurements

The measurement of fungal growth rates has been a topic for much debate over many years. It is difficult to define exactly what is meant by growth and consequently different techniques provide differing sets of results which must be considered both individually and in relation to each other. It should be remembered that what is being quantified is linear hyphal extension, radial growth, biomass increase or some other growth parameter and not simply "growth". There are added difficulties when attempting to measure accurately the amount of fungal 'growth' on straw, this is because, as with many other solid substrates, the close contact between hyphae and substrate makes removal of mycelium for weight increase measurements virtually impossible. The same problem exists when using substrate weight loss as an assessment of growth, although this technique is often made use of where fairly significant weight losses can be expected. The nature of the experiment under consideration requires 'growth' measurement of a single species on non-sterile straw where other species will also be present. Consequently weight changes would be due to the activities of all species and not just *C cinereus*. These objections would also apply to the use of techniques which measure metabolic activity such as respirometry (Umbreit, Burris and Stauffer 1964), enzyme assay (Lenhard, 1956; Kibble, 1966; McGarity, and Myers 1967; Porter, 1965), thermal measurements (Newman and Norman, 1943; Clark, Jackson and Gardner, 1962) and also to those which measure chitin levels. Because of this, it was decided that a comparison of hyphal extension rates would be a valid assessment of 'growth'

under these circumstances, as not only could the reaction of *C cinereus* to different nitrogen sources be observed, but also the effect on growth rates of the presence of competitors under these conditions. Both of these are factors of importance when considering the ability of *C cinereus* to colonise straw.

Evans (1955) used 'growth tubes' to investigate fungal growth rates in soil and McShane (1976) used an adaptation of this technique to study growth rates on straw. In this piece of work a simplified version of McShane's tubes were employed; these were of shorter length and did not have access ports for the removal of straws for microbial examination.

6.2 EXPERIMENTAL

6.2(i) Materials and Methods

A variety of nitrogen sources were investigated to ascertain the ability of *C cinereus* to utilise (when growing on straw) organic nitrogen, nitrate nitrogen, nitrite nitrogen and nitrogen from the ammonium ion as ammonium hydroxide and in compound form as ammonium tartrate. By carrying the work out at an elevated pH it was hoped that the selective effects, if any, of pH alone and in combination with a source of nitrogen could be compared to the previously observed abilities of ammonia solutions. The experiment was carried out under sterile and non-sterile conditions to investigate the effect of the nitrogen sources on the competitive ability of *C cinereus*. Ammonium tartrate was used in an effort to overcome the problems of large pH drifts towards acid conditions which are found when using such sources as ammonium sulphate. This can be prevented by buffering or by adding certain organic acids or by using salts of organic acids (Morton & Macmillan, 1954; Fries, 1955) and the salt of tartaric acid was decided upon so as not to contribute to the carbon pool as the carbon skeleton in ammonium tartrate is not utilised by *C cinereus* (Moore, 1969).

Fig. 6·1 Growth tube

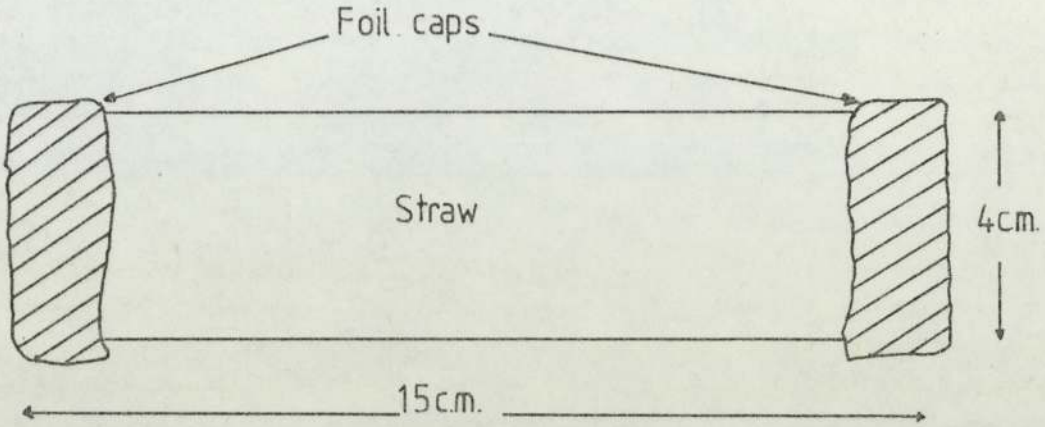
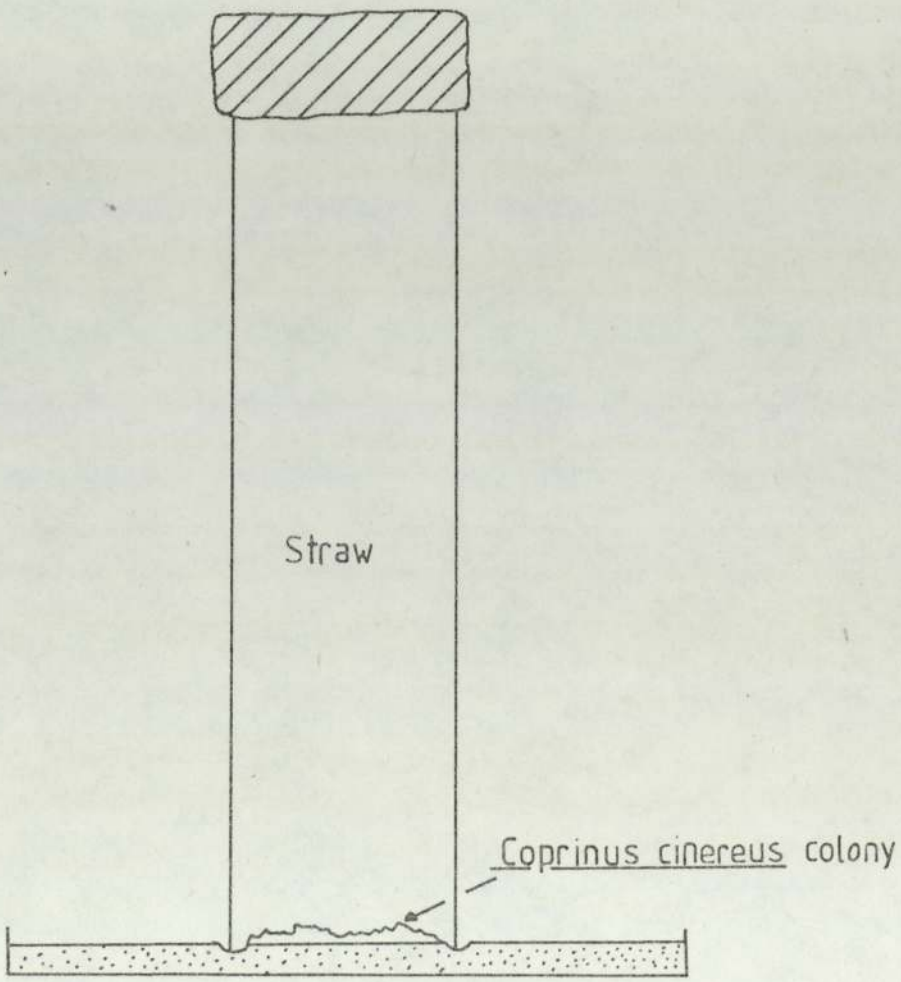


Fig. 6·2 Inoculation of growth tubes



Pyrex glass tubes were produced with a diameter of 4 cm and length of 15 cm for use as growth tubes. These were then packed with 4 g of straw (barley straw of the variety previously quoted) which was arranged so that it lay lengthways along the tubes (Figure 6.1). These were then capped at each end with squares of metal foil and divided into two groups; one group remained non-sterile and the second group was sterilised at 15 lb" for 40 minutes, after first adding 1 ml of distilled water to the straw to moisten and aid sterilisation. Each of these groups was then subdivided into three for inoculation with the three *C cinereus* isolates obtained in previous work. These were AS-1, AS-3 and AS-6, which had been selected after studying the results of the experiments described in Chapter Four as they showed significant differences in their utilisation of cellulose and lignin.

6.2(ii) Inoculation of Growth Tubes

The three isolates were grown from plug inocula on malt extract agar and were incubated for 48 hours at 30°C after which time the colonies had reached a diameter of approximately 4 cm; this was suitable for use in inoculating the growth tubes. Inoculation was carried out under aseptic conditions by removing the foil from one end and pressing the rim of the tube into the agar in a "pastry cutter" fashion (Figure 6.2). The tube was then removed and the inoculum manoeuvred into position in the end of the tube with the aid of a sterile scalpel, the tube was then quickly recapped.

6.2(iii) Addition of Nitrogen Sources

To these sterile and non-sterile tubes six different nitrogen sources were added, three tubes per treatment (Table 6.1). The ammonia solution was added at the rate of 1% weight of ammonia/ weight straw, the other nitrogen sources were added to give equal

amounts of nitrogen per gramme of straw as that in the ammonia treatment. These were first dissolved in 5 ml of distilled H_2O. Stock solutions of the nitrogen sources made up to 150 mls with distilled H_2O were produced and autoclave sterilised. The urea and ammonia solutions were filter sterilised to prevent breakdown.

Preliminary experiments had been carried out to ascertain the amounts of $^{N}/_{10}$ NaOH which would be required to elevate the pH of 5 mls of nitrogen source (post sterilisation) to pH 11, which was that of the ammonia solution. The NaOH was filter sterilised in the same way as the ammonia solution. The required amount was then added to 5 mls aliquots of the nitrogen sources and made up to a total of 17 mls with sterile distilled H_2O and added to the growth tubes which were then well rotated to ensure even distribution of the nutrient-solutions. The amounts which were added are shown in table 6.2. This procedure was carried out aseptically at all stages. Buffers were not added to the nutrient solutions. During the proposed process using ammonium solutions, there is initially a very high pH which drops during the growth period and it was necessary to investigate the possibility that this high pH provides *C cinereus* with an advantage and that the later drop in pH was also of importance.

6.2(iv) Controls

It was possible that autoclave sterilisation would produce somewhat inhibitory compounds in the straw and consequently a control experiment was set up to compare growth rates on autoclave sterilised and propylene oxide sterilised straw. Nine growth tubes with straw were sterilised with propylene oxide by placing them uncapped in a desiccator along with a beaker of propylene oxide. These were then left for 24 hours in a fume cupboard and after this period the tubes were removed, capped with

sterile foils and replaced in the fume cupboard for a further 48 hours to ensure dispersal of any remaining propylene oxide. These were then inoculated with the three isolates, three per isolate and 17 mls of sterile distilled water was added to the straw as above. This inoculation procedure was repeated with nine autoclave sterilised tubes.

A further control was set up to study the effect of pH elevation alone without the addition of nitrogen. Nine autoclave sterilised tubes were inoculated as above and 17 mls of sterile distilled water adjusted to pH 11 with sterile $^{N}/_{10}$ NaOH was added to each tube.

Non-sterile controls were also set up by repeating the above procedures on tubes of non-sterile straw to give nine tubes each at normal and elevated pH.

All inoculated tubes were then incubated at 30°C ± 1°C in separate incubators, this was to prevent cross contamination of nitrogen sources, as ammonia is readily given off at pH above 7 and such a soluble gas would soon appear in other non-ammonia containing tubes. The tubes were removed at 24 hour intervals and the growth of the hyphal front which could be seen clearly was noted. The top edge of the inoculation plug was classed as zero and marked as a future baseline from which all measurements were taken. A rule was considered to be sufficiently accurate for these measurements; hyphal extensions were measured on three sides of the tube to obtain a mean. Growth was measured over a ten day period in the case of sterile tubes or until the hyphal front became indistinct and was lost amongst the hyphae of competing species in the case of non-sterile tubes. After 10 days the final pH of the straw/nutrient mix was taken by the technique described in previous chapters.

6.3 RESULTS

Mean hyphal extensions after each time period are shown in table A.III.2 in appendix 3, and these results are illustrated graphically in figures 6.3 - 6.7. The mean growth rates over the linear growth period are shown in table 6.3 and all statistical calculations are in appendix 4.

Table 6.1 Nitrogen Sources

Ammonia required = 1% ∴ 0.1g/10g straw

or 0.04g/4g straw

In 0.04g ammonia $^{14}/_{17} \times 0.04 = 0.0329$g Nitrogen

∴ 0.033g Nitrogen in 5 ml H_2O added to each 4g of straw.

Nitrogen Source	Gms/150 ml Stock Solution = 0.033g N/5 ml
Ammonia solution	3.42
Urea	2.10
L Asparagine	5.40
Potassium Nitrate	7.20
Sodium Nitrite	4.80
Ammonium Tartrate	6.60

Table 6.2 Composition of Nutrient Solutions

Nitrogen Source 5 ml Stock Solution	mls $^{N}/_{10}$ NaOH	pH of Solution After Sterilisation	Final pH
Ammonia Solution	0	11	)
Urea	1	5.7	)
L Asparagine	6	4.6	)
Potassium Nitrate	1	6.6	) 11 ± 0.2
Sodium Nitrite	1	6.5	)
Ammonium Tartrate	12	5.8	)

Table 6.3 Mean Growth Rates Over Linear Growth Period (mm/hour)

Nitrogen Source	AS-1			AS-3			AS-6		
	1	2	3	1	2	3	1	2	3
Sterile									
Ammonia Solution	0.418	0.426	0.438	0.439	0.442	0.414	0.452	0.520	0.538
KNO_3	0.562	0.564	0.572	0.587	0.591	0.580	0.610	0.548	0.523
$NaNO_2$	0.916	0.334	0.318	0.575	0.586	0.556	0.548	0.519	0.475
Urea	0.608	0.606	0.459	0.609	0.634	0.539	0.655	0.515	0.536
L Asparagine	0.747	0.717	0.755	0.758	0.723	0.801	0.722	0.723	0.798
Ammonium Tartrate	0.594	0.828	0.657	0.703	0.730	0.841	0.735	0.804	0.744
Control Dist H_2O	0.402	0.663	0.492	0.487	0.556	0.531	0.445	0.615	0.524
Control Elevated pH	0.792	0.733	0.667	0.872	0.940	0.759	0.417	0.389	0.417
Non-Sterile									
Ammonia Solution	0.478	0.696	0.511	0.453	0.549	0.521	0.639	0.634	1.145
KNO_2	0.618	0.750	0.507	0.611	0.521	0.500	0.597	0.632	0.583
$NaNo_2$	0.421	0.507	0.546	0.581	0.614	0.767	0.481	0.479	0.452
Urea	0.450	0.426	0.492	0.522	0.464	0.494	0.422	0.502	0.540
L Asparagine	0.701	0.600	0.634	0.593	0.515	0.756	0.585	0.504	0.800
Ammonium Tartrate	0.579	0.656	0.606	0.637	0.668	0.637	0.522	0.586	0.874
Control Dist H_2O	0.387	0.577	0.413	0.505	0.175	0.194	0.627	0.480	0.258
Control Elevated pH	0.389	0.500	0.403	0.375	0.181	0.167	0.292	0.347	0.333

Tables 6.4 and 6.5 show the differences between treatment mean growth rates and their significances on sterile and non-sterile straw. Table 6.7 contains data on the final pH in the growth tubes and table 6.6 shows the time periods after which the *C cinereus* hyphal front could not be distinguished from competitors in the non-sterile tubes.

Figure 6.3 illustrates the growth of the three isolates of *C cinereus* on straw sterilised by autoclave and by propylene oxide. The graphs indicate that after a 24 hour lag, growth on propylene oxide sterilised straw proceeds at a greater rate than on autoclaved straw. This was confirmed by carrying out a Student 't' test (Bishop,1966; Parker,1973) on the mean growth rates of all three isolates and there proved to be significant differences in the growth rates at levels of $p < 0.05$.

Figure 6.4 illustrates the growth of *C cinereus* in the four control treatments. Measurements in all non-sterile tubes were halted when the advancing hyphal front became indistinct and it can be seen that in these controls the fronts were lost in all cases between 90 - 140 hours after inoculation. From the graphs it would appear that under sterile conditions a rise in pH increased the mean growth rate, however, when the data was statistically analysed, no significant differences were found. Growth rates in both non-sterile control treatments were lower than those in the equivalent sterile tubes thus reflecting the presence of competing micro-organisms.

Analysis of variance was carried out on the data shown in Table 6.3 to encompass two experimental factors and replication (Bishop,1966), these factors were sterility and nitrogen sources and also isolate differences and nitrogen source (appendix 4). Under both sterile and non sterile conditions no significant differences were found between the growth rates of the three

6.4 Difference between treatment means and their significances

Sterile

Sterile Treatments	Ammonia	Potassium Nitrate	Sodium Nitrite	Urea	L Asparagine	Ammonium Tartrate	Control	Control Elevated pH
Ammonia	-	0.117	0.060	0.119	0.295*	0.284*	0.070	0.211*
Potassium Nitrate	-	-	0.057	0.003	0.179	0.168	0.047	0.094
Sodium Nitrite	-	-	-	0.059	0.235*	0.224*	0.010	0.151
Urea	-	-	-	-	0.176	0.165	0.050	0.092
L Asparagine	-	-	-	-	-	0.011	0.225*	0.084
Ammonium Tartrate	-	-	-	-	-	-	0.215*	0.660*
Control	-	-	-	-	-	-	-	0.141
Control Elevated pH	-	-	-	-	-	-	-	-

* = Significant difference

6.5 Differences between treatment means and their significances

Non-sterile

Non-sterile Treatments	Ammonia	Potassium Nitrate	Sodium Nitrite	Urea	L Asparagine	Ammonium Tartrate	Control	Control Elevated pH
Ammonia	-	0.034	0.086	0.146*	0.007	0.148*	0.223*	0.293*
Potassium Nitrate	-	-	0.052	0.112*	0.041	0.049	0.189*	0.259*
Sodium Nitrite	-	-	-	0.060	0.093	0.101	0.138*	0.207*
Urea	-	-	-	-	0.153*	0.161*	0.077	0.147*
L Asparagine	-	-	-	-	-,	0.008	0.230*	0.300*
Ammonium Tartrate	-	-	-	-	-	-	0.238*	0.308*
Control	-	-	-	-	-	-	-	0.070
Control Elevated pH	-	-	-	-	-	-	-	-

* = Significant difference

isolates and therefore they do not appear (on the evidence of nitrogen utilisation) to warrant classification as different strains. However, highly significant differences were found under both sterile and non-sterile conditions between growth rates on the different nitrogen sources. Figures 6.5 - 6.7 illustrate the growth of *C cinereus* on the various nitrogen sources and on sterile and non-sterile straws. In all cases the highest growth rates were shown with L Asparagine and ammonium tartrate; these were followed by urea and potassium nitrate and then sodium nitrite and the ammonia solution the latter of which created a significant lag period before growth began. Under non-sterile conditions asparagine and ammonia solution gave the highest growth rates.

The results contained in table 6.6 indicate that ammonia solution greatly extends the length of time over which *C cinereus* could compete successfully with other micro-organisms present in the straw.

Table 6.7 shows that in the tubes treated with ammonia solution the pH drops to a lower level than those found in any other of the nitrogen treated tubes and in fact returns almost to that level found in the controls, where distilled water alone had been added. Overall, the final pHs found in the non-sterile tubes were higher than those in the sterile tubes; this could have been due to the presence of organic acids produced during the autoclave sterilisation of the straw.

6.4 DISCUSSION

In no way can this series of experiments be said to investigate the ability of the *C cinereus* isolates to utilise various compounds as their sole sources of nitrogen. Straw is far from being a controlled homogeneous medium and it does itself contain levels of nitrogen, also the

Table 6.6 Loss of Hyphal front under non-sterile Conditions

Treatment	Time of Hyphal Front Loss (hours)		
	AS-1	AS-3	AS-6
Ammonia Solution	240	280	230
Potassium Nitrate	96	96	96
Sodium Nitrite	143	143	143
Urea	215	143	143
L Asparagine	139	139	139
Ammonium Tartrate	139	139	111
Control dist H_2O	111	111	111
Control elevated pH	96	96	96

Table 67 pH after growth of *C cinereus* for 10 days

Treatment	Final pH (mean of 3 readings)		
Sterile	AS-1	AS-3	AS-6
Ammonia Solution	7.55 ± 0.07	7.65 ± 0.18	7.45 ± 0.07
Potassium Nitrate	8.20 ± 0.40	7.87 ± 0.12	8.33 ± 0.46
Sodium Nitrite	8.93 ± 0.12	8.80 ± 0.36	8.60 ± 0.17
Urea	8.58 ± 0.29	8.90 ± 0.10	8.87 ± 0.15
L Asparagine	9.22 ± 0.16	9.27 ± 0.32	9.47 ± 0.40
Ammonium Tartrate	9.35 ± 0.22	9.22 ± 0.26	9.48 ± 0.28
Control Distilled H_2O	7.25 ± 0.07	6.70 ± 0.24	7.00 ± 0.06
Control Elevated pH	8.50 ± 0.09	7.80 ± 0.11	8.70 ± 0.71
Polypropylene Oxide	8.20 ± 0.35	7.63 ± 0.29	7.87 ± 0.15
Non-Sterile	AS-1	AS-3	AS-6
Ammonium Solution	7.75 ± 0.07	7.85 ± 0.21	7.70 ± 0.01
Potassium Nitrate	9.22 ± 0.03	8.93 ± 0.21	9.12 ± 0.13
Sodium Nitrite	9.33 ± 0.29	9.40 ± 0.09	9.37 ± 0.15
Urea	8.97 ± 0.16	9.15 ± 0.87	9.03 ± 0.05
L Asparagine	8.35 ± 0.35	8.00 ± 0.14	8.40 ± 0.42
Ammonium Tartrate	7.95 ± 0.21	8.13 ± 0.46	8.03 ± 0.18
Control Distilled H_2O	7.05 ± 0.07	7.20 ± 0.07	7.43 ± 0.11
Control Elevated pH	8.20 ± 0.17	8.80 ± 0.14	8.50 ± 0.58

plug of culture medium on which the inoculum was introduced into the growth tubes would have acted as a source of nutrients for quite some time after inoculation. Consequently, this part of the study was not designed as a piece of pure research into nitrogen uptake and utilisation, but as a comparative investigation into the ability of *C cinereus* to metabolise different sources of nitrogen when placed under a set of closely defined environmental conditions on a 'natural' substrate, and hence to assess the competitive ability of *C cinereus* under these conditions when supplied with these sources of nitrogen.

