ECOLOGICAL STUDIES OF THE BIODETERIORATION OF A MODEL CELLULOSE SUBSTRATE BY FUNGI

KAUSER ABDULLA MALIK, B.Sc.(Hons.), M.Sc.

A thesis submitted to the

UNIVERSITY OF ASTON IN BIRMINGHAM

for the Degree of DOCTOR OF PHILOSOPHY

MAY, 1970

SUMMARY

A technique has been described which successfully maintains a controlled microenvironmental condition on a cellulosic substrate by continuous perfusion with a predetermined nutrient solution. This technique has been used to study the effect of different ecological factors namely pH, temperature, nitrogen sources and glucose nutrition on the fungal colonization of a model cellulose substrate in the form of heat rolled polythene backed cellulose chromotography paper, buried in soil. Different successional patterns of fungi colonizing this substrate, have been elucidated and the role of " secondary sugar fungi" has also been elaborated. The extent of biodeterioration of the cellulosic substrate has been determined by its weight loss estimations. Maximum biodeterioration was recorded at $50^{o}C$.

The role of thermophilic actinomycetes in the cellulose biodeterioration has been elucidated by using the perfusion technique for their selective isolation. Five species were identified and their cellulolytic ability was also determined.

The relative cellulolytic ability of all the fungi isolated has been estimated by measuring the depth of clearing of the cellulose agar columns. The effect of pH, temperature, nitrogen sources and glucose nutrition on the relative cellulolytic ability has also been observed.

An alteration of the perfusion technique has been described and used to study the effect of diffusible metabolites of cellulolytic fungi on their linear growth. The effect of non diffusible metabolites of these fungi on

their cellulolytic ability has also been studied by growing them in mixed culture and then observing the clearing of the cellulose agar. There were very few cases of synergism as compared to widespread inhibition.

The perfusion technique has also been used to study the germination and penetration of the spores of ten cellulolytic fungi through different thicknesses of fibre glass cloth. The mode of cellulase production and its diffusion was also analysed by estimating the weight loss of the different layers of a wad of five perfused cellulose strips, after inoculating the top strip.

The perfusion technique has also been used as a test method for screening different biocides commonly used as textile preservatives. Its scope in detoxification studies has also been discussed.

To my parents

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. H. O. W. Eggins for suggesting the topic of this thesis and for his unfailing interest, encouragement and guidance. I am greatly indebted to him for introducing me to biodeterioration research. I also wish to thank Dr. R. F. Sharp for his most enjoyable companionship throughout the period of research. I would also like to acknowledge the co-operation extended by Catomance Ltd. in providing the biocide coated materials. Finally, I would like to thank all the staff of the Biodeterioration Information Centre for their co-operation and help, and especially Miss Vivienne Langran and Mrs. Eleanor Parkes for their patience in reading and typing the handwritten manuscript, without which the present form of this thesis would not have been possible.

CONTENTS

The term "Biodeterioration" has been defined by Hueck (1965) as "any undesirable change in the properties of a material caused by vital activities of organisms". He differentiated biodeterioration from pathology in the sense that materials as substrates for biodeterioration are non living in contrast to living substrates in pathology. Ecology is usually defined as the study of the relations of organisms or groups of organisms to their environment or the science of inter-relations between living organisms and their environment (Odum, 1959). Thus materials (or substrates), organisms and environment which are all intimately concerned in biodeterioration, are also the essentials of ecology.

The work presented in this thesis deals with the ecological aspects of the biodeterioration of a cellulosic substrate. Eggins (1968) has listed three problems within

biodeterioration where ecology has an important role to play: the first is fundamental, being concerned with predicting the susceptibility of a given substrate to attack by various organisms dependent on possibly known environments. The second concerns the accurate assessment of colonization and breakdown of materials and the third is, designing special remedial treatments which will be effective against a predicted population of susceptible deteriogens.

Despite previous studies on the colonization of cellulosic substrates, it is difficult to predict and accurately assess the colonization and breakdown of cellulose under precise microenvironmental conditions. Therefore, if greater emphasis is going to be attached to the above mentioned point of view, it is essential to carefully control the microenvironments around the model cellulosic substrate and then study the effect of different ecological factors on the colonization and biodeterioration of the cellulosic substrate.

In order to control and keep constant the micro-environmental conditions around the cellulosic substrate some new model techniques based on the continuous perfusion of the substrate with known nutrients have been described in this thesis. These techniques have been used to study some of the ecological factors involved in the biodeterioration of cellulose and the use of such techniques as test methods for different biocides has also been discussed.

Before embarking on any such research it is imperative to review briefly the economic losses incurred by the

biodeterioration of cellulosic materials by fungi.

A. ECONOMIC EVALUATION

There is no systematic documentation regarding the cost of biodeterioration of cellulosic materials. Eggins (1967) has stated some of the difficulties in assessing this cost; one of these difficulties is persuading biologists who do understand biodeterioration, to be interested in its economics and, on the other hand, the difficulty of non-biologists in recognizing biodeterioration when they see it. Another difficulty is the natural reluctance of many manufacturers to admit that biodeterioration is a hazard at all.

Though microbiological deterioration of wood is very considerable, the biodeterioration of other cellulosic materials, e.g. cotton, textiles, paper, etc. will be particularly considered in this review since the cellulose in wood, unlike that in cotton, is in association with lignin and thus undergoes a comparatively different pattern of biodeterioration.

The statistics collected as early as 1920, indicated that 10-15 per cent of raw cotton entering England had been disintegrated into short fibres called "fly" (Fleming and Thaysen, 1920). This was thought to be the result of bacterial action during damp storage. The developing organisms were observed to bind themselves and the individual fibres into hard felt-like lumps, which in many cases occupied the entire centre of the bales.

In addition to these losses, Thaysen (1924) estimated that as much as 3% of the total jute import of this country was affected by this type of damage.

Conservative estimates quoted by Thaysen (1924) put the annual loss of cotton in England by microorganisms at about 14 million pounds sterling. In the United States, the annual loss to raw cotton alone equalled 10-30 million pounds sterling in 1926 and damage to finished cellulosic goods was quoted to be above 40 million pounds. (Nixon, 1926).

More recently, the microbial damage to cotton textiles is illustrated by the estimates made on cotton fabrics in the United States, which are quoted by Howard and McCord (1960). These data are summarised in the following table:-

Type of Degradation	Total Cotton Consumption	Portion of Market Affected
	x 1,000 lb.	
1. Microbiological not due to outdoor exposure	451,060	270,492
2. Frequent and lengthy exposures to sunlight.	226,319	189,354
3. Outdoor exposures	309,725	154,650
TOTAL	987,104	614,650

As shown in the table, out of nearly 1 billion pounds of cotton consumption, 0.6 million pounds of cotton products alone go into uses which are subjected to some sort of degradation. Out of these, microbiological deterioration is maximal. It would appear that this volume should be large enough to attract the research needed to develop protective treatments.

Similar evaluations of biodeterioration of different materials have been done by Hueck-van der Plas (1965). She estimated the annual production or consumption of perishable materials to be worth £20.8 billion in the OECD countries (Western Europe and North America). On this figure she calculated an annual loss of £0.4 billion due to biodeterioration. Among these materials the losses due to damage to cellulosic materials are also quite high. Hendey (1967) has quoted detailed data for the annual cost of decay of different materials caused by moulds. The following table represents the loss due to the biodeterioration of cellulosic materials based on the data provided by Hueck van der Plas (1965) and Hendey (1967).

	Annual Production or Consumption.	Annual Cost of Biodeterioration.
	£ x 10^6	£ x 10^6
Cotton	1,166.6	20.8
Jute	58.0	1.2
Rayon	544.0	12.5
Paper	1,750.0	35.0
TOTAL	3,518.6	69.5

With the evidence available, limited in its extent and lacking in specificity, we can roughly estimate the annual cost of biodeterioration of cellulosic materials at 2% of the cost of annual production or consumption. Nevertheless, on this information alone biodeterioration must be seen as an added burden on a country's economy and a reduction in the profits of industry.

B. LITERATURE SURVEY

The economic importance of cellulose fibre has attracted the attention of many workers in the past; the early monographs of Thaysen and Bunker (1927) and Siu (1951) are sufficient to elucidate the importance of cellulose biodeterioration. The majority of the workers in this field have concentrated their efforts to find some preventive procedures in order to overcome biodeterioration. The most common method of prevention has been the application of some kind of chemical preservative known to be toxic to certain microorganisms, to the cellulose material. Normally these preservatives are tested against certain organisms under one set of environmental conditions and in this way their efficiency is evaluated.

The lack of understanding of the varying behaviour of microorganisms under different environmental conditions has lead to the use of an increasing number of different preservatives. All recognised fungicides are able to protect cellulosic materials to some extent but none is completely effective when the material is completely exposed to extreme and varying conditions (Turner, 1967).

This discrepancy in our knowledge arises from a lack of information regarding the ecology of the microorganisms responsible for cellulosic biodeterioration. Previously various workers have compiled lists of fungi isolated from cellulosic materials, particularly textiles, and have also tried to measure the loss of tensile strength caused by individual species (Siu, 1951). One striking characteristic of these fungi is that all of them have also been isolated from soil (Gilman, 1957). In view of this, soil has often been used to determine the microbiological resistance of different protective treatments (Siu, 1951; Lloyd, 1955). The soil burial test has been found to predict rather accurately what may be expected under prolonged exposure of cellulosic materials where microbiological agents are the principal deteriorating factors (Block, 1949). Therefore, in view of such evidence, it is worthwhile to study the ecology of the fungi involved in cellulose decomposition in soil. Moreover, it is widely known that most soils contain an appreciable amount of cellulose and hemicellulose which is generally in the form of residual vegetable material, thus ensuring an active population of cellulolytic microorganisms.

Studies on the decomposition of plant residues in the soil have been carried out as early as 1904 by Van Iterson who used pure cultures of fungi. Similar studies in the soil were later carried out by Waksman and Heukelekian (1925, 1926); Waksman and Skinner (1926) and Waksman (1931). Carbon dioxide evolution was normally taken as a measure of decomposition of organic matter. These studies

led to the conclusion that fungi are most active in the process of decomposition of vegetable residues and in particular cellulose which may be incorporated in soil (Waksman, 1944). This, therefore, makes soil an appropriate substratum to use for studying the ecology of cellulolytic fungi in relation to a model cellulosic substrate.

Isolation of the Active Mycoflora of Soil.

In studying the colonisation of a model cellulosic substrate by a mixed inoculum of fungi in soil, it is essential to study those fungi which are truly active in breaking down the cellulose rather than studying the whole population of potentially cellulolytic fungi (Chesters, 1949; Garrett, 1955). Many efforts in the past had been made to study the active mycoflora from the soil (Conn, 1922; Waksman, 1916; Kubiena, 1932; McLennan, 1928). Rossi (1928) and Cholodny (1930) used a microscope glass slide pressed against a soil surface and then stained. This method, in its original as well as modified form has been widely used (Jensen, 1934; Timonin, 1940; Ryabchenko and Gubanov, 1952; Glathe et al, 1954).

In continuation of these studies, Chesters (1940, 1948) used an agar medium contained within a glass tube pierced with open ended invaginated capillaries to retrieve actively growing fungi from the soil. Mueller and Durrell (1957) used this technique and described a

variant of Chesters' technique. A slide trap for soil fungi was devised by La Touche (1948), using two cavity slides. Thornton (1952) described a screened immersion slide which consisted of a glass slide covered with a thin film of water agar which was screened by another slide having narrow holes. These slides were buried in soil and recovered after intervals whence fungal hyphae had infected the agar through the narrow holes.

All the techniques endeavouring to study the active mycoflora of soil have been proved to be selective in one way or other. The techniques involving the incorporation of agar medium in the soil face the problem of anaerobosity of the agar medium and thus allows only those fungi to be isolated which can tolerate such conditions. Sewell (1956) and Brown (1958) used such techniques and reported on the dominance of Trichoderma viride. Chesters and Thornton (1956) had also earlier concluded that the colonisation of the buried agar medium depends on the ability of one individual species to compete successfully under varying anaerobic conditions of the agar medium. Moreover, it had been already pointed out by Garrett (1951) that all isolation methods employing agar with a carbohydrate as carbon source must inevitably be selective for the ecological group of sugar fungi which are characterised by their rapid germination and exceptionally high growth rate. This fact was also reemphasised by Burges, (1958).

Selective Isolation Techniques.

Another important aspect of the study of the colonisation of substrates is the determination and isolation of individual nutritional groups of fungi which are active in the decomposition of such substrates. The isolation techniques selective for fungi involved in cellulose decomposition in soil can be reviewed under three headings:

a) Soil enrichment,

b) Incorporation of the cellulosic substrate into soil,

c) Selective cellulose agar media.

a) Soil Enrichment.

Among the selective techniques, the soil enrichment method seems to be the oldest. This method has been employed to isolate specific microorganisms concerned in certain processes of importance to soil fertility or of special interest for other reasons (Waksman and Schatz, 1945) The enrichment of soil with cellulosic substrates has found favour with many workers. Waksman and Skinner (1926) found that when finely divided filter paper was mixed with soil, the number of fungi but not of bacteria increased, as determined by the plate method. Dubos (1928), found that fungi were encouraged under anaerobic non-waterlogged and distinctly acidic conditions but in non acid aerated soils, both cellulose decomposing bacteria and fungi greatly increased when paper was added. Later on Skinner and Mellem (1944) statistically confirmed these results. Stapp and Borstels (1934) isolated a number of cellulolytic

fungi and bacteria, using an enrichment culture technique, in which filter paper is placed on a sample of moist soil. A similar technique was also used by Harmsen (1946) for studying the cellulolytic activity of fungi in soil.

b) Incorporation of a cellulosic substrate into soil.

Several general techniques have been devised in which a substrate for microbial growth is placed in soil. Such methods have also been used to study the colonisation of a sterile or non sterile substrate or to follow competition between pure culture and the soil mycoflora. Sadasivian (1939) followed the colonisation of pieces of wheat straw by Fusarium culmorum. Garrett (1956) used inoculated straw buried in soil to follow persistence of soil borne pathogens. Instead of adding materials to soil, the colonisation of root tissues may be followed by excising shoots and leaving roots to become moribund and to decay naturally (Chesters, 1960).

Other cellulosic substrates like rayon cords, cellulose threads, etc., were buried in the soil in order to investigate the cellulolytic activity by measuring their tensile strength (Richard, 1945; Von Goor 1952).

Kohlmeyer (1956) developed a quantitative gravimetric method for determining the cellulolytic activity of fungi, using cellophane foils. Although these methods were originally developed for the measurement of cellulolytic

activity in the natural environment, they are also useful for isolating organisms. An interesting approach in this connection was made by Tribe (1957), who buried pieces of cellophane attached to coverslips in soil. After different periods of time and at different stages of decomposition of the cellophane, the coverslips were removed and examined microscopically. Cellophane has been used in recent experiments (Tribe, 1957, 1960; Went, 1959; Went and de Jong, 1966) to determine the ecology of organisms colonising this substrate. Keynan et al (1961) found this substrate favouring the growth of cellulolytic fungi and not other microorganisms. Griffiths and Jones (1963) used lens tissue as a cellulosic substrate to study fungal colonisation. Eggins and Lloyd (1968) developed a screened substrate immersion tube technique to isolate cellulose decomposing fungi and study their colonisation of a cellulosic substrate. They used fibrous cellulose in the form of polythene backed cellulose chromatography paper, wound round a test tube and screened by an inert woven fibreglass cloth. These tubes are buried in soil. The screening of the substrate ensures that there are no soil particles adhering to the substrate and only growing fungal hyphae can colonise it. Moreover, the form of cellulose used is much more similar to the naturally occurring cellulose in the soil as compared to cellophane which is a highly regenerated form of cellulose.

c) Selective cellulose agar media.

The most commonly used media for the isolation of cellulolytic fungi consist of a cellulosic substrate, combined with a mineral salt solution, solidified with agar or silica gel in most instances.

Van Iterson (1904) described a medium consisting of a mineral salt solution and filter paper for the isolation of fungi. Scale (1916) and McBeth (1916) used agar media containing specially prepared cellulose.

Fuller and Norman (1942) used a cellodextrin medium for isolating cellulolytic fungi from jute and cotton materials. The cellodextrin was prepared by precipitating cellulose dissolved in 70% sulphuric acid and dialysing the precipitate for 72 hours followed by centrifuging. Hungate (1950) used ball milled acid treated cotton for the isolation of anaerobic cellulolytic bacteria. Hazra et al (1958) employed the same technique for fungal isolation and also used chloroiodide of zinc to detect zones of enzymatic activity. Eggins and Pugh (1962) following Hungate (1950) and Hazra et al (1958) used Whatmans chromatography powder and ball milled it for 72 hours to form a 4% suspension in water. They incorporated this suspension into their medium. The finely powdered cellulose becomes evenly distributed when the cellulose agar is poured into the petri dishes, and as it forms a white opaque appearance, the clearings produced by cellulolytic fungi are easily observed. This media has been widely used for isolating cellulolytic fungi from soil (Pugh et al, 1963; Dickinson and Pugh,

1965; Pugh and Dickinson, 1965; Greaves and Savory, 1965; Eggins and Lloyd, 1968).

Dickinson and Pugh (1965) compared the relative abilities of both ball milled cellulose agar and soil extract agar to isolate cellulolytic fungi; their conclusions were that where there was a high incidence of cellulolytic fungi, there was little advantage in using a selective medium, but when there was a high concentration of sugar fungi, then the technique was useful. Greaves and Savory (1965) also tend to confirm these findings.

The Colonisation of Cellulose in Soil.

All the selective isolation techniques described so far give little information as regards the different ecological factors involved in the colonization of the cellulosic substrate in soil. Attempts in this direction were made by Tribe (1966) and Went and de Jong (1966) who buried cellophane in the soil. This substrate was reported to be unsuitable for subsequent colonization by fungi as different metabolic products were accumulated. The techniques required to study the precise ecological factors should be able to control and keep constant all the microenvironmental factors which affect the colonization and biodeterioration of cellulose substrate in the soil. In this way the effect of one ecological factor on cellulose biodeterioration could be studied by keeping all the other factors constant. The technique

described by Eggins and Lloyd (1968) could be used to isolate fungi actively involved in cellulose decomposition, but this technique can not be used to study other ecological factors as its microenvironments cannot be controlled.

Our present knowledge of the ecology of cellulose decomposing fungi comes mainly from the studies in root infecting fungi (Garrett, 1950, 1951, 1956). Such studies led Garrett (1963) to propose a generalized scheme regarding the fungal successions on a corpus of plant tissues lying within or upon the soil. During this proposed succession, the substrate becomes progressively depleted. This depletion occurs in a series of overlapping stages, beginning with sugars and the simpler carbon compounds, which are easiest to decompose, continuing with cellulose and finishing with lignin which is most difficult to decompose. Apparently there exists a correlation between systematic groups of fungi and their ability to decompose; there is an initial phase of Phycomycetes followed by Ascomycetes and finally the Basidiomycetes (Garrett, 1963). This succession has been experimentally analysed by Harper and Webster (1964). In the proposed scheme of succession, Garrett also recognises a secondary group of sugar fungi, which do not live on simple carbon sources initially present in the fresh plant tissues but are dependent on the breakdown products of cellulose decomposing fungi. They absorb a share of these products and hence appear in the succession in association with these. This phenomenon is illustrated

by Chang and Hudson (1967) and Yung Chang (1967) while studying wheat straw compost. In addition to this, there have been successional studies on stems of Agropyron repens (Hudson and Webster, 1958), on leaf litter of Carex paniculata (Pugh, 1958), on the root surface of Halimone portulacoides (Dickinson and Pugh, 1965a) and from aerial stems and leaves of Salsola kali (Pugh and Williams, 1968). Recently such ecological studies on plant remains above the soil have been reviewed by Hudson (1968).

The phenomenon of fungal succession on a specific substrate has normally been considered and studied from an academic point of view. Substrates such as cellulose which are major constituents of the residual vegetation constantly added to the soil, are of great economic importance as illustrated previously. The solution of practical problems in overcoming the biodeterioration of cellulosic wastes involves an understanding of the synecology and autoecology of the microorganisms taking part in these processes. Griffith and Jones (1963) suggested the creation of artificial environments of known characteristics round any substratum whether it be pure cellulose or a piece of root. In this way information about the probable colonists of any postulated habitat may be obtained.

One of the aims of the present investigation is to develop techniques which will enable us to study the effect of different microenvironmental factors on the colonization and successional patterns of fungi on a model cellulose substrate under carefully controlled conditions.

Assay of Cellulolytic Activity.

The evaluation of the qualitative and quantitative cellulolytic activity of fungi has also attracted the attention of many workers in the past. Methods designed specifically for the assay of the activity of cell free enzyme systems have been reviewed by Halliwell in Reese (ed. 1963). Many of the assays of enzyme activity employ soluble cellulose derivatives such as carboxy methyl cellulose, which are often more susceptible to fungal attack than cotton cellulose, thus making direct comparisons with results obtained on cotton cellulose difficult.

A wide range of experimental procedures has been described by Siu (1951) and Marsh, Greathouse, Butler and Bollenbacher (1945) which involve the estimation of tensile strength losses of cotton fabrics, brought about by soil burial and by selected fungi in pure culture. These methods have been adopted as standard test methods for determining the resistance of materials to fungal attack by the American Society for Testing Materials (ASTM 1957).

Several workers have measured the loss in weight of cellulose substrates brought about by microorganisms. Reese (1947) used filter paper as a substrate, whilst Bose (1963) used cellulose powder. Garrett (1963) also used filter paper to estimate the weight loss produced by root inhabiting soil fungi. A similar method has also been employed by Fergus (1969) for thermophilic fungi. A major drawback with this method has been the difficulty of separating mycelial growth from the residual substrate,

making it difficult to obtain accurate weighing of the latter. Apart from this problem, however, this is probably the best method for determining the amount of cellulose utilized by an organism.

Another widely used method is the measurement of the diameter of the clearing zone produced on a cellulose agar medium inoculated with a cellulolytic microorganism (McBeth, 1916; Scales, 1916; Aschan and Norkran, 1953). Eggins and Pugh, 1962 also detected the cellulolytic organism by the production of a clearing zone. Savory et al (1967) measured the clearing zone produced by a cellulolytic fungus on Eggins and Pugh's cellulose agar, an index of cellulolytic activity. They recommended the use of sodium azide to prevent fungal growth but not the cellulase production so that the clearance zone becomes more conspicuous. Merthiolate has also been used previously for inhibition of further fungal growth (Aschan and Norkran, 1953).

Rautela and Cowling (1966) measured the depth of clearing of a cellulose medium instead of the diameter of the clearing zone. They used vertical columns of opaque medium containing cellulose prepared by the procedure of Walseth (1952). As the organisms grow on the cellulose agar they secrete cellulolytic enzymes that act to create a sharply defined clear zone in the opaque medium beneath the growing culture. This clearing is due to dissolution of the cellulose substrate.

Walsh and Stewart (1969), counted the number of cellulose particles left after the dissolution by the cellulolytic enzymes, using Eggins and Pugh's cellulose agar (1962) as a measure of cellulolytic activity.

All the techniques involving the clearing of cellulose do not give a measure of cellulolytic activity in any absolute terms, but are very good means of comparing and screening different organisms for their cellulose decomposing ability. Among these, the technique described by Rautela and Cowling (1966) stands out in its simplicity and ease of operation and in the reproducibility of the results.

Interaction between Microorganisms.

In addition to the studies on isolation, colonization, succession and cellulolytic ability of fungi in soil, there is abundant evidence in the literature as regards to the antagonism and competition between different microorganisms in the soil and which becomes most active during colonization of an organic substrate and its subsequent phases. Garrett (1956) while explaining the competitive saprophytic ability, listed four characteristics which are likely to influence the colonization of the substrate, namely,

1. rapid germination of spores and high rate of hyphal growth;
2. good extracellular enzyme production, which favours rapid and extensive substrate utilization;
3. production of substances toxic to other organisms, which may reduce competition for the available substrates, and,
4. tolerance of antibiotics produced by other organisms.

The first two factors are advantageous to maintain a high saprophytic activity. The third factor is particularly important during early colonization when not many organisms are present and toxic substances produced by the organisms can provide advantages over other organisms. The fourth property is helpful during the later stages of the decomposition of organic substrates when the number of fungi and the amount of toxic substances produced is maximal.

The realisation of the medical significance has made the production of antibiotic probably the most widely investigated area of microbial association. However, Park (1960) thought that tolerance of antibiotics produced by other fungi may frequently be of greater survival value than the actual production of antibiotics.

In addition to this, many different forms of antagonism and other interactions between different microorganisms in the soil have been reported by research workers in the past. Aytoum (1953) described the destruction of Armillaria sp. hyphae by Trichoderma spp. Cellvibrio sp. has been reported to inhibit Strachybotrys sp. when in physical contact (Keynan, Henis and Keller, 1961). Competition for nutrients and space and other interactions has been described by many workers and reviewed by Park (1960).

The interaction of fungi growing on cellulosic substrates have received less attention than other aspects of microbial interaction. Tribe (1966) studied the interaction between various fungi in the soil using cellophane film. In these tests, Trichoderma spp. dominated in all of the combinations in which it was tested but was itself overgrown by Stachybotrys actra. Both these organisms overgrew Fusarium

culmorum. The competitive ability of these fungi was not related to their cellulolytic activity. Wastie (1961) found that F. culmorum had very high competitive ability when tested against soil fungi.

Basu (1948) studying the attack of filter paper by very active cellulolytic fungi found no examples of synergism, the activity of a combined inoculum normally being intermediate to those of pure cultures of the participants. Some synergisms were, however, found among combinations of the less active and highly active fungi. Basu, Bhattacharya and Bose (1950) studied combinations of up to five organisms with respect to their ability to cause tensile strength losses of cotton. Out of all the combinations, a combined inoculum of three organisms was found to be most active. Went and de Jong (1966) reported examples of both antagonism and synergism while studying cellophane degradation by pure and mixed cultures of fungi. Although Trichoderma koningi and Chaetomium sp. were synergistic with regard to their ability to degrade cellophane, a combined inoculum of T. koningi and Alternaria sp. showed less activity than either organism in pure culture. Similar investigations on some other fungal species were previously carried out by Wode (1947) and Brien and Dingley (1946).

Test Techniques for Biocide Efficiency.

A wide number of tests for the resistance of cellulosic materials and the effectiveness of biocides against biological attack are carried out with mixed spore inocula or fungal

species selected at random. Greater knowledge of the interactions between fungi might allow a choice to be made of an organism based on absolute experimental evidence and thus increase the usefulness of test methods.

A survey of biological test methods for materials has been made by Hueck-van der Plas (1965). Some classifications of test methods have also been published by Hueck (1957), Theden (1960) and Hueck van der Plas (1963). From all these surveys of test methods, it is clear that increasingly more factors have to be controlled especially when the test circumstances are further removed from those of the natural environment. This is, of course, well known, as tests are deliberately arranged in such a way as to increase their reproducibility. This goal can only be achieved by controlling as many microenvironmental factors as possible.

C. A BRIEF OUTLINE OF THE PRESENT INVESTIGATIONS.

The work presented in this thesis is primarily concerned with the colonization and biodeterioration of a model cellulose substrate buried in the soil. In this connection, a new technique using heat rolled polythene backed chromatography cellulose paper as a model cellulose substrate has been described. This technique is based on the continuous perfusion of fresh nutrients of known composition onto the model cellulose substrate, thus controlling and keeping constant the microenvironmental conditions.

During the present investigations, this technique has been used to study the effect of different pH values, temperatures, nitrogen sources and additional carbon sources

on the colonization of the cellulosic substrate and subsequent successional patterns produced by the active mycoflora in the soil. The biodeterioration thus produced under different microenvironmental conditions has been measured by estimating the weight loss of the model cellulose substrate buried in the soil, whereas the cellulolytic activity of individual fungal species has been determined by measuring the depth of clearing produced on the cellulose agar columns.

The perfusion technique described in this thesis has also been used as a selective isolation technique for cellulolytic actinomycetes. The weight loss of the cellulose produced by some of the actinomycetes has also been estimated with the help of this technique.

A modification of the perfusion technique has been used to study the fungal interaction produced by their diffusible metabolites. The perfusion technique has also been used to study the germination and penetration patterns of the spores and the mycelia of cellulolytic fungi.

Some work has also been done on the possibility of such techniques being used as test methods for the evaluation of biocide efficiency.

The presence of fungi in a soil can be studied by direct isolation on to glucose agar media (Waksman 1916; Warcup 1950). However, greater selectivity can be achieved by the use of cellulose containing agars, e.g. Eggins & Pugh (1962). Such isolation techniques give little information regarding the active mycoflora in the soil. Chesters (1940, '49) isolated an active mycoflora from the soil by using agar media contained within a glass tube pierced with open ended invaginated capillaries; Thornton (1952) used screened immersion plates to isolate active mycoflora from the soil. Chesters and Thornton (1956) found these methods to favour isolation of fast growing fungi under low oxygen tension.

A number of methods have also been published which involve the burial of cellulosic substrates in soil to study their colonization and deterioration by microorganisms. Such methods have been reviewed by Hazeu and Eggins (1966). The form of cellulose used in these techniques have often not been very comparable with native cellulose. Eggins and Lloyd (1968) described a

screened substrate technique which employs a pure form of cellulose. The cellulosic substrate used in this technique is polythene-coated 3MM Whatman Chromatography paper. The advantages of this substrate are primarily that this relatively undegraded form of native fibrous cellulose is manufactured to a carefully controlled standard and its use in chromatography ensures that the extraneous substances are at a very low level. The resistant polythene backing enables the substrate to be handled even when the cellulose has been considerably deteriorated. This screened substrate technique essentially involved the screening of the buried cellulosic substrate from direct contact with the soil by the use of a finely woven glass fibre tape which, however, does enable fungi to grow through this inert fabric and colonize the underlying substrate.

The polythene backed chromatography paper is used in strips 0.5 - 1.0 cm. wide on which additional nutrients (e.g. Eggins and Pugh's salt solution) may be absorbed. These strips are installed in the soil, wound round test tubes (polythene side innermost). Each strip is then covered with glass fibre tape (E.C.T.101, glass cloth, 2" wide by 0.003" thick; Turner Bros. Asbestos Co.) which is then fastened by an adhesive tape of heat and water resistant resin-backed glass fibre. Such screened substrate tubes are then autoclaved together in covered beakers containing a few mls of water. These tubes are buried in soil and can be sacrificed after regular intervals; the glass fibre fabric tape is removed and the underlying cellulose strip examined for colonization macroscopically and microscopically. The strip may then be cut aseptically into small pieces and plated out on to cellulose agar (Eggins and Pugh 1962) when fungi growing from these pieces can then be

Table 1

Percentage frequency of occurrence of fungi colonizing polythene backed cellulosic paper.

FUNGI	SCREENED PAPER						UNSCREENED PAPER					
	CA*			GS+			CA			GS		
	Days of Incubation											
	2	4	6	2	4	6	2	4	6	2	4	6
Chaetomium globosum	100	100	66	-	-	-	100			33		
Fusarium oxysporum	100	100	100	100	100	100	66	100	100	100	66	100
Mucor globosus										100	66	66
Penicillium funiculosum	100	66	-	100	100	100	100	100	100	66	100	100
Papulaspora sp.	66	33					66	-	33			
Sporotrichium pruinosum	100	100	100	100	100	100	100	100	100		33	
Streptomyces sp.							33					
Rhizopus nigricans								33	100	66	100	100
Trichoderma viride								33	66		33	66
Aspergillus sp.										33	100	66

CA = Eggins and Pugh's cellulose agar

GS = Eggins and Pugh's glucose starch agar

Table 2

Percentage frequency of occurrence of fungi colonizing a cloth strip.

FUNGI	SCREENED PAPER						UNSCREENED PAPER					
	CA			GS			CA			GS		
	Days of Incubation											
	2	4	6	2	4	6	2	4	6	2	4	6
Chaetomium globosum	100	66	100	-	-	-	33					
Fusarium oxysporum	100	100	100	100	100	100	100	100	100	100	100	100
Mucor globosus										33	-	33
Penicillium funiculosum	100			100	100	100	100	100	100	66	66	100
Papulaspora sp.	-	-					66	33	100			
Sporotrichium pruinosum	100	100	66	100	100	100	100	66	100			33
Streptomyces sp.												
Rhizopus nigricans		33					100	66	100	100	33	66
Trichoderma viride						33	33					
Aspergillus sp.										33	33	66

identified.

Eggins and Lloyd (1968) recommended this technique to study the patterns of colonization of a cellulosic substrate and the effects of micro-environmental factors. Furthermore the effects of various protective biocides can also be conveniently studied.

In order to study the advantages of screening the substrate and using a selective medium, a small experiment was performed. Two substrates were used namely polythene coated 3MM chromatography paper and cloth strips; two types of media were also used: Eggins and Pugh's cellulose agar and Eggins and Pugh's glucose starch agar (using 5 gms. of glucose and 5 gms. starch instead of cellulose in the Eggins and Pugh's medium).

Twenty-four substrate test tubes were prepared for each cellulosic substrate (cloth and paper). Out of these twelve tubes were screened and the rest were left unscreened. All these tubes were buried in the soil which was contained in large troughs, and incubated at 25°C. Four substrate tubes were sacrificed for each substrate, after 2,4 and 6 weeks of incubation. Out of the four substrate tubes, two were used for inoculations on to 6 Petri dishes of cellulose agar and the remaining two were inoculated on to 6 Petri dishes of glucose starch agar. These were incubated at 25°C. and observed after 7 days. Similar inoculations were made for both screened and unscreened substrates.

The results are summarized in Table 1 and 2. It is quite apparent from these results that the frequency of sugar fungi increases in the case of unscreened substrates, and furthermore when inoculated on glucose starch medium these fungi become most dominant. Rhizopus nigrican and Mucor globosus were isolated only from the unscreened substrate. The appearance of these and

other fungi on cellulose agar might be due to the adhering soil particles to the unscreened substrate.

Although the use by Eggins and Lloyd (1968) of a glass fibre screened polythene-backed cellulose has facilitated the study of colonization of cellulose by fungi, it was observed that such cellulosic substrates were considerably affected by leaching of nutrients absorbed on the substrate to the soil and by alteration of the substrate by decomposition products of the colonizing fungi. In order to confirm this phenomenon, a small experiment was performed involving the measurement of pH of the substrate before and after burial in the soil.

McIlvaine's buffers ($Na_2 HPO_4$ and citric acid) at pH3 - pH8 were absorbed on to the polythene backed cellulose strips, so that the pH of the paper was the same as that of the buffer. These strips were incorporated into the screened substrate tubes, buried into the soil and then incubated at 25°C. These tubes were taken out after 4 days and the pH of the cellulose strips was measured again. The following results were obtained:

pH of the cellulose strip before burial in the soil.	pH of the cellulose strip after burial in the soil.
3.0	6.0
4.0	6.1
5.0	6.1
6.0	6.1
7.0	6.3
8.0	6.4 - 6.8

pH of the soil was 5.8 and distilled water was 6.5.

The above results point to the fact that there might have been leaching resulting in the escape of the buffers to the soil, thus bringing the pH of the paper to nearly the same value as that of the soil and distilled water which is used for measuring the

pH of the paper. Moreover, additional proof of leaching was obtained when potassium dichromate solution was absorbed onto the paper staining it a yellowish colour. This paper when used for a screened substrate tube and buried in soil, lost its colour after three days, thus confirming the diffusion of potassium dichromate from the paper into the soil and hence altering the original state of the substrate. Similarly it is assumed that the nutrients, initially absorbed onto the cellulose paper in the screened substrate tube are leached into the soil; the substrate is also affected by the decomposition products of the colonizing fungi. This problem is also encountered in the conventional culturing techniques on agar plates, when staling of the medium results in the fall of the fungal rate of growth and certain characteristic changes also occur (Brown 1923, 25). Some of these changes have been shown to be due to alterations in the composition of the medium through the accumulation of substances produced by the growing fungus and not to starvation caused by depletion of food substances (Hawker 1950). Certain fungi may be able to colonize the substrate containing stale nutrients, whereas other species may not have the ability to tolerate such a fungistatic substrate (Dwivedi & Garrett 1968).

In order to alleviate some of these difficulties, a new technique has been evolved which involves a continuous supply of fresh nutrients to the cellulosic substrate in order to overcome the problem of staling and variations in the ancillary conditions of the screened cellulosic substrate. A somewhat similar principle of perfusion with nutrients has been used by some workers in tissue culture and

continuous culture of fungi (Moor , 1945; Seim, 1966). Plunkett (1966) studied branching of the hyphae of Mucor haemalis by perfusing different amino acids.

Description of the Perfusion Technique

The practical details of the technique revolve around the transport principle of chromatography paper and the property of glass fibre sleeving to act as a nutrient conductor. This sleeving is normally used after resin impregnation as an insulant for electrical cables in high temperature machines but since it consists of fine glass fibres woven together, liquids will pass along it by capillarity. Thus the sleeving can be made to perfuse fresh nutrients from a nutrient reservoir to a cellulosic substrate and conduct waste substances away.

The following materials are needed to construct a cellulose paper perfusion system.

Material	Manufacturer
3 MM. Polythene backed cellulose paper	W. & R. Balston Ltd., Springfield Hill, Maidstone, Kent.
Fibre glass cloth ECT 101	Jones Stroud & Co., Long Eaton, Nottingham.
Glass fibre sleeving 4 mm. wide	do.
Silicone rubber tubing 3 mm. wide	do.
Silicone rubber bungs	Jencons (Scientific) Ltd., Mark Road, Hemel Hempstead, Herts.

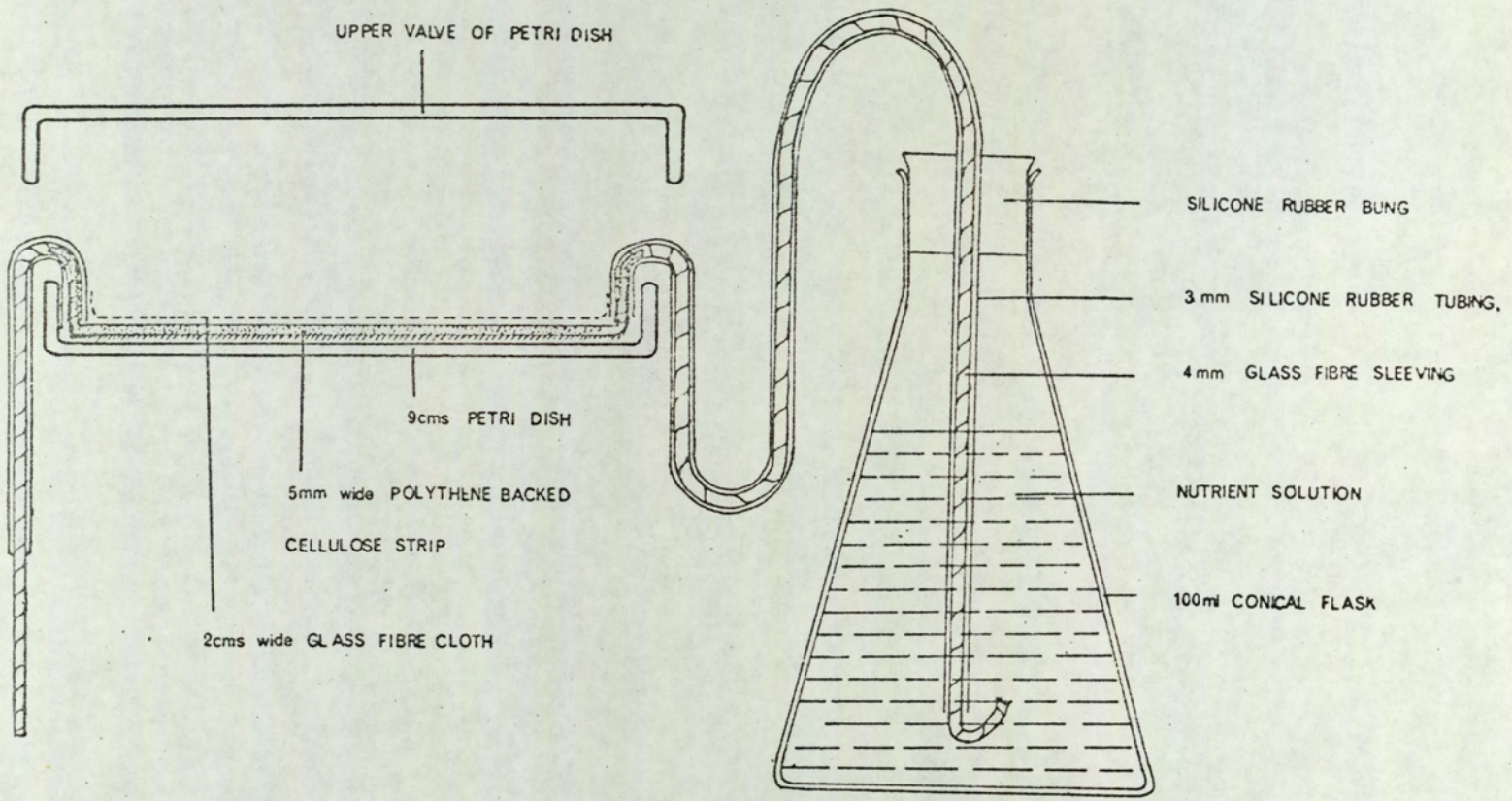


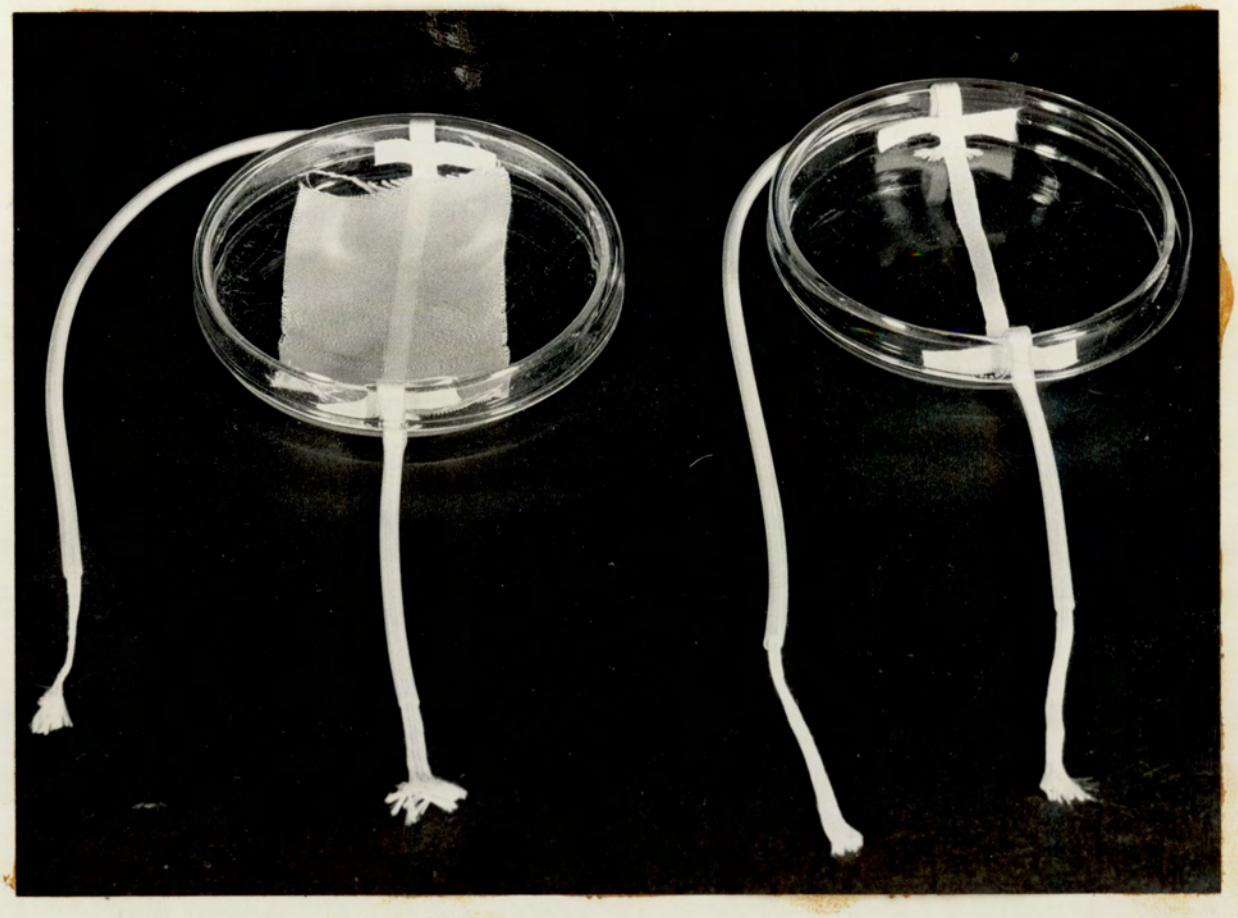
Diagram 1: Diagrammatic representation of a perfusion system.

Adhesive glass fibre tape	General Fabrication Ltd., 26 Orphanage Road, Erdington, Birmingham 24.
9 cm. Petri dish Petri dish carrier 100 ml. conical flask	Baird & Tatlock Ltd., The Laboratory Centre, Slade Road, Birmingham 23.

Before constructing a perfusion system, polythene-backed cellulose paper is cut into strips, 5 mm. wide and 13 cms. long; 4 mm. glass fibre sleeving 30 cms. long,is threaded through 25 cms. of 3 mm. diameter silicone rubber tubing; similarly a 15 cms. length of 4 mm. glass fibre sleeving is threaded through 10 cms. of 3 mm. diameter silicone rubber tubing; a 9 cms. length of 2 cms. wide fibre glass cloth is also cut.

A perfusion system consists of a 9 cms. glass petri dish containing 5 mm. wide polythene backed cellulose strip (with polythene side facing downwards) screened in the petri dish by a fibre glass cloth. One end of the strip is joined to a 30 cms. long 4 mm. wide glass fibre sleeving threaded through 25 cms. long 3 mm. wide silicone rubber tubing (See Diagram 1); this leads to a 100 ml. conical flask containing nutrients. The other end of the cellulose strip is similarly joined to a 15 cms. length of 4 mm. wide glass fibre sleeving threaded through 10 cms. of 3 mm. wide silicone rubber tubing. The glass fibre sleeving at this end is exposed to the atmosphere (See Diagram 1).

The polythene backed cellulose strip is kept in position in the petri dish with the help of a glass fibre adhesive tape (Photograph 1). The connections of the



Photograph 1

Petri dishes containing cellulose strips attached to the glass fibre sleeving threaded through silicone rubber tubing. (A) Cellulose strip screened by a glass fibre cloth; (B) unscreened cellulose strip attached to the petri dish with the help of adhesive fibre glass tape.

cellulose paper strip with the silicone rubber tubings do not interfere with the normal closing of the lid of the petri dish because of the flattened connections and thin wall of the silicone rubber tubing (Photograph 1).

Five such petri dishes containing perfusion systems can be fed from a 100 ml. conical flask containing nutrients. The nutrients in the flask can be kept sterile after autoclaving, by using a silicone rubber bung with a hole for five lengths of silicone rubber tubing. The remaining gaps in the hole can be filled by silicone rubber glue. Fifteen such perfusion systems can be housed in a petri dish carrier with three conical flasks attached to its sides by copper wire (Photograph 2). The whole may be autoclaved after filling the perfusing flasks with an appropriate nutrient solution. All the materials used are heat and pressure resistant and hence can be autoclaved.

When in operation, the petri dishes containing the perfusion system are filled with soil and then incubated at the required temperature. The nutrient solution from the perfusing flask moves due to the capillarity of the glass fibre sleeving and is then perfused through the cellulose strip in the petri dish. The flow of nutrients through the system is maintained by the constant evaporation at the exposed end of the glass fibre sleeving.

After incubation, cellulose strips are sacrificed at intervals, the glass fibre sleeving being cut to release the cellulose strip. Visual observations of colonization are made, then the cellulose strip is cut aseptically into 3 - 4 mm. pieces and plated onto cellulose agar (Eggins & Pugh 1962). The resulting fungi are then recorded.

Photograph 2

A petri dish carrier containing perfusion systems. (Five perfusion devices are fed by one conical flask containing salts solution)

In order to ensure a steady uptake of nutrient, certain checks were made on this perfusion technique. Fifteen perfusion systems were made as described previously. Instead of 100 ml. conical flasks, 5mm. x 18 cm. glass tubes were sealed at one end and are graduated in order to measure the fall in liquid level due to perfusion. McIlvaine's buffer at pH 3.0 was used instead of nutrients and the soil was sterilized before use to avoid any fungal growth on the papers, so that liquid uptake could be measured under constant conditions. The moisture content of the soil was adjusted to 20 - 21%.

As five perfusion systems were placed on top of each other, their height was also taken into account. The petri dish lying at the bottom was numbered 1 and the petri dish at the top was numbered 5. Three replicates were kept for each position of the petri dish. The glass tubes containing perfusing buffers were arranged in such a way that all the petri dishes containing perfusion systems were above the liquid level in the glass tube, so that there was no siphon action working. The uptake rate was observed for seven days and several readings were recorded.

The observations are summarized in Table 3.

The uptake rate in case of all the perfusion petri dishes tends to slow down during the first three days of incubation, whereas after that it was found to be quite constant ranging from 0.31 ml. to 0.36 ml./24 hrs. This constant uptake is presumably achieved when the equilibrium between perfusion of the cellulose strip and

Table 3

Position of Perfusion Petri Dishes	Uptake of liquid ml/24 hrs. * Days of Incubation						
	1	2	3	4	5	6	7
Height No. 1	0.8	0.64	0.46	0.36	0.32	0.33	0.33
.. 2	1.0	0.65	0.46	0.36	0.31	0.31	0.32
.. 3	0.7	0.60	0.44	0.36	0.32	0.32	0.32
.. 4	0.8	0.56	0.42	0.35	0.36	0.33	0.34
.. 5	0.7	0.50	0.44	0.40	0.36	0.34	0.33

* All the figures are average of three observations.

evaporation at the exposed end of the glass fibre sleeving is reached. It can also be seen from the results that the height of the petri dishes did not matter as equal lengths of fibre glass sleeving was used.

After seven days of incubation and perfusion, the pH of the cellulose strip and of the soil was noted. No appreciable change was observed. In addition to this, the moisture content of the soil was also noted at the end of seven days of incubation. The results are summarised as follows.

	Initial	Final
pH of the cellulose strip	3.0*	3.3*
pH of the soil	5.8*	5.8*
% Moisture content of the soil	20.8+	21.2+

* average of three observations

\+ average of five observations

These results show that there has been a negligible change in the pH of the substrate and the soil; thus providing a substrate with constant environmental conditions. The soil moisture content observations also suggest that the perfusion of the cellulose strip does not alter the soil conditions. This technique in addition to eliminating the possible staling hazard by conducting the fungal waste substances away from the cellulosic substrate can also be

employed to study the ecology, especially synecology of fungi responsible for the biodeterioration of cellulose in the soil. It is well known that no one organism is solely responsible for the decomposition of cellulose in the soil, but a population of fungi is involved in this process. With the help of the perfusion technique, the fungal succession developing on a defined cellulosic substrate incorporated into the soil can be studied under controlled and constant microenvironmental conditions. This insight into the different patterns of colonization under varying conditions can very much enlarge our knowledge of cellulose decomposition in the soil by fungi.

In nature, the microenvironmental conditions do not remain constant as is so with the perfusion technique. However, the artificial reproduction of individual environments around a cellulosic substrate helps to obtain information about the probable colonists of any postulated habitat and thus provides a basis for prediction about behaviour in natural systems.

This technique has been used during the present investigation to study the initial and subsequent fungal colonization of a polythene-backed cellulosic substrate in the soil. The effect of different microenvironmental factors (e.g. pH, different nitrogen sources, additional carbon sources, etc.) on the colonization of cellulosic substrate has also been shown by simply changing the perfusing solution. Moreover, the effect of different temperatures (thermophilic and mesophilic) on the decomposition of the cellulose substrate by fungi is also studied by this perfusion technique, without the usual gross changes which often occur

with such experiments.

Certain modifications of this technique have also been used to demonstrate the effect of diffusible metabolites of one cellulolytic fungal species on to the other. Another modification of this technique has been used for studying the competitive saprophytic ability and the cellulose decomposing power of some of the fungi isolated from the soil with the help of the perfusion technique. All these modifications will be described in later chapters.

The soil used for these present investigations was collected from a pasture field in Clent, near Stourbridge, Worcestershire, England. This pasture field has not been treated with any fertilizer or any herbicide for the last forty years. The colonizing flowering plants found on this field have been identified as follows:

Trifolium spp. including T. repens L.
Bellis perennis L.
Taraxacum officinalis
Rumex acetosella L.
Plantago media L.
P. lanceolata L.
Agrostis tenuis Sibth.
Dactylis glomerata L.

The soil was found to be Permo-Triassic in origin, including keuper red and sand stones with pebbly bands. It was brown earth with fine Muscovite micas, namely a ferric brown earth. The top 15 cms. was a dark reddish brown clay loam, with fine block pads. Deeper than

15 cms. the soil conglomerated into coarse block pads.

A soil borer of 6 cms. diameter was used for taking soil up to 15 cms. in depth. The samples were taken at random from 100 sq. yards of the pasture field. The soil core thus obtained were transported in beakers to the laboratory and were used within a month of collection. The soil cores were stored in a humid place and were watered frequently in order to maintain the original level of moisture content. The average pH of the soil was 6.8 and the moisture content was found to be 21% to 23%. Appropriate number of soil cores were broken and thoroughly mixed before use.

Eggins & Pugh's cellulose (Eggins and Pugh 1962) was used for isolating and culturing the cellulolytic fungi. 40 gms. of cellulose chromatography powder was ball milled with two litres of distilled water for 72 hours. 250 mls. of this cellulose suspension was used to make one litre of Eggins and Pugh's cellulose agar. Rose bengal was also added to the medium as a bacteriostat. The autoclaving of the medium was done at 10 lbs pressure for 20 minutes. Glass petri dishes 9 cms. diameter were used for pouring agar plates. These were sterilized by first autoclaving at 10 lbs. for 20 minutes and then oven drying at 160^{o} C. for twelve hours.

The model cellulosic substrate used in these studies, as stated previously was 3 MM polythene coated chromatography papers. The cellulose fibres undergoe a mild treatment to remove the small quantities of impurities such as cotton wax, proteins, pectic and mineral matter

during the purification and paper making process. Physically, the fibres are subjected to a mechanical "beating" process in water which results in varying degrees of polymerisation (on a macro scale) with considerable development of the external surface. (Balston & Talbot 1952). The resistant polythene backing is heat rolled and thus the original purity of cellulose is maintained.

Cellulose decomposition in the soil is governed by a number of environmental factors which determine the cellulolytic decomposing ability of the soils, varying in chemical and physical characteristics. One of these environmental influences is temperature. Many studies on the deterioration of cellulose by fungi seem to ignore this factor, as most of the observations are made at 20-30°C only. If ecology is to play an important role in the prevention of biodeterioration of cellulose, it is essential to take into account such ecological factors; cellulosic materials are exposed to varying temperatures some of which may be caused by insolation.

In order to study the effect of temperature on cellulose biodeterioration, the soil was incubated at various temperatures. Several methods have been used by previous workers for studying the microorganisms associated with cellulose in soil (Van Iterson 1904;

Tribe, 1957; Meyer & Reynolds, 1959; Webley & Duff, 1962). The soil enrichment method is one of the most common procedures employed to isolate specific microorganisms (Waksman & Schatz, 1945).

During the present investigation the soil was enriched with cellulose and incubated at 25° C., 35° C. and 50° C. Time sequential isolations from the incubated soil were made on Eggins & Pugh's cellulose agar. These results were then compared with those obtained by using the perfusion technique at different temperatures.

The soil cores collected from the pasture field were thoroughly mixed with cellulose powder which amounted to 4% of the wet weight of the soil. This enriched soil was put in 18 petri dishes; 9 were incubated at 25° C. and the rest at 35° C. From every petri dish three simple Warcup isolation plates were made using Eggins and Pugh's cellulose agar, after different periods of incubation.

Perfusion sets were also set up as described previously; 45 such sets were incubated at each temperature. Every time 3 perfusion sets were sacrificed, the cellulose strip was released and inoculated on to 6 E & P (Eggins & Pugh) cellulose agar plates. These plates were incubated at the same temperature and observed after 7 days of incubation. The resulting fungi were recorded. Frequency of occurrence was determined by recording its presence or absence in each petri dish.

25° C.

The results of soil enrichment and the perfusion technique isolations at 25° C. are summarized in Tables 4 and 5 Nearly all the common cellulolytic fungi occurring in the soil, have been isolated by the soil enrichment method.

Table 4

Percentage frequency of occurrence of the fungi isolated by Warcup's method from cellulose enriched soil incubated at 25° C.

FUNGI	Days of Incubation of Soil					
	1	2	3	6	9	15
Arthrobotrys sp.				13		
Aspergillus fumigatus Fresenius	13	60			46	
Chaetomium globosum Kunze	60	46	46	13	6	6
Dicoccum asperum Corda	6		6	66	66	33
Fusarium solani (Martius Appel & Wollenweber	100	86	100	93	100	100
Gliocladium roseum (Link) Thom.	13		6	13		
Humicola grisea Traaen			53	13	60	66
Mucor globosus Fischer	80	46	93	33		
Paecilomyces varioti Bainier		6				6
Paecilomyces elegans (Corda) Mason et Hughes apud Hughes (1951)	6		20	6		
Penicillium sp.	33	53	66			
Rhizopus nigricans Ehrenberg	60					
Streptomyces sp.	86			93	80	100
Trichoderma viride Pers. ex Fr.	60	33	80	86	53	33

The most frequently occurring fungi were Fusarium solani and Trichoderma viride. Chaetomium globosum was also fairly common in the beginning but tends to decrease in frequency later. Dicoccum asperum and Streptomyces sp. have also been isolated through out the period of incubation of the soil. It is apparent from Table 4 that most of the fungi isolated, appeared after one day of incubation of the soil. The only exceptions to this are Humicola grisea which appeared after 3 days; Arthrobotrys sp. which was isolated only once after 6 days and Paecilomyces varioti which was occasionally isolated and proved to be non cellulolytic. In addition to the cellulolytic fungi, some sugar fungi namely Mucor globosus and Rhizopus nigricans were also isolated. These fungi were able to grow on E. & P. cellulose agar, presumably utilizing yeast extract and L-asparagine, as these fungi did not produce any clearing of the cellulose agar (Eggins and Pugh, 1962).

The results obtained from this soil enrichment method suggest that there is no fixed succession of fungi associated with cellulose colonisation, as most of the fungi were isolated after one day of incubation of the soil. This makes the delineation of early colonisers from late developers very difficult.

The results of colonisation of cellulose strip by fungi, using the perfusion technique are summarised in Table 5. The initial colonisation started as early as 12 hours but macroscopically visible fungal growth did not appear until after 2 days.

F. solani and T. viride were found to be the main fungi involved in the deterioration of the cellulose strip buried

Table 5

Percentage frequency of fungi colonizing the cellulose strip perfused with E. & P. salt solution unbuffered.

FUNGI	Days of Incubation												
	12 hrs.	1	2	3	5	6	9	12	15	18	21	28	35
Arthrobotrys sp.						64	100	100					
Aspergillus fumigatus													16
Dactylella sp.						16	16						
Fusarium solani	64	100	100	100	100	84	100	100	100	100	100	100	64
Fusidium viride									48	32	32		
Gelasinospora cerealis				64	16		16						16
Gliocladium roseum											32	32	16
Humicola grisea											16	32	48
Mucor globosus			16		16	50							
Paecilomyces varioti								32					
Papulaspora sp.												32	
Penicillium sp.											16		
Trichoderma viride	16	80	80	64	64	64	84	32	84	100	100	64	32
Zygorhynchus moelleri				64									

in the soil. Fusarium solani was isolated after 12 hours of incubation and then remained dominant at all times. T. viride also had similar occurrence but its frequency was comparatively low.

The non-cellulolytic sugar fungi, namely Zygorhynchus moellerii and Mucor globosus, made their appearance between 2 - 6 days of incubation; Gelasinospora cerealis appeared after 3 - 9 days of incubation and was seen growing on the strip. The other fungi isolated between 6 and 18 days of incubation were Arthrobotrys sp., Dactylella sp., Fusarium moniliforme and Paecilomyces varioti. During this period nematodes were also found ramifying in the cellulose fibres. These might explain the presence of the nematode catching fungus Arthrobotrys sp. which is cellulolytic as well as it produced clearing on the cellulose agar plate.

When the strip was highly degraded a variety of fungi were isolated e.g. Papulaspora sp., Gliocladium roseum, Penicillium sp. and Aspergillus fumigatus.

The number of fungal species isolated with the help of the perfusion technique is quite low compared to the soil enrichment method, but the isolations from this technique show some pattern of colonization of the cellulose strip, with distinct early colonizers and late developers. F. solani and T. viride can be called early colonizers which remain dominant throughout the experiment. The late developers comprise G.cerealis, Arthobotrys sp., Dactylella sp.,Fusarium moniliforme . Other fungal species namely G. roseum, Penicillium sp. Aspergillus fumigatus and Papulaspora sp. were isolated when the deterioration

of the cellulose strip was maximum.

The absence of some well known cellulolytic fungi such as C. globosum from the list of the perfusion technique isolations is indicative of the fact that the role of individual species in a pure culture can give misleading information as regards their importance in nature. One of the advantages of the perfusion technique is the isolation of an active cellulolytic mycroflora since the cellulosic substrate is screened from the soil and only actively growing fungi can colonize it.

35°C

The results of the Warcup isolations at 35°C are summarized in Table 6. The numbers of fungi isolated at this temperature was extremely low. Aspergillus fumigatus was the most dominant fungal species occurring on all the cellulose agar petri dishes. Since this fungus is a rapid sporer other fungi face much competition to manifest themselves. The cellulolytic species of Streptomyces have also been frequently isolated by both the soil enrichment and perfusion techniques. During soil enrichment studies Chaetomium elatum, Cephalosporium sp. and Eurotium sp. were isolated after 6 days of incubation whereas Sporotrichum pruinosum was isolated after 9 and 15 days of incubation of the enriched soil.

The colonisation of the cellulose strip as studied by the perfusion technique has enabled the isolation of comparatively more numbers of fungi (Table 7). Aspergillus fumigatus was again dominant and was seen growing on the cellulose strip at the time of sacrificing. Streptomyces sp. was also fairly common throughout the period of incubation

Table 6

Percentage frequency of fungi isolated by Warcup's technique from cellulose enriched soil incubated at 35° C.

FUNGI	Days of Incubation					
	1	2	3	6	9	15
Aspergillus fumigatus	100	100	100	100	100	100
Cephalosporium sp.				6		
Chaetomium elatum				13		
Eurotium sp.				6		6
Mucor globosm						
Streptomyces sp.		50	50	60		
Sporotrichum pruinosum					26	13

It could also be observed on the cellulose strip where it produced small pits by utilizing the cellulose. Chaetomium elatum was isolated once after 2 days of incubation. Among the late developing fungi, Eurotium sp. was isolated between 8 and 15 days of incubation. During the same period T. viride was also isolated but its frequency of occurrence was very low. The fungi isolated between 18 and 30 days of incubation were H.grisea, Penicillium sp.,Graphium sp.,Sporotrichum pruinosum and Coniothyrium fuckeli. At this stage the cellulose strip was found to be fairly deteriorated when observed macroscopically. The percentage frequency of occurrence tends to decrease in the presence of Sporotrichum pruinosum and Penicillium sp. After 26 days of incubation nematodes were found ramifying through the cellulose strip but no nematode catching fungi were observed unlike the previous observations at 25°C.

With the help of the perfusion technique, it has been possible once again to study the successional pattern of colonisation of the cellulose strip by fungi at 35°C. At this temperature as well, there is a distinct early coloniser namely A.fumigatus which by virtue of its fast sporing ability remains dominant throughout the period of incubation. Streptomyces sp. makes its appearance between 2 and 15 days along with Eurotium sp.which occurrs between 8 and 15 days. The remaining fungi namely H. grisea., Graphium sp., C. fuckeli and Penicillium sp.appear when the cellulose strip is fairly deteriorated.

The comparative review of the results of the

Table 7

Percentage frequency of occurrence of fungi colonizing the cellulose substrate when perfused with E. & P. salt solution and incubated at 35°C.

FUNGI	Days of Incubation										
	1	2	3	6	8	10	12	15	18	24	30
Aspergillus fumigatus	60	80	100	100	100	100	100	100	100	100	100
Chaetomium elatum		16									
Coniothyrium fuckeli										50	
Eurotium					16	16	16	32			
Graphium sp.										50	32
Humicola grisea							16			32	32
Penicillium sp.								64			
Sporotrichum primosum								32	64		
Streptomyces sp.		30	30	60	100	80	80	100			
Trichoderma viride				16			16				

perfusion technique isolations at 25°C and 35°C will reveal that each temperature has its own cellulolytic soil fungal flora which has different successional patterns at different temperatures. Some fungi which are common to the lists of fungi isolated at 25°C and at 35°C, play entirely different roles in the colonisation pattern at the different temperatures. These differences in the behaviours make such studies at different temperatures all the more important.

