THE MORPHOLOGY AND PHYSIOLOGY OF

CRYPTOSTROMA CORTICALE

by

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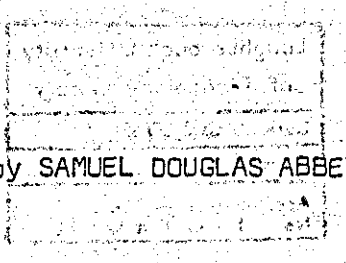

To my
WIFE
AUNT
and
COUSIN

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Summary

Four strains of Cryptostroma corticale have been characterised morphologically and physiologically.

The growth, sporulation and germination were affected differentially by environmental factors. A30 grew rapidly and sporulated abundantly. A32 grew well but not quite as rapidly as A30. A31 and A33 were both equally slow with A33 producing spores in low numbers.

The optimal conditions for growth sporulation and germination under laboratory conditions have been established. The strains were mesophilic, osmoduric, carboxyduric and slightly haloduric.

All the strains showed a non-fastidious nutritional character. Of the carbon sources tested glucose was the best for growth; glucose and sucrose provided the best sources for sporulation while galactose was the poorest carbon source for growth, germination and sporulation. All the nitrogenous sources were efficiently utilized except methionine and cysteine. Arginine, asparagine and urea produced the best growth yield.

The strain, A30, was found to be a prototroph while the other strains were partially auxotrophic to either d-biotin or L-ascorbic acid.

The strains exhibited a high heterotrophic characteristic which could be due to the large number of hydrolytic enzymes they produced.

The pattern the organisms showed in the assimilation and degradation tests may provide a means of differentiating them.

When the cellular proteins were subjected to electrophoresis, the strains showed broadly similar and strain specific patterns.

Electron microscopy studies illustrated no difference in morphology amongst the strains and related genera. Using lytic enzymes it was revealed that the cell wall was possibly three layered and made up of mainly glucan and chitin; cellulose was absent. The carbohydrates were the major polymers composing about 84-90% of the cell walls, proteins were between 5-10% and the lipids 1-3%.

Inhibitory and fungicidal levels have been established for the antimicrobial agents.

The imidazole derivatives produced the greatest lytic effect, followed by the polyenes. Morphogenetic studies were carried out. *Ramihyphin, Rapamycin, CytochalasinA, Aculeacin A* and *Polyoxin D* had the greatest morphogenetic effects causing ballooning of cells and cells of varied ramifications. Polyoxin D, owing to its inhibitory effect on wall synthesis, produced protoplasts which failed to revert to the mycelial form unlike the protoplasts produced with lytic enzymes.

The experimental infection investigation revealed germination in the plant tissue and the fungus was reisolated. This characteristic and the ability of the fungus to produce cell wall degrading enzymes within sycamore cells as well as degrading the cells indicate that C. corticale is probably a facultative parasite.

THE MORPHOLOGY AND PHYSIOLOGY OF CRYPTOSTROMA CORTICALE.

CHAPTER ONE

HISTORICAL BACKGROUND

DISCOVERY AND DISTRIBUTION:

The first description of *Cryptostroma corticale* was made by Ellis and Everhart[1] in 1899. They isolated it from a maple log (*Acer compestre* L.) in North America and named it *Coniosporium corticale*. *C. corticale* is the fungus responsible for the "Sooty Bark Disease" of sycamore (*Acer pseudoplatanus* L.).

In Britain, it was first discovered in Wanstead Park, Essex in 1945 on a dead sycamore.[2] The fungus caused a considerable damage amongst the sycamore population in the Park and a survey was carried out by the Forestry Commission between 1945 and 1951 (Fig 1). The figure shows a cumulative percentage of incidence of the disease and indicates a rate of increase. A further survey between 1951 and 1954 showed a decline in incidence of the disease. This led Peace[3] to describe it as a "disease in eclipse".

The disease was thought to be prevalent only around London. Pawsey[4] reported discovering the disease in Oxfordshire, Norfolk and Northampton. In 1976, there was an unprecendented severe outbreak of the disease in the country. Pavious-Smith[5], Abbott *et al*[6] and C.W.T. Young (personal communication) reported recent cases around Hampshire, Devon and Exeter. There is no reason to suggest the fungus is restricted to these places. Again, its distribution is difficult to ascertain, owing to the insidious nature of the fungus.

CUMULATIVE INCIDENCE OF SOOTY BARK DISEASE IN SELECTED AREAS IN WANSTEAD PARK, ESSEX.

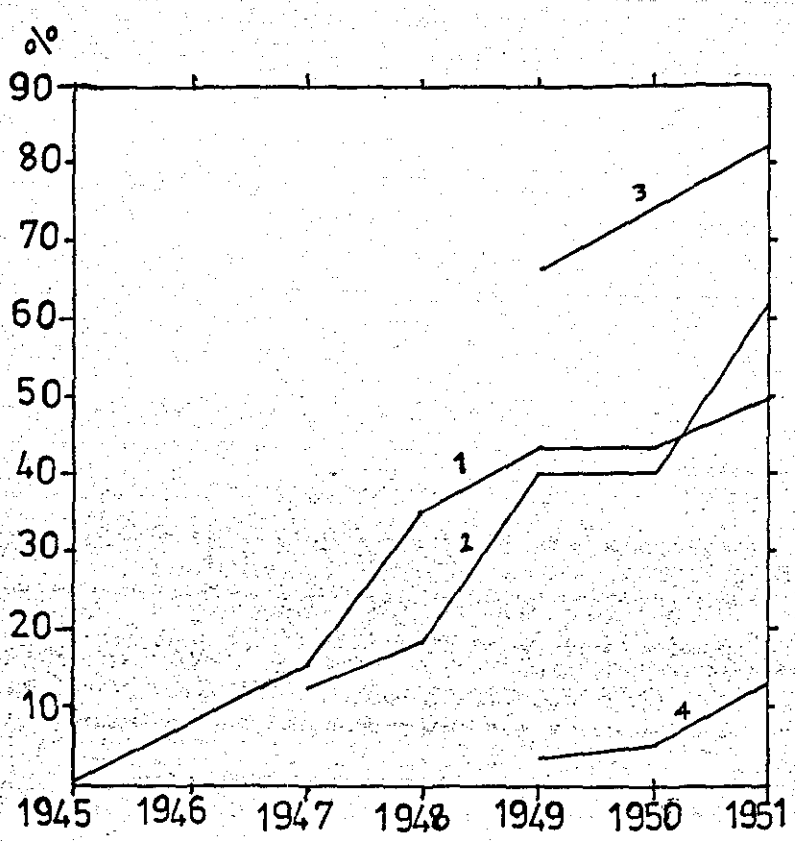


FIG I

FROM GREGORY AND WALLER (9).

The disease has also been reported in France on Norway maple (Acer platanoides L.), on sugar maples (A. campestre L.) and on Box elder (A. negundo L.) in the USA and Canada[7,8].

Gregory and Waller[9] tried to explain the sudden appearance of the fungus in Britain in 1945. They thought it might have been a widely distributed but hitherto overlooked saprophyte which has either produced a mutant pathogenic for A. pseudoplatanus; encountered a local mutant population of susceptible hosts or the fungus has recently been introduced from North America and it was spreading from a centre where it was well established. They regarded the last point as most likely in view of the fact that imported timber came near to Wanstead Park. Also Lister[10] failed to report the disease in her survey. However they said there were verbal reports of sooty trees in the Park which were believed to be due to deposition of London smoke.

THE DISEASE IN PLANTS:

The fungus can gain entry into the plant through small openings and wounds. Initially small branches may be killed or the damage may escalate to the death of the plant. In its virulent form, the fungus spreads through the wood progressively establishing itself extensively leading to wilting of leaves which shows itself notably in the summer. A dark greenish brown to yellow stain develops in the wood invaded by the fungus. It forms dark brown stromata below the bark which is quite cryptic from the initial stage and at a later stage, the bark loosens, exposing a sooty layer of spores.

The disease got its name, "Sooty Bark" from this characteristic appearance. The fungus forms a 2-3 cm thick olivaceous to brownish black sooty layer of minute spores approximately 30 to 170 million per square cm. The spores are dispersed gradually leaving exposed a bluish grey surface.

The infectious process in the plant depends on favourable conditions which occur notably during summer and it can take a minimum of one year. There have been cases when the disease progressed slowly for seven years in the limb before becoming acute.

Summer relationship:

The disease appears to have a relationship with summer temperatures which seem to control the internal spread of the fungus; the higher the temperature the greater the spread. Consequently, internal spread which leads to extensive damage develops for one to two years after a very warm summer weather (C.W.T. Young, personal communication).

Greater London is the area most prone to the incidence of the disease and for much of its history, the greatest damages have been largely confined to this region. This may have a relationship with this area which always records the highest summer temperatures in Britain. In 1975, a notably warm July was followed by an August that, in general, was the warmest since 1947, and in central England was probably the warmest for 300 years and so the unrivalled outbreak of Sooty bark disease followed in 1976. The exceptionally warm summer of 1976 did bring about several epidemics in 1977[11,12].

THE DISEASE IN MAN:

The first human cases due to C. corticale were reported by Towey et al[13] when 35 workers on maple logs went down with typically acute asthmatic conditions. The predominant symptoms here were dyspnea, cough, with varying amounts of expectoration or loss of weight. There were reports of raised temperatures (103°F) night sweating and pains. The x-ray reports showed mottling throughout the lower half of the lungs in the most severe cases with a definite increase in basal trunk and peri-bronchial shadows in all the cases. The picture seemed quite similar to pneumoconiosis.

The disease got its name, "Maple Bark" by being prevalent amongst the maple log workers who were constantly exposed to the spores from infected trees and frequently went down with it.

Similarities have been made between this disease and farmer's lung, an acute granulomatous intersitial pneumonitis[14,15]. This type of pulmonary disorder is widely distributed in Britain. It was first described by Campbell[16] followed by Fawcitt[17,18] and reviewed by Fuller[19] and Williams and Mulhall[20]. Buechner et al[21] in their review on bagassosis (a pulmonary conditions due to inhalation of bagasse - the fibrous material of sugar-cane) drew similarity to maple bark disease and also Cohen et al[22] has shown its connection with sequoiosis, also a granulomatous pneumonitis associated with saw dust inhalation.

Hypersensitivity of individuals to moulds and hays causing allergic conditions and pneumonitis attracted a lot of attention. Emanuel et al[23,24] were involved in this. They received cases of pneumonitis and confirmed them to be due to C. corticale. Tewksbury et al[25] induced the disease, maple bark, in experimental animals. The involvement of C. corticale in extrinsic allergic alveolitis was made by Bulman and Stretton[26] who also showed the capability of the fungus in producing lesions experimentally in rabbits.

CLASSIFICATION:

C. corticale, having been discovered in 1945, remained unidentified. The closest it came to being given a name was in 1948[1]. Ellis and Everhart[1] assumed that the fungus travelled up and down the vessels in the plant producing lesions whenever in contact with the cambium. This they thought, had a close resemblance to the canker stain of plane tree (Platanus) and sugar maple (A. campestre). Both these conditions were caused by fungi of the genus, Endoconidiophora.

Later in the year, the Ministry of Agriculture and Fisheries handled the fungus and following the description of Ellis and Everhart considered naming it Coniosporium corticale (Ellis and Everhart).

In 1950, the type specimen of Coniosporium corticale was examined at the Commonwealth Mycological Institute. Both specimens were found to be similar. From here, the fungus then passed through several genera including being termed the conidial stage of a species of Nummularia.

Gregory and Waller[9], who have done by far the greatest work in this respect, collated results from several authorities and compared to materials of the genus, Coniosporium, Link. This group of fungi contains catenate, dry ellipsoidal, pale to dark brown multi-septate conidia resembling, sporodochia pulvinate; immersed mycelia; stroma present, conidiophores macronematous and meristematic[27]. This description, on the ground of its mode of spore formation and conidiophores arrangement, differed from their two fungi.

It then became clear to them the fungi could not remain in the genus, Coniosporium. After several attempts, they found it convenient to create a new genus, Cryptostroma, and so classified it as below:-

Subdivision:-	Deuteromycotina;
Class:-	Hyphomycetes;
Order:-	Moniliales;
Family:-	Dematiaceae;
Genus:-	Cryptostroma;

The name is descriptive to cover its cryptic stroma. They then put forward the following diagnosis: immersed stroma, consisting of floor or roof connected by stromatic column (only in the plant); short conidiophores ending in phialides; conidia endogenous, dry and produced from phialides. These were accepted and changes were made[28].

C. and M. Moreau[29] suggested that C. corticale is the imperfect stage of Eutypa acharii (Fries) Tul., an Ascomycete of the Order, Sphaeriales. E. acharii is also found on sycamore. No evidence was found to support this claim[8]. The Deuteromycotina (Fungi Imperfecti), as the name implies, houses all fungi with no perfect state as Ainsworth and Bisby[30] rightly pointed out. This makes classification difficult to achieve.

CHAPTER TWO

FUNGAL PARASITISM

Parasitism is the term applied when an organism lives on or in another living organism, deriving at least part of its food from the host[31]. Thus parasitism is basically a nutritional relationship between two living organisms.

FORMS OF PARASITISM:

Facultative Parasitism:

This includes organisms which usually occur as saprophytes i.e. those living on dead organic matter, but can become parasitic when conditions permit[32]. This group of parasites are readily cultivable. Under this, comes the Facultative saprophytes which usually live as parasites but can also grow saprophytically. The difference between these two types lies in the degree. They obtain their nutrients in various ways. Venturia inaequalis, one of the highly specialised of this group, causes little damage, thus, enabling the host cells to remain alive and functional to meet its requirements. This is termed Eusymbiosis[33].

The less specialised parasites, quickly kill the host tissues to live on. Where killing is limited, a Perthophyte is involved; a special case of Necrotrophs, which live on tissues, killed by them[32].

Obligate Parasitism:

This refers to the organisms which grow only in association with a suitable host[34]. The Peronosporaceae (downymildews) Erysiphales (powdery mildews) and the Uredinales (rusts), at one time, were considered as obligate parasites but certain members can now be grown axenically[35]. Gaumann[33] distinguished the obligates into biotrophs, those deriving their nutrients from living cells and necrotrophs.

Symbiosis is regarded as a highly specialised form of parasitism in which both partners benefit from the association[36].

In practice, it is difficult to distinguish one form of parasitism from another and even parasites or predators from symbionts. Hudson[37] pointed this out in Armillaria mellea, the honey fungus. This basidiomycete is capable of forming an endotrophic mycorrhizal association with the orchids, Gastrodia elata[38] and Galeola septentrionalis[39]; of acting as a serious and distinctive facultative parasite of woody plants or of persisting saprophytically[40] Rhizobium, which is normally a symbiont in the roots of legumes, becomes parasitic under certain conditions[41]. It may be right to say that parasitism has originated from mutation or adaptation i.e., organisms living saprophytically on senescent or dead vegetation acquire the ability to attack living tissues.

Colonization of Host by Parasites:

Some parasites can attack almost all parts of the plant but in general, most show some degree of specialisation. Colonization, with eventual infection, is discernable, though not clearly distinctively, into three stages:

(1) Prepenetration:

Spores come into contact with host tissues and germinate under favourable conditions. Invasion is chiefly by germ tube which may be single, aggregated, forming appresoria or haustoria[32, 42].

Penetrations:

Pathogens can invade plants through mechanical penetration, natural openings and wounds. Variations occur amongst fungi which largely depend on genetic and environmental factors. Cladosporium fulvum and C. cucumarinum infect tomato through the stomata[43];

Apple rots are caused by Penicillium expansum invading via the lenticels[32] and Botrytis cinerea penetrates by mechanical process[44].

Entry through wounds which is commonly caused by humans, animals and insects is the means for some fungi. Howell and Wood[45] claimed that infection by Pycnostysanus azaleae (bud blast of Rhododendron) is associated with leaf-hopper, Graphocephala coccinea and animals are also involved in spore dissemination[46]. There have been few reported studies on C. corticale. However, the fungus has been found on dead sycamore wood still attached to the tree with teeth-marks probably made by wood mice or bark voles[5]. It has also been established that the grey squirrel is involved in the entry of C. corticale into the sycamore trees[6]. Entry through wounds seems to be the likely mode of infection for C. corticale.

Post penentration:

Successful entry in the host tissues is followed by establishment and spread of the fungus. This again is dependent on several factors including its aggressiveness, susceptibility of the tissue and environmental factors.

Colonization usually results in infection and this process takes different forms in various diseases[32]. Infection by Venturia inaequalis is localised and the Pythium species are systemic[32]. These modes of invasion also vary. In C. corticale invasion has been seen to be localised at the vascular bundles or it may be systemic[9] and C.W.T. Young (personal communication).

Mechanism of Attack:

Damage to the host cells is usually caused in varying degrees depending on the parasites. The biotrophs, being the more specialised, impart less severe damage while the necrotrophs, the less specialised, cause considerable damage leading to cell death.

The damages are due to products elaborated by the parasites. These products include mainly enzymes and to a lesser extent extra-cellular polysaccharides.

It was DeBary[47] who first suspected the involvement of enzymes in cell wall dissolution, when he detected a macerating enzyme from tissue infected with Sclerotinia libertiana. This was confirmed by Ward[48] with Botrytis species and also Brown[49] who used more active enzymes.

Cell Wall Degrading Enzymes:

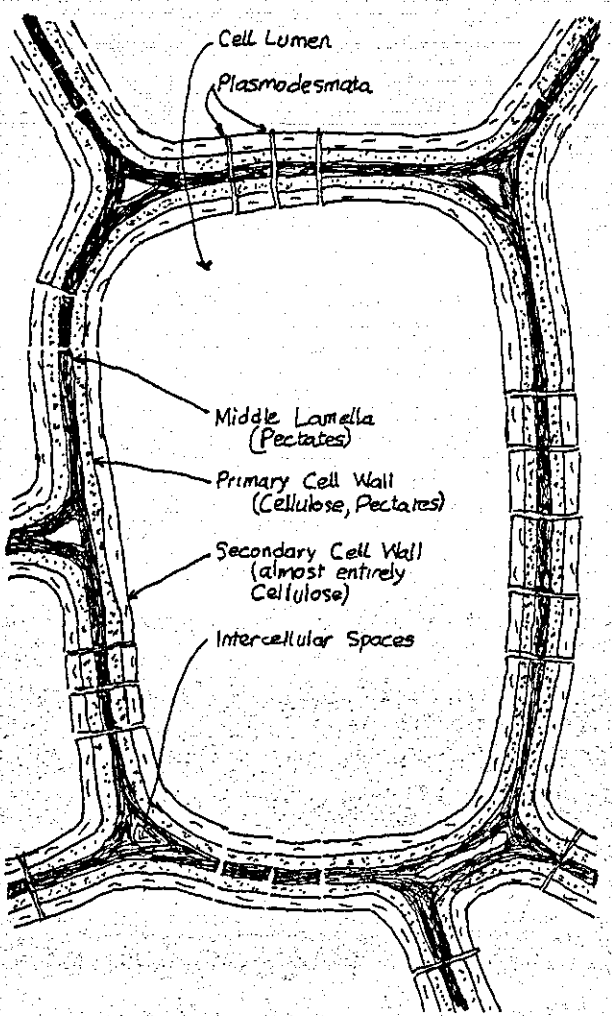
These enzymes are not only concerned with initial entry but also eventual spread of the pathogens in the host tissues. They are capable of degrading complex plant cell wall substances[32,50] and membrane constituents[51]. It is evident that phytopathogens produce these enzymes [51,52]. It does seem that the ability to produce these enzymes and also secreting them within the host should give information about fungal pathogenicity.

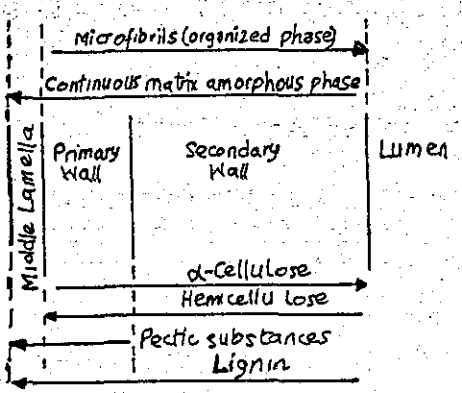
A knowledge of the structure and typical plant cell wall with knowledge of the degrading enzymes, can give a good understanding of pathogenicity.