The results of this work can be considered in two sections, firstly the effects of the nitrogen sources on the growth rates of the *C cinereus* isolates, and secondly a comparison of these growth rates under sterile and non-sterile conditions, so that the ability of *C cinereus* to compete with other fungi when provided with nitrogen at elevated pH can be assessed. But before discussing the data obtained in terms of the above, it is necessary to look at the results of the control experiments, and to explain why certain sections of the experimental design were decided upon after studying indications from the preliminary control experiments. Figure 6.3 compares graphically the growth rates of *C cinereus* on straw which has not been amended with any nitrogen but has been sterilised by propylene oxide and by autoclaving. As already stated there is a significant difference in growth rates on these two straws and it would seem that autoclave sterilisation does cause some inhibitory products to be produced in the straws. This has previously been reported when autoclave sterilisation of vegetable matter has taken place (Lindeberg, 1946). However, it was felt that problems may occur using chemical sterilisation. Comparative studies on sterilisation techniques (Eno and Popenoe, 1964) showed that steam sterilisation alters soil more than irradiation, which in turn causes greater changes than chemical treatments. However they also noted that gas techniques left slight chemical residues in the soil which may be toxic. Even without

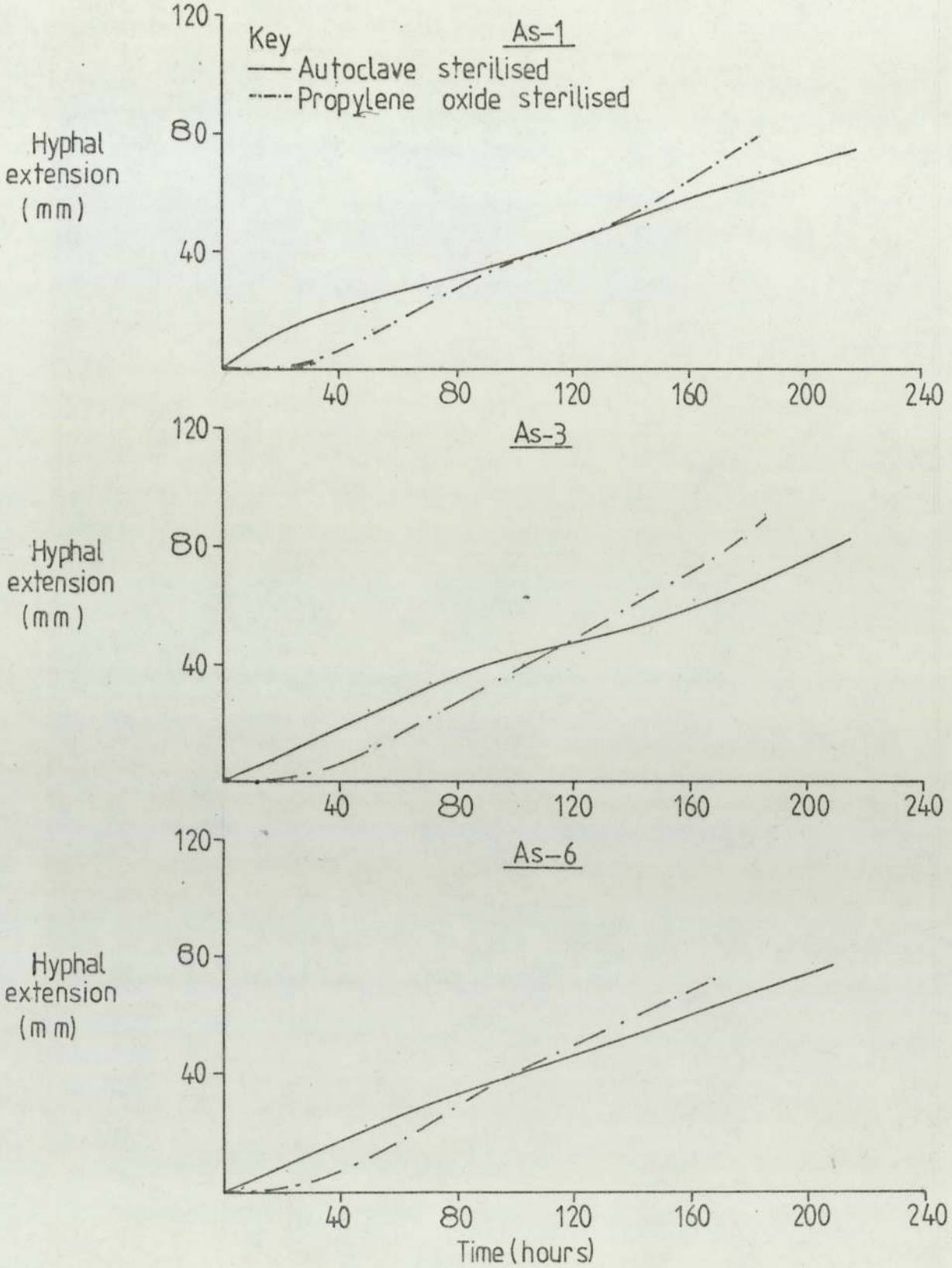


Fig 6·3 Comparison of autoclave with propylene oxide sterilisation

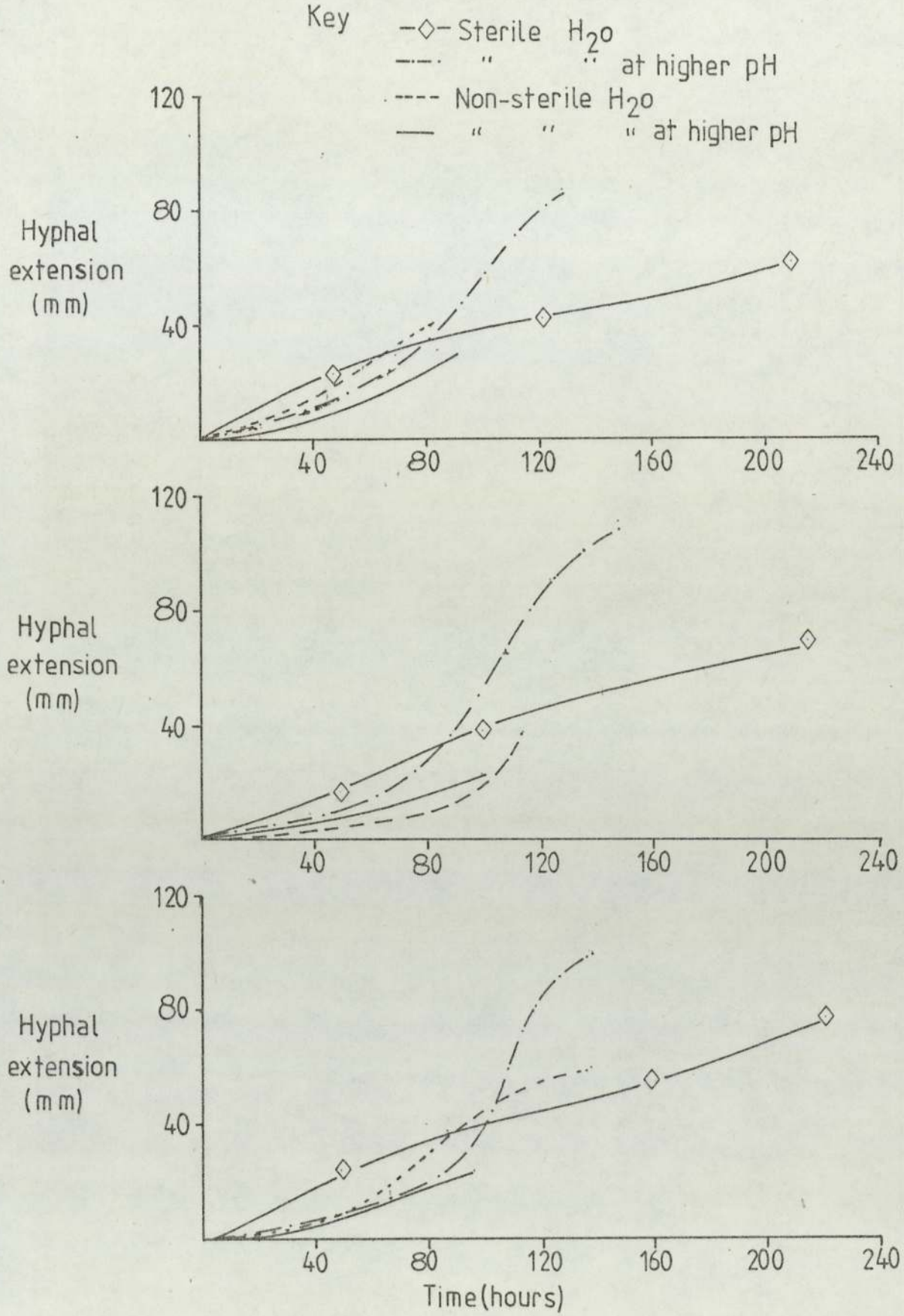


Fig 6·4 Controls — distilled H_2O alone, and at elevated pH

this it is difficult to ensure that all traces of the sterilant are removed before commencing the experimental work, as is illustrated here by the long lag period before *C cinereus* begins to extend. This factor must be considered, particularly in this piece of work where the lag phase brought about by certain conditions is of importance when attempting to discover the action of ammonia. It may have been possible to leave the tubes for longer periods before inoculation, however, with a material such as straw it is difficult to say with confidence that after a certain number of days all traces of growth inhibiting propylene oxide will have disappeared.

Further preliminary work indicated that significant differences in growth rates could be obtained between autoclave sterilised and non-sterile treatments with various nitrogen sources and also between the control treatments where distilled water alone was added. Consequently, it was decided that under these circumstances sterilisation of the straw by autoclave would be valid, as comparison of growth rates between nitrogen treatments would not be effected and when considering differences between sterile and non-sterile straws the problem of slight inhibition was recognised and hence could be taken into account.

6.4(i) Growth on nitrogen sources

Many previous workers have studied nitrogen metabolism in *Coprinus* species and it is worthwhile to compare their results with those obtained here to assist with interpretation and to illustrate how even in a single species numerous variations in nutrient utilisation can be found. One of the earliest and most comprehensive pieces of work on the nitrogen nutrition of the *Coprini* was by Fries (1955) who studied three strains of *Coprinus fimitarius* (synonym *C cinereus*) and found that all strains could utilise asparagine and ammonium nitrogen. L asparagine gave the largest increases in mycelial weights followed by ammonium nitrogen. Two of the three strains utilised nitrate - N, nitrite-N and

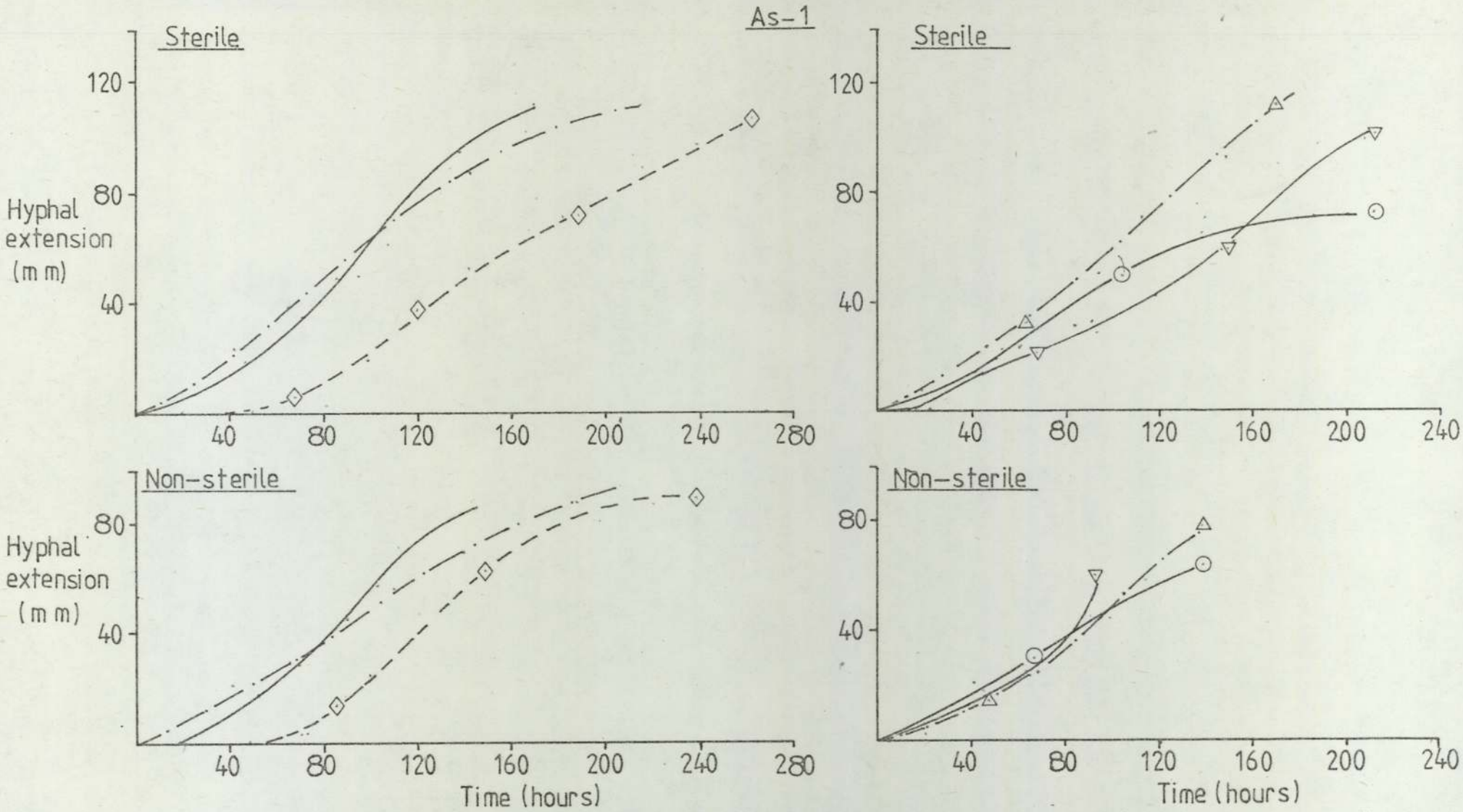


Fig 6-5 C. cinereus growth on nitrogen amended straws

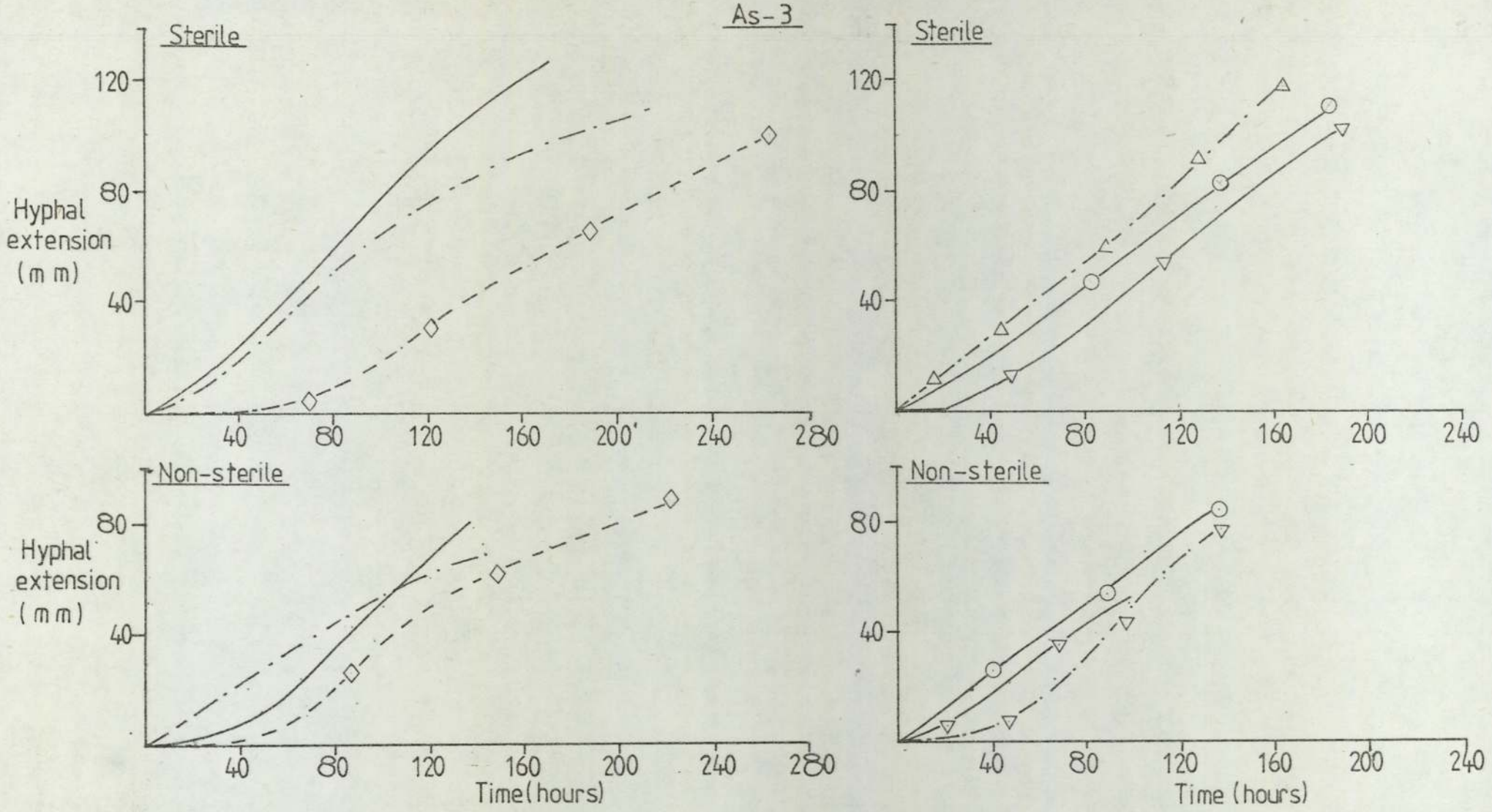


Fig 6-6 C.cinereus growth on nitrogen amended straws

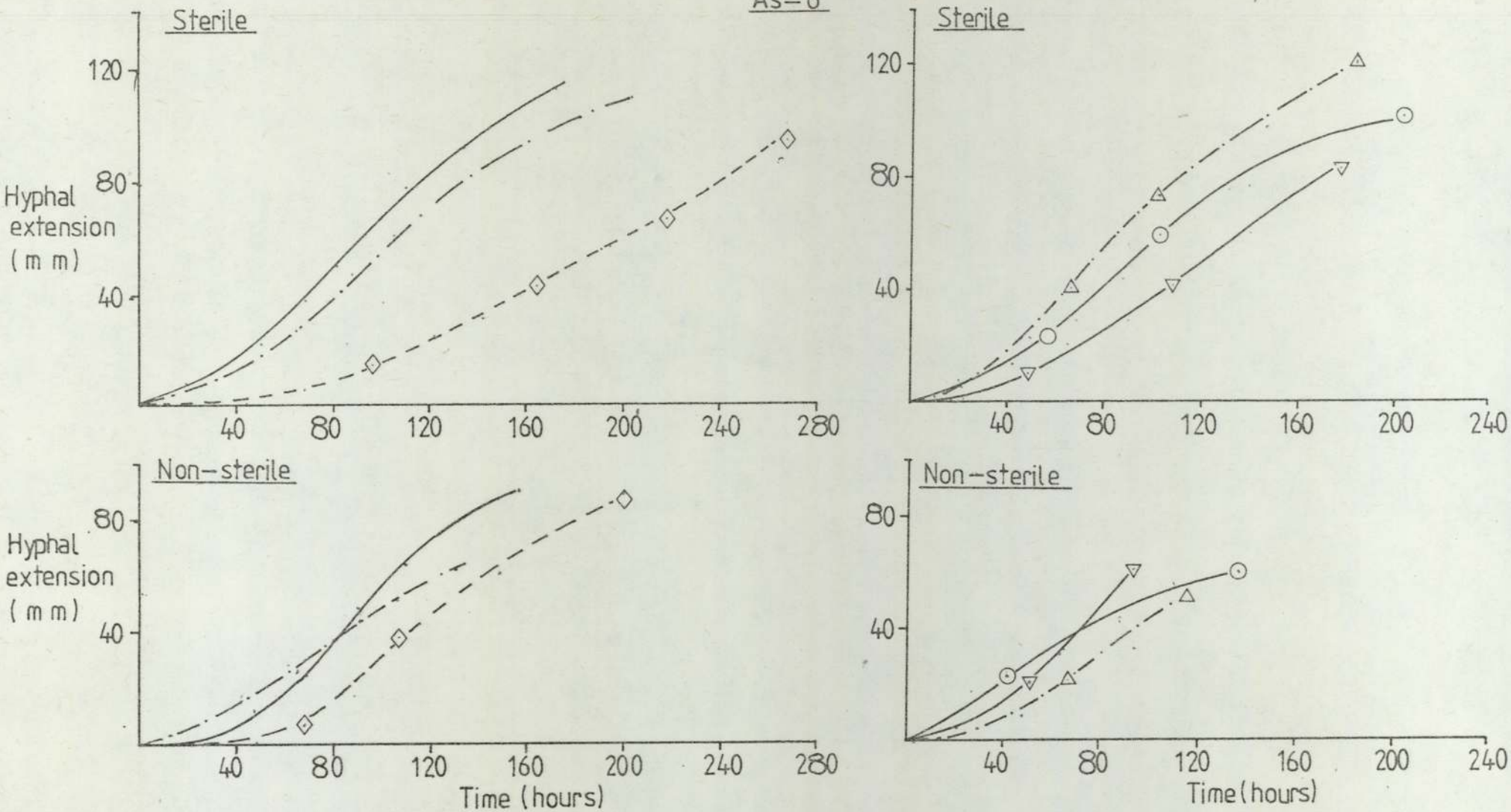


Fig 6-7 C cinereus growth on nitrogen amended straws

ammonium-N equivalently while the third was almost incapable of making any growth on nitrate-N or nitrite-N. Even earlier than this Voderberg (1948) reported that what she called *C lagopus* was able to utilise nitrate-N, ammonium-N and organic-N, whilst the strain studied by Leonian and Lilly (1938) readily utilised amino-acids as sole nitrogen source but was incapable of making use of either nitrate-N or ammonium-N. Madelin (1956) found that his strain was able to utilise both organic and ammonium nitrogen, but not nitrate-N. More recently Moore and Jirjis (1976) studied the growth and sclerotial numbers produced by *C cinereus* with different nitrogen sources and found that asparagine gave the greatest growth and sclerotial formation, closely followed by urea (632 µm/hour and 618 µm/hour respectively) and then ammonium tartrate (414 µm/hour). Yung-Chang-Ho and Yee (1977) reported that the best utilisation of cellulose and hemicellulose by *C cinereus* occurred when the fungus was provided with sodium nitrate; this was followed by ammonium chloride and then by asparagine. McShane (1976) working in these laboratories found significant strain differences when studying the nitrogen nutrition of *C cinereus* in pure culture. Two strains gave greatest mycelial weight increases when grown on L-asparagine while the third grew better on ammonium nitrite; although all three strains showed an ability to utilise a wide range of nitrogen sources.

From these results it is quite evident that if these various isolates are in fact identical (that is warrant the title *Coprinus cinereus*) then, as Moore stated (1969) "either considerable differences exist between strains or the organism is extremely sensitive to alterations in cultural conditions". It would seem that both these factors are probably to some extent correct. Significant differences between strains grown under identical conditions have been found (Fries 1955, McShane 1976) and this, taken together with

the taxonomic difficulties previously noted (Chapter One), could explain many of the conflicting reports published over the years. However, the environmental conditions under which many of these pieces of work have been carried out vary considerably, for example, Madelin used solid media while Moore's work was carried out in liquid media, this difference in substrate also applies to other workers. With regard to Madelin's work it is of interest to note that the strain he used was recorded as having an optimum temperature for growth of only 20-25°C as compared to 35-37°C in most other studies where temperature optima were quoted. This underlines once more the taxonomic problems which have to some extent hindered nutritional and ecological studies of *C cinereus* in the past. The use of pH adjustment and buffers also varies greatly in the reports quoted above. Leonian and Lilly conducted their experiments in media adjusted to pH 5.5 which is well below the optimum of around pH 8 which has been found for *C cinereus* growth (Fries, 1956; McShane, 1976). Although it is interesting to note that the strain studied by Yung Chang Ho and Yee had a reported optimum pH on solid malt media of barely above pH 5. However, in this same piece of work, when growing on cellulose and hemicellulose, *C Cinereus* failed to make good growth at these lower pHs and grew well at a higher pH of between 7 and 8.1. Problems arise when using ammonium salts where often, as the ammonium ion is utilised the remaining negative ion may cause a drift of pH well into acid ranges. Madelin used calcium carbonate to counteract this effect after the method of Morton and Macmillan (1954) while others have used salts of organic acids or the organic acids themselves to overcome the problem (Fries, 1955, Moore and Jirjis, 1976).

Voderberg recorded that all types of nitrogen could be utilised by *C cinereus* and would cause increased fruiting. However, the medium that she used contained ground straw and small amounts of

yeast extract which themselves could act as nitrogen sources, and the size and number of sporophores produced is not necessarily a measure of the ability of that fungus to utilise a particular nutrient. So, it can be seen that it is often difficult to compare the results obtained by different workers under such wide and varied conditions of growth.

After this brief review of studies to date on the nitrogen nutrition of *C cinereus*, it should now be possible to see how (if at all), the results of this present work on 'natural' substrates can be related to these previous reports. As stated, analysis of variance was carried out on the results followed by a closer analysis using Least Significant Differences (LSD) (Parker, 1973) and the results thus obtained are shown in tables 6.4 and 6.5. From these results it can be seen that asparagine and ammonium tartrate give the best growth rates on sterile straw and that in this piece of work no significant differences were found between the two; none of the other nitrogen sources showed a significant increase in growth rate over the controls and an increase in pH alone gave a better growth rate than the addition of ammonia solutions. There is no significant difference between asparagine and ammonium tartrate treatments and potassium nitrate or urea treatments but they are significantly different from ammonia solution and sodium nitrite. So that taking the significant differences overall it would seem that asparagine and ammonium tartrate give the highest growth rates followed by Potassium nitrate and urea, followed by ammonia solution and sodium nitrite. The appearance of asparagine as a better nitrogen source can be expected, as it also acts as a carbon source in a synergistic fashion (Moore, 1969). The effect of ammonium tartrate can be explained as above pH 7 the dissociation of ammonium compounds increases (Culp and Culp, 1972) so that the ammonium ion is readily available, and the uptake of this

ion by fungi is thought to increase at higher pH. Morton and Macmillan (1954) illustrated this using *Scopulariopsis brevicaulis* where the maximum uptake was found to occur above pH 8. When considering the remaining nitrogen sources, without direct significant differences it is difficult to say whether there is any real increase over control rates although other factors would seem to indicate that this is so. However, considering the significant difference between the elevated pH control and ammonia solution growth rates, it is possible that ammonia solutions may fractionally inhibit growth, there is certainly a long lag period before growth commences. These factors could be of importance when considering exactly what the role of ammonia is in situations where the competitive abilities of *C cinereus* appear to be increased. This will be discussed further when considering the growth rates on non-sterile straw, but at this stage ammonia appears to give no advantage to the growth of *C cinereus* and may in fact somewhat inhibit it.

When evaluating nitrogen sources Butcher and Drysdale (1974) found that substrate utilisation, which in their case was cellulose, depended as much on the carbon:nitrogen ratio as on the nitrogen source itself. McShane studied this in relation to *C cinereus* as did Yung Chang-Ho and Yee. McShane found optimum C:N ratios of between 50:1 and 500:1 and from this he suggested that it is primarily a litter fungus since dung tends to have a lower C:N ratio. The C:N ratio of wheat straw as found in this laboratory is roughly 50:1 and hence *C cinereus* should be capable of growing quite well on straw alone without nitrogen amendment. This we see in these results although it should be remembered that barley straw does have a slightly lower C:N ratio than wheat straw and that nutrients were also added in the form of the inoculation plug.

6.4(ii) Growth rates on sterile and non-sterile straw

The loss of hyphal front in this experiment was used as a means of

assessing the competitive ability of *C cinereus* under different conditions, together with the measurement of any changes in growth rates which may occur. The length of time before the front is lost cannot be directly correlated with the length of time that *C cinereus* remains as the sole actively growing coloniser, indeed that experiment provides no evidence that this is in fact so, and isolation work is detailed elsewhere. However loss of the front does provide an objective, measurable factor which is useful for such comparative work.