50°C

Similar studies have also been carried out at 50°C. This temperature falls in the optimal range of the thermophilic fungi. Cooney & Emerson (1964) defined a thermophilic fungus as one that has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or below 20°C. Fungi such as A.fumigatus which have maxima near 50°C but minima well below 20°C have been considered as thermotolerant by them. This definition of thermophily has been accepted here.

Surveys using isolation techniques have generally indicated a relative rarity of these fungi in the soil (Crisan 1959). Therefore it was decided to compare isolations from unamended soil with isolations after enriching the soil with cellulose and incubating at 50°C; comparisons were also made with glucose enriched soil in order to study the behaviour and occurrence of all thermophilic fungi. The cellulose powder and glucose added amounted to 4% of the wet weight of the soil. Each of these amended and unamended soils was put in 9 petri dishes and incubated at 50°C. From

Table 8

Fungi isolated during the present investigation at 50 C

Aspergillus fumigatus Fresenius

Cephalosporium sp.

Chaetomium thermophile la Touche

Humicola insolens Cooney et Emerson

Humicola lanuginosa (Griff. et Maubl.) Bunce

Malbranchea pulchella Saccardo et Penzig

Mucor pusillus Lindt

Myriococcum albomyces Cooney et Emerson

Sporotrichum thermophile Apinis

Streptomyces spp.

Talaromyces duponti (Griff. et Maubl. emend. Emerson) Apinis

Thermoascus aurantiacus Miehe

the soil samples, simple Warcup isolation plates were made after 0 hr., 12 hr., 1 day and 2, 3, 6, 9 and 12 days of incubation. From every petri dish containing soil six total inoculations were made. Out of these six inoculations, 3 were made on Eggins and Pugh's cellulose agar plates and the other 3 inoculations were made on Eggins and Pugh's glucose agar plates. These inoculated plates were also incubated at 50° C. and after 7 days the fungal species were recorded and isolated.

The thermophilic species isolated during this study are listed in Table 8. The results for unamended and unincubated soil are summarised in Table 9 (first column). Five thermophilic species were recorded and isolated but their frequency of occurrence was extremely low. Both on E & P's cellulose agar and glucose agar, Aspergillus fumigatus was most frequent. Cellulose agar (CA) also supported the growth of Chaetomium thermophile, Humicola grisea, Mucor pusillus and Sporotrichum thermophile.

The sequential isolations from unamended incubated soil (Table 6) gave better results. The number of thermophilic species isolated was twice that from the unincubated soil. The frequency of isolation of various species increased with the period of incubation, whereas Cephalosporium sp., Malbranchea pulchella, and Mucor pusillus appeared after 12 days of incubation. Chaetomium thermophile and Humicola grisea were at a maximum after 12 days and 9 days respectively whilst the frequency of Aspergillus fumigatus decreased on cellulose agar. Many of the known thermophilic species grew from incubated cellulose enriched soil (Table 10). Samples

Table 9

Percentage frequency recorded in cultures derived from 9 portions of one sample of soil after 7 days incubation at 50° C., of fungi appearing on cellulose agar (CA) and glucose agar (GA) inoculated with unamended soil, either unincubated or incubated at 50° C. for periods of various lengths.

FUNGI	Period of Incubation of unamended soil							
	0 hr.	12 hr.	1 day	2 day	3 days	6 days	9 days	12 days
	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA
Aspergillus fumigatus	100 100	100				22	77 55	77
Cephalosporium sp.								22
Chaetomium thermophile	22	11		11	22	22	33 33	77 22
Humicola grisea	11	33	22		33	66	100 100	77 100
Humicola insolens						11	22 44	33
Humicola lanuginosa								22
Malbranchea pulchella								44
Mucor pusillus	11 22							
Myriococcum albomyces		11						11
Sporotrichum thermophile	11							
Streptomyces spp.		100	100	100	77	55 66	77	77
Talaromyces duponti								
Thermoascus aurantiacus		11				22		33

Table 10

Percentage frequency recorded in cultures derived from 9 portions of one sample of soil after 7 days incubation at 50° C., of fungi appearing on cellulose agar (CA) and glucose agar (GA) inoculated with cellulose-amended soil incubated at 50° C. for periods of various lengths.

FUNGI	Period of Incubation of soil and cellulose						
	12 hr.	1 day	2 days	3 days	6 days	9 days	12 days
	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA
Aspergillus fumigatus	100			33 22			
Cephalosporium sp.	11	11				44	100
Chaetomium thermophile	33	33	33	33	100	100 44	100 22
Humicola grisea		22	11	11	22	55	77 33
Humicola insolens		33	11	22 11	66	88 44	55
Humicola lanuginosa				11			
Malbranchea pulchella							
Mucor pusillus	11						
Myriococcum albomyces		22	11	22	33	33	11
Sporotrichum thermophile	11	11	33	33 11	100	66 88	77 77
Streptomyces sp.	100	100	100	100	100	100	77 77
Talaromyces duponti							
Thermoascus aurantiacus		11					11

taken over a period of 12 days and inoculated on CA showed a clearly defined development pattern. The incubation at 50°C and the enrichment of the soil with cellulose kept in check the usually fast spore-formers namely Aspergillus fumigatus and Mucor pusillus and at the same time allowed slowly developing fungi which otherwise would not have appeared, to manifest themselves. This phenomenon was very well illustrated by A. fumigatus and Chaetomium thermophile, the former being dominant in the beginning while the latter was dominant after incubation of the soil for 6 days. Similarly Humicola grisea, H. insolens and Sporotrichum thermophile showed a parallel pattern of development. There was a constant rise in the frequency of occurrence of H. grisea and H. insolens starting from the second day to the twelfth and ninth day of incubation respectively and then falling subsequently. Sporotrichum thermophile was quite common in the beginning, having 100% frequency of occurrence after 6 days of incubation, but less frequent later on. Streptomyces spp. were also very common throughout.

Other thermophilic species namely Cephalosporium sp. Mucor pusillus, Myriococcum albomyces and Thermoascus aurantiacus were also isolated on the cellulose medium during the different periods of incubation. Among these only M. pusillus was non-cellulolytic, whereas the others produced clearings on the cellulose agar plates (Eggins and Pugh, 1962)

Inoculations from cellulose-enriched soil on glucose agar yielded a completely different picture. Very few

fungal species were recorded and the non-cellulolytic fungus M. pusillus seemed to have been inhibited by cellulolytic fungi during incubation of the cellulose-enriched soil. Sporotrichum thermophile was the most successful in establishing itself on this medium and had an isolation frequency of 88% after 9 days of incubation.

Inoculations from soil amended with glucose yielded growth of two thermophilic fungi, Talaromyces duponti and Malbranchea pulchella (Table 11). The observations were made both on cellulose and glucose media. Mucor pusillus was quite dominant and attained a frequency of 100% after 3 days of incubation. Talaromyces duponti attained its maximum frequency after 6 days and was dominant throughout on glucose medium. Aspergillus fumigatus was only common in the beginning but did not make its appearance after three days on GA and six days on CA. Other fast sporing late developing fungi may have inhibited it. Malbranchea pulchella was not common but just made its appearance occasionally both on glucose and cellulose media. Sporotrichum thermophile was isolated once on cellulose medium after 12 days and T.aurantiacus occurred both on cellulose and glucose media. T. aurantiacus produced a slight clearing on cellulose agar.

The isolation of nearly all confirmed thermophiles from one locality indicates a wide distribution of these fungi in the soil. This has also been confirmed by more recent investigations by other workers (Apinis 1963, Yung Chang 1967, Fergus 1969) The present results

Table 11

Percentage frequency recorded in cultures derived from 9 portions of one sample of soil after 7 days incubation at 50° C. of fungi appearing on cellulose agar (CA) and glucose agar (GA) inoculated with glucose-amended soil incubated at 50° C. for periods of various lengths.

FUNGI	Period of Incubation of Soil and Glucose						
	12 hr.	1 day	2 days	3 days	6 days	9 days	12 days
	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA	CA GA
Aspergillus fumigatus	80 60	60 60	60 60	60 40	40		
Cephalosporium sp.							
Chaetomium thermophile							
Humicola grisea	20			20 20	40	80	40
Humicola insolens		20				20	40
Humicola lanuginosa							
Malbranchea pulchella	20		20			20	
Mucor pusillus	20	20 20	80 80	80 100	100 100	100 100	80 80
Myriococcum albomyces							
Sporotrichum thermophile							20
Talaromyces duponti	20	20	20	20	40 100	60 100	100 100
Thermoascus aurantiacus	20	20			60		40

demonstrate the advantage of soil enrichment and incubation at 50°C for the detection and isolation of thermophilic fungi. As long as the temperature of the soil is low, all the thermophilic fungi cannot be isolated on a simple sugar medium using Warcup's technique. The sugar medium favours fast growing non-cellulolytic sugar fungi which do not allow growth of slow growing fungi or those which need some form of activation to enable the fungus to manifest itself. Most of the thermophilic fungi have been found to be cellulolytic to some extent and this renders it difficult to isolate them from soil on glucose media. The enrichment of soil with cellulose and glucose and incubation at 50°C provided favourable conditions for both cellulolytic and sugar fungi and thus facilitated isolation.

The fungal colonisation of cellulosic substrates was also studied at 50°C with the help of the perfusion technique. The colonisation of the cellulose strip started after 1 day of incubation (Table 12); deterioration of the strip proceeded very rapidly as the polythene backing of the polythene backed cellulose strip could be observed after 16 days of incubation at 50°C.

Among the early colonisers, C.thermophile made its appearance after 1 day of incubation and had a high frequency of occurrence. Whereas H.insolens also made its appearance after 1 day of incubation but had a very low frequency of occurrence. H. grisea made its appearance after 5 days of incubation when C. thermophile and H. insolens had quite high frequency of occurrence.

Table 12

Percentage frequency of fungi colonizing the cellulosic substrate perfused with E. & P. salt solution at 50° C.

FUNGI	Days of Incubation							
	1	2	3	5	7	9	12	16
Aspergillus fumigatus					16	32		
Cephalosporium sp.				40		16		
Chaetomium thermophile	60	60	100	100	100	100	100	100
Humicola grisea				80		100	100	100
Humicola insolens	16			100	60	80	100	80
Humicola lanuginosa					32	16		
Mucor pusillus								50
Sporotrichum thermophile							32	
Torula thermophile							16	
Streptomyces sp.							100	100

H. grisea was quite dominant after its first isolation. A. fumigatus also appeared between 7 & 9 days but had a very low frequency of occurrence. H.lanuginosa was also isolated at the same time. It was always found along with C. thermophile and therefore it was difficult to isolate H.lanuginosa in pure culture. This species proved to be non cellulolytic when grown on cellulose agar as it did not produce any clearing.

When the cellulose strip was fairly deteriorated a Streptomyces sp.was observed growing on the paper and was found to be producing pits on the strip. It was isolated between 12 and 15 days of incubation and had 100% frequency of occurrence. At the same time a variety of fungi were isolated. Among them were Sporotrichum thermophile, Torula thermophile and Mucor pusillus. Of these fungi only M. pusillus is non cellulolytic. Its appearance on the strips when the strip was completely deteriorated suggests the possible utilization of the breakdown products of cellulose by this fungus.

The comparative results of isolations at 25°C 35°C and 50°C with the help of the perfusion technique and cellulose enrichment method are summarized in Table 13. It is evident from these results that each temperature has its own fungal flora. There are some fungal species which occur at more than one temperature range. A. fumigatus was found to occur at the three different temperatures investigated, but it had a different frequency of occurrence at these temperatures. The percentage frequency was 94-100 at 35°C whereas at 25°C and 50°C it was 1-19; C. globosum was isolated at 25°C by using cellulose enriched soil; C. elatum was isolated

Table 13

Comparison of the percentage frequency of occurrence of fungi isolated at different temperatures by the soil enrichment method and perfusion technique.

FUNGI	25° C.		35° C.		50° C.	
	SE*	PT+	SE	PT	SE	PT
Arthrobotrys sp.	2	22				
Aspergillus fumigatus Fresenius	19	1	100	94	19	6
Cephalosporium sp.			1		17	7
C. elatum Kunze ex Fries			2	2		
Chaetomium globosum Kunze	30					
C. thermophile la Touche					62	90
Coniothyrium fuckeli Saccardo		5		5		
Dactylella sp.		2				
Dicoccum asperum Corda	30					
Eurotium sp.			1	6		
Fusarium moniliforme Sheldon		9				
Fusarium solani (Martius) Appel & Wollenweber	96	93				
Gliocladium roseum (Link) Thom.	5	7				
Graphium sp.				8		
Humicola insolens Cooney et Emerson					30	51
H. grisea Traaen	32	7		5	28	48
H. lanuginosa (Griff. et Maubl.) Bunce						6
Mucor globosus Fischer	42	9				
M. pusillus Lindt					1	6
P. elegans (Corda) Mason et Hughes apud Hughes (1951)	5					
Paecilomyces varioti Bainer	2	3				
Papulaspora sp.		3				
Penicillium sp.		3			6	
Rhizopus sp.	6					
Sordaria fimicola (Rob.) Ces & De Not		8				
S. pruinosum Gilman & Abbott			6	10		
Sporotrichum thermophile Apinis					41	4
Streptomyces spp.	60		26	48	96	22
Torula thermophile Cooney & Emerson						2
Trichoderma viride Pers. ex Fr.	57	54		3		
Zygorhynchus moelleri Vuillemin		7				

* Soil enrichment

\+ Perfusion technique

at 35° C. and C. thermophile occurred at 50° C. H. grisea was also isolated at all the temperatures. It had extremely low frequency of occurrence at 35° C. and was only isolated with the help of the perfusion technique. H. grisea, isolated at 50° C., was placed as a 'variety' of this species under the name Humicola grisea var. Thermoidea because of its thermophilic habit (Cooney and Emerson, 1964). Streptomyces spp. were also fairly common and these occurred at all temperatures, though those isolated at 50° C. were thermophilic species and had different morphology as well. Trichoderma viride was quite common at 25° C. but it had extremely low frequency of occurrence at 35° C. and was only isolated with the help of the perfusion technique.

The comparative review of the total frequency of isolation of fungi at different temperatures shows that at 35° C. fungi have the least percentage frequency of occurrence. This may be due to two reasons. Firstly, this temperature is at the threshold of mesophilic and thermophilic temperatures, thus neither mesophiles nor thermophiles have their optimum growth at this temperature. Secondly, the only fungal species which seem to have its optimum temperature at 35° C. is A. fumigatus which is a confirmed thermotolerant species (Cooney and Emerson, 1964). As this species is a fast sporer, it colonises the whole substrate more effectively than any other species; thus making the isolation of any other colonisers extremely difficult.

Effect of pH and Temperature on Colonisation of the Cellulose Strip by Fungi

One of the microenvironmental factors affecting cellulose colonisation apart from temperature, is the hydrogen ion concentration of the soil and the substrate. In environments of neutral to alkaline pH, some micro-organisms are capable of growing and liberating the appropriate enzymes for the hydrolysis of the polysaccharides; at acid reaction, the disappearence of cellulose is mediated largely by filamentous fungi. Although the process is rapid below pH 5.0 and occasionally below pH 4.0, soils with lower hydrogen ion concentration degrade cellulose more readily. (Schmidt and Ruschmeyer, 1958). This generalisation that fungi usually grow below neutrality and bacteria are limited to alkaline pH's, has been established on the basis of frequent isolations from the soil. Jensen (1931) confirmed these observations by determining the range of pH for growth in pure cultures of bacteria isolated from the soil. Warcup (1951) investigated the relationship between fungal distribution and soil pH by determining the mycoflora of five soils ranging in pH from the extremes of 8.4 to 3.8. He found that fungi showed different distribution in the five soils and was thus able to recognize two large groups of fungi; those common in the acid soil and those common in the alkaline soils. Species of Penicillium and Trichoderma were particularly abundant in the acid soils. Brown (1958) investigated fungal ecology in sand dunes and also found marked differences in fungal populations between acid and

alkaline dunes. Pugh et al (1963) isolated cellulose decomposing fungal species from sand dunes of near neutrality to alkaline reaction. Pugh (1963) also studied the distribution of fungi in sand flats having pH 7.2 - 8.0. Went and Jong (1966) studied the microbiological decomposition of cellulose in the soil of an oak forest with the reaction ranging from 3.5 - 7.2. They traced cellophane deterioration in seven soil samples having different pH values and found that pH was not a dominant factor in the cellophane breakdown as was shown by the quick decomposition in the acid mull in comparison with the much slower decomposition in the mor, both with the same pH. Previously, Tribe (1960) also studied the decomposition of cellulose film (cellophane) buried in soils having different reactions, with special references to ecology of relevant soil fungi.

The information regarding the influence of pH on soil fungi in general and cellulose decomposing soil fungi in particular has been indirectly accumulated by taking into account the reaction of the soil used, as it is evident from the previous investigations. Siu (1951) has reviewed the pH optima of some fungi growing on cellulose. Siu and Sinden (1951) studied the effect of pH on the cellulolytic activity of five fungi grown on bleached cotton sheeting wetted with nutrient solutions maintained at different buffered pH values. The decrease in tensile strength of the cotton sheeting was taken as the index for cellulolytic activity.

As such investigations are done in "pure culture", it is appropriate to consider how far such results can be

applied to pathways of decomposition in soils (Chesters, 1960). Moreover, the differential absorption of nitrogenous ions such as ammonium and nitrate, may result in a considerable pH change. These changes in turn, may have far reaching effects of a secondary nature on the growth and physiology of fungi (Harley, 1960). The release of metabolic end products into the medium also brings about significant changes in the substrate, which may alter the conditions for the growth of fungi.

In order to overcome these difficulties and to study the effect of pH on the fungal population colonizing a cellulosic substrate in the soil, the perfusion technique has been employed. The pH of the cellulosic substrate is changed by changing the pH of the perfusing nutrient salt solution. As this solution is duly buffered and is continuously perfused, it takes all the metabolic end products away; thus keeping the microenvironment around the cellulosic substrates constant. These studies have been undertaken at different temperatures (25° C., 35° C. and 50° C.) in order to elucidate the pH preferences of a wide range of cellulose decomposing fungi in the soil.

The extent of deterioration of the cellulose strip was recorded by estimating the weight loss of the cellulose strip after different periods of incubations. This is the most direct method, since the excess fungal mycelium can be separated as the substrate is screened by a glass fibre cloth. The weight loss method has previously been employed by some workers (Reese, 1947, Siu, 1951, and Garrett, 1963). This weight loss represents only that

proportion of the cellulose actually respired by the fungus and does not include the further proportion converted into fungal substance (Garrett, 1962).

The different pH values investigated were 4.0, 5.0, 6.0, 6.4 (unbuffered cellulose agar) 7.0 and 8.0. The perfusing solution used in these studies was E. & P. salt solution. It has an initial pH of 5.5 and can be adjusted to different pH levels by adding 0.5 N HCl or 0.5 N NaOH (Siu and Sinden, 1951). The solution is then buffered with McIlvaine's buffer containing phosphate and citric acid; the buffers are 0.05M with respect to the phosphate ion concentration.

In order to allow enough replication sixty perfusion sets were set up for each pH value. Four perfusion sets were sacrificed every time for each pH value; out of these, three sets were used for weight loss estimation and the fourth set was used for the isolation of fungi colonising the cellulosic strip. For the weight loss estimation, 7 cms. length of the 5 mm. wide cellulose strip from the perfusion set was cut and oven dried at 120° C. for twelve hours and then weighed. The difference between this weight and that of the control, where no soil had been inoculated was taken as the weight loss.

The cellulose strip was released from the glass fibre sleeving, cut aseptically into small pieces and then inoculated onto six petri dishes containing E. & P. cellulose agar. Four small pieces of cellulose strip were normally inoculated on the cellulose agar petri dish. Out of these six replicate petri dishes, three were buffered

at the same pH as the perfusing solution and the other three were at the normal pH of 6.4.

These inoculated petri dishes were incubated at the same temperature as the perfusion sets and were observed for growth after 7 days of incubation. Frequency of occurrence of a species was determined by recording its presence or absence in each replicate petri dish.

These investigations on the effect of pH were carried at three different temperatures, i.e. 25° C., 35° C. and 50° C. The results have been recorded here under the heading of each temperature.

25° C.

The fungi isolated showed a wide range of pH tolerance but the optimal pH could easily be determined by reviewing the results comparatively. The hydrogen ion concentration also influences deterioration of the cellulose strips.

The initial colonization of cellulose strip perfused with E & P salt solution at pH's 4.0, 5.0, 6.0, 6.4 and 7.0 started as early as 12 hours of incubation, whereas it was one day for pH 8.0. Among the early colonisers, Zygorhynchus moelleri and Mucor globosus were isolated after one day of incubation at pH 4.0, 5.0 and 6.0 only (Table 14a - f). Z. moelleri was the dominant species at pH 4.0. It disappeared after three days and then again made its appearance when the cellulose strip was nearly completely deteriorated. M. globosus was isolated at all pH values but in quite low frequency. Though these organisms were non cellulolytic as demonstrated by E & P cellulose agar (Eggins and Pugh, 1962) they were presumably able to make their appearance by utilising the yeast

extract and L-asparagine from the E & P salt solution in the beginning and then might be living on the breakdown products of the cellulose and of the cellulolytic organisms.

Trichoderma viride and Fusarium solani were found to be the main fungi involved in the deterioration of perfused cellulose strip buried in soil. Fusarium solani was isolated after 12 hrs. at all pH levels except pH 8.0 and was most frequently isolated at all times. T. viride was also similar in occurrence but had a comparatively lower frequency.

Other cellulolytic fungi were isolated between six and eighteen days of incubation. These fungi were Gliocladium roseum, Humicola grisea, Paecilomyces varioti, Arthrobotrys sp., and Dactylella sp. After 6 days nematodes were found on the cellulose strip. This is significant since Arthrobotrys sp., and Dactylella sp., are nematophagous and were found to be cellulolytic as well. G. roseum and P. varioti were found to be absent at pH 4.0 and 8.0 and later was in a low frequency at pH 7.0. G. roseum was isolated after 6 and 9 days of incubation at pH 6.0 but it appeared between 21 and 35 days at pH 5.0, 6.4 and 7.0. Gelasinospora cerealis made its appearance after 3 days at pH 4.0, 5.0, 6.0 and 6.4. Fusarium moniliforme just made its appearance at pH 5.0 and 7.0 between 6 and 12 days of incubation whereas it was isolated between 15 and 28 days of incubation at pH 6.0 and 6.4. It was not isolated at pH 8.0. Papulaspora sp. was isolated at pH 6.0 and 7.0. When the strip was almost completely broken down, a variety of fungi were isolated, e.g. Paecilomyces elegans, Penicillium sp., Aspergillus fumigatus, Coniothyrium fuckeli.

Table 14a

Percentage frequency of the fungi colonising the cellulose strip perfused with E & P salt solution at pH 4.0.

FUNGI	Days of Incubation											
	12 hrs.	1	2	3	6	9	12	15	18	21	28	35
Arthrobotrys sp.						16						
Aspergillus fumigatus									16			
Coniothyrium fuckeli									16			
Dactylella sp.												
Fusarium solani	50	64	64	64	50	50	50	50	84	84	84	84
Fusarium moniliforme												
Gelasinospora cerealis				32								
Gliocladium roseum												
Mucor globosus				50	50						16	
Paecilomyces elegans											50	
Paecilomyces varioti												
Papulaspora sp.												
Penicillium sp.	16	16										
Trichoderma viride	16	32	32	50	32	32	50	50	50	64	50	84
Zygorhynchus moelleri		50	50	64	64	100	100	100	84	64	50	50

Table 14b

Percentage frequency of the fungi colonising the cellulose strip perfused with E & P salt solution at pH 5.0.

FUNGI	Days of Incubation											
	12 hr.	1	2	3	6	9	12	15	18	21	28	35
Arthrobotrys sp.							84	16	16	32		
Aspergillus fumigatus										16		
Coniothyrium fuckeli												
Dactylella sp.					50				50		16	
Fusarium solani	64	50	50	64	84	84	64	50	100	100	100	100
Fusarium moniliforme					16	16	16					
Gelasinospora cerealis				16								
Gliocladium roseum											64	
Mucor globosus	16		16							16	50	
Paecilomyces varioti												
Paecilomyces elegans									50			
Papulaspora sp.												
Penicillium sp.												
Trichoderma viride				16	50	50	50	50	100	64	50	50
Zygorhynchus moelleri		16	50	64	32				32			50

Table 14c

Percentage frequency of the fungi colonising the cellulose strip perfused with E & P salt solution at pH 6.0.

FUNGI	Days of Incubation											
	12 hr.	1	2	3	6	9	12	15	18	21	28	35
Arthrobotrys sp.					32			16			100	100
Aspergillus fumigatus												16
Coniothyrium fuckeli												
Dactylella sp.									64			
Fusarium solani	64	100	100	84	100	100	100	100	84	100	50	50
Fusarium moniliforme								16		32	32	
Gelasinospora cerealis				32	32							
Gliocladium roseum					32	16				16		
Mucor globosus		16		16		16	32		16	16	50	
Paecilomyces elegans												
Paecilomyces varioti							32					
Papulaspora sp.										32		
Penicillium sp.								16				
Trichoderma viride		50	32	32	32	50	64	64	64	50	50	16
Zygorhynchus moelleri		16		50					16			

Table 14d

Percentage frequency of the fungi colonising the cellulose strip perfused with E & P salt solution at pH 6.4 (E & P salt solution unbuffered).

FUNGI	Days of Incubation											
		1	2	3	6	9	12	15	18	21	28	35
Arthrobotrys sp.					64	100	100					
Aspergillus fumigatus												16
Coniothyrium fuckeli												
Humicola grisea												
Fusarium solani	64	100	100	100	100	100	100	100	100	100	100	64
Fusarium moniliforme								48	32	32		
Gelasinospora cerealis				64	16	16						
Gliocladium roseum										32	32	16
Mucor globosus			16	16	50		32					
Paecilomyces elegans												
Paecilomyces varioti							32					
Papulaspora sp.											32	
Penicillium sp.										16	16	
Trichoderma viride	16	80	80	64	64	84	32	84	100	84	64	32
Zygorhynchus moelleri				64								

Table 14e

Percentage frequency of the fungi colonising the cellulose strip perfused with E & P salt solution at pH 7.0.

FUNGI	Days of Incubation											
	12 hr.	1	2	3	6	9	12	15	18	21	28	35
Arthrobotrys sp.								32	32			
Aspergillus fumigatus										16		
Coniothyrium fuckeli												
Humicola grisea												
Fusarium solani	64	100	100	100	64	64	100	84	100	100	100	100
Fusarium moniliforme		50			50	32	16	32				
Gelasinospora cerealis												
Gliocladium roseum											64	32
Mucor globosus	16				16		32				16	16
Paecilomyces elegans											16	32
Paecilomyces varioti								16				
Papulaspora sp.		50								16		
Penicillium sp.												16
Trichoderma viride		32		32	32	50	50	64	32	16	16	32
Zygorhynchus moelleri									32			16

Table 14f

Percentage frequency of the fungi colonising the cellulose strip perfused with E & P salt solution at pH 8.0.

FUNGI	Days of Incubation											
	12 hrs.	1	2	3	6	9	12	15	18	21	28	35
Arthrobotrys sp.					16	16		84	100			
Aspergillus fumigatus												16
Coniothyrium fuckeli												
Humicola grisea												
Fusarium solani												
Fusarium moniliforme												
Gelasinospora cerealis		50	50	32	32	32	50	64	64	64	100	16
Gliocladium roseum												
Mucor globosus										18		
Paecilomyces elegans												32
Paecilomyces varioti												
Papulaspora sp.												
Penicillium sp.												
Trichoderma viride		16	16	16	32	32	16	16	16			
Zygorhynchus moelleri												

Table 15

Isolation of fungi on E & P's cellulose agar at different pH values using Warcup's method.

FUNGI	% frequency of occurrence					
	pH 4.0	5.0	6.0	7.0	8.0	6.4*
Chaetomium globosum	16	16	16	16	32	
Diococcum asperum		16	16			
Fusarium solani	50	85	64	64	85	64
Gelasinospora cerealis						16
Gliocladium roseum	100	100	100	100	100	85
Gliomastix convoluta			64			
Humicola grisea	64	100	50	85	100	85
Mucor globosus	85	64				
Paecilomyces elegans	50	16	64	64		16
Papulaspora sp.				50		16
Stysanus sp.				16		
Trichoderma viride	100	100	85	85		85
Zygorhynchus moelleri	64					

* unbuffered

In order to compare these results with simple soil isolations, Warcup's method was also used (Warcup, 1950). Soil inoculations were made on E. & P.'s cellulose agar maintained at different pH values. Six replicate petri dishes were inoculated for each pH value. The observations for fungal growth on these petri dishes were made after seven days of incubation at 25° C. Frequency of occurrence of a species was determined by recording its presence or absence in each petri dish.

The isolation results obtained by Warcup's method are summarised in Table 15. Trichoderma viride, Fusarium solani, Gliocladium roseum, Humicola grisea and Chaetomium globosum occurred at all pH levels (pH 4-8). Out of these, G. roseum was most frequently isolated at all pH values as reported by Pugh and Dickenson, (1965). Paecilomyces elegans also made its appearance on all pH levels except pH 8.0. Most of the cellulolytic fungi occurred more frequently on cellulose agar petri dishes maintained at pH 7.0 and unbuffered normal pH (6.4).

The pH preferences of the fungi isolated from the cellulose strip are represented in Figure 1a and 1b. The percentage frequency of occurrence of each species is totalled and then divided by the number of times sacrificing was made, to give the average percentage frequency of occurrence.

The weight loss results of the cellulose strip perfused with nutrient solution at different pH values are summarised in Tables 16a - 16f. The comparative results of all pH values are also represented in Figure 2. The maximum deterioration took place at pH 5.0 - 6.0. At pH 6.0 percentage

Figure 1a

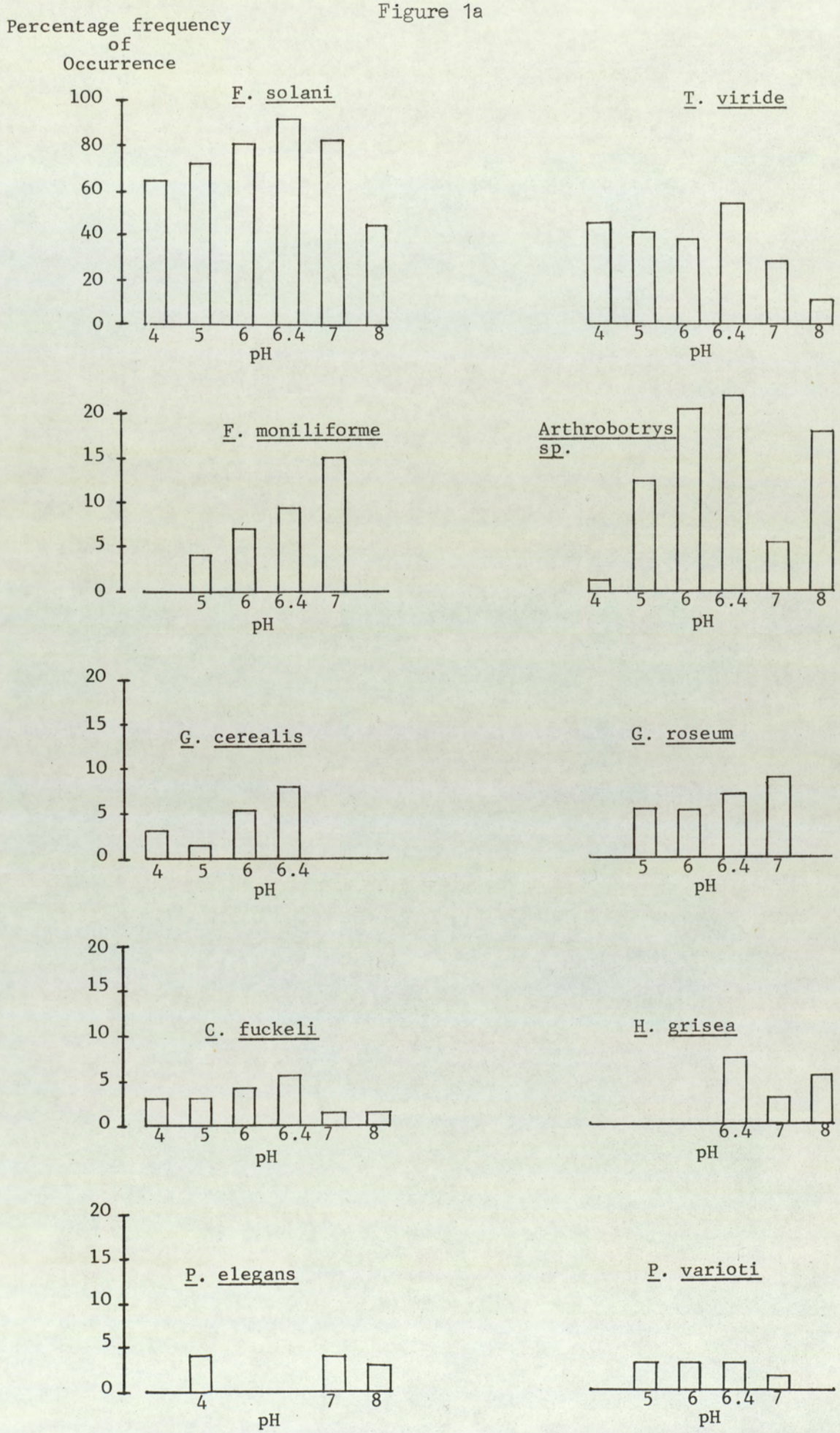

Percentage frequency of Occurrence
F. solani
T. viride
F. moniliforme
Arthrobotrys sp.
G. cerealis
G. roseum
C. fuckeli
H. grisea
P. elegans
P. varioti
pH

Figure 1b

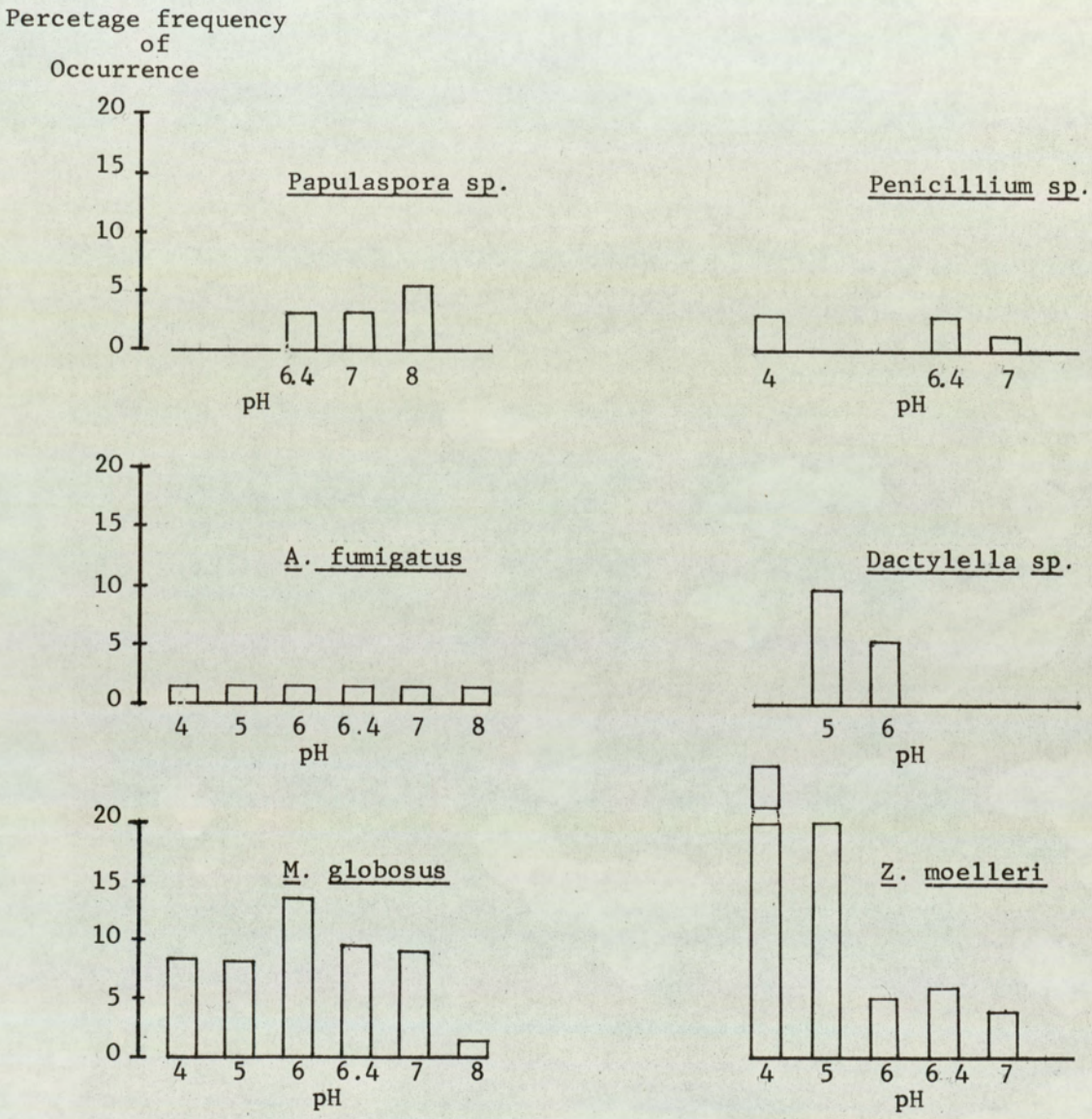


Figure 1a-b: Average percentage frequency of occurrence of fungi isolated from cellulose strips perfused with E.&P. salt solution at different pH values after 30 days of incubation at 25°C.

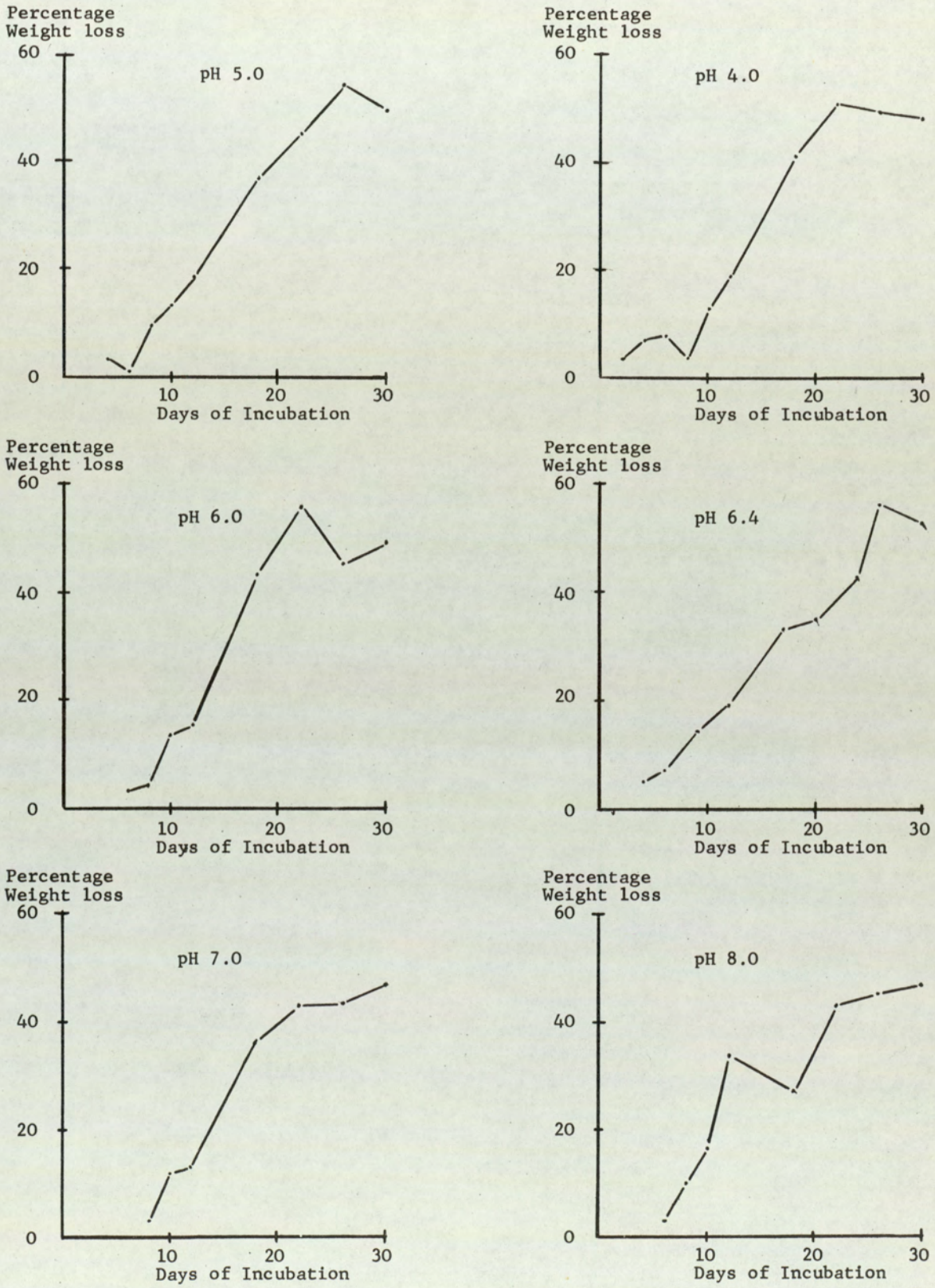


Figure 2: Average percentage weight loss of the cellulose strip perfused with E.&P. saltsoution at different pH values and incubated at 25°C.

Table 16 a-f

Percentage weight loss of the cellulose strip perfused with E. & P. salt solution at different pH values.

(a) pH 4.0

DAYS OF INCUBATION	% WEIGHT LOSS			MEAN	S.D. *
2	3.9	4.1	3.3	3.7	0.4
4	6.2	7.3	6.9	6.8	0.5
6	6.9	3.1	1.8	3.9	2.6
8	6.9	3.1	1.8	3.9	2.6
10	12.4	12.3	14.1	12.9	1.0
12	17.6	19.2	20.1	18.9	1.2
18	42.2	43.4	38.6	41.4	2.4
22	50.4	49.7	53.1	51.0	1.7
26	48.7	49.3	51.7	49.9	1.5
30	46.8	45.6	52.1	48.1	3.4

* Standard Deviation

Table 16

(b) pH 5.0

DAYS OF INCUBATION	% WEIGHT LOSS			MEAN	S.D.*
4	4.3	3.0	2.9	3.4	0.7
6	1.9	0.7	0.0	0.86	0.9
8	9.9	11.3	8.1	9.7	1.6
10	13.4	14.2	12.8	13.4	0.7
12	16.5	18.9	20.0	18.4	1.7
18	37.5	38.5	34.7	36.9	1.9
22	45.2	43.8	47.1	45.3	1.6
26	54.3	53.4	54.6	54.1	0.6
30	48.3	49.3	51.6	49.7	1.6

* Standard Deviation

Table 16

(c) pH 6.0

DAYS OF INCUBATION	% WEIGHT LOSS			MEAN	S.D.*
6	2.7	3.0	2.9	2.8	0.1
8	3.1	4.2	4.8	4.0	0.8
10	13.2	14.3	13.9	13.8	0.5
12	13.2	16.3	15.3	14.9	1.5
18	42.9	44.1	43.4	43.4	0.6
22	56.6	56.1	55.2	55.9	0.7
26	44.8	44.5	46.0	45.1	0.7
30	46.9	48.1	54.0	49.6	3.8

* Standard Deviation

Table 16

(d) pH 6.4

DAYS OF INCUBATION	% WEIGHT LOSS			MEAN	S.D.*
4	2.5	4.2	6.7	4.4	2.1
6	8.8	7.1	6.1	7.3	7.1
8	21.9	7.8	12.7	14.1	7.1
12	15.4	23.2	19.2	19.2	3.9
17	33.4	39.1	26.9	33.1	6.1
20	28.6	35.8	40.8	35.0	6.1
24	37.2	42.4	47.7	42.4	5.2
26	63.0	57.8	49.0	56.0	7.0
30	57.8	47.6	53.7	53.0	5.1

* Standard Deviation

Table 16

(e) pH 7.0

DAYS OF INCUBATION	% WEIGHT LOSS			MEAN	S.D.*
8	2.8	1.9	4.5	3.0	1.3
10	11.9	12.8	14.1	12.9	1.1
12	10.8	13.1	15.5	13.1	2.3
18	34.5	36.4	37.7	36.2	1.6
22	42.1	41.8	45.3	43.0	1.9
26	42.1	45.8	43.0	43.6	1.9
30	48.4	49.4	44.0	47.2	2.8

* Standard Deviation

Table 16

(f) pH 8.0

DAYS OF INCUBATION	% WEIGHT LOSS			MEAN	S.D.*
6	2.9	4.1	1.8	2.9	1.1
8	10.6	11.3	8.1	10.0	1.6
10	15.4	18.1	16.1	16.5	1.4
12	31.8	33.1	26.4	30.4	3.5
18	23.4	28.4	31.4	27.7	4.0
22	42.7	43.5	45.1	43.7	1.2
26	45.9	47.3	42.8	45.3	2.3
30	45.6	48.4	47.3	47.1	1.4

* Standard Deviation

weight loss reached 55.9 in 22 days of perfusion and incubation at 25° C., whereas at pH 5.0 and 6.4 (unbuffered initial pH of E & P cellulose agar) the maximum weight loss was 54.1% and 56.0% after 26 days of incubation. The weight loss tends to decrease after reaching the maximum most probably due to the accummulation of fungal spores and mycelia. At pH 4.0 the maximum weight loss of 51.0% was achieved after 22 days but after 30 days it fell to 48.1%. The weight loss estimations at pH 7.0 and 8.0 did not show much decrease in weight loss but had a maximum weight loss of 47.2% and 47.1% respectively after 30 days of perfusion and incubation.

The fungal colonisation results obtained by the perfusion technique show some kind of colonisation pattern. The results show that there are distinct early colonisers namely Trichoderma viride and Fusarium solani, which were isolated as early as 12 hours and were throughout the experiment frequently isolated. Other fungal species namely Gelasinospora cerealis, Arthrobotrys sp., Gliocladium roseum were normally isolated between 6 and 18 days. A variety of fungi were isolated when the strip was partially or completely deteriorated.

The comparison of the list of fungi isolated by Warcup's method with those isolated by the perfusion technique shows the absence or extremely low frequency of occurrence of certain fungi like Chaetomium globosum, Gliomastix convoluta, Stysanus sp., Dioccum asperum from the list of perfusion technique isolations. Among these fungi C. globosum is most conspicuous because of its accepted cellulolytic nature and its use as a test fungus

in many experiments. The inability of these fungi to colonise the cellulose strip may be attributed to the unfavourable experimental conditions or due to the interaction of different organisms or microenvironmental conditions in the soil; since the behaviour of an organism in pure culture may give little information as regards its reaction in nature.

The other discrepancy in the two lists is the absence of Arthrobtrys sp., and Dactylella sp., from the list of fungi obtained by Warcup's method. Though these fungi come under Garrett's definition of ' soil inhabiting ' fungi (Garrett 1956) their appearance always in association with nematodes has once again pointed towards their nematophagous nature. The most likely explanation of this association of organic matter and predacious activity is given by Linford (1937). According to him the addition of organic matter produces an increase in the number of free living soil nematodes which in turn stimulates the nematophagous fungi to greater activity. This agrees with our observations in the laboratory that these fungi are most active when nematodes are present in abundance. Later investigators (Duddington, 1955, Cooke 1962, Pramer & Kuyama 1963, Olthof and Estey 1966) also tend to confirm this association.

35°C

The colonisation of the cellulose strip at this temperature proceeded comparatively slowly. The number of species isolated was quite small and some of them had quite a low frequency of occurrence.

The initial colonisation started as early as 1 day of incubation (Table 17a-b) Aspergillus fumigatus was observed growing from the edges of the cellulose strip.

Table17a

Percentage frequency of fungi colonizing the perfused cellulose strip at pH 4.0, 5.0 and 6.0 at 35° C.

FUNGI	pH	Days of Incubation										
		1	2	3	6	8	10	12	15	18	24	30
Aspergillus fumigatus	4.0	32	64	64	64	100	100	100	100	80	100	100
	5.0	32	32	64	100	100	100	100	100	100	100	100
	6.0	64	100	100	100	100	100	100	100	100	100	100
Streptomyces sp.	4.0			16			50					32
	5.0		16	32	32		90			64		32
	6.0				32	80	100	100	90	100	64	64
Chaetomium elatum	4.0											
	5.0		16									
	6.0			16								
Mucor sp.	4.0											
	5.0			64	64			16	16			
	6.0											
Trichoderma viride	4.0											
	5.0											
	6.0				16			16				
Humicola grisea	4.0											
	5.0											
	6.0						16		16	16		
Eurotium sp.	4.0											
	5.0											
	6.0							16	33	16		
Penicillium sp.	4.0											
	5.0											
	6.0								64	34		
Sporotrichum pruinosum	4.0											
	5.0			16	33							100
	6.0											
Sorderin fimicola	4.0											
	5.0											
	6.0			16	33							

Table 17b

Percentage frequency of fungi colonizing the cellulose strip at pH 6.4, 7.0 and 8.0 at 35° C.

FUNGI		Days of Incubation										
	pH	1	2	3	6	8	10	12	15	18	24	30
Aspergillus fumigatus	6.4	60	80	100	100	100	100	100	100	100	100	100
	7.0	16	50	32	100	100	100	100	100	100	100	100
	8.0	64	84		100	100	50	69	100	100	64	100
Streptomyces sp.	6.4		30	30	60	100	80	80	100			
	7.0	64	16	32					16	64		
	8.0	16	16				16			32		
Graphium sp.	6.4										50	32
	7.0								16		100	50
	8.0								100		64	50
Humicola grisea	6.4							16			32	32
	7.0		16									
	8.0			16					64			
Penicillium sp.	6.4									64		
	7.0									33	64	
	8.0											
Arthrobotrys sp.	6.4											
	7.0											50
	8.0											
Sporotrichum pruinosum	6.4									32	64	
	7.0										50	64
	8.0											
Trichoderma viride	6.4				16			16				
	7.0				16			32				
	8.0											
Coniothyria fuckeli	6.4										50	
	7.0					32						16
	8.0											
Mucor sp.	6.4											
	7.0					16						
	8.0											

It reached its maximum frequency of occurrence of 100% after 6 days of incubation at all pH values. A. fumigatus remained dominant throughout the experiment and had a profuse growth on the cellulose strip. Streptomyces sp. was also isolated after 1 day of incubation at pH7 and 8, after 2 days at pH 5, 6 & 6.4 and after 3 days of incubation at pH 4.0. This species was subsequently frequently isolated at pH 6-8. Chaetomium elatum was isolated once after 2 days at pH 5.0 and after 3 days at pH 6.0. Mucor sp.was also occasionally isolated between 3 & 15 days of incubation at pH 5.0 only. Humicola grisea was only isolated at pH 6.0, 7.0 & 8.0 between 2 and 18 days of incubation. Its frequency of occurrence was quite low at all the pH values. Eurotium sp. was isolated only at pH 6.0 and 6.4 between 8 and 18 days of incubation and perfusion. Trichoderma viride was also isolated twice at pH 6.0 and 7.0 after 6 and 12 days of incubation. Sordaria fimicola was isolated at pH 6.0 only after 3 and 6 days of incubation.

At the end of the experiment, when the cellulose strip was fairly deteriorated a variety of species were isolated namely Graphium sp at pH 6.4, 7.0 & 8.0, Penicillum sp. at pH 6.0, 6.4 and 7.0,Sporotrichum pruinosum at pH 5.0, 6.0, 6.4 and 7.0. These species were comparatively higher in their frequency of occurrence.

The average percentage frequency of occurrence is calculated as previously and is represented in the Figure 3 It is quite evident that the least number of fungi have been isolated at pH 4.0 whereas most of the fungal species isolated occur at pH 6.0 - 7.0. The percentage frequency of occurrence of all the species isolated is very low as

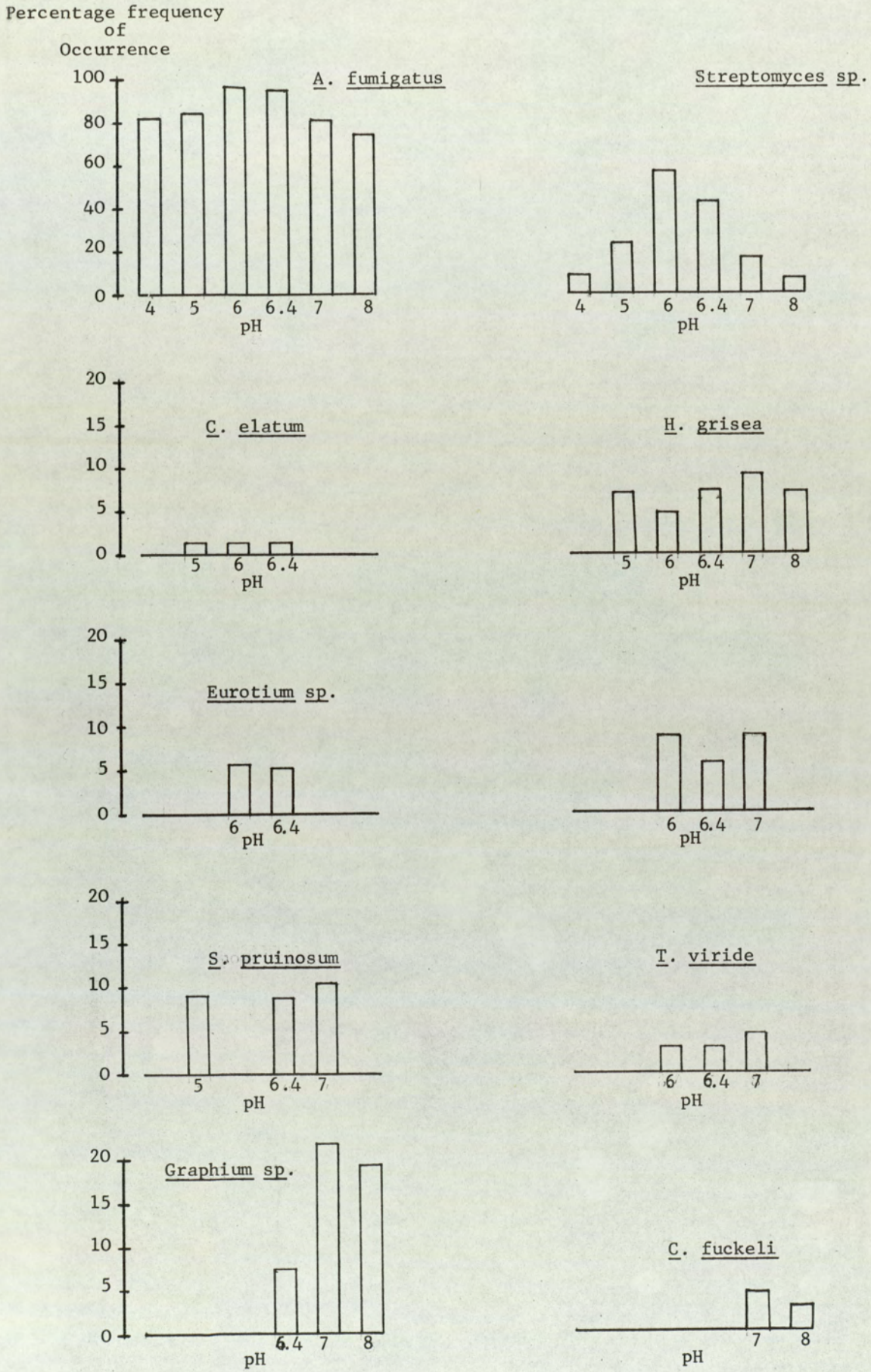


Figure 3: Average percentage frequency of occurrence of fungi isolated from cellulose strip perfused with E.&P. salt solution at different pH values after 30 days of incubation at 35°C.

compared to that of A. fumigatus. The fast growing ability of this fungus might be responsible for its dominance. Moreover, previously the comparative review of isolations at different temperatures has shown 35° C. to be its optimum temperature.

The deterioration of the cellulose strip as measured by the weight loss estimations (Tables 18a-f), has also shown that the weight loss is quite small as compared to the similar studies at 25° C. Maximum deterioration of the cellulose strip took place at pH 6.4 and 7.0; percentage weight loss being 50.7 and 53.0 respectively. The weight loss at other pH values ranged from 20.0% to 33.0%. The minimum percentage weight loss was produced at pH 4.0 (Fig 4).

The colonisation patterns of fungi at this temperature are not as clear as at 25° C. At.pH 4.0 and 5.0 very few fungal species were isolated. Among the early colonisers A. fumigatus and Streptomyces sp. were isolated from all pH values after 1-3 days of incubation at 35° C. H. grisea, Eurotium sp., T. viride and S. fimicola were isolated at pH 6-7 after 6-18 days of incubation. The late developers comprised S. pruinosum, Penicillium sp. and Graphium sp. Arthrobotrys sp. was isolated once after 30 days of incubation. Mucor sp. was also occasionally isolated at pH 5.0 and 7.0. At pH 5.0 it was quite high in frequency of occurrence after 3 and 6 days of incubation. It was only isolated once at pH 7.0 after 6 days of incubation.

All the fungi isolated at this temperature except Mucor sp. produced clearing of the cellulose agar. A. fumigatus proved to be quite cellulolytic but it did not produce as much weight loss of the cellulose strip. Penicillium sp. normally produced a pink pigmentation on the cellulose strip, whereas Eurotium sp.

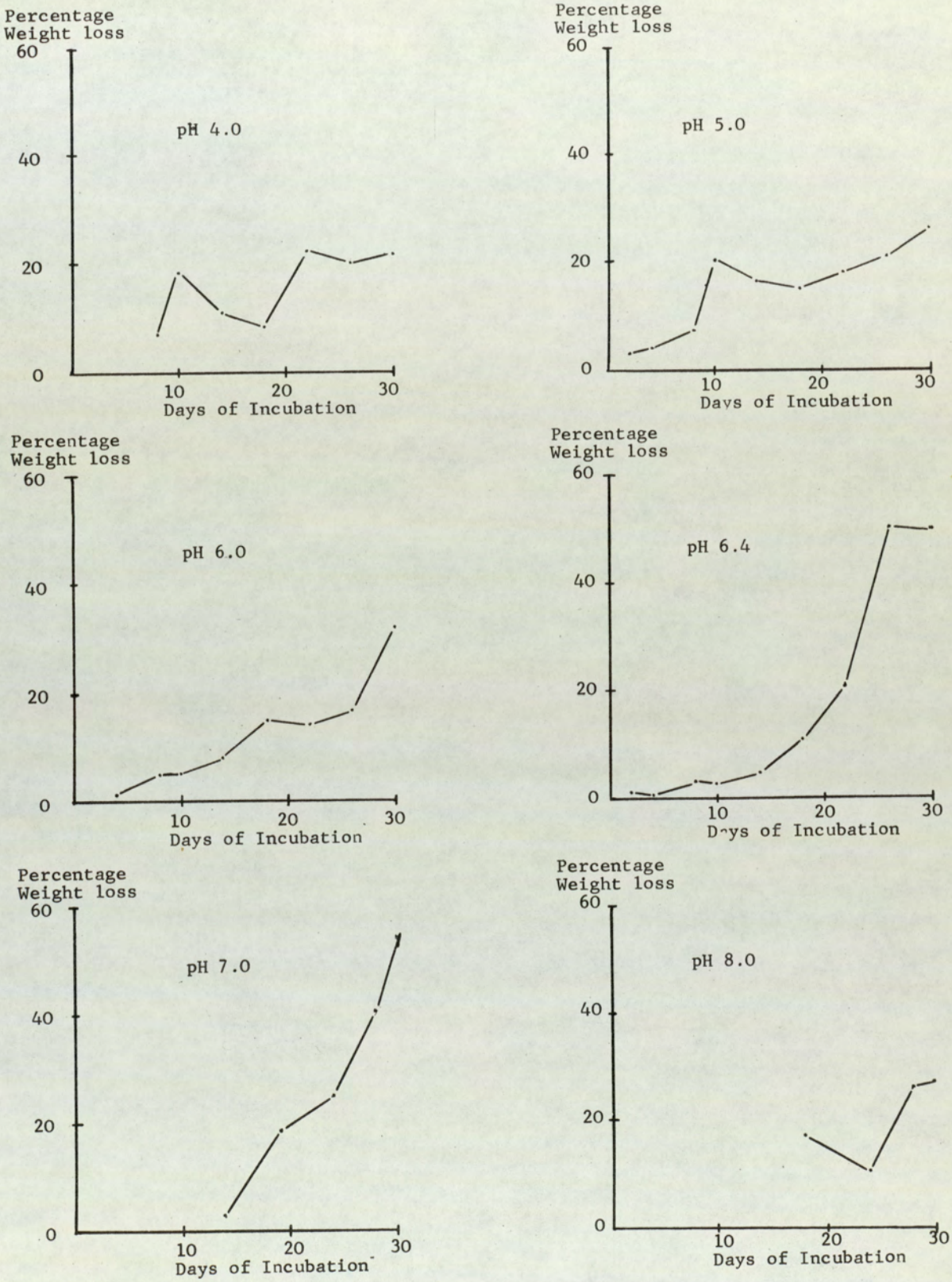


Figure 4: Average percentage weight loss of the cellulose strip perfused with E.&P. salt solution at different pH values and incubated at 35°C.

Table 18 (a-f)

a) pH 4.0

Percentage weight loss of the cellulose strip perfused with E & P salt solution at different pH values at 35° C.

Days of Incubation	% Weight Loss			Mean	S.D.
8	7.3	6.8	7.6	7.2	0.4
10	21.1	15.4	18.6	18.3	2.8
14	8.0	12.1	15.1	11.7	3.5
18	6.6	8.7	9.8	8.3	1.6
22	25.6	23.4	18.0	22.3	3.9
26	24.6	18.1	17.8	20.1	3.8
30	20.9	23.5	21.6	22.0	1.3

Table 18

b) pH 5.0

Days of Incubation	% Weight Loss			Mean	S.D.
2	3.8	4.1	3.3	3.7	0.4
4	3.6	4.9	3.7	4.0	0.7
8	5.9	6.7	9.3	7.3	1.7
10	23.3	20.9	18.1	20.7	2.6
14	12.0	16.4	17.3	15.2	2.8
18	14.2	15.4	13.9	14.5	0.7
22	18.4	19.2	16.5	18.0	1.3
26	23.1	20.9	18.5	21.2	2.0
30	27.4	24.3	28.1	26.6	2.0

Table 18

c) pH 6.0

Days of Incubation	% Weight Loss			Mean	S.D.
4	1.9	2.3	1.3	1.8	0.5
8	5.8	4.4	4.9	5.0	0.7
10	4.3	5.4	5.8	5.1	0.7
14	7.6	8.6	8.1	8.1	0.4
18	15.7	13.1	16.3	15.0	1.7
22	13.4	13.9	16.4	14.5	1.6
26	17.4	18.1	16.9	17.4	0.6
30	33.2	34.1	31.0	32.7	1.5

Table 18

d) E & P salt solution unbuffered (pH 6.4)

Days of Incubation	% Weight Loss			Mean	S.D.
2	0.7	1.3	1.9	1.3	0.5
4	0.1	0.8	1.6	0.8	0.7
8	3.3	3.5	4.3	3.7	0.5
10	2.1	1.9	3.8	2.6	1.0
14	3.5	4.6	5.3	4.4	0.9
18	10.3	9.4	14.1	11.2	2.4
22	21.2	20.0	23.6	21.6	1.8
26	50.3	50.4	51.6	50.7	0.7
30	50.3	52.1	49.3	50.5	1.4

Table 18

e) pH 7.0

Days of Incubation	% Weight Loss			Mean	S.D.
14	2.5	2.8	3.5	2.9	0.5
18	22.3	18.1	15.4	18.6	3.4
24	25.4	27.2	23.1	25.2	2.0
28	38.2	39.4	45.6	41.0	3.9
30	53.2	51.3	54.7	53.0	1.7

Table 18

f) pH 8.0

Days of Incubation	% Weight Loss			Mean	S.D.
18	19.3	16.1	22.0	19.1	2.9
24	8.2	10.3	11.8	10.1	1.8
28	28.3	21.3	29.0	26.2	4.2
30	27.3	28.8	25.6	27.2	1.6

was responsible for yellow patches, as all these fungi were commonly found growing on the edges of the cellulose strip. Among other fungi, Sordaria fimicola and Coniothyrium fuckeli were observed with their fruiting bodies partially embedded in the cellulose fibres. These also produced a fair amount of clearing of the cellulose agar.

50°C

The results of the perfusion of the cellulose strip with E. & P. nutrient solution at different pH levels and incubated at 50°C are summarised in Tables 19a and 19b.