The plant cell wall (Fig 2a) is the structure surrounding the protoplast exterior to the plasmalemma. It is complex but orderly; composing of polysaccharides, glycoproteins and lignin [53,54]. The structure may be viewed as a two phase system - a dispersed phase of cellulose microfibrils and a continuous matrix made up of other polysaccharides grouped as pectic materials, hemi- cellulose and cellulose and a hydroxyproline rich glycoproteins[53]. The cell wall can be divided into three functional regions: the middle lamella which serves as an intra-cellular cement that binds the cells together in a tissue system; the primary wall which is the first formed wall,

FIG 2(a)

SCHEMATIC REPRESENTATION OF THE PLANT CELL WALL AND ITS STRUCTURAL COMPONENTS





Distribution of materials in the mature cell wall. The arrows indicate directions of increasing relative concentration of the particular component.

being the most dynamic of the wall regions and functions to support the protoplast in the young growing cells and then the secondary wall which is deposited after the completion of cell elongation. It functions as a plant body supporting element.

Lignification occurs, in certain tissues, after the deposition of the secondary wall region. This is associated with cessation of metabolic activity and cell death.

PECTINASES:

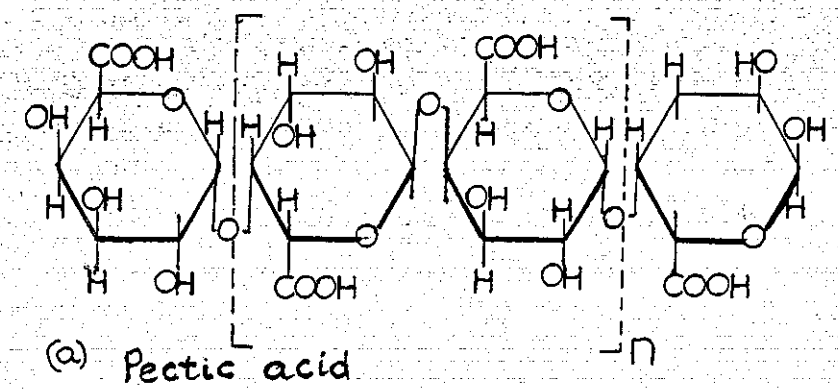
This group of enzymes has received a great deal of attention[48,49,55]. The enzymes degrade pectic substances which are present not only in the middle lamella but also in the primary and secondary walls in which they form an amorphous gel filling the spaces between the cellulose microfibrils. Pectins (Fig 2b) are polymers consisting primarily of α-1-4, linked D-galacturonic acid units. The carboxyl group on C 6 may be unesterified as in the polygalacturonic acids, which if colloidal, are known as pectic acids. The carboxyl groups of pectic acid may be esterified to varying extents with methanol to give pectinic acid[57]. Pectins are pectinic acids of high methoxy contents[50].

The Pectinases fall into two groups:

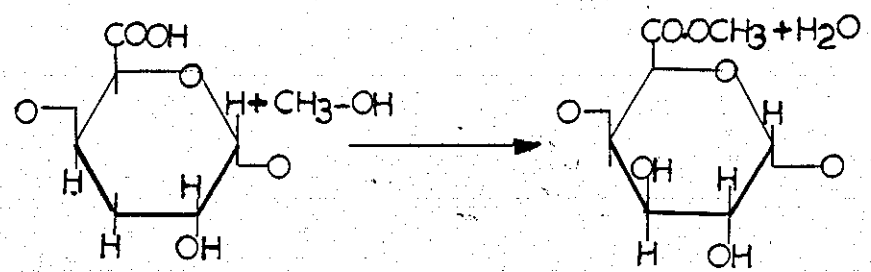
(1) Pectin methyl esterase (PME): These hydrolyse the methyl ester groups of pectinic acids to methanol and pectinic acids of reduced methoxy contents and eventually to pectic acid. They are widely distributed in plants and micro-organisms. Those of fungal origin generally have a lower pH optimum.

(2) Polygalacturonidases (PG): These include the pectic glycosidases and lyases. They break up adjacent galacturonic acid links in pectic acids. These also have widespread distribution in micro-organisms.

FIG 2 b

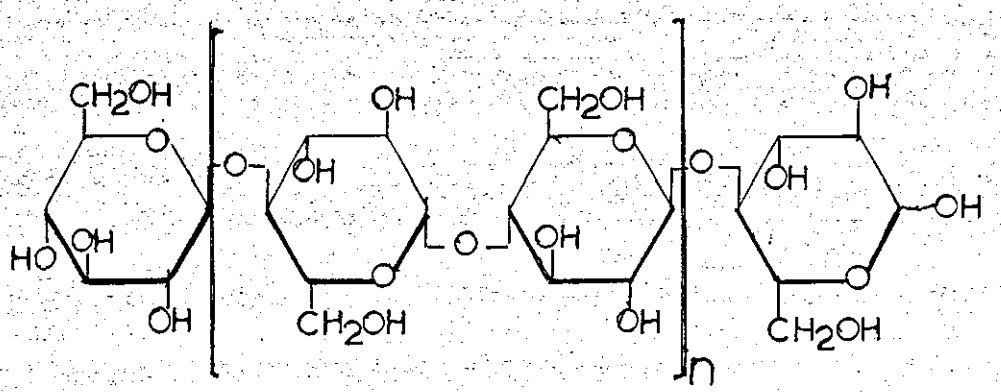


(a) Pectic acid



(b) The methylation of pectic acid

FIG 2 c



Cellulose

CELLULASES:

The cellulase group of enzymes has also been the subject of extensive reviews[58-60]. These enzymes break down cellulose which occurs in higher plants as skeletal structure of cell walls in the form of microfibrils which, in turn, form the basic structural unit of most meristematic cells.

Cellulose (Fig 2c) is a linear polymer of β, 1-4, linked D-glucose residues. The molecules are arranged in parallel bundles forming the microfibrils which group again into larger bundles to form the macrofibrils[57]. The fibres are intertwined to form a lattice-like structure with the inter fibrillar spaces filled with amorphous matrix composed chiefly of pectic substances, hemicellulose, and lignin. Cellulose, thus, forms the basic framework in the plant tissues.

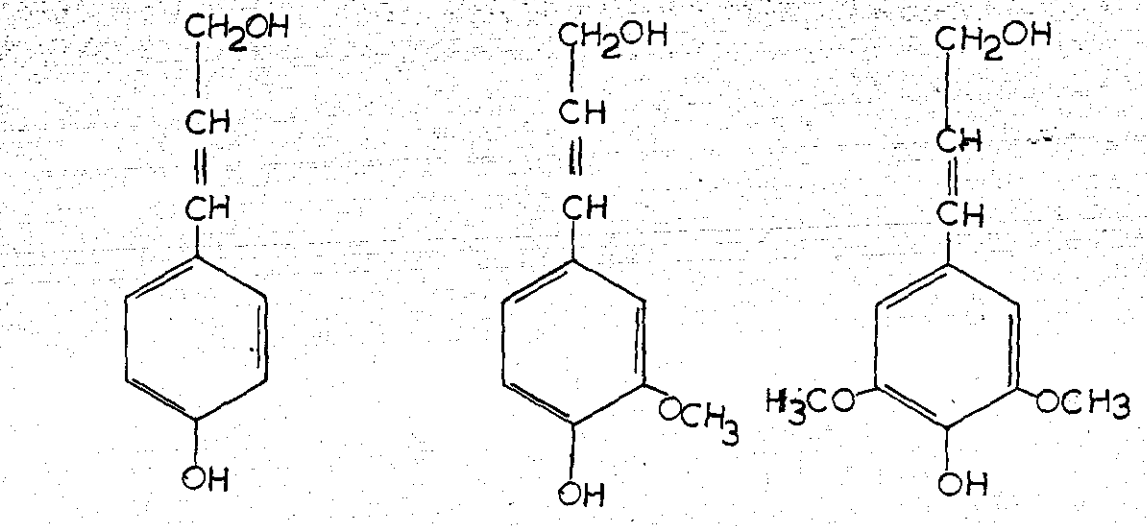
There are several views regarding the breakdown of cellulose. Whitaker[61] suggested the involvement of a single enzyme which converts cellulose into glucose by random cleavage of the molecule. Aitken et al[62] considered that two enzymes were involved and Reese[58] reported a series of enzymes. Many micro-organisms produce cellulase and so are able to convert cellulose into low molecular weight soluble products or glucose[63,64]

LIGNINASE:

Lignin is another important component of cell walls particularly in woody tissues. It has been a topic of several reviews[65-67]. Lignin (Fig 2d) is derived from the enzymic coupling of three aromatic alcohols namely p-coumaryl; coniferyl and sinapyl[68]. The monomers are inter-connected by a variety of C-C and ether bonds, making it difficult to hydrolyse both chemically and enzymatically. It is insoluble and resists attack; properties that have hindered its study. It occurs in the matrix

FIG 2d

Monomers of Lignin

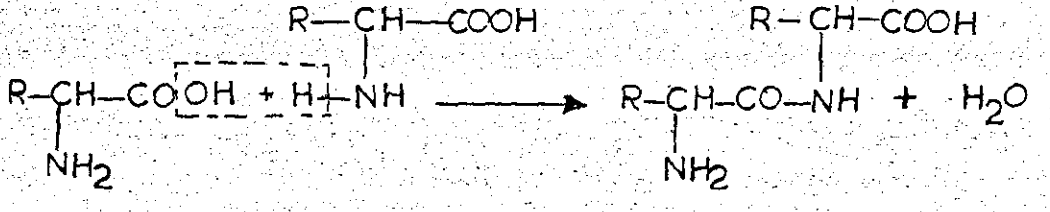


p-Coumaryl alcohol

Coniferyl alcohol

Sinapyl alcohol

FIG 2e



Amino acid

surrounding the cellulose fibrils. In woody cells, lignin constitutes almost all the middle lamella and forms its own framework in the walls, strengthening them so that they remain intact even when the cellulose is removed. Lignin is broken down by ligninase which are apparently polyphenol oxidases. Such enzymes may be present in culture filtration in media where lignin constituted the sole carbon source[69].

PROTEASES:

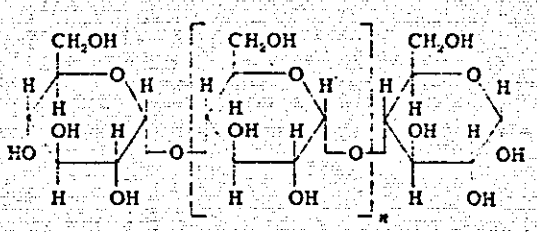
Protein (Fig 2e) is present in all cell walls. Part of this is made up of various enzymes or portions of the plasmadesmata. The remaining part is truly structural cell wall protein, which is similar to other proteins except for the presence of hydroxyproline, an amino acid not found in other plant protein.

Proteases are enzymes that hydrolyse peptide bonds. The breakdown mechanism of cell wall protein will not differ significantly from other proteins. Pathogens are known to degrade protein enzymatically and degradation of protein can profoundly affect the organisation and function of the host cells. Kuc(70) suggested that proteases had *an important role in phytopathogenicity.*

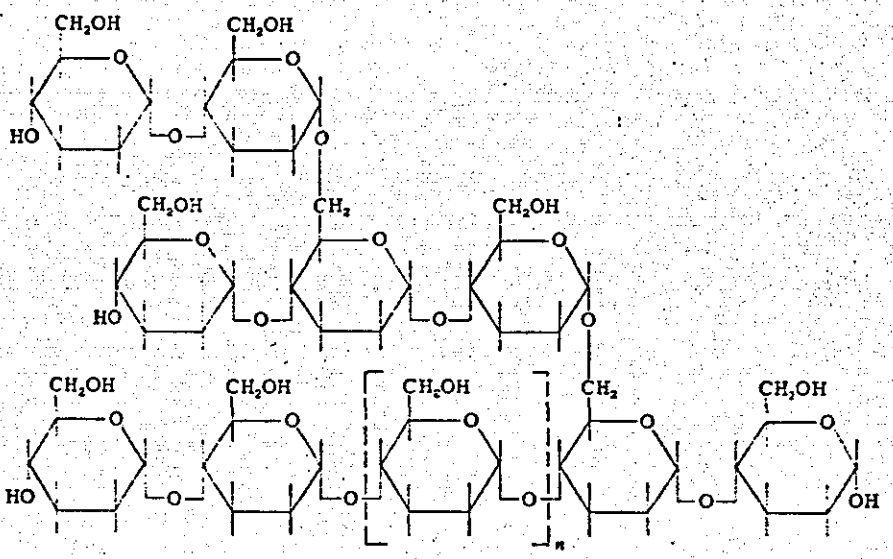
AMYLASES:

Starch is one of the main reserve polysaccharides found in plant cells; synthesised in the chloroplasts and in the amyloplasts of non-photo-synthetic organs.

It is a glucose polymer (Fig 2f) that exists in two forms; amylose, an essentially linear molecule consisting of α 1,4 glycosidic linkages and amylopectin a highly branched molecule of varying chain lengths linked by α 1,6 bonds to the main chain. Many pathogens utilise starch breaking it down by the action of the two enzymes α and β amylases.



Amylose



Amylopectin

Fig 2(f)

LIPASES:

Lipids are present in varying type in plant tissues, the most important being the neutral lipids which function as energy reserves. These include the fats, oils, phospholipids and glycolipids.

Lipases degrade lipids and pathogens are known to break down lipids (44, 71).

NUCLEIC ACIDS:

All cells contain small amounts of both nucleic acids, DNA and RNA. DNA is mainly found in the nucleus, and in very small amounts in chloroplasts and mitochondria. RNA is more widespread; found in the ground cytoplasm, ribosome, mitochrondria, nucleolus and chloroplasts. The DNA carries the genetic code and determines the genetic capabilities of the cell while the RNA translates the genetic message into protein production.

Ribonuclease and deoxyribonuclease are the enzymes that breakdown RNA and DNA respectively. Degradation of nucleic acids apparently occurs in diseased tissues and will obviously lead to distortion of cells but their significance in pathogenicity is not known[36].

MECHANISM OF DEFENSE:

Spores are distributed widely and come in contact with many plants but colonisation is not widespread. C. corticale produces approximately 30 to 170 million spores per square cm of surface[9]. Attempts have failed to colonise plants with fungi other than those to which they are susceptible. This signifies that parasites are only capable of thriving on a few species of plants. It is therefore clear that a mechanism of resistance exists in plants[36]. This topic has been the subject of several reviews[32, 71, 73].

The mechanism of resistance is essentially of two types: The first is the pre-infectional (passive) type which constitutes the first line of defense and tends to be rather non-specific although ineffective against pathogens able to attack the host.

Apart from the obvious mechanical barrier present in the plant, preformed factors that contribute to its resistance exist. These include the nutritional status, presence of antimicrobial agents and physico-chemical factors[36, 74]. Cherewick[75] suggested that specific proteins in the host determines race-specificity.

Kraus[76] was about the first to speculate the preserving and protective role of tannic acid and Cook and Taubenheus[77] demonstrated the toxicity to pathogens of a variety of tannin and phenols and then Rubin and Artsikhouskaya[78] showed that the phenolic compounds play a primary role in resistance.

Resistance conferred by phenolic compounds was demonstrated by Walker and Stahmann[72]. They showed that the resistance to Colletotrichum circinans, of onion varieties with pigmented outer scales is related to the presence of catechol and proto-catechuic acids, phenolic compounds which diffuse from the dead coloured scales and prevent fungal spore germination. Fungi, such as Aspergillus niger, able to withstand the compounds, will attack the pigmented onion[32].

The second type of resistance is post-infectional. Many fungi can enter non-host plants but establishment of disease lesion is gradual and finally ceases[73]. This indicates that resistance is an active and dynamic process. The active response of the cabbage clubroot to infection by Plasmodiophora brassicae[79] supports the active nature of resistance. These dynamic post infectional mechanisms of resistance are now generally considered to be of greater significance than the pre-infectional types.

The first reported case of active defense against a fungus was by Bernard[80]. He described how a fungus stimulated the orchid tissues to secrete an anti-fungal substance that stop further growth of the fungus. The mechanism is homologous to that of animal serology[81] which seemed to support theory of acquired immunity in plants[82].

Muller and Borger[83] reported on the similarity between acquired immunity in plants to induced immunity in animals. Based on studies with hybrids of potatoes resistant to Phytophthora infestans, they demonstrated that the tuber tissue of a partially resistant hybrid, becomes locally immuned against a compatible race of the fungus by prior infection with an incompatible race. They proposed that plant tissues in contact with pathogens produce a substance of non-specific origin that becomes toxic to the fungi. The sensitivity of these fungi is depended on the speed and intensity of the production of the toxic substance which became known as "Phytoalexin". This has been reviewed[84, 86] and several phytoalexins have been isolated including the latest, casbene[87] from castor bean (Ricinus communis).

CHAPTER THREE

MORPHOGENESIS

FUNGAL GROWTH:

A fungal spore will germinate on a solid medium, controlled by some regulatory mechanisms[88], to form a mycelial colony with the outer-most region consisting of young, actively growing and radial hyphae[89]. Provided there are no toxic metabolites, "Staling substances"[90], the colony grows at a steady rate. Fungi can, therefore, be described as Staling and Non-staling types.

The colony continues growing and differentiates into maturity according to the prevailing environmental factors. The mature colony can be said to have four morphological zones[95]:

1. the peripheral zone made up of a sparse network of vegetative hyphae;
2. the productive zone consisting of a dense network of vegetative hyphae;
3. the fruiting zone bearing the reproductive structures and
4. the aged zone with old autolysing hyphae at the centre of the colony.

The above description is one of an ideal colony. However, the morphology varies amongst species within the same genus, and within the same species grown on different media[96] and even in different depths of same media[97].

KINETICS OF COLONY EXPANSION:

Bull and Trinci[88] quoted the need to define;

1. the factors which govern the radial growth rate of microbial colonies;
2. the relationship between radial growth rate and mass growth rate and
3. the factors which cause differentiation within colonies.

Mandels[98] stated that at constant and non-limiting environmental factors with no internal changes other than the normal processes in the cell, growth will occur at a constant rate and since the number of cells present is governed by the time, it can be expressed as:

$$\frac{dN}{dt} = KN \quad \ldots\ldots(1)$$

(where N = the number of cells, t = time and K is a constant).

Equation (1) integrates exponentially to give the organism concentration at a given time (t).

$$N = ce^{kt} \quad \ldots\ldots(2)$$

(where e is the base of natural logarithm). He claimed that exponential growth does not occur in fungi even though Plomley[99] showed that Chaetomium globosum does grow exponentially. Mandels' view was supported by Swanson and Stock[100] who reported that the growth of Microsporum quinckeanum is not logarithmic since individual hyphae grows at a constant rate. However, several workers have reported exponential growth in filamentous fungi [101-103].

Trinci [104] stated that the radial expansion of the fungal colony may be divided into four phases:

(1) Lag, the period between inoculation and emergence of germ tubes;

(2) exponential, during which the colony increases in radius at an exponential rate;

(3) deceleration, the period between the termination of the exponential phase and the onset of the linear phase and

(4) constant growth; during which the colony increases in radius at a constant or linear rate. Thus, for most of its growth, a fungal colony expands at a linear rate.

Righelato [103] in support of this went further to show that the colony growth rate measurements can be related to morphology and specific growth rate in submerged culture.

DIFFERENTIATION:

Organisms pass through an orderly but rather complex cycle of developmental processes of growth and reproduction. They all have a form and so exhibit certain morphological and metabolic patterns; characters which are fundamental and difficult to separate since morphology provides the form of structure needed for metabolism and metabolism, in turn, produces and maintains morphology. Morphogenesis comprises the processes by which the form of an organism arises and changes, which is expressed in the development of differentiated patterns of metabolism and structure [105].

Nickerson and Falcone [106] pioneered an approach to elucidate the biochemical bases of morphogenesis in fungi by first obtaining a better understanding of the cell wall properties and behaviour. Such evidence does indicate that morphological differentiation is correlated with changes directly affecting the cell wall metabolism. This strengthens the main premise of equating morphological differentiation with cell wall differentiation [107-110].