In table 6.5 the significant differences between treatments on non-sterile straw are shown. Initial analysis of variance indicated that there were significant differences between the growth rates on sterile and non-sterile straw and this was followed up by a comparison of LSD between treatments, which gave a clearer insight into where these differences lay. It should be remembered at this stage that the differences between growth on sterile and non-sterile straw may in fact have been greater had it not been for the slight inhibition of growth brought about by steam sterilisation. The growth rates in the controls with elevated pH dropped significantly on non-sterile straw indicating that pH increase alone does not confer any competitive advantage on *C cinereus*. The increase in the number of nitrogen treatments showing significantly different growth rates from the control, indicates that the effects of competition are greater where the straw has not been amended with any nitrogen. This is understandable as a single source of nitrogen has been added which must select against those organisms present which are unable to utilise that source and hence reduce the number of competing fungi. Those nitrogen sources which gave the highest growth rates on sterile straw were not the same sources which gave the best growth on non-sterile straw. The growth rates on ammonium tartrate dropped, so that significant differences appeared between this and the ammonia solution and also between ammonia and urea. Both these nitrogen sources can be used by a wide variety of organisms and ammonium salts

have been reported to be used by a wider spectrum of organisms than are found on asparagine and also more organisms show better growth on ammonium salts than on asparagine (Morton and Macmillan). This could explain the large drops in growth rates on ammonium tartrate. The highest growth rates were found on asparagine and ammonia solutions and no significant differences were evident between these two. Potassium nitrate produced significantly greater growth rates than urea and again this is a nitrogen source which is not used by such a wide spectrum of micro-organisms. Nitrite nitrogen continued to produce the lowest growth rates whilst showing a significantly higher rate than either of the controls.

Closer analysis of the results on sterile and non-sterile straw showed that in most cases the growth rates on non-sterile straw were lower than those on sterile straw which could be expected owing to competition from other organisms. However, between the growth rates on ammonia no significant differences were found except in AS-3 where a slight increase was obtained under non-sterile conditions.

The data contained in table 6.6 indicates that on ammonia treated straw *C cinereus* maintained a distinct hyphal front for a much longer period than with any other nitrogen source; direct observation of the contaminants under the microscope showed that it was made up of mainly *Basidiomycete* hyphae, probably from endemic strains of *C cinereus*. The second longest times were found on urea and this was closely followed by sodium nitrite. It is interesting that these are the two nitrogen sources which show lowest growth rates on non-sterile straw; there is not a direct correlation between the length of time the front is maintained and the growth rates produced by the same nitrogen sources. The next block of nitrogen sources were asparagine and ammonium tartrate although it is probably not accurate to say that there was any real difference between these and the previous two, as the tubes were not observed sufficiently frequently to notice a loss of hyphal front over

a 4 hour period. This also applies to the next block of treatments, potassium nitrate and the two controls. As mentioned previously a high pH alone does not influence the competitive ability of *C cinereus*; neither it would appear does the combination of high pH and a nitrogen source, so that ammonia solution is not just acting as a nitrogen source or pH elevator but it seems to be a unique property of this chemical which is working with *C cinereus* to increase its ability to compete with other organisms.

Taking all these results together a scheme can be suggested to explain how ammonia is selecting out *C cinereus* under non-sterile conditions. From the long lag period and low growth rates observed on ammonia supplemented straw, it would seem that for at least 40-50 hours after inoculation, the ammonia actually inhibits the growth of *C cinereus* and also that of other organisms present. After a period of 50-60 hours the level of ammonia drops sufficiently to allow growth of *C cinereus* to commence, yet it is still at a level which inhibits growth of other organisms; the inhibitory effects of ammonia have been discussed in a previous chapter. As growth rates on ammonia nitrogen are not particularly high, indeed there is some evidence of slight inhibition on sterile straw, it can be assumed that competitive advantage is not conferred because the ammonia solution is a particularly successful nitrogen source for *C cinereus*, but it is the tolerance of *C cinereus* to high ammonia levels which seems to be the key. When comparing growth rates on sterile and non-sterile straw, the growth rates of all but the ammonia treatments drop significantly. The ammonia inhibits the growth of other micro-organisms so that *C cinereus* does not have to compete and maintains the same growth as was observed on sterile straw. From these results the ammonia levels seem to be sufficient to suppress growth of other organisms over the full 10 days of the experiment while the growth on other treatments drops owing to competition from other organisms and eventually even the large inoculum is not sufficient to overcome its competitors and the front is lost.

It could be expected that *C cinereus* is able to tolerate and utilise high levels of ammonia when considering its natural environment of farmyards and straw composts, situations where it is likely to come into contact with high levels of urea and animal wastes. Farmers have reported (personal communications) the frequent and often rapid appearance of *C cinereus* on broken bales which have been left lying around the yards. Chang and Hudson reported the appearance of *C cinereus* on wheat straw composts between 22-28 days. This, it can now be seen, would fit in well with all that is known of its growth parameters. The temperatures were between 25-45°C, the nitrogen levels were beginning to drop and hence increase the C:N ratios, yet the pH was well above 7, ideal for *C cinereus* growth and also ensuring the presence of free ammonia in the compost.

The previous isolation work can now be explained in these terms; the addition of ammonia to the straw or soil inhibits the activities of other organisms and in the case of straw, allows the slower growing *C cinereus* to become dominant or in the case of soil, the addition of straw baits would encourage the germination and growth of any propagules present.

6.5 CONCLUSIONS

It is necessary to study these results in terms of the proposed upgrading process and draw conclusions with regard to these. If this process is to be carried out under non-sterile conditions then as far as fungi are concerned these results indicate that over a fixed period of time it would be possible to suppress contamination and allow *C cinereus* to colonise the straw as the dominant fungus. However, after studying the results of nutritional work it may well be found that a fast, copious growth of *C cinereus* in the target, and in this case ammonia under non-sterile conditions will not fill the requirement. Under these circumstances it would be necessary to put the process on a more

controlled footing requiring higher technology, there would be a need to pre-sterilize the straw and to consider the use of other nitrogen sources, possibly an ammonium salt depending upon the strain requirements, which as shown can vary considerably. However this would markedly affect economic considerations and the feed value of the product would have to be carefully weighed against the loss of production.

There is scope here for further research into the possibiliites of using mixed nitrogen sources; ammonia to suppress competitors and possibly another source to encourage growth rates. However there would be many considerations before commencing such work, for example, the presence of ammonia nitrogen is known to suppress the utilisation of nitrite nitrogen in some micro-organisms (Morton and Macmillan).

CHAPTER SEVEN

THE EFFECT OF ADDED AMMONIA ON THE MICROFLORA OF A BARLEY STRAW

7.1 INTRODUCTION

From the experiments described in the previous chapter it has been concluded that *C cinereus* can tolerate high levels of ammonia and is able to grow on straw at the expense of other species which succumb to these raised levels. However, these other groups will still be present in non-sterile straw, and it was therefore felt necessary to study the effects of ammonia on their development. This would also confirm the ammonia produced inhibition. Knowledge obtained from such a study would be of great importance in the development of an upgrading process involving *C cinereus*. Earlier work (Chapter 2) has included a very limited survey of the microfungi found on straw after ammonia treatments, but the studies described in this chapter expand greatly on that survey to include actively and non-actively growing fungi, aerobic bacteria and actinomycetes, all of which will be inter-related in the ecology of the proposed 'composting' process. A second reason for studying numbers of bacteria and actinomycetes must be the possibility of them causing health hazards to both animals and man.

As in Chapter Two jars were used to predict what may happen in a larger compost, and the microflora was studied in the presence of the natural and inoculated *C cinereus*.

In a further study microbiological analysis was carried out on two small composts, one of which had been inoculated with *C cinereus* spores, both were amended with ammonia and incubated at about 29°C for 12 days.

7.2 BENCH SCALE ANALYSES

7.2(i) Materials and Methods

Two series of jars were prepared each jar containing 5 g of straw. Series 1 was for use as a control, the jars of which were not amended with ammonia and series 2 (which were the test jars) was amended with 1% w/w ammonia. Each series was split into two groups, A & B. To group A was added distilled water to make up the total liquid added to 15 mls and group B was inoculated with 1 ml per jar of a 10^5 spores/ml suspension of *C cinereus* spores (AS-1). These were then topped up to 15 mls as with group A. The jars were well rotated, capped with aluminium foil and incubated at 30°C ± 1°C. Series 1 and 2 were placed in separate incubators to prevent the contamination of series 1 jars with ammonia from series 2. The technique for the culturing of fruit bodies, harvesting of spores and production of spore suspensions are detailed in appendix 2. Jar numbers were such that three from each treatment could be sacrificed at two day intervals over a 16 day period along with a control jar of sterile straw to investigate the natural depletion of ammonia and three jars per treatment to sacrifice for pH and one for ammonia level measurements.

7.2(ii) Microbiological analysis

The plate count method for estimating bacterial and fungal propagule populations is satisfactory for many comparative purposes if relative rather than absolute numbers are required (Marshall Jennisson & Wadsworth, 1940). In this case, as it was intended to compare numbers from various treatments and to follow growth trends over a period of time, it was felt that, providing a number of precautions were observed, dilution plate counts, together with an assessment of actively growing fungi would give a fair approximation of the microbiological activity on the various straws. So although populations per unit of compost are of doubtful value in absolute terms because of dilution errors (Marshall

Jennisson and Wadsworth, 1940; Meiklejohn, 1957) and although false impressions of true fungal activity may be obtained (Warcup, 1960) it was felt that these criticisms would not invalidate comparisons. Also, Hayes (1968) stated that because of rapid changes in populations and species composition, the dilution plate technique was the "most practical and meaningful one to employ in general studies of composts".

At two day intervals 1 g of wet material made up of straw from the three replicates was placed in 250 mls of sterile quarter strength Ringers which contained 1 drop of Tween 80 and a few sterile glass beads, both these were to assist in the removal of propagules from the substrate and to separate clumps of spores and hyphae. This was then shaken mechanically for 30 minutes and from this, dilutions were produced in quarter strength Ringers for plating. Dilutions were made using pipettes, and the propagule suspension was drawn up into these and released ten times before transferring this was to prevent inaccuracies occurring owing to incomplete wetting of the glassware. This was repeated for all treatments. Serial ten fold dilutions were made between 1:100 and 1:1000000 and 0.1 ml aliquots of these were plated out onto the relevant media (5 replicates) as outlined below. Preliminary experiments had indicated which dilution levels would yield the required number of colonies, that is between 30 - 300 for bacteria, between 15-20 colonies for the fungi and between 50 - 200 for actinomycetes at each sampling period.

Bacteria

Dilutions between 1:1000 and 1:1000000 were plated at each sampling period onto nutrient agar pH 7.4. This was unfavourable to most fungal growth and the occasional actinomycete colony which developed was relatively easily identified and excluded from the plate counts. On plating, as throughout the rest of this work, the usual precautions were taken to ensure even distribution of colonies before the media solidified.

Fungi

The full range of dilutions plated out onto 3% malt extract agar which contained 500 ppm aureomycin to suppress bacterial growth. A stock solution of aureomycin was produced, filter sterilised and added to the media after sterilisation. Lacey and Dutkiewicz (1976) found that malt extract was the least selective of the media that they examined for the isolation of fungi from hay and it yielded the highest overall propagule count at mesophilic temperature, therefore malt extract was used here. Dilution plates do not measure actively growing fungi as colonies originate from spores, hyphal fragments or other resting bodies, so that when quantifying and identifying fungi, the heavily sporing species are over represented. Therefore, to supplement these propagule counts ten pieces of straw were selected at random from the combined material, washed as described in chapter 2 and plated out onto malt extract and aureomycin medium. The colonies were assessed for percentage appearance (on ten plates) and subcultured as they appeared for identification, so that qualitative and quantitative data were obtained for the actively growing fungi.

Actinomycetes

The full range of dilutions plated out onto glycerol-casein agar, pH 7 (Hayes, 1968) which contained 500 ppm actidione to suppress fungal growth and this was added to the medium after autoclaving. Lacey and Durkiewicz found that the addition of casein to media aided actinomycete estimation when isolating from hay, and it has also been used by other workers to provide selective media for actinomycete growth (Küster and Williams, 1964).

All plates were incubated at 30°C because, as the process was maintained at that temperature, it was only felt necessary to investigate organisms which were likely to be active under these conditions.

Self heating was not likely to occur with such small quantities of straws and no difficulty was found in maintaining incubator temperatures of 30°C. All plates were counted after 2 and 7 days to ensure no further colony appearance.

Insufficient time was available to identify the bacteria and actinomycetes which were isolated although this would have been useful to help recognise any organisms which may be potentially harmful. However it was felt that at this stage an assessment of numbers alone, in relation to the growth of *C cinereus* and other fungi was of great importance when considering the development of the proposed upgrading process. Identification of the fungi found to be actively colonising the straw was however, as previously stated, carried out.

7.2(iii) pH and Ammonia measurements

Three jars per treatment were sacrificed along with those for microbial analysis; these were to follow the pH of the straws over the 16 day period. To each jar was added 100 mls of distilled water, these were then shaken and left for 1 hour before the pH was measured using a glass electrode and Pye bench pH meter.

A further identical jar per treatment was sacrificed to assess changes in ammonia levels. *C cinereus* has been shown to produce ammonia from the stipe at maturity (Moore and Jirjis), and it was felt that following ammonia levels may help to show under what circumstances, and at what times, ammonia is removed from the growth substrate or alternatively if there is any produced. Again 100 mls of distilled water were added to the jars and the above procedure followed. The ammonia present in the water was measured as ppm using a probe which detected ammonium ions, E L model 8002-2 connected to a log scale meter by Electronic Instruments Ltd model 7030. This was not a very accurate measure of the amounts of ammonia available for utilisation by the micro-organisms, but it did provide overall trends giving useful comparative

data. The sterile jars described previously were amended with 1% w/w ammonia and its depletion was monitored using the probe as outlined. This gave an indication of the amounts of ammonia which may have been lost naturally from the straws. These results however, must be viewed keeping in mind the changes which sterilisation causes in the straw and the differences that these may make to the balance of the dissociation equations of ammonium compounds because of, for example, changed pH and the presence of solutes from the straw.

7.3 STUDY OF LARGER COMPOSTS

Materials and Methods

At the time of this study, larger pilot scale work was being carried out by other workers at these laboratories to obtain material for digestability tests. It was decided to investigate the microflora of these composts to compare results obtained from bench scale studies with those in the actual composts. These composts had been set up by first soaking loose straw in a 1% ammonia solution and then transferring this to large polythene sacks (1 x 1.5 metres). Two composts had been set up, one was inoculated with a spore suspension of *C cinereus* (AS-1) after bagging and the other was left for the endemic organisms to colonise. These were mixed thoroughly, the bags loosely closed and then placed in a cellar at the Biodeterioration Centre where the temperature varied at that time of year from 28 to 30°C. These were left for 12 days, after which time ten samples of about 5g each were taken diagonally through the centres of each compost. These samples were pooled and well mixed and then analysis of the microflora was carried out as previously described for each compost.

7.4 RESULTS

7.4(i) Bench Scale work

Table 7.1 contains the pHs of the straws over the 14 day period

and Table 7.2 shows the measured amounts of ammonia over this same period. The ammonia levels are shown in mg/100 mls of distilled water and therefore show the number of mg of ammonia washed from the straw in each jar. These results are illustrated in figures 7.1 and 7.2. There are no significant differences between the pH in inoculated and non-inoculated straw but there are obviously differences between those which have been amended with ammonia and those not. All statistical analyses quoted are shown in appendix 4. The ammonia curves show, as could be anticipated, that more ammonia is removed than could be expected to be lost by natural depletion and that ammonia starts to become depleted at about day 10, after which, propagule counts begin to drop correspondingly.

Viable propagule counts per gramme wet weight of straw appear in tables 7.3, 7.4 and 7.5 and these are illustrated in figures 7.3, 7.4 and 7.5. The fungal populations were affected by the ammonia in that there was a reduction in numbers in the first 2 days in *C cinereus* inoculated and non-inoculated straws. There followed a fairly slow recovery which never reached the levels attained in non-amended straws. There are significant differences between the results from amended and non-amended straws and also between inoculated and non-inoculated in series 2 straws. This was only seen in series 1 at day 8.

A lag period was not seen in the actinomycete results as there was an immediate logarithmic increase up until day 8 when numbers began to level out into a plateau. The addition of ammonia somewhat increased the number of actinomycete propagules.

Rapid, immediate increases were seen in bacterial numbers which then dropped on days 4 and 6 respectively for non amended and amended straws. This was followed by further increases in numbers until day 8, after which populations began to drop. There were significant differences in numbers between series 1 and 2, those which had been treated with ammonia had lower populations.

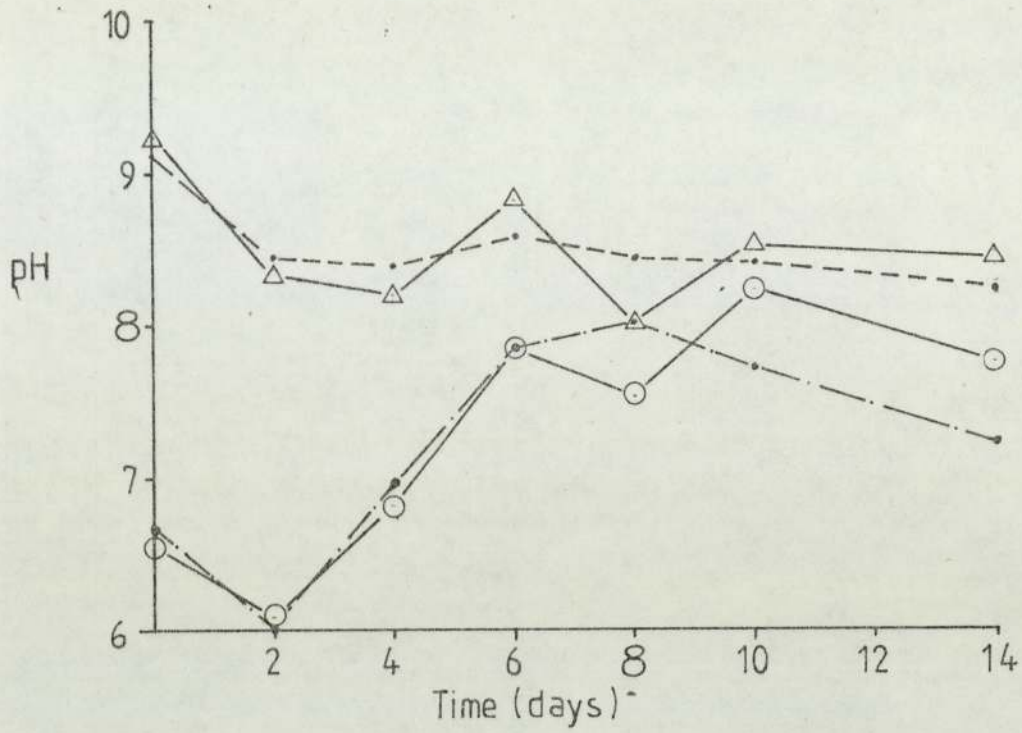


Fig 7·1 pH over 14 day period

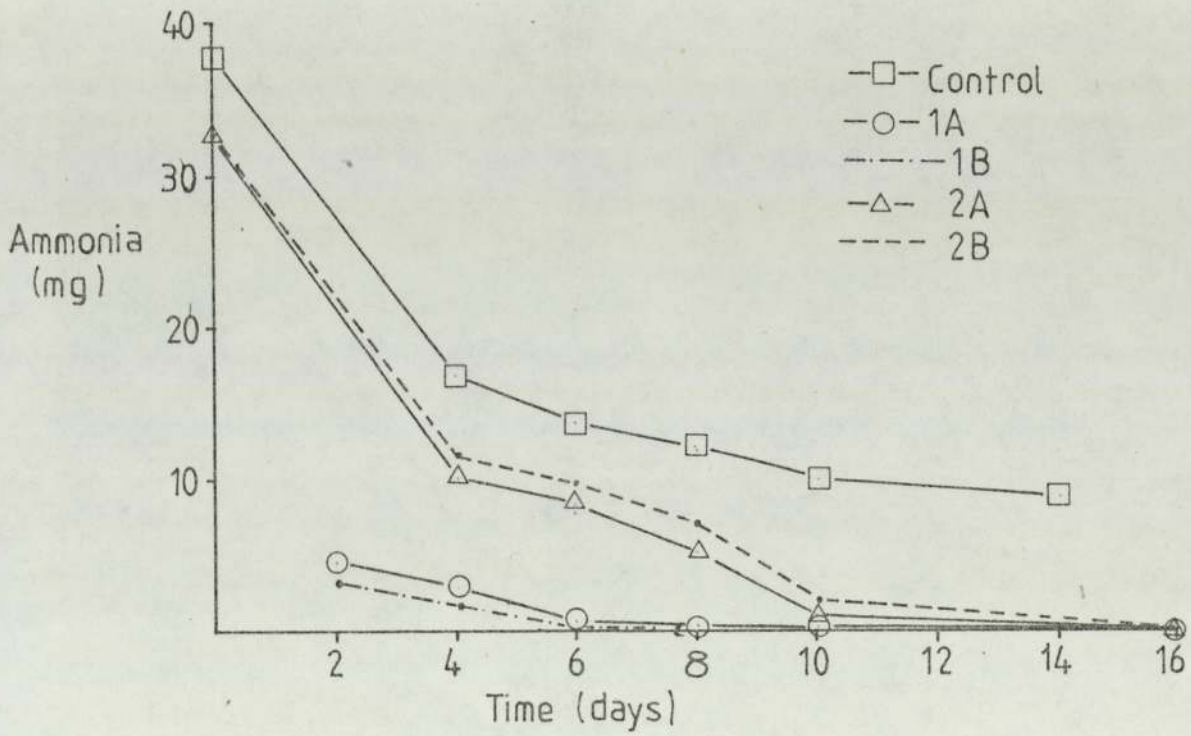


Fig 7·2 Ammonia levels over 16 day period

Tables A III 3 and A III 4 in appendix 3. show the percentage appearance of presumed actively growing fungi isolated from washed straws (ten plates) over the 14 day period. It can be seen that more species were isolated on these malt extract plates than were found in the work described in earlier chapters where cellulose agar alone was used. Histograms were made of these results (Figures 7.6 and 7.7). From these it would seem that ammonia reduces considerably the levels of the phycomycetes, *Absidia sp*, *Mucor sp* and *Rhizopus sp* and levels of *Cephalosporium sp* were also reduced. *Alternaria tenuis* was completely inhibited as was *Aspergillus flavus*, and *Chaetomium globosum* was greatly reduced. *C cinereus* isolations increased significantly and this is illustrated more fully in figures 7.8 and 7.9. Other species which ammonia appeared to favour were *Gliochladium roseum*, *Aspergillus fumigatus* and to some extent *Penicillium cyclopium*. This has not been observed previously when more selective media have been utilised.