The initial colonisation of the cellulose strip started with the appearance of Chaetomium thermophile at pH 6.0, 6.4, 7.0 and 8.0 after 1 day of incubation and later at pH 4.0 and 5.0. Humicola insolens also appeared after 1 day of incubation, but only at pH 6.0, 6.4 and 7.0. C. thermophile and H. insolens were subsequently frequently isolated at all pH values; C. thermophile had the highest frequency of occurrence at pH 6.4 and the lowest at pH 4.0. H. insolens. was comparatively less frequent with the same pH preference (Figure 5).It was isolated only once at pH 4.0 and twice at pH 8.0.

Humicola grisea made its appearance when C. thermophile had established itself. It was first isolated from the cellulose strip at pH 7.0 after 3 days of incubation. It appeared later on other pH values and then remained fairly frequent till the cellulose strip was completely deteriorated. H. grisea had maximum frequency of occurrence at pH 5.0 - 7.0 and a lower frequency at pH 4.0 and 8.0. Aspergillus fumigatus, a thermotolerant fungus (Cooney & Emerson 1964)

Table 19a

Percentage frequency of fungi colonising the cellulose strip at pH 4.0, 5.0 and 6.4* at 50° C.

FUNGI	pH	Days of Incubation							
		1	2	3	5	7	9	12	16
Aspergillus fumigatus	4.0					16			
	5.0			16					
	6.4					16	32		
Cephalosporium sp.	4.0						32		
	5.0								
	6.4				40		16		
Chaetomium thermophile	4.0		16	50	64	50	50	32	32
	5.0		32	100	100	100	100	100	80
	6.4	60	60	100	100	100	100	100	100
Humicola grisea	4.0						80	64	64
	5.0				50		100	100	100
	6.4				80		100	100	100
Humicola insolens	4.0			32					
	5.0		16	16	16	16	32	80	64
	6.4	16			100	60	80	100	80
Humicola lanuginosa	4.0								
	5.0								
	6.4					32	16		
Mucor pusillus	4.0								
	5.0								16
	6.4								50
Talaromyces duponti	4.0				16	16	16		
	5.0					16			
	6.4								
Sporotrichum thermophile	4.0								
	5.0								
	6.4							32	
Torula thermophile	4.0								
	5.0					16			
	6.4							16	
Streptomyces sp. No. 1	4.0							16	
	5.0							80	60
	6.4							100	100
Weight Loss Percentage	4.0	2.73	9.68	10.94	10.7		32.60		68.8
	5.0		9.45	17.88	15.0		49.84	66.07	69.5
	6.4	1.50	4.90	13.10	14.2		34.70	63.60	71.3

* E & P Salt Solution unbuffered

Table 19b

Percentage frequency of fungi colonising the cellulose strip at pH 6.0, 7.0 and 8.0 at 50° C.

FUNGI	pH	Days of Incubation							
		1	2	3	5	7	9	12	16
Aspergillus fumigatus	6.0				50	32			
	7.0				16	100			
	8.0			32	50	50			
Cephalosporium sp.	6.0								
	7.0								
	8.0							64	
Chaetomium thermophile	6.0	80	100	100	64	100	64	100	100
	7.0	100	100	100	64	100	50	64	100
	8.0	80	64	100	80	100	64	100	100
Humicola grisea	6.0					100	50	100	100
	7.0			32	80	50	64	64	100
	8.0					80	32	32	100
Humicola lanuginosa	6.0					16	16		
	7.0					16	32	32	
	8.0								
Humicola insolens	6.0	32	32		16	32	50	50	16
	7.0	16	16	16	32	32	32	16	
	8.0						64		32
Sporotrichum thermophile	6.0								32
	7.0							32	
	8.0								
Streptomyces sp. No. 1	6.0					64		32	80
	7.0			32	50	50		16	80
	8.0			16	32	50	50	16	80
Streptomyces sp. No. 2	6.0						16	16	
	7.0						80		
	8.0				16		50		
Torula thermophile	6.0							16	16
	7.0							32	16
	8.0								
Thermoascus aurantiacus	6.0					16	16		
	7.0								
	8.0								
Talaromyces duponti	6.0				16				
	7.0								
	8.0								
Weight Loss Percentage	6.0	7.3	7.4	15.0	51.3	62.4	75.1		74.6
	7.0	13.1	4.3	17.9	39.5	65.8	66.4		73.1
	8.0	10.2	6.3	17.9	46.1	63.8	64.8		67.8

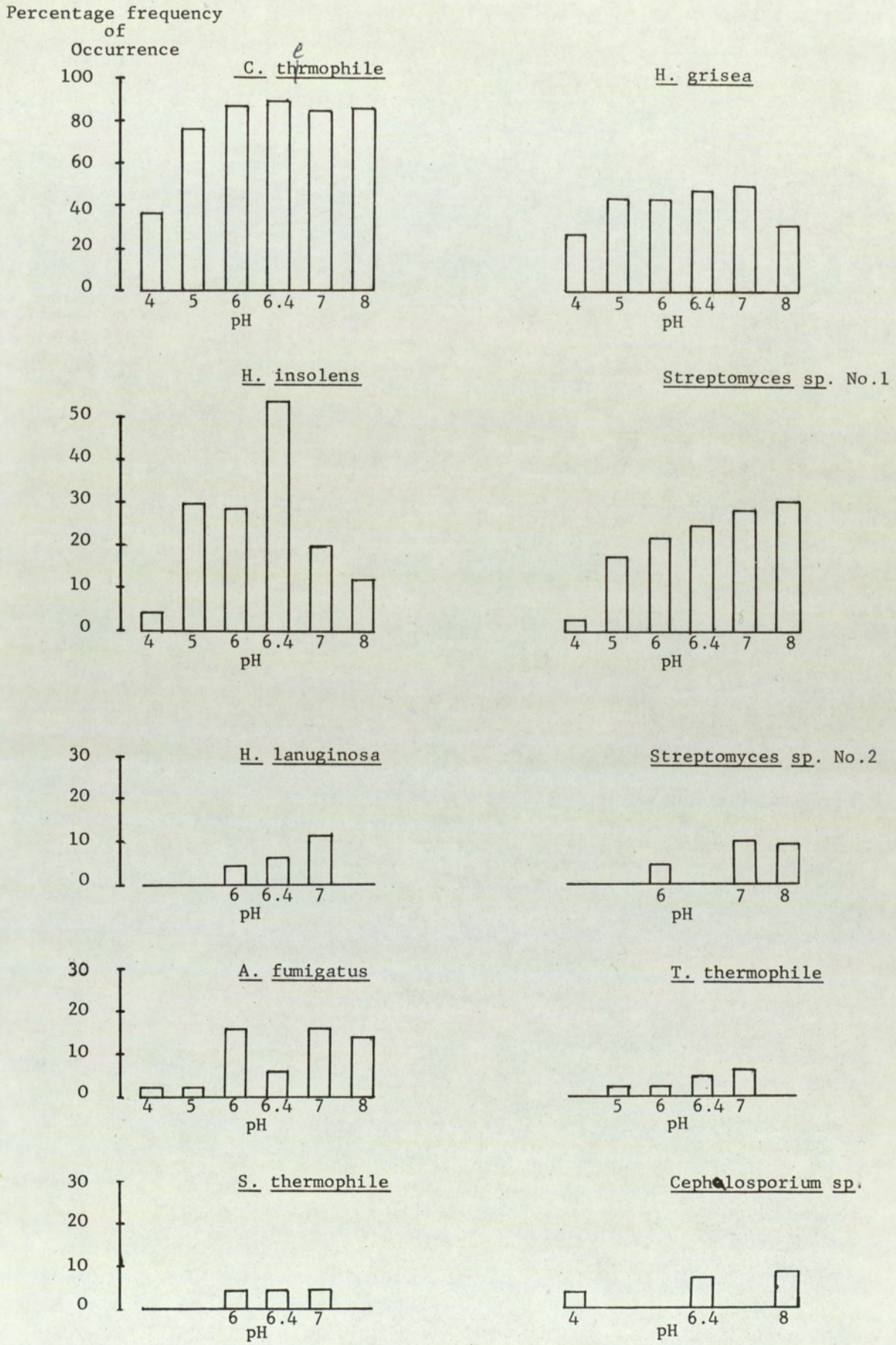


Figure 5: Average percentage frequency of occurrence of fungi isolated from cellulose strip perfused with E.&P. salt solution at different pH values after 30 days of incubation at 50°C.

appeared along with H. grisea but was comparatively short lived and was not isolated from the cellulose strip at any pH, after 9 days of incubation. It had a relatively low frequency of occurrence reaching a maximum of 16% at pH 6.0 and 7.0 whereas at pH 4.0 and 5.0 it was only 2%.

Two cellulolytic species of Streptomyces were isolated from the cellulose strip after 3-5 days of incubation. These were fairly common at pH 7.0 and 8.0 Streptomyces sp. No. 1 was once isolated from pH 4.0 and 5.0 and had quite high frequency of occurrence at other pH values. Streptomyces sp.No.2 was isolated at pH 6.0, 7.0 and 8.0 and had low frequency as compared to Streptomyces sp. No.1.

When the cellulose strip was fairly deteriorated, a variety of fungi were isolated at different pH values. Sporotrichum thermophile was isolated from the cellulose strip at pH 6.0 - 7.0 after 12-16 days of incubation. After the same period of incubation Torula thermophile appeared at pH 5.0 - 7.0 with maximum frequency of 6% at pH 7.0. Cephalosporium sp.was isolated fairly early at pH 4.0 and it was also observed at pH 6.4 and 8.0.

Among the non cellulolytic thermophilic fungi, Humicola lanuginosa was fairly common at pH 6.0 - 7.0 after 7-12 days of incubation. It was normally observed along with C. thermophile and rendered the pure isolation extremely difficult. However, when H. lanuginosa was grown on the E. & P. cellulose agar, it did not produce any clearing of the cellulose (Eggins & Pugh 1962) and was just able to grow; thus confirming its non cellulolytic nature. Other

non cellulolytic thermophilic fungi like Talaromyces duponti and Thermoascus aurantiacus were occasionally isolated from the cellulose strip at different pH values.

The deterioration of cellulose strip was extremely rapid; 60% - 70% of the weight of cellulose strip was lost after 16 days of incubation (Figure 6a-b).The weight loss estimation results are summarised in the bottom columns of Tables 19a-b.Maximum deterioration took place at pH 6.0 and 7.0. C. thermophile seemed to be mainly responsible for the weight loss as it was always observed growing all over the cellulose strip and it produced maximum clearing of the cellulose agar as well.

The fungal colonisation pattern on the cellulose strip at 50° C. shows some interesting features. The initial colonisation started with C. thermophile and H. insolens which are cellulolytic. This was followed by the appearance of H. grisea, H. lanuginosa, T. thermophile and Streptomyces spp. Among these fungi H. lanuginosa was found to be non cellulolytic (Pugh et al, 1964; Yung Chang, 1967; Fergus, 1969). Its association with other cellulolytic fungi was also studied by Yung Chang (1967), who also came to the conclusion that H. lanuginosa was non cellulolytic and was able to use sugars produced by the cellulase of other cellulolytic fungi. Thus it also comes into the category of 'secondary colonisers' as defined by Garrett (1963). The other fungi which appeared when the cellulosic substrate was completely depleted were Cephalosporium sp. and Sporotrichum thermophile. M. pusillus was also isolated once at the end of the experiment. It did not produced any clearing of the cellulose agar thus,

Figure 6a

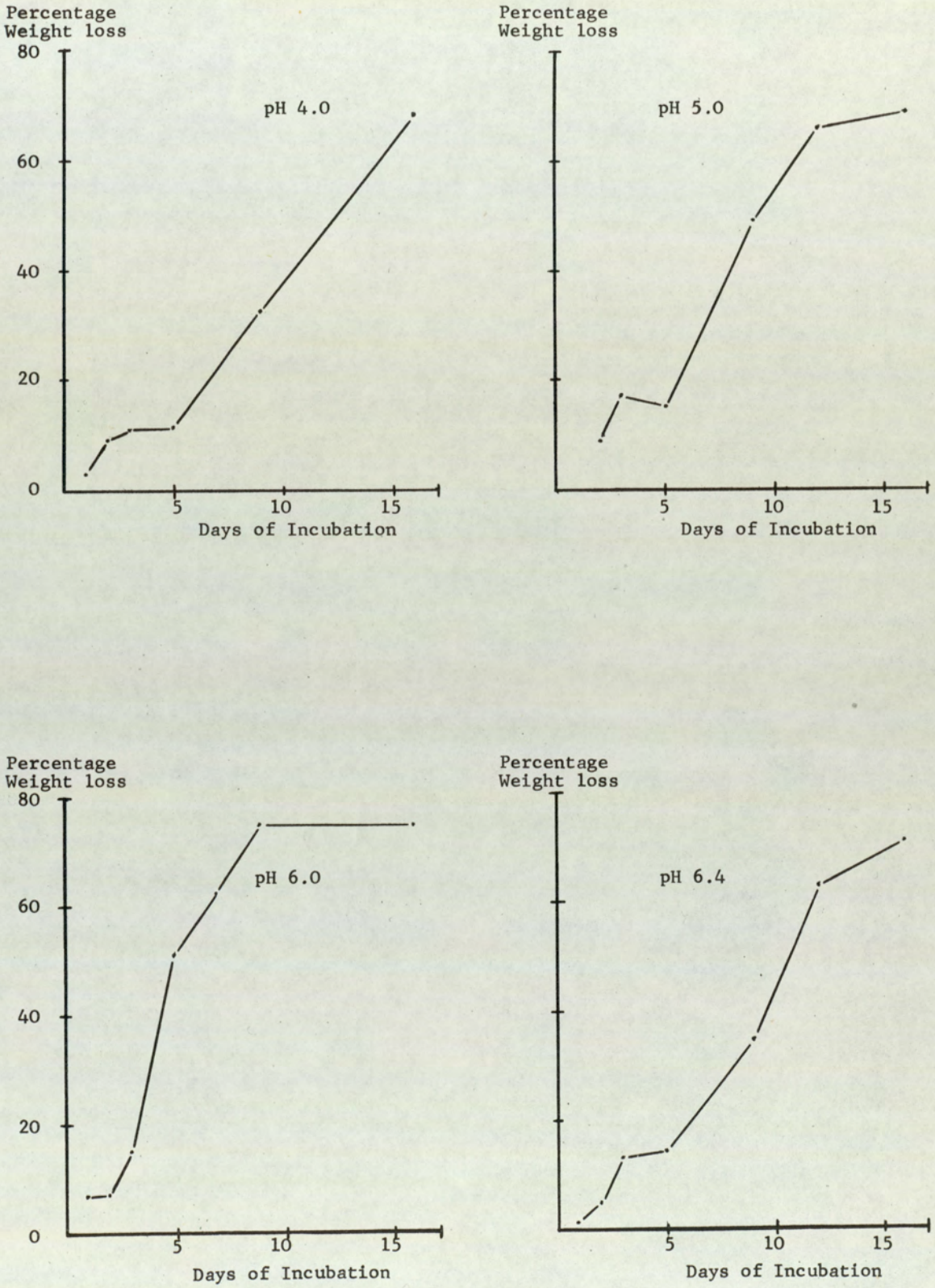
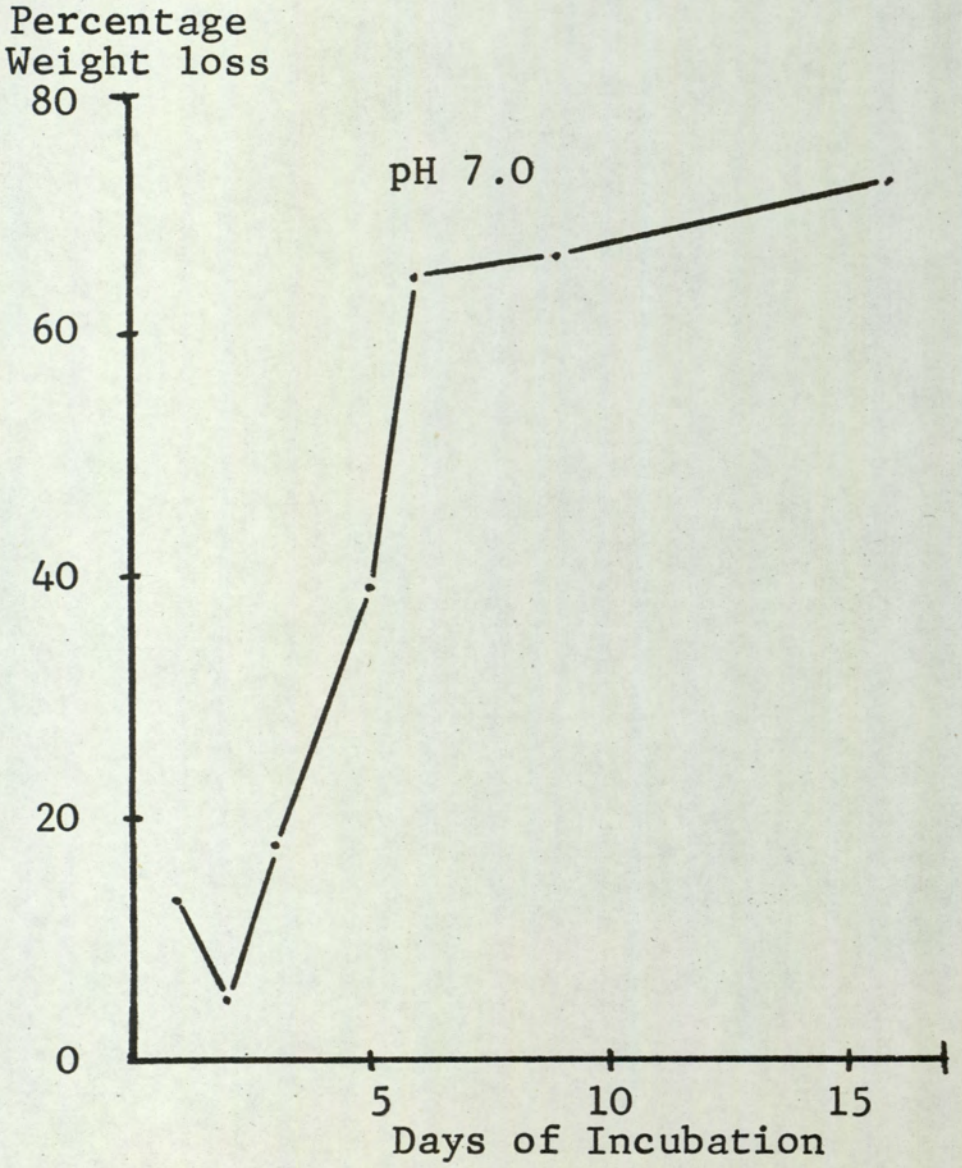


Figure 6a-b: Average percentage weight loss of the cellulose strip perfused with E.&P. salt soluttion at different pH values and incubated at 50°C.


Percentage
Weight loss
80
60
40
20
0
pH 7.0
5
10
15
Days of Incubation

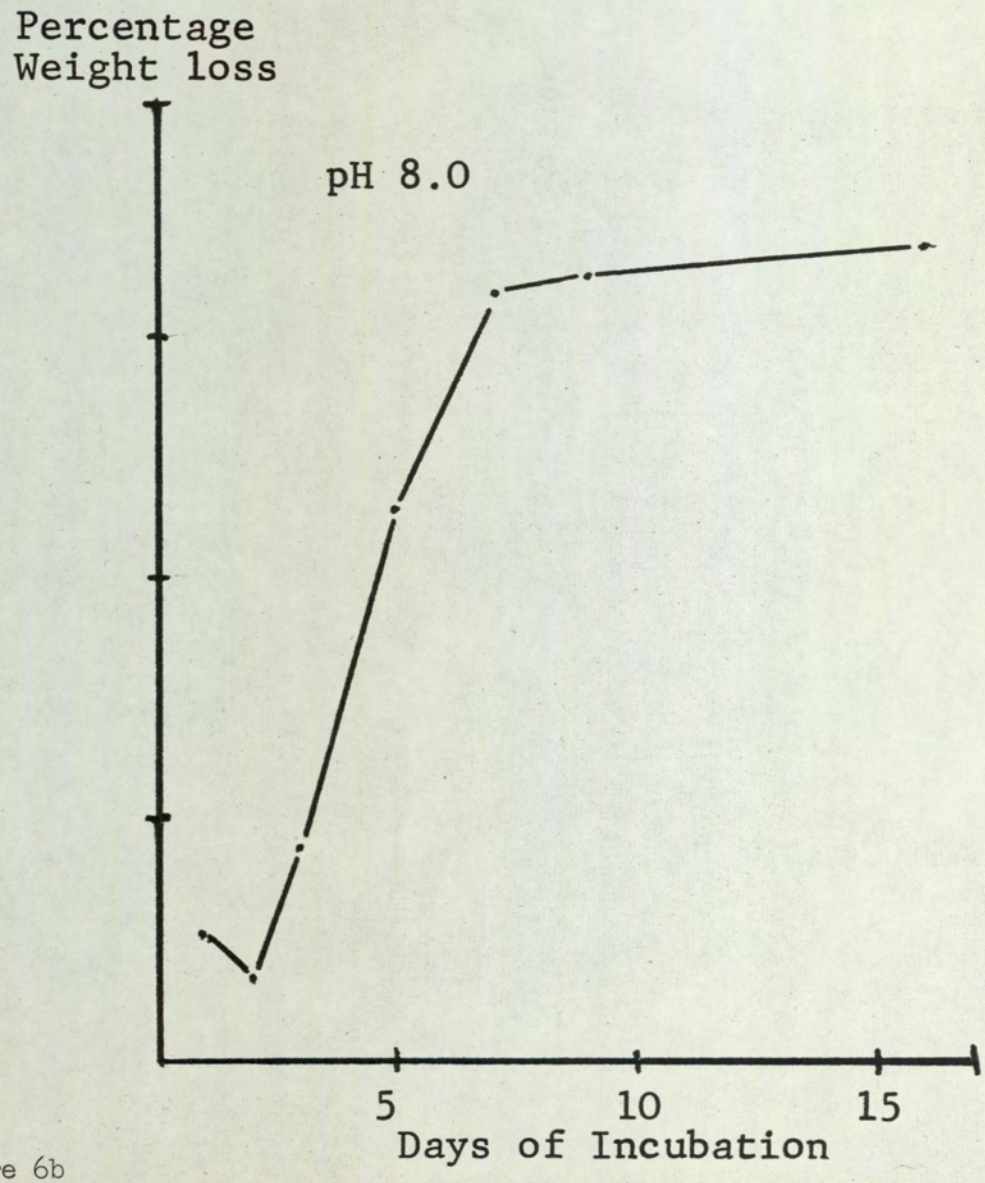

Percentage
Weight loss
pH 8.0
5
10
15
Days of Incubation

Figure 6b

confirming its non cellulolytic nature.

The general pattern of fungal succession varied with different H-ion concentrations but essentially it remained the same except frequency of certain fungi varied at different pH values. On the whole pH 6.0 - 7.0 produced most deterioration of the perfused cellulose strip and most of the thermophilic cellulolytic fungi were isolated at these pH values.

The review of the results at different temperatures and pH values has pointed towards the importance of such studies. The behaviour of fungi colonising the cellulosic substrate in the soil varies with the temperature and pH. It has been elucidated by the fact that biodeterioration of the cellulose strip was quite different at different temperatures and so was the fungal flora. The comparative review of the maximum biodeterioration as estimated by the percentage weight loss at different temperatures and pH has been represented in Figure 7 . The maximum deterioration took place at 50°C with optimum deterioration at pH 6.0. The maximum deterioration at 35°C was at pH 7.0 whereas at 25°C it was at pH 6.0 - 6.4. The deterioration at 50°C was extremely rapid as the maximum weight loss was obtained in 16 days whereas it took 22-30 days at other temperatures.

The fungal colonisation of the cellulose strip was very much influenced by temperatures whereas pH normally only affected the frequency of occurrence of different fungi. The succession of fungi on to the cellulose varied with different H-ion concentration but essentially it remained the same at one temperature.

Most of our present knowledge about cellulose

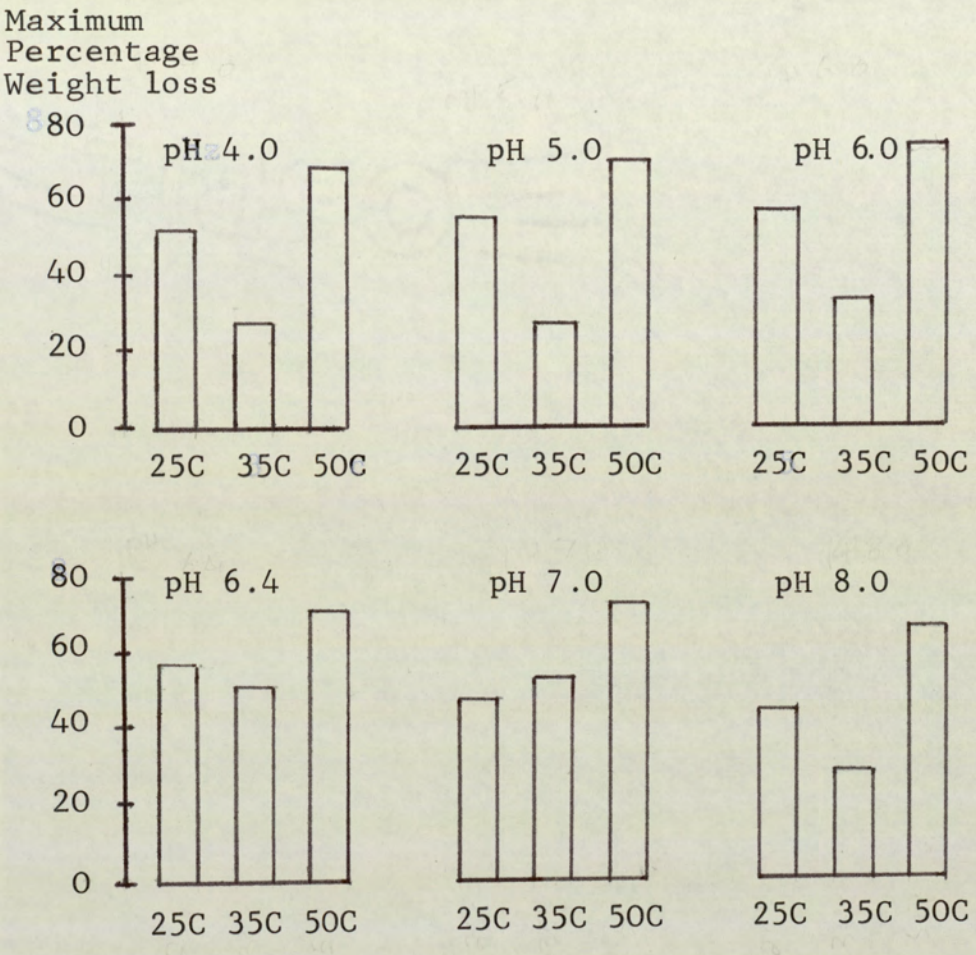


Figure 7: Maximum percentage weight loss of the cellulose strip at different pH values and temperatures.

decomposition in the soil has come through ecological studies of the plant remains above or in the soil (Hudson 1968) as cellulose is one of its constituents. The succession of fungi in the present investigation differs from the previous studies because of the nature of the substrate. In these investigations the substrate used is pure fibrous cellulose whereas previous studies were carried out on mixed substrates i.e. hexose sugars, cellulose and lignin comprising the plant remains and organic matter continuously added to the soil. Apparently, as stated by Garrett (1963) there exists a correlation between systematic groups of fungi and their ability to decompose; as there is an initial phase of Phycomycetes followed by Ascomycetes and then Basidiomycetes. As there is no hexose sugar in the cellulosic substrate used in the present investigation, the first Phycomycetes phase has been eliminated to some extent. Some members of this group were isolated but they did not seem to play an effective role in the succession of fungi on to the substrate. Thus most of the fungi isolated from the cellulose strip were found to be cellulolytic to varying degrees.

The results obtained with the help of the perfusion technique have elucidated a successional pattern within the cellulose decomposing fungi. The role of Garrett's "secondary fungi" has become much more apparent. These fungi may not be "sugar fungi" in the strict sense as stated by Garrett (1963) but produce clearing of the cellulose. Thus these fungi, though cellulolytic, will colonise the cellulosic substrate only when the cellulose has been previously colonised and the breakdown products are at a certain concentration.

Though the nature of these "secondary fungi" changes at each temperature, their overall pattern essentially remains the same.

During the previous studies on the isolation of thermophilic fungi, a number of actinomycetes were also observed. Their isolation was rendered difficult by the relatively rapid rate of growth of some of the fungi.

The interest in actinomycetes has greatly been increased since the discovery of their antibiotic producing capabilities (Waksman and Woodruff 1940). Some attention has also been paid to the thermophilic actinomycetes and their role in the decomposition of cellulose in composts (Waksman, Umbreit and Cordon, 1939). However, their role in the soil has been neglected as their presence is thought to be related merely to their ability to survive under mesophilic conditions rather than to any significant role that they might play at thermophilic temperatures. This discrepancy in knowledge about actinomycetes, especially thermophilic actinomycetes is due to the non availability of reliable isolation technique and difficulty in identifying them even when isolated.

One of the reasons for their difficulty of isolation is their very low competitive ability. Even at very high dilutions of soil suspensions for the dilution plate

technique, the frequency of bacteria and fungi is often so high as to render impossible the isolation of actinomycete colonies. Thus actinomycetes are at a disadvantage, particularly when competing for simple readily available compounds. The only advantage in favour of actinomycetes could be the possible production of antibiotics which help them to compete successfully for a particular substrate. Attempts have previously been made to overcome these difficulties by using various methods, such as the use of selective media, pretreatment of soil samples and soil suspension and incorporation of selective inhibitors.

Several substances have been suggested as selective substrates for actinomycetes. Porter, Wilhelm and Tresner (1960) reported that arginine was a selective nitrogen source and El-Nakeeb and Lechevalier (1963) recommended an arginine - glycerol - salt medium. Media containing chitin as the sole nitrogen and carbon source (Lingappa and Lockwood, 1961, 1962) and a starch - casein medium. (Kuster & Williams 1964) have also been recommended Agre (1964) found that peptone - corn medium supplemented with 1% starch, is most suitable for isolation of pure cultures and for maintaining collections of thermophilic actinomycetes. This was used in conjunction with a technique involving the incubation of inoculated agar media in vials with tightly closed covers. These acted as water containing dessicators to secure optimal humidity for growth.

Methods involving pre-treatment of soil samples involves mixing the soil with compounds such as sodium propionate or calcium carbonate. Crook, Carpenter & Klens (1950) showed that the addition of 0.4% sodium propionate to the soil was optimum for the growth of actinomycetes,

although concentration of 0.8% adversely affected their growth. Tsao, Leben & Keitt (1960) mixed calcium carbonate with soil samples and incubated at 28°C. Pre-incubation of soil at 110°C for 10 mins and the use of dried plates have been advocated by Agate & Bhat (1963). Pre-treatment of soil suspension has also been investigated by Lawrence (1956) , who recommended the addition of 1.4% (w/v) phenol solution. However, phenol has been shown to have a harmful effect on the actinomycetes which inhibit the growth of soil bacteria, (Rehacek, 1957). With the exception of the latter method, all of the techniques mentioned have been used for isolation purposes with some degree of success. However, Rehacek (1957) commented on the considerable inadequacies of all existing isolation techniques and proposed a centrifugation method for isolation of actinomycetes. This method is based on the differences in dimensions of different species of microorganisms. The removal of accompanying microorganisms by this method eliminates their antagonistic effects which could distort any quantitative estimations of actinomycetes in the soil. Moreover, all these isolation techniques do not give any information on the colonisation patterns of actinomycetes.

Antibiotics which inhibit fungi have been found particularly useful in studies on actinomycetes. Corke and Chase (1956) used actidione to inhibit growth of fungi on plates inoculated with an acid forest soil but concluded that agricultural soils with pH 6.0 and above contain relatively few fungi in comparison with the numbers of actinomycetes and bacteria, and thus the addition of actidione

under these circumstances is not necessary. In a comparative survey Porter, Wilhelm and Tresner, (1960) reported that pimaricin, nystatin and cycloheximide were used successfully to suppress fungi on isolation plates. The most successful agent was pimaricin at a concentration of 50 ug/ml of agar. All three antibiotics were also found to be useful in decontaminating fungus infected Streptomyces cultures. Williams & Davies (1965) found that the most satisfactory medium for isolation purposes was Kuster and Williams' starch - casein medium containing sodium penicillin, polyimixin - B - sulphate, nystatin and actidione; the anti-bacterial antibiotics being used to suppress the growth of bacteria in the cultures and thus allow maximum development of actinomycete colonies.

All the above mentioned studies on the effectiveness of antifungal antibiotics has been carried out at mesophilic temperatures. The behaviour of these antibiotics cannot be assumed to be the same at thermophilic temperatures. Moreover, the majority of the isolation techniques using selective substrates or enrichment procedures have also been carried out at mesophilic temperatures. Therefore, it is essential, first of all to test the effectiveness of these antibiotics at thermophilic temperatures in relation to the selective isolation of thermophilic actinomycetes.

The antifungal antibiotics used were as following:

Commercial Name	Antibiotic	Manufacturer
Actidione	Cyclohexamide	Light
Mycostatin	Nyastatin	Squibb
Pimafucin	Pimaricin	Royal Netherland Fermentation Co.

Each of these antibiotics was used independently and actidione and mycostatin were used in combination as well. Throughout the investigations the antibacterial antibiotics used were sodium penicillin and streptomycin . The antifungal antibiotics actidione and mycostatin were both used at 50mg/ml and pimafucin was used at 25 ug/ml. The soil used was the same as described in chapter 2. Two isolation media, Eggins & Pugh's cellulose agar and Kusters and Williams' starch-casein agar were always used. The antibiotics were always incorporated into the medium after autoclaving.

Three methods have been used for the selective isolation of thermophilic actinomycetes these are:

1) direct isolation using dilution soil plate technique and Warcup's soil plate technique (Warcup, 1950)

2) Amending the soil with pimafucin, incubating this soil and making subsequent isolation using Warcup's soil plate technique.

3) Perfusion technique: different antifungal and antibacterial antibiotics were incorporated into either the perfusing flask or directly on to the polythene backed cellulose paper.

Direct Isolation

To prepare the dilution plates, 1 gm of soil was added to 100 ml sterile water in a screw top bottle and shaken for 30 mins on a shaker. From the resulting suspension a further three tenfold dilutions were prepared. 1 ml sample of these four dilutions was used to make the dilution plates. Thirty plates were prepared for each dilution for both media, six plates being used for each

antibiotic and six plates were kept as controls.

For Warcup's soil plate technique, six replicates for each antibiotic and six as control samples were prepared for both media.

All inoculated plates were incubated at 45°C. The relative humidity of the incubator was maintained around 80% in order to prevent the drying of the agar. The plates were observed after 5 and 7 days of incubation. The appearance of fungi and actinomycetes was recorded.

The results of the isolations are summarised in Tables 20 and 21. The appearance of fungi is recorded by a positive (+) sign whereas the appearance of actinomycetes was recorded by the average numbers of colonies per plate.

Table 20

Isolation results obtained by soils dilution plate method using E & P cellulose agar (CA) and starch casein agar (SC)

ANTIBIOTIC		SOIL DILUTION							
		10^{-2}		10^{-3}		10^{-4}		10^{-5}	
		CA	SC	CA	SC	CA	SC	CA	SC
Actidone	Fungi	+	+	+	+	+	+	+	+
	Actinomycetes	-	-	-	-	-	-	-	-
Mycostatin	Fungi	+	+	+	+	+	+	+	+
	Actinomycetes	-	-	-	-	-	-	-	-
Actidione & Mycostatin	Fungi	+	+	+	+	+	+	+	+
	Actinomycetes	-	-	-	-	-	-	-	-
Pimafucin	Fungi	-	-	-	-	-	-	-	-
	Actinomycetes	2	1	2	2	1	2	3	1
Control	Fungi	+	+	+	+	+	+	+	+
	Actinomycetes	-	-	-	-	-	-	-	-

Table 21

Isolation results obtained from E. & P. cellulose agar (CA) and Starch Casein medium (SC) using Warcup's soil plate technique with four antibiotics.

	Actidione		Mycostatin		Actidione & Mycostatin		Pimafucin		Control	
	CA	SC	CA	SC	CA	SC	CA	SC	CA	SC
Fungi	+	+	+	+	+	+	-	-	+	+
Actinomycetes	-	1*	1	2	-	-	1	3	-	-

* Average number of colonies per plate

It is clear from these tables that pimafucin proved to be the most effective antifungal antibiotic. A 100% suppression of fungi on all the plates using both Warcup's soil plate and dilution plate technique was obtained by using this antibiotic. The comparative review of these results also show that more colonies of actinomycetes were observed on the cellulose agar than on the starch-casein medium. This further points towards the active role played by actinomycetes in cellulose decomposition in the soil.

The ineffectiveness of other antibiotics can be attributed to the high temperature (45°C). Williams and Davies (1964), working at mesophilic temperatures, reported that a combination of mycostatin and actidione was most successful in suppression of fungi due to the broad spectrum of antifungal activity of a combination of these two agents. However, at thermophilic temperatures, results

to the contrary effect have been recorded during these experiments. None of the antibiotics except pimafucin was able to suppress the fungi, though actinomycete colonies were observed on Warcup's soil plates containing actidione and mycostatin separately.

Soil Enrichment.

The information about the effectiveness of various antifungal antibiotics from the above mentioned studies was used in these experiments. Therefore, pimafucin was used to amend the soil before incubation. As it has also been indicated that thermophilic actinomycetes may play a role in cellulose decomposition, the soil was also enriched with cellulose. 100 gms of the wet soil was enriched with 4 gms of Whatman chromotography cellulose powder and was thoroughly mixed. From this cellulose enriched soil, 10gm samples were taken and placed in each of the six petri dishes. A 10% suspension of the 2½% pimafucin containing 125mg/ml was prepared in sterile water and 2 ml, 2.5 ml, 3.0 ml, 3.5 ml and 4.0 ml of this were added to each of the petri dishes containing soil and thoroughly mixed. The sixth petridish containing soil was left unsupplemented with pimafucin.

Using Warcup's technique, soil plates were prepared using E & P cellulose agar and glucose-starch agar. Both media contained 25ug/ml of pimafucin and antibacterial antibiotics. Three replicates were prepared for each medium and for each volume of pimafucin added to the soil. The plates were incubated at 45°C. The Petridishes containing enriched soil were also incubated at 45°C and subsequent isolations were made after every 2 days up to 10 days. Observations of all the plates were made after 7 days of

Table 22

Isolation results obtained by Warcup's method by enriching the soil with Pimafucin and cellulose.

Volume of Pimafucin added to soil in mls	Time of Incubation of Soil in Days												Total no. of Isolations	
	0		2		4		6		8		10			
	CA	GS	CA	GS	CA	GS	CA	GS	CA	GS	CA	GS	CA	GS
0	++		+										3	2
2.0		+		+					+		++		3	1
2.5	+	+	+				++		+++		++		9	1
3.0	+++	+					+		+++		++		9	1
3.5	+++	+					+				++		6	1
4.0	++	+					+		+		++		6	1

incubation. The frequency of isolation of actinomycetes was determined by recording the presence or absence of colonies in each sample; a positive record being given if colonies occurred on any of the three replicates.

The results of the frequency of occurrence of actinomycetes are summarised in the Table 22. On cellulose agar containing no pimafucin, the occurrence of actinomycetes ceased after 2 days of incubation of the soil. On plates containing 2 ml pimafucin suspension, no actinomycetes were isolated until 8 days of the incubation of the soil. The total number of isolations was highest on plates containing 2.5 and 3 ml pimafucin (Fig).

On glucose starch agar no actinomycetes were isolated from soil containing no pimafucin. Even from the soil containing pimafucin, actinomycetes were only isolated in the beginning of the incubation of soil and were not isolated later.

From these results it is quite clear that the addition of pimafucin to the cellulose enriched soil allows maximum isolation of thermophilic cellulolytic actinomycetes. The incubation of this enriched soil also helps in their isolation as actinomycetes present in the soil are activated and then can be easily isolated. Moreover, due to the effective suppression of the fungi by the addition of pimafucin, the competitive ability of actinomycetes is raised and enables them to manifest themselves on the cellulose medium.

The advantages of a selective isolation procedure have once again been shown by the low frequency of occurrence of actinomycetes on glucose-starch medium as compared with the selective cellulose medium.

Perfusion Technique

In order to explore further possibilities of the isolation of thermophilic cellulolytic actinomycetes, the perfusion technique was also used. Thirty perfusion devices were made as described previously. The cellulose substrate was perfused with Eggins & Pugh's nutrient salt solution. Due to the insoluble nature of mycostatin and pimafucin, actidione was chosen for use in this technique. Actidione was added to the nutrient solution at a concentration of 50ug/ml. The antibacterial antibiotics sodium benzyl penicillin and streptomycin sulphate were also added to the perfusing solution at a concentration of 1.0 ug/ml.

Another similar experiment was run using pimafucin. As mentioned earlier, due to its insoluble nature it is difficult to incorporate it into the perfusing nutrient solution. Therefore, a suspension of pimafucin was prepared in sterile water containing 25ug/ml pimafucin. Thirty pieces of 9 cms long fibre glass tape used for screening the cellulosic substrate were sterilized and then soaked in the pimafucin suspension overnight. Another 30 perfusion devices were made and the soaked tapes were then placed on to the cellulose substrate, after autoclaving, so that the pimafucin solution was constantly present in contact with the substrate.

In order to allow enough replication, three cellulose strips were sacrificed for each experiment after every day for the first three days, then every following two days for a total period of 16 days. The strips were first

Table 23: Isolation results obtained by perfusion technique using actidione and pimafucin as antifungal antibiotic.

Day of Incubation	ACTIDIONE		PIMAFUCIN	
	Fungi	Actinomycetes	Fungi	Actinomycetes
1	+		-	-
2	+ +		+	+
3	+ +		+	+
5	+ + +		+ +	+
7	+ + +		+	+ +
9	+ + +		+	+ +
11	+ +		+ + +	
13	+ +	+ +	+	+ + +
15	+ + +			+ + +

\+ poor growth

++ moderate growth

+++ excellent growth

observed under a microscope and then infected parts were selected, cut aseptically and plated on to 9 cellulose agar petridishes, all containing pimafucin. The inoculated petridishes were incubated at 45°C and then observed after 7 days for recording the presence or absence of actinomycetes or fungi.

The results of these experiments are summarised in Table 23. Actidione was once again found to be completely ineffective in suppressing the fungi and thus the isolation of actinomycetes was rendered difficult. Actinomycetes were isolated only once after 14 days of incubation. This much frequency of actinomycetes can even be achieved without using any antibiotic. The most common fungus was found to be A. fumigatus

The isolation results obtained by using perfusion sets containing pimafucin showed a different picture. The growth of cellulolytic thermophilic fungi was greatly suppressed. After 9 days of incubation of the perfusion sets, actinomycetes could be seen growing on the cellulose strip. After the same period of incubation A. fumigatus was also observed growing on the edges of some of the cellulose strips, though its frequency of occurrence and growth was quite small as compared to the results obtained by the perfusion of actidione. The fungi growing on the cellulose strip when inoculated on to the cellulose agar, grew only on the piece of cellulose strip and not on the cellulose medium containing pimafucin, whereas the actinomycetes grew both on the inoculated cellulose strips and on the cellulose medium.

These results have also proved the effectiveness of pimafucin for the selective isolation of thermophilic

actinomycetes. The use of pimafucin in the perfusion technique has given quite encouraging results. Nevertheless the incorporation of pimafucin could be made more effective by soaking the cellulose strips in its suspension before inoculating soil on top of it. The observation regarding the growth of A. fumigatus on the piece of cellulose strip and not on cellulose agar containing pimafucin could thus be overcome. In order to eliminate A. fumigatus considerably, it has been found during present investigations, that its frequency of occurrence is very low at 50° C. Thus the selective isolation of thermophilic actinomycetes would be comparatively easier at 50° C. than at 45° C.

The use of the perfusion technique after the above mentioned alteration could then be applied to the hitherto relatively unexplored field of the ecology of thermophilic actinomycetes by working on one variable at a time and keeping other micro-environmental factors constant.

Identification of the Thermophilic Actinomycetes.

The actinomycetes isolated so far, could be differentiated into 6 different isolated on the basis of their cultural morphology. These isolates were numbered from 1 - 5. The method of classification used for the Streptomycetes was the code system of Cross and MacIver (1966). This system is based on a number of characteristics thought to be most useful in the identification of species. These characteristics are melanin production, surface morphology of spores, aerial mycelium morphology, colours of aerial and substrate mycelia and carbon compound utilization.

The results of these tests with isolates No. 1 - 5 are

summarised below:-

Isolate No. 1

Streptomyces M2, S1, A3, C2, G1, U4, 5, 7.

i.e. melanin negative, smooth spores, open loop sporophores, grey aerial mycelium, buff substrate mycelium, ability to utilise mannitol, raffinose, sucrose.

Isolate No. 2

Streptomyces M2, S1, A2, C2, G1, Us, 4, 7, 8.

i.e. melanin negative, smooth spores, flexour sporophores, grey aerial mycelium, buff substrate mycelium, ability to utilise fructose, mannitol, sucrose, xylose.

Isolate No. 3

Streptomyces M2, S1, A2, C2, G1, Us, 3, 7.

i.e. melanin negative, smooth spores, flexous sporophores, grey aerial mycelium, buff substrate mycelium, ability to utilise fructose, inosital, sucrose.

Isolate No. 4

Streptomyces M2, S1, A3, C2, G1, U4, 7.

i.e. melanin negative, smooth spores, open hook sporophores, grey aerial mycelium, buff substrate mycelium, ability to utilise mannitol, sucrose.

Isolate No. 5

Streptomyces M2, S1, A1, C2, G1, U1, 2, 3, 4, 5, 6, 7, 8.

i.e. melanin negative, smooth spores, flexous sporophores, grey aerial mycelium, buff substrate mycelium, ability to utilise arabinose, fructose, inositol, mannitol, raffinose, rhamose, sucrose, xylose.

This method of classification described is an attempt to co-ordinate certain characteristics considered as good taxonomic characters into a working system. Although the laying down of codes to define the characteristics aids in the classification of the techniques and factors used in Streptomycete taxonomy, certain problems, such as the subjective nature of colour determination still remain. However, using this system in conjunction with other literature (Bergey, 1939) isolate nos. 2 and 3 were found to conform to the description of Streptomyces thermoviolaceus var pingens Henssen.

Isolates 1 - 6 were sent to Dr. T. Cross, University of Bradford, England, for further identification. S. thermoviolaceu var pingens was confirmed. Isolates no. 4 and 5 were thought to be the strains of Streptomyces rectus Henssen, although the production of the soluble dark brown pigment usually produced by this organism did not occur in either of the isolates.

Isolate No. 1 could not be identified further than Streptomycetes sp. This culture was catalogued as Streptomyces CUB 59 at the University of Bradford. Isolate No. 6 was identified as Actinomadura glauca. It had light blue colour and grew well on the cellulose agar.

The cellulolytic ability of all the isolates was also examined by estimating the weight loss of the cellulose paper inoculated with these isolates. The perfusion technique was again used for this purpose; 1 cm. wide polythene backed cellulose paper was used and was perfused with E & P salt solution. A standard inoculum using 1 cm. diameter cork borer was used to inoculate the perfused cellulose strip. Fifteen perfusion devices were set up for each isolate

Three cellulose strips were sacrificed for each isolate, after 1, 2, 3, and 4 weeks. Three perfusion devices were kept as controls. From every cellulose strip, 7 cms were cut and dried to constant weight at 110°C.

The results are represented in Table 24.

Table 24

% Weight Loss of the perfused cellulose strip caused by different species of Actinomycetes.

ACTINOMYCETES	Period of Incubation			
	1 week	2 weeks	3 weeks	4 weeks
Actinomadura glanca	1.3%	11.0%	22.3%	33.0%
Streptomyces sp. (CUB 59)	2.6	1.3	4.4	14.0
Streptomyces thermoviolaceus var pingens	0.8	2.6	8.0	15.6
Streptomyces sp. (S. rectus)	0.0	4.1	2.9	3.5
Streptomyces sp. (S. rectus)	0.0	0.0	0.0	0.0

Four out of the five actinomycete species tested, produced weight loss of the perfused cellulose strip. The maximum pecentage weight loss was produced by Actinomadura glauca after 4 weeks of incubation and perfusion of the cellululose strip. S. thermoviolaceus var pingens and a strain of Streptomyces sp. (CUB 59) produced moderate weight loss. A strain of Streptomyces sp.(S. rectus?) produced very little weight loss whereas another strain of the same species produced no weight loss even after 4 weeks of incubation. (Both these strains are thought to belong to S. rectus by Dr. T. Cross, University of Bradford).

The cellulolytic ability of all these species have also been tested using Rautella and Cowling's technique (Rautella and Cowling, 1966) as described previously. Any appreciable clearing was observed only in the case of A. glauca and Streptomyces sp.(CUB 59). The results have been recorded in Chapter 6 where these species are recorded as Streptomyces sp. No. 1 and No. 2 respectively. The clearing in the case of S. thermoviolaceus var pingens could not be observed due to the production of deep pink pigment. Two strains of S. rectus did not produce any measurable clearing at all.

Cellulose decomposition by thermophilic actinomycetes has also been studied by some workers (Henssen, 1957; Waksman, 1959, 1961) but only qualitatively and on a mixture of cellulose and some other readily available organic compound.Very little exact information concerning the ability of many thermophilic actinomycetes to produce cellulolytic enzymes is available. Fergus (1969) found S. thermoviolaceus var pingens to be the only actinomycete to degrade filter paper effectively. Apart from these studies there has been very little work done on the ecology of thermophilic actiomycetes. This lack of

knowledge is mainly due to the inadequate isolation techniques and the use of ineffective antifungal antibiotics. This discrepancy in our knowledge has resulted in the fact that during the formulation of new biocides, the role of actinomyces in biodeterioration is normally ignored as there is not enough information available on their ecology.

The role of these microorganisms in biodeterioration has been reviewed by Williams (1966). It was shown that actinomycetes can attack a wide range of dead plant materials. Some studies have also shown that these microorganisms play a major role in the heating processes causing spoilage of hay (Gregory and Lacey, 1963). On the other hand, this process could be useful for composts in which mushrooms are to be cultivated (Fergus, 1964).

A. Nitrogen Nutrition.

Most of the physiological studies on fungi have been carried out using non cellulolytic fungi growing on sugars as carbon source (Foster 1939; Steinberg 1939) The relationship between nitrogen and carbon assimilation in the formation of humus from cellulose in the soil have been investigated (Jensen 1931; Waksman 1938). Greathouse & Ames (1945) investigated the effects of different nitrogen sources for 16 spp. of Chaetomium. Basu (1948) carried out similar studies on different cellulolytic fungi. Siu & Sinden (1951) studied the effect of various mineral and environmental conditions on the growth of some mesophilic fungi growing on cellulose. There are a few other scattered studies on the mineral nutrition of cellulose decomposing fungi earlier of which are reviewed by Siu (1951) in his monograph.

It is well known that soil fungi use the carbonaceous material of the soil as energy for their growth and the available nitrogen in building their mycelial tissue. This nitrogen may be used in the inorganic form as nitrate, nitrite or amonium or in the various amino forms. The

use of nitrogen by fungi is always associated with the use of energy material and it has been pointed out that this nitrogen, if not an absolute necessity in the decomposition of cellulosic materials is definitely an aid in this process and hastens the utilisation of cellulose by the fungi (Heck 1927). The amount of nitrogen necessary in this decomposition process has been estimated by Waksman & Heukelkian (1925, 26) and Jensen (1931). In general, for each gram of nitrogen taken up about 25-55 grams of cellulose are decomposed. Such figures can be misleading since the growth of fungi may depend on a mobilisation of nitrogen from older hyphae and use of it for new growth, (Cochrane 1958). Morton and Broadbent (1955) observed during their studies with Scopulariopsis brevicaulis that nitrogen is neutralised after exhaustion of available exogenous nitrogen.

Organisms differ in respect to their preferences for sources of nitrogen (Cochrane 1958, Siu 1951). Greathouse & Ames (1945) have shown this difference in different species of Chaetomium. Dawson (1919) found A. fumigatus to prefer inorganic nitrogen to organic, whereas the reverse was found to be for H. grisea. Among the inorganic nitrogen sources there is a differential response of fungi to two utilisable classes of compounds namely nitrate and ammonium. Basu (1948) studied the nutrition of some cellulolytic fungi and compared their cellulose decomposing ability using sodium nitrate, ammonium nitrate, asparegine and peptone as the nitrogen source. Heukelkian and Waksman (1925) found that T. koningi and Penicillium sp. preferred ammonium nitrogen to nitrate nitrogen. Siu (1951) used both ammonium and nitrate nitrogen to study the rate of cellulose breakdown

by Myrothecium verrucaria, Curvularia lunata and A. flavipes. In addition to these studies, there have been a few other scattered studies on the mineral nutrition of cellulose decomposing fungi, earlier work having been reviewed by Siu (1951)

All these studies on nutrition have been carried out on an artificial medium using pure cultures of the fungi. In doing so, as stressed previously, the ancillary environmental conditions around the fungus cannot be kept constant. For instance when a fungus is grown with an ammonium salt as the sole source of nitrogen, the reaction of the medium tends to become more acid. Fungi vary in the rate at which their growth leads to increasing acidity of an ammonium salt medium and also in their toleration of a low pH (Hawker 1950)

In order to study the effect of different inorganic nitrogen and organic nitrogen sources on the fungal colonisation of the cellulosic substrate, perfusion technique has again been used.

In the previous studies on the colonisation patterns of the cellulosic substrates ammonium sulphate has been used in E. & P. salt solution as inorganic source of nitrogen (Eggins & Pugh 1962). In this medium asparagine & yeast extract, provided as an organic nitrogen source. These two sources of nitrogen have been commonly used for culturing cellulolytic fungi.

The effect of different nitrogen sources on the colonisation of a cellulosic substrate has been demonstrated by replacing the nitrogen source from the perfusing E. & P. salt solution. Different nitrogen sources used were

ammonium nitrate and potassium nitrate instead of ammonium sulphate; arginine instead of asparagine. The effect of asparagine was also observed by comparing the results after eliminating it from the perfusing solution.

In order to allow enough replication, 60 perfusion devices were set up as described previously, for each nitrogen source. Every time four such devices were sacrificed; out of these, three were used for estimating the weight loss of the cellulose strip while the fourth strip was used for isolating fungi by cutting the strip aseptically and inoculating it onto six E.&P. cellulose agar petri dishes. Out of these, three petri dishes contained the same nitrogen source as perfused while the rest of the petri dishes contained ordinary cellulose agar. The inoculated petri dishes were incubated at 25°C and observed after 7 days of incubation.

The results of the fungal colonisation of the cellulose strip when perfused with different sources of nitrogen are summarised in Tables 25 - 28.

The colonisation of the cellulose strip perfused with E.&P. salt solution containing ammonium nitrate was quite rapid and the number of fungi and their frequency of occurrence was quite high (Table 25). The initial colonisation started with Fusarium solani which remained dominant through out the process of deterioration of the cellulose strip. Another three species of Fusarium namely F. sporotrichiodes, F. oxysporum and F. moniliforme were also occasionally isolated. Trichoderma viride was isolated after 6 days and it remained fairly common afterwards; but still its frequency of occurrence was much lower as compared to F. solani. The species which occurred after 4 days were

Table 25: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing ammonium nitrate.

FUNGI	Days of Incubation															
	4		6		8		12		16		22		26		30	
	CA*	N+	CA	N	CA	N	CA	N	CA	N	CA	N	CA	N	CA	N
Fusarium solani	100	33	100	100	33	66	33	33	100	100	66		66	33	100	100
Fusarium sporotrichoides		66											33			
Fusarium oxysporium	66	33												66		
Trichoderma viride				33	66	66	66		33	66			33	33	100	100
Humicola grisea								33	66	33	66		66	66		33
Paecilomyces elegans		33	33		33	33	66	100		66	33	33	33	33		
Gliocladium roseum					33	100	100	66	33	66	33	33	66			
Penicillium spp.				33	33	33	100	33	33	33		66		33		33
Chaetomium globosum						33						33				
Mucor globosus	33		33	66			66							33	100	33
Zygorhynchus moelleri				33		100	66	66	66	66	33		100	100		
Diococcum asperum																
Aspergillus fumigatus									100		33	33				66
Arthrobotrys sp.													100	66	33	33

* E & P Cellulose Agar.
+ E & P Cellulose Agar containing Ammonium Nitrate.

Paecilomyces elegans and Mucor globosus. P. elegans remained quite common up to 26 days while M. globosus was occasionally isolated. Humicola grisea, Gliocladium roseum, Penicillium sp. and Chaetomium globosum were isolated after 8 days of incubation. C. globosum was isolated only twice (after 8 and 22 days) on ammonium nitrate medium, whereas P. elegans and G. roseum were fairly common between 8 and 26 days of incubation and were observed growing well on both media containing ammonium nitrate and ammonium sulphate (E & P cellulose agar). Nematodes were observed ramifying through the cellulose strip after 16 days of incubation. Arthrobotrys sp. was isolated between 26 and 30 days of incubation. A. fumigatus was isolated between 16 and 30 days of incubation. When the cellulose strip was completely deteriorated, F. solani and T. viride were still most common in occurrence. H. grisea, Penicillium sp. and Mucor globosus along with P. varioti also made their appearance.

Penicillium sp. produced a pink pigmentation on the paper whereas F. sporotrichiodes produced a dark yellow colouration on the cellulose strip. P. elegans could normally be observed under the stereoscopic microscope growing on the cellulose strip.

The results of the perfusion of the cellulose strip with E & P salt solution containing potassium nitrate instead of ammonium sulphate are summarised in Table 26 . F. solani and T. viride were again very common and were found to be early colonisers. F. sporotrichiodes was also occasionally isolated on E & P cellulose agar containing ammonium sulphate. T. viride and F. solani were equally common on both types of cellulose agar media: one containing ammonium sulphate (CA) and the other containing potassium nitrate (N). Penicillium sp.

Table 26: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing potassium nitrate.

FUNGI	Days of Incubation																	
	2		4		6		8		12		16		20		24		30	
	CA*	N+	CA	N	CA	N	CA	N	CA	N	CA	N	CA	N	CA	N	CA	N
Fusarium solani	66	33	100	66	100	100	100	66	66	100	100	100	66	66	66	66	100	33
Trichoderma viride	66	66	100	66	100	100	100	100	100	66	66	66	66	100	100	33	66	66
F. sporotrichiodes		33					33				66							
Humicola grisea							33	33		66	33	66	33					
Penicillium sp.	33		33	33		33		66			100			66	33	33		
Aspergillus fumigatus				33		66			100		33	33		33			33	66
Chaetomium globosum						33	33	66		33								
Paeciliomyces elegans			33	33	33	33	66	66	100	33	66							
Gliocladium roseum			33	33	66	33	66	33	100	66	66				33	66		33
Sordaria fumicola						33		66	33			33						
Coniothyrium fuckeli											66	33			33	66	33	
Mucor globosus	33	33		33			66					66						
Paeciliomyces varioti													33	33		33	33	33

* E & P Cellulose Agar
\+ E & P Cellulose Agar containing Potassium Nitrate

and M. globosus were also isolated as early as 2 days and were both occasionally isolated through out the period of incubation. After 4 days A. fumigatus appeared on the cellulose strip and had the highest frequency of occurrence after 12 days of incubation. P. elegans was isolated between 4 and 16 days of incubation. C. globosum made its appearance between 6 and 12 days but its frequency of occurrence was not very high. H. grisea was isolated between 8 and 20 days of incubation. Sordaria fimicola was observed between 6 and 16 days and was mainly isolated nitrate medium. Coniothyrium fuckeli appeared when the cellulose paper strip was fairly deteriorated. It was first isolated after 16 days of incubation. When the cellulose strip was completely deteriorated, T. viride, F. solani, A. fumigatus, G. roseum and C. fuckeli could still be isolated. In addition to these P. varioti was also isolated between 20 and 30 days of incubation.

The number of fungi isolated from the cellulose strip when perfused with E.&P. salt solution without L-asparagine and yeast extract (level of inorganic nitrogen was same as normal in E.&P. salt solution) was comparatively low (Table 27). F. solani and T. viride were isolated after 2 days of incubation. T. viride had nearly the same frequency of occurrence on both types of media i.e. E.&P. cellulose agar and E.&P. cellulose agar without L-asparagine and yeast extract whereas F. solani had an extremely low frequency of occurrence on the latter medium. A. fumigatus and M. globosus also made their appearance between 2 and 8 days of incubation. P. varioti was isolated twice after 8 and 10 days only on the medium without L-asparagine and yeast extract. G. roseum was isolated once after 10 days on E.&P. cellulose agar only. Penicillium sp.made its first appearance after 8

Table 27: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution without L-asparagine and yeast extract.

FUNGI	Days of Incubation																			
	2		4		6		8		10		14		18		22		26		30	
	CA	-A*	CA	-A	CA	-A	CA	-A	CA	-A	CA	-A	CA	-A	CA	-A	CA	-A	CA	-A
Fusarium sp.	33	33	100	33			100	33	100	33	66		33		33		66	33	100	33
Trichoderma viride		33	100	100	100	100	33	33	100	100	100	100	100	100	100	100	100	100	100	100
Aspergillus fumigatus				33	33	33	66													
Paeciliomyces varioti								33												
Gliocladium roseum									33											
Penicillium funiculosum								66	66	100	100	100	100	66	33	100			33	100
Paeciliomyces elegans													66		66	66			66	
Mucor globosus	100	100	100				100		100	100										

* E & P cellulose agar without L-asparagine and yeast extract.

days and remained fairly common through out the period of incubation. It was isolated on both media with and without L-asparagine and yeast extract. Paecilomyces elegans was also isolated after 14 days and was continuously isolated mostly on E.&P. cellulose agar up to 30 days of incubation. Trichothecium sp. was also isolated once after 14 days. When the strip was quite deteriorated, Graphium sp., H. grisea and S. fimicola were observed growing on the cellulose strip.

The results of the perfusion of the cellulose strip with E.&P. salt solution containing arginine instead of asparagine are summarised in Table 28. The initial colonisers were again F. solani and T. viride but their frequency of occurrence decreased considerably towards the end of the incubation period. In addition to these F. oxysporum was fairly common in the begining but was occasionally isolated at the end of the incubation period. H.grisea was found to be the most dominant species since it was first isolated after 2 days and was repeatedly isolated through out the experiment. Penicillium funiculosum was isolated between 4 and 20 days of incubation. It had a quite low frequency of occurrence. P. elegans also had a similar frequency but was only isolated between 4 and 12 days of incubation. G. roseum was also occasionally isolated only on E.&P. cellulose agar. S. fimicola was isolated after 16 and 20 days only. Its perithecia were found growing on the cellulose strip. A. fumigatus was also isolated between 16 and 30 days of incubation. When the cellulose strip was completely deteriorated, F. solani, T. viride, G. roseum, H. grisea and A. fumigatus was still present and were isolated on the cellulose agar.

H. grisea normally produced black patches on the cellulose

Table 28: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing L-arginine instead of L-asparagine.