The morphology of the fungus is depended on growth of the hyphae which is dictated by the shape of the hyphal wall. An undertsanding of morphogenesis thus requires a knowledge of the structure of the wall and the apex which generates it. Several workers have shown that hyphal growth is apical [111-113]. These studies still did not reveal whether structural elements were actually inserted at the apex. It was Burnett [114] who, using the information based on the response of hyphal tips to experimental manipulation, suggested that maximum wall synthesis occurred, not at the very tip but in the annular band located immediately behind a non-extensible apical cap.

Bartnicki-Garcia and Lippman[115]; Katz and Rosenberger[116] and Gooday[117] have also shown fairly conclusively by auto-radiographic labelling that microfibrillar constituents are deposited preferentially at the hyphal apex and maximum incorporation occurs at the apical pole.

Furthermore, since a chemically complete cell wall is seemingly essential for normal morphogenesis, it is possible to predict that morphogenetic development may depend to some extent on the specific variations in the relative proportions of the interaction amongst structural components of the cell wall[118]. Of all the aspects of morphogenesis in fungi, the morphology seems to be the most studied. Much valuable information has been accumulated over the years. There are yet many fungi, including C. corticale, for which morphological and cytological details are lacking.

FUNGAL WALL:

The fungal spores germinate by producing hyphae whose branches grow out and fill in the spaces between each other to give the expanding circular colony. Such a mode of growth is well suited on substrates such as wood and animal tissues. The most distinctive part of the fungal cell is the cell wall which is a rigid structure that both protects the fragile protoplast from damages and also maintains the characteristic shape of the cell. Villanueva[119] demonstrated the presence of this structure by treating the mycelia with enzymes that hydrolysed the walls, thus, liberating the protoplasts.

The study of fungal wall composition started many years ago[120,121] showing the wall to consist of mainly polysaccharides and in particular chitin which is found usually in the invertebrates and the fungi where it plays a skeletal role.

Chitin is an aminopolysaccharide, a (1-4) β -homopolymer of N-acetyl glucosamine. It is a strong, inert, macromolecule forming a very stable crystalline structures. Chemically it is analogous to the glucan back bone of peptido-glycan, the (1-4) β - alternate N-acetyl-glucosamine and N-acetyl muramic acid which is the characteristic component of prokaryotic walls. Rudall and Kenchington [122] showed through x-ray diffraction that fungal chitin is in an α -chitin form, a state which is the most stable and that it consists of chains of N-acetyl glucosamine residues linked by CO---NH hydrogen bonds.

Early workers on the fungal cell walls, because of the limitation of the techniques, available, had to rely on qualitative tests on whole cells. This,of course gave limited information about the chemical composition of the cell wall. In recent times the walls have been separated from the other cellular constituents by mechanically disrupting the cell and collecting the walls [123]. Subsequent chemical analysis of the walls showed the nature of its complexity being made of mixtures of polymers and it was also revealed that the nature of polymers varies amongst species and even in the same organism under different conditions [124]. Electron microscopic studies demonstrated that the physical structure is equally complex [125] consisting of network of microfibrils with the space in between filled by matrix polymers such as protein and glucans making it look like a carbon-fibre reinforced resin [126].

There are still many gaps in our knowledge of the chemical composition of fungal walls and in our understanding, of what the physical organisation implies. The walls of relatively few species have been studied but the available findings do form a basis for further research [127] and also understanding many present observations.

ANTIFUNGAL AGENTS AND MORPHOGENESIS:

The fungi are an extremely varied successful group with the ability to live in a wide range of habitats. Their success is partly due to their genetic adaptability. Some fungi undergo dimorphism, a phenomenom by which the organism can exist in both yeast and mycelial forms; conditions which are inducible depending on the environment[128].

Antifungal agents, antibiotics or metabolic inhibitors, used at a suitable sub-inhibitory concentration can modify the growth and development of fungi. Because there is detailed knowledge about the biochemical mechanism of action of many antibiotics, they can be used to elucidate the biochemistry of fundamental cellular processes.

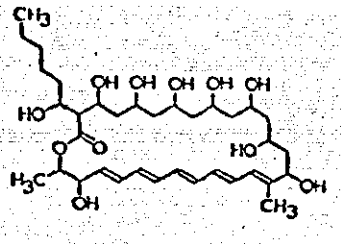
SOME ANTIFUNGAL ANTIBIOTICS:

A major advance in the search for effective antifungal antibiotics (Fig 3a, was the discovery of two agents produced by Streptomyces noursei[129]. One of these, Nystatin, was the first of the Polyene anti-fungal agents to be characterised and used therapeutically.

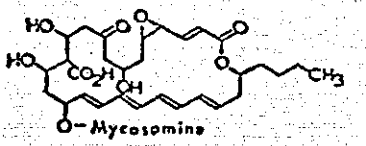
The Polyene antibiotics form a class of macrolide antibiotics consisting of macrocyclic rings closed by a lactone bond, usually with one or more sugars attached glycosidically to the macrolide ring. The ring contains up to 38 carbon atoms, a chromophore with a series of alternating double bonds and usually one amino-sugar moiety. The chromophore gives a rigid planer characteristic to one part of the macrolide ring, which contrasts with the polyhydroxylated hydrophilic region of the rest of the molecules. This amphipathic character accounts for the physico-chemical properties (low water solubility, photolability) and biological activities (interaction with membranes and sterols).

Mode of Action and Biological Activities

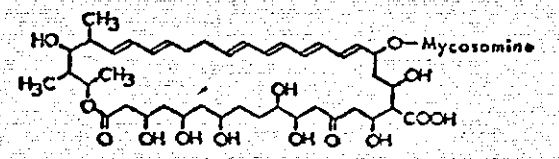
All the polyenes have a similar mode of action which entails, essentially, an irreversible binding to a sterol in the cell membrane with subsequent disfunction of the membrane[130, 131]. It has been established



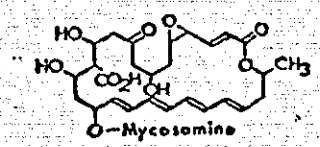
FILIPIN



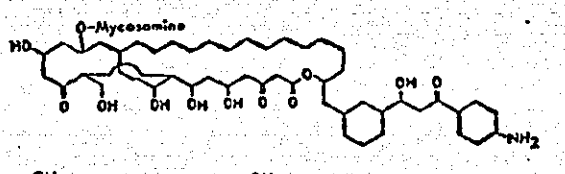
ETRUSCOMYCIN



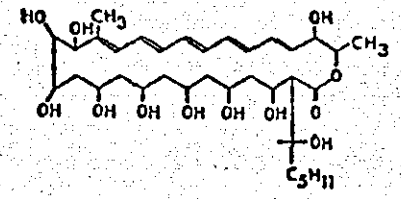
NYSTATIN



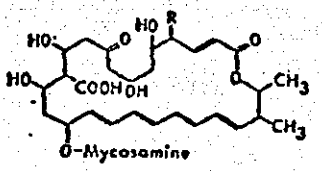
PIMARICIN



TRICHOMYCIN

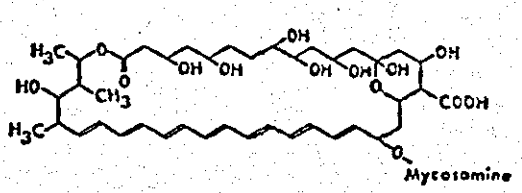


FUNGICHROMIN

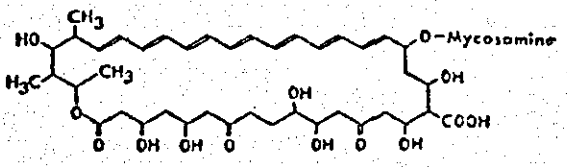


TETRIN A R=H

TETRIN B R=OH



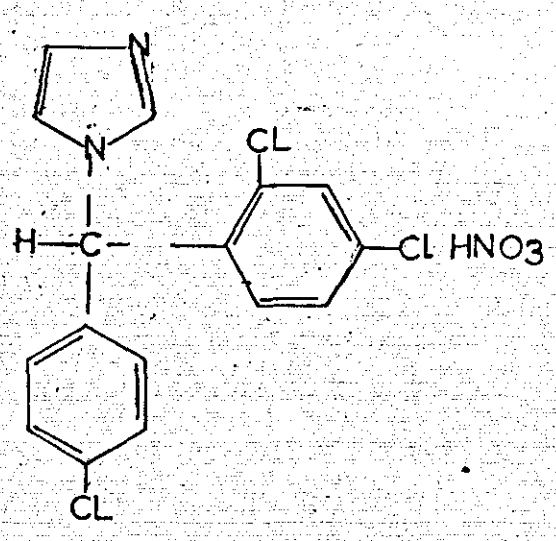
AMPHOTERICIN B



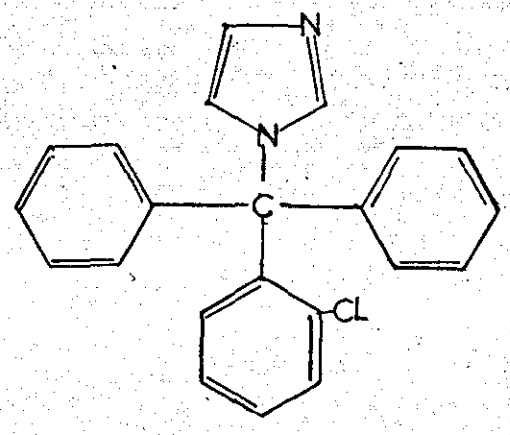
CANDIDIN

Fig 3(a)

FIG 3b



Miconazole



Clotrimazole

that the polyenes induce distortion of selective membrane permeability and this results in leakage of potassium and magnesium ions followed by decreased protein and RNA synthesis[132].

The Imidazole Derivatives:

The initial reports on these anti-fungal agents were published by Seeliger[133] and their activities were confirmed on the dermatophytes and yeast[134, 135]. Clotrimazole and miconazole are the commonest agents. The physico-chemical properties of these drugs have been established[136].

Mode of Action:

It has been suggested that the site of action appears to be in the cell wall and the membrane, making them permeable to intracellular phosphate and potassium, thus inhibiting intra-cellular macro-molecular synthesis[137-14
Van den Bossche[141] suggested there is an enhanced nucleoside transport followed by increased mucleoside incorporation into nucleic acids. Radioactive studies indicated that, in log phase cells, most of the drug was in the cell wall and plasmalemma. Alterations in cell permeability with the leakage of 260nm absorbing materials, amino acids, protein and inorganic cations were also reported[142]. Miconazole is an effective broad spectrum antifungal as well as a bactericidal agent[143].

The Pyrimidine Derivatives:

5-Fluorocytosine, (5-FC), was the first of this group to be synthesised[144]. Scholer[145] suggested that its activity was in its interference with the metabolism of cytosine to uridine. Sensitive cells have a cytosine deaminase that breaks down 5-FC to 5-fluorouracil (5-FU) and then 5-FU gets incorporated into tRNA[146].

Jund and Lacroute[147] showed a further enzyme, cytosine permease to be necessary for its entry into the cells. Polak et al[148] proposed a metabolic pathway and mode of action:

5-FC $\xrightarrow{\text{permease}}$ 5-FC $\xrightarrow{\text{deaminase}}$ 5 FU $\longrightarrow$ 5-FUMP $\longrightarrow$ 5-FUTP $\longrightarrow$ RNA.

Evidence has shown that RNA, heavily substituted with 5-FU functions poorly and leads to abnormal protein synthesis which results in cell death[135]. 5-FU is also a pyrimidine derivative that is related to a block of thymidylate synthetase leading to the inhibition of DNA synthesis[135].

5-FC exhibits no bacteriostasis but showed marked activity against Candida albicans.

Some Newly Characterised Antibiotics:

Aculeacin A:

This was the first of a series of antibiotics isolated from Aspergillus aculeatus.

It is a peptide containing palmitic acid and shows U.V. maximum at 278 nm in methanol. Mizuno et al[149] reported on its isolation and the physico-chemical properties. The mode of action lies in the inhibition of cell wall synthesis which leads to lysis and release of intra-cellular substances[150].

THE CYTOCHALASINS:

These belong to a relatively small group of aglycosidic macrolide antibiotics of fungal origin. They are macrocyclic non-lactone antibiotics with effective antimicrobial activities. They are antifungal and induce morphological changes[151]. The name, cytochalasin, was formulated to describe the effect the compounds have on morphogenesis and cellular movements.

The Polyoxins:

The polyoxins are closely related nucleotide antibiotics, produced by Streptomyces cacaoi. They were first isolated and characterised by Suzuki and co-workers[152].

Polyoxin D, one of the major components of the group, is active against most fungi with chitin[153]. Structural analysis revealed that the highly active forms have a structure similar to UDP-N-acetyl glucosamine, a chitin precursor. Endo and Misato[154] observed competitive inhibition of chitin synthetase of Neurospora crassa by polyoxin D. This was substantiated by Ohta et al[155], who found that UDP-N-acetyl glucosamine accumulates within the cells under the influence of polyoxin D.

Rapamycin:

This antibiotic was isolated from Streptomyces hygroscopicus by solvent extraction[156]. It is a colourless crystalline solid with an empirical formula of C_{56} H89 NO_{14} [157].

The objective of this work, therefore, is to characterise the fungus morphologically as well as physiologically, to examine available strains and show similar and distinguishing features amongst them. It was also intended to investigate the pathogenic state of the organism in the plant and its response to antimicrobial agents and elucidate their effects on morphogenesis.

CHAPTER FOUR

MATERIALS AND METHODS

ORGANISMS:

Four strains of *Cryptostroma corticale* were used. They were A30, isolated from infected maple bark[26], A31 and A32, isolated by R.G. Strouts (Forestry Commission, Surrey) from infected sycamores in Wanstead and Holland Parks, London and A33, from Central bureau voor Schimmel cultures, Baarns, Netherlands.

MEDIA:

MAINTENANCE MEDIA:

Stock cultures were made from single colonies and maintained on Sabouraud agar slopes at 4°C. Fungi are very prone to variation. To safeguard this, several culture slopes were made and a slope used at any one time. Fresh cultures were made every three months.

CULTURAL AND GROWTH MEDIA:

Sabouraud media, SAB, (Oxoid: CM 41); Potato Dextrose Agar, PDA (Oxoid: CM 139); Malt Extract Agar, MEA (Oxoid: CM 59); Corn Meal Agar, CMA, (Oxoid: CM 103); Czapek Dox Agar, CDA, (Oxoid: CM 97); Nutrient Agar, NA (Oxoid: CM 3); Wort Agar, WA, (Oxoid: CM 247); Yeast Extract Agar, YEA, (Oxoid: CM 19); Brain Heart Infusion Agar, BHIA (Oxoid: CM 225); and Glucose Yeast Extract Agar (GYEM) containing (g/l):

Glucose 5

Yeast Extract 1

Agar 20 pH 6.0

This was sterilised by autoclaving at 121°C for 15 mins.

DEFINED MEDIUM:

Georg and Camp (158) medium was modified for use. The basic medium consisted of (g/dm^3):

Glucose	4.0
KNO_3	2.0
$MgSO_4$	0.1
KH_2PO_4	1.8 pH 6.8

Vitamins L-Ascorbic Acid 20 µg

and d-Biotin 20 µg were sterilised separately by filtration.

The medium was dispensed into 500 cm^3 conical flasks in 100 cm^3 volumes, plugged with cotton wool and autoclaved at 115°C for 10 mins. The medium was allowed to cool and the vitamin solutions were then added to the required concentration.

All glassware used in the work was acid washed as described below.

ENZYME ASSAY MEDIUM:

The "Cup plate" method of Dingle, Reid and Solomon[159] was modified to estimate the activity of cell-free extracts.

The medium consisted of:

1% Substrate (w/v)

2% buffered agar solution

0.04% sodium azide.

The substrate and buffer were altered according to the enzyme being tested for. Plates were poured to the depth of 4 mm.

MEDIUM FOR UREASE ACTIVITY:

The medium used was by Christensen[160]. It consisted of (g/dm^3:

glucose	1
peptone	1
sodium chloride	5
KH_2PO_4	2
Agar	20
Phenol red solution (0.2% w/v)	6cm^3, pH 6.8.

The medium was autoclaved at 115^{o}C for 10 mins and cooled to 55^{o}C. Urea solution, sterilised by filtration, was then added to 20% concentration and slopes were made in universal bottles.

Gordon and Mihm medium[161] was modified. It consisted of (g/dm^3:-

NaCl	1
$MgSO_4$ $7H_2O$	0.2
$(NH_4)_2$ HPO_4	1.0
KH_2 PO_4	0.5
Organic compound	1
Agar	20

40cm^3 of 0.2% Bromothymol blue, pH 6.8. The medium was autoclaved at 115^{o}C for 10 mins without the organic compound which was added, as a sterile solution, when the medium cooled to 55^{o}C. Slopes were made in universal bottles.

ANTIMICROBIAL TESTING MEDIUM:

Wickerham's medium as adopted by Shadomy[162] was modified for use. The medium consisted of (g/dm^3):

Bacto Yeast Nitrogen Base	6.7
L. Asparagine	1.5
Dextrose	10
Phenol red	0.025
0.1M phosphate buffer 1000cm^3, pH 7.0.	

The medium was sterilised by filtration.

Medium for Photoplast Studies:

The medium by Peberdy and Gibson[163] was used. It contained (g/dm^3):

glucose	10
sodium citrate	3
KH_2PO_4	5
NH_4NO_3	2
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.1
Yeast extract	0.25 pH 6.5.

The medium was dispensed in $50cm^3$ volume into $250cm^3$ conical flasks and autoclaved.

The generating medium was prepared as above except that glucose was 5%; yeast extract was omitted and 0.4M NH_4Cl was included as an osmotic stabiliser.

CELL CULTURE MEDIUM:

The medium used was based on Street[164]. It comprised of:

Inorganic salts (Heller salts):	mg/dm^3
K Cl	750
$MgSO_4 \cdot 7H_2O$	250
$NaNO_3$	600
$Na\ H_2\ PO_4 \cdot 2H_2O$	130
$CaCl_2 \cdot 6H_2O$	110
$ZnSO_4 \cdot 7H_2O$	1
H_3BO_3	1
$MnSO_4 \cdot 4H_2O$	0.1
$CuSO_4 \cdot 5H_2O$	0.03
Kl	0.01
$FeCl_3 \cdot 6H_2O$	1

Growth Factors and hormones:

Thiamine HCl	1
Pantothenic Acid	2.5
Choline Chloride	0.5
Meso-inositol	100
Cysteine HCl	10
2.4-Dichlorophenoxy acetic acid	1
Kinetin (BDH)	0.25
Carbohydrate (Sucrose)	20,000 pH 6.8

The medium was distributed in 100cm^3 volume into 500cm^3 conical flasks and sterilised at 115^o for 10 mins. Urea (sterilised by filtration was added to the cooled medium to a concentration of 20 mg/100cm^3.

Immunodiffusion Medium:

The medium consisted of:

1% Ionagar No. 2

1M glycine

0.4M sodium barbitone

0.85% sodium chloride, pH 7.4.

Plates were poured on a level surface to a depth of 5 mm.

CHEMICALS:

Enzyme Preparations: pectinase, cellulase, α-amylase, pronase, chitinase (Sigma Chemicals Co. Ltd, London) and snail gut juice (Koch-Light Laboratories, Colnbrook, Bucks). Organic compounds for hydrolytic tests included: adenine, collagen, fibrin, xanthine, tyrosine, guanine, aesculin, (BDH, Ltd, Poole England). Polyphenolic compounds: gallic acid, tannic acid and catechol (Sigma). Organic compounds used as carbon sources: acetamide, benzamide, citrate; formate, lactate, phenol, m-cresol, p-cresol, pyruvate, oxalate, benzoate, malate, propionate, mandelate, malonate, succinate, fumarate (Fisons Ltd).