Table 7.1 Straw pH over 14 days

	pH (MEAN OF 3 REPLICATES)			
DAY	1A	1B	2A	2B
0	6.65 ± 0.08	6.67 ± 0.20	9.32 ± 0.10	9.25 ± 0.15
2	6.08 ± 0.16	5.9P ± 0.28	9.35 ± 0.18	8.37 ± 0.08
4	6.78 ± 0.13	6.92 ± 0.16	8.32 ± 0.12	8.42 ± 0.25
6	7.87 ± 0.34	7.83 ± 0.25	8.78 ± 0.19	8.58 ± 0.10
8	7.55 ± 0.53	8.06 ± 0.28	8.05 ± 0.28	8.40 ± 0.23
10	8.30 ± 0.35	7.80 ± 0.35	8.58 ± 0.20	8.45 ± 0.17
14	7.67 ± 0.92	7.32 ± 0.52	8.47 ± 0.29	8.18 ± 0.18

Table 7.2 Ammonia mg present over 16 days

	AMMONIA LEVELS (mg/100ml)				
DAY	CONTROL	1A	1B	2A	2B
0	38.00	-	-	33.00	33.00
2	-	4.76	3.83	-	-
4	16.80	3.08	2.25	10.45	11.08
6	14.00	0.21	0.43	8.50	8.60
8	13.20	0.21	0.06	5.00	6.50
10	10.80	0.03	0.12	0.65	0.95
16	8.50	0.05	0.06	0.28	0.54

Ammonia content of distilled H_2O from glass still = 0.015 mg/100 ml

Table 7.3 Viable fungal propagules per g wet weight

FUNGI	VIABLE PROPAGULES PER G WET WT STRAW MEAN 5 PLATES			
DAY	1A	1B	2A	2B
0	2.30×10^3	1.20×10^3	4.20×10^3	1.30×10^3
2	1.70×10^7	3.70×10^7	0	0
4	8.80×10^7	5.55×10^7	8.20×10^5	7.80×10^5
6	1.30×10^8	1.45×10^8	8.50×10^5	2.56×10^5
8	1.34×10^8	7.60×10^9	3.60×10^7	1.65×10^7
10	6.00×10^8	1.33×10^8	1.60×10^7	2.00×10^7
14	2.65×10^8	2.40×10^8	1.50×10^7	5.63×10^6

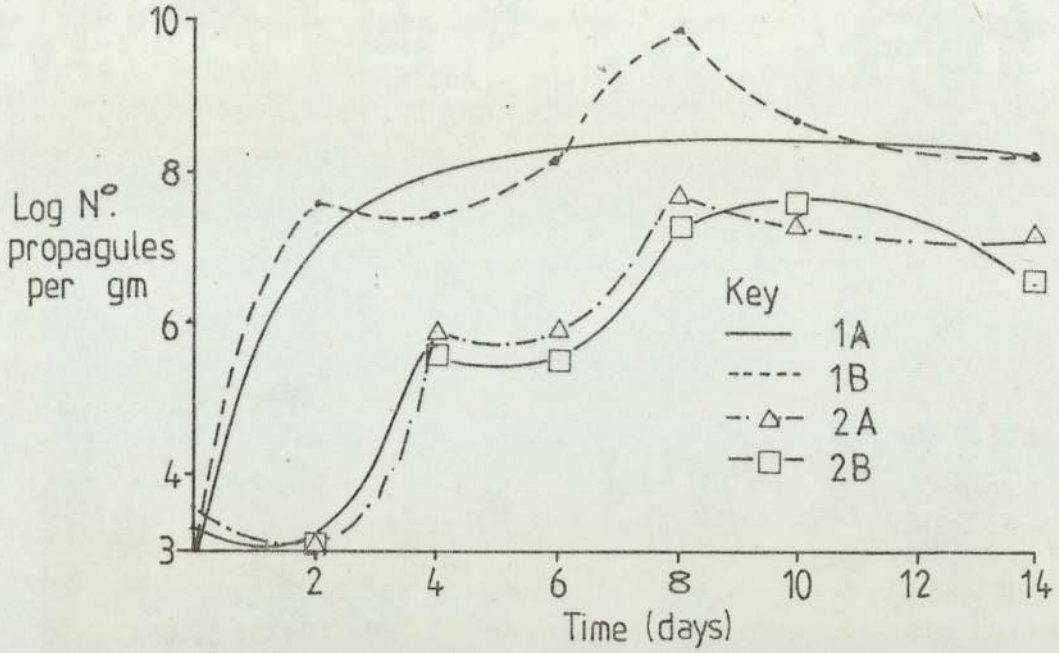


Fig 7-3 Effect of treatment on fungal populations

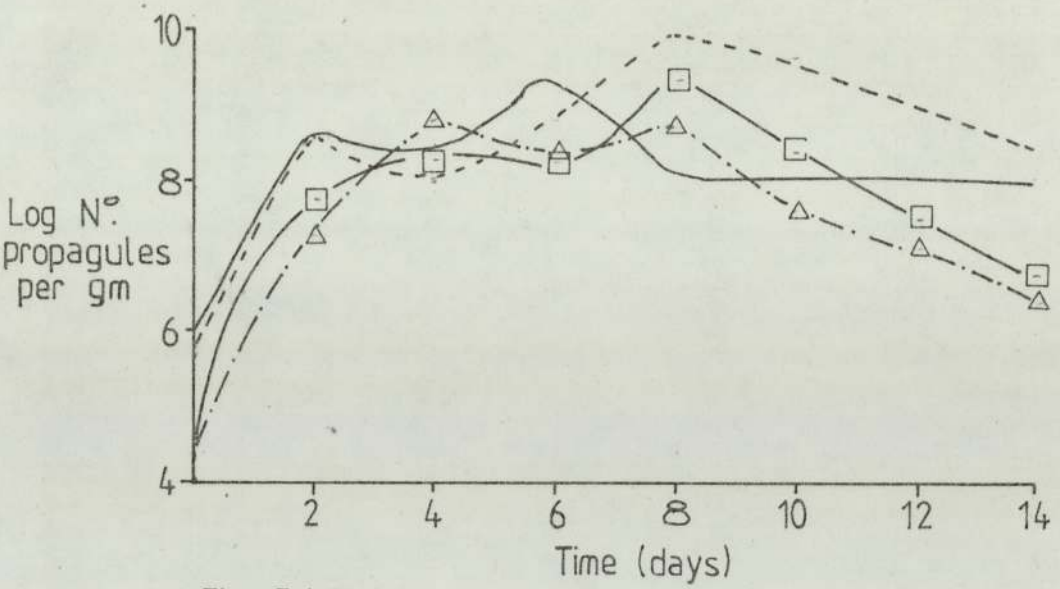


Fig 7-4 Effect of treatment on bacterial populations

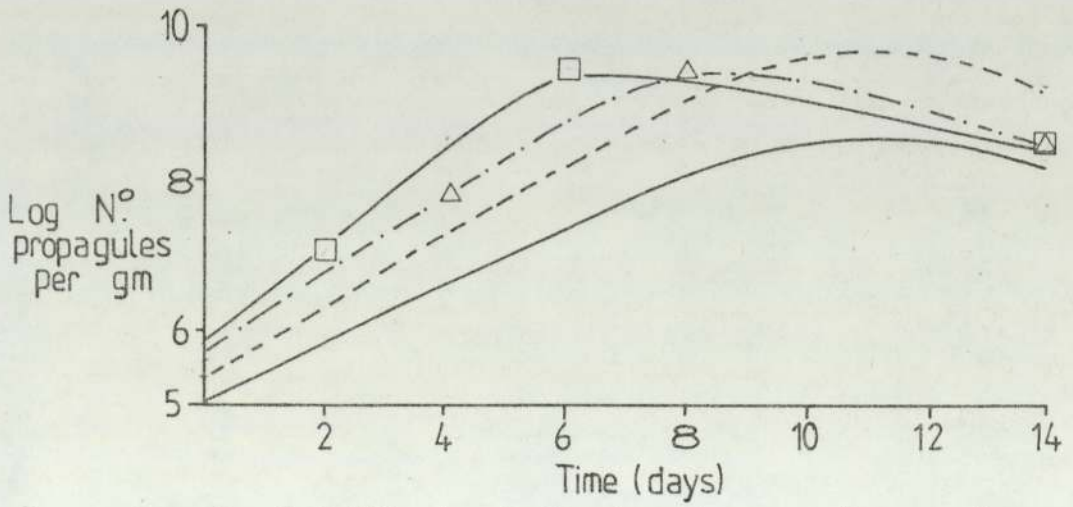


Fig 7-5 Effect of treatment on actinomycete populations

Table 7.4 Viable Bacterial propagules per g wet wt

BACTERIA	VIABLE PROPAGULES PER G WET WT STRAW MEAN 5 PLATES			
DAY	1A	1B	2A	2B
0	6.20×10^5	3.50×10^5	2.50×10^4	3.50×10^4
2	4.90×10^8	1.50×10^9	1.80×10^7	5.80×10^7
4	2.49×10^8	Yeast Contamination	7.00×10^8	1.77×10^8
6	1.90×10^9	7.00×10^8	1.37×10^8	1.25×10^8
8	9.35×10^7	7.75×10^9	6.20×10^8	1.71×10^9
10	9.00×10^7	3.69×10^9	7.50×10^7	1.75×10^8
14	8.00×10^7	1.80×10^8	3.70×10^6	6.5×10^6

Table 7.5 Viable Actinomycete propagules per g wet weight

ACTINOMYCETES	VIABLE PROPAGULES PER G WET WT STRAW MEAN 5 PLATES			
DAY	1A	1B	2A	2B
0	1.05×10^5	2.00×10^5	2.58×10^5	4.71×10^5
2	1.85×10^5	5.13×10^5	-	-
4	3.25×10^6	-	1.60×10^8	2.21×10^8
6	2.65×10^7	7.90×10^7	2.78×10^8	2.17×10^9
8	1.62×10^8	2.44×10^7	1.08×10^9	2.08×10^9
10	2.68×10^8	3.50×10^9	1.70×10^8	6.00×10^9
14	1.50×10^8	4.75×10^8	2.20×10^8	2.20×10^8

7.4(ii) Results from larger composts

Table 7.6 contains the viable propagule counts after 12 days from the bagged composts and these results are shown in histogram form (Fig 7.10).

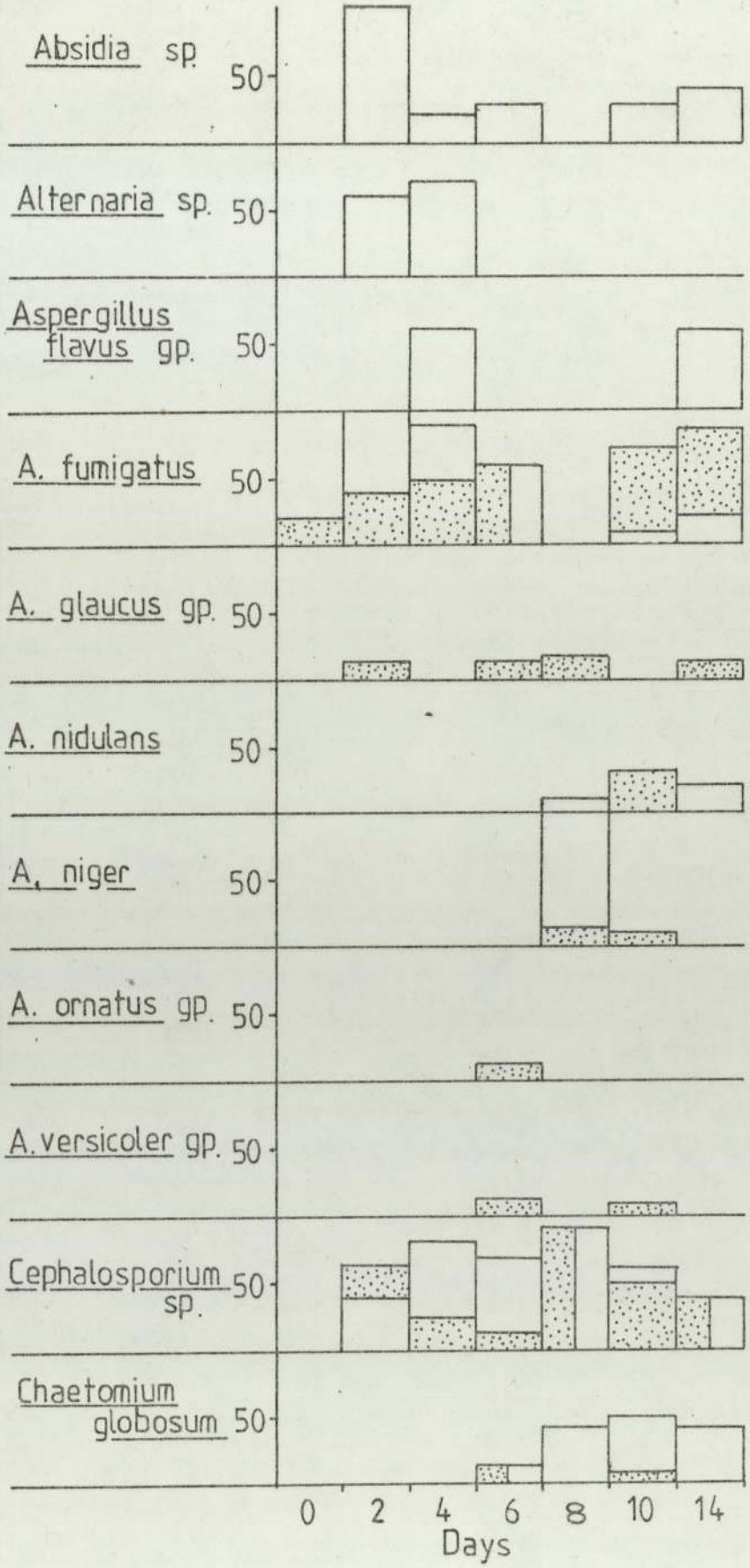


Fig 7-6 Effect of ammonia on isolations from non-inoculated straw

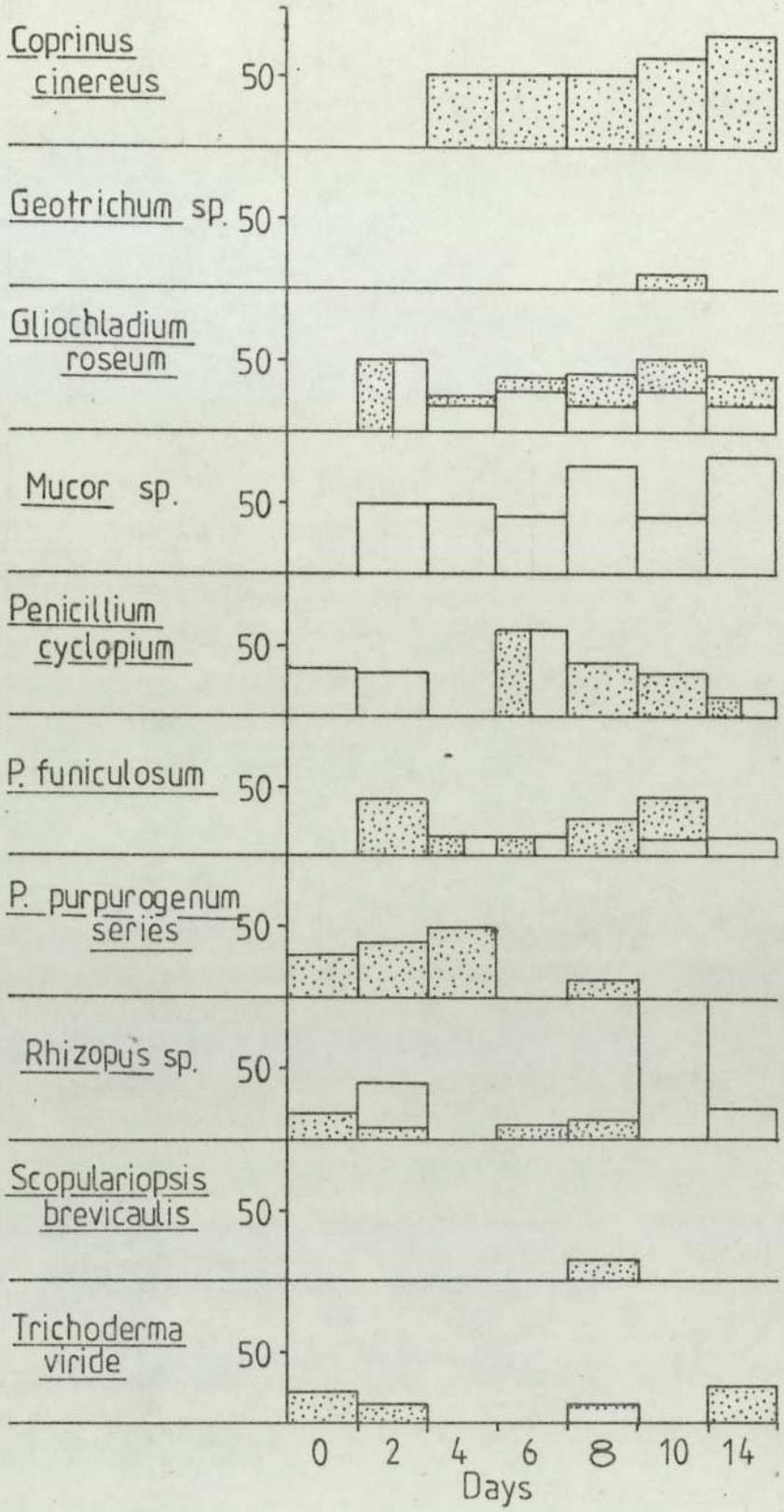


Fig 7-6 Con't

Key

Treatment. 1a.

" 2a.

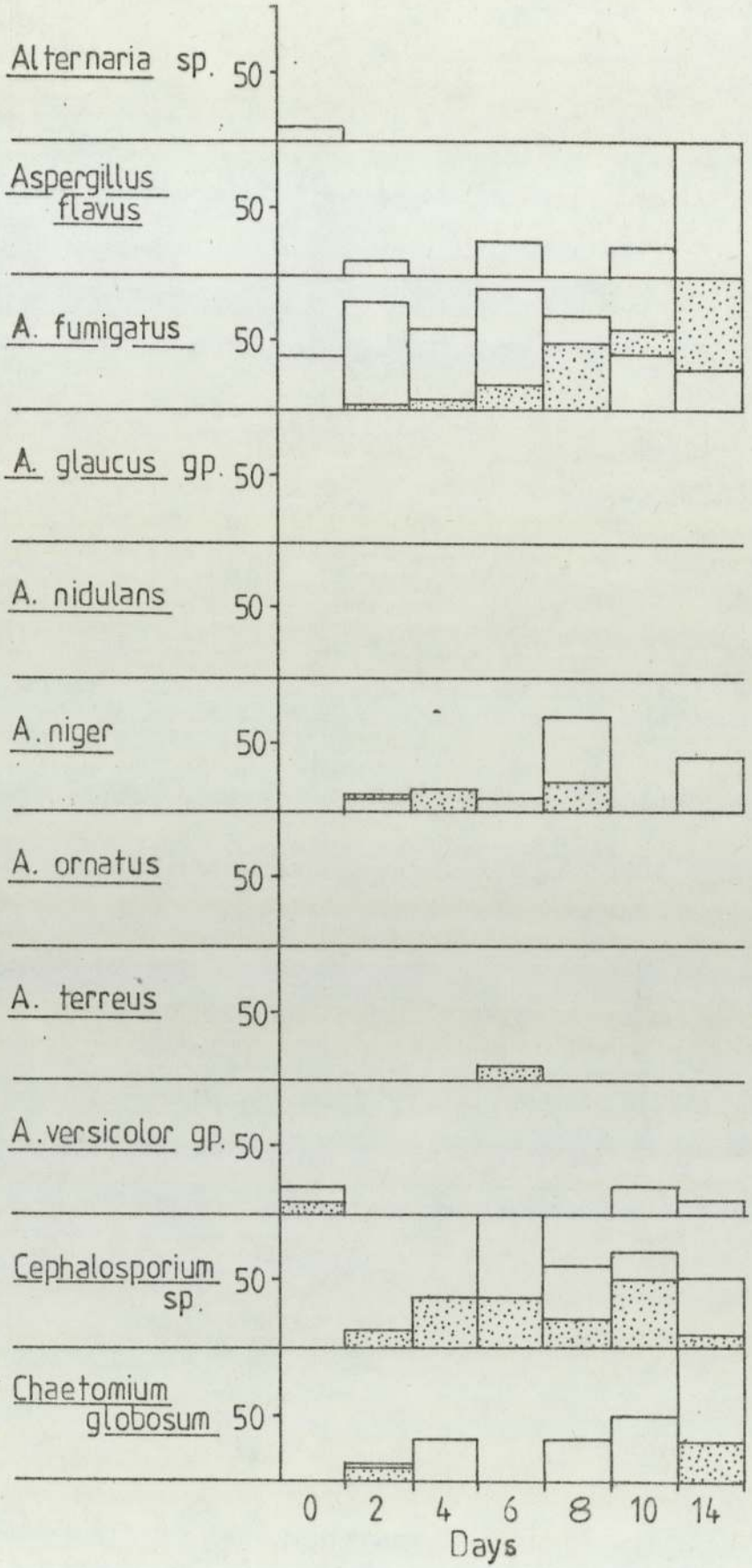


Fig 7-7 Effect of ammonia on isolations from inoculated straw

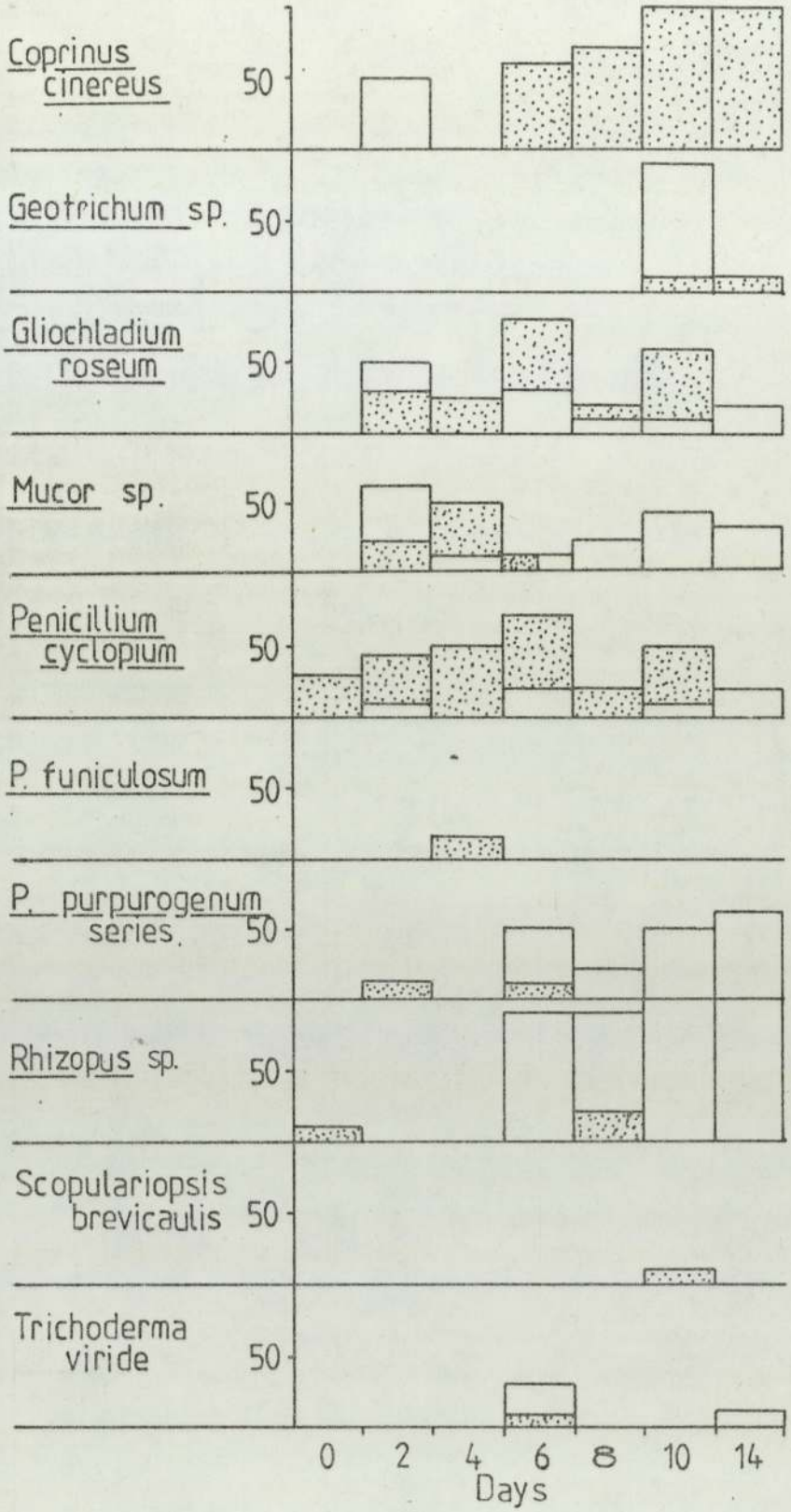


Fig 7-7 con't

Key

Treatment. 1b.

" 2b.

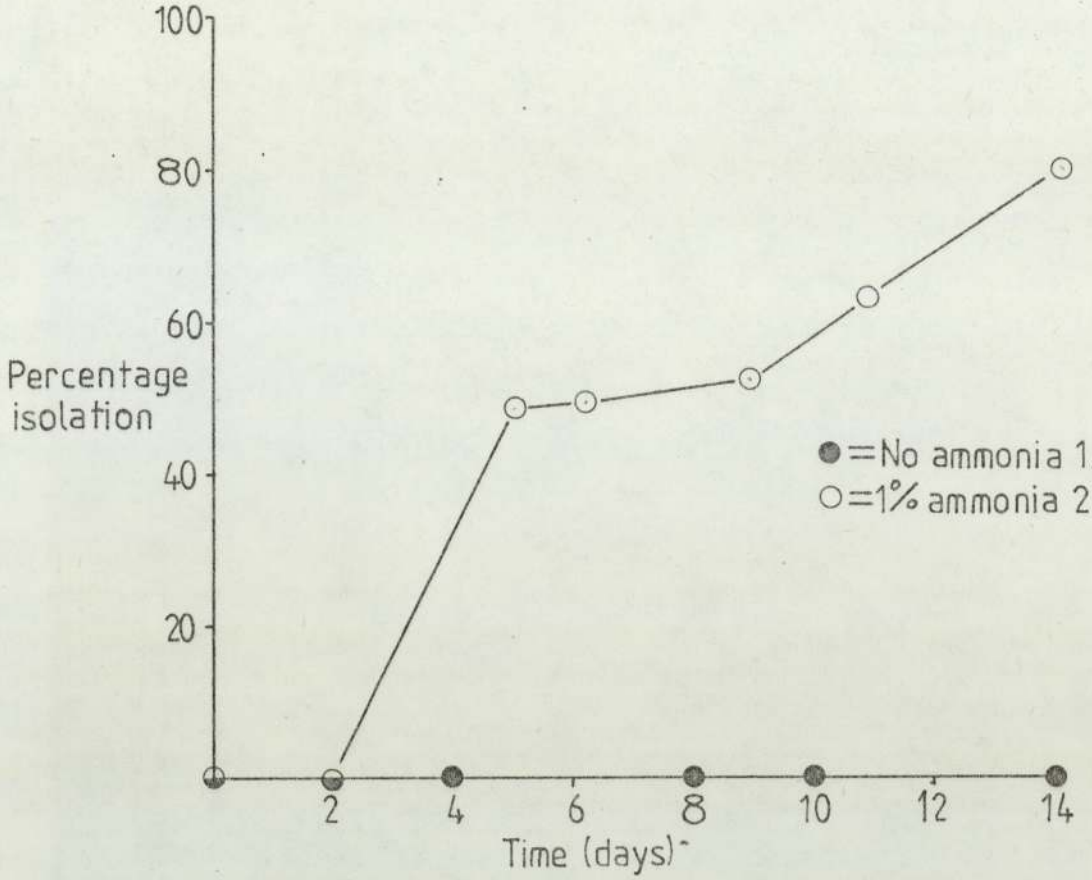


Fig 7·8 Effect of ammonia on the isolation of C. cinereus over 14 days

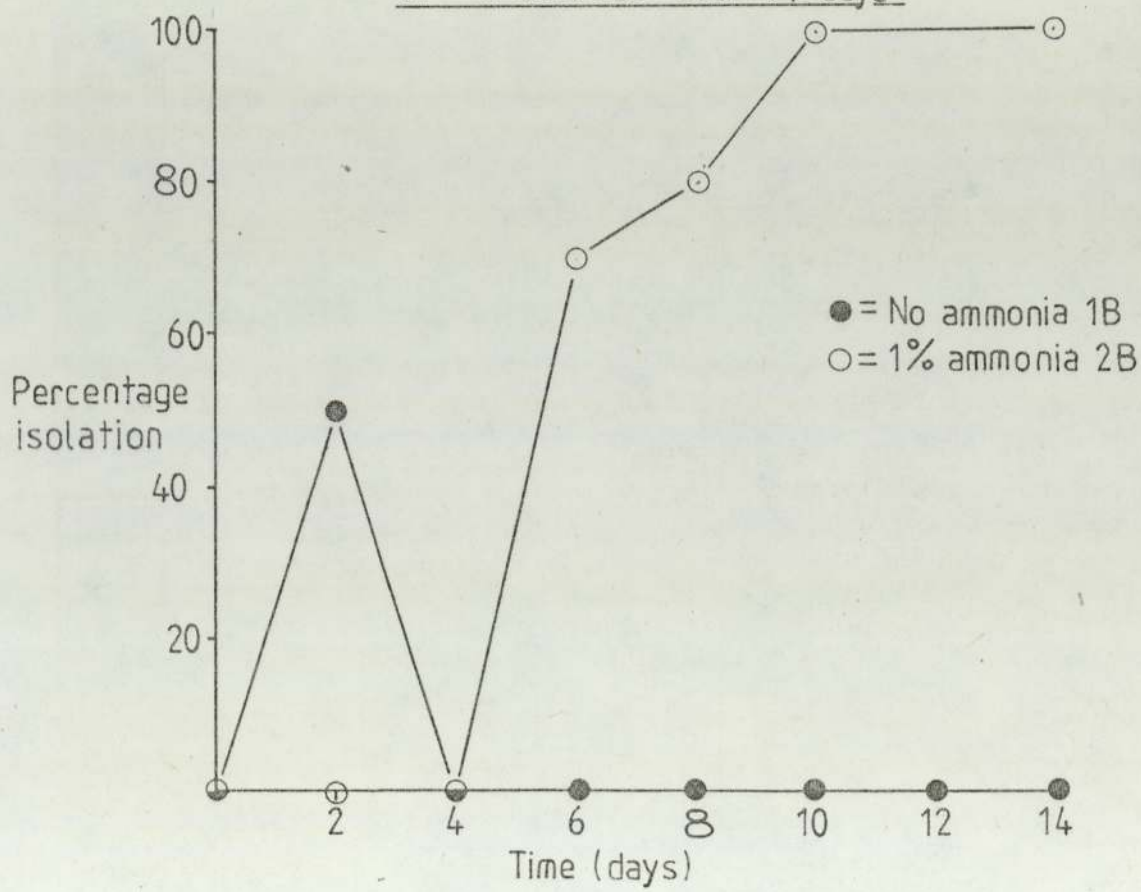


Fig 7·9 Effects of ammonia on the isolation of C. cinereus from inoculated straws over 14 days

There were no significant differences between the inoculated and non-inoculated bacteria counts. There were however significant differences between the fungal counts and between the actinomycete counts in the two composts. In both cases the non-inoculated compost showed higher counts than the inoculated compost. Table 7.7 shows the percent appearance of actively growing fungi isolated from the two composts. *C cinereus* was isolated from 100% of the plates from the inoculated composts and the only other isolate was *Penicillium cyclopium* (10%), whereas five species were isolated from the other composts. However, *Scopulariopsis brevicaulis* was found on dilution plates from the *C cinereus* inoculated compost.