FUNGI	Days of Incubation																			
	2		4		6		8		12		16		20		24		28		30	
	CA	Arg*	CA	Arg	CA	Arg	CA	Arg	CA	Arg	CA	Arg	CA	Arg	CA	Arg	CA	Arg	CA	Arg
Fusarium solani	100	66	100	66	100	100	100	66	100	33	100	100	66		33		33		33	
T. viride	66		100	33	33	33	66	66			33	33	33		33		33	33		33
F. oxysporum	100	33		66	66	33	33				66				33					
G. roseum		33					33				66						33		33	
Mucor globosum					33		33				33				33					
H. grisea	33		66	66	33	100	100	33	66	66	66	33	100	33	100	66	66	66	100	33
P. funiculosum			66	33	33	33	33	66	33	33			33	33						
Paecilomyces elegans			33				33		33	33										
Penicillium sp.				33									33							
Zygorhynchus moelleri					66	66	66		100		66									
P. varioti					33															
Sordaria fimicola											100	100		33						
Aspergillus fumigatus											33		33			33			33	

* E.&P. cellulose agar containing L-arginine instead of L-asparagine

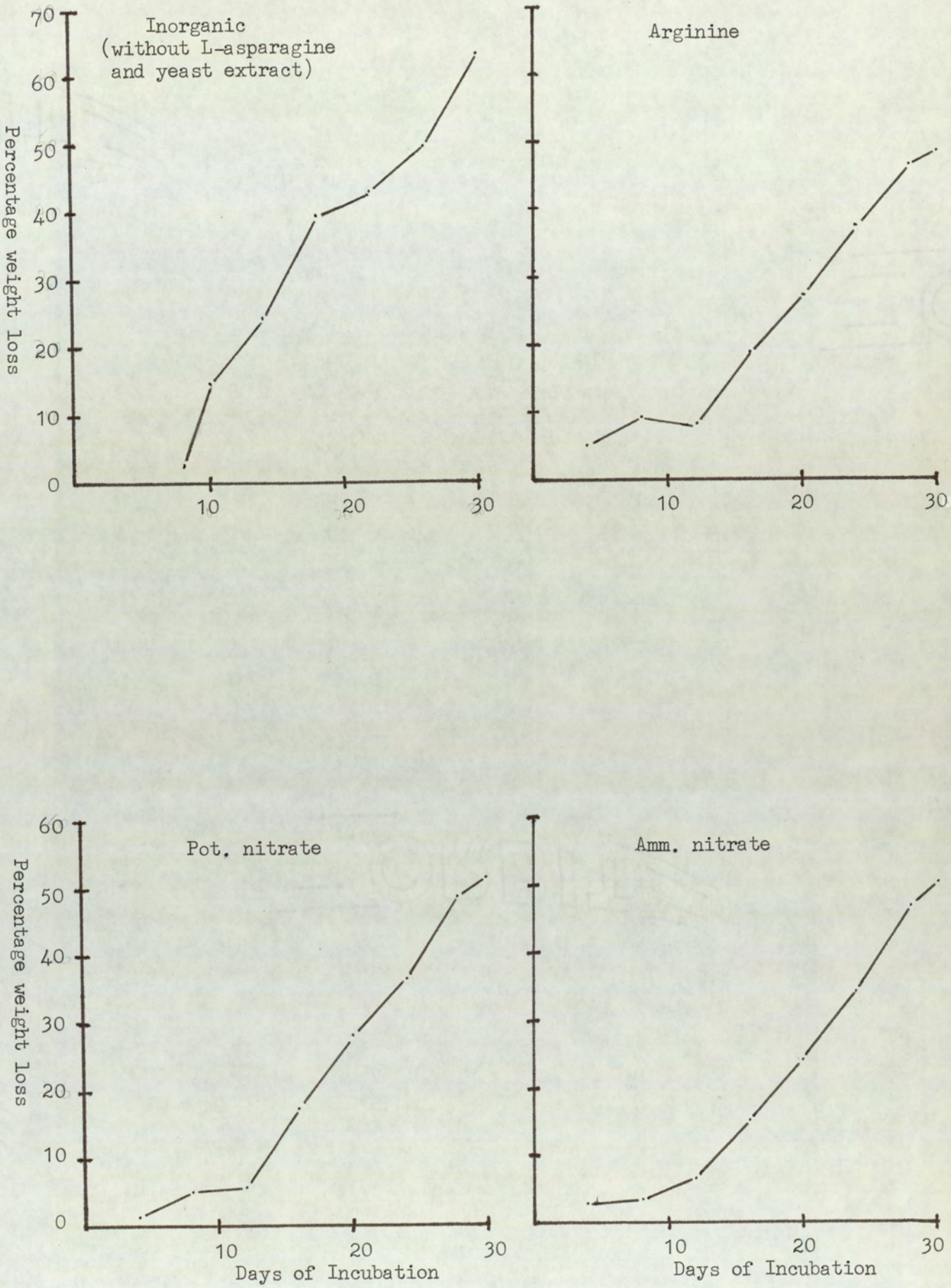


Figure 8: Average percentage weight loss of the cellulose strip perfused with E.&P. salt solution containing different nitrogen sources.

strip and its dark brown chlamydospores could be observed under the stereoscopic microscope.

The weight loss results of the cellulose strip perfused with varying sources of nitrogen are summarized in Tables 29a-d and are represented in Figure 8. There was not much difference in the maximum percentage weight loss of the cellulose strip at different nitrogen sources. Most weight loss was recorded when the cellulose strip was perfused without any organic nitrogen source. It was as high as 63.1 $\pm$ 2.5 per cent. It is evident from Table 27, that T. viride and F. solani were most dominant during this perfusion; these species might be responsible for this maximum weight loss. Bravery (1968) found L. asparagine and yeast extract to have an inhibitory effect on the cellulose decomposing ability of the fungi and he recomended their omission from the medium. Perfusion of ammonium nitrate and potassium nitrate produced nearly the same extent of deterioration of the cellulose strip. The weight loss estimated after the perfusion of arginine as the organic source of nitrogen was comparatively low as it reached only 48.5 per cent.

The relatively small difference in the deterioration of the cellulose strip, produced by perfusing different nitrogen sources does not imply that the fungal flora isolated and their frequency of occurrences were also the same. The average frequency of occurrence of all the fungi isolated are represented in Figure 9. It is evident from these results that the fungi which have been isolated at all the nitrogen sources have a varying frequency of occurrence. T. viride had quite high frequency with potassium nitrate and when perfused without any organic nitrogen, whereas when perfused

Table 29 (a-d): Percentage weight loss of the cellulose strip perfused with E.&P. salt solution containing different nitrogen sources.

a) KNO_3

Days of Incubation	% Weight Loss			Mean	S.D.
4	1.6	3.4	0.5	1.8	1.4
8	2.8	5.3	7.1	5.1	2.1
12	6.3	2.9	8.6	5.9	2.8
16	14.5	18.1	20.1	17.5	2.8
20	31.6	24.6	27.8	28.0	2.0
24	29.8	37.5	41.2	36.2	5.8
28	44.8	49.7	51.1	48.5	3.3
30	49.3	51.8	53.4	51.5	2.0

Table 29

b) NH_4NO_3

Days of Incubation	% Weight Loss			Mean	S.D.
4	2.8	1.8	3.1	2.6	0.6
8	2.9	2.1	4.3	3.1	1.1
12	6.4	7.1	5.1	6.2	0.9
16	16.8	10.1	18.3	15.1	4.3
20	28.2	24.3	19.7	24.1	4.2
24	34.7	38.1	29.8	34.2	4.1
28	43.4	48.5	49.6	47.2	2.7
30	54.3	46.1	51.1	50.5	4.1

Table 29

c) E.&P. salt solution without L-asparagine and yeast extract

Days of Incubation	% Weight Loss			Mean	S.D.
8	1.9	2.8	3.1	2.6	0.6
10	13.0	14.7	15.5	14.4	1.2
14	19.6	20.2	21.5	20.4	0.9
18	37.6	39.5	40.3	39.1	1.3
22	42.7	41.5	44.1	42.7	1.3
26	48.2	49.1	50.8	49.3	1.3
30	66.0	61.1	62.3	63.1	2.5

Table 29

d) Arginine

Days of Incubation	% Weight Loss			Mean	S.D.
4	1.8	7.6	5.6	5.0	2.9
8	10.8	8.2	9.6	9.5	1.3
12	11.4	7.8	5.6	8.2	2.9
16	17.5	23.8	16.4	19.0	3.5
20	16.7	15.3	20.5	17.5	2.6
24	31.4	44.0	38.6	38.0	6.3
28	48.8	54.3	36.7	46.6	9.0
30	50.6	47.8	47.1	48.5	1.8

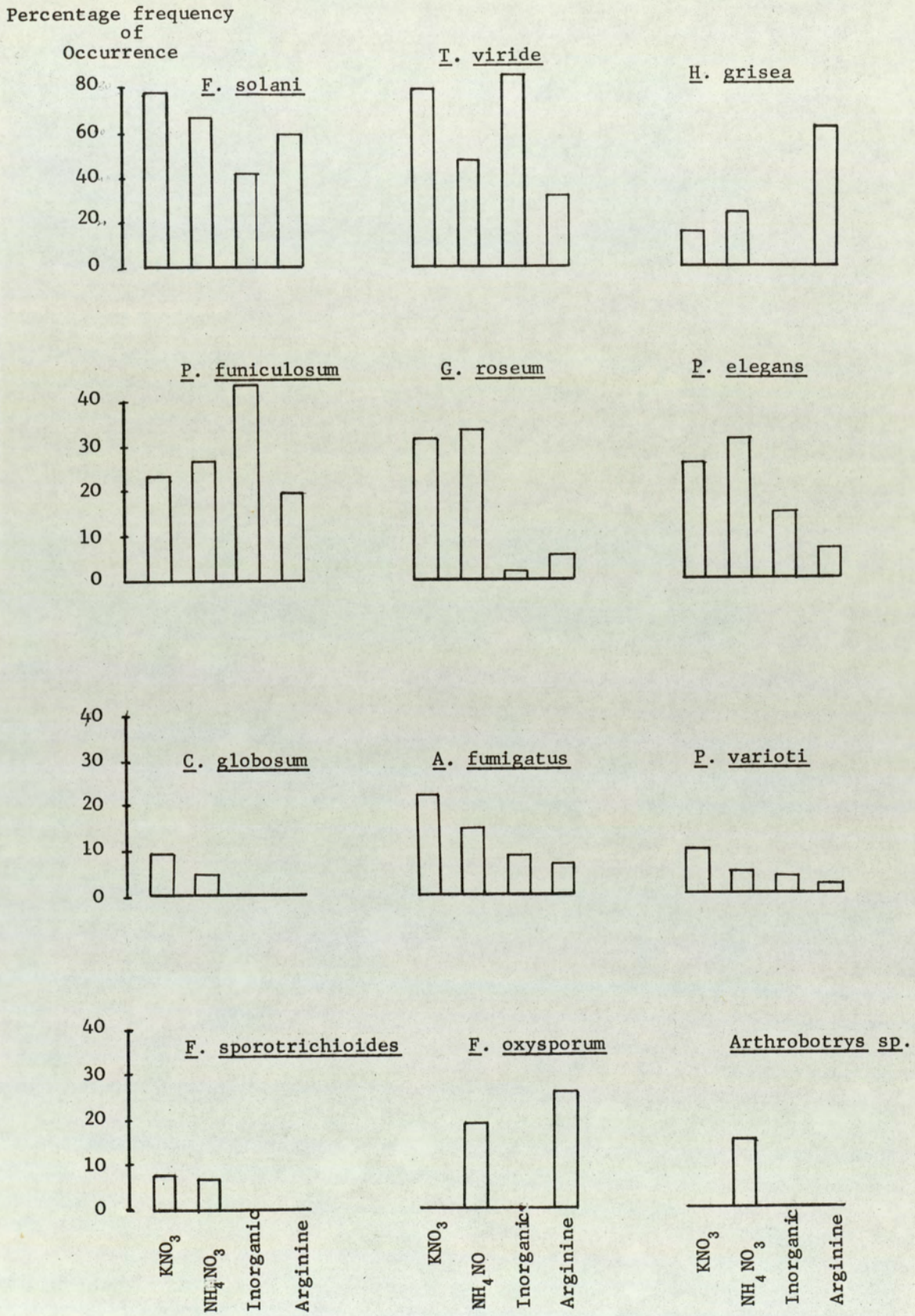


Figure 9: Average percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing different nitrogen sources.

with ammonium nitrate and arginine it was quite low in occurrence. Similarly F. solani had its maximum frequency of occurrence with potassium nitrate and minimum with inorganic nitrogen source (ammonium sulphate only). F. sporotrichioides have been recorded with potassium nitrate and ammonium nitrate while F. oxysporum was found with ammonium nitrate and arginine. Chaetomium globosum was also found with potassium nitrate and ammonium nitrate whereas S. fimicola was present with potassium nitrate and arginine. Coniothyrium fuckeli was isolated with potassium nitrate only. All these fungi have been reported previously to utilize nitrate nitrogen (Robins, 1937). This does not mean that these fungi can not grow on ammonium nitrogen but it simply indicates their preference (Lilly and Barnett,1951). H. grisea had the highest percentage frequency of 61.0 when perfused with arginine whereas it was 14.0% and 22.0% with potassium nitrate and ammonium nitrate respectively. Dawson (1919) and Reese (1946) also found similar results. Paecilomyces elegans was also isolated with all the four nitrogen sources; it had its maximum frequency of occurrence with ammonium nitrate. P. varioti was also found to be present with all the nitrogen sources but its frequency was much lower than P. elegans. Arthrobotrys sp. was only isolated with ammonium nitrate and had a frequency of 14.0%.

All these fungi isolated with varying nitrogen sources proved to be cellulolytic except P. varioti, to a varying degree as demonstrated by clearing on the cellulose agar (Eggins & Pugh, 1962). Detailed studies on their relative cellulolytic ability will be presented in the next chapter.

Glucose Nutrition

Certain carbon sources such as glucose and sucrose are generally utilizable by most microorganisms. On the other hand cellulose is not a satisfactory source for many fungi but is an excellent substrate for a relatively restricted number of species (Siu 1951). Most of the cellulolytic fungi also grow quite well on simple sugars.

It is believed that the presence of such simple sugars along with cellulose might inhibit utilization of cellulose by fungi. However, instances have also been recorded in which the presence of such sources leads to stimulation of cellulolytic activity in vitro (Mandle and Reese 1957, 1965; Talboy 1958; Johansson 1966). Horton and Keen (1964) found that glucose inhibited enzyme (cellulase) synthesis at high concentrations and regulates it at low concentrations. Siu and Sinden (1951) had found that in the presence of sugars with cellulose, no cellulolytic action occurred until the sugar was consumed. Arakawa (1934) reported that the same sugar elicits varying reactions from different fungi. Thus, while xylose,arabinose, glucose and sucrose inhibited the action of Aspergillus oryzae 30 - 49% on filter paper, they actually stimulated the cellulolytic activity of T. koningi.

In view of the probable inhibitory role of these sugars, their effect has been studied on the colonization of the cellulose strip and its deterioration by fungal populations.

In order to study the effect of the perfusion of additional carbon sources on to the cellulosic substrate, three concentrations of glucose were perfused along with the normal E & P salt solution. These concentrations of

glucose were 0.25%. 0.5% and 1.0%. For adequate replication 60 perfusion devices were set up as described previously, for each glucose concentration. Every time 4 such devices were sacrificed; out of these, there were used for weight loss determination and the fourth one was inoculated on to 6 cellulose agar plates, 3 of which contained the same concentration of glucose along with cellulose. The inoculated agar plates were incubated at the same temperature as the perfusion sets, i.e. 25° C., and were observed after 7 days of incubation.

The results of the perfusion with varying concentration of glucose are summarized in Tables 30-32.

The number of fungi isolated at the 0.25% concentration of glucose was relatively low. However, the frequency of occurrence of sugar fungi namely Mucor sp. and Zygorhynchus moelleri was quite high (Table 30). These were quite common throughout the period of incubation. Among the cellulolytic fungi, Fusarium solani was isolated as early as 2 days and had a very high frequency of occurrence throughout the process of deterioration of the cellulose strip. Trichoderma viride, another well known cellulose decomposer, had a much lower frequency of occurrence and was first isolated as late as 8 days of incubation. It only became dominant towards the end of the incubation period. Humicola grisea appeared only between 4 and 8 days. Gliocladium roseum and Paeciliomyces elegans were also isolated after 4 days but remained dominant throughout the experiment. G. roseum had a higher frequency of occurrence as compared to P. elegans. Penicillium funiculosum was observed growing in the cellulose strip after 6 days of incubation and it was then after

Table 30: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing 0.25% glucose.

FUNGI	Days of Incubation																	
	2		4		6		8		12		16		22		26		30	
	CA	G*	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G
Aspergillus fumigatus												100	33	33				
Chaetomium globosum																		33
Diplococcum asperum		33																
Fusarium oxysporum									33	33								
Fusarium solani	66	33	100	100	66	66	100	100	33	33	100	100	100	33	100	66	66	66
Gliocladium roseum				66	33	33	66	33	100	100	33	66	33	33	100	66	100	100
Humicola grisea			66			66	66											
Mucor globosus	33	66	33		66	100		33	33	33	66	33	33	33	66	33	100	100
Paecilomyces elegans		33	33	33		33			66	33	33		33	66				33
Penicillium funiculosum						33	33	66	66	100	33	100	66	100		66		66
Trichoderma viride								33	33	66	66	33	33	100	100	66	100	100
Zygorhynchus moelleri					66	100		100		100	100	100	33	66	66	66		

* E & P cellulose agar containing 0.25% glucose

repeatedly isolated. Among other fungi, Dicoccum asperum was once isolated after 4 days and Chaetomium globosum was isolated after 30 days of incubation. A. fumigatus was also observed after 16 and 22 days of incubation of the perfused cellulose strip.

The colonization pattern obtained by the perfusion of 0.5% glucose concentration in the E & P salt solution, revealed interesting results. (Table 31). At this concentration of glucose T. viride was found to be very dominant in frequency of occurrence whereas F. solani had a much lower frequency. T. viride reached a 100% frequency of occurrence and it was the same till the cellulose strip was completely deteriorated. Mucor globosus was isolated after 2 days of incubation and it had quite high frequency of occurrence throughout the period of incubation. Penicillium funiculosum was isolated after 6 days and it was again very high in frequency till the complete deterioration of the cellulose strip. Paecilomyces elegans was also isolated during the same period but was comparatively low in frequency of occurrence. G. roseum also made its appearance between 6 and 22 days of incubation. A. fumigatus also made its appearance after 6 and 14 days. Among other species, Rhizopus sp. and P. varioti were isolated occasionally during the period of incubation. Eurotium sp. was also isolated towards the end of the experiment. During the period of incubation, some nematodes were observed on the cellulose strip, but no nematode trapping fungi were recorded.

The perfusion of the cellulose strip with E & P salt solution containing 1% glucose enabled the isolation of more species as compared to similar isolations at lower glucose

Table 31: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing 0.5% glcose.

FUNGI	Days of Incubation																	
	2		4		6		8		10		14		18		22		26	30
	CA	G*	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA G	CA G
Aspergillus fumigatus						66					33	33						
Fusarium sp.			33				33	33	100	100	66	33	66					33 33
Gliocladium roseum					33	66		66			33	33	66	66	33			
Mucor globosus	100	66	100	100	33	66	33		100	66	100	100						100
Paeciliomyces elegans					100	100	33							100	100	33		33 66
Penicillium funiculosum						100		100	66	66	33	100	100	100	100	100		100 100
Trichoderma viride		100	66	100	100	100	100	100	100	100	100	100	100	100	100	100	100 100	100 100

* E & P cellulose agar containing .5% glucose

Table 32: Percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing 1.0% glucose.

FUNGI	Days of Incubation																	
	2		4		6		8		12		16		20		24		28		30	
	CA	G*	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G	CA	G
Aspergillus fumigatus				33							33	33								
Chaetomium globosum											33									
Coniothyrium fuckeli										33										
Eurotium sp.			66											33		66				
Fusarium oxysporum			33		66															
Fusarium solani	100	66	33	66	100	100	66	100	33	33	100	100								
Gliocladium roseum				66		33		33			66					66				33
Humicola grisea				33					33	100	100	66			33	66	33	33		66
Mucor globosus	66	33	33	66		100		33	66	66		33								33
Paeciliomyces elegans			33	66	66		33	66	100	100	33	100	33	66		33	33		33	33
Paeciliomyces varioti			33									33								
Penicillium fumiculosum			100	66	66	66	66	100	100	66	66	66	66	66	33	33		33	33	66
Penicillium sp.			33										33	33					33	
Rhizopus sp.	33						66	100												
Trichoderma viride	66	100	100	66	100	100	33	33	33	33		66	33		33				33	33
Zygorhynchus moelleri	66	100			66	66	100	100	100	100	100	66			66	66	33	66	33	33

* E & P cellulose agar containing 1.0% glucose

concentrations (Table 32). The early cellulolytic colonizers namely, F. solani and T. viride were isolated after 2 days and had nearly the same frequency of occurrences. These species had maximum frequency of occurrence 100% between 8 and 16 days whence it decreased towards the end of the incubation period. Among the sugar fungi, Zygorhynchus moelleri, Mucor globosus and Rhizopus sp. were isolated after 2 days and were common throughout the experiment. Most of the remaining fungi were isolated after 4 days of incubation. Among the most frequent species were Penicillium funiculosum, Paecilomyces elegans and Humicola grisea. Among these P. elegans and H. grisea reached a maximum frequency of 100% after 12 and 16 days when after it declined. P. funicolosum had a similar maximum frequency of occurrence but it was between 4 and 12 days where after it just made its appearance. G. roseum was also isolated after 4 days and was occasionally isolated afterwards with quite a low frequency of occurrence. It was mostly isolated on cellulose agar containing 1% glucose. F. oxysporum was also isolated on this medium after 4 and 6 days of incubation. Penicillium sp., Paecilomyces varioti, A. fumigatus and Eurotium sp. were also isolated after 4 days but were only isolated once or twice later on. C.fuckeli and Chaetomium globosum were also isolated once, after 12 and 16 days respectively.

The results of the extent of biodeterioration of the cellulose strip as estimated by its weight losses are summarized in Tables 33a-c. There was again not much difference in the weight loss produced at varying glucose concentrations (Figure 10). The percentage weight loss

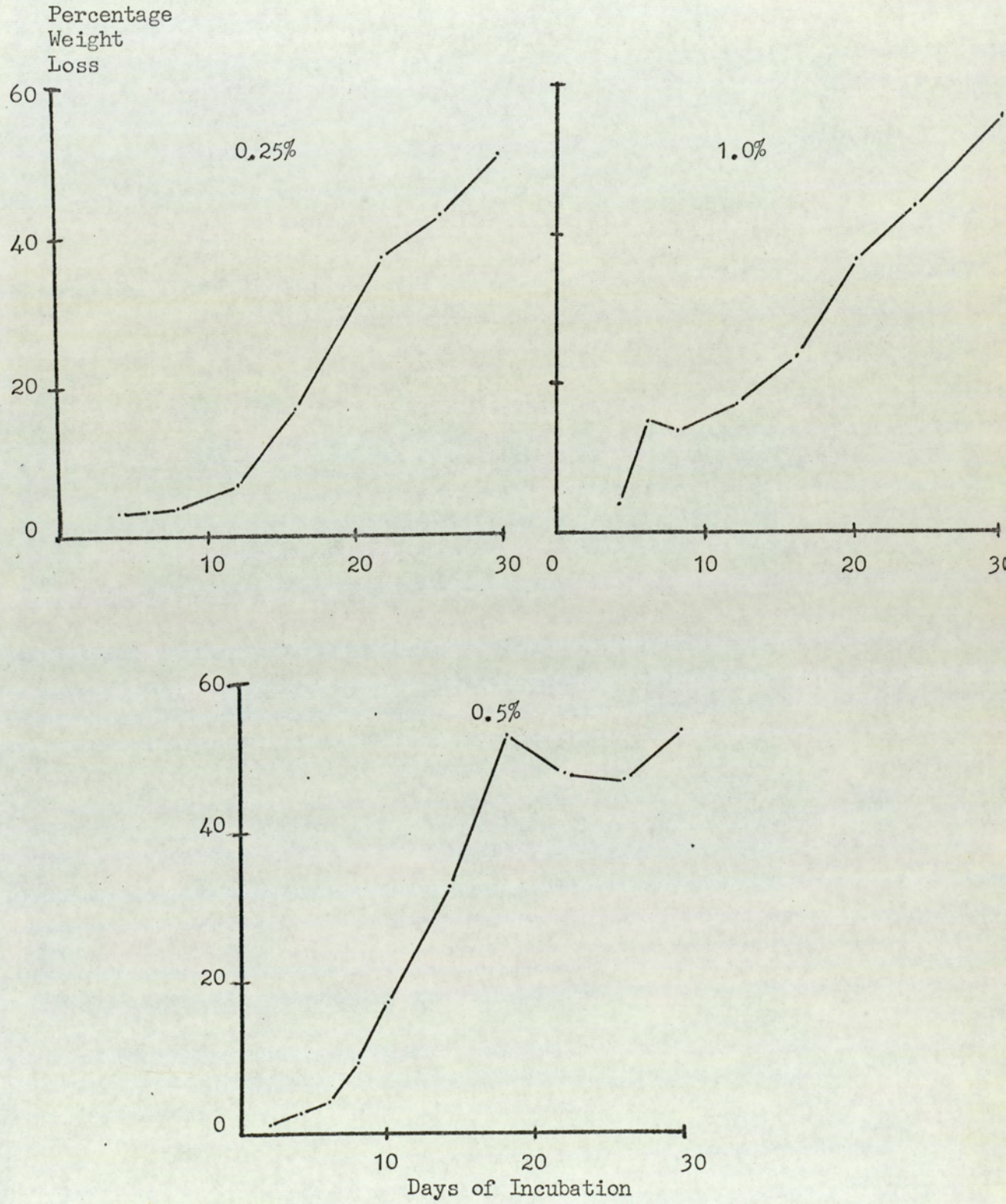


Figure 10: Average percentage weight loss of the cellulose strip perfused wth E.&P. salt solution containing different concentrations of glucose.

Table 33a-c: Percentage weight loss of the cellulose strip perfused with E.&P. salt solution containing various amounts of glucose.

a) 0.25% glucose

Days of Incubation	% Weight Loss			Mean	S.D.
4	2.2	3.1	4.1	3.1	0.9
6	1.9	4.3	2.8	3.0	1.2
8	3.6	5.1	2.9	3.8	1.1
12	6.7	6.1	7.3	6.7	0.5
16	15.8	16.1	18.3	16.7	1.3
22	31.3	38.6	40.7	36.8	4.9
26	44.6	39.6	43.2	42.5	2.5
30	49.8	52.1	50.4	50.7	1.1

Table 33

b) 0.5% glucose

Days of Incubation	% Weight Loss			Mean	S.D.
4	1.8	2.1	7.7	3.8	3.3
6	16.5	12.1	14.4	14.3	2.2
8	11.8	15.5	14.1	13.8	1.5
12	19.2	12.1	17.3	16.2	3.6
16	23.1	21.3	26.1	23.5	1.9
20	37.5	37.5	33.6	36.2	1.8
24	44.6	45.2	40.6	43.4	2.5
30	56.4	51.5	58.6	55.5	2.9

Table 33

c) 1.0% glucose

Days of Incubation	% Weight Loss			Mean	S.D.
2	1.2	0.54	0.8	0.84	0.33
4	2.8	1.9	2.1	2.2	0.4
6	4.6	5.3	3.2	4.3	1.0
8	9.1	8.5	10.1	9.2	0.8
10	15.9	17.8	20.1	17.9	2.1
14	32.2	34.1	35.1	33.8	1.4
18	53.0	53.5	54.8	53.7	0.9
22	48.3	47.5	49.5	48.4	1.0
26	46.9	48.1	47.8	47.6	0.6
30	55.8	56.1	52.1	54.6	2.2

ranged from 49.7 to 54.6. The validity of some of these results can be doubted as in these cases quite thick "mats" of P. funiculosum were observed growing from the edges of the cellulose strip. It was very difficult to remove fungal mycelia from the cellulose strip without damaging it or removing some cellulose fibres with it. This effect does not seem to be too great to invalidate the results. Moreover the standard deviation of most of the observations is quite low and fairly constant.

In spite of little difference in the extent of deterioration of the cellulose strip at varying concentrations of glucose, the colonization patterns have shown interesting results. The average percentage frequency of occurrence of different fungi at different concentrations of glucose are represented in Figure 4. At 0.25% glucose, F. solani had the maximum frequency of occurrence whereas T. viride had the lowest frequency of occurrence. This phenomenon was completely reversed at 0.5% glucose where T. viride had the highest and F. solani the lowest frequency of occurrence. It is interesting to note that maximum weight loss of the cellulose strip also took place at 0.5% glucose. This fact indicates that T. viride is the main fungus involved in the cellulose decomposition. This conclusion is also born out in the preceding results, when the cellulose strip was perfused with E & P salt solution without any organic nitrogen; it produced maximum weight loss. In this case T. viride also had highest frequency of occurrence (Figure 2) and F. solani the lowest. This phenomenon may be due to two factors. Firstly, the low concentration of glucose (0.25%) may be able to stimulate the growth of

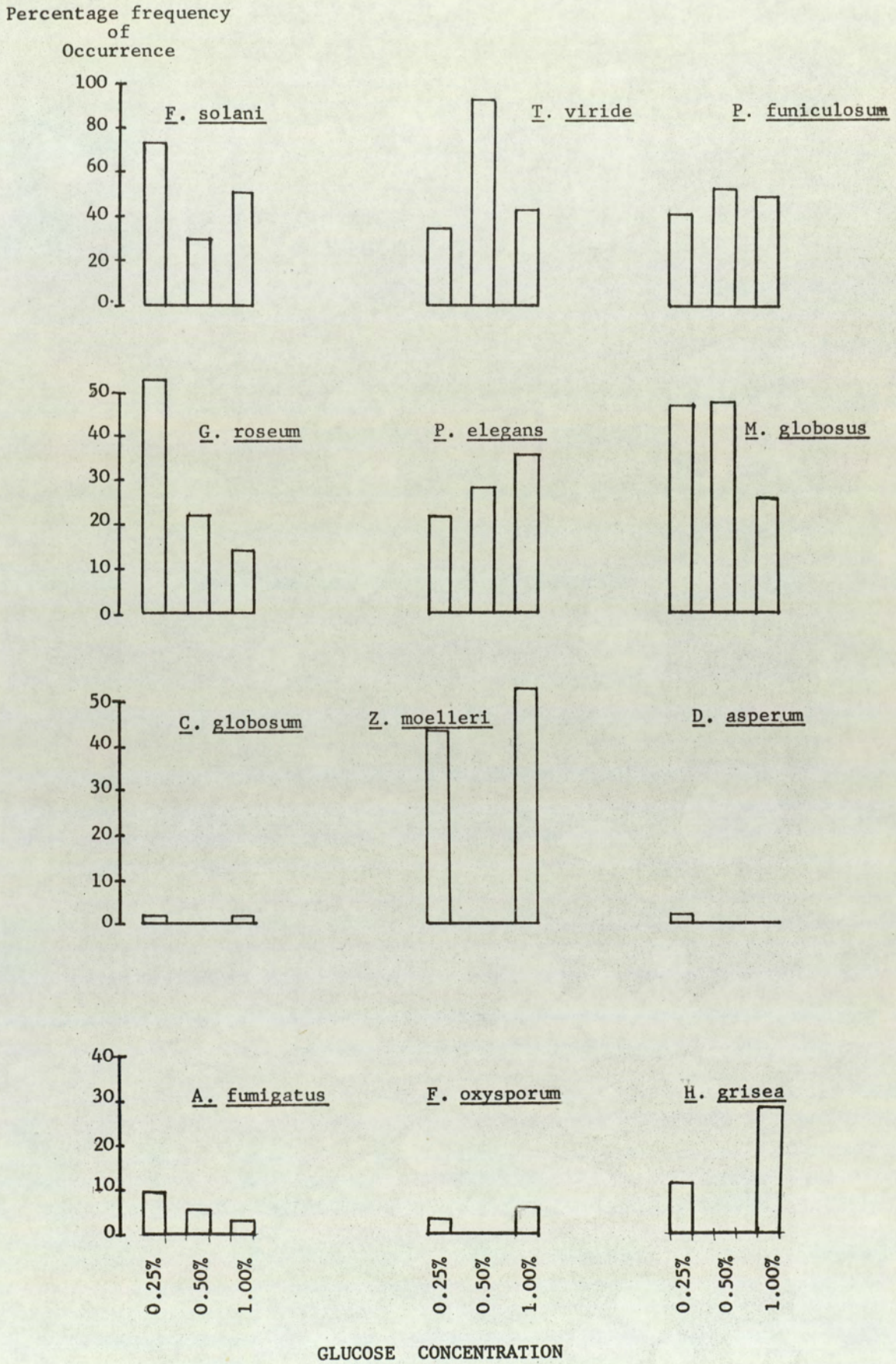


Figure 11: Average percentage frequency of occurrence of fungi isolated from the cellulose strip perfused with E.&P. salt solution containing various amounts of glucose.

F. solani and not T. viride. Secondly, the dominant position of F. solani may in turn act as an antagonist to T. viride. At 0.5% glucose, T. viride is also presumably stimulated as it has maximum frequency of occurrence. This high frequency may in turn inhibit F. solani which might explain its low frequency of occurrence. At 1% glucose concentration, it seems that some part of equilibrium has been reached as both these species have nearly the same frequency of occurrence. Another interesting example is that of G. roseum. It had a maximum frequency of occurrence of 53.0% at 0.25% glucose. This frequency decreased with the increase of glucose concentration. The frequency of this species was quite low when the cellulose strip was perfused with E & P salt solution only. These observations can best be explained by the assumption that glucose when present in very low concentrations stimulates the growth of certain microorganisms whereas these might be inhibited at higher concentrations of glucose. A. fumigatus also belongs to this category of fungi as its frequency decreased with the increase in concentration of glucose. This may be due to the high frequency of occurrence of T. viride at 0.5% glucose. Certain other cellulolytic fungi such as H. grisea, P. funiculosum and Paecilomyces elegans showed different behaviour. It was found that their frequency of occurrence was directly proportional to the glucose concentration. It seems that glucose, in this case is acting both as a stimulatory factor and as a substrate. This phenomenon has previously been demonstrated in cellulolytic bacteria by Stainies (1942). On the other hand Siu (1951) has quoted Fahraens (1942) who found that 0.1% solution of

glucose retarded the growth of Cytophaga globosa and 0.25% glucose stopped its growth altogether. This discrepancy in the results can be explained on the assumption that the low concentration value of glucose needed for the stimulation of fungi varies with different fungi.

The majority of the fungi isolated so far, using the perfusion technique at different pH values, temperatures and nutrients, produced a clearing of the cellulose agar (Eggins & Pugh, 1962), thus confirming their cellulolytic nature. The extent of the clearing of the cellulose varies with different fungi and their micro-environmental conditions.

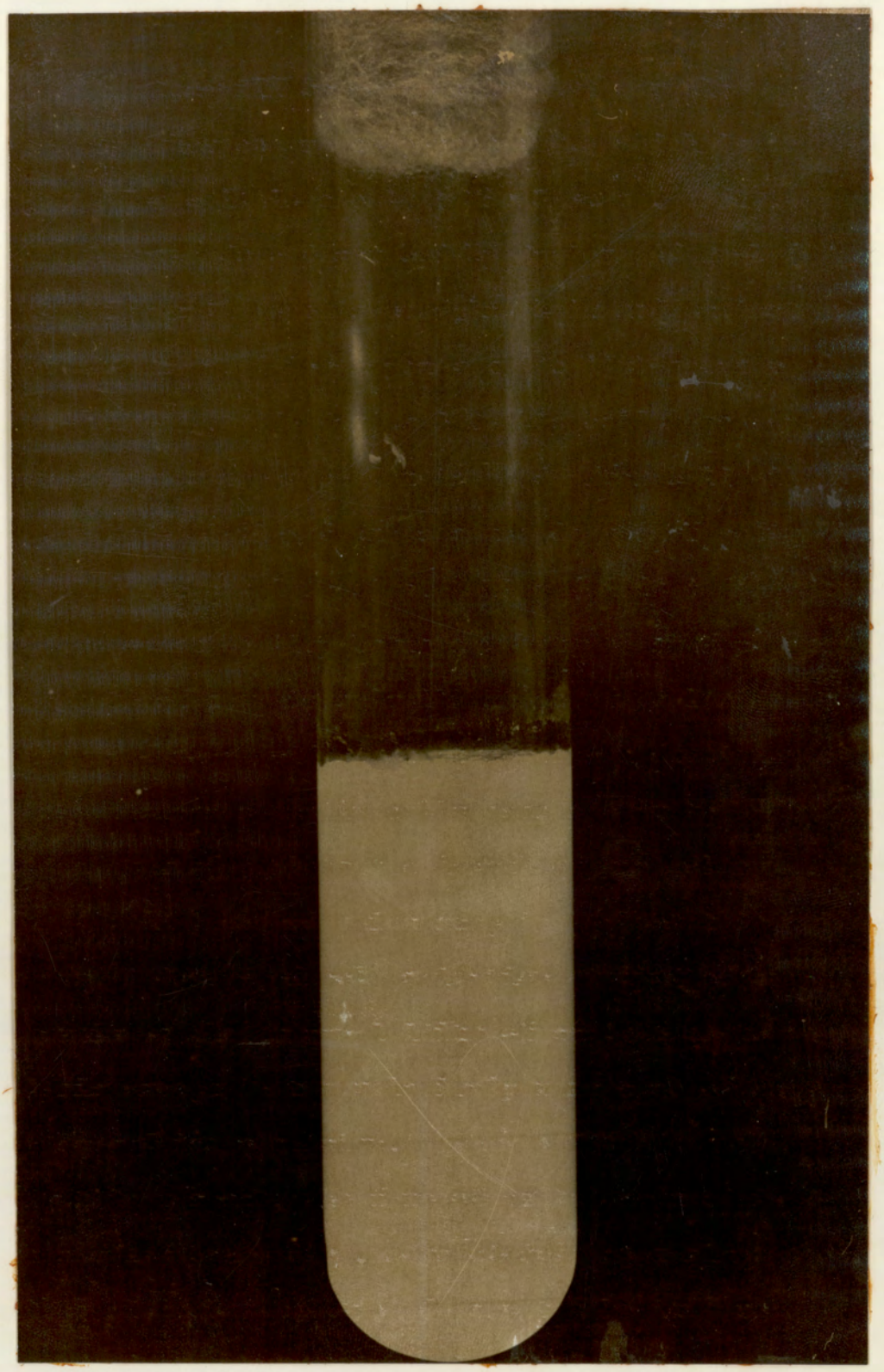
A simple method for determining the relative cellulolytic activity of fungi which was originally described by Rautela and Cowling (1966) has been employed here, after some modifications. This method essentially involves the measurement of the depth of clearing of an opaque column of cellulose agar medium contained in a boiling tube. Rautela and Cowling (1966) used cellulose prepared by the procedure of Walseth (1952). In this process Whatman powdered cellulose is made 'reactive' before incorporating into the media by swelling the cellulose in o-phosphoric acid and then it is regenerated and washed in cold water. Thus according to the C_1C_x concept (Siu & Reese 1953), during the hydrolytic dissolution of the cellulose, the componement C_x of the

cellulase system will be most active but may also involve the other enzyme component (C_1) which is active on crystalline cellulose only (Siu & Reese, 1953).

In the procedure described by Eggins and Pugh (1962) a crystalline cellulose is used in their medium instead of partially crystalline regenerated cellulose as used by Walseth (1952). In Eggins & Pugh's cellulose agar Whatman chromotography powder is used after ball milling for 72 hrs. Ball milling decreases the degree of polymerization by reducing the particle size. King (1966) demonstrated a direct proportionality between substrate surface area and the rate of enzymic solubilization of cellulose. He has discussed some evidence that endwise hydrolysing cellulases do exist; whereas normally cellulases appear to hydrolyse cellulose at random along the length of the molecule. Cowling (1963) seemed quite sceptical about this evidence and regarded degree of polymerization of limited significance in determining the susceptibility of cellulose to enzymatic hydrolysis except in the relatively rare case of enzymes that act by an endwise mechanism.

In this respect, the form of cellulose used in Eggins & Pugh's cellulose medium, more closely approximates native cellulose since ball milling does not make it ' reactive ' but only reduces the particle size to help dispersion in the medium. Thus dissolution of this cellulose may involve both C_1& C_x factors of the cellulase enzyme system.

Sterile, uniformly opaque, vertical columns (4 - 6 cms in height) of Eggins & Pugh's cellulose agar medium were prepared in 18mm diameter test tubes. This was easily done by pouring sterile molten cellulose agar into the sterile



Photograph 3

A test tube containing an opaque column of E. & P. cellulose agar.

S. thermophile C. thermophile H. insolens A. fumigatus

Photograph 4
Opaque columns of cellulose agar showing depth of clearing.

test tubes standing in cold water so that the cellulose agar set rapidly and therefore the cellulose could not settle down. These opaque columns were inoculated with standard discs of fungal mycelium and agar from the pure culture plates. The inoculum was always taken from the growing edge of the colony with the help of a 5mm diameter cork borer. The inoculated tubes were then incubated standing upright, at the required temperature. The measurement of the depth of clearing was taken after different intervals of incubation by measuring the clearing against a strong light. In order to standardise the observation and to minimise the visual error, the level of the cellulose agar column was marked on the test tube with a sharp wax pencil, before incubation. The subsequent measurements of the depth of clearing were always taken from the same point. (Photo 3)

This simple technique has been employed in the present investigation to study the effect of different micro-environmental factors on the relative cellulolytic ability of the fungi previously isolated with the help of the perfusion technique. The effect of microenvironmental factors namely pH, temperatures and nutrients, on the colonisation of the cellulosic substrate has been investigated and discussed in the previous chapters. The effect of similar environmental factors on the relative cellulolytic ability of fungi has been studied here by simply changing the pH of the medium, or the chemical composition of the medium, or by incubating the inoculated tubes at different temperatures.

The results of these investigations are presented in this chapter under the heading of different microenvironmental factors.

Effect of pH and Temperature on the Relative Cellulolytic Ability of the Fungi Isolated at Different Temperatures.

Eggins and Pugh's cellulose agar media was prepared at different buffered pH values (pH 4.0, 5.0, 6.0, 6.4, 7.0 and 8.0) as described previously in Chapter 3. The opaque columns of cellulose agar were made out of this cellulose medium maintained at different pH levels. Three replicate test tubes containing cellulose agar columns were set up for each fungus at every pH level. The fungi isolated at various temperatures were tested for their relative cellulolytic ability at the same temperature. The results, hereafter, are summarised under various temperature headings.

25° C.

The maximum relative cellulolytic activity of the fungi at different pH values, after 30 days of incubation is represented in Figures 12 a - b.

Out of all the fungi tested, Trichoderma viride proved to be the most cellulolytic. The pH did not have much effect on its cellulolytic activity as the depth of clearing varied from 25.5 mm. to 18.0 mm. The maximum depth of clearing was produced at pH 4.0 and minimum at pH 8.0. Fusarium solani and F. moniliforme also seemed to be quite cellulolytic with their maximum clearing of 18.0 mm. and 19.5 mm. respectively at pH 6.4. The other fungus which reached a comparable degree of cellulolytic activity was Aspergillus fumigatus. It had as high degree of clearing as 21.0 mm. at pH 6.4. The depth of clearing here again was not very much affected by different pH values.

Among other cellulolytic fungi Chaetomium globosum Gliocladium roseum, Humicola grisea, Arthrobotrys sp.,

FIG. 12a

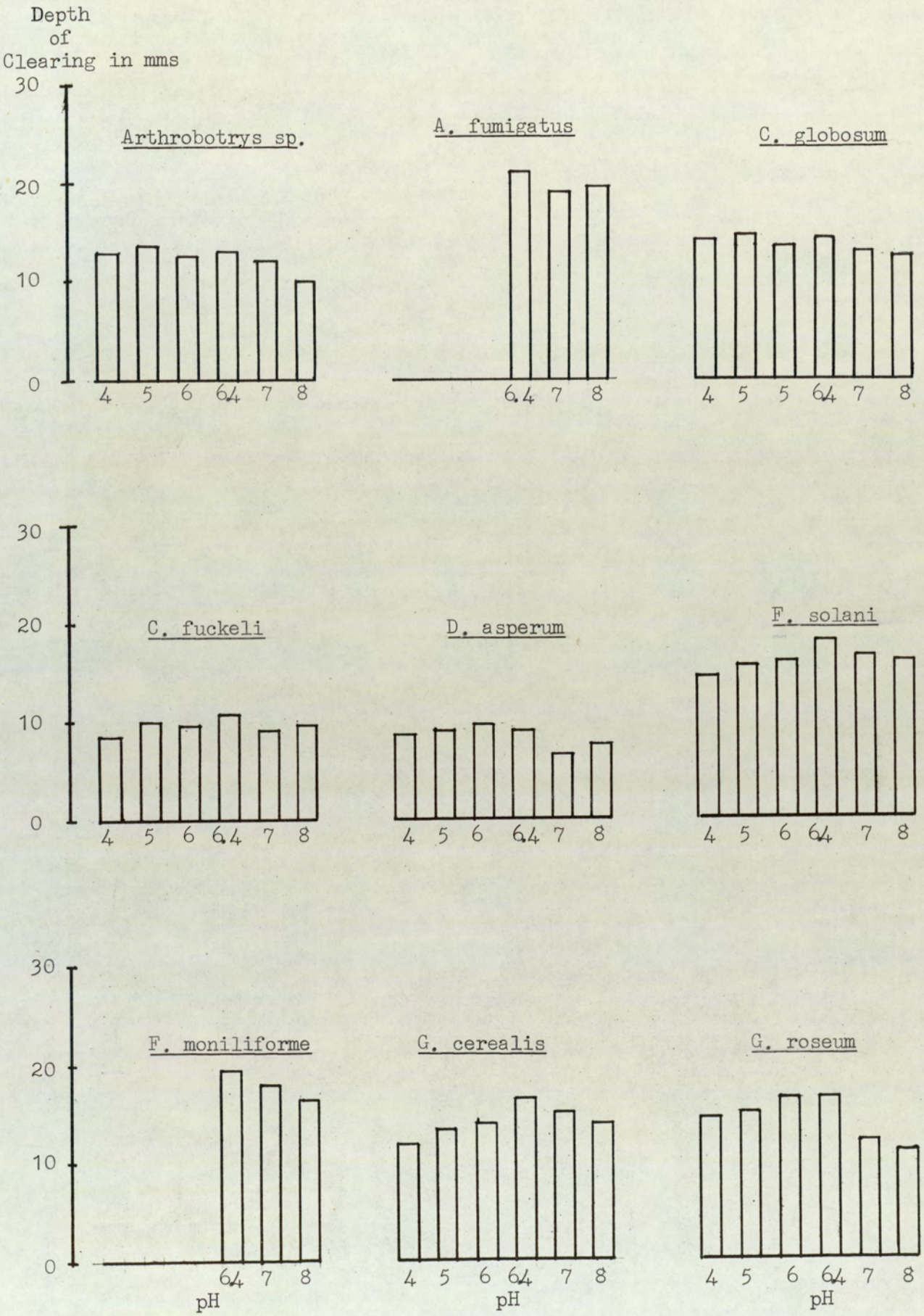
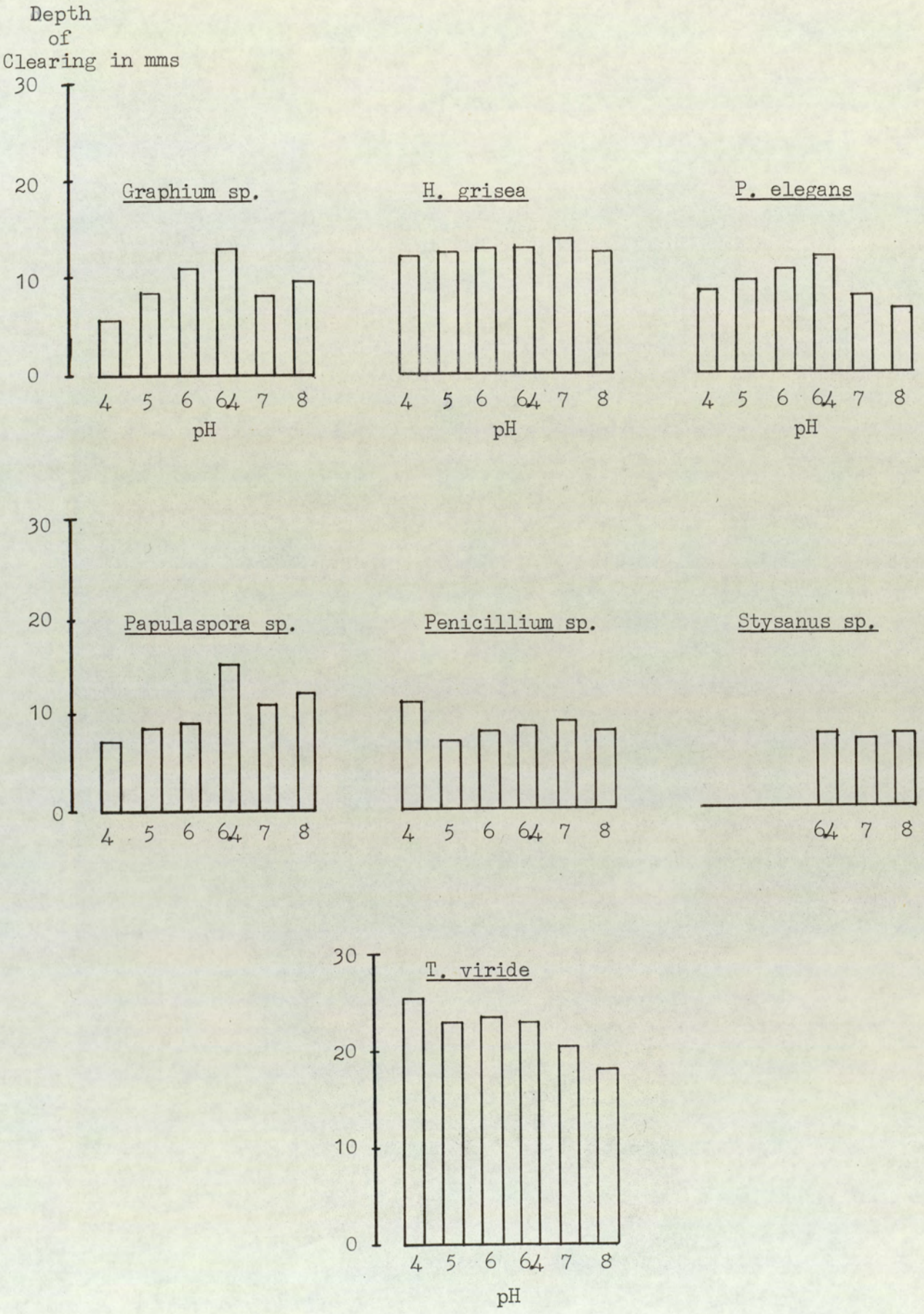


Figure 12a-b: Maximum depth of clearing produced by different fungi after 30 days of incubation at 25°C at different pH values.

FIG. 12b


Depth of Clearing in mms
30
20
10
0
Graphium sp.
H. grisea
P. elegans
Papulaspora sp.
Penicillium sp.
Stysanus sp.
T. viride
4 5 6 6½ 7 8
pH

Gelasinospora cereali, Paecilomyces elegans and Papulaspora sp. produced clearing of the cellulose in the range of 10 mm to 15 mm. The optimum pH for maximum clearing varied with various fungi; but still there was not much difference at different pH values.

There were certain fungi which produced less than 10 mm clearing of the cellulose agar column after 30 days of incubation. Such fungi were comprised of Dicoccum asperum, Penicillium sp., Stysanus sp. and Graphium sp. Most of these fungi showed their maximum cellulolytic ability between pH 6 and 7. Penicillium sp. and Graphium sp. produced a clearing of 11.0mm at pH 4.0 and 13.0 at pH 6.4 respectively.

Some fungi produced no clearing of the cellulose agar column and thus proved to be non cellulolytic. These were Mucor globosus, Paeciliomyces varioti, Rhizopus sp. and Zygorhynchus moelleri.

The comparative review of the relative cellulolytic activity and the percentage frequency of occurrence of these fungi showed some interesting features. T. viride and F. solani had the highest frequency of occurrence (Fig 1a Chapter 3), similarly these two fungi have also proved to be highly cellulolytic, the only difference being that optimum pH for frequency of occurrence was 6.4, whereas T. viride had optimum pH 4.0 for maximum cellulolytic activity. A.fumigatus was extremely low in its frequency of occurrence whereas it had a very high cellulolytic activity. This might point towards its nature as an airborne contaminant, playing an insignificant role in the cellulose deterioration in the soil at 25^{o}C.

Arthrobotrys sp. and F. moniliforme were fairly common at pH 6-6.4 and pH 7.0 respectively. The relative

cellulolytic activity of F. moniliforme was quite high and can be placed in the category of F. solani and T. viride, whereas Arthrobotrys sp. proved to be moderately cellulolytic.

The remaining cellulolytic fungi had a very low frequency of occurrence ranging from 3% to 10%. Their cellulolytic ability was also comparatively lower than the above mentioned species but not as low as their frequency of occurrence.

Any discrepancy in the frequency of occurrence and the relative cellulolytic ability of the fungi might be due to different experimental conditions. The percentage frequency of occurrence is calculated after recording the abundance of the fungi associated with cellulose deterioration under comparatively natural conditions; whereas the relative cellulolytic activity of the fungi is estimated by measuring the depth of clearing of a cellulose agar medium which provided comparatively artificial conditions.

35°C

The relative cellulolytic ability of the fungi isolated at 35°C is represented in Fig 13.

Among the fungi tested for cellulolytic activity at this temperature, A. fumigatus proved to be most cellulolytic, producing maximum clearing of 20.0 mm at pH 6.4. Minimum clearing of 16.5mm was produced at pH 4.0 and 8.0. Among other fungi tested at this temperature, Eurotium sp. and Sprotrichum pruinosum were also quite cellulolytic, producing 14.5 mm - 18.0 mm and 11.5mm - 15.0mm clearing respectively at different pH values. At pH 5.0 the cellulolytic activity of Eurotium sp.was maximum whereas optimum pH for S. pruinosum was 6.4. Humicola grisea

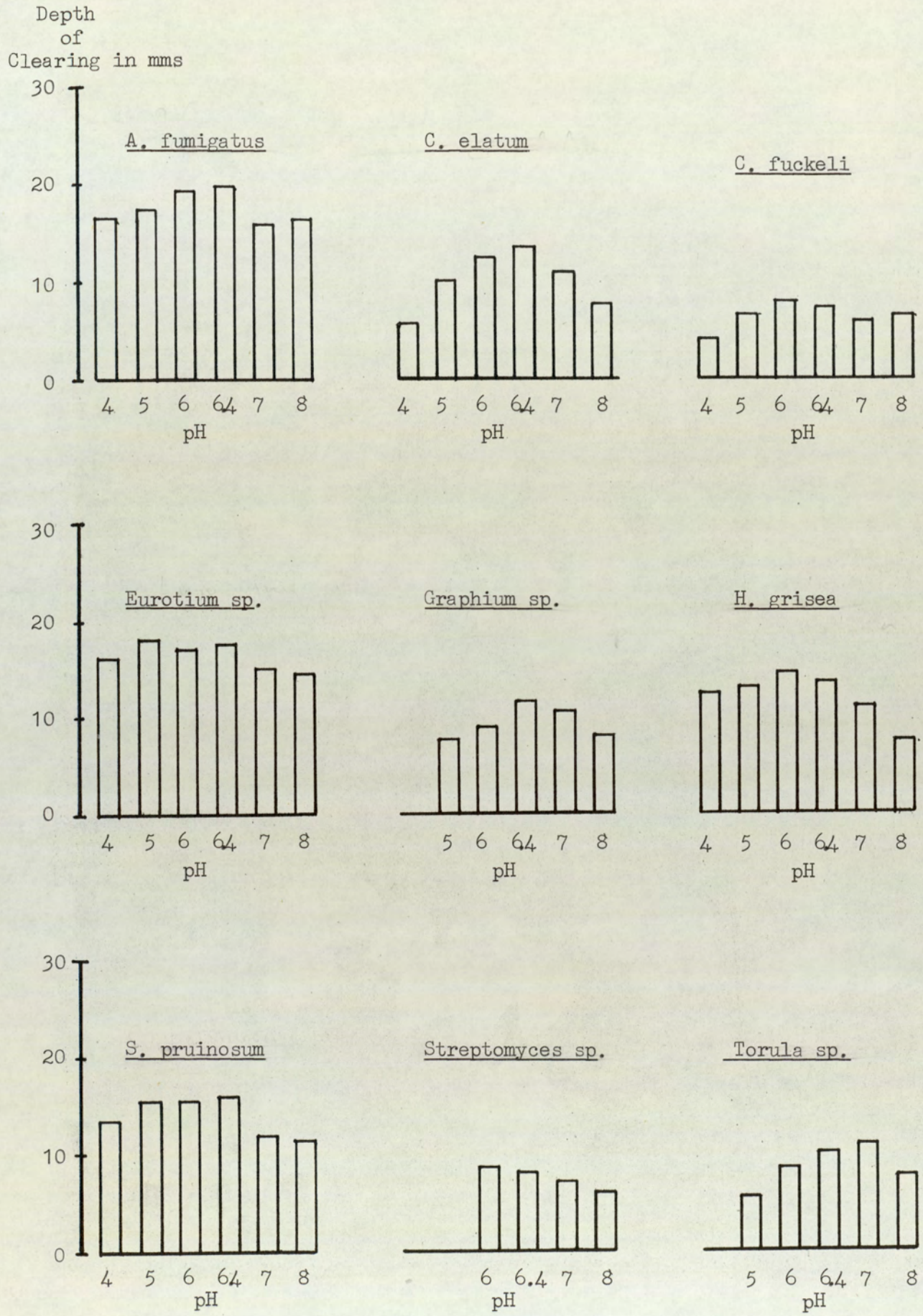


Figure 13: Maximum depth of clearing produced by different fungi after 30 days of incubation at 35°C at different pH values.

and Chaetomium elatum also produced a fair amount of clearing on the cellulose agar column. The clearing produced by H. grisea ranged from 10. 0 mm to 14.5 mm; the maximum being at pH 6.0 and minimum at pH 8.0. C. elatum had quite low cellulolytic activity at pH 4.0 and pH 8.0. Its optimum pH was 6.4 where it produced 13.5 mm clearing of the cellulose agar. Torula sp. and Graphium sp. produced comparatively less clearing. Torula sp. had a maximum clearing of 11.0 at pH 7.0 and minimum 5.5 mm at pH 5.0 and it did not grow at pH 4.0. Graphium sp. also did not grow at pH 4.0 but had maximum clearing of 11.5 mm at pH 6.4 and minimum 7.5 mm at pH 5.0. Streptomyces sp. tested for cellulolytic activity did not grow at pH 4.0 and pH 5.0 but produced a clearing ranging from 8.5 mm to 6.0 mm with maximum clearing at pH 6.0 and minimum at pH 8.0. Coniothyrium fuckeli also produced very little clearing and it managed to grow on all pH values, having its maximum clearing at pH 6.0.

The role of A. fumigatus at this temperature will be much elucidated, if these results are compared with those of the percentage frequency of occurrence (Fig 3 Chapter 3). A. fumigatus had an extremely high frequency of occurrence and so was the case with its cellulolytic activity at this temperature. This strongly points towards its association with cellulose deterioration in the soil at 35°C unlike its negligible role at 25°C. Most of the remaining cellulolytic fungi had a comparatively low frequency of occurrence and low pH also seemed to be the limiting factor. This phenomenon was also depicted in their relative cellulolytic ability where it was extremely low at low pH values. Streptomyces sp.

did not produce any clearing at pH 4.0 and 5.0 and Graphium sp. and C. fuckeli also did not grow at pH 4.0 when inoculated for testing their cellulolytic ability at this pH. C. elatum had a very low frequency of occurrence but its cellulolytic activity was fairly high whereas reverse was the case with Streptomyces sp.

50°C

The results of the relative cellulolytic activity are presented in Fig 14 and some in Photograph 5.

Chaetomium thermophile, Cephelosporium sp. and Sporotrichum thermophile proved to be the most cellulolytic fungi at this temperature. The maximum clearing of the cellulose agar produced after 30 days of incubation was 26.0 mm at pH 6.4 for C. thermophile, 26.5 mm at pH 4.0 for Cephelosporium sp. and 25.0 mm at pH 5.0 for S. thermophile. The depth of clearing at other pH values did not vary much from the maximum except that S. thermophile did not grow on the cellulose agar column at pH 4.0. Humicola grisea var thermoidea was also fairly cellulolytic. It produced clearing ranging 13.5 mm to 16.0 mm at different pH values. The maximum clearing was produced at pH 5.0 and 7.0 . H. insolens did not produce any clearing at pH 4.0 and 5.0 whereas at other pH values it produced clearing ranging from 10.8mm to 12.5mm; maximum clearing being at pH 6.4. Torula thermophile also did not grow at pH 4.0 and at other pH values the degree of clearing ranged from 8.5 mm to 11.5 mm with the maximum clearing at pH 7.0 and 8.0. Streptomyces sp. No.1. and Streptomyces sp. No.2 produced clearing only at pH 6 - 8 . The depth of clearing ranged from 7.5 mm to 8.5 mm. Maximum depth of clearing

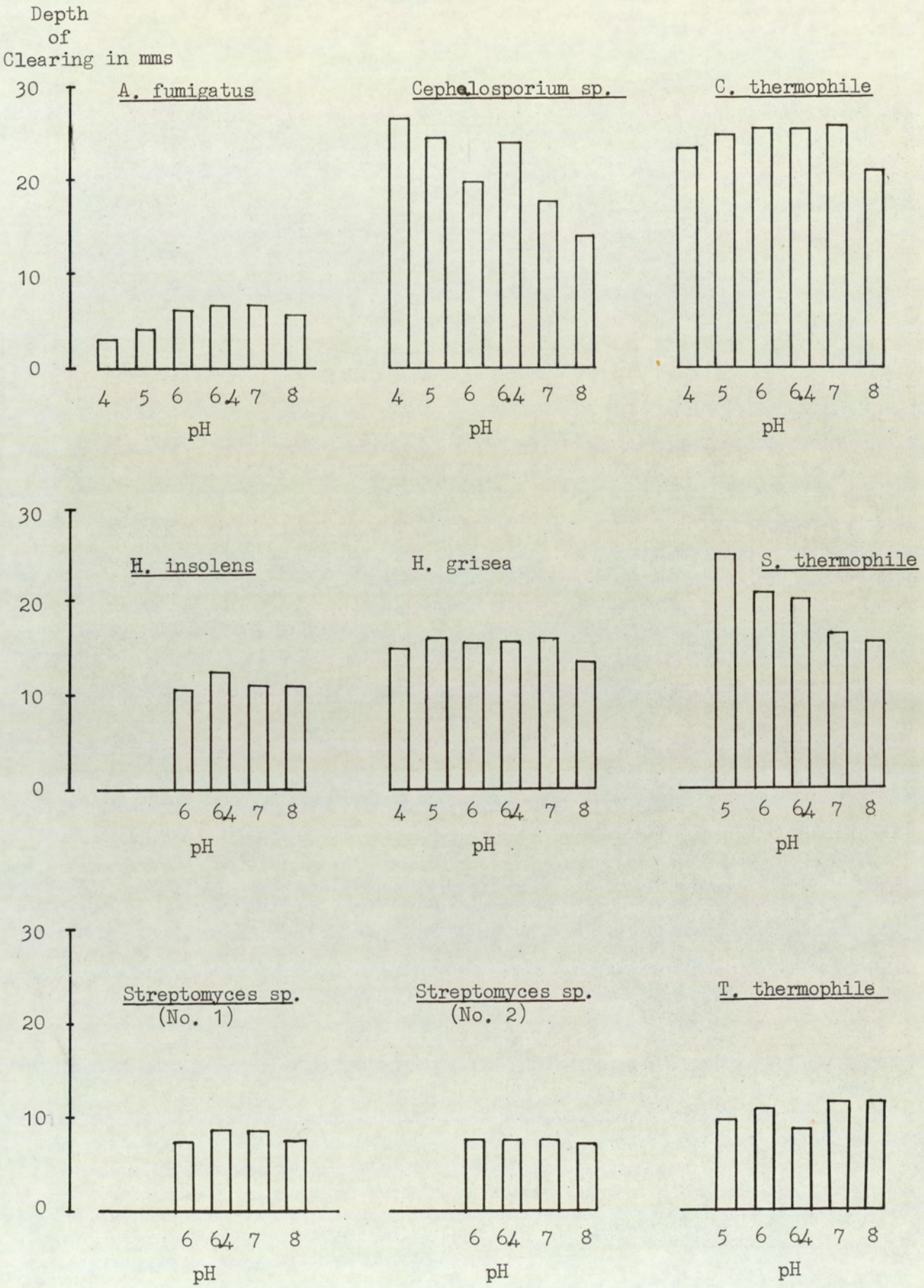


Figure 14: Maximum depth of clearing produced by different fungi after 30 days of incubation at 50°C at different pH values.

was produced at pH 7.0 A. fumigatus was able to grow on the cellulose agar column at all pH values but its depth of clearing was extremely low. Maximum clearing was 6.5 mm and was produced at pH 6.4 - 7 0.

The comparison of the relative cellulolytic activity with the frequency of occurrence of these fungi has shown some interesting results. Out of the three most cellulolytic fungi, only C. thermophile had the equivalent high percentage frequency of occurrence. S. thermophile and Cephalosporium sp. had an extremely low frequency of occurrence. H. grisea and H. insolens had a fairly high frequency of occurrence and so was their cellulolytic activity. Torula thermophile was also extremely low in its frequency of occurrence where it cleared a fiar depth of the cellulose agar. Streptomyces sp. No. 2 was only isolated at pH 6.0, 7.0 and 8.0 and it also produced clearing at the same pH values.

Effect of different nitrogen sources on the Cellulolytic activity of Mesophilic Fungi

The effect of different nitrogen sources on the relative cellulolytic ability of the fungi at 25° C. has been studied by simply adding or replacing the appropriate nitrogen source from Eggins and Pugh's cellulose agar used for making Rautela and Cowling's cellulose agar columns. The effect of four combinations of sources have been studied. These are: 1) potassium nitrate and L. asparagine; 2) ammonium nitrate and L. asparagine; 3) ammonium sulphate only, and 4) ammonium sulphate and L. arginine. Yeast extract was present in all the combinations. The effect of these nitrogen sources on the fungal colonisation of the cellulosic substrate has been discussed in the preceding chapte

The fungi tested for these nitrogen sources have been

isolated by the perfusion technique, perfusing varying nitrogen sources. Three replicate cellulose agar tubes were set up for each fungus for each nitrogen source. These tubes were incubated at 25°C and the depth of the clearing was observed over a period of 30 days.