ANTIMICROBIAL AGENTS:

Amphotericin B, Nystatin and Miconazole (E.R Squibbs & Sons Ltd, Twickenham); Griseofulvin (May & Baker Ltd, Dagenham, England); Natamycin (Brocades, Great Britain Ltd, West Byfleet, Surrey); Trichomycin (Fujisawa Pharmaceutical Co. Ltd., Osaka Japan); Clotrimazole and 5. Fluorocytosine were kindly donated by Dr. Holt, Queen Marys Hospital Carshalton Surrey); Gentamicin (Nicholas Laboratories Ltd Slough, England); Phanquone (4,7-phenanthroline-5,6-quinone (Ciba Laboratories Ltd, Horsham England);Rifampicin (Lepetit Pharmaceuticals Ltd Maidenhead, England); Rapamycin (Ayerst Research Laboratories, Montreal Canada); Tetracycline (Pfizer Ltd, Sandwich, England); Ethidium Bromide (Boots Ltd, Notts, England); 5-Fluorouracil and Cytochalasin D (Sigma); 2-4 Dinitrophenol (BDH Ltd, Poole, England); Cycloheximide (Actidione-Upjohn Co. Kalamazoo, Michigan); Aculeacin A (Toya Jozo Co. Ltd, Shizuoka Japan); Polyoxin D (Kaken Chemical Co. Ltd Bunkyo-Ku, Tokyo); Ramyhyphin A (Biological Institute, Slovak Academy of Science, Bratislava).

SINGLE COLONY ISOLATION:

Fungal isolates from plants are often contaminated with other fungi. It is, therefore, essential to obtain single colonies of the fungus. This has its advantages in presenting pure cultures and again, it reveals, if more than one strain or variant is involved.

A series of 10 fold dilutions were made down to 10^{-4} from a broth culture of the original specimen. Pour plates were made using $1cm^3$ from the dilution and the plates incubated. Colonies were selected on a similarity basis.

WASHED CELLS:

In order to prevent the transfer of contaminating nutrients into defined media and also for inoculating liquid cultures, it was necessary to keep a stock of washed cells. The fungi were grown in liquid SAB. The cells were harvested in the active growth phase and washed three times in ¼ strength Ringers solution (Oxoid: BR 52) and then stored in universal containers at 4°C for subsequent use.

SPORE SUSPENSION:

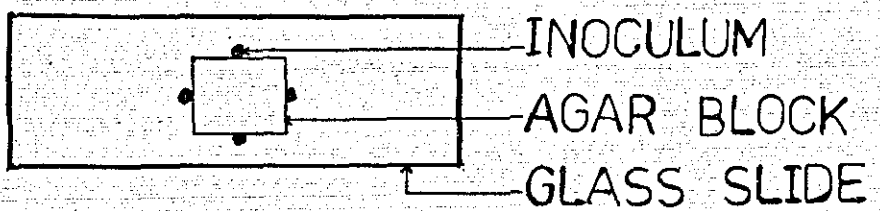
Spores from a 3 day SAB. agar culture were washed off into a 200cm^3 bottle of ¼ strength Ringer's solution. This was thoroughly agitated on a whirl-mixer and filtered through three layers of glass wool to remove the mycelial particles. The number of spores present was counted using the neubauer haemocytometer and the volume was adjusted to contain 10^6 spores/cm^3.

SLIDE CULTURE:

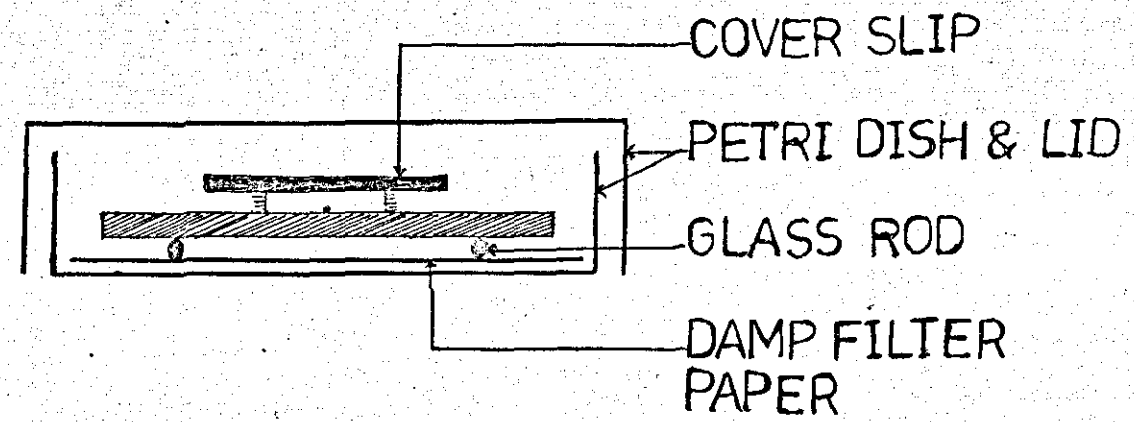
From a suitable medium an agar block (1 cm cube) was cut out and placed on a sterile microscopic slide. The block was inoculated with the culture on all four sides as in Fig 4. A sterile coverslip was then placed on the block and the slide inserted into a petri dish. The petri dish was incubated at 25°C until mycelial growth reached the coverslip, which was then removed and placed over a drop of water. The above technique was employed to prepare needle-mounts for microscopic morphological studies and photomicrography. If a stained preparation was required, lactophenol was used.

FIG 4

SCHEMATIC ILLUSTRATIONS OF SLIDE CULTURE



(a)



(b)

Preparation of Glassware:

All glassware for media preparation was soaked in dichromate-sulphuric acid cleaning solution for 3 h. Then removed and washed four times in running hot water, allowed to drain dry or dried in the oven.

Dichromate-sulphuric acid fluid:

63g sodium (or potassium) dichromate was dissolved by heating in 35 cm^3 water. This was made up to 1 litre with concentrated sulphuric acid.

GROWTH CURVE:

For growth curves of the organisms, liquid SAB. was dispensed in 100 cm^3 volume into 500 cm^3 conical flasks. These were inoculated with standardised washed cell inoculum. The cultures were shaken at 150 rpm in an orbital shaker (Gallenkamp) for 12 days, at 25°C. Measurement of growth was by dry weight as described below.

MEASUREMENT OF GROWTH, SPORULATION AND GERMINATION:

Growth was measured by linear growth rate on agar plates and dry weight in liquid medium.

LINEAR GROWTH RATE:

This was performed by measuring the colony diameter (cm) in two directions at right angles to each other[165].

DRY WEIGHT:

After a suitable period of incubation, growth was dislodged from the walls of the flasks, filtered through Whatman's No. 1 filter paper which had been previously dried at 120°C for 4 hrs, cooled and weighed. The growth was then dried overnight at 90°C, cooled in a desiccator and the weight determined.

SPORULATION:

At the end of an incubation period, a known volume of ¼ strength Ringers solution was added to the flask of medium; the growth dislodged from the walls of the flask and the cotton-plug replaced with parafilm. The flask was then shaken vigorously to free the spores from the mycelium. The suspension was filtered through glass wool and the spores counted using a haemocytometer.

GERMINATION:

This was determined by percentage germination (i.e. the percentage of spores that form a germ tube in a given time).
An aliquot from the medium was put on a slide and examined under the microscope. The mean spore germination was assessed in twenty fields and the percentage of germinating spores calculated.

BIOCHEMICAL ANALYSIS:

(1) CARBOHYDRATE (REDUCING SUGARS:)

The anthrone method [166] was used to estimate reducing sugars throughout.

Reagents:

Anthrone reagent (0.2% w/v anthrone-9, 10-dihydro-9 oxanthracene dissolved in concentrated H_2SO_4)

Glucose standards (0-100 $\mu g/cm^3$); used to prepare a standard curve of O.D. at 620 nm.

To 1 cm^3 test sample, 4 cm^3 anthrone reagent was added and mixed quickly. The tubes were covered with marbles and heated in a boiling water bath for 10 mins; allowed to cool and the absorbance read at 620 nm against a reagent blank. The test was performed in duplicates and the glucose equivalents read off a standard curve.

NITROGEN DETERMINATION:

The nitrogen content was determined by the Macrokjedahl method[167].

To 5cm^3 sample in a kjedahl flask, 10cm^3 concentrated H_2SO_4 was added, followed by one kjedahl tablet (Thomas and Capper Ltd, Liverpool). This was allowed to digest and then diluted with water when cooled. 80cm^3 of 40% NaOH was added to the flask and also a little Devardas alloy. The flask was set up for distillation; the distillate collected in a conical flask containing 50cm^3 saturated boric acid and 2 drops of methyl orange. This was titrated against 0.2 N hydrochloric acid (HCl).

PROTEIN DETERMINATION:

This was performed by the method of Hartree[168].

Reagents:

Solution A: 2 g Potassium Sodium tartrate and 100g Na_2CO_3 dissolved in 500cm^3 N NaOH and diluted to 1 dm^3 with water.

Solution B: 2g Potassium Sodium tartrate and 1g $CuSO_4 \cdot 5H_2O$ were dissolved in 90cm^3 water and 10cm^3 N NaOH added.

Solution C: Freshly prepared Folin-ciocalteu reagent (1 volume to 15 volume water)

Samples were diluted to 1 cm^3 and treated with 0.9cm^3 solution A. The tubes were heated in a water bath at 50^{o}C for 10 mins; cooled and 0.1cm^3 solution added. They were left at room temperature for 10 mins; 3cm^3 solution C was added and then heated again for another 10 mins; cooled and the absorbance read at 650nm. Reagent blank and standards (0-100 ug/cm^3 bovine albumin) were similarly treated. The concentration of protein was estimated from a standard curve.

CELL WALL PREPARATION:

Hyphae were harvested and disrupted using an X-press (LKB) with a pressure of 15,000 lb/in^2 at -30^{o}C. The procedure was repeated three times. The hyphal fragments were washed six times in 1 M phosphate buffer. Microscopic examination showed the preparation was free from cytoplasmic materials. The preparation was then lyophilised and kept at 4^{o}C for further analyses.

AMINO SUGAR DETERMINATION:

The method of Stewart-Tull[169] was applied.

Reagent: Acetyl acetone (1cm^3 in 100cm^3 bicarbonate buffer, pH 9.8 prepared fresh before use) and Erhlich's reagent.

The test was carried out by hydrolysing 50 mg sample of dried cell wall at 105^{o}C for 18h in concentrated HCl; drying off the hydrolysate and dissolving the residue in 2cm^3 water. 5.5cm^3 acetyl acetone was mixed with the hydrolysate and heated in boiling water for 20 mins. 4cm^3 of this was then added to 6cm^3 Erhlich's reagent and the absorbance read at 545 nm. Reagent blank and standards (glucosamine and galactosamine) were similarly treated. The concentration of amino sugars was read off a standard curve.

IDENTIFICATION OF CHITIN:

Chitin was identified by the method of Applegarth[170]. 10 mg sample (cell wall preparation) was extracted with 5cm^3 1N acetic acid at 100^{o}C for 20 mins. The acid extract was removed by centrifugation and grams iodine added dropwise. No reaction at this stage meant the absence of chitosan. The residue was then extracted with 24% potassium hydroxide at 160^{o}C. The resulting solution was acidified with 0.5cm^3 concentrated H_2SO_4 and iodine solution added again. A red colour indicated the presence of chitosan, formed by alkaline degradation of chitin. Chitosan and chitin were used as controls.

Chitin was quantitatively estimated by the fractionation procedure of Mahadevan and Tatum[171]. 100mg of cell wall preparation was hydrolysed in 2N NaOH for 16 h at 25°C. The residue was treated with IN H_2SO_4 for 16 hr at 90°C and the residue from here, was furthermore treated with 2N NaOH for 30 mins at 25°C. The final residue, chitin, was then dried and weighed.

ENVIRONMENTAL AND NUTRITIONAL FACTORS AFFECTING GROWTH, SPORULATION AND GERMINATION

Effect of Media Composition and Temperatures on:

Growth:

All the growth media given above were inoculated with an agar block culture (2mm) of each strain. The plates were incubated at the following temperatures; 0°, 5°, 10°, 15°, 20°, 25°, 30°, 37°, 40°, and 45°C for 7 days and examined daily.

For each determination, 6 replicates were carried out except when otherwise stated.

Sporulation:

It was found that sporulation was higher on a solid medium than in a liquid medium. Therefore a solid medium for sporulation studies was used. The defined medium was solidified with 2% (w/v) Oxoid agar No. 3. This medium was prepared in 50cm^3 volumes in 250cm^3 conical flasks covered with cotton-wool plugs. The flasks were inoculated by flooding the surfaces with 1cm^3 of spore suspensions containing 10^6 spores per 1cm^3. Incubation was carried out at the specified temperatures for 7 days.

Germination:

The liquid defined medium was inoculated with 1cm^3 of spore suspension and incubated at the specified temperatures. Samples were withdrawn at timed intervals and examined under the microscope for germ tube production.

EFFECT OF pH ON GROWTH, SPORULATION AND GERMINATION

GROWTH:

The pH of SAB agar was adjusted after autoclaving with 4N NaOH or 4N HCl to cover the pH range 2 to 12. The pH adjustment could not be made before autoclaving since the agar failed to set below pH 4. Furthermore any drop in pH during autoclaving could also be compensated for. Plates were poured and inoculated using an agar block culture and incubated at 25°C for 7 days and the radial growth measured daily.

SPORULATION:

The solidified defined medium was used and the pH adjusted as above. The surface of the medium was flooded with $1cm^3$ spore suspension and incubated for 7 days at 20°C.

GERMINATION:

The liquid defined medium was used and the pH adjusted as above. The medium was inoculated with $1cm^3$ spore suspension and incubated at 25°C and the germination of the spores examined.

EFFECT OF CARBON DIOXIDE ON GROWTH AND SPORULATION:

SAB agar plates were inoculated with agar block culture and incubated in various concentrations of carbon dioxide. The BBL Gaspak system was used to give the required carbon dioxide atmosphere. The plates were incubated at 25°C for 3 days with A30 and 6 days for the other strains. Growth was compared to culture grown in air which was taken as 100% growth.

SPORULATION:

The defined agar medium was poured into petri dishes. These were inoculated by flooding the surfaces with $1cm^3$ spore suspension and incubated as above at 20°C for 7 days.

EFFECT OF LIGHT ON SPORULATION AND GROWTH:

SPORULATION:

The defined agar medium was inoculated with spore suspension ($10^6/cm^3$) and incubated at 20°C for 7 days in sets of 6 replicates, one set was in continuous light; another in continuous darkness and a third set alternately in light and dark at 12 hourly intervals. The light intensity applied was 300 foot candles/$inch^2$ (172).

The effect of light on growth was determined as above on SAB agar but with an incubation temperature of 25°C.

From the growth test on various media it was observed that A30 had the tendency to grow radially in zones and this was most pronounced on SAB. The strain was, therefore, used to investigate the effect of light on zonation. The test was performed on SAB agar medium, in which the various sources of nutrients were either substituted or omitted. The plates were inoculated and incubated in sets of 6 replicates under varying conditions:

(a) exposure to direct day light;
(b) in continuous darkness;
(c) in continuous light;
(d) 3 h exposure to light followed by 21 h darkness;
(e) incubated alternately at 12 hourly intervals at 25°C and 30°C.

All the plates except (a) and (e) were incubated at 20°, 25°, 30° and 37°C.

EFFECT OF GLUCOSE AND SODIUM CHLORIDE:

Graded concentrations of either glucose or sodium chloride were prepared in SAB agar and inoculated with an agar block culture. They were then incubated at 25°C for 21 days and examined regularly.

HEAT RESISTANCE OF SPORES:

Washed spore suspensions ($10^4/cm^3$) were made in $10cm^3$ saline contained in 6 x ¾ in pyrex glass test tubes. These were incubated at various temperatures in water baths. At timed intervals, $0.03cm^3$ of each sample was spread over SAB plates and incubated at 25°C for 24 h . The colonies produced were counted and the percentage of surviving spores calculated.

NUTRITION:

The defined medium was used throughout and compounds were omitted or altered according to the experiment.

The nutritional factors under investigation were left out before autoclaving the medium and added subsequently as sterile solutions. In all cases control systems, in which the nutritional factors were omitted, were included.

To test for the ability to utilize a certain nutrient, the appropriate medium was inoculated with $1cm^3$ washed cells and incubated in an orbital shaker at 25°C for 7 days except where specified. The residual nutrients were analysed. For the sporulation test the defined agar medium was used as previously described. The germination rate was studied in the liquid defined medium which was inoculated with $1cm^3$ spore suspension ($10^6/cm^3$), incubated at 25°C and samples examined after 24 h .

BIOCHEMICAL CHARACTERISATION:

CELL WALL DEGRADING AND OTHER HYDROLYTIC ENZYMES:

The test organisms were grown in the liquid medium incorporating 1% w/v of appropriate subtrate at 25°C for 7 days. The amount of growth was determined by dry weight. The cell free extract was assayed for the presence of the enzymes. The protein and reducing sugar content of the extract, as well as the reducing power, were estimated by the methods described earlier.

ENZYME ASSAY:

Cups, 4mm in diameter, were cut out in the assay plates and the cups were filled with 0.5cm^3 of a range of standard enzyme preparations, as well as test enzyme extract. After suitable periods of incubation, the plates were developed with appropriate agents where applicable and the zone diameter measured. The activities were read off a standard curve.

For Pectinase activity, the substrate used was sodium pectate (BDH) in 0.2M phosphate buffer (pH 5.3) with 0.5% (w/v) ammonium oxalate added. The plates were developed with 5N HCl, which gave clear zones around the cups on an opalescent background.

The substrate for Cellulase activity was sodium carboxymethyl cellulose suspended in sodium acetate buffer at pH 4.5. The plates were developed with 10% (w/v) solution of copper acetate. When cellulase was produced a clear zone on an opalescent background was observed.

α-amylase: Soluble starch in 0.3M phosphate buffer at pH 4.5 was used as the substrate and iodine solution used to develop the plates. A clear colourless zone on a blue background indicated a positive test.

Protease: Casein and gelatin were used as substrates.

The organisms were grown in the defined medium with 1% casein and the cell-free extract was used in the cup plate, which was made with 1% skimmed milk as the substrate. Where a protease was present a clear zone with a hazy background was observed.

Gelatin: Nutrient agar deeps with 1% gelatin were prepared. These were stabbed with the cultures and incubated at 25^{o}C. After 7 days, the bottles were left overnight at 4^{o}C. Liquefaction of the medium indicated hydrolysis of the gelatin.

Lipase Production: This was examined using 1% of the substrate incorporated in YEA. The substrates include the tweens (20, 40, 60 and 80), tributyrin and triolein. Plates were incubated for 7 days with daily examination for precipitates around the colonies.

Oils. (clove, olive, palm and rape) were also used to test for the production of lipase. YEA medium containing 5% concentration of the substrate and 10% (v/v) Victoria Blue B salt (1:1500 in water) was prepared. Before pouring, the medium was shaken vigorously to disperse the substrate. The plates were inoculated and incubated for 7 days with daily examination for blue surround to colonies on a pinkish background.

Phospholipase Production: Production of phospholipase was examined on YEA with the addition of 1% (w/v) sodium chloride and 10% (v/v) egg yolk (Oxoid: SR 47). Enzyme production was indicated by opacity around the colonies.

Nuclease: activity was tested using 1% (w/v) deoxyribonucleic and ribonucleic acids in YEA. At the end of the incubation period the plates were flooded with 5N HCl. A clear zone around the colonies showed hydrolysis of the nucleic acids.

Urease Activity: Urea hydrolysis was indicated by the production of a red colouration in the medium by the test organisms.

Production of Polyphenyl oxidase:

YEA medium supplemented with 0.1% (w/v) of substrate was used for the test. Brownish colouration around colonies signified enzyme production.

Hydrolysis of Organic Compounds:

YEA medium incorporating 1% w/v of substrate was used to demonstrate the hydrolytic activity. Clearance of the suspension under colonies indicated activity.

Aesculin hydrolysis was examined separately on YEA medium to which were added 0.1% (w/v) aesculin and 0.05% (w/v) ferric citrate. Production of a brownish colouration showed hydrolysis.

Utilization of Organic Compounds as Sole Carbon and Energy Source:

The medium of Gordon and Mihm [161] was modified for use. The slopes were inoculated with washed cells, incubated at 25°C for 21 days and examined daily for an indicator change from green to blue demonstrating the utilization of the compound. 6 replicates including control systems were carried out.

PROTEIN PROFILE AND ZYMOGRAMS:

Cellular proteins were prepared by the method of Gill and Powell [173]. Mycelia were harvested and homogenised in 0.1M phosphate buffer (pH 7.0) at 4°C. The supernatant fluid from the homogenate was centrifuged at 48,000g for 1 hr at 4°C. The final supernatant fluid was then lyophilised. The protein level was standardised to 300 µg/cm^3 for electrophoresis.