7.5 DISCUSSION

The changes in pH observed in the non-amended straws follow closely those seen by Chang and Hudson (1967) over the first few days of their wheat straw composts. It is interesting to note that although the pH reached a suitable level for *C cinereus* growth in these jars it was in fact only isolated once, further indicating the selective nature of ammonia. The ammonia curves show that the low levels present in non-amended jars are soon depleted and the amounts in the amended jars reach low levels, as previously stated at a time corresponding to the plateau regions on the growth curves, as could be expected. There are no measurable increases in levels of ammonia which could be explained by its excretion from *C cinereus*. However this could be predicted as *C cinereus* has only been observed to give off ammonia from the stipes after maturity and in this situation fruit bodies were only just beginning to appear by the end of the study period and few had actually reached maturity.

From the fungal propagule counts it can be seen that ammonia greatly reduces the number of viable spores and that the inoculation of the straws with *C cinereus* also reduces numbers. This latter is only

Table 7.6 Viable propagule counts after 12 days:

from bagged composts

	FUNGI		BACTERIA		ACTINOMYCETES	
	Inoculated	Non Inoculated	Inoculated	Non Inoculated	Inoculated	Non Inoculated
Propagules per g wet weight	* 1.50×10^7	* 5.17×10^7	NS 3.39×10^9	NS 3.63×10^9	* 4.42×10^8	* 1.42×10^9

* Significant differences between treatments at P = 0.05

NS NOT SIGNIFICANT

Table 7.7% appearance of actively growing fungi

on washed straws (from 10 plates)

	INOCULATED	NON INOCULATED
C cinereus	100	10
A fumigatus	0	50
Aspergillus sp	0	50
Penicillium sp	10	20
Scopulariopsis brevicaulis	0	50

Scopulariopsis brevicaulis was found on dilution plates from the inoculated bag.

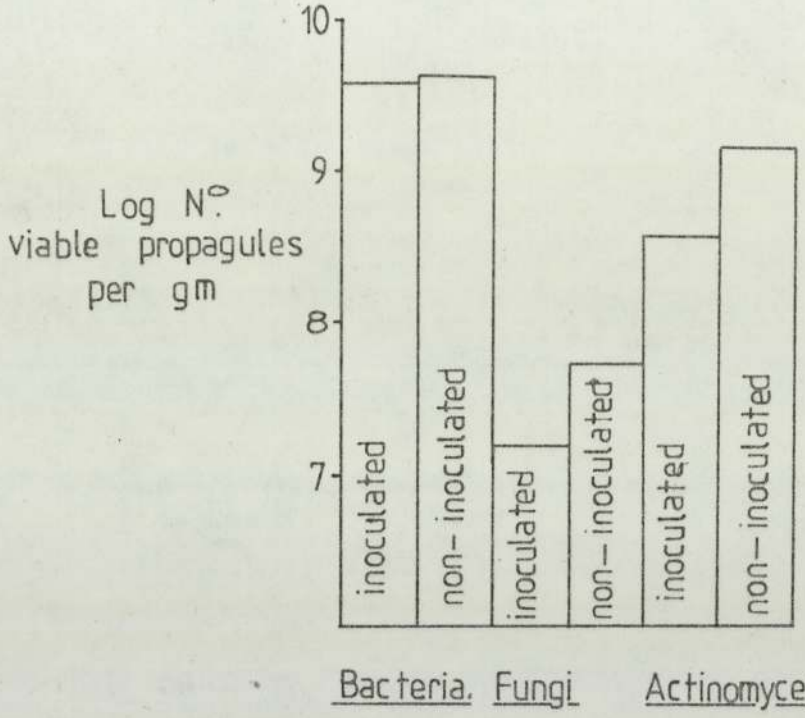


Fig 7-10 Comparison of propagule counts on C. cinereus inoculated + non-inoculated composts

significant after day 6 which corresponds to the rapid increase in *C cinereus* isolations shown in figures 7.6 and 7.7. In figure 7.3 we again see the 2 day lag period in growth brought about by ammonia. This confirms further the hypothesis that ammonia is selecting by first inhibiting the growth of micro-organisms. It would seem that as there is a rapid logarithmic increase after day 2, many of the fungal spores are not killed but germination is inhibited, so that as the levels of ammonia drop, germination begins, producing a situation analogous to fungistasis as described in soils. However, actively growing fungi were isolated at low levels from ammonia amended straws on day 2. It is possible that these were deep seated hyphae which may have been shielded from the major effects of ammonia by the straw and began immediate growth when placed on suitable media away from the influence of ammonia. The active role of ammonia in soil fungistasis has been investigated by Ko and Hora (1972) and Ko *et al* (1974) and it would seem that a similar situation is occurring here.

Actinomycete levels were increased by the addition of ammonia but they appear to be decreased by *C cinereus* inoculation. This is probably because competition from *C Cinereus* is increased. It has been shown that some *Streptomyces sp* produce ammonia (Hora and Baker, 1972; Ko and Hora, Ko *et al*) and this volatile inhibitor is capable of suppressing conidial growth in soils. Such actinomycetes must be able to tolerate raised levels of ammonia themselves and when examined microscopically, the dominant actinomycetes isolated here were grey-white speckled Streptomyces colonies. The lack of any lag period produced by ammonia treatments would also seem to confirm the tolerance of these actinomycete species. Chang and Hudson found increased levels of actinomycetes in straw composts up to peak heating and Hayes reported a rapid re-colonisation by actinomycetes after peak heating in mushroom compost, increases which were not observed in the fungal or bacterial propagules.

Growth of bacteria does not appear to be suppressed completely at any stage by ammonia, however, overall growth levels were somewhat reduced; no significant differences were seen between inoculated and non-inoculated straws. High pHs above 11 have been shown to completely inhibit the growth of some bacteria (Doyle, 1967) and it was also found that a pH of 9-10 will reduce the growth rates of these organisms, so the effect of the pH elevation in this work may have reduced the number of viable bacterial propagules.

The initial counts of all organisms agreed closely with those reported by Chang and Hudson and in the case of fungi also by Eastwood (1952). The types of organisms isolated are also consistent with those found by other workers on straw and straw composts. The only species appearing in significant numbers in ammonia amended straws and not in the others was *C cinereus*. However although *C cinereus* isolation was greatly increased, it is obvious from this more complete study of the microflora that other micro-organisms are present when *C cinereus* is at its peak of isolation. Earlier work utilising cellulose agar did not detect these, although as the more searching programme shows they may have been present. Other species which had an increased percent isolation from ammonia amended straws were *Aspergillus fumigatus* which can tolerate a wide range of conditions and is a fast growing, high sporing species and *Gliocladium roseum* which is known to have antagonistic properties which may help its growth here. An antagonistic ability may also help the selection of *C cinereus* as Penn (1977) has reported that *C cinereus* shows antagonism towards other fungi under alkaline conditions, and hyphal interference caused by this fungus on others present in straw composts has been reported by Hedger and Hudson (1974).

7.6 CONCLUSION

The appearance of these other fungi on the ammonia treated straws is not encouraging when the proposed upgrading process is considered.

The possible dangers to man and animal from mouldy fodders have been well documented (Pepys *et al* 1963, Lacey 1975). Fungi and actinomycetes may cause infections, provoke allergic responses or poison with toxic metabolites. Two potentially pathogenic fungi were isolated from these straws after ammonia treatment, these were *A fumigatus* and *Scopulariopsis brevicaulis*. The increase in actinomycete numbers should not be encouraged as some are implicated as the causative agents of Farmers' Lung disease. However, the results from the larger composts are interesting in that only a single isolation of a single species other than *C cinereus* was made from the inoculated compost and inoculation also significantly reduced the numbers of actinomycetes. There could be at least two reasons for this; (1) a much larger inoculum was used which allowed *C cinereus* to quickly swamp the other species as was used in the tubes of the previous chapter where *C cinereus* alone completely covered the straw in the ammonia treated tubes and (2) the closed bag helped to keep a higher, more constant level of ammonia than with the more open jars. There may also be changes in temperature levels within the composts which affect the microflora.

It would seem that if a non-sterile process is to be considered, a large inoculum of *C cinereus* will be necessary and this provides a basis for further work to detail the size and nature of the inoculum. It would also be useful to study the manner in which ammonia should be added. Further small ammonia supplements after the initial addition may maintain levels inhibiting the competitors of *C cinereus*. It will also be necessary to interrelate such results as shown here with those obtained from nutritional studies. Such work at present indicates that the best time to harvest the fodder for optiumum feed value is only 4-5 days after inoculation (Burrows, unpublished data) at which time actinomycete levels as shown in this work are still below levels indicated as "mouldy" hay by Lacey and Durkiewicz. Obviously further work will be required

to ensure that the product and means of production are completely under control at all times and that there is no possibility of health hazards occurring at any stage.

CHAPTER EIGHT

DISCUSSION

8.1 DISCUSSION AND CONCLUSIONS

The work reported here should be viewed from two angles. Firstly, the pure aspects of the study. These are the contributions made to the body of knowledge regarding *C cinereus* by the investigation of its widespread presence in agricultural environments and also the study of its responses to ammonia and other nitrogen sources at elevated pH. Information has also been gained on the ecology of other species which have been isolated from straw at different stages of the work. The second facet of this study is the relationship of the above to the proposed straw biodegradation process and as such these results provide guidelines to the feasibility of such a process as it was conceived at the beginning of this project. The results also indicate parameters for the composting and point to problems which may be met with and modifications which may be necessary on a larger scale pilot scheme. This is the more applied aspect of the research programme.

From the pure research point of view the observation that *C cinereus* can be isolated from straw and soils using ammonia and the inference that this places the fungus in Sagara's 'chemoecological' grouping of 'ammonia fungi' is of great interest. Sagara isolated other fungi usually classed as coprophiles by using ammonia and it could be suggested that the response to ammonia may, along with other nutritional factors be important in the metabolic activities which define coprophiles. Free ammonia will be present in composts, dung heaps and other such environments.

It has been shown that ammonia solutions could be used as the selective agent in a upgrading process and that a level of 1% w/w at 30°C will provide optimum selective conditions. From the work reported

in chapter six it would seem that this ammonia actually inhibits the growth of *C cinereus* and other fungi for up to 50 hours after inoculation and amendment. After a period of 50-60 hours the level of ammonia drops sufficiently to allow the growth of *C cinereus* to commence. This is still either inhibitory to the growth of other organisms or lasting damage may have occurred to the propagules of other species at these high ammonia levels. There is evidence that ammonia actually inhibits *C cinereus* growth rates so that it can be assumed that competitive advantage is not conferred by enhancing the speed of colonisation, it is the tolerance of *C cinereus* to high ammonia levels which seems to be the key.

The work reported in chapter 7 where a more comprehensive isolation programme was undertaken showed that at the time of peak growth of *C cinereus,* other species were also present in the bench scale experiments. However, when larger composts were considered which had been inoculated with *C cinereus* spores, these fungal competitors were not isolated. Bacterial and actinomycete levels were still high and over a period of days reached numbers that were classed as unsafe by Lacey and Dutkiewicz (1976). However these levels must be viewed in relation to time periods required for optimum increases in digestibility as pointed out in chapter 7. The indications throughout this thesis are that the action of ammonia is unique. It is not simply acting as a nitrogen source, nor an agent to elevate pH. As demonstrated when these factors were introduced using other agents, useful *C cinereus* selection was not observed (Chapter 6).

The effect of ammonia on straws and soils can to some extent be compared to fungistasis in the soil. Dobbs and Hinson (1953) declared that the soil is an antagonistic medium for soil fungi and that isolated pockets of activity may "break-through" this inhibition around areas of

dead organic matter. The exact nature of the phenomenon of soil fungistasis is not known being the subject of considerable controversy. However, recent work (Hora and Baker, 1972) has suggested that one of the major causes may be volatile ammonia produced by species of streptomyces in the soil. Hence the comparison here - ammonia is creating a hostile environment for all fungi but as the levels drop slightly *C cinereus* activity can "break out" then, as levels drop further and the ammonium ion is utilised, if a sufficiently large inoculum of *C cinereus* is not present other fungi can overcome the ammonia barrier and 'break out'.

A further link indicating the regulatory nature of ammonia in some situations was the work of Moore and Jirjis. This showed that *C cinereus* used ammonia to regulate its own life cycle. Ammonia is a primary metabolite of *C cinereus* and it was found that raised levels inhibited the production and maturation of sclerotia. This is correlated with the ability of the sporophore to excrete ammonia so as to provide a mechanism for sequencing the two pathways. During sporophore development the ammonia concentrations will be maintained at a level which prevents sclerotial maturation, thus avoiding competition for metabolites between the two morphogenic states.

Ammonia appears to play a very important role in the ecology of *C cinereus* and possibly other coprophilic fungi and hence also in the ecology of composting organic material.

The limited investigation of the cellulolytic and ligninolytic abilities of some *C cinereus* isolates showed that it may be possible to utilise this organism to break the lignocellulose complex. However its utilisation of lignin appears to be a long term reaction, and a fine balance must be reached between the time taken for *C cinereus* to increase digestibilities and longer periods after which the fungus may begin to deplete cellulose and actively decrease digestibility. Unpublished enzymatic data from these laboratories (Seal, unpublished data) indicate that *C cinereus* will preferentially utilise hemicelluloses prior

to degrading cellulose, a phenomenon which would lend credence to the reported observations that maximum digestibility increases occur at about 4 days after incubation and inoculation, as after this period cellulose will begin to be utilised. The removal of hemicelluloses from the lignocellulose complex will, as noted previously (chapter 1) facilitate the breakdown of the other components, so that the ability of *C cinereus* to utilise and break the bonds binding hemicelluloses may be of far greater importance than its cellulolytic and ligninolytic characteristics.

This work has served to indicate the large number of interrelated factors which must be understood when considering the growth of micro-organisms on natural substrates - particularly under non-sterile conditions. The ecology of such environments is extremely complex, however it is possible to utilise some of the naturally occurring limiting factors to control the environment and hence obtain a system which will eventually lead to the desired product. The work reported in this thesis has been an attempt to do just that. The results show greater and lesser degrees of success at various stages of the project, however the final indications must point to a technique which requires further research, refinement and attention to safety aspects but which however does have possibilities of success.

As with the aims of the work, the conclusions can be summarised as follows:-

1. *C cinereus* can be isolated from straw and agricultural soils using techniques based on ammonia.

   This fungus was isolated from 100% of all field and bale straw samples and from over 70% of all soils examined which had been under cereals. The exact nature of this inoculum was not identified.

2. The presence of free ammonia produces a selective pressure in favour of *C cinereus* and the optimum levels to bring this about on straw appear to be in the region of 1% w/w at 30°C.

3. Ammonia inhibits the growth of many fungi but not actinomycetes, yet it would appear that where a heavy inoculum of *C cinereus* spores is also introduced, axenic cultures could be maintained. *C cinereus* is also marginally inhibited by ammonia at levels of 1% though as levels drop this organism recovers and colonises more speedily and successfully than its competitors and hence becomes the dominant fungal coloniser.

4. Bacteria do not appear to be greatly affected by levels of 1% ammonia and the populations at various times must be considered with the upgrading process in view.

5. Growth rates, cellulose and lignin utilisation can vary with strain therefore this should be taken into account when considering the one for use in the straw biodegradation process. The possibility of genetic manipulation of the species to provide more of the required enzymes could be considered.

6. This work cannot be considered in isolation. When laying down parameters for the process it must be studied in conjunction with results from nutritional and biochemical studies, pilot plant investigations, feeding trials and close scrutiny of the implications of changing economic situations.

8.2 CRITICISMS OF THE WORK

There are a number of points of adverse criticism which could be aimed at this work; possibly the main one is the validity of extrapolating results obtained in bench scale work to the situation which may occur in large farmyard composting. It could be argued that there would be numerous changes in pH, temperature and O_2 and CO_2 tensions in the large compost which would grossly affect the microflora yet cannot be reflected by a series of jars in a controlled environment. This argument would be totally valid if a non controlled, mixed culture compost was being

contemplated. Chang and Hudson (1967) found the temperatures in such composts rose to 70°C, almost the peak temperature by day four. However, far from this the technique envisaged utilises environmental control and inoculation of a dominant fungus which will grow throughout the compost in a very short time. As previously mentioned, evidence indicates that the optimum time for harvesting may be as little as 4 days after inoculation (Burrows unpublished data); combining this with the ammonia induced lag period observed in this work gives a harvest time of 6 days after incubation begins. It is felt that little self heating and oxygen depletion would have occurred by this period in a loosely packed, high ammonia level compost. This is shown by the results obtained from larger composts in chapter 7 where the isolates obtained compared well with those from the bench scale experiments. However pilot scale work is being undertaken by the BIC and the size of the process will depend largely on the time for optimal increase in digestibility. On present evidence it would appear that the short time span would allow the farmer to store straw as normal and compost as and when required.

The main reason however for using bench scale, rather than larger composts was that it allowed many more nitrogen sources and levels of ammonia to be investigated than could possibly have been carried out using larger amounts of material. Hence this technique was employed.

Other problems which could be envisaged when considering the transfer of the system from laboratory to farm is that of handling the ammonia. However the farmer in this age of pesticides is familiar with the regular wearing of protective clothing to handle chemicals, and ammonia is far less caustic than the concentrated sodium hydroxide which is used in some chemical upgrading techniques on the farm.

Criticism could be levelled at the project on economic feasibility. There is no question that such a process would be of use in the areas where mixed farms can still be found and in areas where monoculture

interests overlap, however the problem of excess straws in the East Anglian region would not be alleviated as there is very little requirement for an animal feedstuff from straw in this area. However should the process be sufficiently successful there could be economic arguments for reintroducing the mixed farm to these areas. Any attempt to put this process on a more centralised footing would involve all the problems of straw transfer so that further investigation into the economics of the process would be necessary.

8.3 SUGGESTIONS FOR FURTHER WORK

Possible lines of further research have been put forward throughout the thesis, this section is intended to bring together and extend those ideas.

Probably the largest question which has been posed rather than answered by this work is the origin and nature of the *C cinereus* isolates which were obtained from the straw and soils in the agricultural environment. Further investigations in this area could yield interesting information. It was suggested in chapter 3 that a study of *C cinereus* occurrence on growing cereal stems and on straw litter may provide evidence as to the origin of the inoculum. It may also be of use to combine this with extended investigations of the air spora using an impinger which collects the spores in liquid rather than impaction techniques where they are collected on agar plates. As previously shown it is difficult to identify colonies of *C cinereus* from spore origins without further investigation into selective media. An impinger would allow spores to be observed, isolated and cultured for easier identification. Difficulties can be forseen when attempting to identify the nature of the inoculum, some form of screened substrate may be used in enriched soils. However, the addition of enrichments may simply stimulate resting bodies to germinate. The evidence that ammonia selects for *C cinereus* by inhibiting competitors may preclude this, and that obtained by Mosely (1977) which indicated that

ammonia may actually inhibit *C cinereus* spore germination suggests that any isolations made by this method would be from actively growing hyphae. A second possibility is to use the technique of Warcup as previously outlined and examine soil and straw samples microscopically for hyphae, spores and sclerotia. This would be a very time consuming process and large amounts of material would need to be examined. It may also be possible to identify some physiological difference between the spores and hyphae of *C cinereus* which may enable one to be isolated at the expense of the other. However, these all require much further detailed examination.

It would be of great interest to investigate further the physiological responses of *C cinereus* and some of its competitors to ammonia in pure culture. The effect on growth rates and any antagonistic or other properties which may enable the fungus to compete successfully in mixed culture could be studied. This has been investigated at raised pH by Penn (1977) but not at increased pH and ammonium ion levels brought about by amendment with ammonia solutions.

Enzymatic studies have been carried out on *C cinereus* over a range of pH (Seal, unpublished data) to observe the kinetics of cellulose and hemicellulose breakdown. It would be useful to carry out similar investigations after *C cinereus* had been grown in the presence of various percentages of ammonia and the carbohydrate under investigation. Aspects of this work are being carried out by Burrows who is investigating the breakdown of the various components of straw by *C cinereus* after amendment with ammonia.

The responses of other fungi normally classed as coprophilic (or which could be expected to be found in composts) to ammonia would be of value. It is possible that their presence and that of *C cinereus* itself in a compost at a certain time is related to ammonia levels as well as temperature and nutritional levels as has previously been shown.

Further investigations extending the work of Moore and Jirjis may also be rewarding.

There is need and scope for further investigations into the project as a whole. The prospect of keeping the method as an on-farm process must be viewed against the possibility of linking the system with a slurry stripping operation of a more centralised nature.

Finally, feeding trials are an obvious requirement together with more large scale studies to ensure that axenic cultures of *C cinereus* can be maintained without risk of outside contamination. *C cinereus* is an edible fungus related to the often eaten *C comatus*. Feeding trials to date using rats and rabbits have shown no ill effects to either. However, the health factor obviously figures large when new feedstuffs are under consideration, so that the health and safety aspects of the process still require much investigation, especially with the increased concern for the well being of trained and non-trained workers alike when handling microorganisms.

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APPENDIX I

MEDIA USED IN EXPERIMENTAL WORK

A. EGGINS AND PUGH CELLULOSE AGAR

Potassium dihydrogen orthophosphate	1.0 g
Ammonium sulphate	0.5 g
Potassium chloride	0.5 g
L-Asparagine	0.5 g
Yeast Extract	0.5 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Agar	20.0 g
Ball milled cellulose -4% w/v	250 ml
Distilled water	750 ml

B. POTATO MALTOSE AGAR

Cooked potatoes	200 g
Maltose	5 g
Agar	10 g
Distilled water to:-	1 litre

Potatoes are peeled, boiled and squeezed through muslin before adding to the other ingredients.

C. LANGE'S HORSE EXTRACT AGAR

Magnesium sulphate	0.5 g
Calcium nitrate	0.5 g
Potassium dihydrogen orthophosphate	0.25 g
Peptone	0.1 g
Agar	10 g
Dung extract	100 ml
Distilled water up to:-	1 litre

Dung extract: One 'horse apple' is heated (not boiled) in 200 ml or water for a few minutes and filtered before use.

D. TANNIC AND GALLIC ACID MEDIA

Base medium:	Glucose	10 g
	Ammonium chloride	0.5 g
	Potassium dihydrogen orthophosphate	0.5 g
	Magnesium sulphate	0.5 g
	Malt extract	2.5 g
	Agar	15 g
	Distilled water	1 litre
Added post-autoclaving:	Gallic acid	850.6 mg
	or	
	Tannic acid	500 mg

Stock solutions of these were filter sterilised and stored at 4°C until required.