The measurement of the depth of clearing after 30 days of incubation is represented in Fig 15 a-b.

A majority of the fungi grew well on all nitrogen sources but produced varying depths of the clearing of the cellulose agar column. Potassium nitrate, ammonium nitrate and arginine did not have much effect on the relative cellulolytic activity of these fungi. In the case of ammonium sulphate some fungi failed to produce any clearing or the clearing was very small. The exceptions to this generalisation were T. viride and P. funiculosum. Both these species had their maximum clearing of 24.0 mm and 11.5 mm respectively with ammonium sulphate as the only nitrogen source. Fusarium solani and F. oxysporum preferred potassium nitrate nitrogen as these had maximum relative cellulolytic activity for 16.5mm and 17.0 mm respectively. F. sporotrichiodes had maximum clearing of 11.5 mm with organic nitrogen i.e. arginine. H. grisea, Dicocum asperum and Papulospora sp. also had their maximum depth of clearing with arginine as its organic nitrogen source. Paecilomyces elegans, Gliocladium roseum and Sordaria fimicola had their maximum relative cellulolytic activity with ammonium nitrate as the inorganic nitrogen source. Chaetomium globosum and Aspergillus fumigatus had their optimum clearing with potassium nitrate as the inorganic nitrogen source. The difference

Figure 15a-b: Maximum depth of clearing produced by different fungi after 30 days of incubation at 25 C with different nitrogen sources.

Legend: Inorganic = ammonium sulphate only

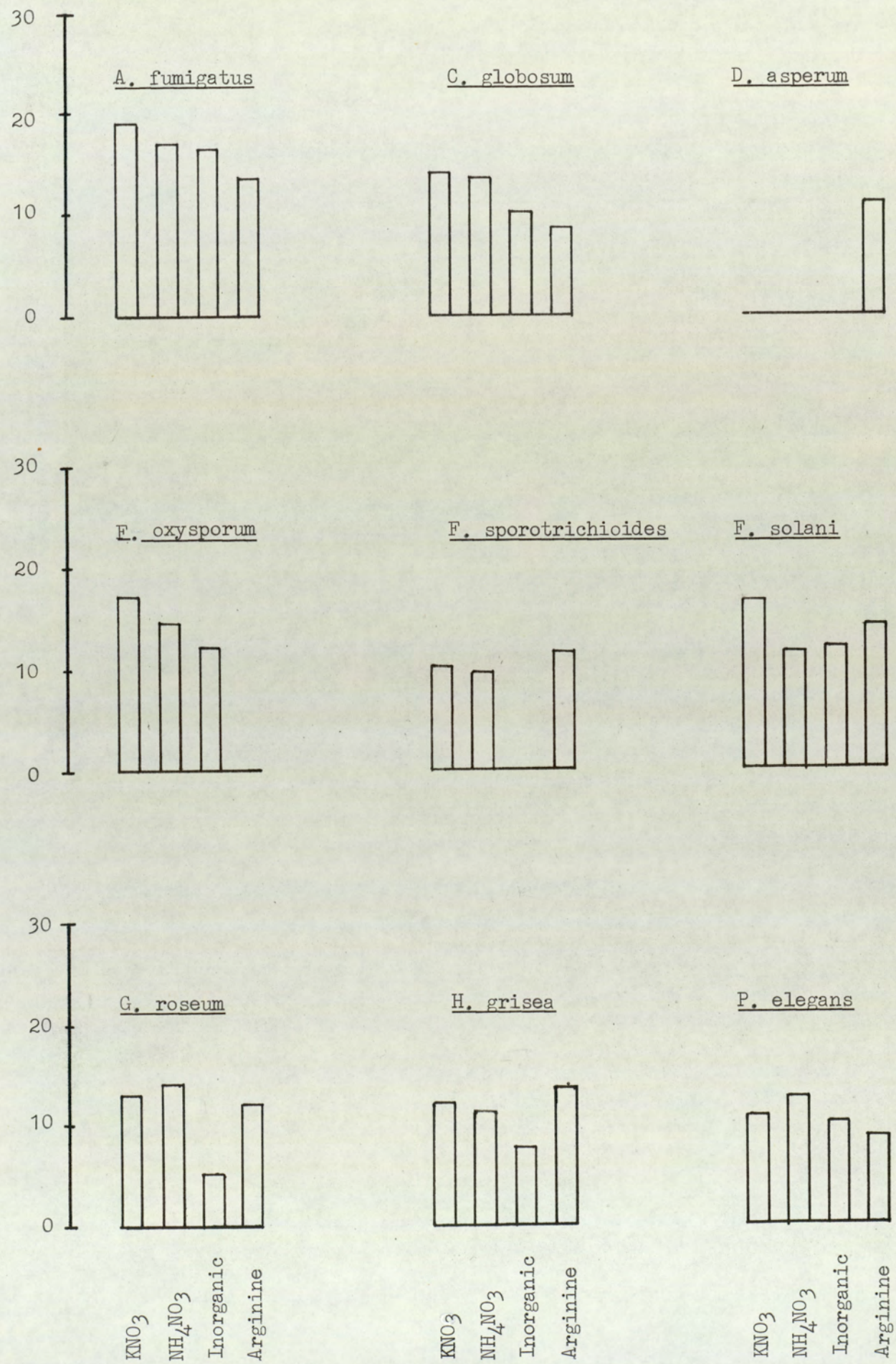
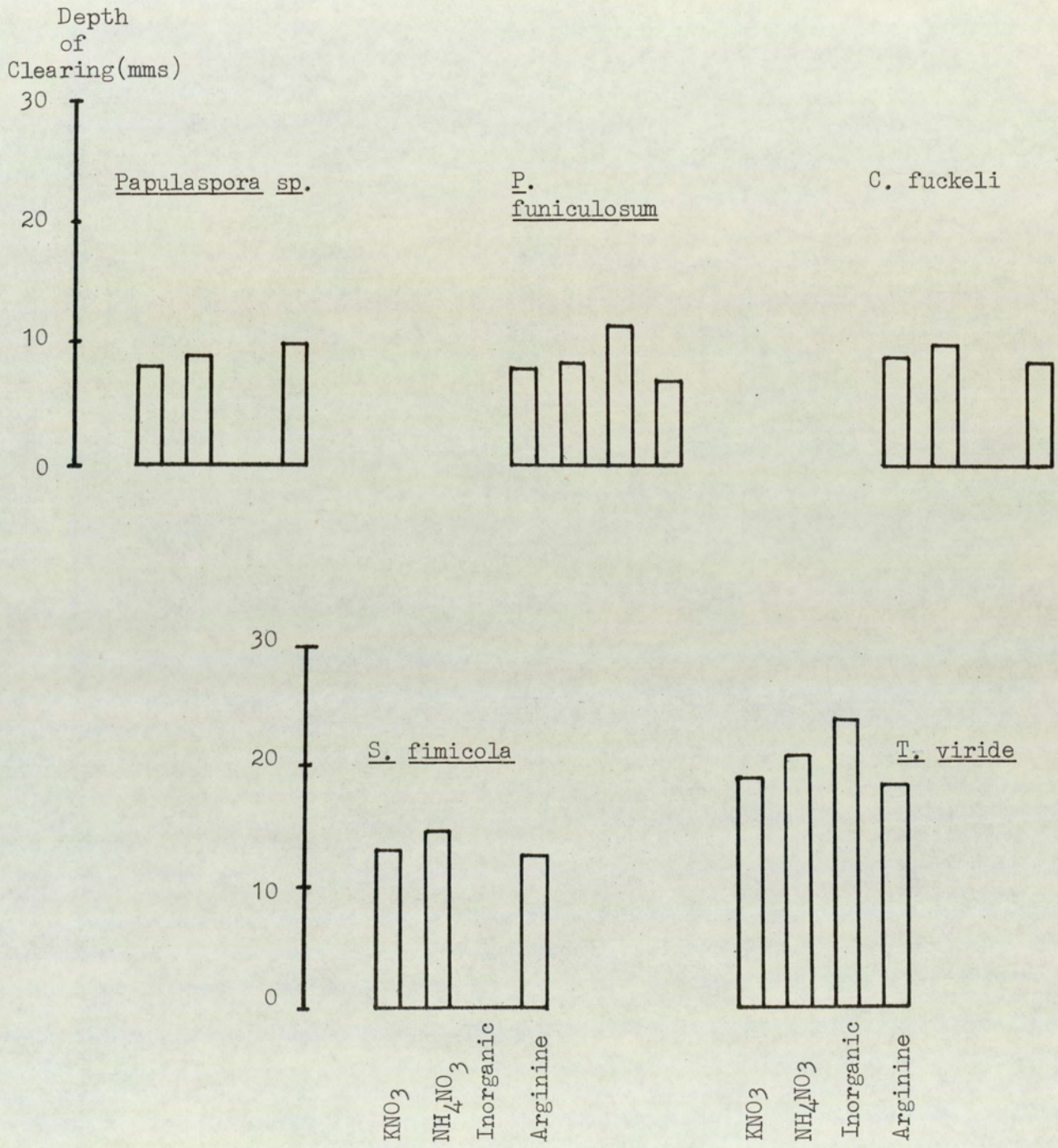


Figure 15b


Depth of Clearing(mms)
30
20
10
0
Papulaspora sp.
P. funiculosum
C. fuckeli
S. fimicola
T. viride
KNO3
NH4NO3
Inorganic
Arginine

between the depth of clearing with potassium nitrate and ammonium nitrate was quite small as compared to that of ammonium sulphate only and arginine.

The comparison of the relative cellulolytic ability of these fungi with their percentage frequency of occurrence has revealed interesting results. A similar preference for the nitrogen source has been found both in the case of percentage frequency of occurrence and the relative cellulolytic ability for most of the fungi tested. Among such fungi are F. solani, T. viride, P. funiculosum, G. roseum, Paecilomyces elegans, H. grisea and A. fumigatus. In case of these fungi the nitrogen source producing maximum frequency of occurrence also produced maximum depth of clearing. Among other fungi C. globosum was only isolated when the cellulosic substrate was perfused with E. & P. salt solution containing potassium nitrate and ammonium nitrate. The maximum frequency of occurrence was at potassium nitrate. In the case of it with relative cellulolytic activity, it produced maximum clearing with potassium nitrate and it also produced comparatively less clearing at other nitrogen sources. F. oxysporum produced maximum clearing of the cellulose agar with potassium nitrate and less with other nitrogen sources, whereas it was only isolated from the cellulose substrate when perfused with ammonium nitrate and arginine. Similarly F. sporotrichiodes had quite a low frequency of occurrence with potassium nitrate and ammonium nitrate and was not isolated from the cellulose when perfused with ammonium sulphate only and arginine, whereas it produced maximum clearing with arginine.

Effect of Additional Carbon Source on the Relative Cellulolytic Ability of Mesophilic Cellulolytic Fungi

Glucose was used as an additional carbon source to observe its effect on the depth of the clearing of the cellulose agar by cellulolytic fungi. It was used in different concentrations in addition to the ball milled cellulose. These concentrations were 0.25%, 0.5% and 1.0% of glucose in the E & P cellulose agar.

The effect of these concentrations of glucose on the colonisation of the cellulosic substrate by fungi, has also been studied with the help of the perfusion technique, the results of which have been discussed in the previous chapter. The fungi isolated with this technique have been tested for their cellulolytic ability with varying concentrations of glucose.

Three cellulose agar tubes were inoculated for every fungal species tested at each concentration of glucose. The inoculated tubes were incubated at 25° C. and the depth of clearing was measured over a period of 30 days.

The results for this experiment are represented in Figures 16a-b. The relative cellulolytic ability of most of the cellulolytic fungi tends to decrease with the increase in concentration of glucose. This is particularly true in the case of A. fumigatus, C. globosum, D. asperum, F. oxysporum, G. roseum, Papulaspora sp., P. funiculosum, S. fimicola and T. viride. The degree of the decrease in the depth of clearing varies with different cellulolytic fungi tested. G. roseum had a profuse growth on all the concentrations of glucose but it failed to produce any clearing on 1% glucose. Papulaspora sp. only produced clearing of the cellulose agar

Figure 16a-b: Maximum depth of clearing produced by different fungi after 30 days of incubation at 25 C at different concentrations of glucose.

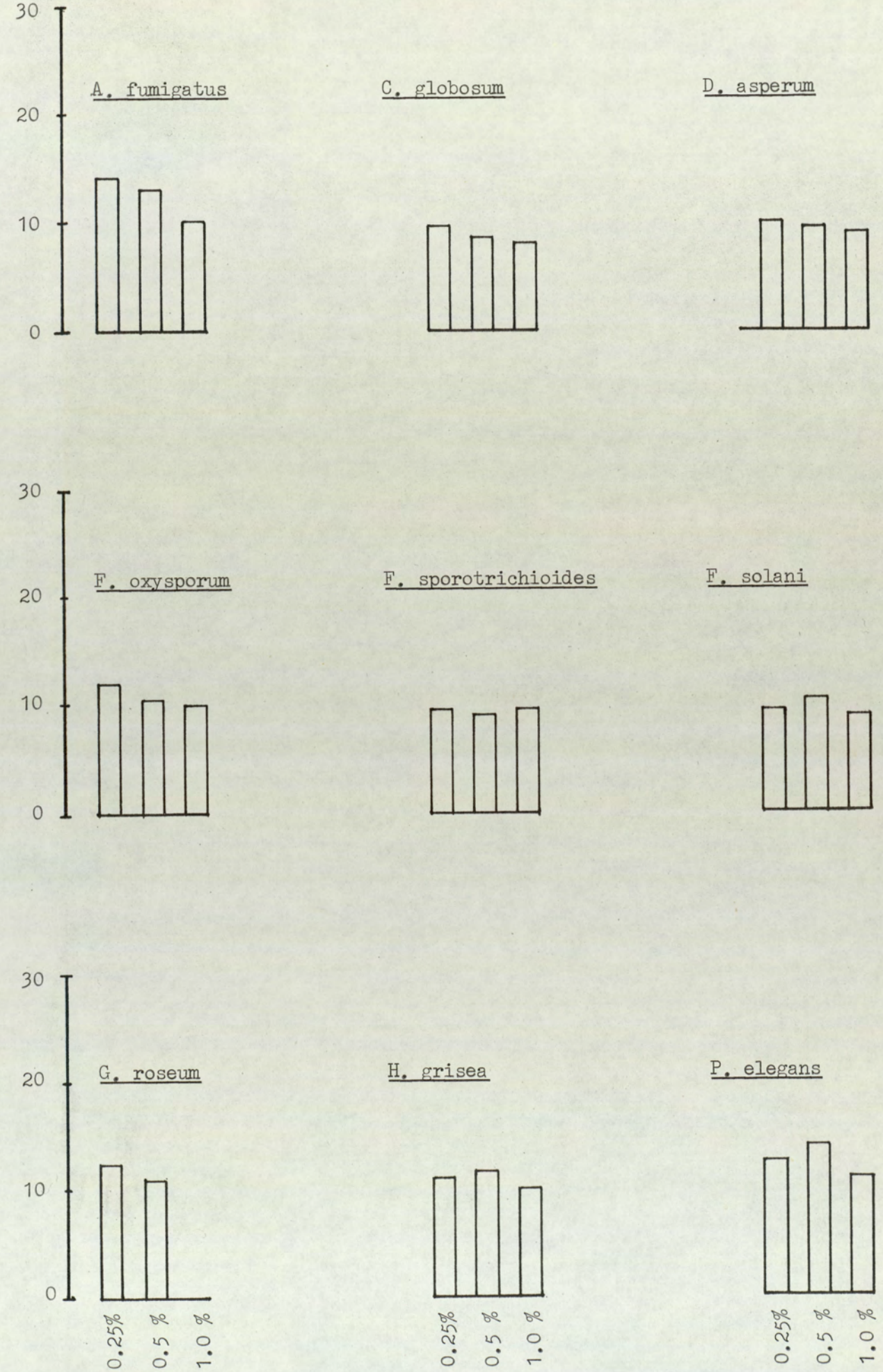
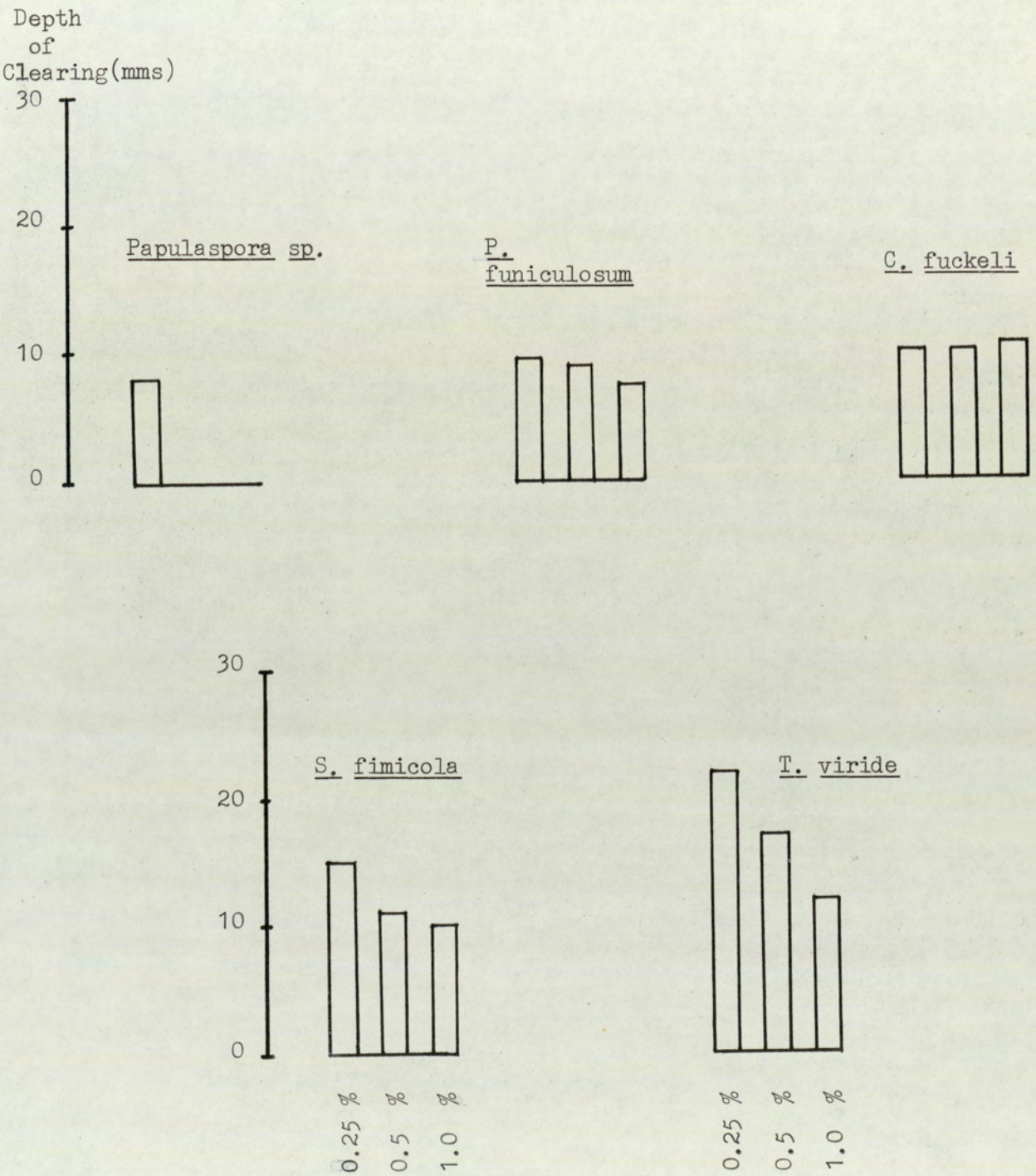


Figure 16b


Depth of Clearing(mms)
30
20
10
0
Papulaspora sp.
P. funiculosum
C. fuckeli
S. fimicola
T. viride
0.25 %
0.5 %
1.0 %

with 0.25% glucose but there was no clearing with 0.5% and 1% glucose. Higher concentrations of glucose seemed to inhibit the growth of this species, whereas in most of the cases glucose allowed luxuriant growth but inhibited the cellulolytic activity of the fungi. F. sporotrichiodes did not have much difference in the depth of clearing of the cellulose agar with varying concentrations of glucose. It had a profuse growth and produced deep yellowish pigment on all the glucose concentrations. F. solani was on the whole very much inhibited with regard to its cellulolytic activity. The relative cellulolytic activity was maximum at 0.5% glucose whereas it was nearly the same at 0.25% and 1% glucose. Humicola grisea also produced maximum depth of clearing with 0.5% glucose whereas it was comparatively less on 1% glucose. Paecilomyces elegans also produced maximum clearing on 0.5% glucose. This depth of clearing was nearly the same as produced on cellulose agar without any additional carbon source. Different concentrations of glucose did not seem to have much effect on the cellulolytic activity of C. fuckeli. The depth of clearing increased slightly with 1% glucose which was equal to the clearing produced on the cellulose agar without any glucose. Nearly all the fungi tested their the relative cellulolytic ability with varying concentrations of glucose, were found to have an inhibitory effect on their cellulolytic ability. Some other fungi produced nearly the same amount of clearing with glucose as without any glucose. No concentration of glucose tested, was found to enhance the cellulolytic activity of the fungi under observation.

The comparative review of the relative cellulolytic activity of the fungi with varying concentrations of glucose

and their percentage frequency of occurrence (Figure 11) has shown that there does not exist any definite correlation between these two results. The most commonly occurring soil fungi namely, F. solani and T. viride had their maximum frequency when the cellulosic substrate was perfused with 0.25% and 0.5% glucose respectively, whereas their optimum cellulolytic activity was found to be at 0.5% and 0.25% respectively. Among other fungi, G. roseum had quite a high frequency of occurrence at 0.25% glucose and it was much lower at 1% glucose. Similarly G. roseum produced maximum clearing at 0.25% glucose and no clearing was observed at 1% glucose. The frequency of occurrence of P. elegans increased with the increase in the concentration of glucose whereas its maximum clearing of the cellulose was produced at 0.5% glucose. C. globosum was only isolated at 0.25% and 1% glucose and it had extremely low percentage frequency of occurrence. Its cellulolytic power also decreased with increase in the glucose concentration. The cellulolytic activity and the percentage frequency of occurrence both decreased with the increase in the glucose concentration in the case of A. fumigatus.

Interaction between Cellulolytic Fungi

The cellulolytic ability of the fungi which have been isolated at 25° C. with the help of the perfusion technique, have already been studied by measuring the depth of the clearing produced on the cellulose agar column. Out of these cellulolytic fungi, the ten most cellulolytic species were inoculated in combinations of two in order to observe whether such combinations resulted in a depth of clearing greater or less than could be produced by either organisms alone All the inoculations were made with a 5 mm. diameter cork borer from a 7 days old culture. The depth of clearing was measured as previously. Three cellulose agar tubes were inoculated for each combination in order to allow enough replication. All the inoculated tubes were incubated at 25° C. and the depth of clearing was measured over a period of 30 days.

The average depth of clearing of various combinations of these fungi, after 30 days of incubation have been summarised in Table 34. Certain features of the behaviour of fungi in association with other fungi have emerged from these results. Table 34 also contains the results of visual observation, where possible, regarding which organism was dominant in the combinations and it is represented along with the measurement of the depth of clearing. Other symbols are explained in the key to the Table 34 .

In majority of the cases, the depth of clearing produced by fungi in combinations, was found to be intermediate between that of the two organisms in pure culture. There was only one example of synergism among the combination

Table 34

LEGEND

i = intermediate depth of clearing.

- = inhibition of depth of clearing.

initials represent dominant of the two fungi (wherever possible to ascertain)

	Arthrobotrys sp.	Chaetomium globosum	Fusarium solani	Gelasinospora cerealis	Gliocladium roseum	Humicola grisea	Papulaspora sp.	Coniothyrium fuckeli	Aspergillus fumigatus	Trichoderma viride
Arthrobotrys sp.	13.0									
Chaetomium globosum	i 12.5	14.0								
Fusarium solani	F.s. i 16.0	F.s. i 17.0	18.0							
Gelasinospora cerealis	- 10.3	C.g. i 13.5	F.s. i 14.6	15.5						
Gliocladium roseum	G.r. - 10.0	- 12.0	i 16.5	- 11.3	16.5					
Humicola grisea	- 9.0	i 14.0	F.s. i 13.0	- 9.0	- 11.0	13.0				
Papulaspora sp.	- 8.0	- 11.5	i 16.5	- 7.8	- 9.6	- 7.3	15.0			
Coniothyrium fuckeli	- 9.2	i 11.5	F.s. i 14.5	- 9.2	- 10.5	- 8.0	- 9.2	10.6		
Aspergillus fumigatus	Asp i 17.1	i 15.0	F.s. i 16.0	Asp i 18.0	i 18.5	i 20.0	- 10.5	i 17.5	21.5	
Trichoderma viride	T.v. i 19.3	T.v. i 17.5	T.v. i 20.0	T.v. i 19.5	- 12.5	T.v. - 11.8	- 11.3	- 10.3	i 18.0	23.0

tested. Fusarium solani and F. moniliforme when grown together produced slightly more clearing of the cellulose agar than that of either grown in pure culture (Table 44).

There were many examples where the cellulolytic activity of the combined fungi was less than the activity of either of the fungi grown separately. This phenomenon was mostly represented when fungi such as Groseum, Humicola grisea, Papulaspora sp. and to some extent Coniothyrium fuckeli were grown in combination with other fungi. Trichoderma viride and F. solani were largely affected by the presence of these fungi as their depth of clearing decreased to quite a large extent. Aspergillus fumigatus produced this effect only in combination with Papulaspora sp. when the depth of the clearing of both these fungi, especially A. fumigatus decreased considerably.

The visual observations of the growth on the cellulose agar columns suggested that T. viride, F. solani, C. globosum tended to be dominant when inoculated in combination with other organisms. Some fungi e.g. H. grisea, G. cerealis, and Papulaspora sp. made their appearance quite well even when the other species was dominant.

The type of clearing of the cellulose agar produced by various fungi in combination was different with different fungi: C. globosum produced a clearing with a brownish tinge and this light coloration was present with all the fungi tested. T. viride produced a very distinct nearly transparent clearing of the cellulose agar and was always the same with all other fungi tested. F. solani produced a comparatively hazy clearing, both in pure culture and in combination with other fungi. Arthrobotrys sp., S. fimicola and P. elegans produced more or less similar type of clearing. G. roseum produced quite

light clearing of the cellulose and the degree of this lightness varied with the fungus in combination. Among other fungi tested, Papulaspora sp. and H. grisea produced very thick mycelial mats and comparatively less distinct clearing; the thickness of the mycelial mat also varied with different fungi in combination.

All the investigations discussed above have been done by inoculating two fungi together on to a cellulose agar column and then measuring their effect on the depth of clearing. Any increase or decrease in the cellulolytic activity may be due to a number of factors. One of them may be the mycoparasitism of various fungi, such as G. roseum and Papulaspora sp.(Shigo 1958, Barnett & Lilly 1962). The other factor may be the production of antibiotics or other toxic metabolites by one fungus which might be active against the other. The action of these substances may be localised or these substances might only be produced at the point of contact between the two fungi under test. The third factor may be the low oxygen tension below the surface of the cellulose agar. The fourth possible factor for any influence on the cellulolytic activity might be the rapid staling of the cellulose agar medium. This factor has been also reported by many early workers who demonstrated the inhibition of fungi by autotoxic bye-products or staling substances and not by the depletion of nutrients or the organic substrate. Tribe (1957) has also reported the unsuitability of his cellophane film buried in soil for further growth after sometime. In view of these observations by other workers, it seems plausible that the decrease in cellulolytic activity may be due to the staling of the medium as it might be very rapid since two fungi were inoculated

on the cellulose agar medium.

This phenomenon of inhibition of a fungal culture due to staling has been termed iso-antagonism by Waksman (1937) as compared to hetero-antagonism existing in the soil. In order to study any real interaction between different fungal species, it is essential therefore, to continually remove all the autotoxic substances responsible for the iso-antagonism and to allow these substances to be used by other known fungal species, so that any real effect could be observed. In doing so, the effect of diffusible metabolite of one fungus on to the other will be studied, as compared to the effect of non-diffusible metabolites on different fungi as investigated by measuring the depth of clearing on the cellulose agar column.

In order to investigate this particular phenomenon of interaction between different fungi involving their diffusible metabolites and eliminating the hazard of staling and mycoparasitism, a technique has been evolved which appears to overcome succesfully the above mentioned snags.

The technique to be described here is based on the measurement of the linear growth of different fungi on a cellulosic strip. The effect of diffusible metabolites of one fungus is observed on the linear growth of the other. This technique essentially involves continuous perfusion of the cellulose strip with fresh nutrients. The form of cellulose used here is 3MM polythene coated chromatography paper (Eggins & Lloyd 1968) which in this work, is to cut into U shaped strips, the arms being 5mm in width; the base of the U is covered by a coverslip which is kept in place by silicone adhesive glue (See Diag. 2). One arm of the U strip is attached to a 12 inches length of a 4mm wide glass fibre

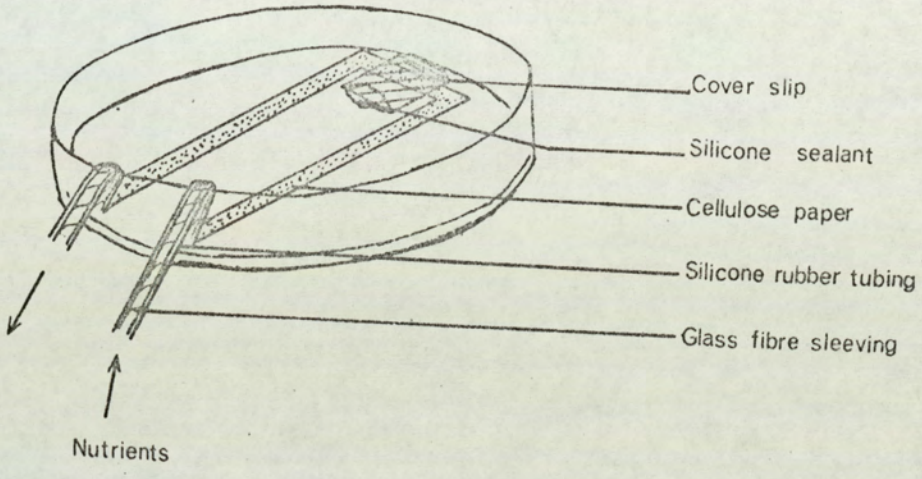
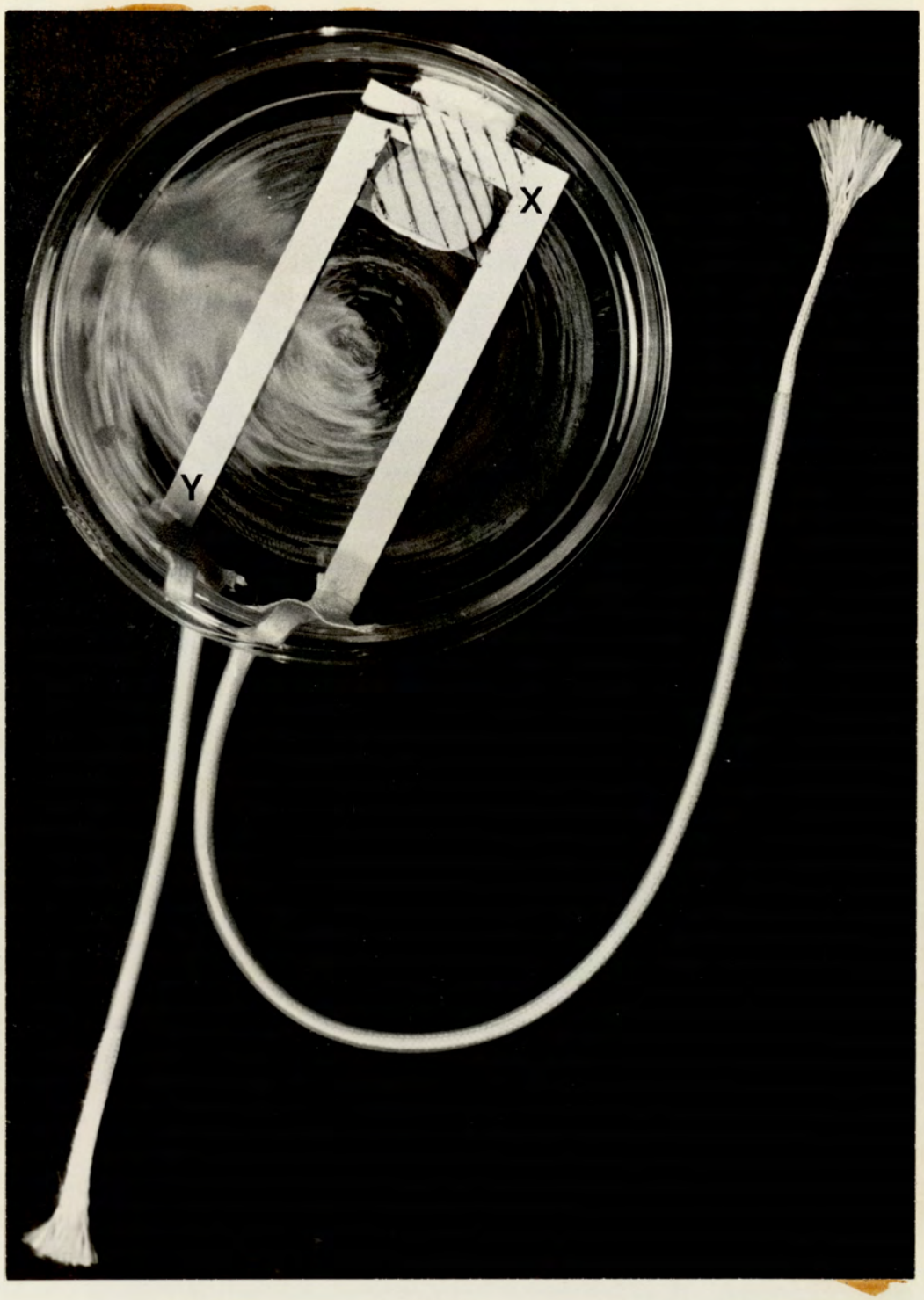

Cover slip
Silicone sealant
Cellulose paper
Silicone rubber tubing
Glass fibre sleeving
Nutrients

Diagram 2

sleeving, threaded through 10 inches of 3mm bore silicone rubber tubing. This leads to a 100 ml flask containing nutrients. The other arm of the U strip is attached to a 6 inches similar glass sleeving which is threaded through 4 inches of silicone rubber tubing so that a part of the glass fibre sleeving is exposed to the atmosphere. The nutrients are taken up from the flask by the capillarity of glass fibres and are perfused on to the U shaped cellulose strip, where it is perfused all the way and is evaporated on the other side.

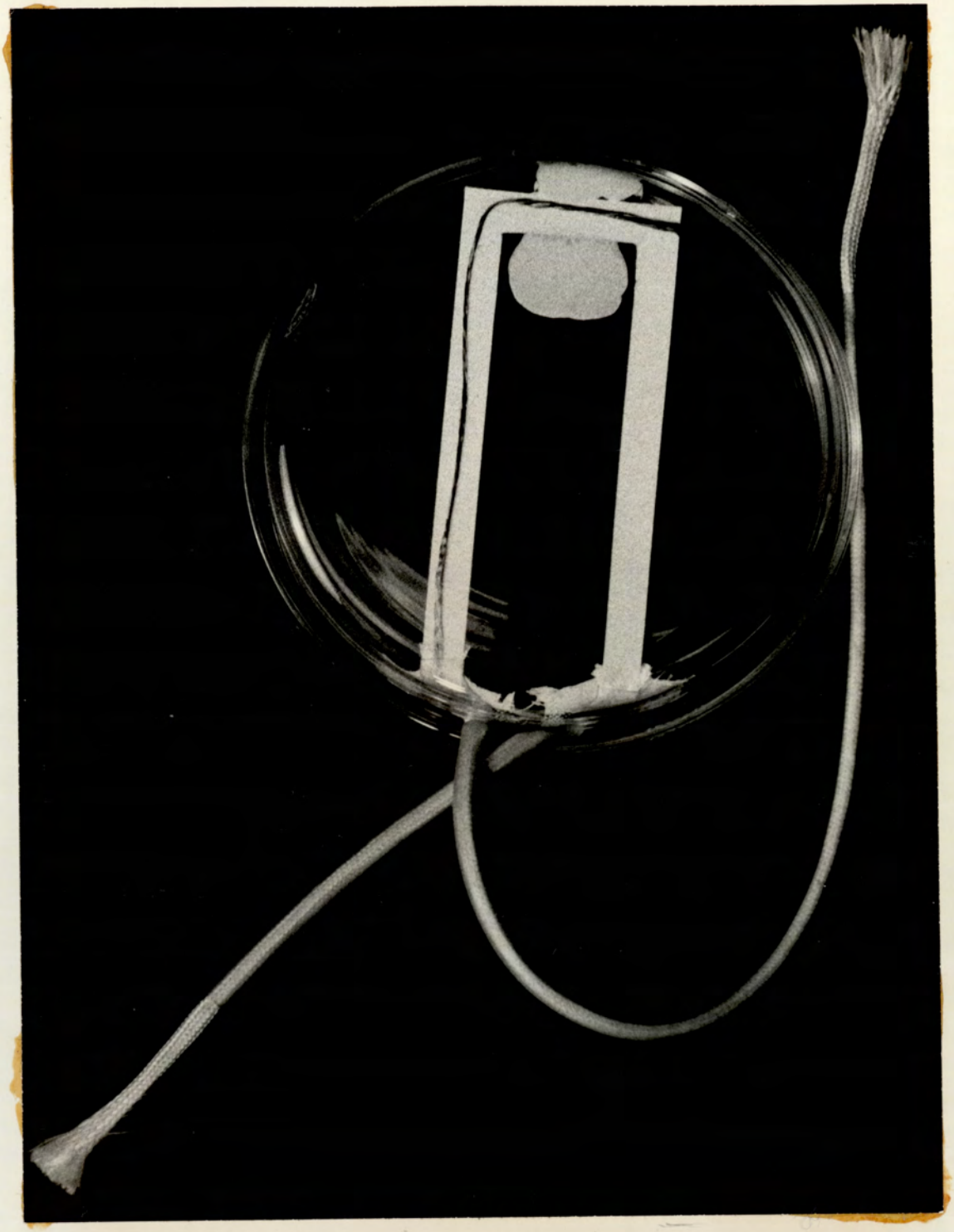
Two different fungal species are inoculated on the opposite arms at X and Y (Photo 5). The fungus inoculated at X can not cross the cellulose strip covered by the coverslip becuase of the low oxygen content and therefore, it will preferably grow towards fresh nutrients. Thus only the metabolites of the fungus at X will be passed on to the fungus at Y.

The level of nutrients is kept constant by having a very thin fibre cord running beneath the U shaped polythene backed cellulose strip. The cord is only in contact with the second arm of the U strip (Photo 6). This extra nutrient supply takes into account the utilisation of nutrients by the fungus inoculated at X. After inoculation of the fungal species on both the arms of the U strip, linear growth of the species inoculated at Y is measured at different intervals. The comparison of this linear growth is made with controls (no inoculum at X), to show any effect of the diffusible metabolites of the species inoculated at X. The initial point of inoculation is marked on the back of the glass petri dish and subsequent readings are taken from it. The linear growth can easily be measured by simply holding the petri dish



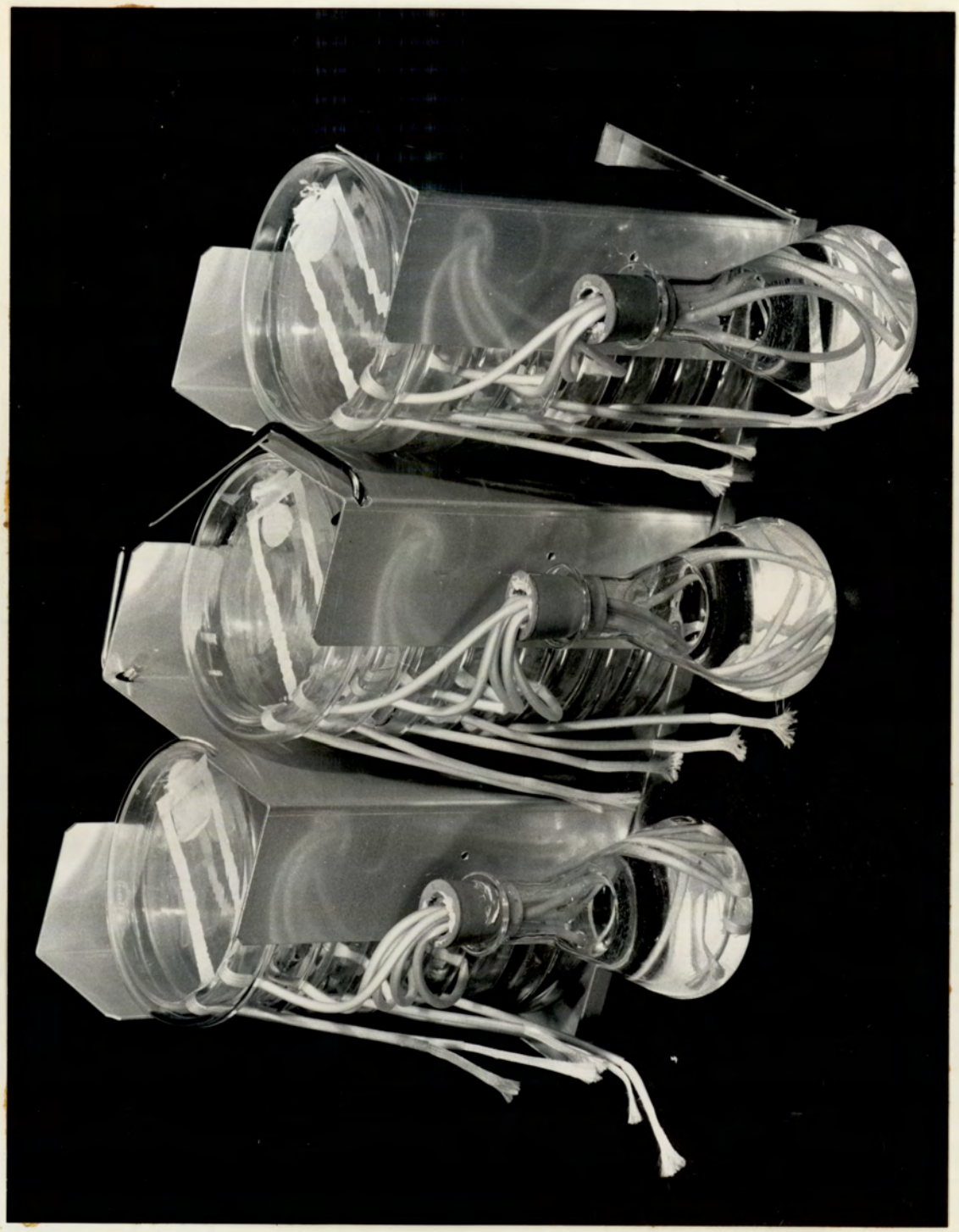
Photograph 5

A petri dish containing interaction polythene backed cellulose strip: shaded portion represents the position of the coverslip which is kept in position with the help of silicone rubber glue. It also illustrates the contact of the auxillary fibre glass cord with the cellulose paper.



Photograph 6

Same petri dish upside down, showing the fibre glass cord (dyed black) running beneath the polythene backed cellulose interaction strip. The white blob is the silicone rubber glue.



Photograph 7

A petri dish carrier containing cellulose interaction perfusion systems.

containing the inoculated interaction strip against strong light.

During the previous investigations with mixed cultures of fungi, the inhibitory role of Gliocladium roseum was most striking. Therefore, it was considered worthwhile to study the effect of diffusible metabolites of this species on the linear growth of different cellulolytic fungi. The fungi tested for this effect were Arthrobotrys sp., Chaetomium globosum, Coniothyrium fuckeli, Humicola grisea, Sordaria fimicola and Trichoderma viride.

G. roseum was always inoculated at point X on the interaction strip and the test fungi were inoculated at Y after 2 days of the inoculation of G. roseum. Three replicates were kept for each test fungus and a similar number of controls were also kept where only test fungus was inoculated.

The inoculum was taken from a 7 days old fungal colony growing on E & P cellulose agar. A 5 mm. diameter cork borer was used for all the inoculations. Before inoculation E & P salt solution was added to the perfusing flasks and the petri dishes containing the U strip were put in petri dish carriers (Photo 7). After autoclaving the strips were then inoculated and incubated at 25° C. The linear growth of the test fungi were recorded every day for a period of about two weeks depending upon the growth rate of the test fungi.

All the results are expressed as an average of three observations. The results of the effect of the metabolites of G. roseum on Arthrobotrys sp., C. fuckeli and T. viride are represented in Figure17a. The linear growth per 24 hours and percentage inhibition or otherwise is calculated and summarised in Table 35. Arthrobotrys sp. reached a linear

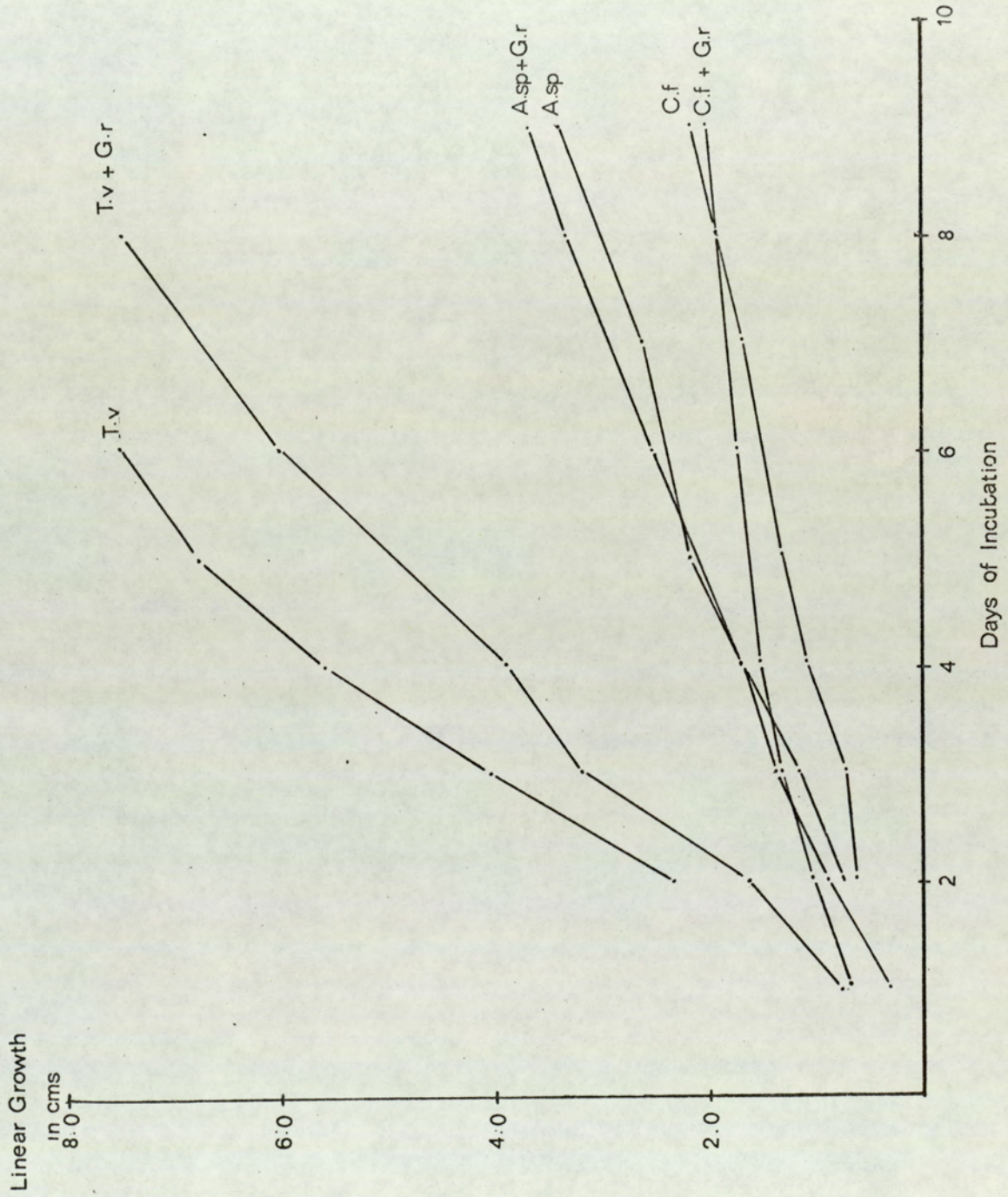


Figure 17a: Linear growth of different fungi when perfused with the diffusible metabolites of G. roseum (G.r)

Legend: Arthrobotrys sp. (A.sp); Coniothyrium fuckeli (C.f); Trichoderma viride (T.v).

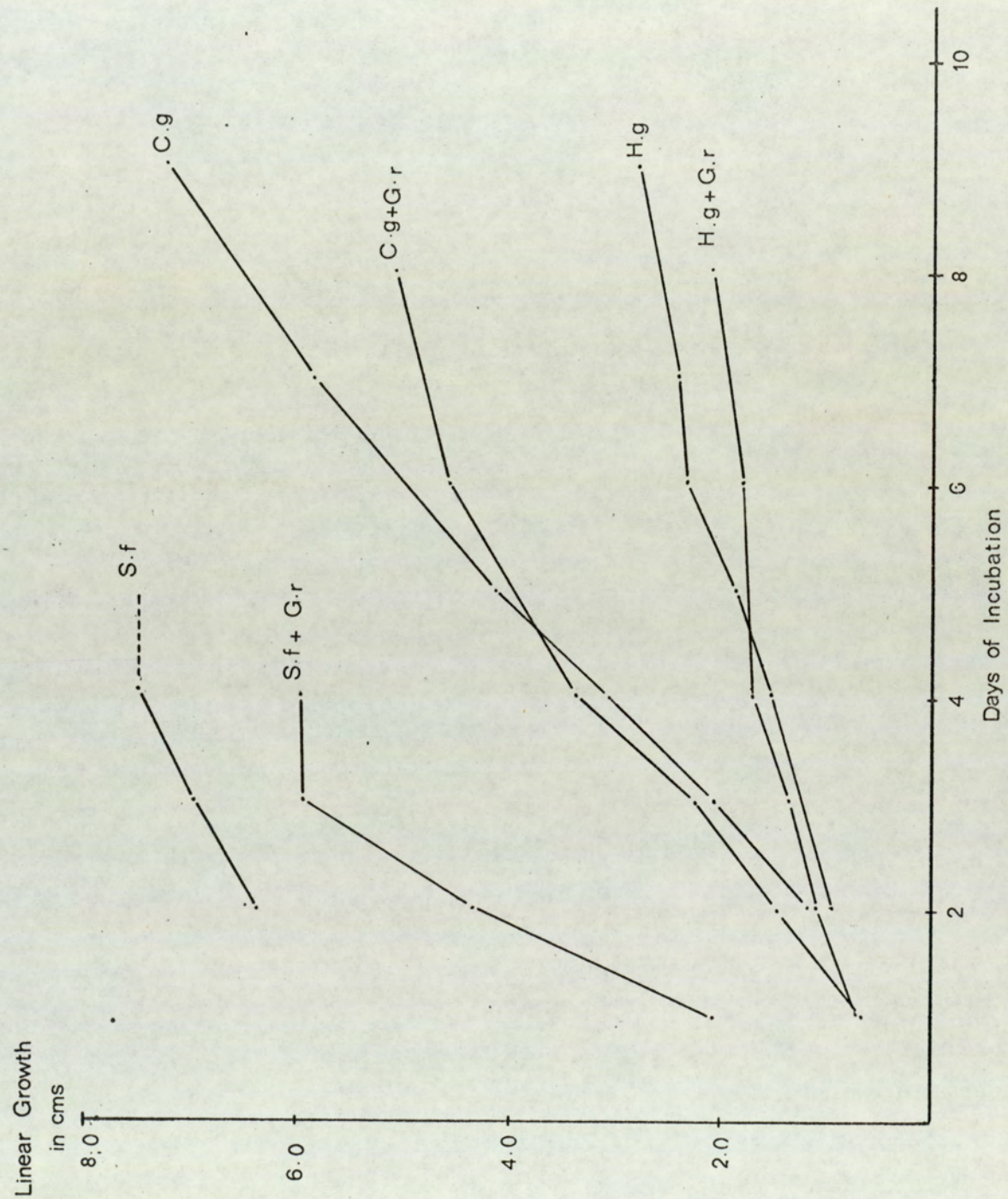


Figure 17b: Linear growth of different fungi when perfused with the diffusible metabolites of G. roseum.

Legend: Chaetomium globosum (C.g); Humicola grisea (H.g); Sordaria fimicola (S.f).

Table 35

Effect of the diffusible metabolites of Gliocladium roseum on the linear growth of different fungi.

FUNGI	Linear Growth / 24 hrs.*			
	Control (C)	with G.roseum (T)	Inibition C - T = I	% Inhib. I/CX100
Arthrobotrys sp.	0.37 cm.	0.41 cm.	- ~~0.11~~ 0.04	- 29.0+
Chaetomium globosum	0.81 cm.	0.64 cm.	0.17	21.0
Coniothyrium fuckeli	0.23 cm.	0.22 cm.	0.01	4.3
Humicola grisea	0.31 cm.	0.26 cm.	0.05	16.1
Sordaria fimicola	1.8 cm.	1.2 cm.	0.6	33.3
Trichoderma viride	1.2 cm.	0.93 cm.	0.27	22.5

* All figures are average of three readings

\+ Percentage increase of linear growth

growth of 3.4 cms. whereas when it was perfused with the metabolites of G. roseum, its growth was enhanced and it reached 3.7 cms. in 9 days. This increase was calculated to be 9.8%. C. fuckeli proved to be extremely slow growing and reached 2.1 cms. after 9 days as compared to 2.0 cms. under the influence of G. roseum. As this was an extremely small difference, the percentage inhibition was 4.3 as compared to 29% increase for Arthrobotrys sp. T. viride which is a well known fast growing fungus, achieved its maximum linear growth of 7.5 cms. in 6 days, whereas when it was grown with the metabolites of G. roseum it grew comparatively slowly reaching 7.5 cms. in 8 days instead of 6 days. This inhibition was calculated to be 22.5%.

Figure 17b represents the results for S. fimicola, C. globosum and H. grisea. S. fimicola is also a fast growing fungus as its mycelium reached 6.4 cms. in 2 days but it was until 4 days that it started producing perithecia; there was no fixed pattern of perithecial production. The broken lines in Figure 17b represent the formation of perithecia. S. fimicola when perfused with the metabolites of G. roseum grew comparatively slowly, reaching its maximum linear growth of 6.0 cms. in 3 days after which it slowed down completely; the significant difference being the lack of perithecia even after 5 days. Perithecia did eventually appear on the cellulose strip but their number was drastically smaller than those of the controls. This fungus had a maximum inhibition value of 33.3% (Table 35). C. globosum showed a steady linear growth reaching 7.3 cms. in 9 days. Here the perithecia were produced from the very beginning and the

entire strip was covered with them. Its linear growth slowed down under the influence of G. roseum and was only able to reach 5.8 cms. This slowing down process began after 4 days. The inhibition percentage calculated for this fungus was 21.0. Like C. fuckeli, H. grisea is another slow growing fungus which reached 2.8 cms. linear growth in 9 days. This fungus was further slowed down when perfused with the metabolites of G. roseum; it reached a linear growth of 2.1 cms. The inhibition value for this fungus was calculated to be 16.1%.

Out of all the above mentioned fungi tested, the linear growth of Arthrobotrys sp. was enhanced whereas all other fungi were inhibited by G. roseum by varying degrees.

In order to compare these results with those of the non diffusible metabolites of G. roseum, percentage decrease in the depth of clearing of these fungi is calculated from Table 34 and is summarized in Table 36 .

The percentage inhibition obtained from both experiments (depth of clearing and linear growth) is represented in Figure 18 . There was no marked difference between the inhibition of cellulolytic activity and linear growth of C. globosum, C. fuckeli, H. grisea and S. fimicola.

Arthrobotrys sp. had a 23% inhibition of cellulolytic activity whereas its linear growth increased by only ~~29.0~~ 9.8% in the presence of G. roseum. T. viride showed a maximum inhibition of cellulolytic activity of 45.6% whereas its linear growth was suppressed by only 22.5%. This result is significant since T. viride is the most common soil fungus and is mostly responsible for the cellulose decomposition in the soil.

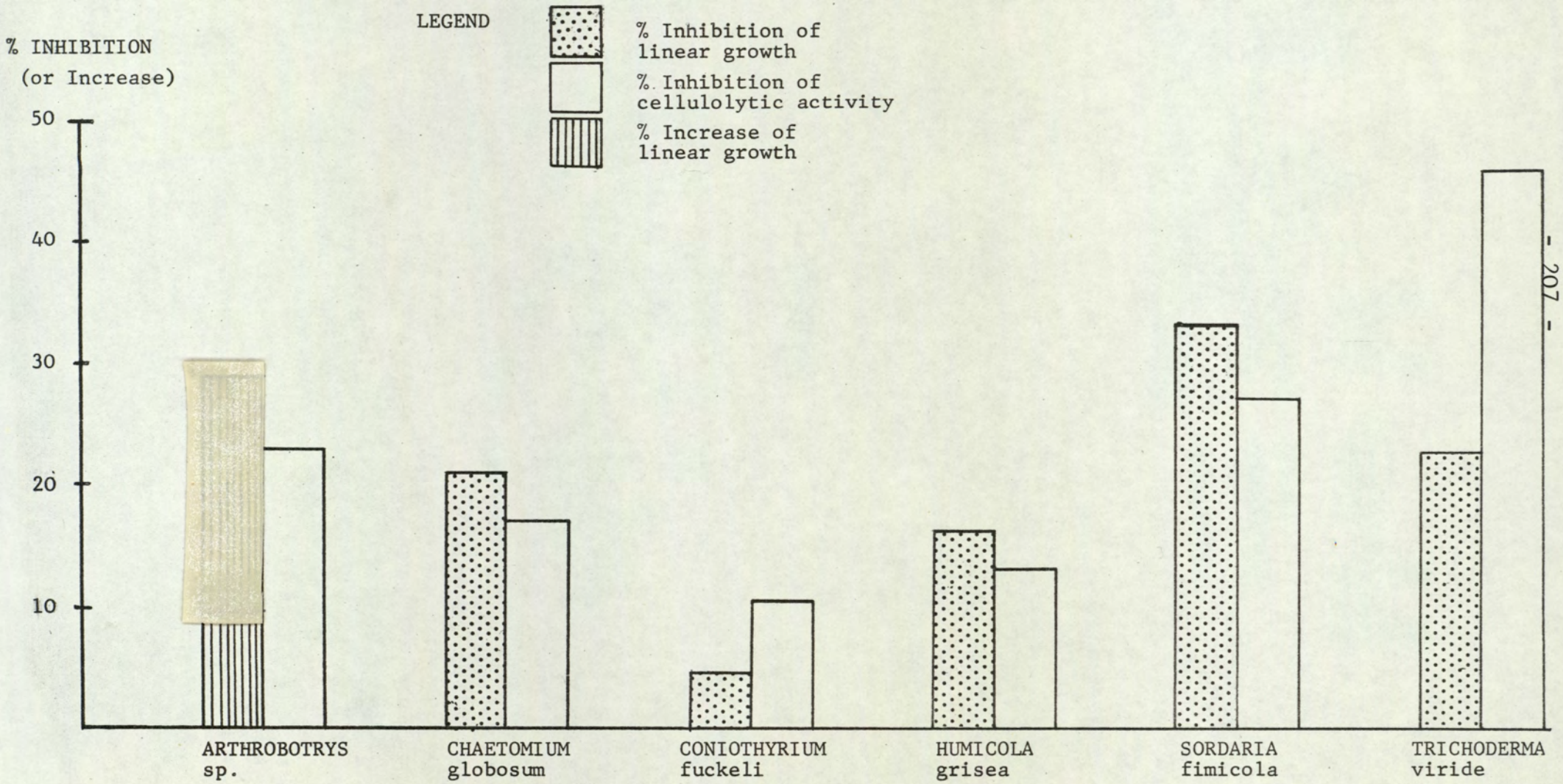
Table 36

The percentage decrease or increase of the depth of clearing produced by different fungi when grown in combination with Gliocladium roseum.

FUNGI	Depth of clearing in 30 days *			
	Control (C)	with G.roseum (T)	Inhibition C - T = I	% Inhib. I/Cx100
Arthrobotrys sp.	13.0 mms.	10.0 mms.	3.0	23.0
Chaetomium globosum	14.0 mms.	11.6 mms.	2.4	17.1
Coniothyrium fuckeli	10.6 mms.	9.5 mms.	1.1	10.3
Humicola grisea	13.0 mms.	11.3 mms.	1.7	13.0
Sordaria fimicola	15.5 mms.	11.3 mms.	4.2	27.0
Trichoderma viride	23.0 mms.	12.5 mms.	10.5	45.6

* All figures are average of three readings

Figure 18: The percentage inhibition or increase in the linear growth and depth of clearing of different fungi when grown in combination with Gliocladium roseum.



With all the test fungi, the cellulolytic activity of G. roseum also decreased. The possible explanation of this may be its mycoparatic nature which might be affecting the cellulose enzyme production. The other explanation for this decrease in the depth of clearing of the cellulose might be the rapid staling of the cellulose agar medium to which G. roseum seems to be very sensitive.

Trichoderma viride and Fusarium solani have been found to be the most commonly occurring fungi in the soil and their association with cellulose decomposition has also been elucidated in the previous chapters. Some workers have also reported antibiotics from these fungi which are active against other microorganisms. (Bilai 1963). Therefore two similar experiments were carried out using the perfusion interaction technique to observe the effect of the diffusible metabolites of T. viride and F. solani on the linear growth of different cellulolytic fungi. These results were then compared to those obtained by inoculating these fungi in combination on to the cellulose agar column and then measuring the depth of clearing.

T. viride was inoculated at point X (See Photo. 5) on the interaction U strip at the same time as the test fungi which were inoculated at point Y. The controls for test fungi were also kept where T. viride was not inoculated.

The results of the effect of diffusible metabolites of T. viride on the linear growth of eight cellulolytic fungi namely Arthrobotrys sp., C. globosum, H. grisea, F. solani, G. roseum, C. fuckeli, Papulaspora sp. and S. fimicola are represented in Figures 19a-b. The percentage inhibition of

Figure 19b: Linear growth of different fungi when perfused with the metabolites of Trichoderma viride.

Legend: Fusarium soalni (F.s); rest same as Fig. 17a-b.