The electrophoresis was carried out by the Disc Acrylamide method as modified by Tombs and Akroyd [174] using the Shandon Disc Electrophoresis apparatus.

Reagents:

Buffer: Tris-glycine, pH 9.4, (0.08M Tris and 0.022M glycine)

Gel: Cyanogum 41 (BDH)

Initiator: β-dimethyl-amino-propionitrile

Catalyst: 7% w/v ammonium persulphate

15% w/v sucrose solution in buffer.

Procedure: The gel was prepared by adding 1cm^3 initiator and 2cm^3 catalyst to 30cm^3 of 8% cyanogum 41 solutions in buffer. Running tubes (Shandon) were filled with this solution.

Sample Application: Samples (lyophilised) were solubilised in the sucrose solution and applied in appropriate volumes.

Electrophoresis: An initial current of 1.25 mA per tube was applied for 5 mins to concentrate the protein on the gel and then increased to 5mA.

Staining: The gels were stained in 1% amido black and destained in 2% acetic acid:

An estimate of the relative amounts of protein in each band was obtained by densitometry.

ZYMOGRAM:

The procedure for Amidases was carried out by the method of Hagenmaier[175]. After electrophoresis, the gels were immersed in a solution of L-leucyl-β-naphthyl amide HCl for 30 mins at 37°C, washed and put in Fast Blue B salt solution until the colour was fully developed. The method of Uriel and Berges[176] was applied for the Esterases. The gels were immersed in a solution of α-naphthyl-acetate or acetyl-DL-phenylalanine β-naphthyl ester in which Fast Blue B salt was dissolved for 1 h and washed off in 2% acetic acid.

MICROSCOPY:

Light: Photomicrographs were taken from needle-mount preparations or slide culture from the various media using Vickers M 25 series microscope. If preparations were required stained, lactophenol blue was used.

ELECTRON MICROSCOPY: Scanning (SEM) and Transmission (TEM);

Hyphae were harvested at the active phase from liquid media by centrifuging, at 4000g at 4°C and washed with ¼ Ringers solution. Spores were washed off solid media; washed thrice and filtered through glasswool to remove the hyphae. Subsequent examinations by light microscopy showed the absence of hyphal fragments.

SEM materials were prepared by the method of Bulmann and Stretton[177]. Glutaraldehyde (G.T. Curr, Bucks) was added to washed cell suspensions to give a concentration of 1.5% (v/v). After 2 mins contact, the cells were removed by centrifugation at 3000g at 4°C for 15 mins. and resuspended in 5% (v/v) glutaraldehyde for 16 h at 4°C.

The cells were centrifuged, washed three times in distilled water and resuspended to an appropriate density. One drop of this suspension was then air-dried on a cover-slip in a vacuum desiccator. The samples were then coated with gold-palladium in a high vacuum unit to obtain a coating of approximately 10nm thickness and examined under Cambridge Stereoscan (Mk 11A Cantab Instruments Co. Ltd) with a beam angle of 45^{o} using a 30 Kv voltage.

TEM:

Reagents:

Fixatives: Glutaraldehyde

Potassium permanganate, $KMnO_4$

Osmium tetroxide, $Os\ O_4$

Buffer: Phosphate buffer (0.1M pH 7.2)

Cacodylate - HCl. 0.1M (pH 7.0)

Solution A: Sodium cacodylate: 47.8g
($Na\ (CH_3)\ As\ O_2 - 3H_2O$).
in 1000 cm^3 water

Solution B: 0.2M HCl conc (36-38%) 10cm^3
in 603cm^3 water.

Solution B was added to solution A to obtain the required pH.

Dehydrating agent: Graded alcohol - 20,50,70,80,95 and 100%

Clearing agent: Propylene oxide

Embedding agents:

Araldite mixture consists of:

Araldite (Polaron Equip Ltd Watford) GY 212: 27cm^3

Hardener (Polaron) HY 964: 23cm^3

Acelerator (N-Benzyl-dimethylamine-Polaron): 1cm^3

Two techniques for TEM were applied.

(1) O_sO_4: Samples (washed) were suspended in 3% glutaraldehyde in phosphate buffer for 1 h ; The cells were centrifuged and resuspended in 1% O_sO_4 for another 1 h ; washed three times and dehydrated. The cells were suspended in 2% agar and the agar cut into small cubes (1mm) and treated as samples. After dehydration the samples were cleared and embedded in capsules. These were kept at 60° for 48 h. Sections were then cut with a Cambridge Huxley Mk 11 Ultra microtome equipped with glass knives and picked up on copper grids. They were then examined with AEI EM6G electron microscope unstained or post stained for 20 mins in uranyl acetate (70% v/v in ethanol) and 5 mins in lead citrate (0.04% in 0.1N NaOH carbonate free - Taab Laboratories, Reading).

(11) $KMnO_4$:

Samples were fixed in 2.5% glutaraldehyde in 0.01M sodium cacodylate buffer at 4°C for 2 h, centrifuged and resuspended in freshly prepared 2% $KMnO_4$ solution at 4°C for 2 h and the procedure repeated at room temperature. Samples were centrifuged and washed four times in distilled water; pre-stained in a 1:1 solution of 1% potassium dichromate and 1% uranyl acetate for 2 h at 4°C. They were washed again, dehydrated; cleared and embedded as previously described.

Shadow-Casting

Hyphae were harvested washed in distilled water and dried on grids coated with carbon. The grids were then shadowed with Au/Pd (60/40) at an angle appropriate to the thickness of the cells (45°) and examined under the electron microscope. All the processes were performed under high vacuum except the mounting of samples.

MYCELIAL WALL COMPONENTS:

Amino acids

The amino acids were liberated from the cell walls by hydrolysing 20 mg of cell wall preparation with 5cm^3 6N HCl in a sealed ampoule at 100^{o}C for 18 h. The hydrolysate was centrifuged and the supernatant evaporated to dryness. A solution of this was made in water. 2 µl of each sample and standards were examined by paper chromatography (Whatmans No. 1). The chromatograms were developed with n-butanol-acetic acid-water (12:3:5) for 18 h and sprayed with 0.1% ninhydrin in acetone. The amino acid spots were located by heating at 105^{o}C for 3 mins.

Quantitative analysis was performed at Unilever Research Laboratories, Bedford. The samples were hydrolysed for 18 h under reflux in 6N HCl (0.15 mg/cm^3) in a nitrogen atmosphere and aliquots of this analysed.

Carbohydrates:

Qualitative analysis of the carbohydrates was performed by thin layer chromatography (TLC). 50 mg of cell wall preparation was hydrolysed with 3cm^3 INH_2SO_4 in a sealed ampoule for 2 h at 100^{o}C. Saturated barium hydroxide solution was added dropwise until the pH was between 5.2 and 5.5. The precipitate was removed by centrifugation and the supernatant evaporated to dryness. A solution of the residue was made in water and used.

The sugars were separated by TLC using silica gel C plates of 0.20 mm thickness (E. Merck, Dermstadts). The chromatogram was developed in n-butanol-pyridine-water (6:4:3) and the spots visualised with acid aniline phthalate. Ninhydrin spray was used to detect the amino sugars.

Quantitative analysis was carried out by gas liquid chromatography (GLC) based on the method of Sweeley et al[178].

Trimethylsilyl (TMS) derivatives were made from dried cell wall preparations and also dried hydrolysates using Tri-sil (Pierce Chemical Co. Ltd). The derivatives were separated in a Pye series 104 chromatograph, fitted with a 6 ft glass column packed with 3% SE 30 CQ (Phase Separations Ltd., Deeside) and also 10% Apiezon L on Chromasorb W (Applied Science Laboratories).

Individual sugars were identified by comparing their retention times with those of standards and were estimated by the triangulation method[179]. The area under each peak was estimated by multiplying the peak weight and the distance the base makes with the centre of the peak. This was then expressed as a percentage of the total content.

Lipids:

The method applied was based on the procedure of Bartnicki-Garcia and Nickerson[124].

"Readily Extractable Lipid" was determined by extracting 100mg cell wall sample with 50cm^3 ethanol-ether(1:1) for 24 h at room temperature. The supernatant from three successive treatments was further extracted with chloroform and evaporated to dryness in nitrogen and the weight determined.

"Bound Lipid", was determined on the previous residue by extracting twice with hydrochloric acid/ethanol/ether mixture; twice with the ethanol/ether mixture and again with chloroform. The residue was then dried and weighed. The procedure was carried out in duplicate. Blank determinations were also carried out.

ENZYMATIC TREATMENT OF CELLS:

Hyphae were harvested and washed in ¼ Ringers solution. The cells were incubated at 30°C in the enzymes cellulase; pronase; chitinase and snail gut juice separately and sequentially [125]. Control experiments were carried out for each treatment with boiled enzyme to ensure that buffer alone has no effect on the cells. The cells were examined by the Shadow-casting technique.

ANTIMICROBIAL ACTION ON CELLS:

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Cidal Concentration (MCC):

The MIC values were determined by the tube dilution method using the modified Shadomy's medium.The test was performed in duplicate including control systems to check;

(a) sterility of the drugs and materials (drug in medium without organism) and

(b) the efficiency of the medium (medium without drug plus organisms).

The MCC was achieved by plating out the tubes showing no growth on the solid medium.

Morphological changes effected by the agents were studied by using drugs at different sublethal concentrations and examined by microscopy.

PRODUCTION AND REVERSION OF PROTOPLASTS:

Cells were harvested and washed in 0.2M phosphate buffer at pH 6.0 and resuspended in the same buffer with 0.4M NH_4Cl added. Equal volume of cell suspension was added to the lytic enzymes (snail gut juice and chitinase separately and to both combined) and incubated at 30°C. Release of the protoplasts was determined microscopically at an interval of 3h and left overnight. The protoplasts were collected by centrifuging at 500g for 10 mins.

Reversion of Protoplasts:

The protoplasts were washed three times with 0.2M phosphate buffer (pH 6.0) containing 0.4M NH_4Cl as a stabiliser and finally resuspended in the generating medium. The culture was incubated at 25°C and examined three hourly for revertants.

PLANT INOCULATION AND PHYTOALEXIN DETECTION

Potted sycamore (Acer pseudoplatanus) saplings were grown in the laboratory and allowed time to adjust.

Procedure For Inoculation:

The plant surfaces were cleaned with alcohol. Using a sterile scalpel, a v-shaped piece of wood was cut; lifted backwards and the spot infected with a moistened spore block from A30. The piece of wood was replaced and the plants left to grow. The saplings were sectioned at intervals of one, three and six months and examined for the presence of phytoalexin and the effect of the fungus on the plant. Some of the sections were planted in agar medium for reisolation of the fungus.

EXTRACTION AND DETECTION OF PHYTOALEXIN:

The method of Letcher et al[180] was modified for use. Sections were macerated in 80% ethanol (500g to 200 cm^3 w/v) and shaken at 4°C. The extract was filtered off and evaporated to about 50cm^3. To this, was added 50cm^3 5% Na_2CO_3 and extracted four times with ether. The ethereal extract was evaporated to near dryness. 1 cm^3 CH_2Cl_2 was added and the soluble portion was used for chromatography in methanol - CH_2Cl_2 (1:5) solvent on silica gel plates. After running the plates were dried and viewed under U.V. light for blue fluorescent bands.

A second technique was also tried. Extraction was started in methanol (500g: 200 ml w/v) shaken at 4°C overnight. The extract was decanted off and process repeated three times. The extract was combined and evaporated to dryness under nitrogen. The residue was then dissolved in ether and chromatography performed in Hexane: acetone (2:1) solvent, followed by another run in chloroform:petrol (2:1). The plates were examined under UV. light.

STAINING OF SECTIONS:

Sections (5mm) were fixed in 5% formalin overnight. These were further sectioned on the microtome to 10µ; passed down to water and stained by Stoughtons method.

Technique:

(i) Stains for 1 h in 0.1% thionin (BDH) solution in 5% aqueous phenol

(ii) Dehydrate in successively stronger alcohol

(iii) Differentiate in a saturated solution of Orange G (BDH)

(iv) Wash in absolute alcohol

(v) Clear in xylene and mount in Canada Balsam.

Parasites:	Violet Purple
Cellulose Wall:	Yellow or Green
Xylem:	Blue
Chromosomes:	Blue
Spindle:	Purple

CELL CULTURE:

The cell culture medium was inoculated with a callus of sycamore cells (kindly supplied by Dr. N. Robertson, Leicester University) and shaken at 25°C.

Cell growth was determined by dryweight and packed cell volume (PCV). The cells were also examined microscopically.

Fungal Growth: The fungus was grown in the medium and the cell-free extract was tested for the production of cell wall degrading enzymes.

INFECTION OF SYCAMORE CELLS:

A large suspension of cells (200 mg/cm^3 dry weight) was infected with a spore suspension and incubated at 25^{o}C in an orbital shaker. The effects on the cells were examined microscopically and production of enzymes was again tested. Fresh cell suspension was also treated with the enzymic extract.

SEROLOGY:

Preparation of Antisera:

Actively growing cells were harvested and washed in saline. The mycelia were homogenised in a blender and the spores adjusted to contain 10^6 spore/cm^3. Thiomersalate (0.04 % w/v) was used as a preservative. These suspensions were diluted and used as the antigens for injection starting with the lowest dilution up to the undiluted suspension. Following the regime of injection, 1cm^3 from each series of suspension was injected into the ear lobe of a rabbit at an interval of 7 days. The regime was completed by injecting with 1cm^3 of live suspension. The rabbits were bled after 7 days and the serum collected in bottles containing thiomersalate. Necropsy was then performed on the rabbits.

Antigen Preparation:

The cell suspensions were prepared as for injection but were shaken in 0.2M pyridine-acetic acid buffer (pH 5.0) for 48 h at room temperature.

IMMUNODIFFUSION:

The technique of Ouchterlony[181] was used. Wells, 4mm in diameter, were cut on the plates 5mm apart and filled with $0.2cm^3$ fluid. The plates were incubated at 37^oC for 18 h. An extract of Staphylococcus aureus and saline were included as controls.

Antigenic Components:

Mycelia and spores suspensions were treated with the enzymes, cellulase, pronase, α-amylase, separately and sequentially for 24 h at 37^oC. The extracts from these treatments were used as antigens in the diffusion plates.

CHAPTER FIVE

RESULTS

THE GROWTH AND CULTURAL CHARACTERISTICS OF C. CORTICALE:-

The growth on all the growth media used was compared.(Fig 5a,b) Strain, A30, produced very light, faint and mainly mycelial growth on CMA and CDA. On NA, YEA and BHIA, growth was thick and slow. Growth on PDA, MEA and GYEM was similar; fast and sparse mycelia were produced with floccose hyphae and a large number of spores (Plate 1) and on SAB and WA growth was also fast and floccose. The mycelium was thicker with extensive stroma and spores were produced abundantly (Plate 2).

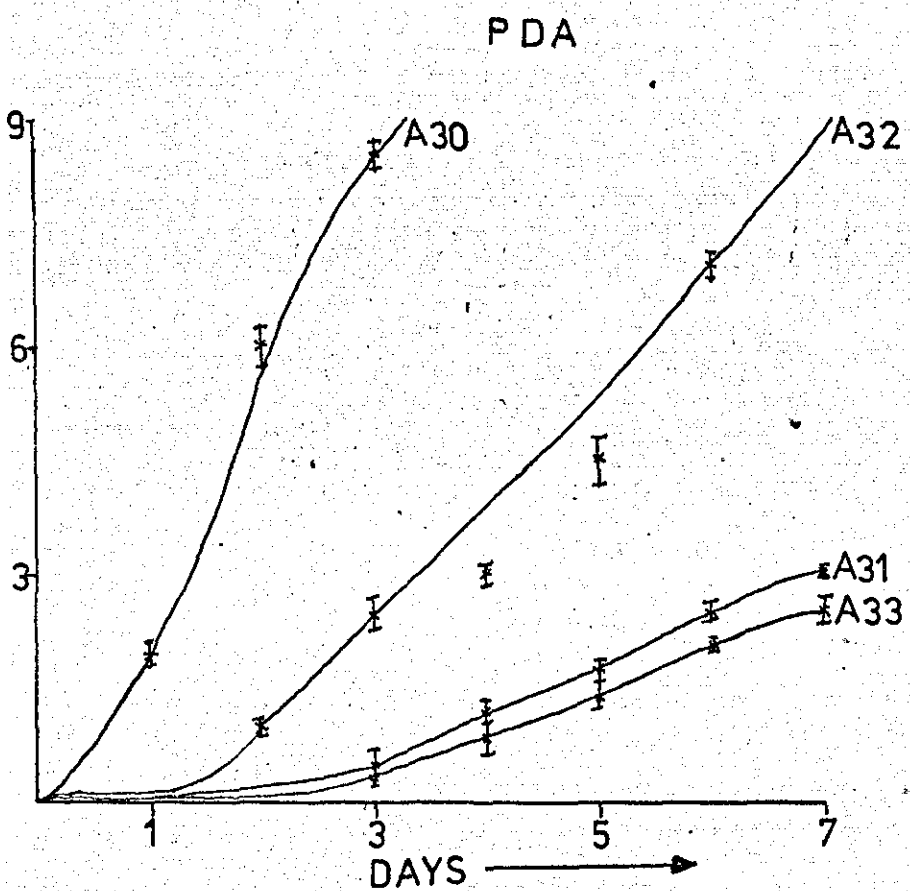
The other strains also produced better growth on SAB. SAB was, therefore, made the medium of choice for future experiments.(Fig 6)

THE CULTURAL FEATURES ON SAB AT 25^{o}C:

A30: This strain produced a fast growth under optimum conditions covering a 9cm diameter plate in 4 days. The colony started quite floccose and radial on the periphery with a powdery (spores) centre and a whitish mat-like structure, the Stroma,covering the base of the mycelium. The spores were white at first and progressively turned dark green. Thus, giving the colony its characteristic appearance of a thick greenish central spore zone in the centre with a buff coloured floccose and peripheral growth having moderately long hyphae. No pigment was produced. This appearance, however, differs from that seen on infected sycamores, where it has a dark brown colour.