E. MALT EXTRACT AGAR

Oxoid Ltd

F. NUTRIENT AGAR

Difco Ltd 'Bacto' nutrient agar.

G GLYCEROL-CASEIN AGAR

Glycerol	10 ml
Soluble white casein	2 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulphate	0.2 g
Ferrous sulphate	0.01 g
Agar	15 g

APPENDIX II

PRELIMINARY EXPERIMENTS AND TECHNIQUES EMPLOYED IN THIS THESIS

A. THE HARLEY AND WAID WASHING TECHNIQUE

Method

Five pieces of straw of about 5 cm in length were removed aseptically from the material under examination and placed into a sterile 'Universal' bottle along with 15 ml of sterile distilled water. The bottle was then capped and shaken mechanically for 2 min; after this period the liquid was decanted off and replaced by 15 ml of fresh sterile water, this was repeated the required number of times. The pieces were then removed and blotted onto sterile filter paper to remove excess water and hence prevent the spread of bacteria. The straw was then roughly chopped into 7-10 pieces and plated onto the relevant medium. This technique is designed to remove surface hyphae and spores, so leaving the actively growing, deep seated hyphae to be isolated.

A preliminary piece of work was carried out to assess the number of water changes which would be required to remove surface spores from the straw. The above technique was used on 'fresh' (F) straw and 'mouldy' (M) straw. These were washed with twenty changes of water and 1 ml aliquots of washwaters 1,2,3,5,10,15 and 20 were plated out in triplicate onto Eggins and Pugh cellulose agar and Eggins and Pugh glucose/starch agar. These were then incubated at 30°C and counted after 4 days.

Results and Conclusions

The results are shown in table A 2.1. In all cases by the fifth wash a constant low level of fungal propagules retrieved from the wash-water had been achieved, so that this was the number of washes which were employed in all later work.

TABLE A.2(1) Fungal Colonies Retrieved From Washwaters

Wash No.	E & P Glucose/Starch			E & P Cellulose		
	1	Plate 2	3	1	Plate 2	3
M_1	64	132	112	120	88	116
M_2	7	8	14	24	32	33
M_3	15	12	6	17	20	12
M_5	0	0	0	4	0	2
M_{10}	0	0	0	5	2	7
M_{15}	0	0	0	5	0	3
M_{20}	0	0	0	1	4	4
F_1	6	5	0	10	16	13
F_2	0	0	0	7	4	9
F_3	0	0	1	5	2	0
F_5	1	0	0	2	1	1
F_{10}	1	0	1	1	3	4
F_{15}	0	0	0	0	2	1
F_{20}	0	0	1	6	0	0

B. THE EFFECT OF 'BENLATE' ON THE GROWTH OF *C CINEREUS*

Method

Eggins and Pugh cellulose agar was produced and amended with 'Benlate' at a range of levels (Table A 2.2). These were then inoculated with 5 mm plugs of *C cinereus* isolate As-1, 5 plates per treatment and incubated at 30°C, the growth was measured at 5 and 6 days and these results are also shown in Table A.2.2.

TABLE A 2.2 Growth of *C cinereus* on Eggins and Pugh Cellulose Agar Amended With Benomyl

Benomyl ppm	Radial Growth (mm)		
	Day 2	Day 5	Day 6
Control 0	20.66 ± 4.69	69.50 ± 3.37	85.00 Plate covered
0.5	21.40 ± 1.64	77.20 ± 3.27	85.00 Plate covered
2.0	16.40 ± 2.63	70.10 ± 3.96	84.70 ± 0.67
10.0	15.70 ± 3.87	66.80 ± 4.76	84.70 ± 0.45
15.0	16.90 ± 5.18	53.90 ± 5.27	70.30 ± 5.75
20.0	19.60 ± 3.44	54.00 ± 4.30	62.80 ± 5.30
25.0	17.40 ± 2.10	49.80 ± 6.31	64.30 ± 7.22
30.0	10.40 ± 3.03	23.30 ± 3.53	27.10 ± 5.75
40.0	No growth	No growth	No growth
50.0	No growth	No growth	No growth

Results

Above 20-25 ppm benomyl, growth rates of *C cinereus* were seriously inhibited and the experimental work reported in chapter 3 was pursued with consideration to these results.

C. PRODUCTION AND HARVESTING OF *C CINEREUS* SPOROPHORES

Many workers in the past who have required fruiting cultures of *C cinereus* have used complex natural substrate mixes of dung and cellulose, and have inoculated these with hyphae in milk bottles (Anderson 1971). This is a time consuming and messy process. A technique using solid media was developed at the BIC to overcome these problems, the method allowed mature caps to be harvested for spores and made it possible to photograph sporophore growth and development.

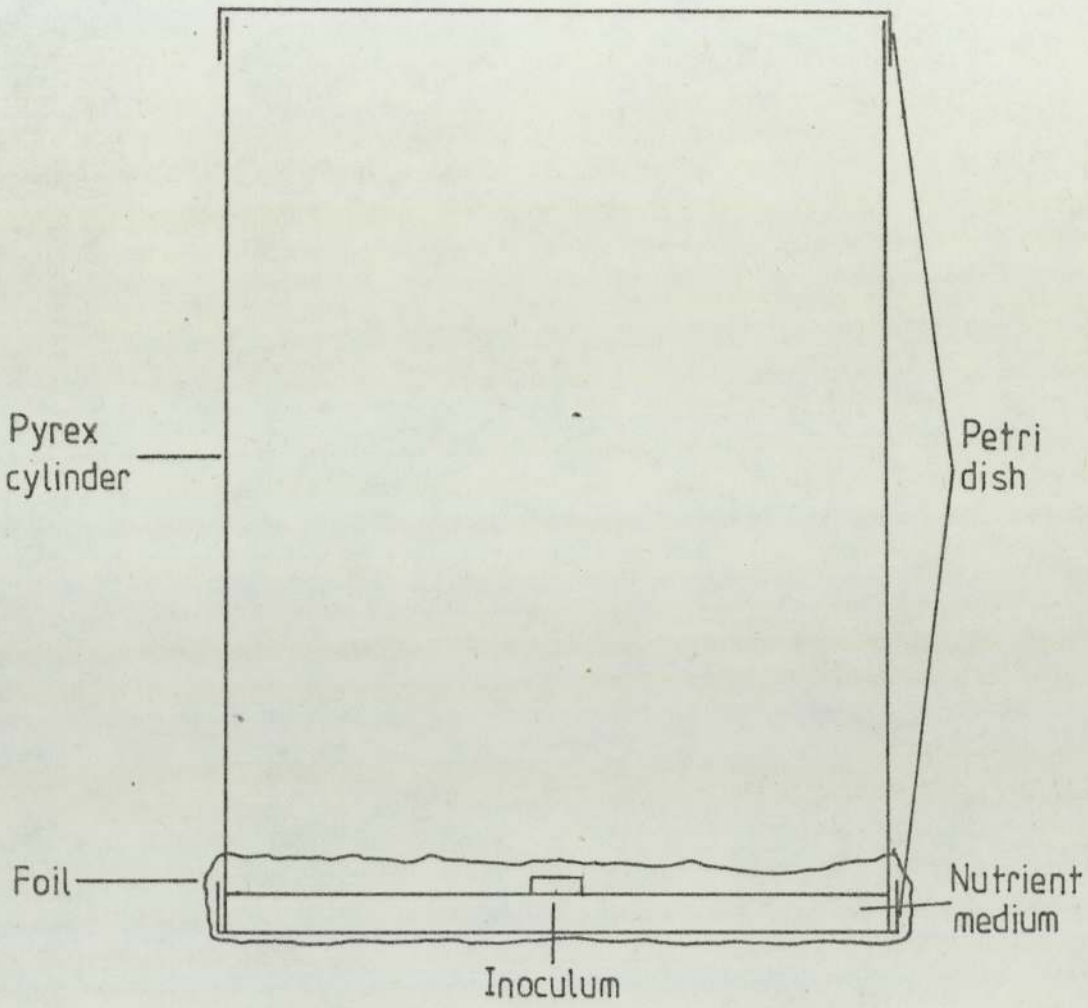

Fig. A.2.1 Apparatus for the production of *C. cinereus* sporophores

Method

Potato maltose agar was found to be an exellent medium for the fruiting of *C cinereus* and this was used in the process. Pyrex glass cylinders which just fitted into 90mm petri dishes were utilised, these were about 120mm high. The cylinders were wrapped and autoclaved at 15 lb/sq in and then placed into petri dishes (Fig A 2.1), the foil which had wrapped the cylinder during autoclaving was placed round the dish base to prevent entry of organisms to any media which may escape from the cylinder. Media were placed into the base and the extended petri dish was inoculated with *C cinereus* and the lid replaced. These were incubated at 30°C until sporophore initials were well developed and then brought into the light to mature. When the *C cinereus* caps began to darken indicating the onset of autolysis they were aseptically removed and placed into a clean, sterile petri dish. Autolysis then continued and spores were liberated into the petri dish leaving spore prints. Sterile distilled water was then added and the spores stirred into this, the remains of the gills were usually easily removed in one piece leaving a spore suspension which could be filtered and used for inoculations. If photographs were required at any stage, the cylinder was removed leaving the sporophores free from distorting glass. (Plates A 2.1 and A 2.2).

D. IDENTIFICATION OF *C CINEREUS* ISOLATES BY MONOKARYON CROSSING

Identification of those isolates which were to be used for further experimentation was confirmed by attempting to cross a monokaryon of the isolate in question with one from a known *C cinereus* strain. This was used purely as a confirmatory test, as should a dikaryon fail to be produced from the cross, it may simply mean that the monokaryons are not of compatible mating strains. Therefore a successful cross confirmed that the isolate was *C cinereus* but an unsuccessful one was not necessarily proof of it being another species.

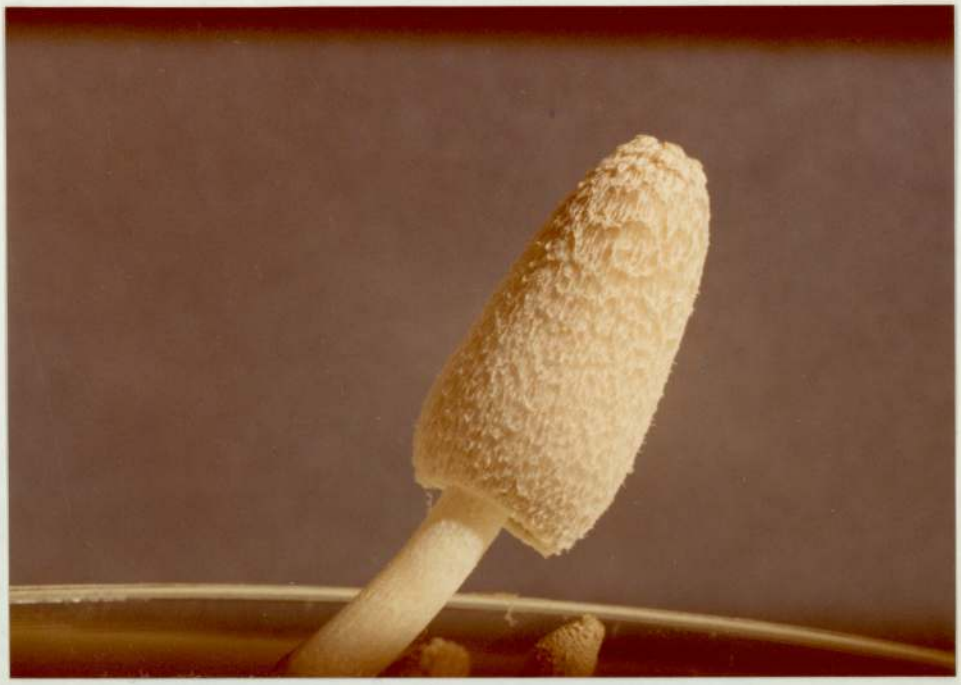

PLATE A.2.1 Developing Sporophores of *C cinereus*

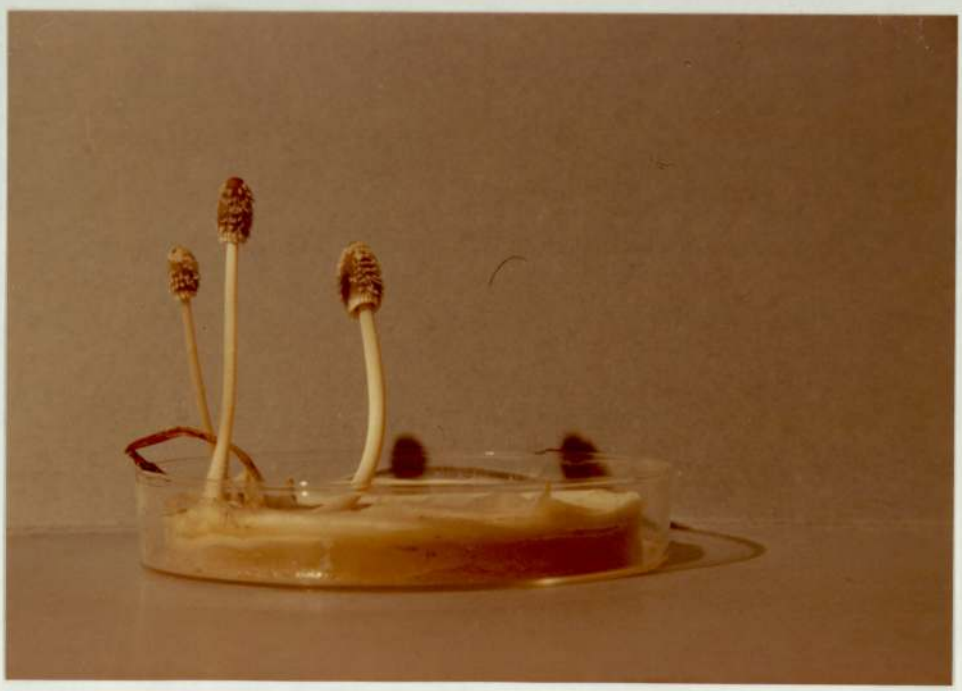

PLATE A.2.2 Mature Sporophores of *C cinereus* Showing Onset of Autolysis

Method

A known monokaryon was obtained from the collection of Roger Kemp at Edinburgh University. Monokaryons of the isolates under test were produced from single spore cultures. Spore suspensions were obtained as previously described, these were diluted 1:100 and 0.1 ml of this suspension was spread onto a malt extract agar plate. The spores were then incubated at 30°C and kept under close observation until germination occurred. At this stage, five individual spores were picked off and subcultured onto further malt agar plates. This medium was used as it allowed the spores to be observed microscopically, it being less opaque than Eggins and Pugh cellulose or Potato Maltose agars, yet it still produced successful spore germination. Pieces of the growing edge of the colonies thus obtained were then crossed with the known monokaryon.

The two inocula (about 1 mm^3 in size) were placed in the centre of a malt agar plate, about 2-3 mm apart, these were then incubated at 30°C and the junction line was examined at regular intervals for the production of dikaryotic hyphae (plate A.11.3). These were easily identified by the appearance of clamp connections, and by the macroscopic appearance of the colonies, the dikaryon having a much fluffier colony edge and aerial growth while the monokaryon has flatter aerial growth and a smooth colony outline.

Results

Dikaryons were obtained from crosses between all examined isolates, ie As-1, As-2, As-3, As-4 and As-6 giving positive identification of these as *C cinereus*.

Plate A.2.3 Identification cross between *C cinereus* monokaryons after 24h incubation.

APPENDIX III

TABLES OF RESULTS

Table	Source	Title
A III 1	From Chapter Two	Isolations from straw after incubation with nitrogen sources.
A III 2	From Chapter Six	Hyphal Extensions
A III 3	From Chapter Seven	Appearance of fungi on inoculated straws
A III 4	From Chapter Seven	Appearance of fungi on non-inoculated straws.

TABLE A III 1 ISOLATIONS FROM STRAW AFTER INCUBATION WITH NITROGEN SOURCES

		Percent appearance (15 plates)									
Treatment	Straw	*A fumigatus*	*A versicolor* series	*Alternaria tenuis*	*Botrytis cinerea*	*Chaetomium globosum*	*Coprinus cinereus*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*	*Scopulariopsis brevicaulis*
1	A	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0
	E	0	0	0	0	0	0	0	0	0	0
2	A	0	0	0	0	0	100	0	0	0	0
	B	0	0	0	0	60	100	0	0	0	0
	C	0	0	0	0	0	100	0	0	0	0
	D	0	0	0	0	0	100	0	0	0	0
	E	0	0	0	0	0	100	0	0	0	0

TABLE A III 1 ISOLATIONS FROM STRAW AFTER INCUBATION WITH NITROGEN SOURCES (Cont'd).....

		Percent appearance (15 plates)									
Treatment	Straw	*A fumigatus*	*A versicolor* series	*Alternaria tenuis*	*Botrytis cinerea*	*Chaetomium globosum*	*Coprinus cinereus*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*	*Scopulariopsis brevicaulis*
3	A	0	0	0	0	60	100	0	0	0	0
	B	0	0	0	0	100	93.33	0	0	0	0
	C	0	0	0	0	80	100	0	0	0	0
	D	0	0	0	0	100	100	0	0	0	30
	E	0	0	0	0	100	100	0	0	0	0
4	A	0	0	0	0	100	100	0	0	0	6.67
	B	0	0	0	0	100	40	0	0	0	0
	C	20	0	0	0	100	100	0	0	0	0
	D	0	0	0	0	100	100	0	0	0	0
	E	30	0	0	0	100	100	0	0	0	0

TABLE A III 1 ISOLATIONS FROM STRAW AFTER INCUBATION WITH NITROGEN SOURCES (Cont'd) ...

Percent appearance (15 plates)											
Treatment	Straw	*A fumigatus*	*A versicolor* series	*Alternaria tenuis*	*Botrytis cinerea*	*Chaetomium globosum*	*Coprinus cinereus*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*	*Scopulariopsis brevicaulis*
5	A	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0	0	0	0
	E	0	0	0	0	0	0	0	0	0	0
6	A	0	0	0	0	0	100	0	0	0	0
	B	0	0	0	0	0	100	0	0	0	0
	C	0	0	0	0	0	100	0	0	0	0
	D	0	0	0	0	0	100	0	0	0	0
	E	0	0	0	0	0	100	0	0	0	0

TABLE A III 1 ISOLATIONS FROM STRAW AFTER INCUBATION WITH NITROGEN SOURCES (Cont'd) ...

		Percent appearance (15 plates)									
Treatment	Straw	*A fumigatus*	*A versicolor* series	*Alternaria tenuis*	*Botrytis cinerea*	*Chaetomium globosum*	*Coprinus cinereus*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*	*Scopulariopsis brevicaulis*
7	A	0	0	0	0	0	100	0	0	0	0
	B	0	0	0	0	100	100	0	0	0	0
	C	0	0	0	0	93.33	100	0	0	0	0
	D	0	100	0	0	100	30	0	0	0	100
	E	0	0	0	0	0	100	0	0	0	0
8	A	0	0	0	0	40	20	0	0	0	0
	B	0	0	0	0	100	100	66.6	0	0	0
	C	0	0	0	0	93.33	100	0	0	0	0
	D	0	100	0	0	100	0	0	0	0	100
	E	0	66.67	0	6.67	20	66.67	0	0	0	0

TABLE A III 1 ISOLATIONS FROM STRAW AFTER INCUBATION WITH NITROGEN SOURCES

		Percent appearance (15 plates)									
Treatment	Straw	*A fumigatus*	*A versicolor* series	*Alternaria tenuis*	*Botrytis cinerea*	*Chaetomium globosum*	*Coprinus cinereus*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*	*Scopulariopsis brevicaulis*
9	A	6.67	0	0	0	46.67	46.67	0	0	0	0
	B	6.67	0	0	0	66.67	46.67	0	6.66	0	0
	C	40	0	60	0	100	46.67	0	0	0	0
	D	0	60	0	0	100	20	0	20	6.67	100
	E	0	20	0	0	20	100	0	0	0	0
10	A	0	0	0	0	30	100	0	0	0	0
	B	0	0	0	0	40	100	0	0	0	0
	C	6.67	0	0	0	66.67	100	0	0	0	0
	D	0	0	0	0	40	66.67	0	0	0	0
	E	6.67	0	0	0	30	93.33	0	0	0	0

TABLE A III 1 ISOLATIONS FROM STRAW AFTER INCUBATION WITH NITROGEN SOURCES (Cont'd) .....

		Percent appearance (15 plates)									
Treatment	Straw	*A fumigatus*	*A versicolor* series	*Alternaria tenuis*	*Botrytis cinerea*	*Chaetomium globosum*	*Coprinus cinereus*	*Fusarium sp*	*Trichoderma viride*	*Sordaria fimicola*	*Scopulariopsis brevicaulis*
11	A	100	0	0	0	66.67	0	0	0	0	0
	B	20	0	6.67	0	100	46.67	0	0	0	0
	C	66.67	0	30	0	93.33	20	0	0	0	0
	D	0	100	0	0	100	30	0	0	0	30
	E	30	100	0	6.67	100	100	0	0	0	0
12	A	100	0	0	0	30	0	0	6.67	0	0
	B	20	0	20	0	30	0	66.67	6.67	0	0
	C	30	0	0	0	100	66.67	0	0	0	0
	D	0	30	0	0	100	20	0	6.67	6.67	0
	E	30	10	0	0	20	100	0	0	0	0

Table A III.2 Hyphal Extensions - *C cinereus* on straw in growth tubes

Treatment	Sterile Straw				Non-Sterile Straw			
	Time (hours)	Growth mm ± SD			Time (hours)	Growth mm ± SD		
		As-1	As-3	As-6		As-1	As-3	As-6
1% ammonia	24	0	0	0	24	0	0	0
	48	0	0	0	46	0	0	0
	72	5.1 ± 1.39	4.7 ± 2.39	5.1 ± 2.78	70	4.0 ± 1.10	12.0 ± 2.83	6 ± 1.41
	98	23.8 ± 2.60	21.7 ± 5.16	10.6 ± 5.36	82.5	15.0 ± 1.31	25.0 ± 1.14	24.30 ± 5.1
	122	38.8 ± 2.00	32.8 ± 4.30	20.2 ± 4.24	103	34.5 ± 6.36	38.33± 8.5	40.67 ± 7.3
	142	49.4 ± 1.01	44.5 ± 3.96	32.9 ± 8.83	151	65.7 ± 3.06	60.7 ± 9.02	69.7 ± 10.02
	165	60.5 ± 2.83	53.8 ± 2.83	44.2 ± 7.02	199	81.5 ± 2.12	79.0 ± 2.83	88.0 ± 4.24
	190.5	72.7 ± 3.68	65.7 ± 1.65	55.7 ± 1.07	220	86.0 ± 3.71	83.0 ± 1.98	-
	220.5	84.1 ± 1.69	79.3 ± 0.11	73.1 ± 6.83				
	237.5	88.6 ± 0.98	84.33± 1.41	78.8 ± 7.36				
	269.0	97.1 ± 6.15	98.3 ± 4.84	94.80± 9.36				
KNO_3	24	0	0	0	24	5.5 ± 0.71	5.5 ± 0.92	6.5 ± 2.12
	48	10.80± 1.98	12.5 ± 1.50	11.4 ± 0.70	48	18.0 ± 1.41	20.0 ± 2.83	17.5 ± 3.54
	74.3	24.50± 2.17	29.7 ± 1.45	24.4 ± 2.77	72	34.8 ± 1.77	38.5 ± 6.36	34.5 ± 6.36
	91.8	31.9 ± 4.67	39.8 ± 0.84	32.3 ± 3.67	96	58.5 ± 4.95	55.0 ± 1.40	53.0 ± 3.01
	117.8	45.8 ± 4.48	55.1 ± 2.67	46.6 ± 5.34				
	145.8	64.0 ± 4.70	73.8 ± 2.22	61.33± 5.81				
	175.8	87.0 ± 1.00	91.1 ± 2.70	82.20± 1.60				
	191.3	95.3 ± 0.58	103.2 ± 1.18	-				
	220	98.5 ± 0.47	-					
$NaNO_2$	41.50	12.7 ± 1.85	15.0 ± 0.88	14.6 ± 0.77	41.50	18.7 ± 1.15	20.0 ± 1.94	20.1 ± 6.43
	48.00	15.5 ± 4.7	-	-	69.00	35.0 ± 0.88	38.2 ± 3.25	34.9 ± 5.80
	69.00	24.6 ± 6.30	32.2 ± 0.69	29.4 ± 0.51	90.25	44.7 ± 2.52	52.4 ± 2.22	41.7 ± 1.85
	90.25	33.9 ± 1.87	45.6 ± 2.50	42.3 ± 2.18	114.25	56.7 ± 5.77	68.7 ± 1.41	52.6 ± 6.20
	114.25	47.8 ± 1.15	63.9 ± 2.37	59.1 ± 1.17	143.00	65.5 ± 7.78	82.5 ± 3.54	57.2 ± 6.36
	143.00	63.3 ± 2.37	82.3 ± 1.06	75.0 ± 4.36	215.00	-		
	215.00	77.5 ± 3.54	110.6 ± 5.35	103.3 ± 2.47				