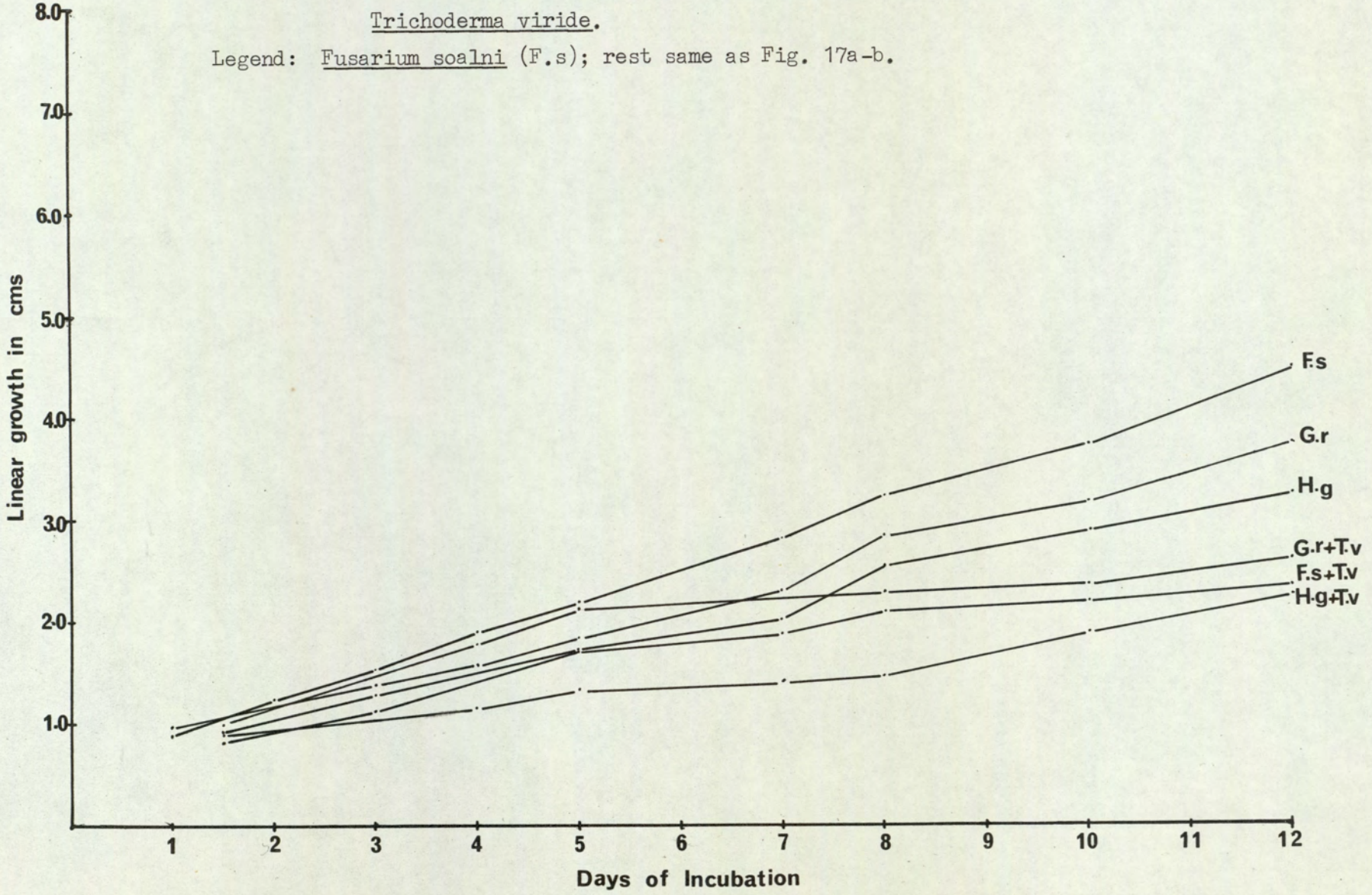
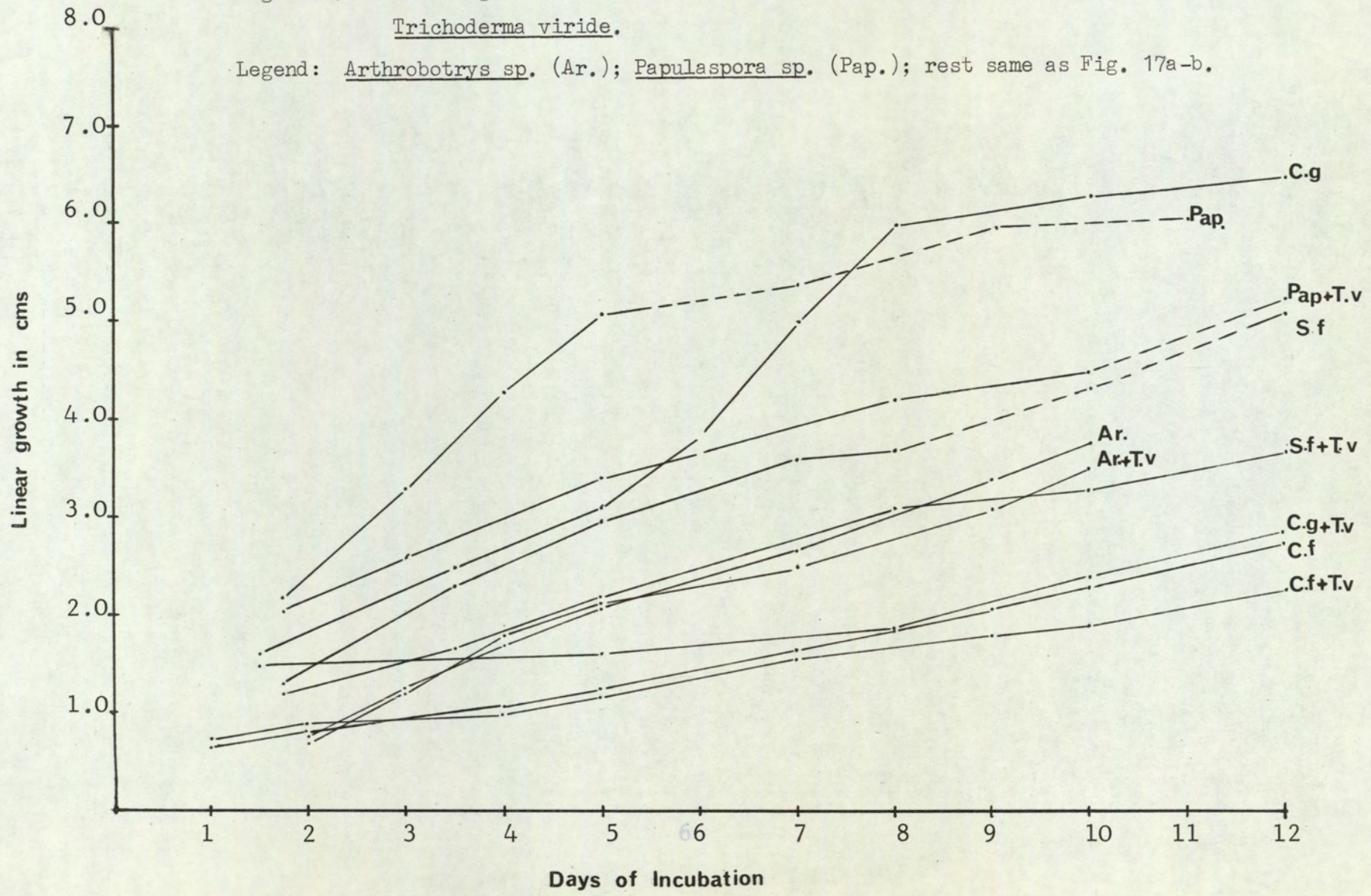


Figure 19a: Linear growth of different fungi when perfused with the metabolites of Trichoderma viride.

Legend: Arthrobotrys sp. (Ar.); Papulaspora sp. (Pap.); rest same as Fig. 17a-b.



the linear growth of these fungi has been calculated and is represented in Table 37. The linear growth of Arthrobotrys sp. and C. fuckeli were not very much suppressed by the metabolites of T. viride. The degree of inhibition of their linear growth was calculated to be 5.4% and 17.0% respectively. C. globosum seemed to be very much inhibited as its linear growth was only 2.8 cms. after 12 days of incubation as compared to 6.0 cms. after 8 days of incubation. This inhibition was calculated to be 69.3%. The linear growth of H. grisea was also suppressed by 34.4%. This was found to be a slow growing species and its linear growth reached 3.5 mms. whereas under T. viride's influence it was reduced to 2.3 cms. Papulaspora sp. was found to be a fast growing species covering the whole cellulose strip in about 8 days which is then followed by blackening of the strip due to the penetration of the mycelium into the cellulose fibres. This blackening of the strip started after 5 days. The metabolites of T. viride slowed down the linear growth of Papulaspora sp. and also delayed the blackening of the cellulose strip. The degree of inhibition was comparatively small as it was calculated to be 20.4%. Fusarium solani also experienced inhibition of its linear growth under the influence of the metabolites of T. viride. The extent of inhibition was calculated to be 46.0%. Among other fungi tested, G. roseum and S. fimicola underwent nearly similar degree of inhibition of their linear growth when perfused with the metabolites of T. viride. The degree of inhibition was 26.0% and 28.5% respectively. S. fimicola was previously found to be quite fast growing species but during this experiment it was comparatively slow growing and its linear growth was 5.1 cms. after 12 days.

Table 37

Effect of the diffusible metabolites of T. viride on different fungi.

FUNGI	Linear Growth in cms / 24 hrs. *		Difference C-T=I	% Inhibition I/C X 100
	Control (C)	Perfused with metabolites of T. viride (T)		
Arthrobotrys sp.	0.37 cm.	0.35 cm.	0.02	5.4
Chaetomium globosum	0.75 cm.	0.23 cm.	0.52	69.3
Coniothyrium fuckeli	0.23 cm.	0.19 cm.	0.04	17.3
Fusarium solani	0.37 cm.	0.20 cm.	0.17	46.0
Gliocladium roseum	0.31 cm.	0.23 cm.	0.08	26.0
Humicola grisea	0.29 cm.	0.19 cm.	0.10	34.4
Papulaspora sp.	0.54 cm.	0.43 cm.	0.11	20.4
Sordaria fimicola	0.42 cm.	0.30 cm.	0.12	28.5

* All the figures are average of three readings.

The production of perithecia was also quite slow and they appeared on the cellulose strip as late as 10 days of incubation. The perfusion of S. fimicola with the metabolites of T. viride slowed the linear growth slightly but it somehow produced perithecia earlier than the controls. The perithecia, in this case were observed after 8 days of incubation.

The percentage inhibition of the cellulolytic activity of the test fungi has been calculated and represented in the Table 38. It can be seen from this table that the cellulolytic activity of only 4 species namely C. fuckeli, H. grisea, G. roseum and Papulaspora sp. was inhibited to varying degrees. The maximum inhibition was produced by Papulaspora sp. and G. roseum. All the other species tested had their cellulolytic activity in between the cellulolytic power of T. viride and the test fungi. These results are contrary to the results obtained with the metabolites of G. roseum where the activity of all the fungi tested decreased.

The percentage inhibition of the linear growth and the cellulolytic ability has been represented in Figure 20. The linear growth of all the fungi tested was inhibited whereas the cellulolytic activity of some of the test fungi was increased. The comparison of C. globosum was quite marked as its linear growth inhibition was as high as 69.3 while the cellulolytic activity increased by 25.0%.

Due to this widespread inhibition of the linear growth of all the fungi tested by the diffusible metabolites of T. viride, another similar experiment was devised in order to observe the effect of the diffusible metabolites of these fungi on the linear growth of T. viride. The interaction perfusion strips were set up as described previously. This

Table 38

The percentage inhibition or increase in the depth of clearing of different fungi when grown in combination with T. viride.

FUNGI	Depth of clearing in 30 days		Difference	% Inhibition
	Control (C)	with T.viride (T)	T - C	
Arthrobotrys sp.	13.0 mms.	19.3 mms.	5.7	43.8
Chaetomium globosum	14.0 mms.	17.5 mms.	3.5	25.0
Coniothyrium fuckeli	10.6 mms.	10.3 mms.	- 0.3	- 2.8*
Fusarium solani	18.0 mms.	20.0 mms.	2.0	11.1
Gliocladium roseum	16.5 mms.	12.5 mms.	- 4.0	- 24.2*
Humicola grisea	15.0 mms.	11.8 mms.	- 1.2	- 9.2*
Papulaspora sp.	15.0 mms.	11.5 mms.	- 3.7	- 24.6*
Sordaria fimicola	15.5 mms.	19.5 mms.	4.0	25.8

* = % inhibition

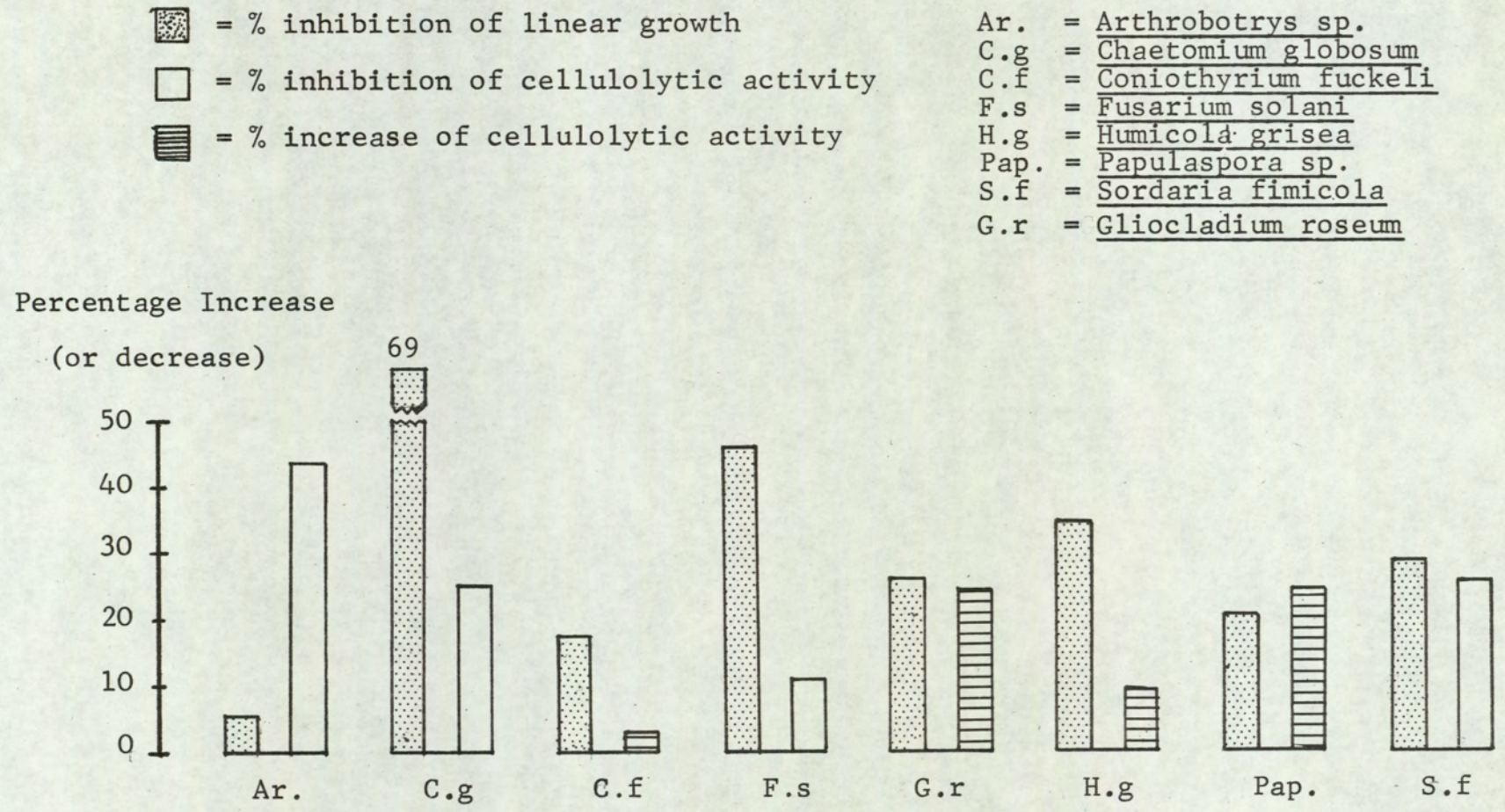


Figure 20: Percentage increase or decrease of the linear growth and depth of clearing of cellulose of different fungi when grown with Trichoderma viride.

time the test fungi were inoculated at point X and T. viride at point Y in the cellulose U shaped strip (Photo. 5). Appropriate controls were again kept for this experiment. T. viride was inoculated after 2 days of the inoculation of the test fungi, so that enough time was allowed for the diffusible metabolite of the test fungi to reach the point Y where T. viride was inoculated.

The results of the measurement of the linear growth of T. viride growing on the metabolites of different cellulolytic fungi are presented in the Table 39. The diffusible metabolites of Arthrobotrys sp. F. solani and Papulaspora sp. did not produce any effect on the linear growth of T. viride. (Fig. 21) S. fimicola and H. grisea were just able to suppress its linear growth to a small extent whereas C. fuckeli and C. globosum inhibited the linear growth of T. viride to a relatively large extent. The degree of inhibition was calculated to be 26.6% and 46.6% respectively.

The inhibition of the cellulolytic activity of T. viride when grown with other fungi, have also been calculated and represented in Table 40. The depth of clearing produced by T. viride in combination with other fungi, decreased with all the fungi tested. Maximum inhibition was produced by C. fuckeli which was 55.2% whereas minimum inhibition was 13.0% produced by F. solani.

The comparison of both the inhibitions, i.e. linear growth and cellulolytic activity have been presented in Figure 22 . C. globosum, C. fuckeli, H. grisea and S. fimicola produced inhibition of the both, linear growth and the depth of clearing of the cellulose agar produced by T. viride, whereas Arthrobotrys sp., F. solani and Papulaspora sp.

Table 39

Effect of the diffusible metabolites of different fungi on the linear growth of T. viride. Linear growth of T. viride = Control = 1.5cms/24 hrs. (C)

FUNGI	Linear growth of T. viride perfused with metabolites of test fungi, per 24 hours.* (T)	C-T Difference	% Inhibition
Arthrobotrys sp.	1.5 cms.	0	0
Chaetomium globosum	0.8 cms.	0.7	46.6
Coniothyrium fuckeli	1.1 cms.	0.4	26.6
Fusarium solani	1.5 cms.	0	0
Humicola grisea	1.25 cms.	0.25	16.5
Papulaspora sp.	1.5 cms.	0	0
Sordaria fimicola	1.4 cms.	0.1	6.6

* = All figures are the average of three readings.

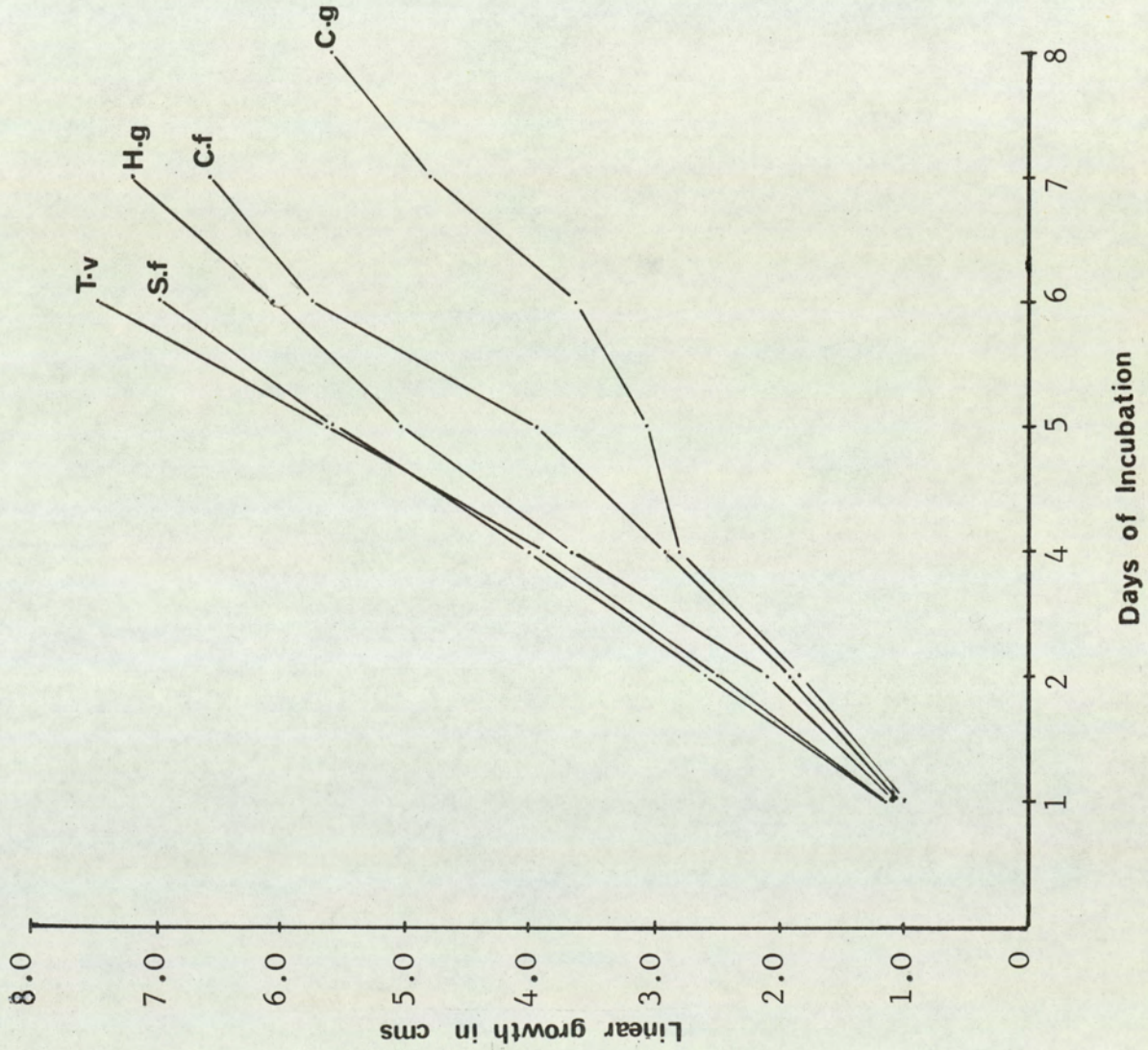


Figure 21: Linear growth of Trichoderma viride when perfused with the diffusible metabolites of different fungi.

Legend: Same as Fig. 17a-b.

Table 40

The percentage inhibition or increase in the depth of clearing produced by T. viride when grown in combination with test fungi.

T. viride = 23.0 mm.

TEST FUNGI	Depth of clearing with the test fungi (T)	Difference TV - T	% Inhibition of T.viride
Arthrobotrys sp.	19.3 mms.	3.7	16.0
Chaetomium globosum	17.5 mms.	5.5	23.9
Coniothyrium fuckeli	10.3 mms.	12.7	55.2
Fusarium solani	20.0 mms.	3.0	13.0
Humicola grisea	11.8 mms.	11.2	48.7
Papulospora sp.	11.3 mms.	11.7	50.7
Sordaria fimicola	19.5 mms.	3.5	15.2

Legend: same as Fig. 20

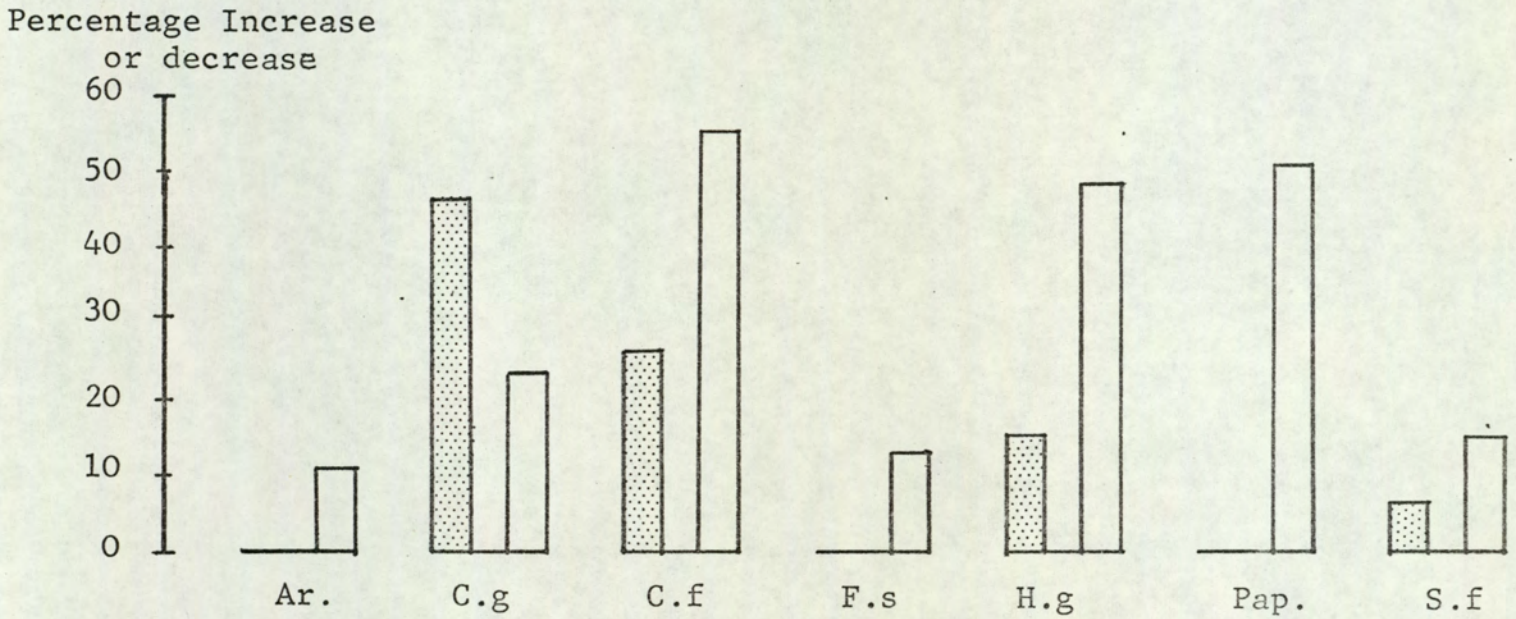


Figure 22: Pecentage increase or decrease of the linear growth and depth of clearing of Trichoderma viride when grown with different fungi.

produced inhibition of the depth of clearing but had no effect on the rate of linear growth of T. viride.

Similar experiments have been performed to demonstrate the effect of diffusible and non diffusible metabolites of Fusarium solani on the growth of 5 other cellulolytic fungi. These were Arthrobotrys sp., C. fuckeli, F. moniliforme, H. grisea and S. fimicola. During the isolation and colonization studies of these fungi, these were found to be commonly occurring and showed some sort of interaction on the cellulose agar plates.

The interaction perfusion strip were made as described previously. F. solani was inoculated at point X on the interaction cellulose strip whereas the test fungi were inoculated at point Y so that the metabolites from X could be perfused on to the point Y (See Photo 5).

Figure 23 represents the results of the linear growth of cellulolytic fungi under the influence of F. solani. The percentage inhibition or enhancement of the linear growth of these fungi have also been calculated and is summarized in Table 41.

F. solani did not seem to have much effect on the linear growth of the fungi tested as compared to G. roseum and T. viride. The linear growth of Arthrobotrys sp. was enhanced by 30% when perfused with the metabolites of F. solani. It did not have any effect on F. moniliforme. The maximum inhibition occurred in the case of S. fimicola when the inhibition was 45.3% whereas H. grisea had an inhibition of 21%. Very little inhibition of the linear growth of C. fuckeli was observed as it was only 3%.

The percentage decrease or increase in the depth of clearing

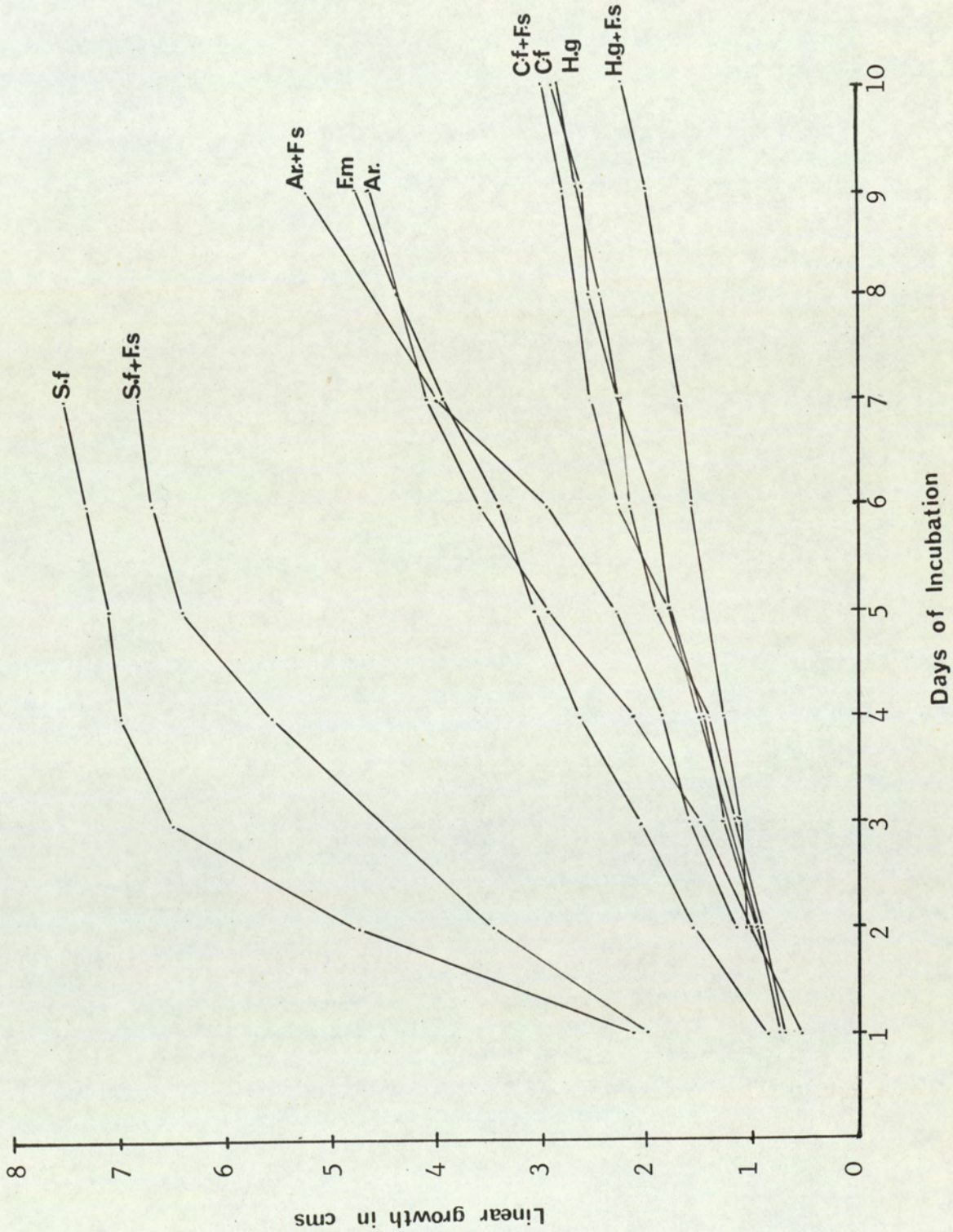


Figure 23: Linear growth of different fungi when perfused with the diffusible metabolites of Fusarium solani.

Legend: Fusarium moniliforme (F.m); rest same as in previous figures.

Table 41

Effect of the metabolites of Fusarium solani on different fungi.

FUNGI	Linear Growth/24 hrs.		Difference	%
	Control	with F. solani		
Arthrobotrys sp.	0.52	0.68	- 0.16	- 30.8
Fusarium moniliforme	0.53	0.53	0	0
Coniothyrium fuckeli	0.29	0.30	- 0.01	- 3.4
Humicola grisea	0.28	0.22	0.06	21.4
Sordaria fimicola	1.7	0.93	0.77	45.3 8.2

Table 42

The percentage decrease or increase in the depth of clearing of different fungi when grown in combination with F. solani.

FUNGI	Depth of clearing in 30 days in mms.		Difference	Inhibition
	Control (C)	with F. solani (T)	C - T	%
Arthrobotrys sp.	13.0	16.0	- 3.0	- 23.0*
Fusarium moniliforme	19.5	21.5	- 2.0	- 10.0*
Coniothyrium fuckeli	10.6	14.5	- 3.9	- 37.0*
Humicola grisea	13.0	13.0	0	0
Sordaria fimicola	15.5	14.5	1.0	6

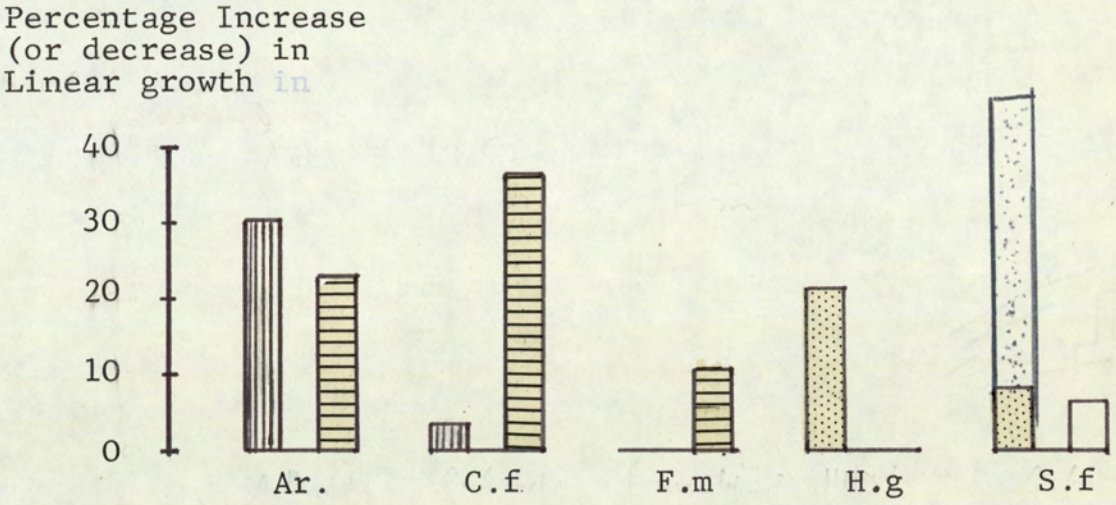
* = Percentage increase in the depth of clearing.

of the cellulose by test fungi when grown with F. solani has been summarized in Table 42 . The depth of clearing of Arthrobotrys sp. and C. fuckeli has been increased to some extent when grown in the presence of F. solani. There was no effect on the clearing produced by H. grisea in combination with F. solani. The depth of clearing produced by F. moniliforme and S. fimicola was decreased to a small extent when grown with F. solani.

The comparison of the cellulolytic activity and the effect on the linear growth of the test fungi of the metabolites of F. solani has been represented in Figure 24. Arthrobotrys sp. had an increased cellulolytic activity and linear growth in association with F. solani. There was very little decrease in the linear growth of C. fuckeli but there was qiute a large increase in the depth of clearing of the cellulose agar.

The effect of the diffusible metabolites of the test fungi has also been observed on the linear growth of F. solani using the interaction perfusion technique. The measurement of the linear growth of F. solani perfused with the metabolites of the test fungi are represented in Figure 25 and are summarized in Table 43 .

The diffusible metabolites of H. grisea and C. fuckeli enhanced the linear growth of F. solani by 5.5% and 3.1% respectively. Arthrobotrys sp. whose linear growth was enhanced with the metabolites of F. solani, produced an inhibitory effect on the linear growth of F. solani. The inhibition produced by F. moniliforme was maximum as it ranged to 34.7%. S. fimicola did not produce any effect on the linear growth of F. solani.



Legend

Ar. = Arthrobotrys sp.
C.f = Coniothyrium fuckeli
F.m = Fusarium moniliforme
H.g = Humicola grisea
S.f = Sordaria fimicola

▥ = % increase in linear growth

(rest same as Fig 20)

Figure 24: Percentage increase or decrease of the linear growth and depth of clearing of cellulose by different fungi when grown with Fusarium solani.

Figure 25: Linear growth of Fusarium solani when perfused with the diffusible metabolites of different fungi.

Legend: Same as in previous figures.

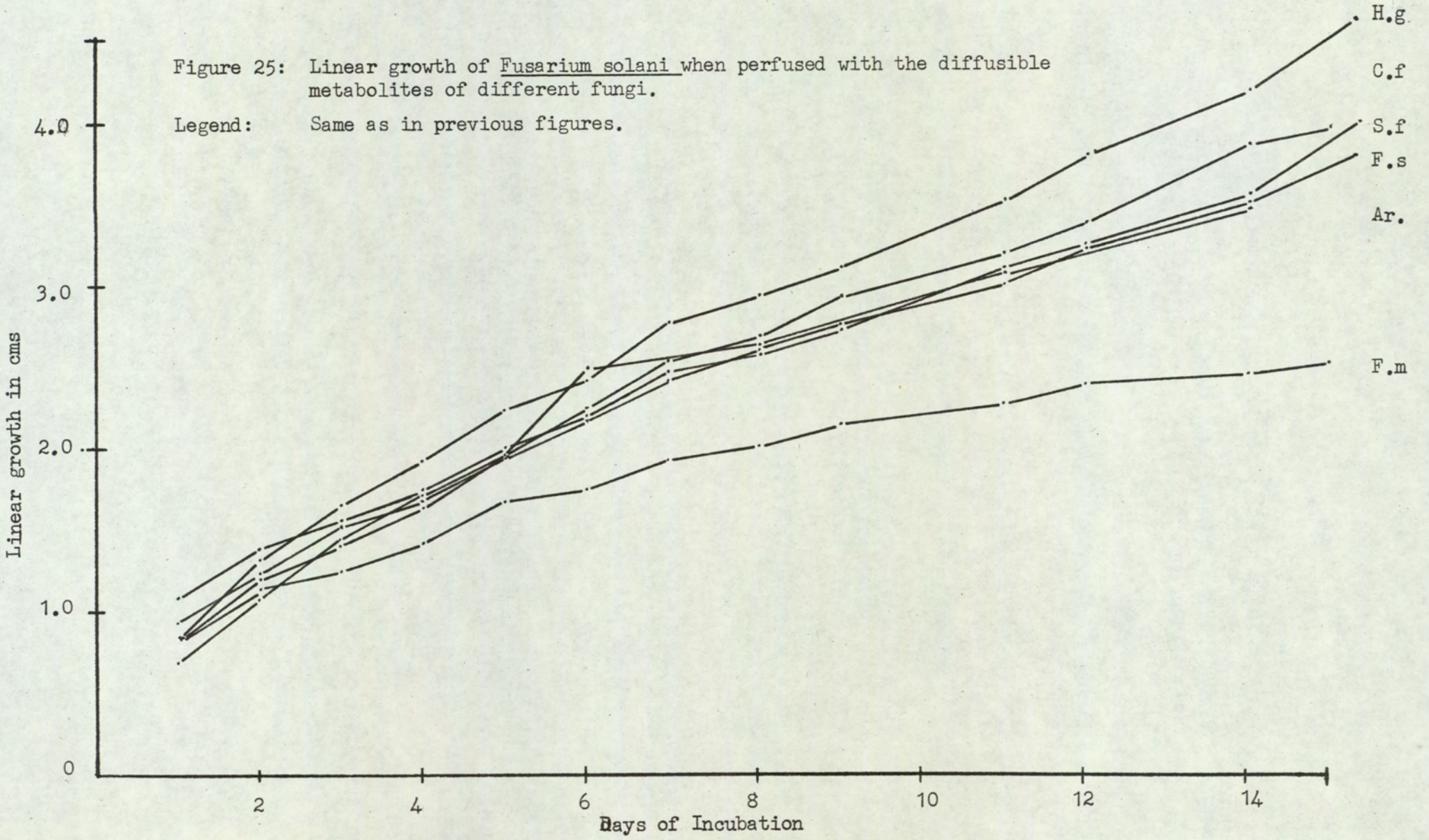


Table 43

Effect of the diffusible metabolites of different fungi on the linear growth of Fusarium solani.

Linear growth of F. solani = 0.31 mm./24 hrs. (C)

FUNGI	Linear Growth (T)	Difference C - T	% Difference
Arthrobotrys sp.	0.28	0.03	10.7
Fusarium moniliforme	0.23	0.08	34.7
Coniothyrium fuckeli	0.32	- 0.01	- 3.1
Humicola grisea	0.34	- 0.02	- 5.5
Sordaria fimicola	0.31	0	0

Table 44

The percentage decrease or increase in the depth of clearing of Fusarium solani when grown in combination with other test fungi.

Depth of clearing after 30 days of F. solani = 18.0 cms. (C)

FUNGI	Depth of clearing with test fungi (T)	Difference C - T	% Inhibition
Arthrobotrys sp.	16.0 mms.	2.0	11.1
Fusarium moniliforme	21.5 mms.	- 3.5	- 19.4*
Coniothyrium fuckeli	14.5 mms.	3.3	19.4
Humicola grisea	13.0 mms.	5.0	27.7
Sordaria fimicola	14.5 mms.	3.5	19.4

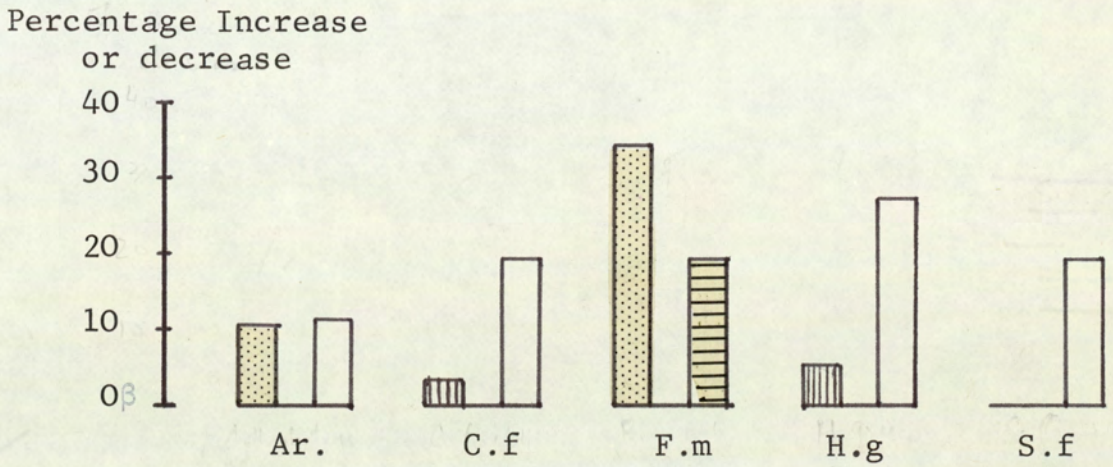
* = Percentage increase in the depth of clearing.

The percentage inhibition or otherwise of the cellulolytic activity of F. solani in association with other test fungi has been calculated and summarized in Table 44 . The comparison of the effect on the linear growth and cellulolytic activity has been represented in Figure 26 .

Among all the fungi tested F. moniliforme and F. solani when grown together resulted in increased cellulolytic power as the depth of the clearing of the cellulose agar increased by 19.4%. The decrease of the depth of the clearing of the cellulose agar occurred in all other instances when F. solani was grown with different test fungi. As C. fuckeli and H. grisea enhanced the linear growth of F. solani, its cellulolytic activity was decreased in their presence. The extent of inhibition was 19.4% and 27.7% respectively.

In addition to these experiments, some investigations were made on the interaction of A. fumigatus with G. roseum and Papulaspora sp. A. fumigatus occurred on the cellulose agar plates quite frequently and it showed some sort of interaction with these two fungi. Therefore a similar experiment was run using perfusion interaction technique.

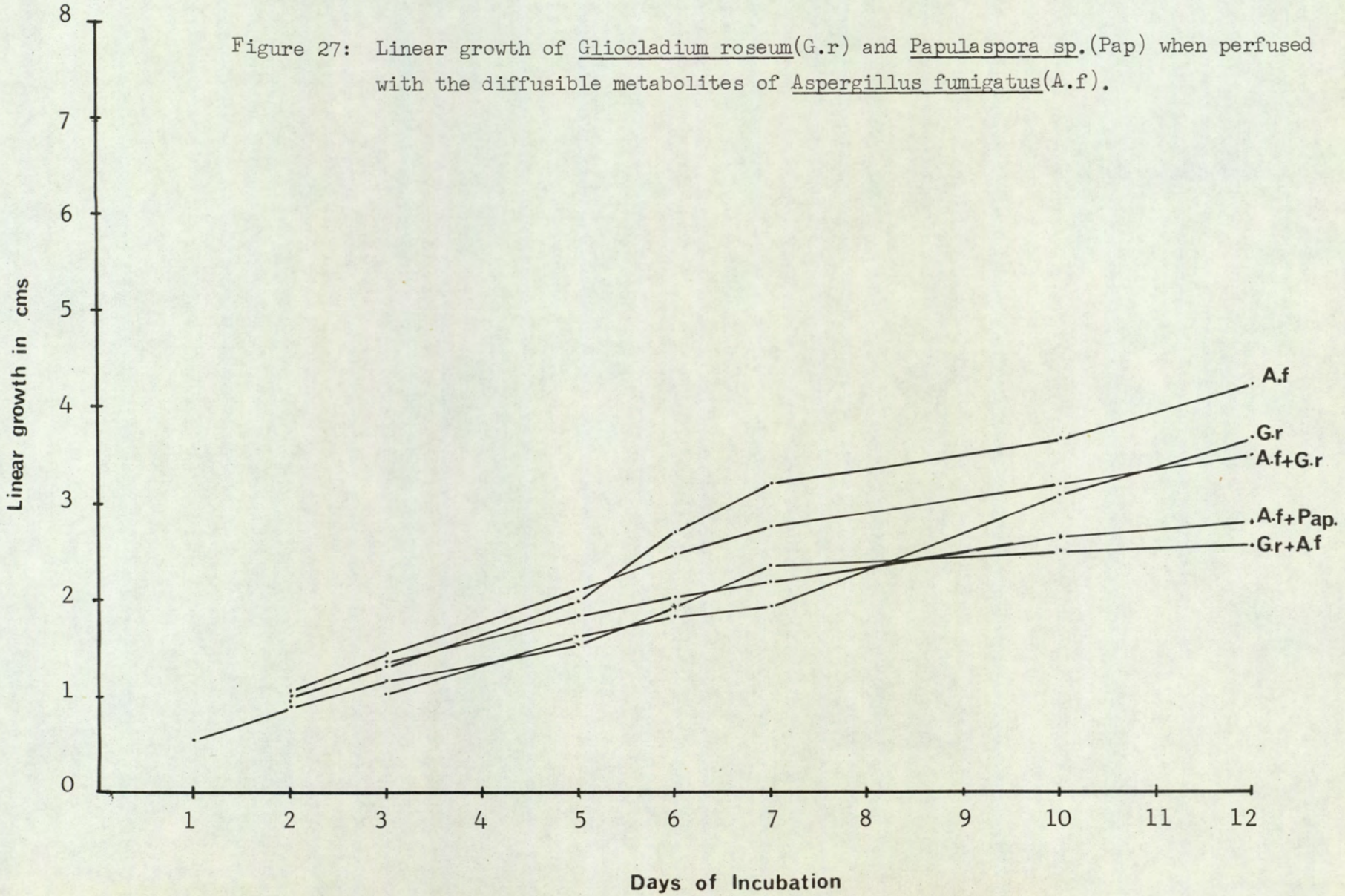
The results of the interaction between A. fumigatus and G. roseum are represented in Figure 27 . The diffusible metabolites of A. fumigatus suppressed the linear growth of G. roseum by 1.1 cms. after 12 days of incubation at 25° C. The percentage inhibition of the linear growth of G. roseum was calculated to be 19.6%. The diffusible metabolites of G. roseum when perfused to A. fumigatus suppressed its linear growth as well. This suppression was only 0.8 cm. after 12 days of incubation. This inhibition was calculated to be 11.1%.



Legend: same as Figs 20 and 24.

Figure 26: Percentage increase or decrease of the linear growth and depth of clearing of cellulose by Fusarium solani when grown with other fungi.

Figure 27: Linear growth of Gliocladium roseum(G.r) and Papulaspora sp.(Pap) when perfused with the diffusible metabolites of Aspergillus fumigatus(A.f).



The effect of the diffusible metabolites of A. fumigatus was also observed on Papulaspora sp. using the same interaction perfusion technique. There was no inhibition of the linear growth of Papulaspora sp. but its growth was very poor as compared to the controls and it did not produce much blackening of the cellulose strip as is normally produced by the dark brown hyphae of Papulaspora sp.

The metabolites of Papulaspora sp. were also perfused on to A. fumigatus to observe the effect on its linear growth. The results are represented in Figure 27 . The linear growth of A. fumigatus proceeded steadily up to 6 days of incubation after which it was slowed down to a large extent. After 12 days it reached 2.8 cms. as compared to 4.2 cms. of the control. This inhibition was calculated to be 33.3%.

In addition to the inhibition of the linear growth of A. fumigatus, its cellulolytic activity also decreased to quite a large extent when grown in combination with Papulaspora sp. (Table).

From these results obtained, its is quite apparent that the perfusion interaction technique has worked quite successfully thus allowing the study of any quantitative effect on the linear growth of one fungus by the diffusible metabolites of another.

Reviewing the results thus obtained, it is quite clear that there are very few examples of the increase of the linear growth as compared to the inhibition of test fungi. Arthrobotrys sp. was the only species which produced a notable increase as its linear growth was enhanced by ~~29%~~ 9.8 when perfused with the

diffusible metabolites of G. roseum whereas metabolites of F. solani produced 30.3% increase in its linear growth. Coniothyrium fuckeli had its linear growth enhanced by 3.4% when perfused with the metabolites of F. solani. The diffusible metabolites of T. viride did not produce any increase of the linear growth of test fungi. On the other hand, the linear growth of T. viride was suppressed by 46.6% and 26.6% when perfused with the metabolites of C. globosum and C. fuckeli respectively. The linear growth of F. solani was slightly increased by 3.1% and 5.5% when perfused with the metabolites of C. fuckeli and H. grisea respectively.

The ecological studies carried out on the soil fungi can be better understood with the help of several concepts formulated by Garrett (1950, '56, '63.) All these concepts elucidate the synecology of the fungi colonising a specific substrate. The studies of many workers (Brian 1957; Gottlieb & Siminoff 1951; Wright 1955) have pointed towards the antagonism, interaction and competition between different microorganisms in the soil, emphasising those which become most active during the colonisation of an organic substrate and its subsequent phases. This behaviour of the microorganisms in the soil is referred to as ' competitive saprophytic ability ' by Garrett (1950) who defined it as " the summation of physiological characteristics that make for success in competitive colonisation of dead organic substrates ". In this connection Garrett listed four characteristics that are likely to contribute to a high degree of saprophytic ability, namely : 1) rapid germination of spores and a high rate of hyphal growth, both favouring rapid colonisation; 2) good enzyme production, which favours rapid and extensive substrate utilisation; 3) production of substances toxic to other organisms, which may reduce competition for available

substances; and 4) tolerance of antibiotic substances produced by other organisms. The third factor is important during early colonisation when not many organisms are present and toxic substances produced by the organisms can provide advantages over other organisms. The fourth property is is helpful during the later stages of the decomposition of organic substrates when the number of fungi and the amount of toxic substance is maximal. These factors have been studied to some extent in the previous chapter, using the perfusion interaction technique and mixed culture inoculations on to the cellulose agar columns (Rautela & Cowling 1966). The first two factors pertaining to spore germination and enzyme production are advantageous to maintain a high saprophytic activity (Park 1968).

The factors involved in the spore germination and fungal viability have been discussed by Sussman (1966) Park(1965), and Sussman & Halvorsun (1966). Some workers have also observed inhibition of fungal spores in the soil which was termed fungistasis by Dobbs & Hinson (1953). They observed a widespread fungistasis in the soil which was later termed as mycostasis by Dobbs, Hinson & Bywater (1957). Although the soil provides an environment suitable for growth of many fungi, the mycostatic activity may result in the inhibition of spore germination and mycelial growth which was found to be due to biological factors (Dobbs, Hinson & Bywater 1957). Lockwood (1964) considered this inhibition was completely removed by sterilizing or air drying the soil. Brian (1960) indicated that only antibiotic production was not a possible cause of widespread

fungistasis. In order to apply these synecological concepts in the particular situation of the germination, colonization and subsequent succession on the perfused cellulosic substrate, a small experiment was performed using the perfusion technique.

Spore suspension of the following ten cellulolytic fungi as there were commonly isolated from the soil was prepared by using 0.5% Tween 80 (Admek 1965). The fungi used were Arthrobotrys sp., Chaetomium globosum, Fusarium solani, Gliocladium roseum, Humicola grisea, Paecilomyces elegans, Graphium sp. Sordaria fimicola and Trichoderma viride. These fungi were grown on E & P's cellulose agar slopes for 7 days. The spore suspension was prepared by adding 10 mls. of 0.5% Tween 80 in sterile water to the agar slope, shaking the slope and then recovering the suspension. The number of spores in the suspension were counted using a haemocytometer. The spore suspension was diluted if necessary and then varying volumes of suspension of various fungi were mixed in order to have equal numbers of spores of all the above mentioned fungi.

The perfusion devices were made as described in Chapter 2. The fibre glass cloth used to screen the cellulose strip was 2 cms. wide and 9 cms. long. It was used in a thickness of 1, 2, 3, 5, and 10 layers. For each number of layers 10 perfusion devices were made. E & P salt solution was again used for perfusion. After autoclaving, all the perfusion devices were inoculated with 2 mls. of the spore mixture suspension. In order to prevent the spore suspension filtering through the different layers of fibre glass cloth

the 2 mls. spore suspension inoculum was pipetted on to 2 cms. x 9 cms. glass fibre cloth contained in a sterile petri dish. The fibre glass was allowed to absorb the suspension for 5 minutes and then extra suspension was removed by simply tilting the petri dish. This inoculated fibre glass cloth was then transferred aseptically on to the perfusion devices containing different number of layers of fibre glass cloth. The inoculated perfusion devices were then incubated at 25° C.

After different intervals of incubation, one perfusion device was sacrificed for each thickness of fibre glass cloth covering the cellulose strip. All the layers of fibre glass cloth were removed aseptically and the cellulose strip was released from the fibre glass sleevings, cut into small pieces and inoculated on to E & P cellulose agar (as described previously). These plates were observed after 7 days of incubation at 25° C. and all the fungi appearing were recorded.

The results of this experiment are summarized in Table 44 The most rapidly germinating fungi were found to be T.viride and G. roseum. These were isolated from all the cellulose paper strips covered with 1, 2, 3, 5 and 10 layers of fibre glass cloth after 8 hrs. of incubation. The spores of T. viride could be seen germinating on the fibre glass cloth and giving rise to mycelium which penetrated the different layers of fibre glass cloth to reach the cellulose paper strip. Its mode of growth was also observed through various layers of fibre glass cloth. The mycelium thus formed after germination of the spore, produced spores in turn while still

Table 44

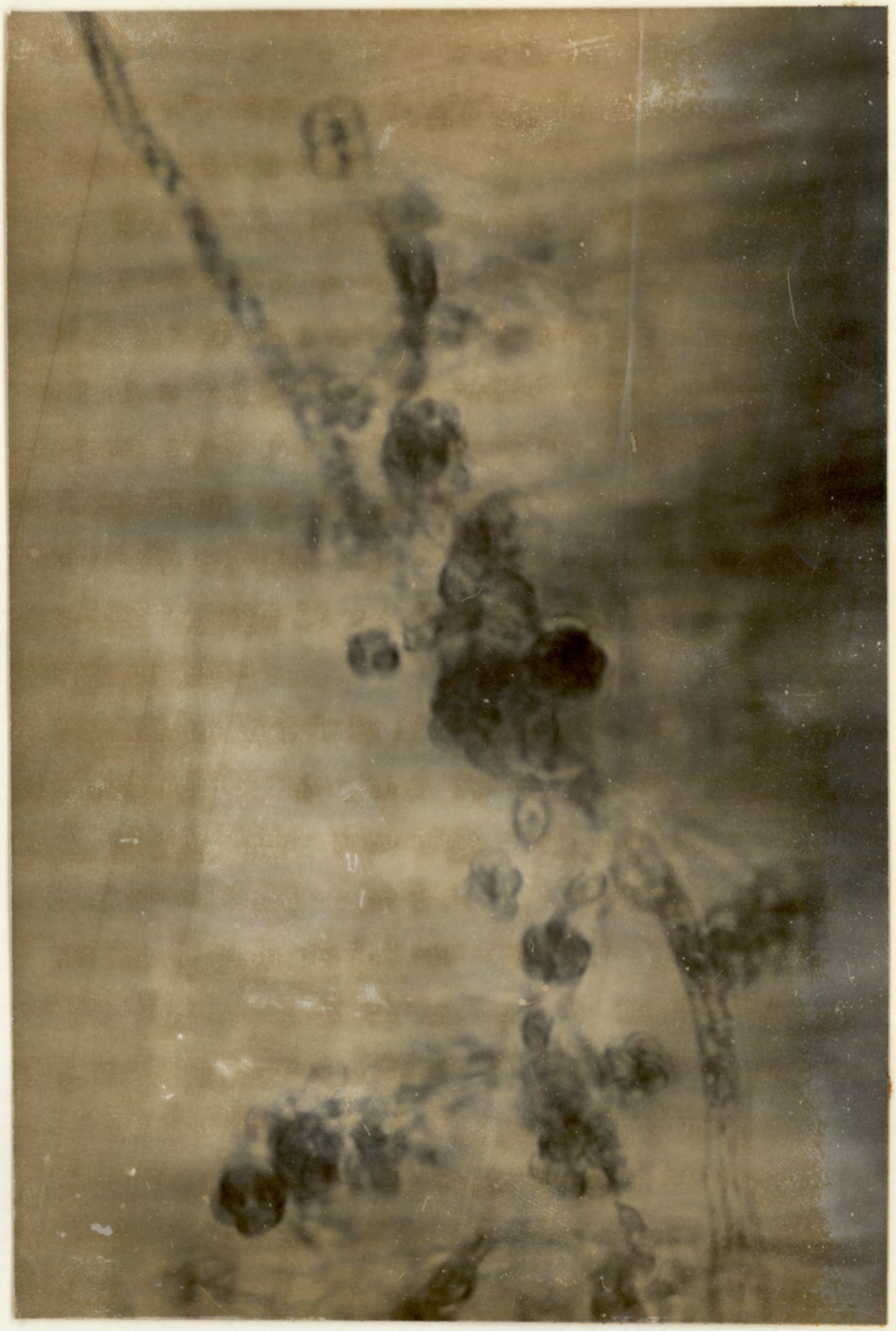
Percentage frequency of isolation of the fungi colonising the cellulose strip through various thicknesses of the fibre glass cloth.

Days of Incubation	No. of layers of Fibre Glass Cloth	Arthrobotrys sp.	C. globosum	F. solani	F. sporotrichiodes	G. roseum	Graphium sp.	H. grisea	P. elegans	Penicillium sp.	S. fimicola	T. viride
8 hrs.	1	-	-	-	-	62	-	-	-	-	-	100
	2	-	-	-	-	38	-	-	-	-	-	100
	3	-	-	-	-	12	-	-	-	-	-	100
	5	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-
1 day	1	-	-	12	12	77	-	-	66	-	-	100
	2	-	-	25	12	50	-	-	100	-	-	100
	3	-	-	12	-	-	-	-	100	-	-	100
	5	-	-	-	-	-	-	-	66	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-
2 days	1	-	-	25	-	83	-	-	100	-	-	100
	2	-	-	25	-	50	-	-	100	-	-	100
	3	-	-	12	-	50	-	-	66	-	-	100
	5	-	-	12	-	-	-	-	100	-	-	83
	10	-	-	-	-	-	-	-	50	-	-	-
6 days	1	62	12	25	-	62	-	62	-	-	-	100
	2	25	-	-	-	77	-	-	-	-	-	100
	3	-	-	12	-	77	-	12	-	-	-	100
	5	-	-	-	12	37	-	-	50	-	-	100
	10	-	-	-	-	-	-	-	50	-	-	100
10 days	1	-	-	25	-	77	-	-	-	8	-	100
	2	-	-	33	-	77	-	-	-	-	-	100
	3	-	-	25	-	77	-	-	-	12	12	100
	5	77	-	-	19	91	-	-	-	19	12	100
	10	12	-	-	-	-	-	-	-	-	-	100
16 days	1	91	25	-	-	33	-	-	-	33	-	100
	2	75	-	-	-	55	-	-	-	25	-	83
	3	83	-	-	-	83	-	-	-	-	-	100
	5	100	-	-	-	67	-	-	-	-	37	50
	10	-	8	-	-	-	-	-	-	75	-	100
22 days	1	-	-	-	-	50	-	-	-	-	-	50
	2	-	-	-	-	53	-	-	-	-	-	83
	3	-	-	-	-	77	-	-	-	-	-	100
	5	100	8	37	-	100	-	-	-	-	50	91
	10	50	8	-	-	100	-	-	-	-	83	100
30 days	1	-	-	-	-	50	-	-	-	-	-	50
	2	-	-	-	-	50	-	-	-	-	-	83
	3	-	-	37	-	50	-	-	-	-	-	100
	5	50	-	37	-	77	-	-	-	75	37	100
	10	37	-	-	-	50	-	-	-	37	-	60

on the fibre glass cloth (Photograph8). This characteristic gave an added advantage to T. viride for successful colonization of the cellulose strip.

The spores of G. roseum also germinated readily but were able to reach the cellulose strip through up to 3 layers of fibre glass cloth after 8 hrs. of incubation. Its frequency of occurrence decreased with the increase in the number of the layers of fibre glass cloth. After 6 days of incubation G. roseum was also isolated from the cellulose strip covered with 5 layers of fibre glass cloth whereas after 22 days it was isolated from that of 10 layers of fibre glass cloth.

G. roseum had a fairly high frequency of isolation from the cellulose strip throughout the period of incubation, whereas T. viride had a very high frequency of occurrence in the early days of incubation but it decreased towards the end of the incubation period and was just able to make its appearance. Moreover, at the end of the incubation period, T. viride had a sparse growth on the cellulose agar which also had a staled appearance. G. roseum had been recorded as a genuine soil inhabiting fungus (Pugh and Dickinson 1965) and a destructive mycoparasite (Barnett and Lilly 1962) and has been shown capable of killing hyphae of Rhizoctonia solani in paired cultures (Pugh and van Enden 1969). In view of such observations by many research workers, it can be presumed that the sparse and unhealthy growth of T.viride on the cellulose agar plate might be due to the activities of G. roseum.



Photograph 8

The sporulating mycelium of T.viride produced immediately after the spore germination on the fibre glass cloth.

Paeciliomyces elegans was able to penetrate the first five layers of fibre glass cloth covering the perfused cellulose strip after 1 day of incubation. After 2 days of incubation it penetrated 10 layers of fibre glass cloth as it was isolated from the cellulose strip screened by 10 layers of fibre glass cloth. P. elegans was not isolated again during this experiment except after 6 days when it was isolated from the perfused cellulose strip screened by 5 and 10 layers of fibre glass cloth.

The frequency of isolation of F. solani and F. sporotrichiodes seemed to be very low. F. solani was never isolated from the cellulose strip covered with 10 layers of fibre glass cloth. F. sporotrichiodes had a much lower frequency and was only isolated between 1 and 10 days of incubation.

C. globosum was first isolated after 6 days of incubation from cellulose strip covered with only 1 layer of fibre glas cloth. It was also isolated after 16 and 22 days of incubation. Arthrobotrys sp. was isolated from 1 and 2 layers thickness after 6 days of incubation and was isolated from all thicknesses of fibre glass cloth after 10 days of incubation. After 22 and 30 days of incubation, it was only isolated from the cellulose strip covered with 5 and 10 layers of fibre glass cloth.

Penicillium sp. and S. fimicola were isolated for the first time after 10 days of incubation. Penicillium sp. was isolated from all thicknesses of the fibre glass cloth after 16 days of incubation, whereas it was only isolated from 5 and 10 layers of fibre glass cloth after 30 days of incubation. S. fimicola was never isolated from the cellulose

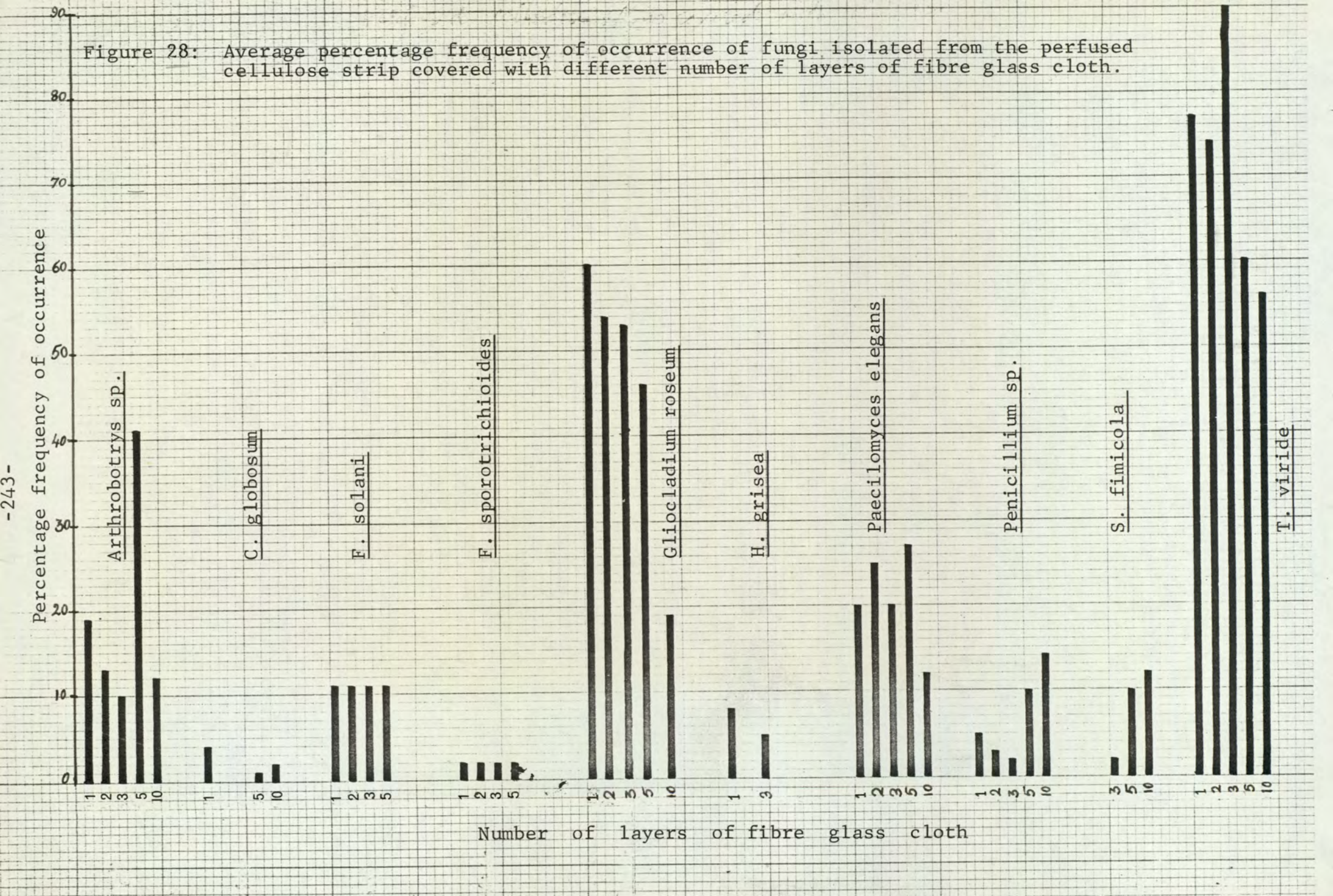
strip covered with 1 and 2 layers of fibre glass cloth. It was only isolated from 3 - 10 layers of fibre glass cloth after 16 - 30 days of incubation. The percentage frequency of occurrence of these fungi was quite low.