Under the microscope, the mycelium consisted of septate hyphae which were hyaline when young and darkly stained when old. Conidiogenous cells (phialides) either grew directly from the hyphae or were produced at the apex of short lateral conidiophores singly or in clusters. The conidia were endogenous with hyaline walls and typically

FIG 5(a) GROWTH ON VARIOUS MEDIA AT 25°C



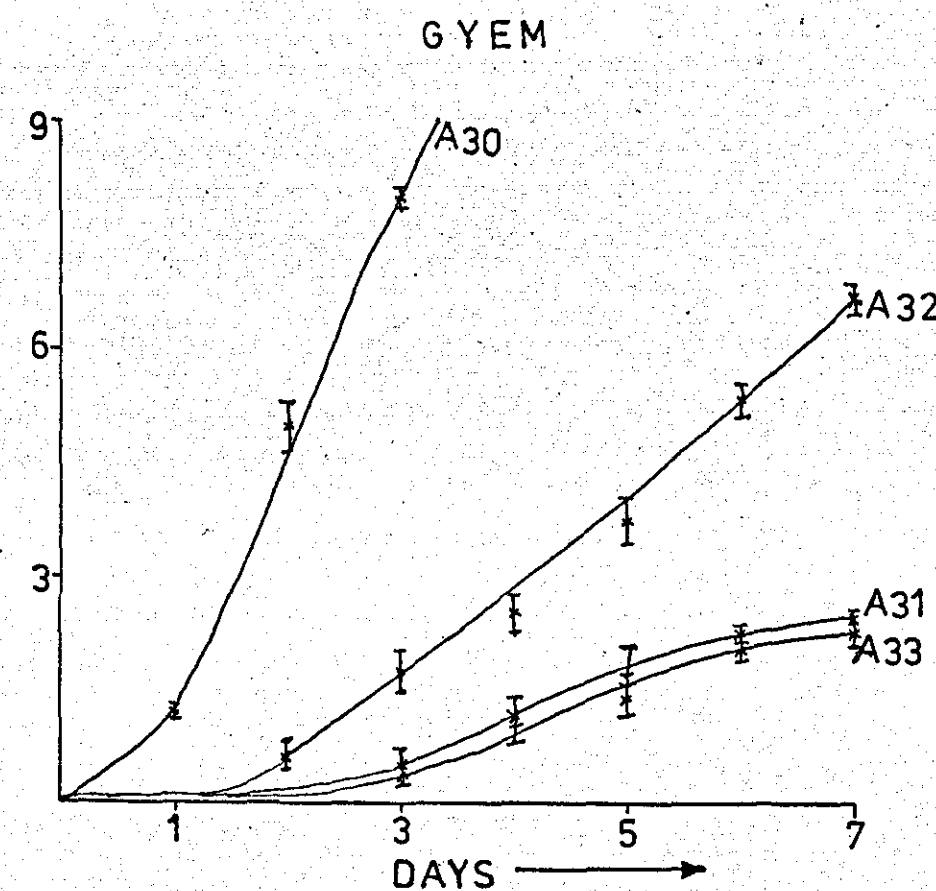
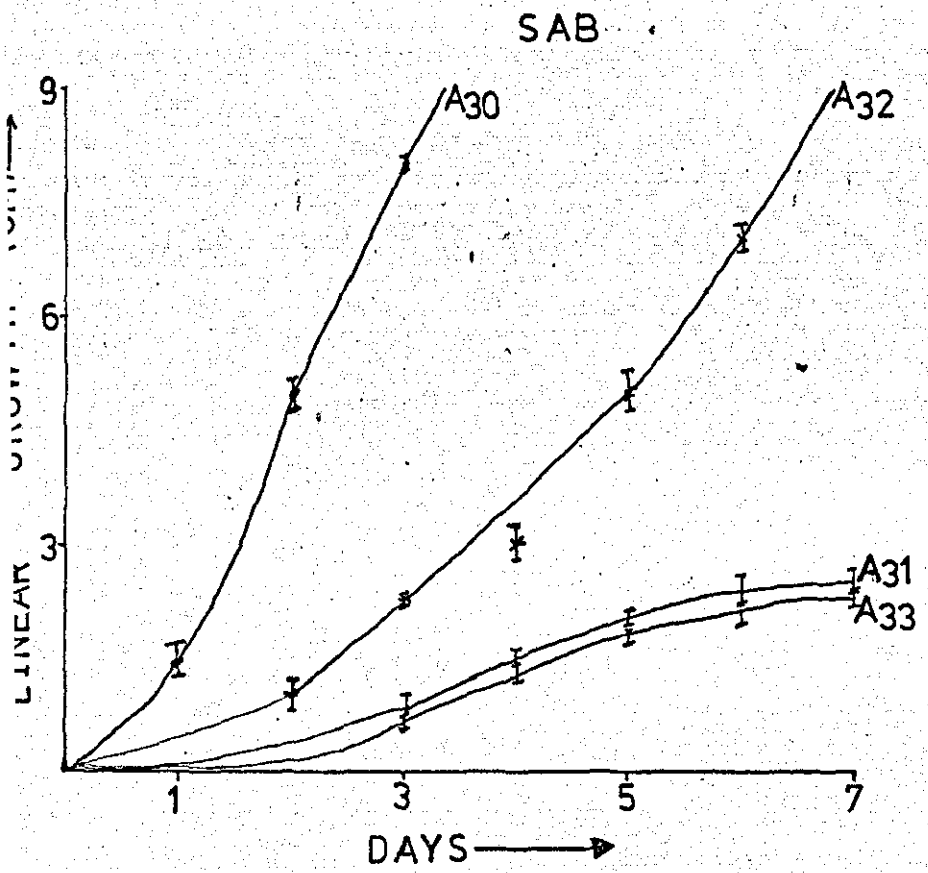


FIG 5(b) GROWTH ON VARIOUS MEDIA AT 25°C



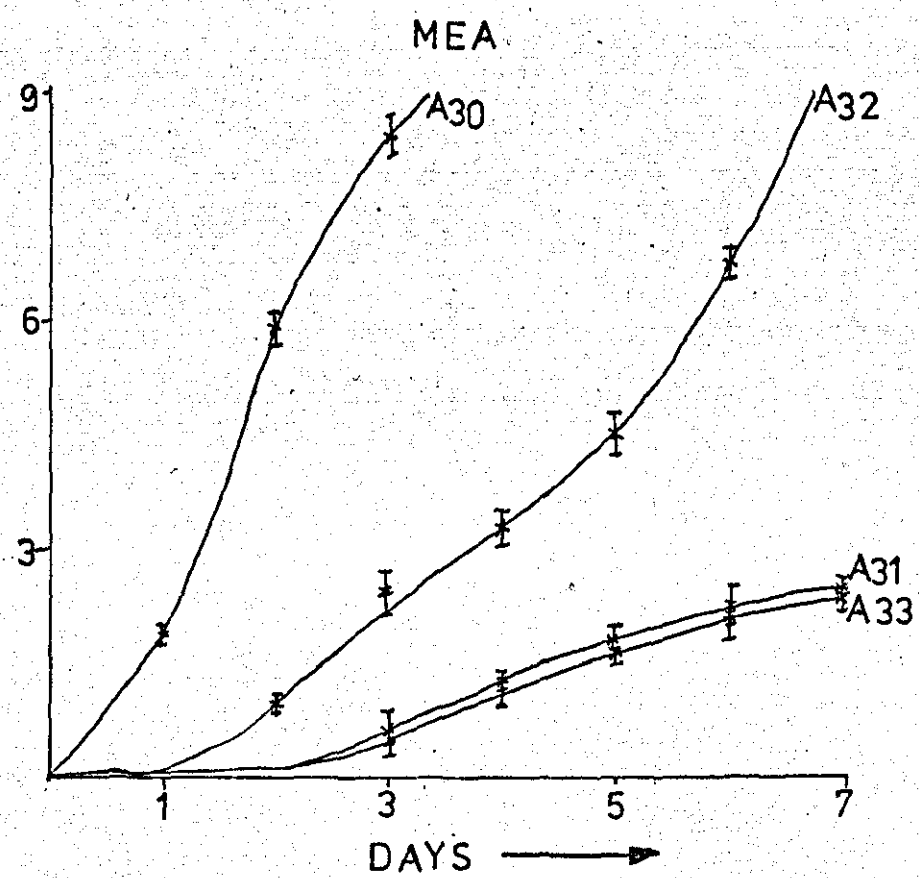


FIG 6

GROWTH CURVE ON SAB AT 25°C

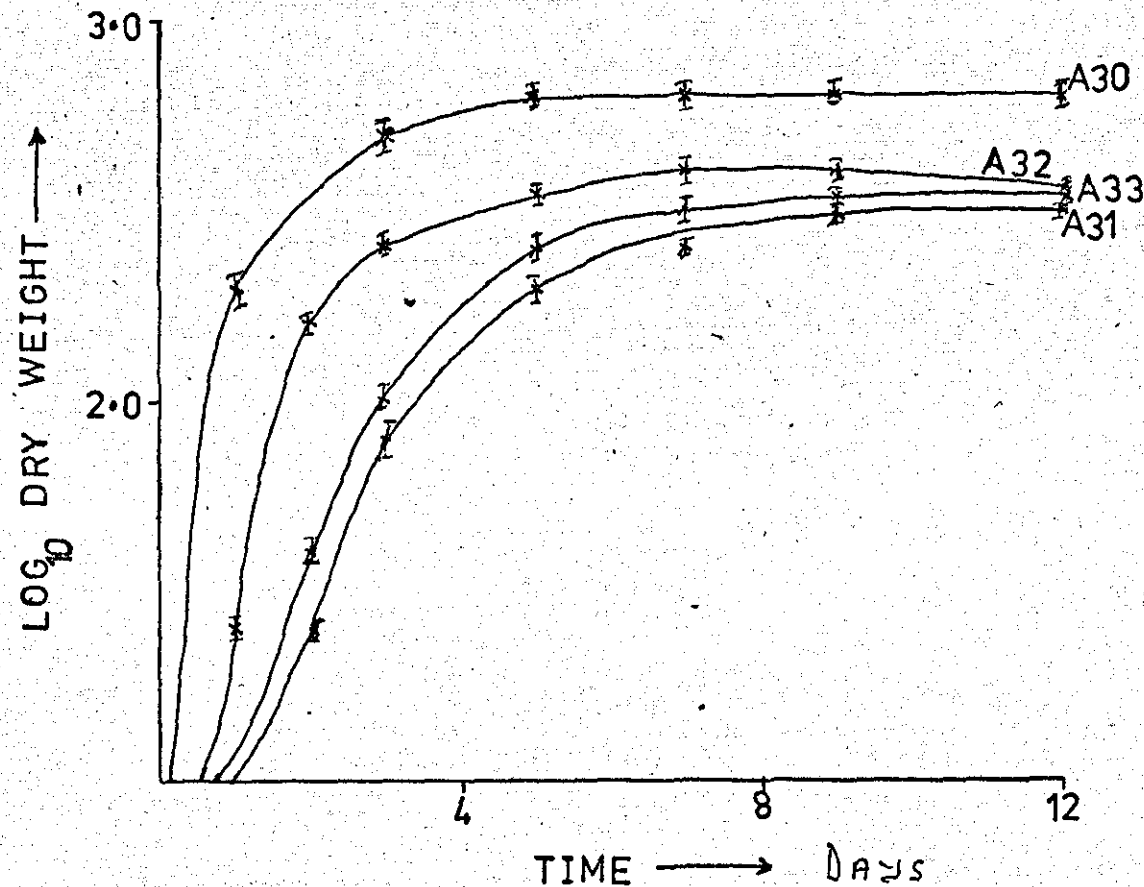
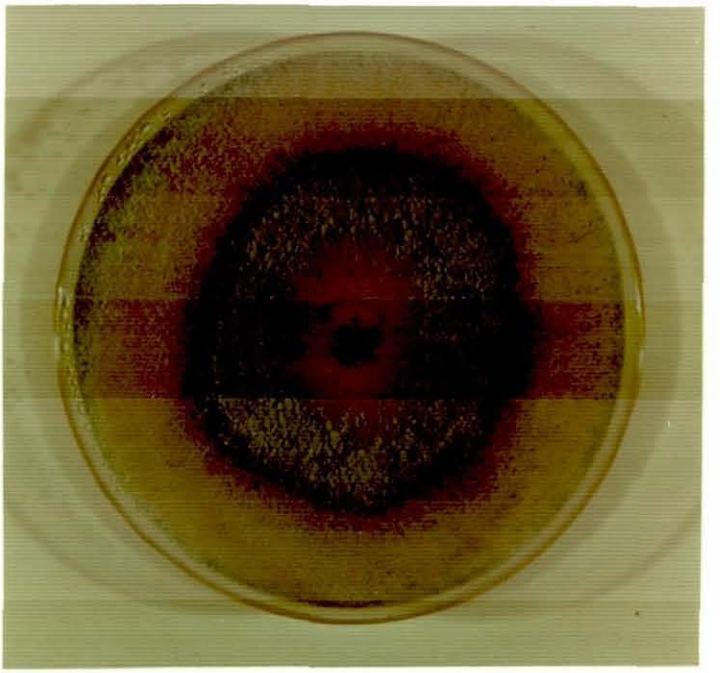
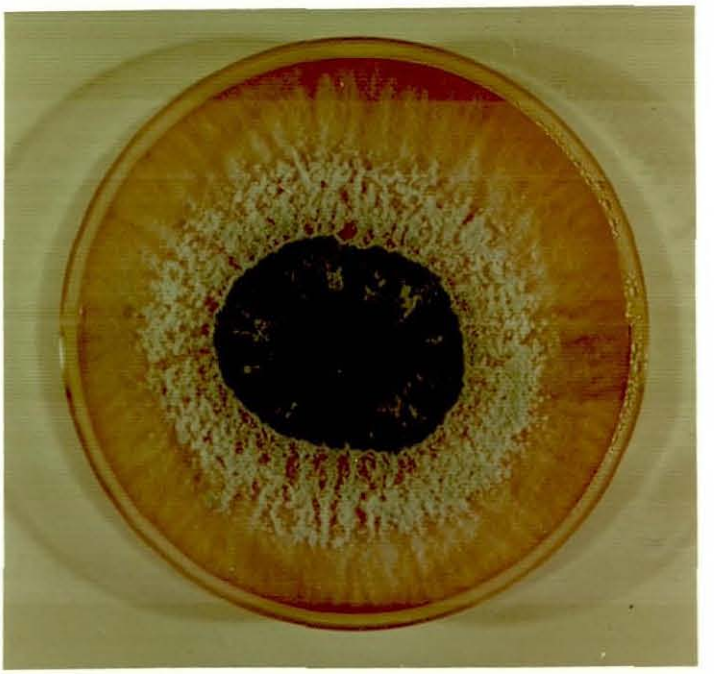


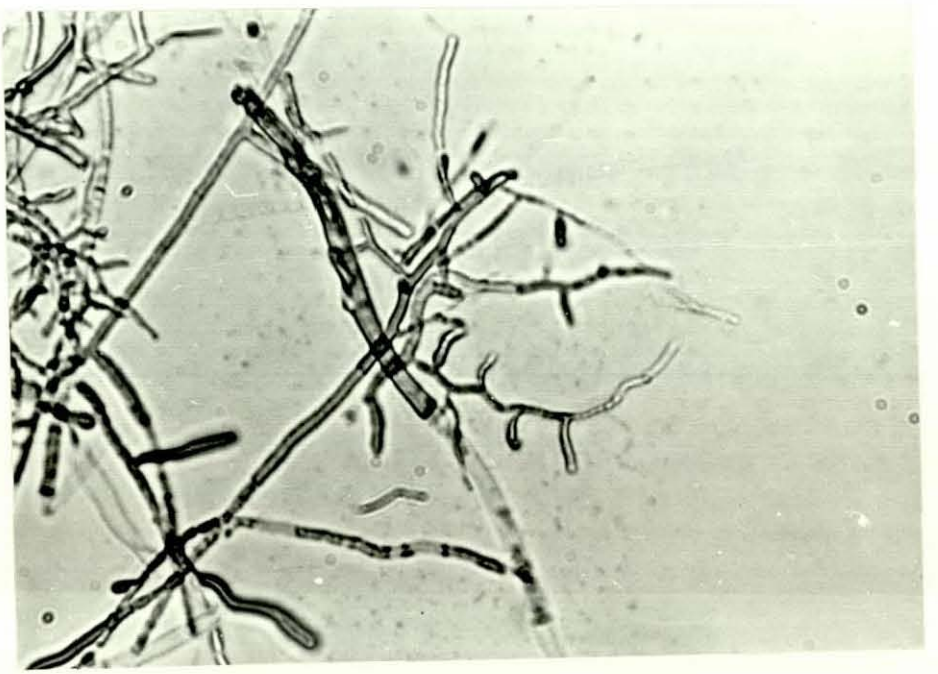
Plate 1. - Cultural plate showing a colony of strain, A30, on PDA incubated for 4 days at 25°C.

Plate 2. - Cultural plate showing a colony of A30 on SAB. incubated for 4 days at 25°C.

Plate 3. - Light micrograph of A30 grown on SAB for 4 days at 25°C. Septate hyphae and phialides borne singly are present x 400.







ovoid shape but could be globose owing to external pressure. They were amerosporous and glabrous; produced singly, in clusters or in chains and varied in size; the smallest measuring (on the average) 2.5 x 4μ and the largest 4.5 x 6μ (Plate 3) .

A31: This grew slowly attaining only a maximum of 3 cm in diameter in a 7 day period under optimum conditions. The organism grew into a thick whitish and slightly floccose colony with a characteristic elevation at the centre. A depression occurred around the agar which was an indication of the thickness of the colony. The organism neither sporulated readily nor produced pigment. However, the reverse surface was darkly coloured, (Plate 4).

The mycelium consisted of septate hyphae. There was an absence of conidia but abundant hyphal swellings (Plate 5).

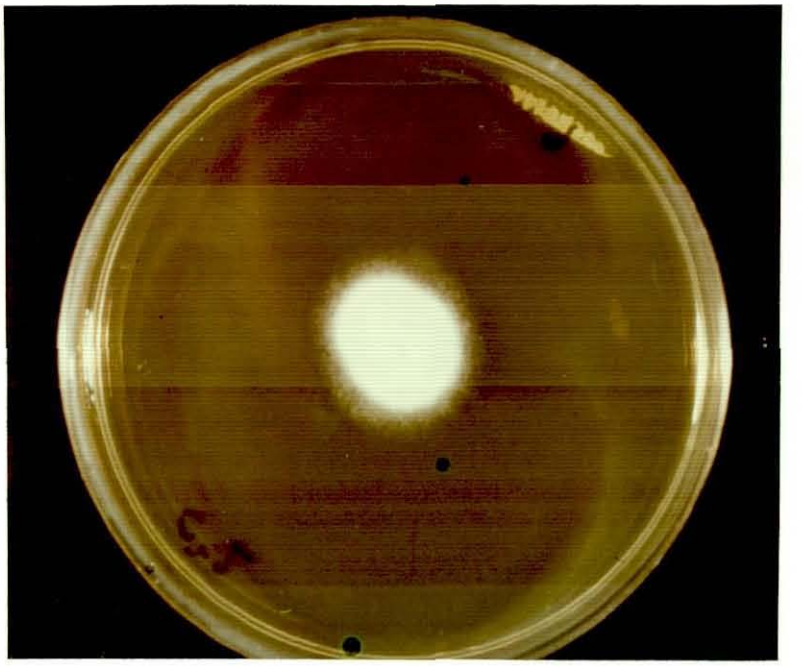
A32: This grew moderately fast covering the dish (9 cm) in 7 days. The fungus produced a small whitish floccose colony in 2 days. This developed into a thick and fairly granular form with an extensive stroma and a central dark coloured zone. On prolonged incubation the colony turned brown but there was no diffusible pigment (Plate 6).

The microscopic appearance was similar to A30 but no spores were formed. This strain sporulated only when grown with small fragments of sycamore twigs in the medium (C.W.T. Young - personal communication) (Plate 7).

A33: This strain, like A31, also grew slowly covering only 2.8 cm in diameter in 7 days under optimum conditions. It developed a thick, fairly hard and elevated colony accompanied by a depression in the medium. There was a small peripheral growth that was hardly floccose. The colony had a characteristic buff colour with its elevation crumbling into crusty forms. Spores were produced readily but the number was low (Plate 8).

Plate 4. - Cultural plate showing a colony of strain A31 on SAB after 7 days incubation at 25°C

Plate 5. - Light micrograph of A31 from SAB culture after 7 days incubation at 25°C. Septate hyphae and hyphal swellings are present. x 400



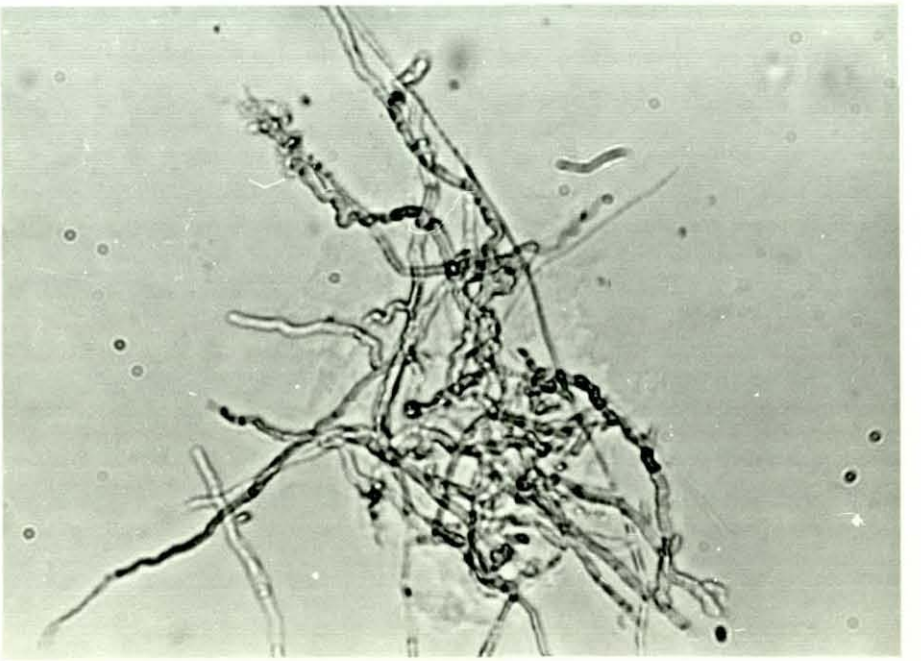


Plate 6. - Cultural plate showing a colony of strain A32 on SAB after 7 days incubation at 25°C.