Treatment	Sterile Straw				Non-Sterile Straw			
	Time (hours)	Growth mm ± SD			Time (hours)	Growth mm ± SD		
		As-1	As-3	As-6		As-1	As-3	As-6
Urea	41.50	26.6 ± 1.67	20.3 ± 1.53	17.2 ± 1.90	41.50	19.7 ± 5.51	18.7 ± 2.52	17.0 ± 0.50
	69.00	39.6 ± 2.52	38.6 ± 3.34	32.6 ± 1.57	69.00	34.6 ± 8.30	37.6 ± 2.67	33.1 ± 1.40
	90.25	53.9 ± 3.24	53.8 ± 2.59	46.9 ± 2.68	90.25	45.6 ± 8.06	49.1 ± 3.42	42.5 ± 1.59
	114.25	71.7 ± 6.12	72.3 ± 4.93	63.6 ± 4.14	114.25	57.8 ± 8.57	59.6 ± 4.14	52.2 ± 2.25
	143.00	85.6 ± 1.72	88.8 ± 5.72	82.4 ± 7.10	143.00	69.9 ± 7.81	69.8 ± 4.37	66.0 ± 5.29
	215.00	110.7 ± 2.79	111.5 ± 3.61	109.6 ± 1.36	215.00	91.2 ± 3.54		
L Asparagine	24.00	8.7 ± 1.06	11.3 ± 0.33	7.6 ± 1.15	24.0	0	0	0
	51.50	23.1 ± 1.09	28.5 ± 0.72	22.1 ± 1.44	46.0	18.0 ± 1.01	13.5 ± 2.12	11.0 ± 1.41
	77.50	39.3 ± 0.44	46.7 ± 0.58	39.7 ± 0.88	70.0	30.0 ± 2.40	23.3 ± 5.77	27.0 ± 1.73
	95.00	50.6 ± 0.70	57.8 ± 1.48	50.8 ± 0.17	92.5	52.1 ± 1.83	42.7 ± 5.51	46.1 ± 0.90
	117.50	79.9 ± 6.03	88.5 ± 2.41	80.3 ± 2.52	111.0	66.7 ± 0.97	54.5 ± 6.36	61.5 ± 4.95
	145.50	92.3 ± 6.18	105.6 ± 1.93	96.1 ± 2.27	139.0	88.0 ± 3.21	75.0 ± 3.27	93.0 ± 3.50
	173.50	113.7 ± 3.28	123.2 ± 1.54	112.0 ± 0.47				
Ammonium tartrate	24.0	48.3 ± 2.12	7.7 ± 1.34	5.2 ± 0.51	24.0	2.0 ± 3.46	2.3 ± 2.08	1.3 ± 2.31
	51.5	19.1 ± 0.51	26.0 ± 1.20	23.3 ± 0.25	46.0	13.0 ± 3.50	10.0 ± 5.00	9.0 ± 7.94
	77.5	34.5 ± 0.87	41.9 ± 2.17	41.1 ± 1.25	70.0	28.7 ± 5.03	28.3 ± 4.90	24.3 ± 1.10
	95.0	45.2 ± 1.32	55.8 ± 1.26	53.8 ± 2.22	92.5	43.7 ± 4.73	46.0 ± 3.00	35.0 ± 1.41
	117.5	72.5 ± 5.60	84.1 ± 5.98	77.1 ± 5.82	111.0	57.7 ± 2.08	59.3 ± 1.15	53.0 ± 4.24
	145.5	86.9 ± 9.68	100.1 ± 6.36	94.8 ± 4.80	139	77.0 ± 3.00	79.7 ± 4.04	
	173.5	114.5 ± 14.42	118.3 ± 7.13	118.0 ± 4.00				
Control- Distilled Water	24.0	8.0 ± 0.46	6.4 ± 0.34	6.4 ± 0.40	24.0	2.1 ± 1.04	2.7 ± 1.02	4.3 ± 0.39
	50.0	20.4 ± 0.40	18.3 ± 0.45	19.0 ± 1.10	46.0	9.7 ± 4.24	6.0 ± 4.30	8.0 ± 4.11
	74.0	29.2 ± 0.99	27.7 ± 0.25	27.9 ± 1.25	70.0	22.5 ± 2.12	13.0 ± 5.20	17.7 ± 3.79
	95.0	34.9 ± 3.12	35.0 ± 3.97	34.3 ± 2.54	92.5	41.2 ± 0.95	24.5 ± 3.44	52.1 ± 4.24
	118.0	42.2 ± 4.60	43.3 ± 5.77	43.0 ± 4.41	111.0	53.2 ± 1.18	41.5 ± 9.19	61.5 ± 2.33
	143.5	49.9 ± 5.07	51.8 ± 6.83	51.8 ± 4.76	139			
	173.5	58.2 ± 6.26	61.3 ± 6.33	61.8 ± 7.28				
	190.5	63.4 ± 7.91	66.0 ± 7.09	66.0 ± 4.58				
	222.0	71.6 ± 9.58	73.7 ± 6.96	78.8 ± 8.11				

	Sterile Straw				Non-Sterile Straw			
	Time (hours)	Growth mm ± SD			Time (hours)	Growth mm ± SD		
		As-1	As-3	As-6		As-1	As-3	As-6
Control-Distilled H_2O at elevated pH	24.0	3.0 ± 1.01	1.0 ± 0.77	2.3 ± 0.58	24	1.5 ± 0.71	1.7 ± 0.58	3.7 ± 2.08
	48.0	7.3 ± 2.52	8.7 ± 2.08	9.4 ± 0.77	48	8.7 ± 2.08	6.3 ± 3.06	8.1 ± 4.24
	72.0	21.4 ± 2.65	28.3 ± 2.89	19.3 ± 1.15	72	19.0 ± 7.50	12.7 ± 4.8	14.3 ± 3.61
	96.0	33.5 ± 3.50	42.7 ± 2.31	30.3 ± 0.70	96	32.3 ± 3.46	21.5 ± 10.6	24.5 ± 2.17
	120.0	68.7 ± 7.77	90.1 ± 1.73	83.9 ± 1.37				
	144.0	87.7 ± 7.50	109.3 ± 2.89	101.4 ± 0.87				
Propylene Oxide Sterilised	24.00	0	0	0				
	47.75	10.3 ± 2.65	11.5 ± 2.27	12.5 ± 2.41				
	74.00	19.3 ± 4.91	24.3 ± 1.15	22.4 ± 3.20				
	91.00	32.0 ± 7.13	35.0 ± 1.67	30.0 ± 6.08				
	117.00	42.4 ± 10.50	49.5 ± 2.80	46.0 ± 3.79				
	145.00	56.2 ± 15.80	60.4 ± 2.84	56.7 ± 2.20				
	174.00	75.4 ± 22.11	75.8 ± 5.89	74.5 ± 7.78				
	189.50	77.5 ± 20.03	90.0 ± 3.78	74.5 7.78				

TABLE A III 3 Percent appearance of fungi over 0-14 days on inoculated straws amended and not amended with ammonia

Species	Treatment	Day						
		0	2	4	6	8	10	14
Alternaria sp	1b	10	0	0	0	0	0	0
	2b	0	0	0	0	0	0	0
Aspergillus flavus	1b	0	10	0	25	0	20	100
	2b	0	0	0	0	0	0	0
A fumigatus	1b	40	80	60	90	70	40	30
	2b	40	2	7	20	50	60	100
A glaucus gp	1b	0	0	0	0	0	0	0
	2b	0	0	0	0	0	0	0
A nidulans	1b	0	0	0	0	0	0	0
	2b	0	0	0	0	0	0	0
A niger	1b	0	10	0	10	70	0	40
	2b	0	12	13	0	20	0	0
A ornatus gp	1b	0	0	0	0	0	0	0
	2b	0	0	0	0	0	0	0
A terreus	1b	0	0	0	0	0	0	0
	2b	0	0	0	10	0	0	0
A versicolor gp	1b	20	0	0	0	0	20	10
	2b	10	0	0	0	0	0	0
Cephalosporium sp	1b	0	0	0	100	60	70	50
	2b	0	12	37	40	20	50	10
Chaetomium globosum	1b	0	10	30	0	30	50	100
	2b	0	12	0	0	0	0	30
Coprinus cinereus	1b	0	50	0	0	0	0	0
	2b	0	0	0	70	80	100	100
Geotrichum sp	1b	0	0	0	0	0	90	0
	2b	0	0	0	0	0	10	10
Gliocladium roseum	1b	0	50	0	30	10	10	20
	2b	0	33	25	80	20	60	0
Mucor sp	1b	0	60	10	10	20	40	30
	2b	0	22	50	10	0	0	0

Species	Treatment	Day						
		0	2	4	6	8	10	14
Penicillium cyclopium ..	1b	0	10	0	20	0	10	20
	2b	30	44	50	70	20	50	0
P funiculosum	1b	0	0	0	0	0	0	0
	2b	0	0	12.5	0	0	0	0
P purpurogenum	1b	0	0	0	50	20	50	60
	2b	0	12	0	10	0	0	0
Rhizopus sp	1b	0	0	0	90	90	100	100
	2b	10	0	0	0	20	0	0
Scopulariopsis	1b	0	0	0	0	0	0	0
brevicaulis	2b	0	0	0	0	0	10	0
Trichoderma viride	1b	0	0	0	30	0	0	0
	2b	0	0	0	10	0	0	0

TABLE A III4. The percent appearances of fungi over 0-14 days on ammonia treated and non treated

Species	Day							Treatment
	0	2	4	6	8	10	14	
Absidia sp	0	100	20	30	0	30	40	1a
	0	0	0	0	0	0	0	2a
Alternaria.....	0	60	70	0	0	0	0	1a
	0	0	0	0	0	0	0	2a
Aspergillus Flavus gp ..	0	0	60	0	0	0	60	1a
	0	0	0	0	0	0	0	2a
A fumigatus	0	100	90	60	0	10	20	1a
	20	37	50	60	0	70	87	2a
A glaucus gp	0	0	0	0	0	0	0	1a
	0	12	0	12	12.5	0	12.5	2a
A nidulans	0	0	0	0	10	0	20	1a
	0	0	0	0	0	30	0	2a
A niger	0	0	0	0	100	0	0	1a
	0	0	0	0	12.5	10	0	2a
A ornatus gp	0	0	0	0	0	0	0	1a
	0	0	0	12	0	0	0	2a
A versicolor gp	0	0	0	0	0	0	0	1a
	0	0	0	12	0	10	0	2a
Cephalosporium sp	0	40	80	70	90	60	30	1a
	0	62	25	12	87	50	37.5	2a
Chaetomium globosum	0	0	0	10	40	50	40	1a
	0	0	0	12	0	10	0	2a
Coprinus cinereus	0	0	0	0	0	0	0	1a
	0	0	50	50	50	62	80	2a
Geotrichum sp	0	0	0	0	0	0	0	1a
	0	0	0	0	0	10	0	2a
Gliocladium roseum	0	50	20	30	20	30	20	1a
	0	50	25	37	37.5	50	37.5	2a
Mucor sp	0	50	50	37	75	40	80	1a
	0	0	0	0	0	0	0	2a

Continued/...

Species	Day							Treatment
	0	2	4	6	8	10	14	
Penicillium cyclopium ..	33	30	0	60	0	0	10	1a
	0	0	0	60	37.5	30	12.5	2a
P funiculosum	0	0	10	10	0	10	10	1a
	0	37	12	12	25	40	0	2a
P purpurogenum	0	40	0	0	0	100	20	1a
series	30	37	50	0	12.5	0	0	2a
Rhizopus sp	0	30	90	80	30	0	10	1a
	20	12	0	10	12.5	0	0	2a
Scopulariopsis	0	0	0	0	0	0	0	1a
brevicaulis	0	0	0	0	12.5	0	0	2a
Trichoderma viride	0	0	0	0	10	0	0	1a
	20	12	0	0	12.5	0	25	2a

APPENDIX IV

STATISTICAL ANALYSIS OF EXPERIMENTAL DATA

ANALYSIS OF RESULTS FROM CHAPTER TWO

The total number of isolates other than *C cinereus* obtained from straws amended with nutrient salts and those not were compared statistically. The Mann-Whitney 'U' test was used as the data being of an appearance - non appearance nature does not conform to normal distribution. This non-parametric 't' test equivalent was therefore employed.

	Straw	A	B	C	D	E
Treatments	1-4	25	39	30	34	34
Treatments	5-8	9	25	28	9	14

Ranking gives:-

	A	B	C	D	E	
1-4	4.5	10	7	8.5	8.5	$R_1 = 38.5$
5-8	1.5	4.5	6	1.5	3	$R_2 = 16.5$

$U_1 = n_1n_2 + \frac{1}{2}n_2(n_2+1) - R_2$ $\quad$ $U_2 = n_1n_2 + \frac{1}{2}n_1(n_1+1) - R_1$

$= 25 + 15 - 16.5 = 23.5$ $\quad$ $= 25 + 15 - 38.5 = 1.5$

From tables, when $n_2 = 5$, $U \leq 1.5$ $n_1 = 5$, there is a probability of occurrence of $p = 0.008$. A previously set significant level of 0.05 was thus not exceeded so that the null hypothesis that results from both sets of treatments were the same can be rejected. Therefore there are significant differences.

Chapter Three

A. STATISTICAL ANALYSIS OF RESULTS FROM SOIL ISOLATIONS

These results are expressed as numbers of isolations out of ten samples. The distributions of such proportions or percentages are not usually normal and because of this direct analysis of variance cannot be carried out with any confidence. The use of a transformation followed by parametric tests, or a non-parametric test alone should therefore be considered.

Kent (1972) states that non-parametric tests are often of greater value when dealing with fungal populations. However Bishop (1966) suggests that the use of the arcsin transformation is adequate. It was decided to use this followed by analysis of variance. The significant differences thus obtained would seem to justify this choice.

To make analysis of variance possible the two missing values from a woodland and a grassland soil were first calculated.

Farms		1	2	3	4	5
Edwards	1	10	10	2	x	4
Gorringe	2	7	10	4	4	4
Evans	3	10	7	4	3	y

Estimated $x = 4$

Soil area

1. Wheat soil
2. Barley soil
3. Pastureland
4. Woodland
5. Grassland

(i) $$y = \frac{rR + cC - T}{(r-1)(c-1)}$$

where r = No. rows

c = No. columns

R = Total of row with missing value

T = Grand total

C = Total of column with missing value.

$$y = \frac{(3 \times 24) + (5 \times 8) - 83}{2 \times 4}$$

$$y = 3.625$$

Replace y in (i) and calculate x

$$x = \frac{rR + cC - T}{(r-1)(c-1)}$$

$$x = \frac{(3 \times 26) + (5 \times 7) - 82.63}{8}$$

$$x = 3.796$$

$$y = \frac{rR - cC - T}{(r-1)(c-1)}$$

$$y = \frac{(3 \times 24) + (5 \times 8) - 82.796}{8}$$

$$y = \underline{3.6505}$$

These are sufficiently close to warrant these values of x and y as whole numbers are involved, these can be rounded off to give $\underline{x = y = 4}$

Analysis of Variance

The above data were first converted to percentages

	1	2	3	4	5
1	100	100	20	40	40
2	70	100	40	40	40
3	100	70	40	30	40

These were then transformed

	1	2	3	4	5	Totals	Sum of sqs
1	90	90	26.6	39.2	39.2	285.0	19980.84
2	56.8	90	39.2	39.2	39.2	264.4	15936.16
3	90	56.8	39.2	33.2	39.2	258.4	15501.76
Totals	236.8	236.8	105.0	111.6	117.6	807.8	

Sum of sq. 19426.24 19426.24 3780.84 4175.52 4609.92 $\underline{51418.76 = A}$

$\underline{\text{Sum of sq of Col tot}}$ / No. in col. 56074.24 56074.24 11025 12454.56 13829.76 $\underline{49819.27 = B}$

$\underline{\text{Sum of sq of row total}}$ / No. in row $\underline{43580.58 = C}$

$\underline{\text{Sum of Grand Total}}$ / Total No. observations $\underline{43502.72 = D}$

Sum of Squares

Between column SOS = B-D = 6316.55

Between row SOS = C-D = 78.58

Total SOS = A-D = 7916.04

Residual SOS = Total - (column + row) = 1520.63

Table of Analysis of Variance

Source of Variance	Sum of sq	°Freedom	Mean sq
Between columns	6316.55	U-1 = 4	1579.14
Between rows	78.58	V-1 = 2	39.43
Residual	1520.63	(U-1)(V-1) = 8	190.08
Total	7916.04	UV-1 = 14	

Variance Ratio Test

°Freedom $^{4}/_{8}$ $F = \frac{\text{Between columns mean sq}}{\text{residual mean sq}} = \frac{1579.14}{190.08} = 8.308$

°Freedom $^{2}/_{8}$ $F = \frac{\text{Between rows mean sq}}{\text{residual mean sq}} = \frac{39.43}{190.08} = 0.207$

At $p = 0.01$ $F = 7$ $\therefore$ between columns variation is significant at this level.

Significant difference is not shown between rows.

From this it can be seen that there is no significant difference between the number of isolates found on different farms, but there are highly significant differences between numbers isolated from different sites on these farms.

Without further analysis being necessary it is obvious that the largest differences lie between those soils which had been under cereals and those which had not. There is no significant difference between numbers isolated from wheat and barley soils.

B. STATISTICAL ANALYSES OF RELATIONSHIPS BETWEEN SOIL FACTORS AND THE ISOLATION OF *C CINEREUS*

CORRELATION COEFFICIENTS

1. Moisture Content

Moisture Content x	No. of isolations y	x^2	y^2	xy
14.65	90.0	214.62	8100.00	1318.50
16.43	90.0	269.94	8100.00	1478.7
23.30	26.6	542.89	707.56	619.78
22.91	39.2	524.87	1536.64	898.07
18.43	90.0	339.67	8100.00	1658.70
23.28	56.8	541.96	3226.24	1322.30
24.63	39.2	606.64	1536.64	965.50
22.86	33.2	522.58	1102.24	758.95
27.59	56.8	761.20	3226.24	1567.11
19.07	90.0	363.66	8100.00	1716.30
15.65	39.2	244.92	1536.64	613.48
24.23	39.2	587.09	1536.64	949.82
20.89	39.2	436.39	1536.64	818.89
Totals 273.92	729.4	5956.43	48345.48	14684.9

n = 13

$$\Sigma d_x^2 = \Sigma x^2 - (\Sigma x)^2/_n = 5956.43 - \frac{75032.17}{13}$$

$$= 5956.43 - 5771.71 = \underline{184.72}$$

$$\Sigma d_y^2 = \Sigma y^2 - (\Sigma y)^2/_n = 48345.48 - \frac{532024.36}{13}$$

$$= 48345.48 - 40924.95 = \underline{7420.53}$$

$$\Sigma dx dy = \Sigma xy - \Sigma x \Sigma y/_n = 14684.9 - \frac{(273.92 \times 729.4)}{13}$$

$$= 14684.9 - 15369.02 = \underline{-684.12}$$

Correlation coefficient, $r = \frac{-684.12}{\sqrt{184.72 \times 7420.53}} = \frac{-684.12}{1170.78} = \underline{-0.584}$

With n-1 degrees of freedom when p = 0.1, r = 0.458, the calculated value of 'r' exceeds this. Therefore there appears to be slight correlation between *C cinereus* isolation and moisture content of the soil.

2. Soil pH

Soil pH x	No. of isolations y	x^2	y^2	xy
7.00	90	49	8100.00	630.00
6.13	90	37.58	8100.00	551.70
6.59	26.6	43.43	707.56	175.29
7.61	39.2	57.91	1536.64	298.31
6.79	90	46.10	8100.00	611.10
7.06	56.8	49.84	3226.24	401.00
6.52	39.2	42.51	1536.64	255.58
4.99	33.2	24.90	1102.24	165.67
6.76	56.8	45.70	3226.24	383.97
6.98	90	48.72	8100.00	628.20
6.52	39.2	42.51	1536.64	255.58
7.47	39.2	55.80	1536.64	292.82
7.19	39.2	51.70	1536.64	281.85
Total 87.61	729.4	595.70	48345.48	4931.05

$$\Sigma d_x^2 = \Sigma x^2 - (\Sigma x)^2/_n = 595.70 - \frac{7675.51}{13}$$

$$= 595.70 - 590.42 = \underline{5.28}$$

$$\Sigma d_y^2 = \Sigma y^2 - (\Sigma y)^2/_n = 48345.48 - \frac{532024.36}{13}$$

$$= 48345.48 - 40924.95 = \underline{7420.53}$$

$$\Sigma dxdy \quad \Sigma xy - \Sigma x\, \Sigma y/_n = 4931.05 - \frac{(87.61 \times 729.4)}{13}$$

$$= 4931.05 - 4915.59$$

$$= \underline{15.46}$$

$$r = \frac{15.46}{\sqrt{5.28 \times 7420.53}} = \frac{15.46}{197.94} = \underline{0.0781}$$

With n-1 degrees of freedom the calculated value of r at no point exceeds those in correlation coefficient tables. Therefore no correlation was found between soil pH and appearance of *C cinereus*.

3. Soil Organic Content

Organic Content x	No. of isolations y	x^2	y^2	xy
3.06	90	9.36	8100.00	275.40
1.96	90	3.84	8100.00	176.40
5.61	26.6	31.47	707.56	149.23
4.63	39.2	21.44	1536.64	24.70
3.58	90	12.82	8100.00	320.40
4.03	56.8	16.24	3226.24	228.90
6.05	39.2	36.60	1536.64	237.16
4.44	33.2	19.71	1102.24	147.41
8.62	56.8	74.30	3226.24	489.62
2.38	90	5.66	8100.00	214.20
2.12	39.2	4.49	1536.64	83.10
7.37	39.2	54.32	1536.64	288.90
9.05	39.2	81.90	1536.64	354.76
Totals 62.9	729.4	372.15	48345.48	3213.18

$$\Sigma d_x^2 = \Sigma x^2 - (\Sigma x)^2/_n = 372.15 - \frac{3956.41}{13}$$

$$= 372.15 - 304.34 = \underline{67.81}$$

$$\Sigma d_y^2 = \Sigma y^2 - (\Sigma y)^2/_n = 48345.48 - \frac{532024.36}{13}$$

$$= 48345.48 - 40924.95 = \underline{7420.53}$$

$$\Sigma dx dy = \Sigma xy - \Sigma x \Sigma y/_n = 3213.18 - \frac{(62.9 \times 729.4)}{13}$$

$$= 3213.18 - 3529.17 = \underline{-315.99}$$

$$r = \frac{-315.99}{\sqrt{67.81 \times 7420.53}} = \frac{-315.99}{709.36} = \underline{-0.445}$$

With n-1 degrees of freedom the calculated value of r at no point exceeds those in correlation coefficient tables. Therefore no correlation was found between soil pH.and appearance of *C cinereus*.

4. Soil Temperature

Soil temperature x	No. of isolations	x^2	y^2	xy
6.2	90	38.44	8100.00	558.00
6.7	90	44.89	8100.00	603.00
3.6	26.6	12.96	707.56	95.76
6.4	39.2	40.96	1536.64	250.88
8.3	90	68.89	8100.00	747.00
7.0	56.8	49.00	3226.24	397.60
8.3	39.2	68.89	1536.64	325.36
7.7	33.2	59.29	1102.24	255.64
5.6	56.8	31.36	3226.24	318.08
7.1	90	50.41	8100.00	639.00
5.1	39.2	26.01	1536.64	199.92
6.5	39.2	42.25	1536.64	254.80
5.0	39.2	25.00	1536.64	196.00
Totals 83.5	729.4	558.35	48345.48	4841.04

$$\Sigma d_x^2 = \Sigma x^2 - (\Sigma x)^2/_n = 558.35 - \frac{6972.25}{13}$$

$$= 558.35 - 536.33 = \underline{22.02}$$

$$\Sigma d_y^2 = \Sigma y^2 - (\Sigma y)^2/_n = 48345.48 - \frac{532024.36}{13}$$

$$= 48345.48 - 40924.95$$

$$= \underline{7420.53}$$

$$\Sigma dx dy = \Sigma xy - \Sigma x \Sigma y/_n = 4841.04 - \frac{(83.5 \times 729.4)}{13}$$

$$= 4841.04 - 4684.99 = \underline{156.05}$$

$$r = \frac{156.05}{\sqrt{22.02 \times 7420.53}} = \frac{156.05}{404.23} = \underline{0.386}$$

With n-1 degrees of freedom the calculated value of r at no point exceeds those in correlation coefficient tables. Therefore no correlation was found between soil temperature and the appearance of *C cinereus*.

CHAPTER FOUR

1. ANALYSIS OF VARIANCE BETWEEN LENGTH OF FINAL CLEARED ZONE IN 'COWLING' TUBES

Replicate	As-1	As-2	As-3	As-4	As-6	
1	5.85	7.50	11.17	8.60	8.80	
2	5.50	6.50	9.17	9.70	10.50	
3	6.00	8.33	8.00	9.00	6.50	
4	5.83	8.17	8.50	8.90	12.50	
5	5.83	8.83	8.01	9.20	7.20	Grand Total
Totals	28.99	39.33	44.85	45.40	45.50	204.07
Mean	5.80	7.87	8.97	9.08	9.10	
Sum of sq.	168.22	312.61	409.27	412.90	438.03	1741.03 A
	a_1	a_2	a_3	a_4	a_5	
Sq.Column Totals	168.02	309.37	402.30	412.23	414.05	1706.03 B
No.observations	b_1	b_2	b_3	b_4	b_5	
Diff. (a-b)	0.14	3.24	6.97	0.67	23.98	A-B = 35

$$\frac{\text{Grand Total}}{\text{No. observations}} = D = \frac{204.07}{25} = \frac{1665.78}{}$$

Total SOS = A-B 1741.03 - 1665.78 = 75.25

SOS Between Treatment = B-D = 1706.03 - 1665.78 = 40.25

Residual SOS = A-B 1741.03 - 1706.03 = 35

Table of Analysis of Variance

Source of Variance	Sum of sq	°Freedom	Mean sq
Between treatments	40.25	U-1 = 4	10.06
Residual	35.00	U(V-1)= 20	1.75
Total	75.25	UV-1 = 24	3.14

Variance Ratio

$$F = \frac{\text{Between treatment mean sq.}}{\text{Residual mean sq.}} = 5.75$$

From tables F = 4.4 at 0.01 level, therefore variance due to strain difference is significantly greater than random error.