H. grisea was also isolated once after 6 days of incubation from the cellulose strip covered with 1 - 3 layers of fibre glass cloth. The only fungal spores which either failed to germinate or if they germinated the hyphae produced, failed to penetrate in order to reach the cellulose paper strip was that of Graphium sp. The first 3 layers of the 10 layers of fibre glass cloth covering the cellulose strip, produced Graphium sp., when inoculated into the cellulose agar whereas there was no trace of any further penetration by Graphium sp.

All the fungal spores except Graphium sp. were able to germinate and colonize the cellulose substrate. A careful analysis of the results can illustrate a succession of fungi colonizing the cellulosic substrate, which is composed of early colonizers and late developers.

Under these experimental conditions, as roughly equal number of spores were mixed, no one organism had any preadvantage over the others. Thus any fungus from the early colonizers of the cellulose strip, must have a high competitive saprophytic ability as the successful colonization can only take place due to the 'rapid germination of spores and a high rate of hyphal growth'. The spores having such characteristic could be referred to T. viride and G. roseum and P. elegans which comprise the early colonizers. Among these T. viride and G. roseum were continuously isolated

Figure 28: Average percentage frequency of occurrence of fungi isolated from the perfused cellulose strip covered with different number of layers of fibre glass cloth.



throughout the experiment whereas P. elegans was isolated only up to 6 days of incubation. This disappearance of P. elegans may be attributed to the interaction with other organisms which by now had been able to germinate and colonize the cellulose strip. Among these fungi are Arthrobotrys sp., C. globosum and H. grisea. Among the late developers are S. fimicola and Penicillium sp. which were isolated after 10 days of incubation.

The average total percentage frequency of isolation of different fungi isolated from the cellulose strip covered with different layers of fibre glass cloth is represented in Figure 28 .

The delineation of these fungal spores into early developers and late developers might be due to certain factors which influence their germination in spite of the constant environmental conditions. This phenomenon has also been observed and pointed out by other workers (Dobbs and Hinson, 1953; Dobbs, Hinson and Bywater, 1957; Dwivedi, 1968; Park, 1968). These factors could also be explained by applying Garrett's concept of inoculum potential. Garrett (1956) defined it as "the energy for growth of a fungus available for colonization of a substrate at the surface of the substrate to be colonized". This concept has been commonly used in plant pathology. A host exerts a physical and chemical resistance to fungal penetration and the extent to which this penetration takes place depends on the growth energy or inoculum potential of the pathogen. Park (1968) discussed similar phenomenon with regards to dead substrates. He termed it "substratum resistance" as an ecological equivalent

to host resistance. There are at least three possible sources of substratum resistance. Firstly, it may be a residual resistance carried over from the living host to the dead substratum. Secondly, there may be some toxic substances produced by the microorganism already active in the early stages of substratum succession. Thirdly, it may be due to the initial nature of the substrate and thus microorganisms might need some "starter" energy in order to successfully colonize the substrate (Park, 1968).

In the present investigation, the first source of resistance is irrelevant, as the cellulosic substrate used has been purified and contained negligible impurities. The remaining two factors can well be used for explaining the late or early germination of various fungal spores.

Penetration

As has been stated previously, for the effective utilization of the substrate by fungi, one of the factors is good enzyme production which favours extensive substrate utilization. In order to investigate this phenomenon, another small experiment was performed, again using the perfusion technique.

In this case, some cellulolytic fungi which had been isolated previously, were used to study the enzyme production and the penetration of their mycelium by inoculating the fungal species on top of 5 layers of 3 mm. Whatman cellulose paper and then estimating the weight loss of different layers of the cellulose paper.

The cellulose paper used in this experiment was the same as used in the previous experimens, i.e. 3 mm. Whatmans chromatography paper. The only difference being that it did not have any polythene backing. This cellulose paper was cut into 1.0 cm. wide strips. A wad of 5 such strips was incorporated into the perfusion system. The length of the strips was 9 cms. except the middle strip which was 13 cms. long. This strip was attached to 6 mm. wide glass fibre sleeving threaded through 5 mm. wide silicone rubber tubing which then led to 100 ml. conical flask containing the nutrient solution (Diagram 3). The nutrients were conducted on to the cellulose strip which was in the middle of the wad of 5 cellulose strips. From this, the nutrients were diffused on to the cellulose strips above and below it. All the sides of the wad of the cellulose strips were sealed with silicone rubber glue and the top of the cellulose strips was also covered with glass fibre tape. A known

Table 45

FUNGI	Cellulose Strip Layer No.				
	1	2	3	4	5
C. globosum	2*	2	2	4	11
C. fuckeli	2	4	4	11	11
F. solani	2	4	4	4	4
G. roseum	2	2	4	4	4
H. grisea	2	2	4	4	11
Papulaspora sp.	2	2	2	4	4
S. fimicola	2	4	11	11	20
T. viride	2	2	2	2	2

* All figures represent the days of incubation after which first isolation was made.

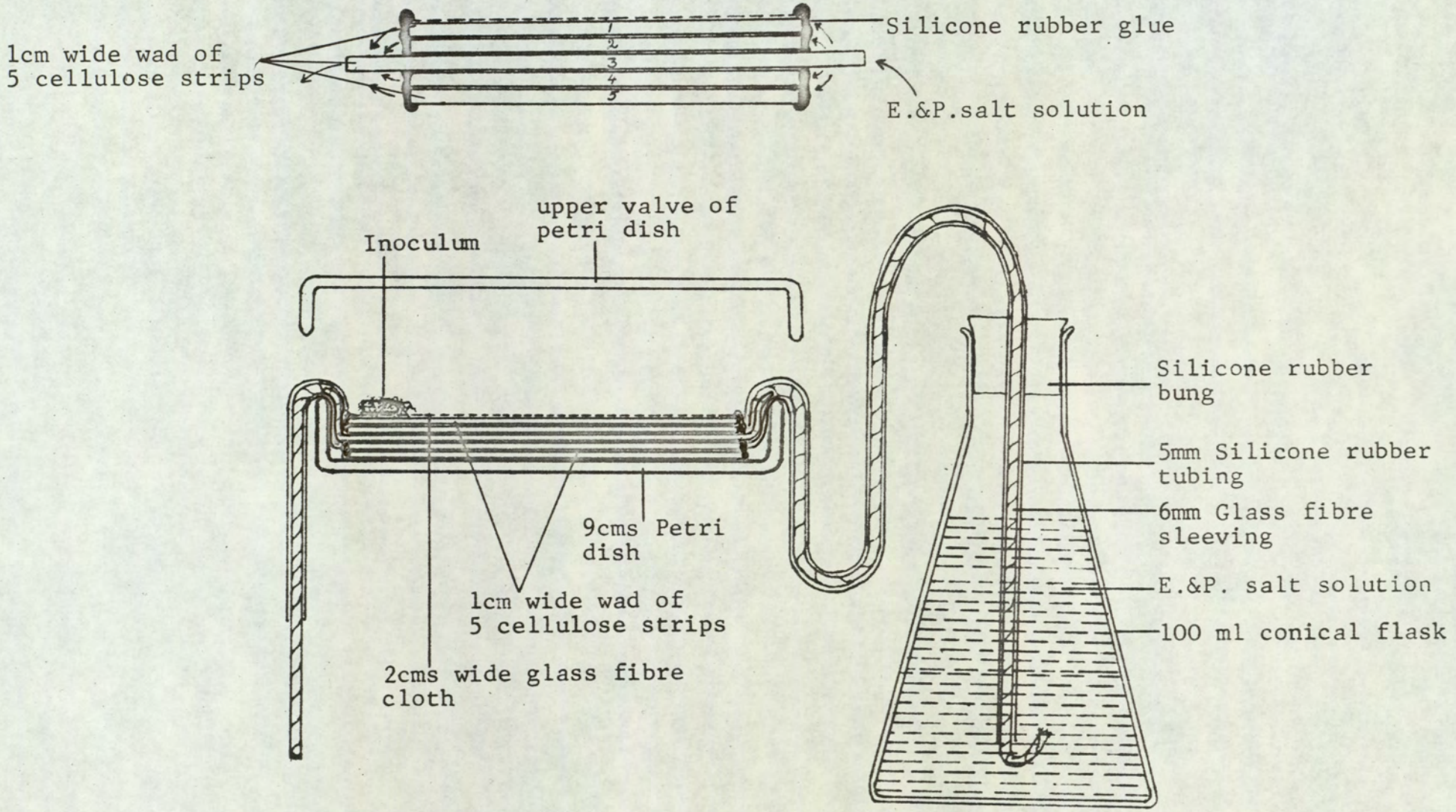

1cm wide wad of
5 cellulose strips
Silicone rubber glue
E.&P.salt solution
upper valve of
petri dish
Inoculum
Silicone rubber
bung
5mm Silicone rubber
tubing
6mm Glass fibre
sleeving
E.&P. salt solution
100 ml conical flask
9cms Petri
dish
1cm wide wad of
5 cellulose strips
2cms wide glass fibre
cloth

Diagram 3

fungal species was inoculated on the glass fibre tape towards the evaporation end of the perfusion system so that the fungus could grow towards the fresh nutrients. Moreover, the fungal mycelium could only penetrate the cellulose strips from the top surface as all the sides of the cellulose strips were sealed.

In order to allow enough replication 15 such perfusion devices were made for each fungal species. Three such devices were sacrificed each time after 2, 4, 11, 20 and 32 days of incubation. The wad of cellulose strips was released from the glass fibre sleeving and a 5 mm. diameter was removed with the help of an alcohol flamed cork borer from each layer of the wad and inoculated onto the E & P cellulose agar. A standard 5 mm. x 50 mm. punch was also removed from each wad and the weight loss of each layer was estimated separately.

The eight fungi tested were Chaetomium globosum, Fusarium solani, Humicola grisea, Gliocladium roseum, Papulaspora sp., Coniothyrium fuckeli, Sordaria fimicola and Trichoderma viride. E & P salt solution was used to perfuse. The incubation temperature was 25° C.

The weight loss results are represented in the various graphs in Figure 29 and 30 . Each reading is a mean of 3 observations. The isolation results are summarised in Table 45. All the figures represent the day of its first isolation.

F. solani and T. viride produced maximum weight loss. T. viride was isolated from all the layers of the wad of 5 cellulose strips after 2 days of perfusion and incubation; whereas F. solani was isolated from all the layers after 4 days of incubation. C. globosum and C. fuckeli produced a

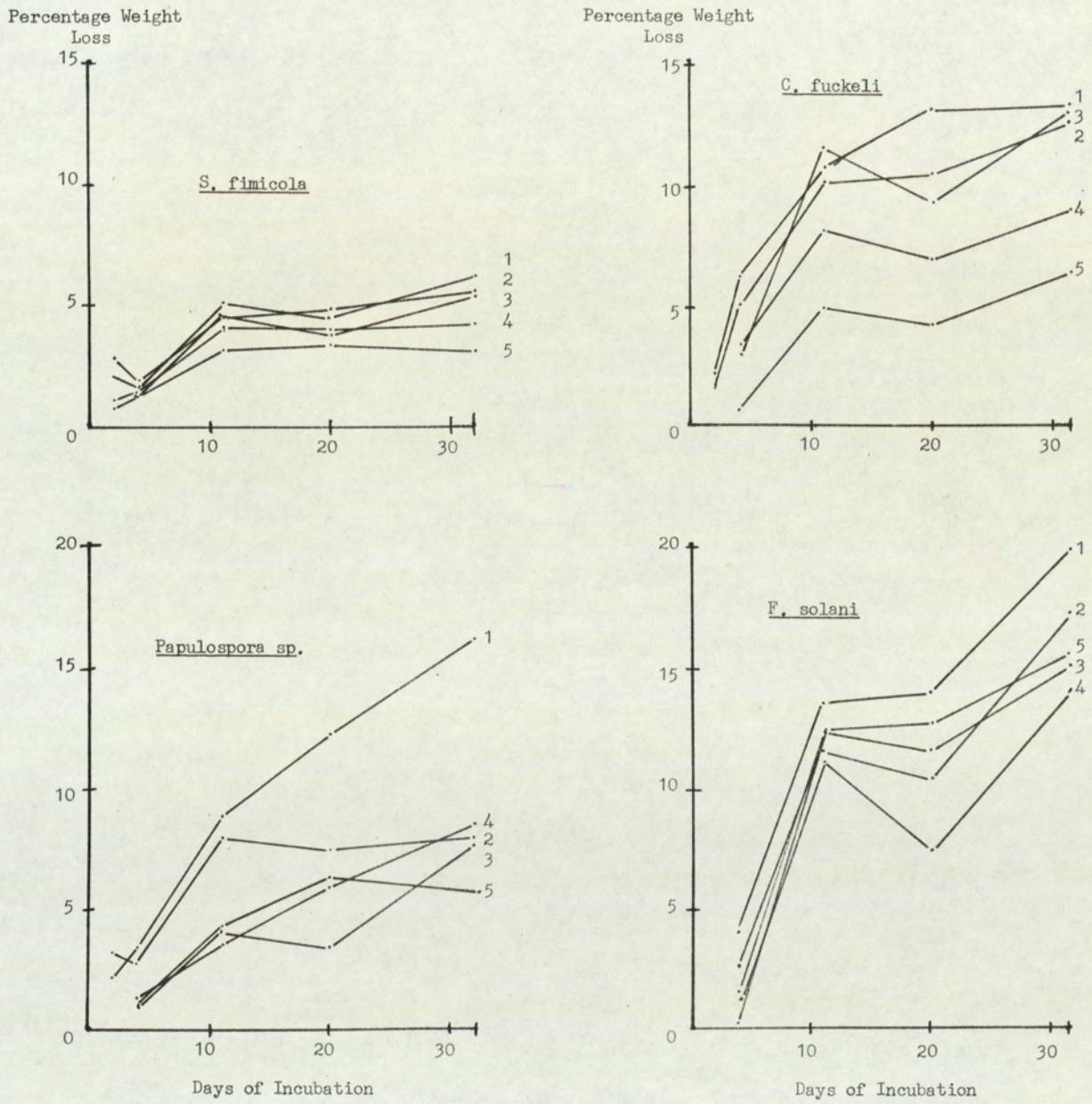


Figure 29: Average percentage weight loss of different layers of the wad of cellulose strips produced by different fungi.

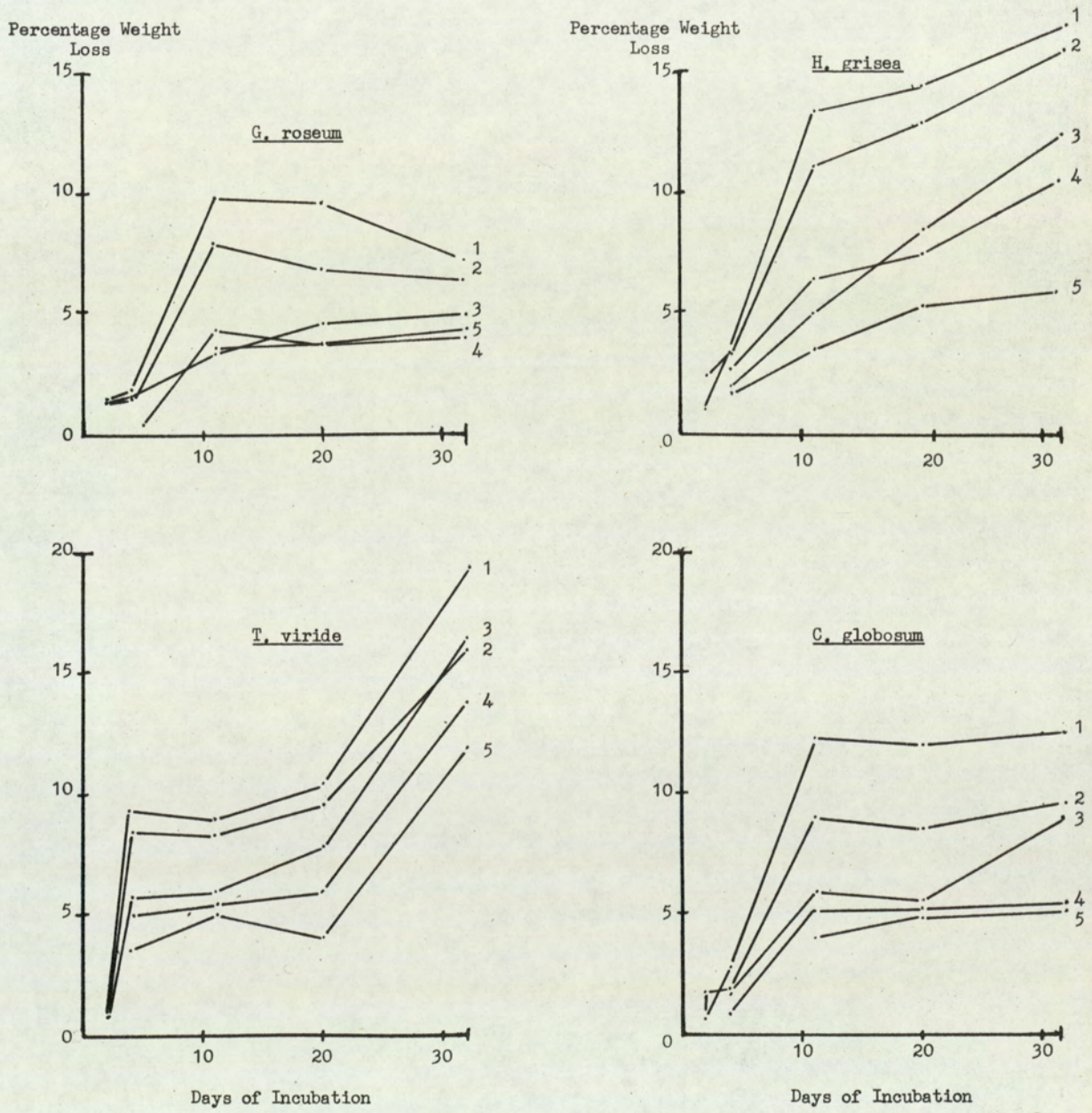


Figure 30: Average percentage weight loss of different layers of the wad of cellulose strips produced by different fungi.

moderate weight loss in the first 3 layers of the wad of cellulose strips whereas little weight loss was recorded in the fourth and fifth cellulose strip. C. globosum was able to penetrate the first 3 layers in 2 days, the fourth layer in 4 days and the first isolation from the fifth layer was made after 11 days of incubation. The penetration rate of C. fuckeli was not as rapid as C. globosum. C. fuckeli was isolated from the first layer after 2 days, from the second and third layer it was isolated after 4 days, whereas it took 11 days to penetrate the fourth and fifth layer of the wad of cellulose strips. Papulaspora sp. produced 16.3% weight loss in the first layer whereas in all the other lower layers the weight loss ranged from 5.7% to 8.5%. On the contrary, the rate of penetration of Papulaspora sp. was quite rapid. It penetrated the first 3 layers in 2 days and the fourth and fifth layer after 4 days of incubation and perfusion. H. grisea can also be grouped with Papulaspora sp. as it also produced quite high weight loss in the first two layers but quite low in the fifth layer of the wad of cellulose strips. H. grisea was also able to penetrate the first 2 layers in 2 days, third and fourth layer in 4 days while the fifth layer was penetrated after 11 days of incubation. G. roseum and S. fimicola produced little weight loss in the various layers of the wad of the cellulose strips. The rate of the mycelial penetration of these two fungi differed with each other as the rate of penetration of G. roseum was quite rapid and it was able to penetrate first 2 layers in 2 days and the remaining after 4 days of incubation. The rate of penetration of S. fimicola was comparatively slow as it

took 2 days to penetrate the first layer, 4 days for the second layer, 11 days for the third and fourth layer and 20 days to penetrate the fifth layer of the wad of cellulose strips.

It is apparent from the graphs in Figures 28 and 29 that the percentage weight loss decreases from layer number 1 to 5; maximum weight loss was produced in the layer number 1 and the minimum was normally produced in the layer number 5. The extent of this difference varies with various fungi. These differences have been summarised in Table 46

The maximum percentage difference has been calculated to be 65.2 and 63.5 in the case of H. grisea and Papulaspora sp. respectively. The least percentage difference was estimated with F. solani where it was 22.0%. All sorts of variations on percentage difference were found between these two extremes (Figure 31).

This difference in the weight loss of the various layers of the wad of the cellulose strips presumably depends on the following three factors:-

a. The rate of diffusion of the enzyme cellulase.

b. The rate of penetration of the fungal mycelium.

c. The enzyme production under a tendency to anaerobic conditions.

The degree of penetration of these fungi has been demonstrated (Table 46) whereas the weight loss results represented in the Figures 29 and 30 are summation of the 3 above mentioned factors. For example, H. grisea had a maximum percentage difference in weight loss between the first and fifth layer of the wad of the cellulose strips; its penetration rate was comparatively high as it penetrated the first 4 layers in

Figure 31: Average percentage weight loss of the different layers(1-5) of perfused cellulose strips after 32 days of incubation at 25°C.

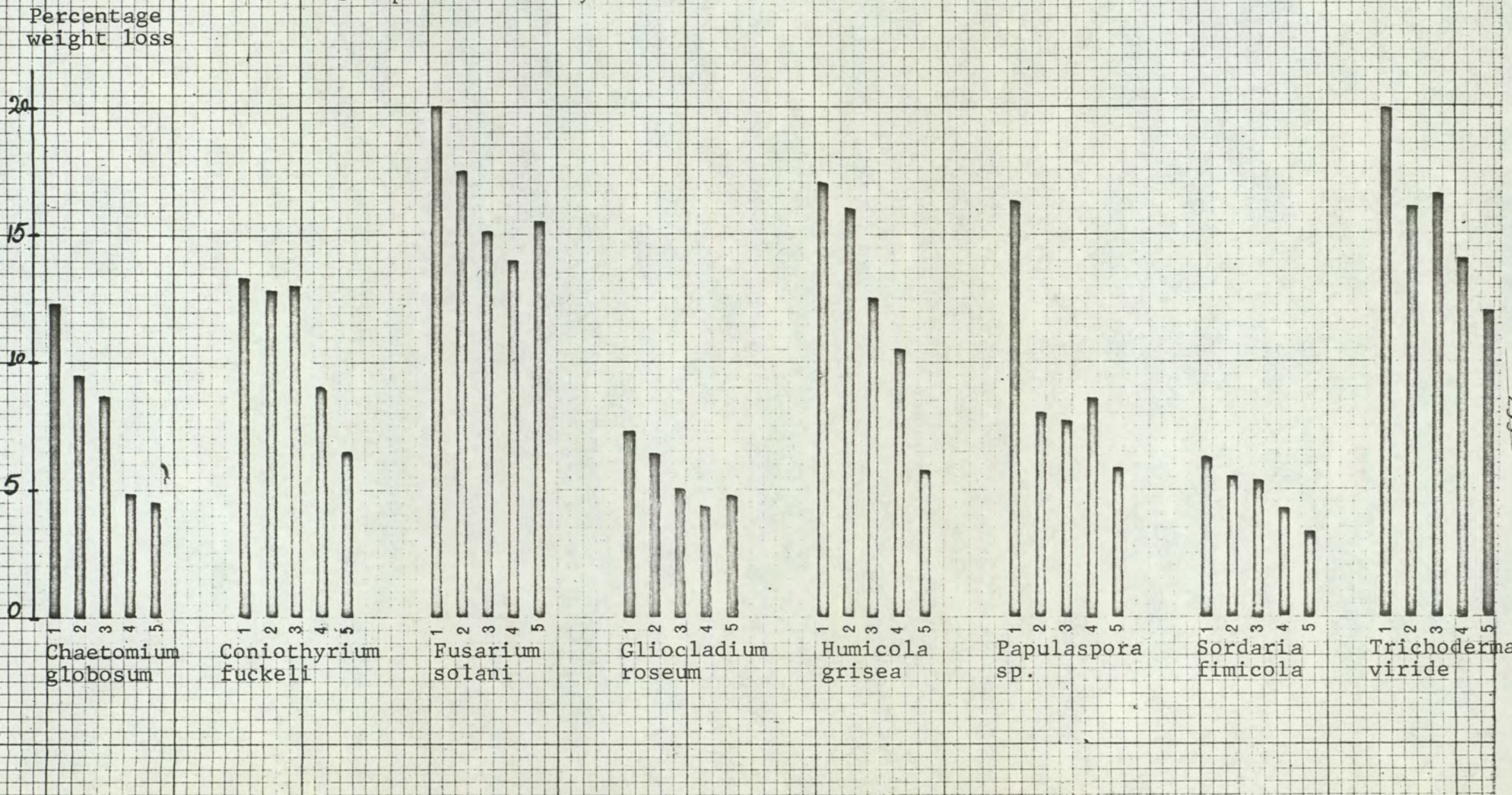


Table 46

Maximum and Minimum weight loss after 32 days of incubation

FUNGI	Maximum % Wt. Loss	Minimum % Wt. Loss	Difference	% Difference $\frac{\text{Diff.}}{\text{Max.Wt.Loss}} \times 100$
C. globosum	12.3	5.1	7.2	58.5
C. fuckeli	13.4	6.0	7.4	55.2
F. solani	20.0	15.6	4.4	22.0
G. roseum	7.2	4.3	2.9	40.2
H. grisea	17.0	5.9	11.1	65.2
Papulaspora sp.	16.2	5.9	10.3	63.5
S. fimicola	6.3	3.2	3.1	49.2
T. viride	19.9	12.0	7.9	39.7

four days of incubation. This discrepancy in these two observations might be due to the low enzyme production under an atmosphere of low oxygen tension as more free oxygen is available to the first layer than to the subsequent ones. Papulaspora sp. and C. globosum also seem to be in the same category. C. fuckeli produced maximum percentage weight loss in the first three layers of the wad of cellulose strip. This observation coincides with the fact that these three layers were also penetrated by its mycelium after 4 days of incubation whereas the fourth and fifth layers were penetrated as late as 11 days and hence quite a low percentage weight loss. The percentage difference in the case of G. roseum and T. viride was nearly the same. The rate of penetration was also quite rapid as T. viride was isolated from all the layers after 2 days and G. roseum after 4 days. The weight loss produced in the lower layers seem to be due to the diffusion of cellulase produced on the first and presumably second layer of the wad of cellulose strips and the mycelium present in the 3-5 layers does not seem to have produced enough cellulase in order to produce weight loss comparable to the first layer.

The percentage difference in the weight loss in the case of S. fimicola seems to be due to a very slow rate of penetration. The minimum difference in weight loss was produced in the case of F. solani as it also had a quite rapid rate of penetration.

The realisation of the problem of biodeterioration and recent advances made towards its control have resulted in a number of fungicides being used to preserve cellulosic materials from microbial attack. Wessel and Bejuki (1959) have prepared a list of industrial fungicides (including wood preservatives) mentioning 403 products representing 140 different chemicals or combinations being marketed by 171 different companies. The nature and type of fungicide used depends on the nature of the material and its potential use which varies from one product to another.

Considering the great variety and number of these fungicides, it is obvious that a wide array of tests pertaining to the biodeterioration of cellulosic materials exists; many of these test methods have been described and reviewed by Siu (1951) and Hueck van der Plas (1965b).

During the present investigation, the perfusion technique has been used as a test method to demonstrate the effectiveness of some of the commonly used paper and textile preservatives. The following were the preservatives used in various concentrations:

1. Copper naphthenate 0.2%, 0.4% and 0.8%
2. Sodium pentachlorophenol 0.1%, 0.3% and 0.6%

3. Lauryl pentachorophenol 2%.

The ready availability, cheapness, ease of application and dependability of copper naphthenate made it the fungicide of almost exclusive use for tents, gun covers, tarpaulins etc., during the second World War (Block 1967). Later on numerous other copper salts like copper - 8 - quinolinolate, copper formate copper borate etc., have been used as textile preservatives.

Pentachlorophenol, because of its powerful fungicidal activity, low cost and lack of colour has found use as a textile preservative. Sodium pentachlorophenate is also used in the manufacture of paper board resistant to microbial degradation (Shapiro & Volina 1961). It is used in boxboard such as is employed for packaging electrical equipment for shipment to the tropics and the effective concentration was found to be 0.3 - 0.6% of the weight of the board.

The use of pentachlorophenols as textile preservatives has however, several disadvantageous characteristics among which is the liberation of hydrochloric acid upon decomposition in sunlight, a reaction which accelerates deterioration (Bertolet 1943). Lauryl pentachlorophenol has been found to meet some of the objections to pentachlorophenol especially those based on its skin irritating properties. Lauryl pentachlorophenol has been shown to give satisfactory decay resistance in soil burial tests at 0.5 - 2.0% when used with a water repellant (Hueck and La Brijn 1960).

The polythene backed cellulose paper was coated with all the above mentioned fungicides separately at different concentrations. This coated paper was used in the perfusion

devices in order to study the effectiveness of these fungicides. The resultant observations have also been compared with similar observations obtained by Eggins & Lloyd's screen substrated immersion tube technique (Eggins & Lloyd 1968).

With the substrate immersion tube technique, both treated cloth and paper were used. Only two fungicides, namely copper naphthenate and sodium pentachlorophenate, were tested. Their concentrations were the same as mentioned previously. The difference between the screened and unscreened substrates was also noted. Therefore, to allow enough replication, 24 immersion tubes were prepared for each concentration of a fungicide. Out of these, 12 were kept screened and the remaining were left unscreened. These immersion tubes were buried in troughs of soil and incubated between 25 - 29°C. The sacrificing was made after 1 week, 3 weeks, 5 weeks and 7 weeks of incubation. Each time, 6 tubes were sacrificed for each concentration of the fungicide; 3 for the unscreened and 3 for the screened substrate. The cellulose paper, thus recovered was cleaned with a sterile brush in the case of the unscreened substrate; aseptically cut into small pieces and inoculated on to 9 cellulose agar petri dishes. The species were recorded after 7 days of incubation at 25°C. A similar experiment was run for the treated cloth strip.

The results for both paper and cloth treated with sodium pentachlorophenol and copper naphthenate are recorded in the Tables 47-50.

Fusarium solani and Sporotrichum pruinosum seemed to be the most commonly occurring fungi on the paper treated with 0.1%, 0.3% and 0.6% sodium pentachlorophenate.

Table 47: Percentage frequency of occurrence of fungi colonising the cellulose strip coated with varying concentrations of sodium pentachlorophenate (NaPCP) using screened substrate immersion tubes.

FUNGI	Conc. of Na PCP	1 week		3 weeks		5 weeks		7 weeks	
		US	S	US	S	US	S	US	S
Fusarium solani	0.1%	100	100	100	100	100	100	100	100
	0.3%	100	66	100	100	100	33	100	100
	0.6%	100	33	100	100	66	33	100	66
Sporotrichum pruinosum	0.1%	100	66	66	33	33		100	100
	0.3%			66	33	66		100	66
	0.6%			66	33			100	100
Penicillium fumiculosum	0.1%	100	66			33	66		
	0.3%	100	33				33		
	0.6%	100	-			33	33		
Aspergillus sp.	0.1%	33	-					33	
	0.3%								
	0.6%								
Chaetomium globosum	0.1%	100	66			33	33	33	33
	0.3%	33	-			33	66	66	33
	0.6%	33	-			33		-	-
Trichoderma viride	0.1%							-	-
	0.3%							100	100
	0.6%							100	66
Gliocladium roseum	0.1%					66	33		
	0.3%								
	0.6%								
Paecilomyces varioti	0.1%								
	0.3%		33						
	0.6%								

US unscreened; S screened.

Table 48: Percentage frequency of occurrence of fungi colonising the cloth strip coated with varying concentrations of sodium pentachlorophenate(NaPCP) using screened substrate immersion tubes.

FUNGI	Conc. of Na PCP	1 week		3 weeks		5 weeks		7 weeks	
		US	S	US	S	US	S	US	S
Fusarium	0.1%	100	100	100	100	100	100	100	100
solani	0.3%	66	33	100	100	100	100	100	66
	0.6%	-	-	100	33	66	33	66	33
Sporotrichum	0.1%	100	100	100	100	66	66	66	66
pruinosum	0.3%	66	-	100	66	66	33	66	33
	0.6%	-	-	66	-	-	33	66	-
Penicillium	0.1%	100	66				33		
fumiculosum	0.3%					33	33	33	
()	0.6%					100	-	66	33
Aspergillus	0.1%				33				
sp.	0.3%	66							
	0.6%			100					33
Chaetomium	0.1%	66							
globosum	0.3%					33			100
	0.6%						33		

Table 49: Percentage frequency of occurrence of fungi colonising the cellulose strip coated with varying concentrations of copper naphthenate using screened substrate immersion tube.

FUNGI		1 week		3 weeks		5 weeks		7 weeks	
		US	S	US	S	US	S	US	S
Fusarium solani	0.2%	100	100	100	100	100	100	100	100
	0.4%	100	100	100	100	100	100	100	100
	0.8%	100	50	100	33	100	100		
Sporotrichum pruinosum	0.2%	100	100	100	100	33	66	66	100
	0.4%	66	33	33	-	-	66	100	100
	0.8%	-	-	-	-	-	-	66	33
Gliocladium roseum	0.2%					33		33	
	0.4%							33	
	0.8%								
Penicillium fumiculosum	0.2%	66	33	33	66	66	66	66	66
	0.4%			33			66	66	66
				66		33	33	66	-
Aspergillus sp.	0.2%			66	66	66	33	100	33
	0.4%			33	-		66	100	100
	0.8%			33	-	100	33	100	-

The frequency of occurrence was comparatively high among the unscreened isolations compared with the screened ones. The majority of the species isolated were recorded after 1 week of soil burial of the paper treated with 0.1% sodium pentachlorophenate. S. pruinosum was first isolated after 3 weeks of soil burial of the paper treated with 0.3% and 0.6% of the fungicide. Chaetomium globosum was isolated after 1 week from the paper treated with all 3 concentrations. It was not isolated after 3 weeks but reappeared only at 0.1% and 0.3% concentration of the fungicide, after 5 weeks and 7 weeks of soil burial: it had quite a low frequency of occurrence at this time. Penicillium funiculosum also made its appearance after 1 week and then after 5 weeks of soil burial. Its frequency decreased with the increas in time of the soil burial. Gliocladium roseum was isolated only once from the paper treated with 0.1% sodium pentachlorophenate after 5 weeks of soil burial. Trichoderma viride was also isolated from the paper treated with 0.3% and 0.6% fungicide, after 7 weeks soil burial at 25^{o} C. At this time its frequency of occurrence was quite high. In addition to these fungi Paecilomyces varioti was also isolated from 0.3% fungicide treated paper after 1 week of soil burial.

The results of the similar experiment performed with the cloth strips are summarised in Table 48. The pattern of isolation was generally the same with some difference in the frequency of occurrence of certain fungi. F. solani and S. pruinosum were again the most commonly occurring fungi but the cloth treated with 0.6% fungicide resulted in the decrease of the frequency of occurrence of S. pruinosum. P. funiculosum was isolated first after 5 weeks of soil burial of the cloth strips. C. globosum was also occasionally isolated but its frequency of occurrence

was quite low. Among other fungi observed on the petri dish but are not recorded in the table, were A. niger, G. roseum, Rhizopus nigricans, Scopulariopsis brevicaulis and T. viride.

The results of the colonisation of the paper treated with varying concentrations of copper naphthenate are summarised in Table 49. At 0.2% and 0.4% copper naphthenate, F. solani had the highest frequency of isolation. This frequency decreased at 0.8% concentration of the fungicide. S. pruinosum also had a similar effect, as at 0.8%, copper nephthenate, it was first isolated after 7 weeks of soil burial. P. funiculosum and Aspergillus sp. were quite high in their frequency of isolation as compared to the previous experiments. Aspergillus sp. and P. funiculosum were first isolated after 3 weeks of soil burial except at 0.2% copper naphthenate. P. funiculosum was isolated after the first week. G. roseum had a very low frequency and was first isolated from the paper treated with 0.2% and 0.4% fungicide, after five weeks of soil burial at 25^{o} C.

The results of similar experiment with the cloth strips are summarised in the Table 50. Again the same type of fungi were isolated. The relative frequency of isolation of all the fungi isolated was higher than the similar isolations from the paper. G. roseum was isolated from the cloth at all concentrations of copper nahthenate. T. viride was isolated only once after seven weeks from the cloth treated with 0.8% copper naphthenate.

The average percentage frequency of occurrence of the individual fungi has also been calculated by adding the percentage frequencies and then dividing them by the total

Table 50 : Percentage frequency of occurrence of fungi colonising the cellulose strip coated with varying concentrations of copper naphthenate using screened substrate immersion tubes.

FUNGI	Conc. of Cu. Nephth.	1 week		3 weeks		5 weeks		7 weeks	
		US	S	US	S	US	S	US	S
Fusarium solani	0.2%	100	100	100	100	100	100	100	100
	0.4%	100	100	100	100	100	100	100	100
	0.8%	50	50	100	100	100	100	100	100
Sporotrichum pruinosum	0.2%	100	-	100	-	66	-	66	-
	0.4%	100	-	100	-	33	-	66	-
	0.8%	100	-	33	66	66	-	-	-
Penicillium funiculosum	0.2%	-	-	66	66	33	-	-	-
	0.4%	33	-	66		66	66	66	-
	0.8%	66	66	100	66	60	33	33	-
Gliocladium roseum	0.2%	-	-	-	33	-	-	-	-
	0.4%	33	-	-	-	33	-	33	-
	0.8%	-	-	-	-	33	33	33	33
Aspergillus sp.	0.2%	33	33	100	-	100	100	33	-
	0.4%	-	33	-	-	100	100	30	-
	0.8%	-	-	-	-	100	66	-	-
Trichoderma viride	0.2%								
	0.4%								
	0.8%								33

number of isolations made. The results are represented in the Figs 32-35. It will be seen, as stated earlier, that the frequency of occurrence of the fungi was quite high among the unscreened substrates. A maximum number of fungi were recorded from the paper treated with sodium pentachlorophenate. Some of the fungi such as T. viride, G.roseum and C. globosum sometimes appeared on the substrate towards the end of the soil burial period, when a fair amount of fungicide had leached into the soil. This leeching was quite apparent in the case of copper naphthenate, as the paper or the cloth started losing the colour.

The fungicide treated polythene backed cellulose paper was also used in the perfusion technique. In order to allow enough replication, 40 perfusion devices were set up for each concentration of fungicide. Four perfusion devices were sacrificed each time; out of these three cellulose strips were used to estimate weight loss and the fourth strip was aseptically cut into small pieces and inoculated onto 6 E. & P. cellulose agar petridishes. 7 cms of each cellulose strip was cut and oven dried at 110°C for 12 hours for the estimation of weight loss. The treated paper strips were perfused with E.& P. salt solution. The soil used was the same as described previously. It had 19-23% moisture content and its pH was 6.8. The perfusion sets were incubated at 25°C.

The results of the fungi colonising the perfused treated cellulose paper strip are summarised in the Tables 51-52.

In the case of paper treated with varying concentrations of copper naphthenate, the initial colonisation started after 6 days at 0.2% of the fungicide; after 9 days at 0.4% and 12 days at 0.8% of the fungicide (Table 51). The initial

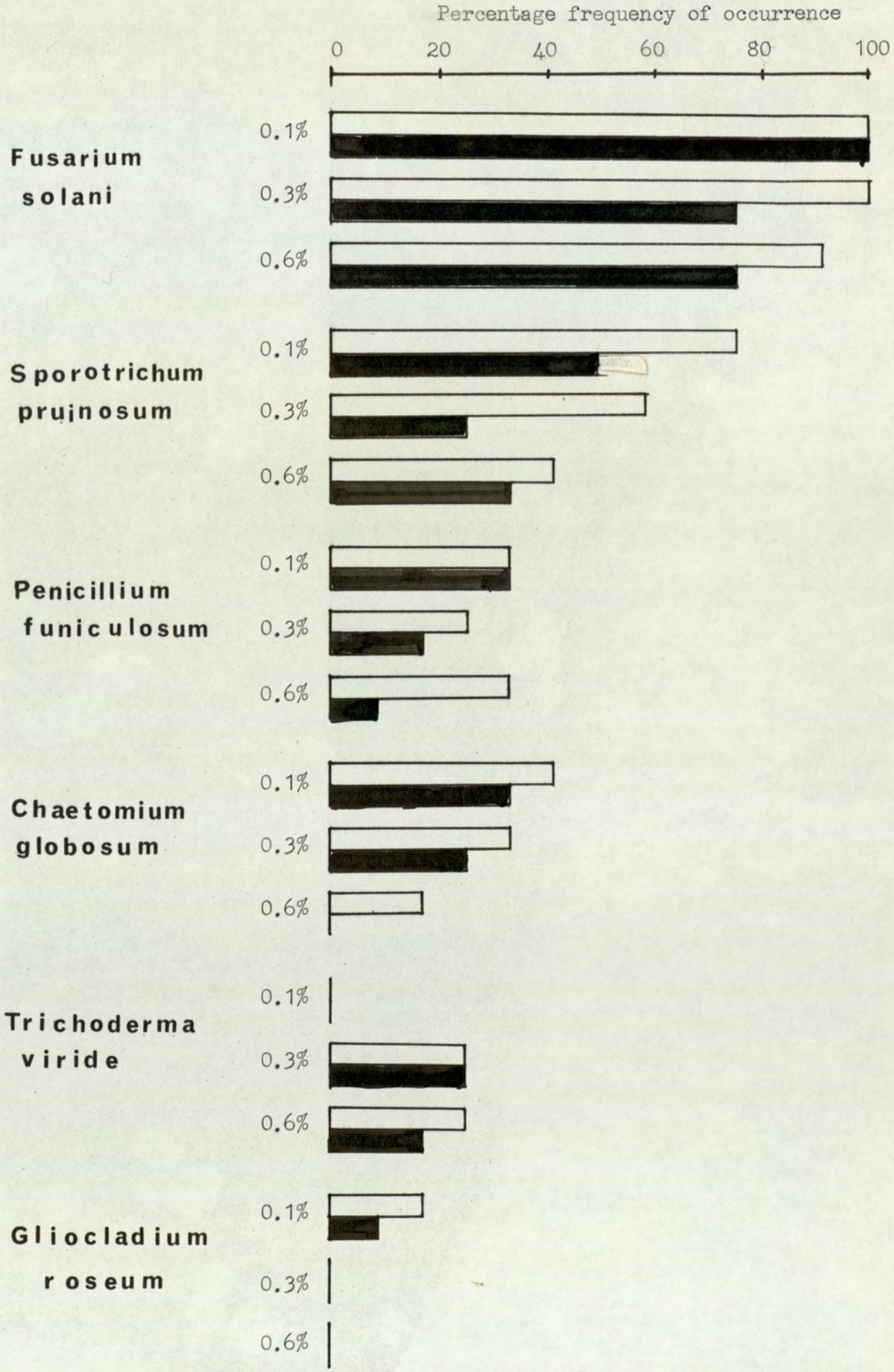


Figure 32. Average percentage frequency of fungi isolated by Eggins and Lloyd's immersion tube technique from the cellulose strip coated with various concentrations of Sodium pentachlorophenate.

Legend: ■ screened □ unscreened

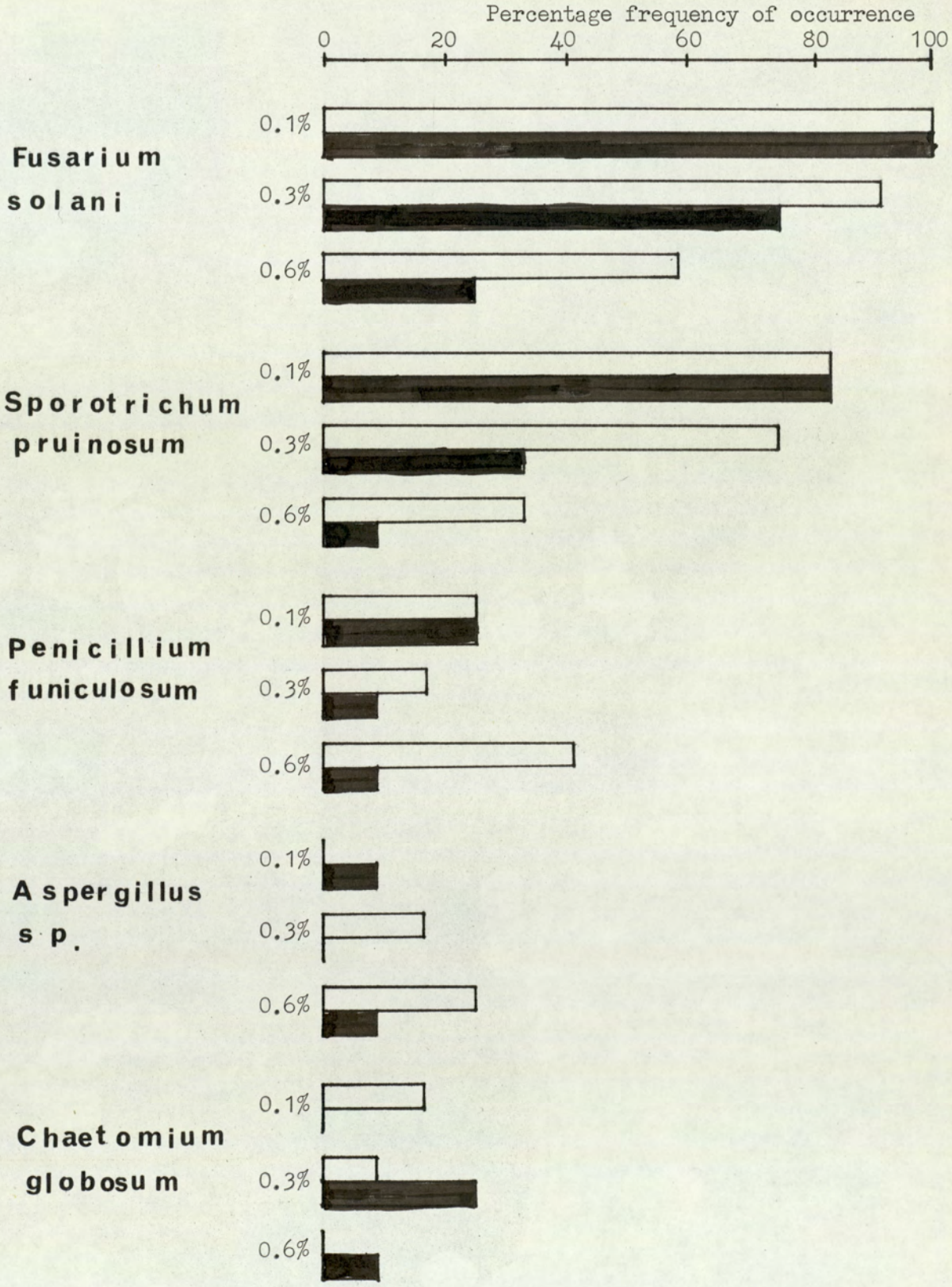


Figure 33: Average percentage frequency of fungi isolated by Eggins and Lloyd's immersion tube technique from the cloth strips coated with various concentrations of sodium pentachlorophenate.

Legend: same as in Fig. 32

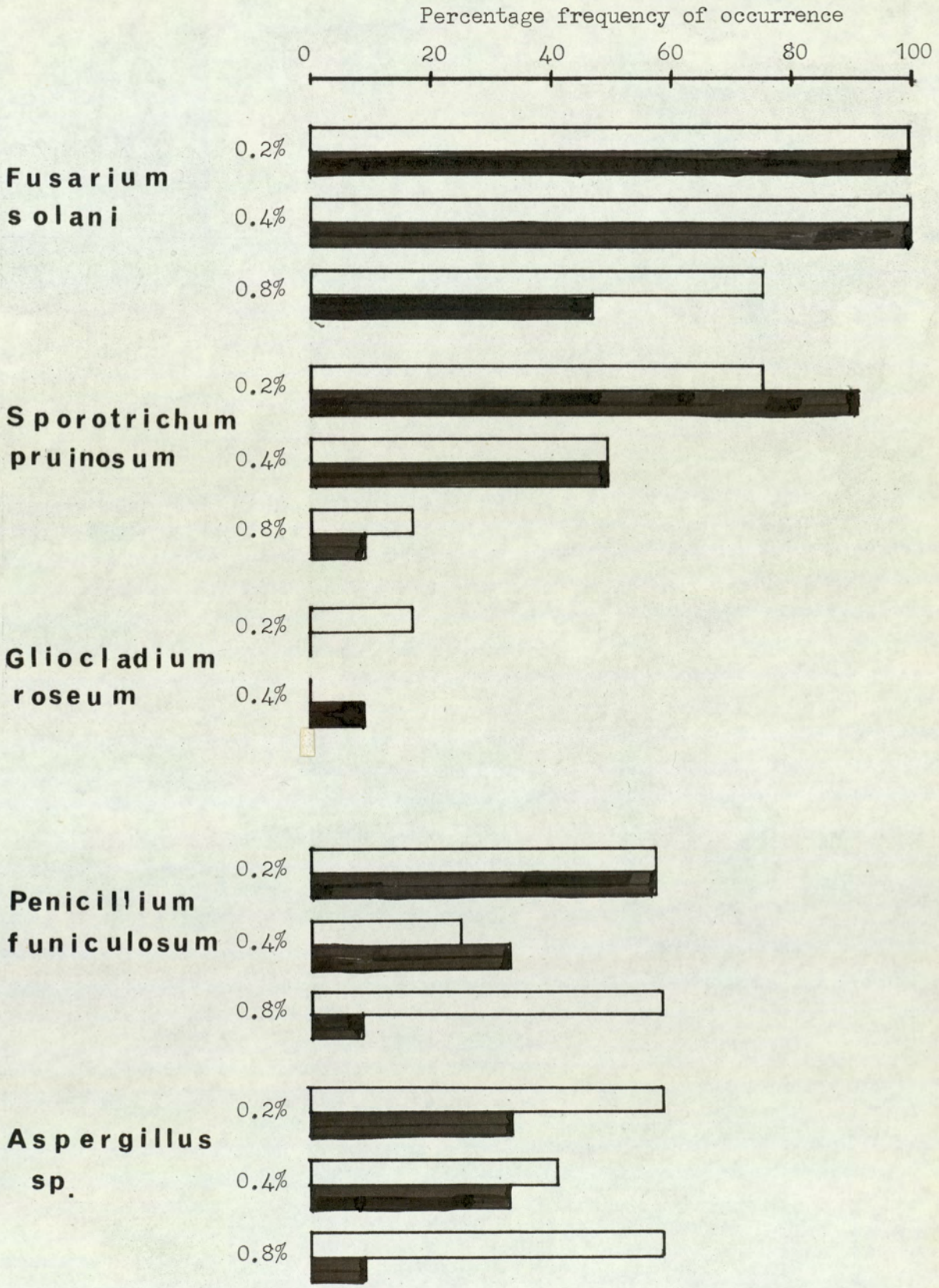


Figure 34: Average percentage frequency of fungi isolated by Eggins and Lloyd's immersion tube technique from the cellulose strip coated with various concentrations of copper naphthenate.

Legend: same as Fig. 32

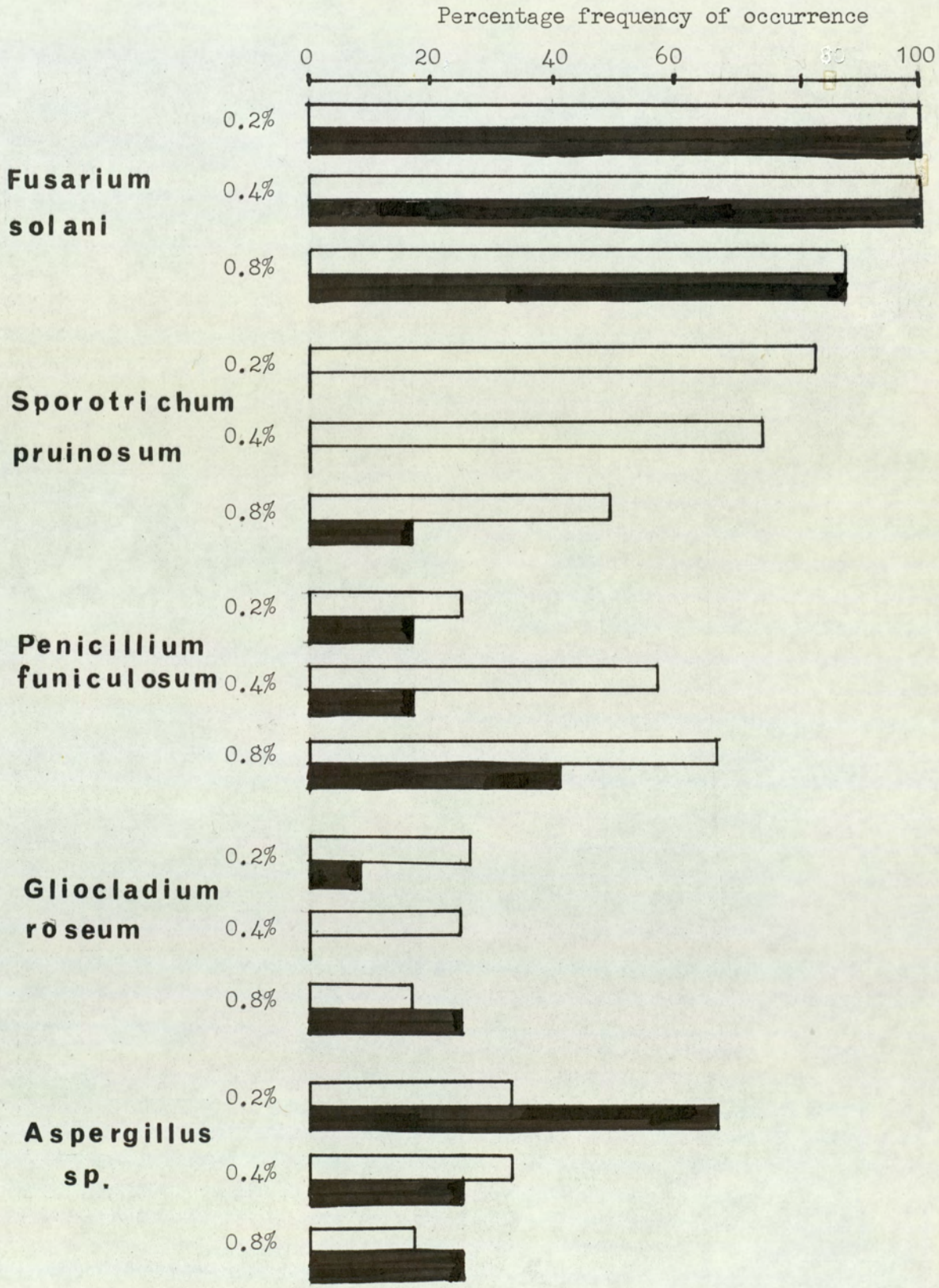


Figure 35: Average percentage frequency of fungi isolated by Eggins and Lloyd's immersion tube technique from the cloth strips coated with various concentrations of copper naphthenate.

Legend: same as fig. 32

Table 51

Percentage frequency of fungi colonizing the cellulose strip coated with different concentrations of copper naphthenate.

FUNGI	Conc. of Cu-Napth.	Days of Incubation									
		3	6	9	12	15	18	21	24	27	30
Fusarium	0.2%		33	16	100	66	16		16		
solani	0.4%			50	100	50	16	33			
	0.8%				33	16		16		16	16
Trichoderma	0.2%		16	50		16	50	83	83	50	
viride	0.4%			16	16	16	33	16			
	0.8%						33	16		16	33
Arthrobotrys	0.2%						83	50		48	83
sp.	0.4%								33	16	16
	0.8%										
Penicillium	0.2%						16				
sp.	0.4%										
	0.8%										
Sordaria	0.2%							33	48		
fimicola	0.4%										
	0.8%										
Paecilomyces	0.2%								48	33	50
elegans	0.4%							16			16
	0.8%										
Zygorhynchus	0.2%										
moelleri	0.4%				50	33	16				
	0.8%										

colonisers, were invariably F. solani and T. viride. Both these species had quite a low frequency of occurrence. After about 18 days, nematodes were found ramifying through the fibres of the cellulose treated with 0.2% and 0.4% fungicide This observation was followed by the isolation of Arthrobotrys sp. which was quite high in its frequency and it remained common till the end of the experiment. Dactylella sp. was also observed on the strip but was not isolated. During the same period (i.e. 18-30 days) Penicillium sp.and Sordaria fimicola were also isolated from the cellulose strips at 0.2% fungicide only. Paecilomyces elegans occurred consistently from 24-30 days at 0.2% fungicide whereas it was occasionally isolated at 0.4% fungicide. In the course of this experiment Zygorhynchus moelleri was also isolated from the paper at 0.4% fungicide, after 12-18 days of incubation. In addition to these species Rhizopus sp.and Gliocladium roseum were also observed on the paper but were never isolated.

The results of the colonisation of the paper strip treated with varying concentrations of sodium pentachlorophenate are summarised in Table 52 .

The number of fungi isolated was quite low and so was their percentage frequency of occurrence. T. viride and F. solani were again found to be more common and had a higher frequency of occurrence than other species. P. funiculosum was isolated after 21 days from the paper treated with 0.1% sodium pentachlorophenate, whereas Aspergillus sp.just made its appearance on all the concentrations of the fungicide after 15 days of the incubation of the perfusion sets at 25^{o}C.

Table 52

Percentage frequency of fungi colonizing the cellulose strip coated with different concentrations of sodium pentachlorophenate.

FUNGI	Na PCP	Days of Incubation									
		3	6	9	12	15	18	21	24	27	30
Trichoderma	0.1%		16	16	32	32		64	32	32	16
viride	0.3%				16	64	32	16			
	0.6%								32	16	16
Fusarium	0.1%				16	16	16	32	16	32	64
solani	0.3%				32	16		16	16		
	0.6%					16	32		16	16	32
Penicillium	0.1%							16		16	
funiculosum	0.3%										
	0.6%										
Aspergillus	0.1%								16		32
sp.	0.3%							16		16	32
	0.6%					16	16				

The results obtained from the paper treated with 2.0% lauryl pentachlorophenol are summarised in the Table 53. The colonisation of the treated cellulose strip started after 6 days when T. viride and F. solani were isolated. Both these species were regularly isolated till the end of the experiment. T. viride reached its highest percentage frequency after 12 days whereas F. solani had the highest frequency of occurrence after 27 days of incubation. F. oxysporum was also isolated after 15 and 18 days. Penicillium funiculosum also made its appearance after 21 and 24 days and A. fumigatus was also occasionally isolated.

The average percentage frequency of occurrence of the fungi isolated from the treated perfused paper has been represented in the Figs 36-37. The maximum frequency of occurrence was reached by T. viride from the paper treated with 0.2% copper naphthenate. At this concentration, Arthrobotrys sp. also had quite a high frequency. It was not isolated from the cellulose paper treated with the other fungicides. The number of fungi isolated from the cellulose paper treated with copper naphthenate was quite high as compared to those of isolated from the paper treated with the other two fungicides. The number and frequency of occurrence of the fungi was extremely low at the maximum concentration of the fungicide used. Only 2 species (F. solani and T. viride) were isolated from the paper treated with 0.6% sodium pentachlorophenate and 0.8% copper naphthenate. In general T. viride and F. solani can be regarded as the species tolerant to some extent to the lower concentrations of the fungicides used. The majority of the remaining species either occurred very occasionally or were

Table 53

Percentage frequency of fungi colonizing the cellulose strip coated with different concentrations of lauryl pentachlorophenol.

FUNGI	Days of Incubation									
	3	6	9	12	15	18	21	24	27	30
Trichoderma viride		16		66	16	32	32	66	16	32
Fusarium solani		16	16	32	16	16	32	32	66	66
Fusarium oxysporum					32	32				
Aspergillus fumigatus			16			16		16		
Penicillium sp.							16	32		

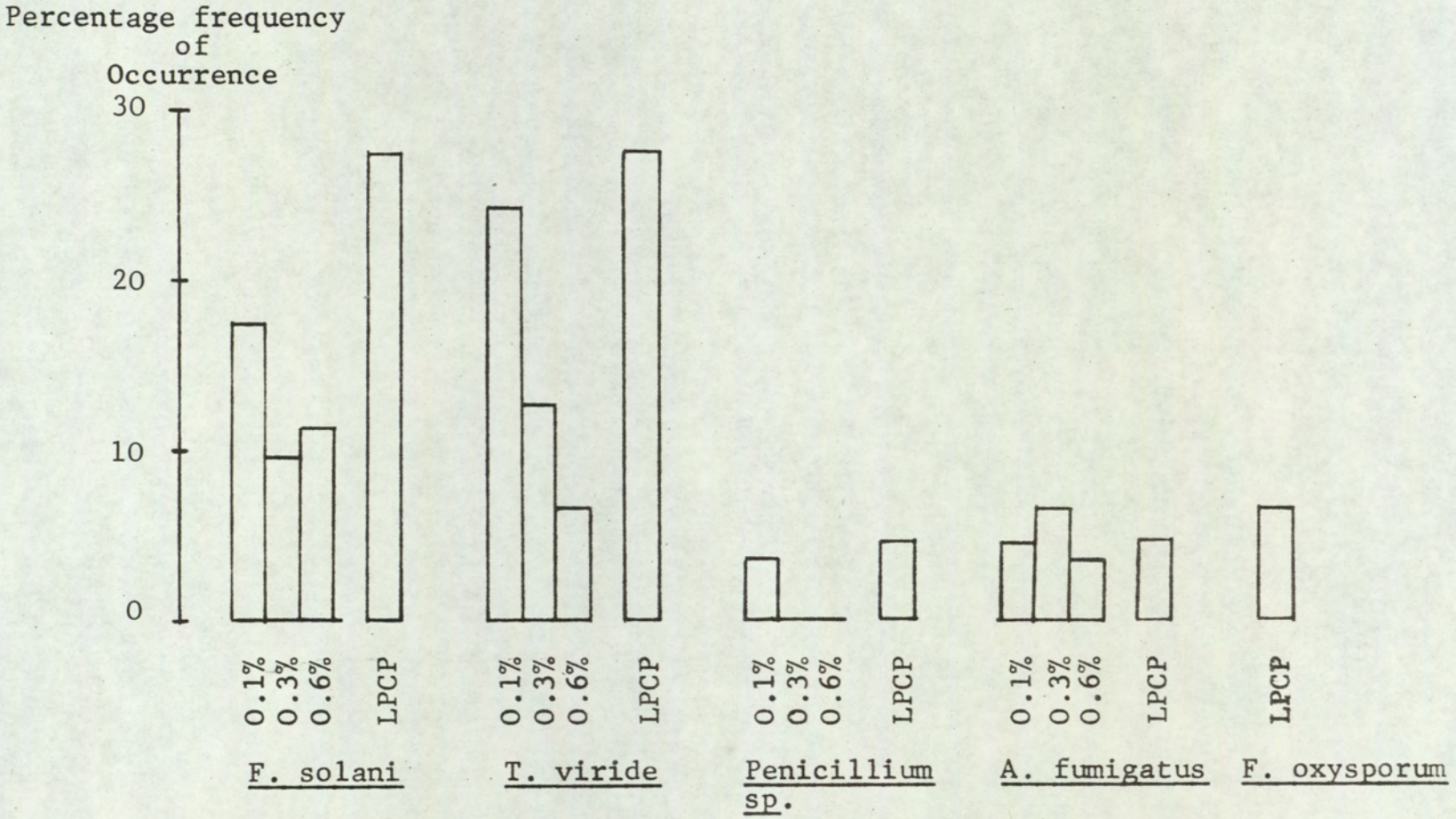


Figure 36: Average percentage frequency of occurrence of fungi isolated from the cellulose strip coated with different concentrations of sodium pentachlorophenol and lauryl pentachloro phenol.