Plate 7. - Light micrograph of A32 from SAB culture after 7 days incubation at 25°C. The hyphae are septate. x 400.

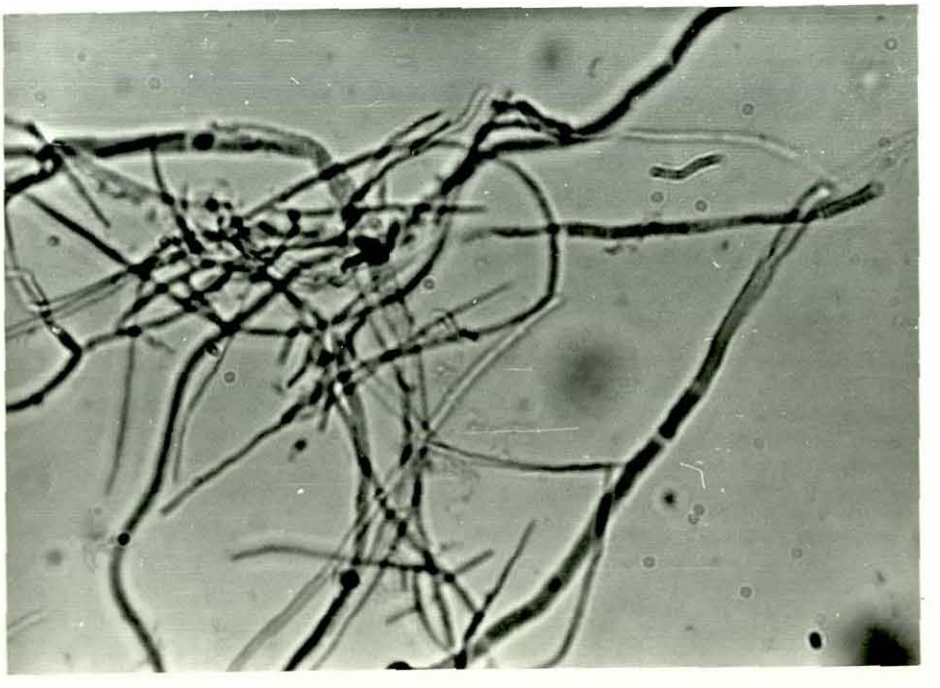
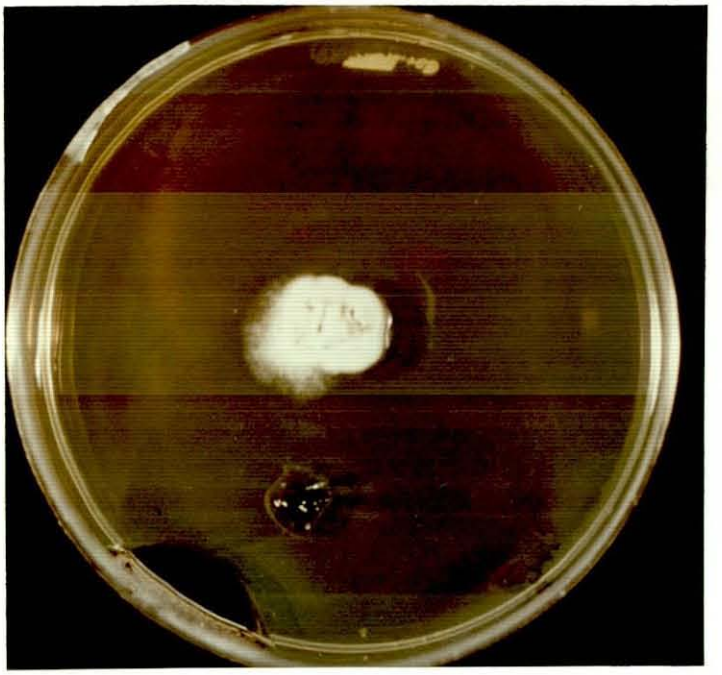
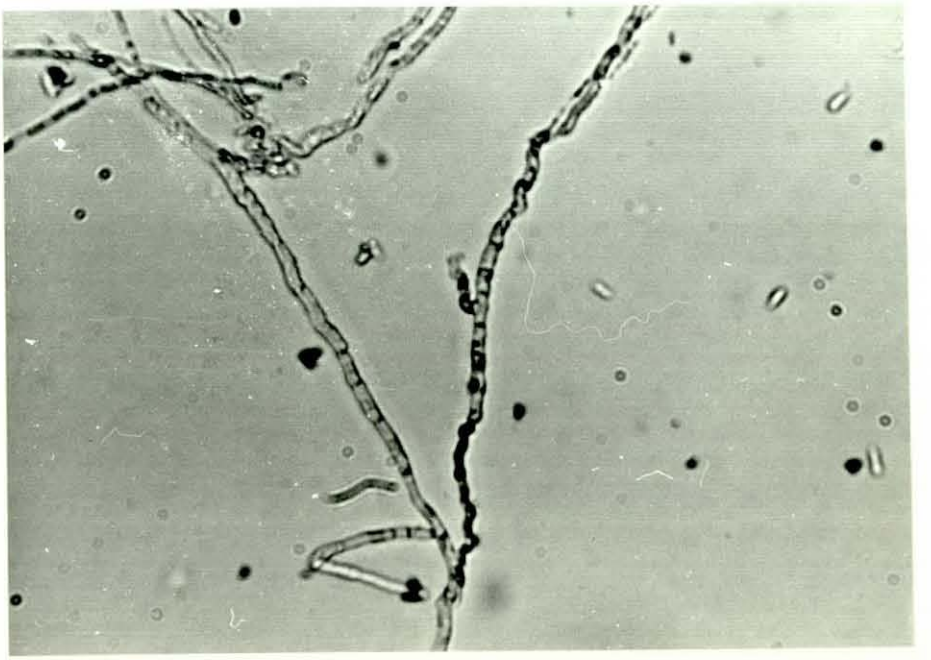


Plate 8. - Cultural plate showing a colony of strain A33 on SAB after 7 days incubation at 25°C.

Plate 9. - Light micrograph of A33 from SAB culture after 7 days incubation at 25°C. The hyphae are septate with swellings and few conidia are present. x 400.





On microscopic examination this strain was similar in appearance to A31 but there were spores present. The average dimension of the spores was 4 x 6μ and were usually fusoid (Plate 9). Other factors such as temperature, pH, carbon dioxide and osmotic pressure affecting growth, germination and sporulation are presented in Figs 7 to 15.

THE EFFECT OF LIGHT ON GROWTH, SPORULATION AND ZONATION:

The growth was not significantly affected by light but on sporulation, a varied effect was obtained (Table 1). Continuous light had no zoning effect neither did continuous darkness (Plate 10). The best defined zonation was obtained from treatment in alternate light and darkness and then fluctuation in temperature (Plate 11 and 12).

Alteration of nitrogen sources and their concentrations had no zoning effect. However, with carbon sources, zones were produced. Concentration of glucose up to 4% (w/v) produced distinct zones but above this concentration, the zones became diffused (Plate 13). Growth from the different zones, was used in a repeat experiment and similar results were achieved.

THE EFFECT OF SODIUM CHLORIDE ON GROWTH:

A30 grew in the presence of sodium chloride up to 2% (w/v) concentration. After 14 days incubation a scanty growth was observed at 4% (w/v) concentration.

The other strains grew well up to 1% and produced poor growth on 2% after 14 days (Table 2).

NUTRITION:

The strains were examined for their ability to utilize various sources of carbon for growth and also the effect these sources had on sporulation and germination. (Tables 3 and 4). The rate of utilization was followed over a period of 12 days at an interval of 3 days. This was to allow for adaptation of the strain to the substrate in order to obviate false utilization[183]. Residual sugars were determined after incubation to give an indication of efficient utilization of each source. This is also necessary as it is possible for growth to occur without a good utilization of the source

TIME COURSE OF SPORE GERMINATION AT 25°C.

(EACH POINT IS A MEAN OF SIX REPLICATES)

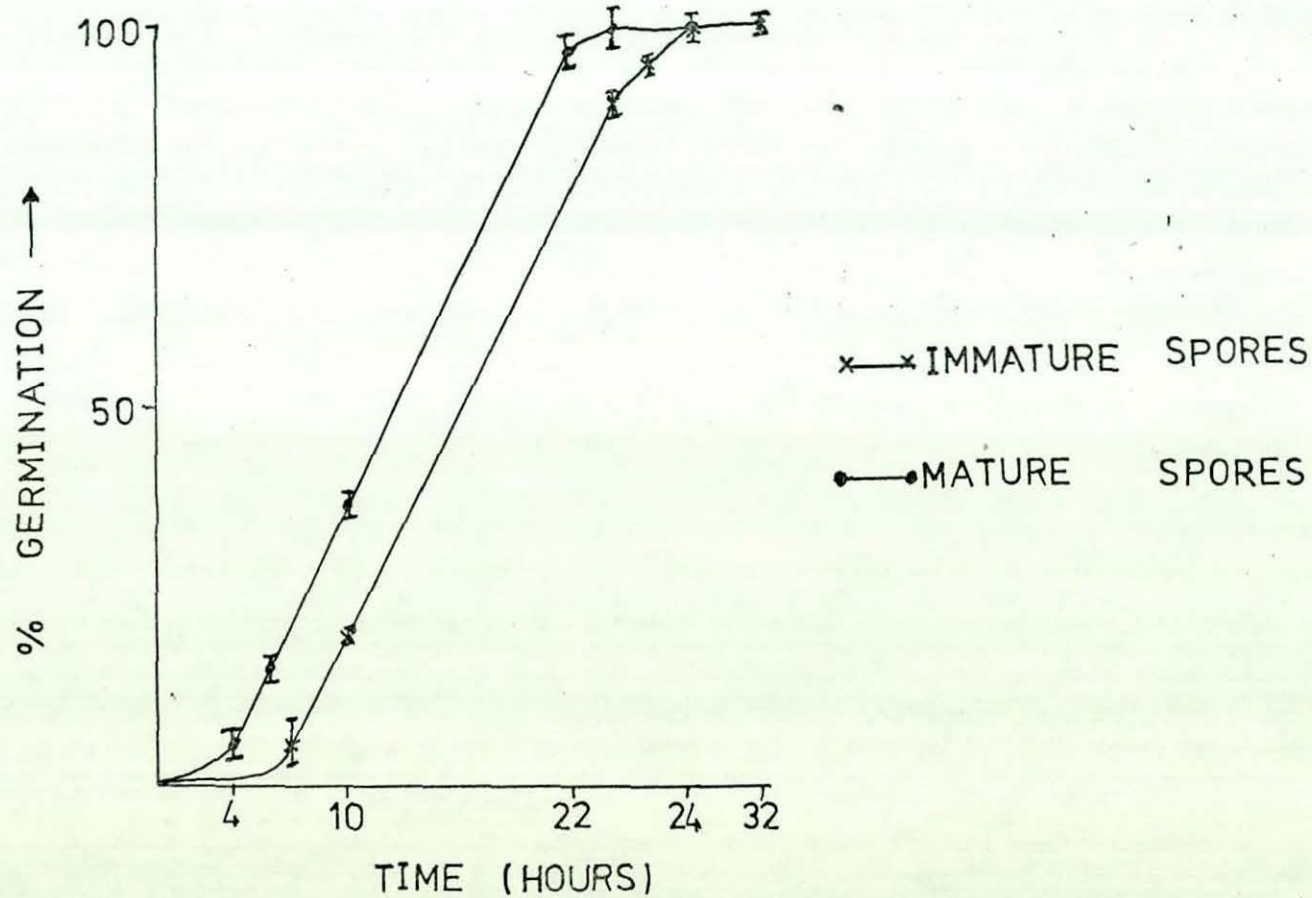
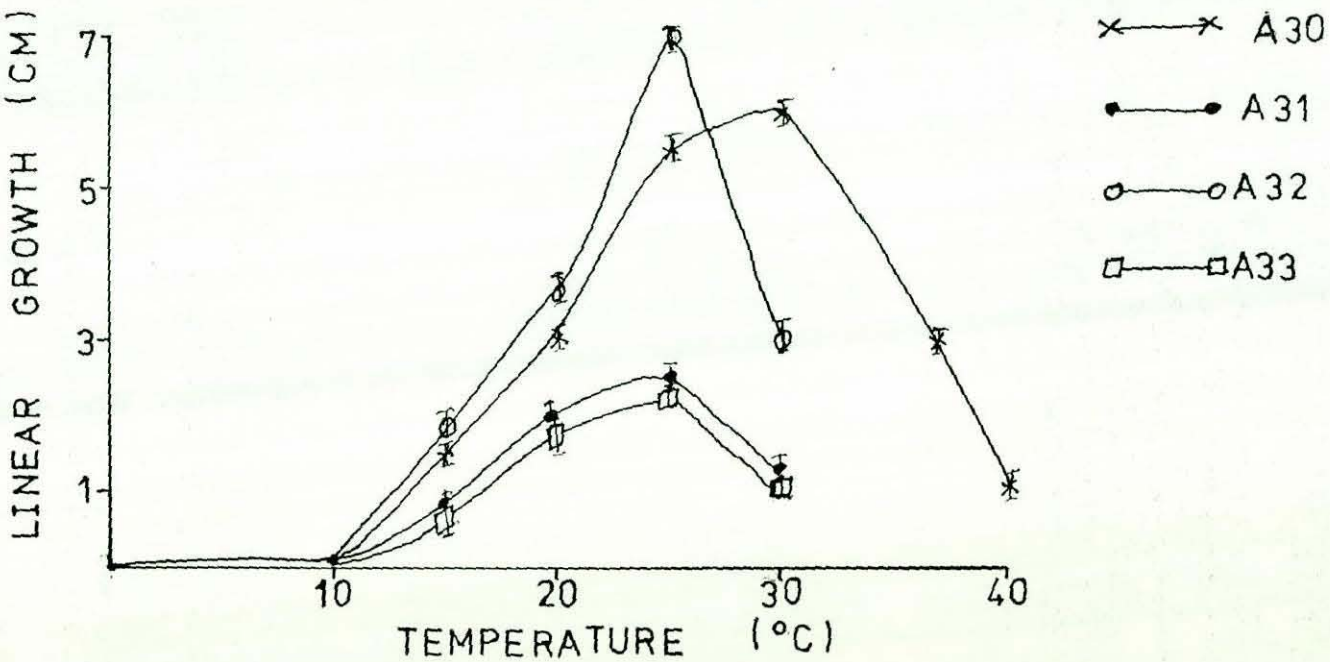


FIG 7

FIG 8

EFFECT OF TEMPERATURE ON GROWTH ON SAB.

(EACH POINT IS A MEAN OF SIX REPLICATES)



READINGS WERE TAKEN ON THE 3RD DAY FOR A30, 6TH DAY FOR A32 & 7TH FOR THE REST

TEMPERATURE EFFECT ON SPORULATION AND GERMINATION

(EACH POINT IS A MEAN OF SIX REPLICATES)

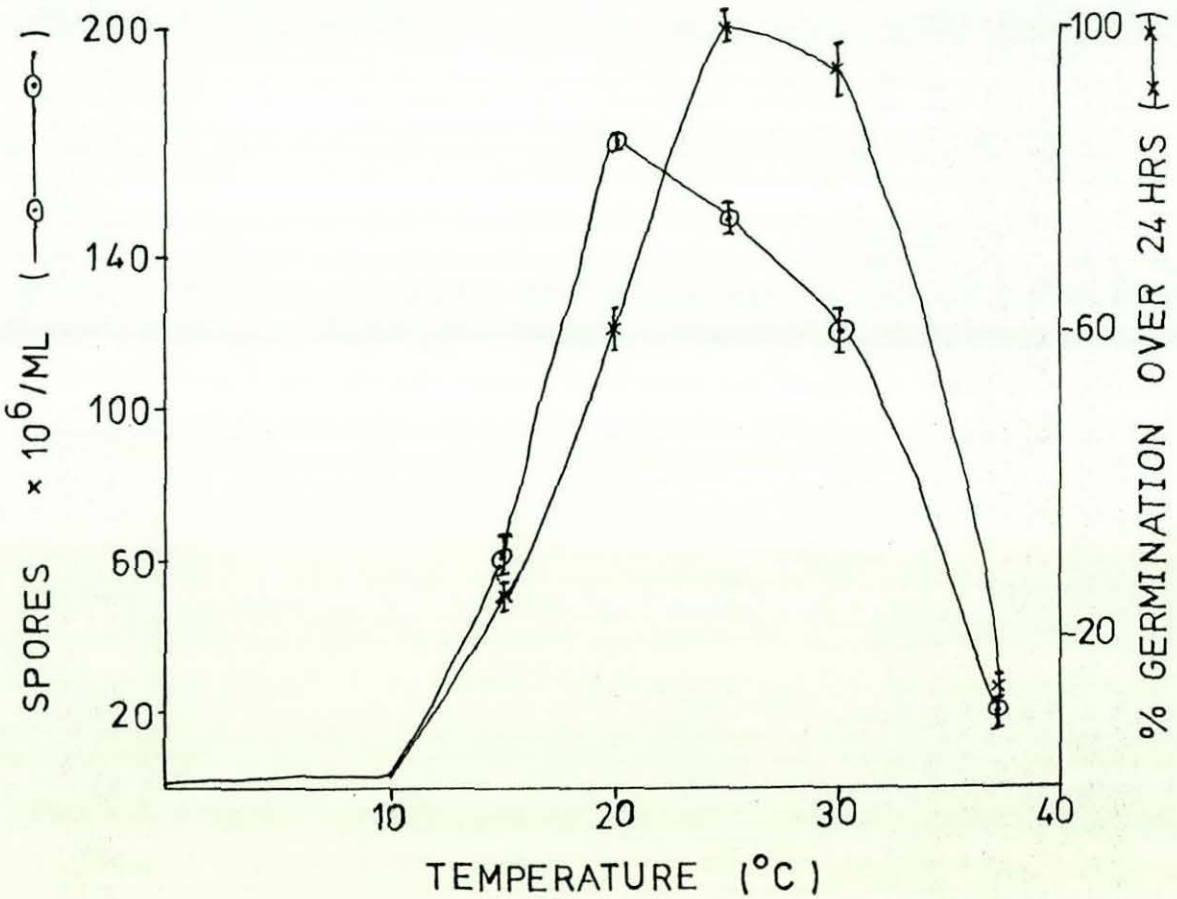


FIG 9

FIG 10

SPORES: HEAT SURVIVAL CURVE

(EACH POINT IS A MEAN OF SIX REPLICATES)