But this does not show where the differences lie, therefore further analysis using the method of least significant difference was undertaken.

$$LSD = t_{0.05} \sqrt{\frac{(2s^2)}{n}}$$

Where t = the figure extracted from "t" tables at p = 0.05 and 20° Freedom

$$LSD = 2.09 \times \sqrt{\frac{2 \times 1.75}{5}} = \underline{1.75}$$

Therefore to be a significant difference between the mean cellulose clearing, that difference must exceed 1.75.

Differences Between Means

	As-1	As-2	As-3	As-4	As-6
As-1	-	2.062*	3.16*	3.27*	3.29*
As-2	-	-	1.1	1.21	1.23
As-3	-	-	-	0.11	0.13
As-4	-	-	-	-	0.02
As-6	-	-	-	-	-

* = Significant difference in cellulose clearing between isolates.

2. ANALYSIS OF VARIANCE BETWEEN CLEARING RATES ON CELLULOSE AGAR PLATES

Replicate	As-1	As-2	As-3	As-4	As-6	
1	0.128	0.191	0.407	0.299	0.341	
2	0.206	0.168	0.445	0.237	0.475	
3	0.248	0.225	0.334	0.321	0.349	
4	0.287	0.161	0.503	0.305	0.165	
5	0.204	0.331	0.378	0.287	0.293	Grand Totals
Totals	1.073	1.076	2.067	1.449	1.623	7.288
Means	0.215	0.215	0.413	0.290	0.325	
Sum of sq of totals	0.244	0.251	0.871	0.424	0.577	2.367 A
	a_1	a_2	a_3	a_4	a_5	
Sq column totals	0.230	0.231	0.854	0.420	0.527	2.262 B
No.observations	b_1	b_2	b_3	b_4	b_5	
Diff (a-b)	0.014	0.020	0.017	0.004	0.05	= 0.105

D = 2.1246

Total SOS 0.242

Between treat. SOS = 0.137

Residual SOS = 0.105

Table of Analysis of Variance

Source of Variance	Sum of sq	°Freedom	Mean Sq
Between treatment	0.242	4	0.061
Residual	0.105	20	0.005
Total	0.242	24	

Variance Ratio:- $\frac{\text{Between treatment mean sq}}{\text{Residual mean sq}} = \frac{0.061}{0.005} = 12.2$

From tables F = 7.1 at p = 0.001 ∴ variance due to difference in strains is of significance. However LSD analysis gives:-

$$LSD = 2.09 \times \sqrt{2 \times \frac{0.005}{5}} = 0.093$$

So that means must differ by more than 0.093 to be seen as significant.

Differences Between Means

	As-1	As-2	As-3	As-4	As-6
As-1	-	0	0.198*	0.075	0.110*
As-2	-	-	0.198*	0.075	0.110*
As-3	-	-	-	0.123*	0.088
As-4	-	-	-	-	0.035
As-6	-	-	-	-	-

* = Significant difference between rates of cellulose clearing caused by the isolates.

3. ANALYSIS OF VARIANCE BETWEEN GROWTH RATES OF *C CINEREUS* isolates on CELLULOSE MEDIUM

Replicate	As-1	As-2	As-3	As-4	As-6	
1	0.459	0.427	0.465	0.442	0.488	
2	0.483	0.418	0.482	0.432	0.534	
3	0.513	0.482	0.459	0.484	0.508	
4	0.495	0.424	0.483	0.438	0.501	
5	0.498	0.437	0.506	0.449	0.510	Grand Total
Totals	2.448	2.188	2.395	2.245	2.541	11.817
Means	0.490	0.438	0.479	0.449	0.508	
Sum of sq.	1.200	0.960	1.149	1.010	1.292	5.611 A
	a_1	a_2	a_3	a_4	a_5	
Sq Column totals	1.199	0.957	1.147	1.008	1.291	5.602 B
No observations	b_1	b_2	b_3	b_4	b_5	
Diff (a-b)	0.001	0.003	0.002	0.002	0.001	0.009

D = 5.5857

Total SOS = 0.025

Between treatment SOS = 0.016

Residual SOS = 0.009

Analysis of Variance Table

Source of Variance	Sum of sq	°Freedom	Mean sq
Between treatment	0.016	4	0.004
Residual	0.009	20	0.00045
Total	0.025	24	

Variance Ratio:- $\frac{\text{Between treatment mean sq}}{\text{Residual mean sq.}} = \frac{0.004}{0.00045} = \underline{8.889}$

From F tables = 7.1 at 0.001 level ∴ variance due to strains is of significant difference. Further analysis using LSD gives:

$$LSD = 2.09 \times \sqrt{2 \times \frac{0.00045}{5}} = \underline{0.028}$$

So that means must differ by greater than 0.028 to be seen as significant.

Differences Between Means

	As-1	As-2	As-3	As-4	As-6
As-1	-	0.052*	0.010	0.041*	0.019
As-2	-	-	0.041*	0.011	0.071*
As-3	-	-	-	0.030*	0.029*
As-4	-	-	-	-	0.059*
As-6	-	-	-	-	-

* = Significant differences between rates of growth of the *C cinereus* isolates were obtained.

CHAPTER SIX

1. COMPARISON OF *C CINEREUS* GROWTH RATES ON AUTOCLAVE AND POLYPROPYLENE OXIDE STERILISED STRAW

Student 't' tests were carried out on the data shown in table A.IV These were from the linear growth period, ie 24 h until the completion of the experiment.

Table A.IV. Hyphal extension rates (mm/h) on autoclave (A) and polypropylene oxide (P) sterilised straw.

As-1		As-3		As-6	
A	P	A	P	A	P
0.333	0.435	0.267	0.482	0.267	0.524
0.475	0.343	0.469	0.487	0.485	0.381
0.400	0.745	0.392	0.633	0.370	0.445
0.271	0.402	0.348	0.556	0.305	0.615
0.316	0.492	0.362	0.393	0.377	0.381
0.304	0.663	0.334	0.531	0.346	0.615
0.277	0.133	0.315	0.914	0.333	-
0.294	-	0.270	-	0.241	-
0.258	-	0.240	-	0.400	-

't' tests

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad \text{where } S = \sqrt{\frac{\Sigma(X - \bar{X}_1)^2 + \Sigma(X - \bar{X}_2)^2}{n_1 + n_2 - 2}}$$

As-1

$$S = \sqrt{\frac{0.248 + 0.039}{14}} = \sqrt{0.0205}$$

$$t = \frac{0.459 - 0.325}{\sqrt{0.0205 \times 0.254}} = \frac{0.134}{0.0722} = 1.856$$

$$^{\circ}F = n_1 + n_2 - n_2 = 14$$

From 't' tables this is significant at P = 0.1 but no greater.

As-3

$$s = \sqrt{\frac{0.17 + 0.04}{14}} = \sqrt{0.015}$$

$$t = \frac{0.571 - 0.333}{\sqrt{0.015 \times 0.254}} = \frac{0.238}{0.062} = \underline{3.839}$$

$$^{\circ}F = n_1 + n_2 - 2 = 14$$

From 't' tables this is significant at p = 0.05

As-6

$$s = \sqrt{\frac{0.058 + 0.032}{13}} = \sqrt{0.0069}$$

$$t = \frac{0.494 - 0.347}{\sqrt{0.0069 \times 0.278}} = \frac{0.147}{0.044} = \underline{3.341}$$

$$^{\circ}F = n_1 + n_2 - 2 = 13$$

From 't' tables this is significant at p = 0.05

Conclusions

There were significant differences between the growth rates of *C cinereus* on the straws sterilised by these two differing techniques. These differences ranged from barely significant (As-1) to very highly significant (As-6).

2. ANALYSIS OF VARIANCE ON REMAINING GROWTH RATE DATA

Analysis of variance was carried out on the data shown in table 6.3 to encompass two experimental factors and replication. These were to investigate differences between growth rates on different nitrogen sources and on sterile and non-sterile straw. A set of analyses were also carried out to study differences between isolates on the various nitrogen sources.

A closer analysis of these results was then carried out using 'Least Significant Differences (LSD).

Analysis of the effect of nitrogen source and sterility of straw on growth rates

	As-1									
	1% Ammonia	KNO_3	$NaNO_2$	Urea	L Asparagine	Ammonium tartrate	Control	Control at elevated pH	Totals	Sums of squares of cell totals
Sterile Straw	0.418 0.426 0.438	0.562 0.564 0.572	0.716 0.334 0.318	0.608 0.606 0.459	0.747 0.717 0.755	0.594 0.838 0.657	0.402 0.663 0.492	0.792 0.733 0.667	14.078	25.714 α_1'
Cell Totals	1.282	1.698	1.368	1.673	2.219	2.089	1.557	2.192		
Non Sterile Straw	0.478 0.698 0.511	0.618 0.750 0.507	0.421 0.507 0.546	0.450 0.426 0.492	0.634 0.600 0.701	0.579 0.650 0.606	0.387 0.577 0.413	0.389 0.500 0.403	12.841	21.076 α_1'
Cell Totals	1.685	1.875	1.474	1.368	1.935	1.835	1.377	1.292		
Column Totals	2.967	3.573	2.842	3.041	4.154	3.924	2.934	3.484	26.919	
Sum of square totals	4.483 α_1	6.399 α_2	4.044 α_3	4.670 α_4	8.668 α_5	7.731 α_6	4.320 α_7	6.474 α_8		46.790 α

A $\left(\frac{\alpha}{\text{No.replicates}}\right)$ = 15.597, B $\left(\frac{\text{Sum of sq column totals}}{\text{No.observations - column}}\right)$ = 15.382, C = $\left(\frac{\text{Sum of sq of row totals}}{\text{Row observations}}\right)$ = 15.128

D $\left(\frac{\text{Grand total}}{\text{total observations}}\right)$ = 15.097 L (Sum of sq of observations) = 16.080

Total SOS (sum of squares) = L-D = 0.983

Between column SOS B-D = 0.285

Between row SOS C-D = 0.031

Residual SOS L-A = 0.483

Interaction SOS 0.983 -

(0.285 + 0.031 + 0.483) = 0.184

Analysis of variance table

Source of Variance	Sum of sq	°Freedom	Mean sq
Between columns (-N)	0.285	U-1 = 7	0.041
Between Rows (Sterility)	0.031	V-1 = 1	0.031
Interaction (N x Sterility)	0.184	(U-1)(V-1) = 7	0.026
Residual	0.483	UV(p-1) = 32	0.015
Total	0.983	UVp-1 = 47	

Variance Ratio Test

$$F = \frac{\text{Between column mean sq}}{\text{Residual mean sq}} = \frac{0.041}{0.015} = 2.733$$

$$F = \frac{\text{Between row mean sq}}{\text{Residual mean sq}} = \frac{0.031}{0.015} = 2.067$$

$$\text{Interaction } F = \frac{0.026}{0.015} = 1.733$$

These are then looked up in F tables.

Between columns there was no significant difference at p = 0.05 levels, and thus no difference in As-1 growth rates on different nitrogen sources.

Between rows slight significant difference was obtained at p = 0.20 levels, and therefore there was a little significant difference between growth rates on sterile and non-sterile straws.

There was a slight significance in the interaction test at p = 0.20 and therefore possibly slight interaction.

Analysis of the effect of nitrogen source and sterility of straw on growth rates

	As-3									
	1% Ammonia	KNO_3	$NaNO_2$	Urea	L Asparagine	Ammonium tartrate	Control	Control at elevated pH	Totals	Sums of squares of cell totals
Sterile Straw	0.442 0.414 0.439	0.587 0.591 0.580	0.578 0.586 0.556	0.609 0.634 0.539	0.758 0.723 0.801	0.703 0.730 0.841	0.487 0.556 0.531	0.872 0.940 0.759	15.253	30.357 α_1'
Cell Totals	1.295	1.758	1.717	1.782	2.282	2.274	1.574	2.571		
Non Sterile Straw	0.453 0.549 0.521	0.611 0.521 0.500	0.581 0.614 0.767	0.522 0.464 0.494	0.593 0.515 0.756	0.637 0.668 0.637	0.505 0.175 0.194	0.375 0.181 0.167	12.000	19.555 α_2''
Cell Totals	1.523	1.632	1.962	1.480	1.864	1.942	0.874	0.723		
Column Totals	2.818	3.390	3.679	3.262	4.146	4.216	2.448	3.294	27.253	
Sum of square totals	3.997 α_1	5.754 α_2	6.798 α_3	5.366 α_4	8.682 α_5	8.942 α_6	3.241 α_7	7.133 α_8		49.912 α

A = 16.637 B = 15.903 C = 15.694 D = 15.473 L = 16.836

Total SOS - L-D = 1.365

Between column SOS B-D = 0.430

Between row SOS C-D = 0.221

Residual SOS L-A = 0.199

Interaction SOS. 1.365 - (0.43 + 0.221 + 0.199) = 0.515

Analysis of Variance Table

Source of Variance	Sum of Squares	°Freedom	Mean Sq
Between column	0.430	U-1 = 7	0.0614
Between Row	0.221	V-1 = 1	0.221
Interaction	0.515	(U-1)(V-1) = 7	0.074
Residual	0.199	UV(p-1) = 32	0.006
Total	1.365	UVp-1 = 47	

Variance Ratio Test

$$F \frac{\text{Between column mean sq}}{\text{Residual mean sq}} = \frac{0.061}{0.006} = 9.903$$

$$F \frac{\text{Between Row mean sq}}{\text{Residual mean sq}} = \frac{0.221}{0.006} = 35.645$$

$$\text{Interaction } F \frac{\text{Interaction mean sq}}{\text{Residual mean sq}} = \frac{0.074}{0.006} = 11.935$$

Significant differences at p = 0.001 levels were found between columns, rows and interaction ∴ highly significant differences.

Effect of Nitrogen Source and Sterility - As-6

Total SOS - L-D = 1.201

Between column SOS B-D = 0.580

Between rows SOS C-D = 0.005

Residual SOS L-A = 0.414

Interaction SOS 1.201 -(0.58 + 0.005 + 0.414) = 0.202

Analysis of the effect of nitrogen source and sterility of straw on growth rates

	As-6									
	1% Ammonia	KNO_3	$NaNO_2$	Urea	L Asparagine	Ammonium tartrate	Control	Control at elevated pH	Totals	Sums of squares of cell totals
Sterile Straw	0.452 0.520 0.538	0.610 0.548 0.523	0.548 0.519 0.475	0.655 0.515 0.536	0.722 0.723 0.798	0.735 0.804 0.744	0.445 0.615 0.524	0.417 0.389 0.417	13.772	24.642 α_1'
Cell Totals	1.510	1.618	1.542	1.706	2.243	2.283	1.584	1.223		
Non Sterile Straw	0.639 0.634 1.145	0.597 0.632 0.583	0.481 0.479 0.452	0.422 0.502 0.540	0.585 0.504 0.800	0.522 0.586 0.874	0.627 0.480 0.258	0.292 0.347 0.333	13.314	23.572 α_2''
Cell Totals	2.418	1.812	1.412	1.464	1.889	1.982	1.365	0.972		
Column Totals	3.928	3.493	2.954	3.170	4.132	4.265	2.949	2.195	27.086	
Sum of square totals	8.127 α_1	5.109 α_2	4.372 α_3	5.054 α_4	8.599 α_5	9.140 α_6	4.372 α_7	2.441 α_8		48.214 α

A = 16.071 B = 15.864 C = 15.289 D = 15.284 L = 16.485

Analysis of Variance Table

Source of Variance	Sum of Squares	°Freedom	Mean Sq
Between columns	0.580	U-1 = 7	0.089
Between rows	0.005	V-1 = 1	0.005
Interaction	0.202	(U-1)(V-1) = 7	0.029
Residual	0.414	UV(p-1) = 32	0.013
Total	1.201	UVp-1 = 47	

Variance Ratio Test

$$F \frac{\text{Between column mean sq}}{\text{Residual mean sq}} = \frac{0.089}{0.013} = 6.846$$

$$F \frac{\text{Between Row mean sq}}{\text{Residual mean sq}} = \frac{0.005}{0.013} = 0.385$$

$$F \frac{\text{Interaction mean sq}}{\text{Residual mean sq}} = \frac{0.029}{0.013} = 2.231$$

Highly significant differences were found between columns at p = 0.001 level. No significant differences were found between rows. Significant interaction was found at p = 0.20 level.

Strain Difference and Nitrogen Source - Sterile Straw

Total SOS = 3.076

Between columns SOS = 0.735

Between Row SOS = 0.051

Residual SOS = 1.975

Interaction SOS 3.076 -(0.735 + 0.051 + 1.975) = 0.315

Analysis of strain differences and nitrogen source on growth rates

	Sterile Straw									
	1% Ammonia	KNO_3	$NaNO_2$	Urea	L.Asparagine	Ammonium tartrate	Control	Elevated pH control	Totals	Sum of squares of cell totals
As-1	0.418 0.426 0.438	0.562 0.564 0.572	0.716 0.334 0.318	I,608 0.606 0.459	0.747 0.717 0.755	0.594 0.838 0.657	0.402 0.663 0.492	0.792 0.733 0.667	14.078	25.714 α_1'
Cell Totals	1.282	1.698	1.368	1.673	2.219	2.089	1.557	2.192		
As-3	0.442 0.414 0.439	0.587 0.591 0.580	0.575 0.586 0.556	0.609 0.634 0.539	0.758 0.723 0.801	0.703 0.730 0.841	0.487 0.556 0.531	0.872 0.940 0.759	15.253	30.357 α_2'
Cell Totals	1.295	1.758	1.717	1.782	2.282	2.274	1.574	2.571		
As-6	0.452 0.520 0.538	0.610 0.548 0.523	0.548 0.519 0.475	0.655 0.515 0.536	0.722 0.723 0.798	0.735 0.804 0.744	0.445 0.615 0.524	0.417 0.389 0.417	13.772	24.642 α_3'
Cell Totals	1.510	1.681	1.542	1.706	2.243	2.283	1.584	1.223		
Column Totals	4.087	5.137	4.627	5.161	6.744	6.646	4.715	5.986	43.103	
Sum of square totals	5.601 α_1	8.800 α_2	7.197 α_3	8.885 α_4	15.165 α_5	14.747 α_6	7.410 α_7	12.911 α_8		80.715 α

A = 26.905 B = 26.539 C = 25.855 D = 25.804 L = 28.88

Analysis of Variance Table

Source of Variance	Sum of Squares	°Freedom	Mean Sq
Between columns	0.735	U-1 = 7	0.105
Between Rows	0.051	V-1 = 2	0.026
Interaction	0.315	(U-1)(V-1)=14	0.023
Residual	1.975	UV(p-1) = 48	0.041
Total	3.076	UV p-1 = 71	

Variance Ratio Test

$$F \frac{\text{Between column mean sq}}{\text{Residual mean sq}} = \frac{0.105}{0.041} = 2.555$$

$$F \frac{\text{Between row mean sq}}{\text{Residual mean sq}} = \frac{0.026}{0.041} = 0.620$$

$$F \frac{\text{Interaction mean sq}}{\text{Residual mean sq}} = \frac{0.023}{0.041} = 0.547$$

Significant difference between columns at p = 0.05 level.

No significant difference between rows ∴ no strain difference.

No significant difference in interaction means ∴ no interaction.

Strain Difference and Nitrogen Source - Non Sterile Straw

Total SOS = 1.809

Between Column SOS = 0.843

Between Row SOS = 0.031

Residual SOS = 0.633

Interaction SOS, 1.809 - (0.843 + 0.31 + 0.633) = 0.023

Analysis of strain differences and nitrogen source on growth rates

	Non-Sterile Straw									
	1% Ammonia	KNO_3	$NaNO_2$	Urea	L.Asparagine	Ammonium tartrate	Control	Elevated pH control	Totals	Sum of squares of cell totals
As-1	0.478 0.696 0.511	0.618 0.750 0.507	0.421 0.507 0.546	0.450 0.426 0.492	0.634 0.600 0.701	0.579 0.650 0.606	0.387 0.577 0.413	0.389 0.500 0.403	12.841	21.076 α_1'
Cell Totals	1.685	1.875	1.474	1.368	1.935	1.835	1.377	1.292		
As-3	0.453 0.549 0.521	0.611 0.521 0.500	0.581 0.614 0.767	0.522 0.464 0.494	0.593 0.515 0.756	0.637 0.668 0.637	0.505 0.175 0.194	0.375 0.181 0.167	12	19.555 α_2''
Cell Totals	1.523	1.632	1.962	1.480	1.864	1.942	0.874	0.723		
As-6	0.639 0.634 1.145	0.597 0.632 0.583	0.481 0.479 0.452	0.422 0.502 0.540	0.585 0.504 0.800	0.522 0.586 0.874	0.627 0.480 0.258	0.292 0.347 0.333	13.314	23.572 α_3'
Cell Totals	2.418	1.812	1.412	1.464	1.889	1.982	1.365	0.972		
Column Totals	5.626	5.319	4.854	4.312	5.688	5.759	3.616	2.987	38.160	
Sum of square totals	11.005	9.462	8.016	6.205	10.787	11.067	4.521	3.139		64.202 α

A = 21.401 B = 21.068 C = 20.256 D = 20.225 L = 22.034

Analysis of Variance Table

Source of Variance	Sum of Squares	°Freedom	Mean Sq
Between column	0.843	U-1 = 7	0.120
Between row	0.031	V-1 = 2	0.016
Interaction SOS	0.023	(U-1)(V-1)= 14	0.0016
Residual SOS	0.633	UV(p-1) = 48	0.013
Total	1.809	UVp-1 = 71	

Variance Ratio Test

$$F \quad \frac{\text{Between column mean sq}}{\text{Residual mean sq}} = \frac{0.120}{0.013} = 9.236$$

$$F \quad \frac{\text{Between row mean sq}}{\text{Residual mean sq}} = \frac{0.016}{0.013} = 1.231$$

$$F \quad \frac{\text{Interaction mean sq}}{\text{Residual mean sq}} = \frac{0.0016}{0.013} = 0.1231$$

Highly significant differences were found between columns at p = 0.001 level.

No significant difference was found between rows.

No significant interaction was found.

The implications of these significant or non-significant results cannot be assessed without further analysis. In theory, to compare any two treatments means 't' tests should be performed, so that to be accurate and to compare all possible pairs of means, the variances for each of the treatments should be calculated separately. However, as a rough approximation, it is possible to use the method of 'Least Significant Difference' (LSD) where it is possible to calculate a figure which must be exceeded before a significant difference between two means can be accepted.

A. Differences between growth rates with various nitrogen sources on sterile straw

Significant interactions were not observed between strains and nitrogen sources, therefore it is possible to make a quantitative statement about the difference between treatments which is applicable to all blocks (As-1, As-3 and As-6).

$$LSD = t_{0.05}\sqrt{\frac{(2s^2)}{(n)}}$$ where $t_{0.05}$ is the value for t

for n treatments, 48° of freedom and for P = 0.05 that is 95% confidence limits.

$$= 2.021\sqrt{\frac{2 \times 0.0411}{9}} = \underline{0.193}$$ and S^2 = residual mean square

Thus the difference between the two experimental means must exceed 0.193 before being considered of significant difference.

B. Differences between growth rates with various nitrogen sources on non-sterile straw

$$LSD = 2.021\sqrt{\frac{2 \times 0.013}{9}} = 0.109$$

∴ the difference between the two experimental means must exceed 0.109 before being considered of significant difference.

The significant differences thus assessed and differences between treatment means are shown in tables 6.4 and 6.5 in Chapter 6.

ANALYSIS OF SOME DATA FROM CHAPTER SEVEN

Significant differences between ammonia amended and non amended straws were apparent from the graphs of the experimental data. Statistical analysis on the bench scale studies was therefore not performed beyond obtaining population standard deviations.

Differences between inoculated and non-inoculated straws in the larger scale work were investigated using the Mann-Witney 'U' test for reasons previously explained.

1. Bacteria after 14 days growth

Inoculated	3.75×10^9	3.45×10^9	2.98×10^9	3.75×10^9
non-inoc.	3.48×10^9	3.70×10^9	3.78×10^9	3.25×10^9

Ranking gives

6.5	3	1	6.5	$R_1 = 17$
4	5	8	2	$R_2 = 19$

$U_1 = n_1n_2 + \frac{1}{2}n_2 (n_2 + 1) - R_2$ $\quad U_2 = n_1n_2 + \frac{1}{2}n_1 (n_1 + 1) - R_1$

$U_1 = 16 + 10 - 19 = 7$ $\quad U_2 = 16 + 10 - 17 = 9$

When $n_1 = n_2 = 4$ and $U = 7$, probability is 0.443 this is greater than previously set limits, therefore there is no significant difference between the two populations.

2. Fungi after 14 days growth

Inoc	2.0×10^7	1.25×10^7	1.25×10^7	1.5×10^7
Non inoc	5.0×10^7	5.5×10^7	5.0×10^7	5.25×10^7

Ranking gives

4	1.5	1.5	3	$R_1 = 10$
5.5	8	5.5	7	$R_2 = 26$

$U_1 = 16 + 10 - 26 = 0$ $\quad U_2 = 16 + 10 - 10 = 16$

When $n_1 = n_2 = 4$, $U = 0$ probability is 0.014 which is less than the previously set limit of 0.050. Therefore these are from two different populations.

3. Actinomycetes after 14 days growth

Inoculated	4.25×10^8	5.0×10^8	4.0×10^8
Non-inoc.	1.20×10^9	1.25×10^9	1.80×10^9

Ranking gives

2	3	1	$R_1 = 6$
4	5	6	$R_2 = 15$

$U_1 = 9 + 6 - 6 = 9$ $\quad$ $U_2 = 9 + 6 - 15 = 0$

When $n_1 = n_2 = 3$, U = 0, probability = 0.050, which equals previously set levels and therefore the data are drawn from two significantly different populations.