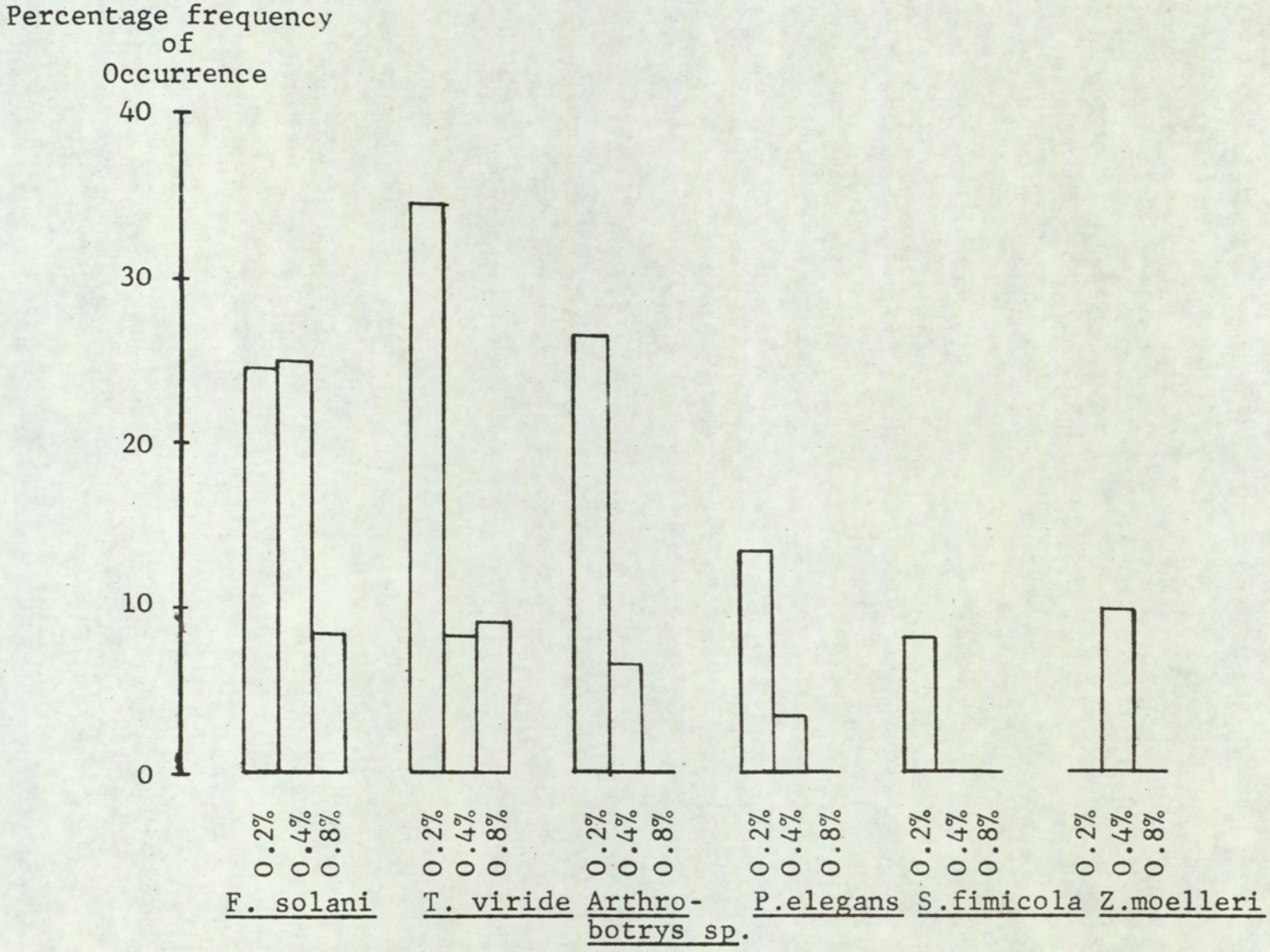


Figure 37: Average percentage frequency of fungi isolated from the cellulose strip coated with different concentrations of copper naphthenate.

able to colonise when enough fungicide had been leached out. Nematodes also seem to play a role in the cellulose deterioration in the soil, especially in the case of copper naphthenate where these were invariably followed by the isolation of nematode catching fungi such as Arthrobotrys sp and Dactylella sp.

The percentage weight loss of the treated cellulose paper is summarised in the Tables 54-60. The maximum percentage weight loss was estimated in the case of 0.2% copper nephthenate. The weight loss increased steadily over 30 days of incubation and reached a maximum of 36.0%. The percentage weight loss in the case of 0.4% and 0.8% copper naphthenate was much smaller. The maximum weight loss recorded was 5.6% and 4.6% respectively after 30 days of incubation.

Similar results were also obtained from the cellulose paper treated with various concentrations of sodium pentachloraphenate. The maximum weight loss of 8.7% was recorded in the case of 0.1% NaPCP after 30 days of incubation. The weight loss estimated for 0.3% and 0.6% of NaPCP was relatively smaller as the maximum percentage weight loss recorded was 7.1% and 5.7% respectively after 30 days of incubation.

The use of the perfusion technique as a test method for the effectiveness of these fungicides, has given quite encouraging results. The number of fungi decreased with the increase of the concentration of the fungicide and so was the percentage weight loss. The use of this technique has thus enabled us to comparatively review the effectiveness of a particular biocide at a particular concentration.

There has been very little work done on the detoxification

Table 54: Percentage weight loss of the cellulose strip coated with 0.2% copper naphthenate and perfused with E.&P. salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
12	2.4	1.2	1.9	1.8	0.4
15	2.8	2.6	2.1	2.5	0.8
18	3.8	4.6	6.0	4.8	0.9
21	6.7	5.1	6.9	6.2	0.8
24	14.1	16.1	11.2	13.8	2.0
27	25.0	28.1	22.7	25.2	2.2
30	31.0	39.3	36.0	35.4	3.4

Table 55: Percentage weight loss of the cellulose strip coated with 0.4% copper naphthenate and perfused with E.&P. salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
15	2.9	1.4	1.9	2.1	0.6
18	1.7	2.9	2.1	2.2	0.4
21	1.2	3.1	2.7	2.3	0.8
24	2.7	4.3	3.9	3.6	0.6
27	2.9	4.9	5.5	4.4	1.1
30	4.1	5.8	6.9	5.6	1.1

Table 56

Percentage weight loss of the cellulose strip coated with 0.8% copper naphthenate and perfused with E & P salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
18	2.9	1.8	2.5	2.4	0.4
21	4.9	3.9	5.1	4.6	0.5
24	5.2	4.3	4.8	4.8	0.3
27	4.0	4.6	4.8	4.4	0.3
30	4.1	5.0	4.7	4.6	0.3

Table 57

Percentage weight loss of the cellulose strip coated with 0.1% sodium pentachlorophenate and perfused with E & P salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
15	1.3	0.9	1.5	1.2	0.2
18	2.1	1.1	1.0	1.4	0.4
21	4.1	4.9	5.8	4.9	0.6
24	3.1	6.9	4.1	4.7	1.5
27	8.1	7.9	9.1	8.3	0.5
30	9.1	6.8	10.3	8.7	1.4

Table 58: Percentage weight loss of the cellulose strip coated with 0.3% sodium pentachlorophenate and perfused with E.&P. salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
18	0.9	0.5	1.6	1.0	0.4
21	2.1	0.5	1.0	1.2	0.6
24	2.6	1.8	1.3	1.9	0.5
27	4.3	4.1	3.2	3.9	0.4
30	6.7	7.1	7.4	7.1	0.2

Table 59: Percentage weight loss of the cellulose strip coated with 0.6% sodium pentachlorophenate and perfused with E.&P. salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
18	1.1	1.8	3.1	2.0	0.8
21	1.0	1.3	1.9	1.4	0.3
24	3.3	2.8	2.1	2.7	0.4
27	3.1	4.3	1.9	3.1	0.9
30	5.3	5.8	6.1	5.7	0.3

Table 60: Percentage weight loss of the cellulose strip coated with 2.0% lauryl pentachlorophenate and perfused with E.&P. salt solution.

Days of Incubation	Percentage Weight Loss			Mean	S.D.
9	0.5	1.1	1.9	1.2	0.5
12	1.1	0.5	0.9	0.8	0.2
15	2.3	3.1	1.2	2.2	0.7
18	3.3	4.3	2.6	3.4	0.6
21	4.7	5.9	5.6	5.4	0.5
24	6.5	7.1	4.8	6.1	0.9
27	8.3	10.2	9.1	9.2	0.7
30	11.4	9.8	9.7	10.3	0.7

of different fungicides. Some workers have studied the detoxification of phenolic biocides (Cserjesi, 1967 & Loos et al 1967), but it is always preferable to devise test techniques which will also enable the study of detoxification of biocides and thus the information obtained could be employed to improve the biocide or will help in the formulation of new biocides. The importance of such dual purpose techniques was stressed by Hueck & Brijn, (1965). The perfusion technique here used as a test method can also be employed for detoxification studies. This can be done by simply cutting off the evaporating end of the glass fibre sleeving of the perfusion system after incubation and perfusion and then extracting and analysing the residue which contains the breakdown products of biocide coated cellulose. Such studies have been undertaken by Allsopp et al (1970).

The role of ecology related to biodeterioration and the importance of techniques used to study it has been stressed by Eggins (1968). Many workers in mycology have contributed to the understanding of fungal ecology but this knowledge has seldom been related to the problem of overcoming biodeterioration. The scope and the limits of accuracy of available techniques (Garrett, 1955) for studying this ecology from a non applied point of view has, furthermore, made information thus obtained somewhat remote from the problem of biodeterioration.

It is generally agreed that in order to have a clear understanding of the problems of soil fungal ecology, three major factors should be studied;

1. differentation between the active and dormant mycoflora,
2. estimating the activity of the fungi, and,
3. ascertaining some patterns of succession of various fungi on the substrate.

The perfusion technique described in this thesis is therefore, a continuation to the overall understanding of the ecology of cellulose decomposition in soil and also in particular is a contribution to our understanding in preventing the biodeterioration of cellulosic materials. This technique has successfully been employed to study the above mentioned factors relating to the ecology of soil fungi. The screening of a model cellulosic substrate ensures that only actively growing fungi can colonize the substrate. The activity of fungi colonizing the cellulosic substrate can be estimated by measuring the weight loss of the cellulosic substrate as it can be handled even when highly deteriorated. The successional patterns of these fungi colonizing the substrate can also be studied with the help of this technique. The continuous enrichments and perfusion of the cellulosic substrate ensures constant microenvironmental conditions. In this way the staling of pure culture media due to the presence of autoxic substances (Waksman, 1937) and the problem of non suitability of a buried substrate for colonization before its depletion as experienced by Tribe (1957) and discussed by Robinson (1969) can be overcome by the continuous perfusion of the cellulosic substrate with fresh nutrients. Thus the successional patterns of fungi colonizing the substrate could be studied under constant and controlled microenvironmental conditions while altering one parameter at a time.

The conditions thus created with the help of this technique are not wholly similar to those in Nature. But in order to have an insight into the complexities of soil fungal ecology, it is essential to accumulate knowledge

about all the ecological factors in a systematic fashion by studying one factor at a time and then correlating the results.

The forms of cellulose (like cellophane) previously used by various workers (Tribe, 1957; Went and de Jong, 1966) do not compare very closely with the form of cellulose occurring in Nature. Thus cellophane, a highly regenerated form of cellulose, although it has advantages of transparancy and ease of staining, cannot be used for the study of precise ecological conditions because of the difficulty in its handling when deteriorated, the difficulty of isolating fungi from it and also because of its artificial form. The model heat rolled polythene backed chromatography cellulose paper used in the perfusion technique, resembles more closely native cellulose and stands out because of its ease of handling even when completely deteriorated. Moreover, this cellulosic substrate enables the easy isolation of active cellulolytic fungi due to the fibre glass cloth screening.

Most of the work presented in this thesis revolves around the use of this perfusion technique. It has been successfully used to study the effect of pH, temperature, different nitrogen sources and glucose on the colonization of the cellulosic substrate buried in soil, by fungi.

In comparing the results regarding pH, with those of other workers, it should be borne in mind that by employing the perfusion technique, the pH of the substrate is changed whereas previously various workers have noted the similar effect by studying the decomposition of cellulose in soils of different reactions (Tribe, 1960; Went and de Jong, 1966; Pugh et al, 1963).

The general conclusion reached by these workers has been that fungi have a wide range of pH tolerance and minor changes in the pH do not effect their growth (Park, 1968). The results presented in this thesis also confirm this generalization as it was found that most of the fungi were isolated from pH 4.0 - 8.0 but their optimum pH could also be determined by comparing the percentage frequency of occurrences at different pH values. Cochrane (1958) and Siu (1951) have given lists of micro-organisms whose pH optima have been studied. These lists show that the majority of the cellulolytic fungi studied prefer an acidic pH. This has been reported earlier by many workers (Dubos, 1928; Jensen, 1931). These studies have been carried out with individual species and therefore cannot be easily compared with the results in this thesis as every fungus has been studied during the present investigations as a part of a population in the soil and not in isolation from it. The importance of this has been previously stressed by many fungal ecologists (Chesters, 1949; Garrett, 1955; Burges, 1958).

The colonization of the model cellulose substrate by fungi can also be compared to those of the plant remains above or in the soil which have been reviewed by Hudson (1968). The results presented in this thesis differ from these studies as the substrate used here is pure cellulose whereas plant remains are composed of other compounds in addition to cellulose. Despite such differences, a successional pattern of the fungi colonizing this pure

cellulosic substrate can be observed. This succession is somewhat different from that proposed by Garrett (1963) as most of the fungi involved are cellulolytic and there is no effective initial Phycomycete phase. The role of "Secondary fungi" has also been furthermore elucidated in these studies. It has been found that "secondary fungi" may not necessarily be saprophytic sugar fungi as initially proposed by Garrett (1963) but may be cellulolytic fungi which are activated by a certain concentration of metabolites of initial colonizers of the cellulosic substrate. This phenomenon has been demonstrated by the colonization results obtained at 25° C., 35° C. and 50° C. At all temperatures the delineation between early colonizer and late developers could be easily made. The early colonizers when tested for their cellulolytic ability, produced maximum clearing of the cellulose agar. Trichoderma viride and Fusarium solani were the early colonizers and they produced maximum clearing at 25° C. Similarly Aspergillus fumigatus and Chaetomium thermophile were the early colonizers at 35° C. and 50° C. respectively. Both fungi had a maximum depth of clearing among the fungi isolated at that particular temperature.

Therefore, on the basis of information presented in this thesis, it can be assumed that the fungi having maximum frequency of occurrence have a relatively similar cellulolytic activity as determined by the depth of clearing of the cellulose. The reverse generalization, that fungi having high cellulolytic activity have a high frequency of occurrence does not hold true, as it has been stated by

many workers and confirmed in this thesis that the behaviour of a species in pure culture is no index of its activity in the soil. This phenomenon can be explained by the insignificant role played by C. globosum in the colonization of the model cellulosic substrate at 25° C. as compared to the production of a relatively large depth of clearing. A. fumigatus can also be included in the same category at 25° C. but it was observed to be an active colonizer at 35° C. and also produced a maximum depth of clearing of the cellulose agar. Similarly Chaetomium thermophile was the early colonizer at 50° C. and had a maximum frequency of occurrence; the depth of clearing of cellulose produced by this species was also quite high. On the other hand, S. thermophile produced as much depth of clearing as C. thermophile but its frequency of occurrence was extremely low.

Therefore, it can be concluded, that we can only regard a fungal species as actively cellulolytic under known environments in the soil if it has a comparable frequency of occurrence and cellulolytic ability, the latter being estimated in this thesis by measuring the depth of clearing of the cellulose agar columns.

A similar behaviour of fungi was also observed during the other nutritional studies. The perfusion of different nitrogen sources effected the successional pattern of fungi colonizing the cellulose strip in two ways; firstly, the percentage frequency of occurrence was affected and, secondly, the time taken by a species to colonize the cellulose strip may also be affected. These effects have also been demonstrated

by the perfusion of different concentrations of glucose onto the cellulose strip. The optimal nutritional requirements of the fungi colonizing the cellulose strip could be studied by comparatively reviewing their percentage frequency of occurrences. In majority of the cases, the percentage frequency of occurrence of a species is supported by a comparably depth of clearing, thus confirming its optimum activity at a particular nitrogen or additional carbon source. This phenomenon has been demonstrated by nearly all the fungi isolated, and in particular, G. roseum, P. funiculosum, T. viride and H. grisea, as they had the same nitrogen source producing a maximum frequency of occurrence and depth of clearing of the cellulose. Similarly, the perfusion of different concentrations of glucose effected the percentage frequency of occurrence of certain fungi and their role in the succession onto the cellulose strip. The early appearance of G. roseum and P. elegans at 0.25% glucose, which were previously observed when the cellulose strip was nearly deteriorated, suggests that these fungi might be activated by simple sugars perfused, which are normally available because of the cellulolytic activity of early colonizers. Similar variation also occurred during the perfusion of E & P salt solution with higher concentration of glucose (0.5% and 1.0%).

The effect of different environmental factors like pH, temperature, nitrogen sources and glucose nutrition, on the biodeterioration of the cellulose strip has also been discussed in the previous chapters. Reviewing these results, it becomes clear that the overall biodeterioration as

estimated by the weight loss method has been very little affected by varying pH values. The most remarkable effect on the extent of deterioration has been found to be due to temperature. Out of the three temperatures (25° C., 35° C. and 50° C.) investigated least deterioration took place at 35° C. and the most at 50° C. The contention that thermophilic fungi are no more cellulolytic than mesophiles (Reese, 1946) has certainly been proved wrong during the present investigation. The rate of deterioration at 50° C. has been found to be much faster than that at 25° C. During these investigations it was also found that the weight loss of the cellulose strip produced by one known fungus was much smaller than that produced by a fungal population in the soil. This has been explained by the maximum weight loss of 15 - 20% produced by T. viride in 30 days at 25° C., whereas at the same temperature and incubation period 56% weight loss of the cellulose strip was produced by the fungi present in soil.

The information thus obtained can be very helpful in understanding and overcoming biodeterioration. It can help in the devising of different test methods for biocides to be used under different environmental conditions. As previously discussed by Eggins (1968) such information can be helpful in predicting and assessing the extent of biodeterioration under a known environmental conditions and thus it can serve as a basis for the development of test techniques and biocide design. In this connection the studies on association and interaction between fungi involved in biodeterioration can also be of great help.

The effect of interaction between different fungi on their cellulolytic ability has been studied by inoculating two known fungal species, on top of the opaque cellulose agar column. There were many examples of inhibition of the cellulolytic activity of both the fungi when grown together, whereas there was only one instance of synergism, which was observed between F. solani and F. moniliforme. Even in this case the increase in depth of clearing of the cellulose was very small. The fungi producing the most inhibition comprised of C. fuckeli, G. roseum and Papulaspora sp. only. T. viride and C. globosum are confirmed cellulolytic fungi and are also reported to produce antibiotics as well (Bilai, 1963). Similarly, G. roseum has been reported to produce three antibiotics which are mildly active against bacteria and fungi. Barnett and Lilly (1962) while investigating the destructive mycoparasitic nature of G. roseum against different fungi, reported that the killing of the host fungi was not due to any highly diffusible antibiotic since only the cells contacted by the parasitic hyphae of G. roseum were killed. Warren (1948) and Butler (1957) working with Rhizoctonia solani seemed to have found similar results. Therefore, in order to ascertain whether inhibition of any activity of the fungi is due to any diffusible metabolic product (which may be an antibiotic) or it is due to the physical contact between two fungi, a new technique has been used to study the effect of diffusible metabolites of one fungus onto the linear growth of another. This technique, as described in this thesis, is also based on the perfusion principle.

The results obtained by this technique have shown the effect produced by the diffusible metabolites of G. roseum, T. viride and F. solani on the linear growth of some cellulolytic fungi. The importance of such studies is stressed by the fact that tolerance to antibiotics is an important character in ecological success and it has been demonstrated in a number of review papers (Garrett, 1950; Hawker, 1957; Park, 1960). Garrett (1956) has also listed this character as one of the four likely to favour high competitive saprophytic ability.

There are also very few examples of synergism among the results obtained with the interaction perfusion technique. The linear growth of Arthrobotrys sp. was enhanced by 9.8 ~~29.0~~% when perfused with the metabolites of G. roseum whereas all other fungi tested, including T. viride were inhibited to varying degrees. The diffusible metabolites of T. viride seemed to be more effective in inhibiting the linear growth of the cellulolytic fungi. The inhibition of C. globosum which is also a confirmed soft rot fungus of wood, was also estimated to be 69.3%. T. virde has been reported to produce a fungistatic metabolic product, namely Gliotoxin, which is also moderately toxic to a wide range of bacteria and actinomycetes (Brian and Hemming, 1945). This fungus has also been previously studied as a possible antagonist of wood inhabiting Hymenomycetes (Hashioka, et al 1961). Shields (1963) and Shields and Atwell (1968) studied the effect of T. viride on the decay of birch logs and possible control of the decay by this fungus. They recommended the use of antagonistic fungi to reduce discoloration and decay in

land stored logs as an alternate method of controlling deterioration where other means of protection are not possible. The diffusible metabolites of T. viride inhibited the linear growth of F. solani by 46.0%. Chi (1960) reported some interaction between T. viride and three species of Fusarium including F. solani. His observations were mainly restricted to mycoparasitism but he reported inhibition of F. solani in gliotomium fermentation medium. Arthrobotrys sp. which increased the role of its linear growth with G. roseum, was inhibited to a very small extent (5.4%) by T. viride. But the linear growth of Arthrobotrys sp. was again enhanced by 30.3% when perfused with the metabolites of F. solani. Arthrobotrys sp. seems to be quite tolerant to the metabolic products of the fungi tested. There is information available on the nematophagous activity of this species (Duddington, 1955) but no work on interaction has been reported. F. solani was also able to enhance the linear growth of C. fuckeli by 3.4% only. This species (C. fuckeli) also produced increase in the linear growth of F. solani by 6.6% when its diffusible metabolites were perfused. But the diffusible metabolites of C. fuckeli produced 26.6% inhibition of the linear growth of T. viride. On the other hand, diffusible metabolites of C. globosum produced 46.6% inhibition of the linear growth of T. viride. Broadbent (1968) compiled a list of fungi producing antibiotics. He has quoted four species of Chaetomium (not C. globosum) as producing antibiotics against fungi and bacteria. He also quotes Voros (1958) for C. fuckeli, also producing antibiotics active against fungi.

The results obtained by the interaction perfusion technique have increased our knowledge concerning interaction between different fungi and it has also elucidated the role of diffusible metabolites as compared to non diffusible by-products. The majority of techniques used to study the antagonistic effects, use solid media and involve inoculation of test organisms on the same plate, followed by observation and measurement of mutual effects of their growth. Such studies have been reviewed in detail by Wood and Tveit (1955). These studies do not differentiate between the antibiotics or any antagonistic compound produced at the point of contact of two test organisms and any genuine toxic or non toxic metabolic product produced by different organisms. Moreover, measurement of the growth of the test organism on the agar plate is very difficult to standardise as all the fungi do not grow in exact circles. In view of these shortcomings in the techniques generally used, the interaction perfusion technique has clear advantages over the previous techniques. It takes into account only the effect of diffusible metabolites of one fungus onto the other; the measurement of linear growth is more absolute and simple than measuring the diameters of a colony which may or may not be growing in circles.

This technique for studying interactions can easily be employed for screening fungi for the production of diffusible antifungal metabolites. Interaction between different sugar fungi can also be studied by simply adding simple sugars to the perfusing solution and thus using the cellulose paper strip as a supporting and conducting substrate.

In order to successfully contribute towards the problem of overcoming biodeterioration, it is essential to study to the fullest extent the different ecological factors effecting the biodeteriogens. Without such a knowledge, there might be pitfalls in biodeterioration control development. In this connection the information concerning the interaction between different fungi involved in cellulose deterioration can be effectively used in selecting the test organisms for biocide development.

The use of the perfusion technique, selective for thermophilic cellulolytic actinomycets has once again elucidated the importance of isolating techniques. The role of actinomycetes in biodeterioration is normally ignored due to the difficulty in their isolation and their identification. This discrepancy in our knowledge is responsible for the fact that actinomyces are normally neither taken into account while formulating biocides nor these are used as test organisms. Their role in biodeterioration has recently been reviewed by Williams (1966).

The perfusion technique has shown promising results for the selective isolation of thermophilic cellulolytic actinomycetes by perfusing antifungal and antibacterial antibiotics along with other nutrients, onto the cellulose strip. Among the antifungal antibiotics used, Pimafucin has been found to be the most effective at thermophilic temperatures.

The widespread fungistasis in soil whereby the spores of soil saprophytes having all the normal nutrient requirements and moisture, fail to germinate (Dobbs and Hinson, 1953)

has also been observed by many other workers (Caldwell, 1958; Chinn, 1953; Chinn and Ledingham, 1957; Dobbs, Hinson and Bywater, 1957; Jackson, 1957, 1958; Park, 1955). Park (1960) while giving an explanation of this phenomenon compared it with staling in pure cultures (Robinson, 1969). As in pure cultures, where the fungus is inhibited by its autotoxic by-products and not by depletion of nutrients (Boyle, 1924; Pratt, 1924; Waksman and Foster, 1937), so similarly a soil can be viewed as a medium which has been inoculated with complex mixed cultures of fungi and incubated for a long time under varying cultural conditions. Therefore, any soil at a particular time will represent the end product of immense biological activity which had been going on in the soil for presumably a very long period. The soil solution in water has also shown that the nutrients present are enough to keep the fungal population going (Park, 1960). In this respect fungistasis presented by inhibition of spore germination in the soil has been explained on the basis of toxic metabolic compounds (antibiotics) produced by the fungal activity in the soil. Brian (1960) did not agree with these explanations and noted a lack of definite information regarding the biological and specifically microbial origin of fungistasis.

The results presented in this thesis regarding the germination of spores and the colonization of a cellulosic substrate by a number of fungal spores were also obtained by using an alteration of the perfusion technique (Chapter 7). Instead of soil, a suspension containing an equal number of spores of ten cellulolytic fungi was inoculated. The

nutrients were supplied by perfusion. The germination of T. viride spores was very rapid and it also had nearly one hundred percent frequency of isolation throughout the incubation and perfusion period. Similarly G. roseum and P. elegans germinated quite early but had a comparatively low frequency of isolation. Surprisingly the rate of germination of F. solani was extremely low and it was only occasionally isolated and it did not seem to have been able to penetrate ten layers of fibre glass cloth covering the cellulose strip.

The successional patterns depicted by these observations make T. viride, G. roseum and P. elegans early colonizers as compared to the very late appearance of G. roseum and P. elegans on the cellulose substrate in soil. Among the late developers were Arthrobotrys sp., Penicillium sp. and S. fimicola. C. globosum and H. grisea were only isolated twice and had a very low frequency of occurrence.

Any difference between this successional patterns and that obtained with soil, must be due to the complex microenvironmental condition in the soil. Moreover, these results also help to explain the different factors contributing to competitive saprophytic ability. In this case the factors concerning the rapid germination of spores and a high rate of hyphal growth has been elucidated. But all these factors cannot be viewed in isolation as Garrett (1950) defined competitive saprophytic ability as the total of four factors which have been mentioned earlier in this thesis.

Another factor contributing to the saprophytic ability is good enzyme production and extensive substrate utilization. This factor has been demonstrated experimentally by using another alteration of the perfusion technique. The enzyme production and extent of utilization of cellulose was estimated by measuring the loss of weight of different layers of a wad of cellulose strips. Such studies also demonstrated the ability of certain fungi to penetrate a thick cellulosic substrate and the production of cellulose at different depths. Trichoderma viride and Fusarium solani produced maximum weight loss and the variation in the extent of weight loss at different depths (i.e. layers of the wad of five cellulose strips) was quite small. As the fungal mycelium of these two species was found to penetrate the whole thickness of the wad of cellulose strips in two to four days the diffusion of the enzyme away from the hyphae was not considered as an important factor in this case. This suggests that the hyphae of these species are capable of substrate penetration, good enzyme production and substrate utilization. In the case of Papulospora sp., although the mycelium was able to penetrate the wad of cellulose strip, only the topmost layer of the wad was utilized effectively as the weight loss produced on the top layer was much more than that produced in the subsequent layers. A similar situation was observed in H. grisea and C. globosum.

The use of the perfusion technique, with its different modifications has resulted in the accumulation of quite useful information regarding the ecology of fungi colonizing

cellulose. The perfusion technique has also been used as a test method for biocides as it can keep the microenvironment constant; a condition strongly recommended by Hueck van der Plas (1965). Different biocides were tested at 25°C. and quite encouraging results have been obtained. The advantage of the perfusion technique as a test method, apart from controlling the environmental conditions, is the ease of testing a biocide at different temperatures and other environmental conditions. Moreover, this technique can effectively help in detoxification studies and thus play a vital role in the development of new biocides.

In conclusion, it can be said that the perfusion technique and its variants have enabled us to study a wide range of ecological problems concerning cellulose biodeterioration. Garrett (1951), while discussing substrate relationships remarked " Isolation of soil fungi may have appeared a deceptively simple problem to the early students of soil mycology, but increasing knowledge of the complex and delicately balanced microbiological equilibrium of the soil has now explained why slight alterations in isolation techniques have frequently produced remarkable changes in the results obtained". In addition to the isolation techniques, there is a general lack of standardised techniques for studying various ecological problems. Some new techniques have been described in this thesis, which it is hoped, will go a long way in helping to understand the "complex and delicately balanced microbiological equilibrium of the soil", whilst providing valuable information which can be used to overcome biodeterioration and help to formulate new biocides.

REFERENCES

ADAMEK, L. (1965)
Submerse cultivation of the fungus Metarrhizum anisopliae (Metsch.)
Folia Microbiologica, 10 255-257.

AGATE, A. D. and BHAT, J. V. (1963)
A method for the preferential isolation of actinomycetes.
Antonie van Leeuwenboek, 29 297-304.

AGRE, N. S. (1964)
Method of isolating and culturing thermophilic actinomycetes.
Mikrobiologiya, 33 913-917.

ALLSOPP, D., HUECK, H. J., LLOYD, A. O. and EGGINS, H. O. W. (1970)
Evidence of the hydrolysis of pentachlorophenyl laurate by soil microorganisms.
To be published.

APINIS, A. E. (1963)
Occurrence of thermophilous microfungi in certain alluvial soil near Nottingham.
Nova Hedwigia, Zeitschr, Kryptogamenk, 5 57-58.

ARAKAWA, S. (1934)
Tottori Nogaku Kwaiho, 5 27.

ASCHAN, K. and NORKRANS, B. (1953)
A study in the cellulolytic variation for wild types and mutants of Collybia velutipes.
Physiologia Pl. 6 564-583.

A.S.T.M. (1957)
Supplement to tbe book of A.S.T.M. standards including tentatives. Part 7. Methods of test for the resistance of textile materials to microorganisms.
(D 684-57, pp. 47-53).

AYTOUN, R. S. C. (1953)
The genus Trichoderma: its relationship with Armillaria mellea (vahl. ex. Fries) Ond. and Polyporus schweinitzii Fr. together with preliminary observations on its ecology in wood lands.
Trans. Proc. Bot. Soc. Edinb. 36 99-114.

BALSTON, J. N. and TALBOT, B. C. (1952)
A guide to filter paper and cellulose chromatography.
Reeve Angel, Maidstone, W. & R. Balston.

BARNETT, H. L. and LILLY, V. G. (1962)
A destructive mycoparasite, Gliocladium roseum.
Mycologia, 54 72-77.

BASU, S. N. (1948)
Fungal decomposition of jute fibre and cellulose. Part II.
The effect of some environmental factors.
J. Text. Inst. 39 T237-T248.

BASU, S. N., BHATTACHARYA, A. and BOSE, R. G. (1950)
A multiple culture technique for testing rot resistance,
particularly suited to jute materials.
J. Text. Inst. 41 465-488.

BERGEY, D. H., BREED, R.S., MURRAY, E.G.D. and HITCHENS, P.A.
Mannual of determinative bacteriology. (1939)
5th ed. Williams and Wilkins Co., Baltimore.

BERTOLET, E. C. (1943)
The finishing of army ducks with special reference to
mildew proofing.
Am. Dyestuff Rept. 32 214-219.

BILAI, V. I. (1963)
Antibiotic producing microscopic fungi.
Amsterdam, Elsevier Pub. Co.

BLOCK, S. S. (1949)
Fungicide-treated cotton fabric. Outdoor exposure and
laboratory test.
Ind. Eng. Chem. 41 1783-1789.

BLOCK, S. S. (1967)
Application and use of fungicides as industrial preservatives.
In Fungicides, Vol. 1, pp. 379-423.
Ed. D. C. Torgeson, Academic Press, New York, London.

BOSE, R. G. (1963)
A modified cellulosic medium for the isolation of
cellulolytic fungi from infected materials and soils.
Nature, Lond. 198 505-506.

BOYLE, C. (1924)
Studies in the physiology of parasitism: The growth reactions of certain fungi to their staling products.
Ann. Bot. 38 113-135.

BRAVERY, A. F. (1968)
Microbiological breakdown of cellulose in the presence of alternate carbon sources.
J. Sci. Fd. Agric. 19 133-135.

BRIAN, P. W. (1957)
The ecological significance of antibiotic production.
7th Symp. Soc. Gen. Microbiol. 168-188.
Cambridge Univ. Press.

BRIAN, P. W. (1960)
Antagonistic and competitive mechanisms limiting survival and activity of fungi in soil.
In The Ecology of Soil Fungi, pp. 115-129.
Ed. Parkinson, D. and Waid, J. S., Liverpool Univ. Press.

BRIEN, R. M. and DINGLEY, J. M. (1946)
Rot proofing of fabrics and comparative experiments using different test fungi.
N.Z. J. Sci. Tech. 28 131-135.

BROADBENT, D. (1968)
Antibiotics produced by fungi.
Pest Articles and News Summaries, 14 (2): 120-141.

BROWN, W. (1923)
Experiments on the growth of fungi on culture media.
Ann. Bot. 37 105-129.

BROWN, W. (1925)
Studies in the genus Fusarium. II. An analysis of factors which determine the growth forms of certain strains.
Ann. Bot. 39 373-408.

BROWN, J. C. (1958)
Soil fungi of British sand dunes.
J. Ecology, 46 641-664.

BURGES, A. (1958)
Microorganisms in the soil.
Hutchinson Univ. Library, London.

BUTLER, E. E. (1957)
Rhizoctonia solani as a parasite of fungi
Mycologia, 49 354-373.

CALDWELL, R. (1958)
Fate of spores of Trichoderma viride introduced in to soil.
Nature, Lond., 181 1144-1145.

CHANG, YUNG (1967)
The fungi of wheat straw compost.
Trans. Br. mycol. Soc. 50 667-677.

CHANG, YUNG and HUDSON, H.J. (1967)
The fungi of wheat straw compost.
Trans. Br. mycol. Soc. 50 649-666.

CHESTERS, C.G.C. (1940)
A method for isolating fungi.
Trans. Br. mycol. Soc., 24 352.

CHESTERS, C.G.C. (1948)
A contribution to the study of fungi in soil.
Trans. Br. mycol. Soc., 30 100-117.

CHESTERS, C.G.C. (1949)
Concerning fungi inhabiting soil. Presidential address.
Trans. Br. mycol. Soc., 32 197-216.

CHESTERS, C.G.C. and THORNTON, R.H. (1956)
A comparison of techniques for isolating fungi.
Trans. Br. mycol. Soc., 39 301-313.

CHESTERS, C.G.C (1960)
Certain problems associated with the decomposition of soil organic matter by fungi.
In The Ecology of Soil Fungi, pp. 223-238,
Ed. D. Parkinson and J.S. Waid, Liverpool Univ. Press.

CHI, C.C. (1960)
Effects of Strptomyces and Trichoderma on Fusarium.
Phytopath., 50 631.

CHINN, S.H.F. (1953)
A slide technique for the study of fungi and actinomycetes in soil with special reference to Helminthosporium sativum.
Can. J. Bot., 31 718-724.

CHINN, S.H.F. and LEDINGHAM, R.J. (1957)
Studies on the influence of various substances on the germination of Heminthosporium sativum spores in soil.
Can. J. Bot., 35 697-701.

CHOLODNY, N. (1930)
Uber eine nene Methode zur Untersuchung der Boden-mikroflora.
Arch. Mikrobiol., 1 620-652.

COCHRANE, V.W. (1958)
Physiology of Fungi.
J. Wiley & Sons, New York.

CONN, H.J. (1922)
A microscopic method for demonstrating fungi and actinomycetes in soil.
Soil Sci., 14 149-151.

COOKE, R. C. (1962)
Ecological characteristics of nematode trapping Hyphomycetes.
Ann. appl. Biol. 52 431-437.

COOKE, C. T. and CHASE, F. W. (1956)
The selective enumeration of actinomycetes in the presence of large numbers of fungi.
Can. J. Microbiol. 2 12.

COONEY, D. G. and EMERSON, R. (1964)
Thermophilic Fungi, an account of their biology, activities and classification.
W. H. Freeman & Co., San Francisco and London.

COWLING, E. B. (1963)
Structural features of cellulose that influence its susceptiblity to enzymatic hydrolysis.
In Advances in Enzymic Hydrolysis of Cellulose and related materials, pp. 1-32.
Ed. E. T. Reese, Pergamon Press, London and New York.

CRISAN, E. V. (1959)
The isolation and identification of thermophilic fungi.
M.Sc. Thesis, Purdue University.

CROOK, P., CARPENTER, C. C. and KLENS, P. F. (1950)
The use of sodium propionate in isolating actinomycetes from soils.
Science, 112 656.

CROSS, T. and MACIVER, A. M. (1966)
An alternative approach to the identification of Streptomycetes - a working system.
In Identification Methods for Microbiologists.
Ed. Gibbs and Skinner. Academic Press, London.

CSERJESI, A. J. (1967)
The adaptation of fungi to pentachlorophenol and its biodegradation.
Can. J. Microbiol. 13 1243-1249.

DAWSON, A. I. (1919)

J. Bact. 4 133.

DICKINSON, C. H. and PUGH, G. J. F. (1965)
Use of selective cellulose agar for isolation of soil fungi.
Nature, Lond. 207 440-441.

DICKINSON, C.H. and PUGH, G.J.F. (1965)
The mycoflora associated with Halimone portulacoides.
Trans. Br. mycol. Soc. 48 381-390.

DOBBS, C.G. and HINSON, W.H. (1953)
A widespread fungistasis in the soil.
Nature, Lond., 172 197-199.

DOBBS, C.G., HINSON, W.H. and BYWATER, J. (1957)
Mycostasis in soil.
J. gen. Microbiol. 193 xi.

DUBOS, R.J. (1928)
Influence of environmental conditions on the activities of cellulose decomposing organisms in the soil.
Ecology, 9 12-27.

DUDDINGTON, C.L. (1955)
The predacious fungi: Zoopagles and Moniliales.
Biol. Rev. 31 152-193.

DWIVEDI, R.S. (1968)
Ecology of soil fungi with emphasis on fungistasis.
Proc. Symp. Recent adv. Trop. Ecol., pp. 288-294,
Ed. Misra, R. and Gopal, B.

DWIVEDI, R.S. and GARRETT, S.D. (1968)
Fungal competetion in agar plate colonization from soil inocula.
Trans. Br. mycol. Soc. , 51 95-101.

EGGINS, H.O.W. (1967)
The economics of biodeterioration.
Environmental Engineering, No. 29 15-16.

EGGINS, H.O.W. (1968)
Ecological aspects of biodeterioration.
Proc. Ist Int. Biodetn. Symp. pp. 22-27.
Elsevier Publishing Co.

EGGINS, H.O.W. and LLOYD, A.O. (1968)
Cellulolytic fungi isolated by the screened substrate method.
Experientia, 24 749.

EGGINS, H.O.W. and PUGH, G.J.F. (1962)
Isolation of cellulose decomposing fungi from the soil.
Nature, Lond. 193 94-95.

EL-NAKEEB, M. A. and LECHEVALIER, H. A. (1963)
Selective isolation of aerobic actinomycetes.
Applied Microbiology, 11 75-77.

FAHRAENS, G. (1942)
Zentr. Bakt. Parasitenk. Abt. II, 104 264.

FERGUS, C. L. (1964)
Thermophilic and thermoltolerent molds and actinomycetes of mushroom compost during peak heating.
Mycologia, 56 267-284.

FERGUS, C. L. (1969)
The cellulolytic activity of thermophilic fungi and Actinomycetes.
Mycologia, 61 120-129.

FLEMING, N. and THAYSEN, A. C. (1920)
On the deterioration of cotton in wet storage.
Biochem. J., 14 25-28.

FOSTER, J. W. (1939)
The heavy metal nutrition of fungi.
Bot. Rev. 5 207-239.

FULLER, W. H. and NORMAN, A. G. (1942)
Cellulose decomposition by microorganisms.
Advances Enzymol. 2 239-264.

GARRETT, S. D. (1950)
Ecology of root inhabiting fungi.
Biol. Rev. 25 220.

GARRETT, S. D. (1951)
Ecological groups of soil fungi: A survey of substrate relationships.
New Phytol. 50 149-166.

GARRETT, S. D. (1955)
Presidential address: Microbial ecology of the soil.
Trans. Br. mycol. Soc. 38 1-9.

GARRETT, S. D. (1956)
Biology of root infecting fungi.
Cambridge Univ. Press.

GARRETT, S. D. (1962)
Decomposition of cellulose in soil by Rhizoctinia solani.
Trans Br. mycol. Soc. 45 115-120.

GARRETT, S. D. (1963)
A comparison of cellulose decomposing ability of five fungi causing cereal foot rots.
Trans. Br. mycol. Soc. 46 572-576.

GILMAN, J. C. (1957)
A manual of soil fungi.
Second Eddition. Iowa State Univ. Press. Ames, Iowa.

GLATHE, H., BERNSTOFF, C. V. and ARNOLD, A. (1954)
Zentr. Bakteriol. Parasitenk, Abt. II, 107 481-488.

GOTTLIEB, D. and SIMINOFF, P. (1951)
The production and role of antibiotics in the soil.
Phytopath. 41 5.

GREATHOUSE, G. A. and AMES, L. M. (1945)
Fabric deterioration by thirteen described and three new species of Chaetomium.
Mycologia. 37 138-155.

GREAVES, H. and SAVORY, J. G. (1965)
Studies of the microfungi attacking preservative treated timber with particular reference to methods of thin isolation.
J. Inst. Wood Sci. 15 45-50.

GREGORY, P. H., LACEY, M. E., FESTENSTEIN, G. N. and SKINNER, F. A. (1963)
Microbial and biochemical changes during the moulding of hay.
J. Gen. Microbiol. 33 147-174.

GREGORY, P. H. and LACEY, M. E. (1963)
Mycological examination of dust from mouldy hay associated with farmers lung disease.
J. Gen. Microbiol., 30 75-88.

GRIFFITH, E. and JONES, D. (1963)
Colonization of cellulose by fungi.
Trans. Br. mycol. Soc. 46 285-294.

HARLEY, J. L. (1960)
The physiology of soil fungi.
In The Ecology of Soil Fungi, pp. 265-276. ed. Parkinson, D. and Waid, J. S., Liverpool University Press.

HARLEY, J. L. and WAID, J. S. (1955)
A method of studying active mycelia on living roots and other surfaces in the soil.
Trans. Br. mycol. Soc. 38 104-118.

HARMSEN, G. W. (1946)
Onderzoekingen over de aerobe celluloseontleding in den grond.
Wageringen Dissert., 229 p. J.B. Wolters: Groningen.

HARPER, J. E. and WEBSTER, J. (1964)
An experimental analysis of the coprophilous fungus succession.
Trans. Br. mycol. Soc. 47

HASHIOKA, Y., KOMATSU, M. and ARITA, I. (1961)
Trichoderma viride, as an antagonist of the wood inhabiting hymenomycetes.
Reports of the Tottori Mycological Inst. No. 1. pp.1-8.

HAWKER, L. E. (1950)
Physiology of Fungi. University of London Press.

HAWKER, L. E. (1957)
Ecological factors and the survival of fungi.
Seventh Symp. Soc. gen Microbiol. pp. 238-258.
Cambridge University Press.

HAZEU, W. and EGGINS, H. O. W. (1966)
Isolation methods for cellulolytic fungi.
Int. Biodetn Bull., 2 (2): 135-145.

HAZRA, A. K., BOSE, S. K. and GUHA, B. C. (1958)
A rapid method for survey of cellulolytic power of fungi.
Sci. cult., 24 39-40.

HECK, A. F. (1929)
Nitrogenous compounds in fungus tissue.
Soil Sci., 27 1-46.

HENDEY, N. I. (1967)
Fungicides in industry.
Engineer (London), 224 (5819): 155-158.

HENSSEN, A. (1957)
Uber die Bedeutung der thermophilen Mikroorganismen fur die Zesetzung des Stallmistes.
Arch. Mikrobiol., 27 63-81.

HEUKELEKIAN, H. and WAKSMAN, S.A. (1925)
Carbon and nitrogen transformation in the decomposition of cellulose by filamentous fungi.
J. Biol. Chem., 66 323-34o

HORTON, J.C. and KEEN, N.T. (1964)
Glucose inhibition of cellulase synthesis by Pyrenochaeta terrestris.
J. Iowa Acad. Sci., 71 79-81.

HOWARD, J.W. and McCORD, F.A. (1960)
Cotton quality study. IV: Resistance to weathering.
Text. Res. J. 30 75-117.

HUDSON, H.J. (1968)
The ecology of fungi on plant remains above the soil.
New Phytol. 67 837-874.

HUDSON, H.J. and WEBSTER, J. (1958)
Succession of fungi on decaying stems of Agropyron repens.
Trans. Br. mycol. Soc. 41 165-177.

HUECK, H.J. (1957)
A study of the determination of rotproofness. General Introduction.
Report Centeral Laboratory TNO, Holland.

HUECK, H.J. (1965)
The biodeterioration of materials as a part of hylobiology.
Material und Organismen, 1 5-34.

HUECK, H.J. and HUECK van der PLAS, E.H. (1963)
Comparison of biological test methods for materials.
OECD Report DAS/RS/63.91

HUECK, H.J. and LA BRIJN, J. (1960)
Pentachlorophenol and dodecylpentachlorophenol as mildew preventives for cotton.
Texil-Rundschau, 15 467-472.

HUECK, H.J. and LA BRIJN, J. (1965)
Moth proofing properties of insecticides: 1) insecticides containin a chlorinated bridged cyclohexane nucleus.
J. Soc. Dyers Colourists, 81 158-161.

HUECK van der PLAS, E.H. (1965)
Co-operative research in biodeterioration.
TNO-Nieuws, 20 945-959.

HUECK van der PLAS, E.H. (1965b)
A survey of biological test methods for materials.
Int. Biodetn. Bull., 1(2) 38-45.

HUNGATE, R.E. (1950)
The anaerobic mesophilic cellulolytic bacteria.
Bat. Rev., 14 1-49.

JACKSON, R.M. (1957)
Fungistasis as a factor in the rhizosphere phenomenon.
Nature, Lond. 180 96-97.

JACKSON, R.M. (1958)
An investigation of fungistasis in Nigerian soils.
J. gen. Microbiol., 18 248-258.

JENSEN, H.L. (1931)
The microbiology of farmyard manure decomposition in soil.
II. Decomposition of cellulose.
J. Agric. Sci., 21 81-100.

JENSEN, H.L. (1934)

Proc. Linnean Soc. N. S. W., 65 543

JOHANSSON, M. (1966)
A comparison between the cellulolytic activity of white and brown rot fungi. 1. The activity on insoluble cellulose.
Physiol. Plant., 19 709

KEYNAN, A., HENIS, Y. and KELLER, P. (1961)
Factors influencing the composition of soil microflora on soil crumb plates.
Nature, Lond. 191 307.

KING, K.W. (1966)
Enzymic degradation of crystalline hydrocellulose.
Biochem. Biophys. Res. Comm. 24 295-298.

KOHLMEYER, J. (1956)
Ueber den Cellulose-abbau durch einige phytopathogene Pilze.
Phytopath. Zeitschr. 27 147-182.

KUBIENA, W. (1932)
Über Frruchtkörperbildung und engere Standortwahl von Pilzen in Bodenhoheräumen.
Arch. Mikrobiol., 3 507-542.

LINGAPPA, Y. and LOCKWOOD, J.L. (1961)
A chitin medium for growth, isolation and maintenance of actinomycetes.
Nature, Lond. 189 158

LINGAPPA, Y. and LOCKWOOD J.L. (1962)
Chitin media for selective isolation and culture of actinomycetes.
Phytopath., 53 317-323.

KUSTER, E. and WILLIAM, S.T. (1964)
Selection of media for isolation of Streptomyces.
Nature, Lond., 202 928-929.

LA TOUCHE, C.J. (1948)
Slide traps for soil fungi.
Trans. Br. mycol. Soc. 31 281-284.

LAWRENCE, C.H. (1956)
A method for isolating actinomycetes from scabby potato tissue and soil with minimal contamination.
Can. J. Bot., 34 44.

LILLY, V.G. and BARNETT, H.L. (1951)
Physiology of the Fungi.
McGraw Hill Book Co. New York and London.

LINFORD, M.B. (1937)
Stimulated activity of natural enemies of nematodes.
Science, 85 123-124.

LLOYD, A.O. (1955)
A soil infection method for the testing of textiles for resistance to microbiological attack.
J. Text. Inst., 46 T653-T661.

LOCKWOOD, J.L. (1964)
Soil fungistasis.
Ann. Rev. Phytopath., 2 341-362.

LOOS, M.A., BOLLAG, J.M. and ALEXANDER, M. (1967)
Phenoxyacetate herbicide detoxification by bacterial enzymes.
J. Agric. Food Chem. 15 858-860.

McBETH, I.C. (1916)
Studies on the decomposition of cellulose in soils.
Soil Sci., 1 437-487.

McLENNAN, E. (1928)
The growth of fungi in soil.
Ann. appl. Biol. 15 95-109.

MANDLE, M. and REESE, E.T. (1957)
Induction of cellulase in Trichoderma viride as influenced by carbon sources and metal.
J. Bact. 73 269- 278.

MANDLE, M. and REESE, E.T. (1965)
Inhibition of cellulases.
Ann. Rev. Phytopath. 3 85-102.

MARSH, P.B., GREATHOUSE, G.A., BUTLER, M.L. and BOLLENBACHER, K. (1945)
Testing fabrics for resistance to mildew and rot.
U.S. Dept. Agric. Tech. Bull. No. 892.

MEYERS, S.P. and REYNOLDS, E.S. (1959)
Effects of wood and wood products on perithecial development by lignicolous marine Ascomycetes.
Mycologia, 51 138-145.

MOOR, W.A. (1945)
The continuous cultivation of microorganisms.
Science, 102 594.

MORTON, A.G. and BROADBENT, D. (1955)
The formation of extracellular nitrogen compounds by fungi.
J. gen. Microbiol., 12 248-258.

MUELLER, A.E. and DURRELL, L.W. (1957)
Sampling tubes for soil fungi.
Phytopathology, 47 243

NIXON, R.L. (1926)
Bull. No. 1438, U.S. Dept. of Agriculture.

ODUM, E.P. (1959)
Fundamental of Ecology.
W.B. Saunders Co., Philadelphia and London.

OLTHOF, T.H.A. and ESTAY, R.H. (1966)
Carbon and nitrogen level of a medium in relation to growth and nematophagous activity of Arthrobotrys oligospora Fres.
Nature, Lond. 209 1158.

PARK, D. (1955)
Experimental studies on the ecology of fungi in soil.
Trans. Br. mycol. Soc., 38 130-142.

PARK, D. (1960)
Antagonism - the background to soil fungi.
In The Ecology of Soil Fungi, pp. 148-159, ed. Parkinson, D. and Waid, J.S., Liverpool University Press.

PARK, D. (1968)
The ecology of terrestrial fungi.
In The Fungi, pp. 5-40, ed. Ainsworth, C.G. and Sussuman, A.S.
Academic Press, New York and London.

PLUNKETT, B.E. (1966)
Morphogenesis in the mycelium; control of lateral hypha frequency in Mucor hiemalis by amino acids.
Ann. Bot. 30 133-151.

PORTER, J.N., WILHELM, J. and TRESNER, H.D. (1960)
Method for the preferential isolation of actinomycetes from soil.
Appl. Microbiol. 8 174-178.

PRAMER, D. and KUYAMA, S. (1963)
Nemin and nematode trapping fungi.
Bact. Rev., 27 282-292.

PRATT, C.A. (1924)
The staling of fungal cultures: 1. General chemical investigation of staling by Fusarium.
Ann. Bot. 38 563-595.

PUGH, G.J.F. (1958)
Leaf litter fungi found in Carex paniculata L.
Trans. Br. mycol. Soc. 41 185

PUGH, G.J.F. (1963)
Ecology of fungi in developing coastal soil.
In Soil Organism, pp. 439-445,
Amsterdam, North Holland Publishing Co.

PUGH, G.J.F., BLAKEMAN, J.P., MORGAN-JONES, G. and EGGINS, H.O.W. (1963)
Studies on fungi of coastal soil. IV. Cellulose decomposing species in sand dunes.
Trans. Br. mycol. Soc. 46 565-571.

PUGH, G.J.F., BLAKEMAN, J.P. and MORGAN-JONES, G. (1964)
Thermomyces verrucosus sp. nov. and T. lanuginosus.
Trans. Br. mycol. Soc., 47 115-121.

PUGH, G.J.F. and DICKINSON, C.H. (1965)
Studies on fungi in coastal soils. VI. Gliocadium roseum Bainer
Trans. Br. mycol. Soc. 48 279-286.

PUGH, G. J. F. and VAN EMDEN, J. H. (1969)
Cellulose decomposing fungi in polder soils and their possible influence on pathogenic fungi.
Neth. J. Pl. Path. 75 287-295.

PUGH, G. J. F. and WILLIAMS, G. M. (1968)
Fungi associated with Salsola kali.
Trans. Br. mycol. Soc. 51 389-396.

RAUTELA, G. S. and COWLING, E. B. (1966)
Simple cultural test for relative cellulolytic activity of fungi.
Appl. Microbiol. 14 892-896.

REESE, E. T. (1946)
Decomposition of cellulose by microorganism at temperatures above 40° C.
Thesis, Penn. State College.

REESE, E. T. (1947)
On the effect of aeration and nutrition on cellulose decomposition by certain bacteria.
J. Bact. 54 389-400.

REESE, E. T. (1963)
Advances in enzymic hydrolysis of cellulose and related materials.
Oxford, New York, Pergamon Press.

REHACEK, Z. (1957)
Isolation of actinomycetes and determination of their spores in soil.
Microbiology, 28 222-225.

RICHARD, F. (1945)
Der biologische Abban von Zellulose-und Eiweiss-Testschnuren im Boden von Wald-und Rasengesellschaften.
Mitt. Schweiz Anst. forstl. Versuchsw., 24 297.

ROBBINS, W. J. (1937)
The assimilation by plants of various forms of nitrogen.
Amer. J. Bot. 24 243-250.

ROBINSON, P. M. (1969)
Aspects of staling in liquid cultures of fungi.
New Phytol. 68 351-357.

ROSSI, G. M. (1928)
Il terreno agrario nella teoria e nella realta.
Italia agric. 4 247.

RYABCHENKO, I. M. and GUBANOV, Y. V. (1952)
Sovet. Agron. 4 73-77.

SADASIVIAN, T. S. (1939)
Succession of fungi decomposing wheat straw in different soils, with special reference to Fusarium culmorum.
Ann. appl. Biol. 26 497-508.

SAVORY, J. G., MATHER, B., MAITLAND, C. C. and SELBY, K. (1967)
Assay of fungi for cellulolytic activity.
Chem. Ind. No. 4 153-154.

SCALES, F. M. (1915)
Some filamentous fungi tested for cellulose destroying power.
Bot. Gaz. 60 149-153.

SCHMIDT, E.L. and RUSCHMEYER, O.R. (1958)
Cellulose decomposition in soil burial beds.
Applied Microbiolgy, 6 108-114.

SEIM, J. (1966)
Perfusion chambers for small scale culture of microorganisms.
Nature, Lond. 212 94.

SEWELL, G. W. F. (1956)
A slide trap method for the isolation of soil fungi.
Nature, Lond. 17 708.

SHAPIRO, A. D. and VOLINA, T. L. (1961)
The manufacture of board resistant to biological degradation.
Bumazhn. Prom. 36 12-13.

SHIELDS, J. K. (1968)
Role of Trichoderma viride in reducing storage decay of Birch logs.
Research notes, 24 (1):
Canadian Dept. of Forestry and Rural Development.

SHIELDS, J. K. and ATWELL, E. A. (1963)
Effect of a mold, Trichoderma viride, on decay of birch by four storage-rot fungi.
Forest Products J. 13 (7): 262-265.

SHIGO, A. L. (1958)
Fungi isolated from oak-wilt trees and their effect on Ceratocystis fagacearum.
Mycologia, 50 757-769.

SIU, R. G. H. (1951)
Microbiological decomposition of cellulose.
New York, Reinhold.

SIU, R. G. H. and REESE, E. T. (1953)
Decomposition of cellulose by microorganisms.
Botanical Review. 19

SIU, R. G. H. and SINDEN, J. W. (1951)
Effects of pH, Temperature and mineral nutrition on cellulolytic fungi.
Am. J. Botany. 38 284-290.

SKINNER, C. E. and MELLEM, E. M. (1944)
Further experiments to determine the organisms responsible for decomposition of cellulose in soils.
Ecology, 25 360-365.

STAINIER, R. Y. (1942)
Are there obligate cellulose decomposing bacteria?
Soil Sci. 53 (6): 479-480.

STAPP, C. and BORSTELS, H. (1934)
Mikrobiologische Untersuchungen uber die Zersetzung von Waldstren.
Centralblatt Bakt. Parasitenk. abt. II 90 28-66.

STEINBERG, R. A. (1939)
Growth of fungi in synthetic nutrients solution.
Bot. Rev. 5 327-350.

SUSSMAN, A. S. (1966)
Dormany and spore germination.
In The Fungi.
Ed. Sussman, A. S. and Ainsworth, L. G., Academic Press, New York, London.

SUSSMAN, A. S. and HALVORSON, H. O. (1966)
Spores; their dormancy and germination.
Harper and Row, New York.

TALBOYS, P. W. (1958)
Degradation of cellulose by Verticullium Albo-Atrum.
Trans. Brit. mycol. Soc. 41 (2): 242-248.

THAYSEN, A. C. (1924)
J. Soc. Dyers Colourists. 40 101.

THAYSEN, A. C. and BUNKER, H. J. (1927)
The microbiology of cellulose, hemicelluloses pectin and gums.
Oxford Univ. Press, London.

THORNTON, R. H. (1952)
The screened Immersion plate: A method of isolating soil microorganisms.
Research, Lond. 5 190-191.

THEDEN, G. (1960)
Vergleichende Erprobung von Verfahren zur Prufung der Pilz-Wilderstandsfahigkeit von Wirkstoffen.
Materialprufung. 2 88-97.

TIMONIN, M. I. (1940)
The interaction of higher plants and soil microorganisms. II. Study of the microbial population of the rhizosphere in relation to resistance of plants to soil borne diseases.
Can. J. Res. C., 18 444-456.

TRIBE, H. T. (1957)
Ecology of microorganism in soils as observed during their development upon buried cellulose film.
In Microbial Ecology, 7th Symp. Soc. Gen. Microbiol., pp. 287-298. Cambridge Univ. Press.

TRIBE, H. T. (1960)
Aspects of decomposition of cellulose in Canadian soils.
Can. J. Microbiol. 6

TRIBE, H. T. (1966)
Interaction of soil fungi on cellulose filuse.
Trans. Br. mycol. Soc. 49 457-466.

TSAO, P. H., LEBEN, C. and KEITT, G. W. (1960)
An enrichment method for isolating actinomycetes that produce diffusible antifungal antibiotics.
Phytopath. 50 88-89.

TURNER, J. N. (1967)
The microbiology of fabricated materials.
J. & A. Churchill Ltd. London.

ITERSON, VAN, G. (1904)
Zentrbl. f. Bakt., Abt. II. 11 689.

VON GOOR, C. P. (1952)
Een kwantitatieve methode voor het bepalen van de biologische bodemactiviteit.
T.N.O. - Nieuws, 7 11-14.

VOROS, J. (1958)
Fungistatic activity of the Sphaeropsidales and Melanconiales.
Acta Microbiol. Acad. Sci. Hung. 5 261-266.

WADE, G. C. (1947)
Laboratory methods for testing the resistance of textiles to attack by fungi.
J. Coun. Scient. ind. Res. Anst. 20 445-450.

WALSETH, C. S. (1952)
The influence of the fine structure of cellulose on the action of cellulases.
Tappi, 35 233-238.

WALSH, J. H. and STEWART, C. S. (1969)
A simple method for the assay of the cellulolytic activity of fungi.
Int. Biodetn Bull. 5 15-20.

WAKSMAN, S. A. (1916)
Soil fungi and their activities.
Soil Sci. 2 103-155.

WAKSMAN, S. A. (1931)
Decomposition of the various chemical constituents, etc. of complex plant materials by pure cultures of fungi and bacteria.
Arch. Mikrobiol. 2 136-154.

WAKSMAN, S. A. (1937)
Associative and antagonistic effects of microorganisms.
Soil Sci. 43 51-60.

WAKSMAN, S. A. (1938)
"Humus".
2nd Ed., William and Wilkins, Baltunore.

WAKSMAN, S. A. (1944)
Three decades with soil fungi.
Soil Sci. 58 89-116.

WAKSMAN, S. A. (1959)
The Actinomycetes. Vol. 1. Nature, occurrence and activities.
William and Wilkins, Baltimore.

WAKSMAN, S. A. (1961)
The Actinomycetes. Vol. 2. Classification, identification and description of genra and species.
William and Wilkins, Baltimore.

WAKSMAN, S. A. and FOSTER, J. W. (1937)
Associative and antagonistic effects of microorganisms. II. Antagonistic effects of microorganisms grown on artificial substrates.
Soil Sci. 43 69-76.

WAKSMAN, S. A. and HEUKELEKIAN, H. (1925)
Carbon and nitrogen transformations in the decomposition of cellulose by filmentous fungi.
J. Biol. Chem. 66 323-342.

WAKSMAN, S. A. and HEUKELEKIAN, H. (1926)
Cellulose decomposition by various groups of soil microorganisms.
Fourth International Soils Sci. Conf., Rome. 3 216-228.

WAKSMAN, S. A. and SCHATZ, A. (1945)
Soil enrichment and development of antagonistic microorganisms.
J. Bact. 51 305-316.

WAKSMAN, S. A. and SKINNER, C. E. (1926)
Microorganisms concerned in the decomposition of cellulose in the soil.
J. Bact., 12 57-84.

WAKSMAN, S. A., UMBREIT, W. W. and CORDON, T. C. (1939)
Thermophilic actinomycetes and fungi in soils and in composts.
Soil Sci. 47 37-54.

WAKSMAN, S. A. and WOODRUFF, H. B. (1940)
The soil as a source of microorganisms antagonistic to disease producing bacteria.
J. Bacteriol. 40 581-600.

WARCUP, J. H. (1950)
The soil plate method for isolation of fungi from soil.
Nature, Lond., 166 117-118.

WARCUP, J. H. (1951)
The ecology of soil fungi.
Trans. Br. mycol. Soc. 34 376-399.

WARREN, J.R. (1948)
An undescribed species of Papulaspora parasitic on Rhizoctonia solani Kuhn.
Mycologia, 40 391-401.

WASTIE, R.L. (1961)
Factors affecting competitive saprophytic colonisation of the agar plate by various root infecting fungi.
Trans. Br. mycol. Soc., 47 535-540.

WEBLEY, D.M. and DUFF, R.B. (1962)
A technique for investigating localised microbial development in soils.
Nature, Lond. 194 364-365.

WENT, J.C. (1959)
Cellophane as a medium to study the cellulose decomposition in forest soils.
Acta. bot. neerl. 8 490-491.

WENT, J.C. and JONG, F.D. (1966)
Decomposition of cellulose in soil.
Antonie van Leeuwenhoek, 32 39-56.

WESSEL, C.J. and BEJUKI, W.M. (1959)
Industrial fungicides.
Ind. Eng. Chem., 51 52-53.

WILLIAMS, S.T. (1966)
The role of actinomycetes in biodeterioration.
Int. Biodetn. Bull. 2(2): 125-134.

WILLIAMS, S.T. and DAVIES, F.L. (1965)
Use of antibiotics for selective isolation and enumeration of actinomycetes in soil.
J. gen. Microbiol., 38 251-261.

WOOD, R.K.S. and TVIET, M. (1955)
Control of plant diseases by use of antagonistic organisms.
Bot. Rev., 21 441-491.

WRIGHT, J.M. (1955)
The production of antibiotics in the soil.
Ann. appl. Biol., 43 288-296.