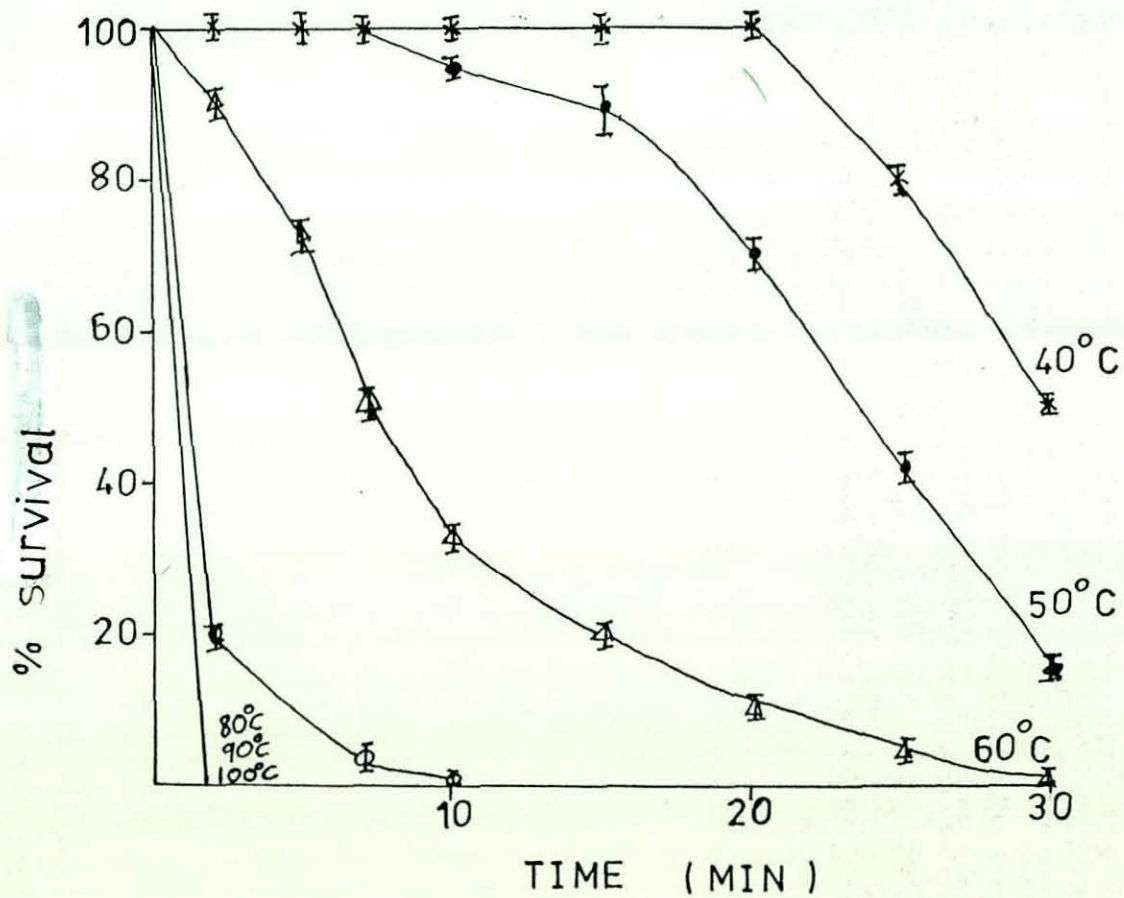
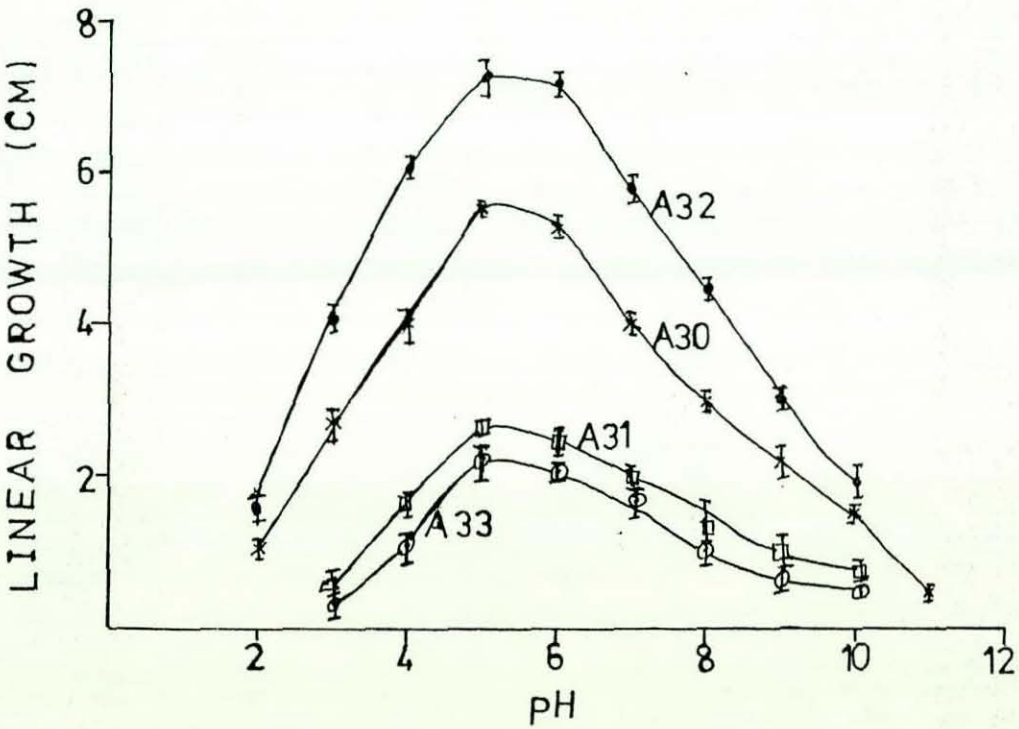


FIG 11 EFFECT OF PH ON GROWTH ON SAB AT 25°C

(EACH POINT IS A MEAN OF SIX REPLICATES)



READINGS FOR A30 & A32 WERE TAKEN ON 3RD & 6TH DAY, OTHERS ON THE 7TH.

pH EFFECT ON SRORULATION AND GERMINATION

(EACH POINT IS A MEAN OF SIX REPLICATES)

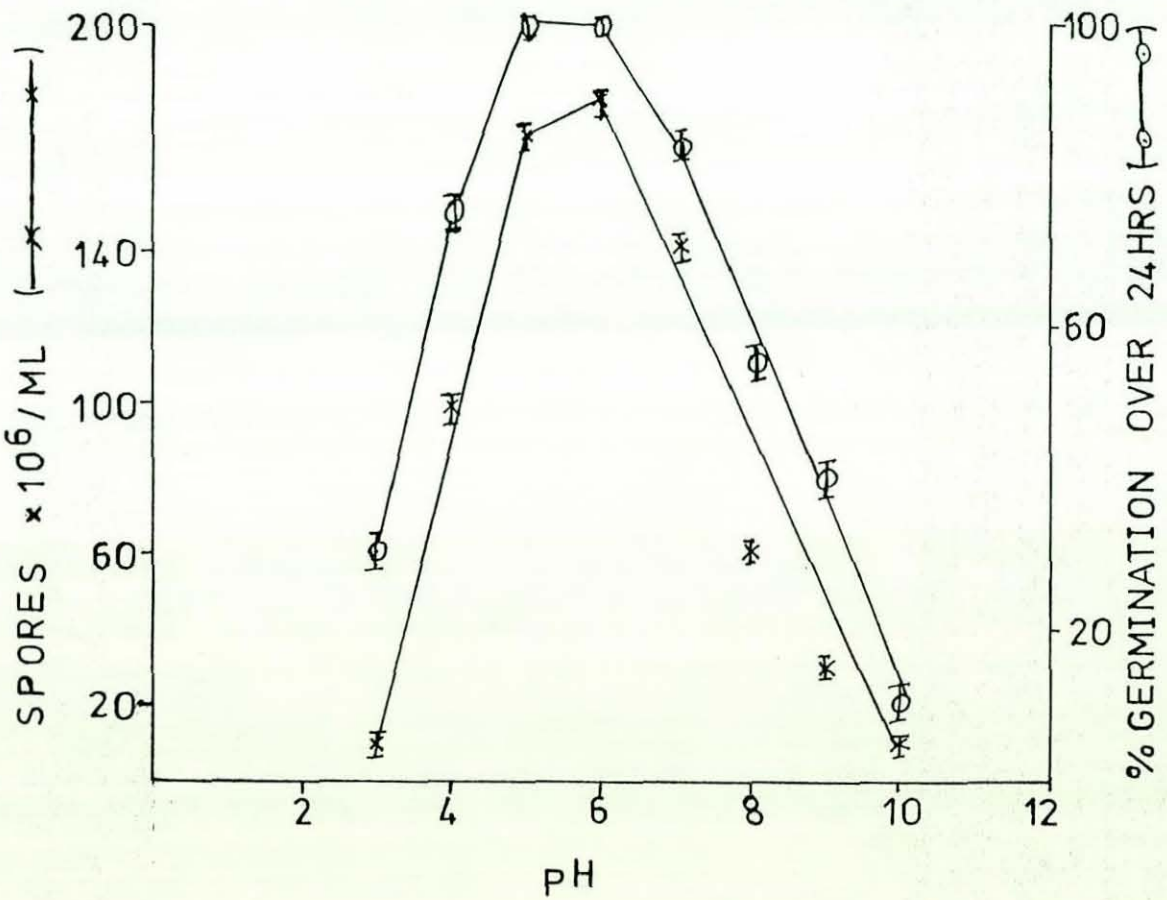


FIG 12

CO_2 — LINEAR GROWTH RELATIONSHIP

(VALUES ARE ± STD ERROR OF MEAN OF SIX REPLICATES
READINGS TAKEN AS IN TEMPERATURE TEST)

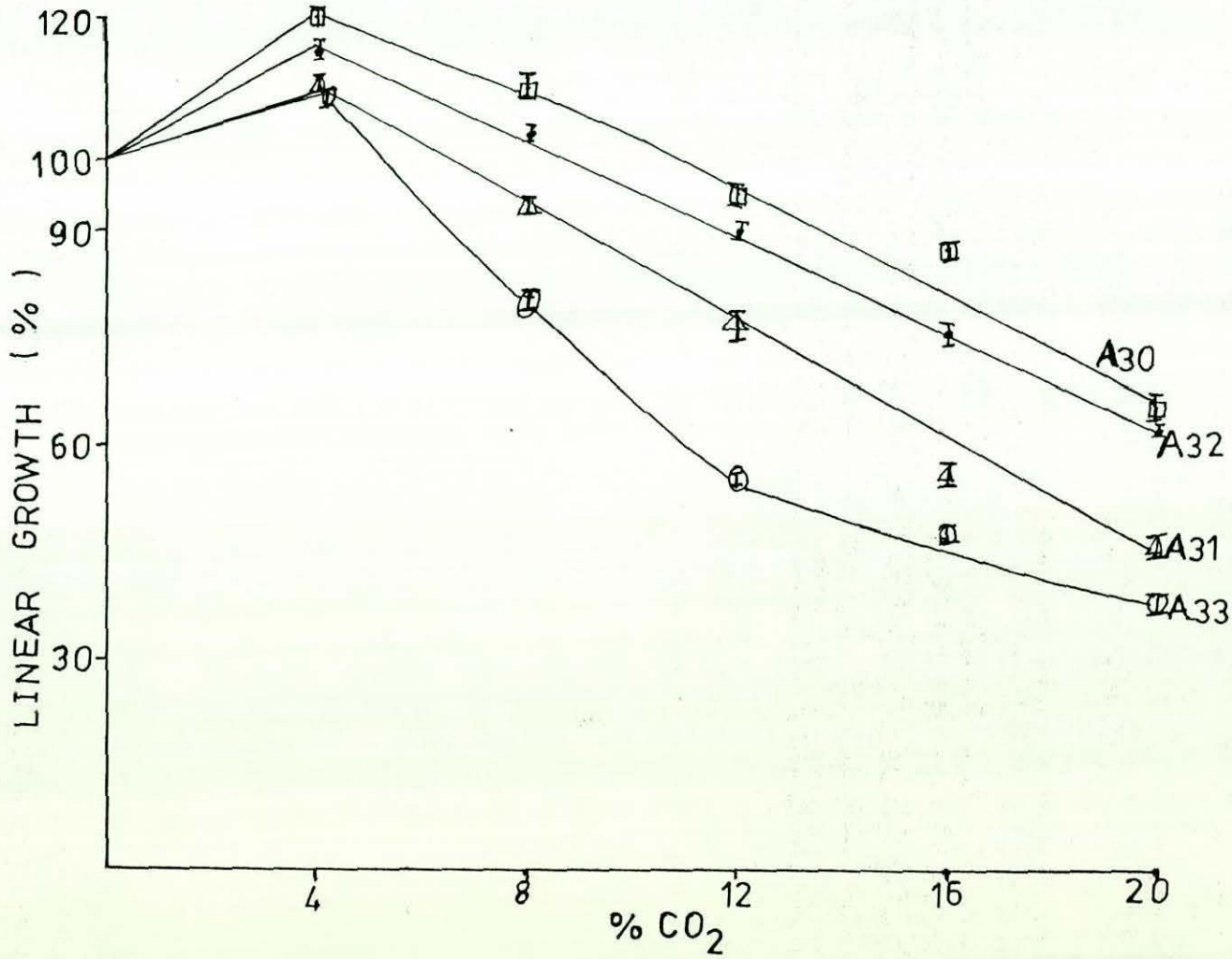


FIG 13

THE EFFECT OF CARBON DIOXIDE ON SPORULATION

(EACH POINT IS A MEAN OF SIX REPLICATES)

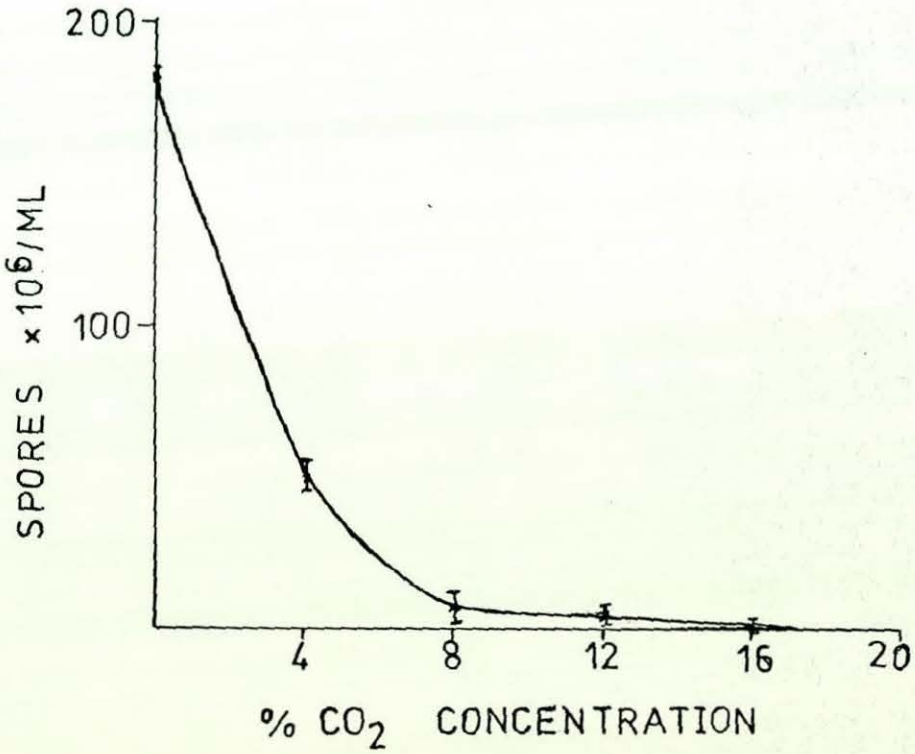


FIG 14

THE EFFECT OF GLUCOSE CONCENTRATION ON GROWTH AFTER 7 DAYS ON SAB

(EACH POINT IS A MEAN OF SIX REPLICATES)

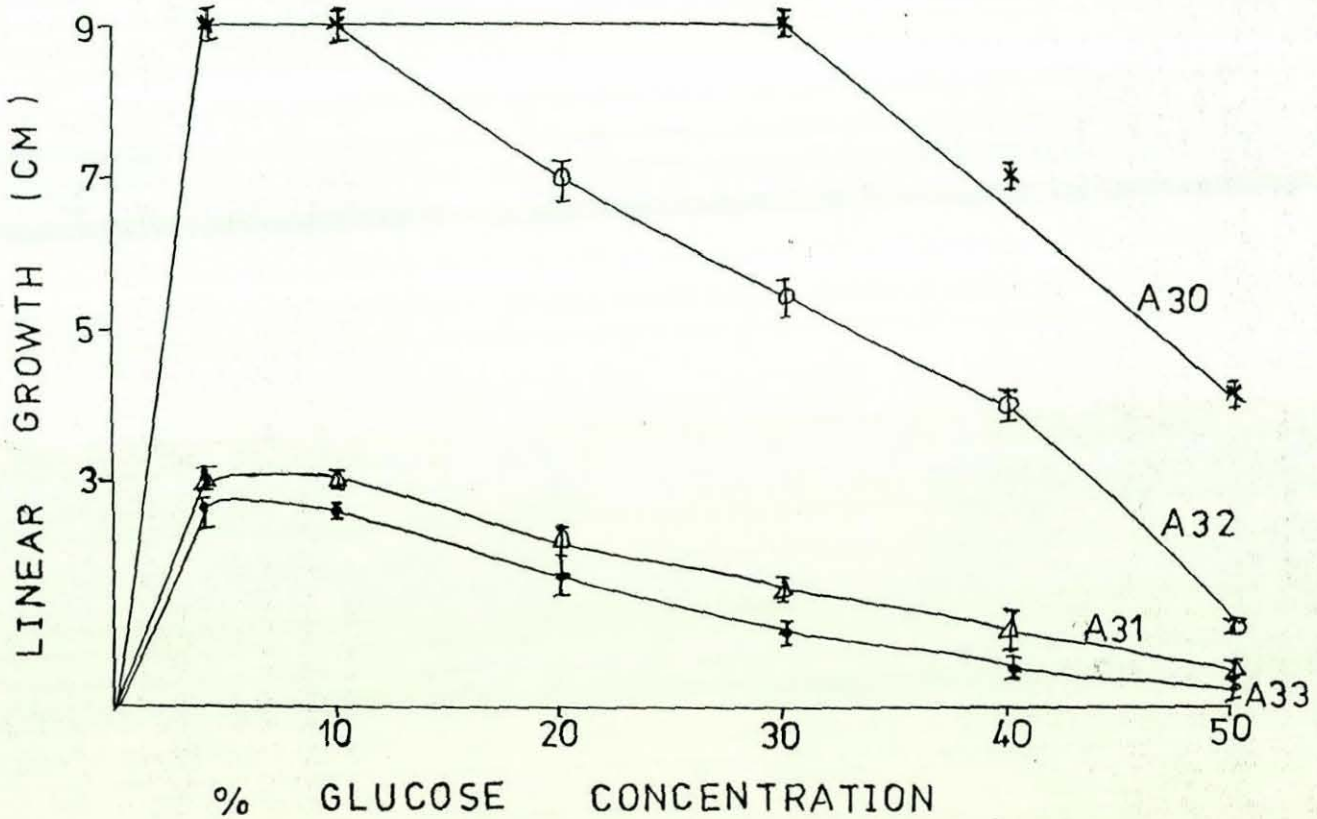


FIG 15

TABLE: 1

EFFECT OF LIGHT ON SPORULATION AT 25°C
ON DEFINED MEDIUM

TREATMENT	SPORES X $10^6/cm^3$
CONTINUOUS LIGHT	40.1 ± 1.5
CONTINUOUS DARKNESS	160.5 ± 2.3
ALTERNATE LIGHT AND DARKNESS	170.3 ± 1.7

EACH VALUE IS A MEAN OF 6 REPLICATES.

STANDARD ERRORS ARE SHOWN.

TABLE: 2

EFFECT OF SODIUM CHLORIDE ON GROWTH AT 25°C ON SAB

	SODIUM CHLORIDE CONCENTRATION (% w/v)						
STRAINS	0	0.5	1.0	2.0	4.0	8.0	16.0
A30	+	+	+	+	± ◇	-	-
A31	+	+	+	± ◇	-	-	-
A32	+	+	+	± ◇	-	-	-
A33	+	+	+	± ◇	-	-	-

6 REPLICATES WERE CARRIED OUT

KEY:

+: Good growth

±: Scanty growth

-: No growth

◇: 14 days incubation

TABLE: 3 THE UTILIZATION OF CARBON SOURCES.

ARBON OURCES	DAYS OF INCUB-ATION	A30			A31			A32			A33		
		DRY WEIGHT (mg)	FINAL pH	RESIDUAL SUGARS (%)*	DRY WEIGHT (mg)	FINAL pH	RESIDUAL SUGARS (%)	DRY WEIGHT (mg)	FINAL pH	RESIDUAL SUGARS (%)	DRY WEIGHT (mg)	FINAL pH	RESIDU SUGARS (%)
GLU-COSE	3	121.7 (1.5)	6.5		43.4 (1.2)	6.5		91.7 (1.2)	6.8		55.7 (1.1)	6.8	
	6	145.4 (1.1)	6.3		60.8 (1.5)	6.5		112.4 (1.2)	6.0		87.9 (1.1)	6.5	
	9	146.1 (1.2)	6.3		107.4 (1.4)	6.0		117.4 (1.2)	6.0		118.3 (1.5)	6.0	
	12	100.8 (1.5)	6.3	1	110.2 (1.5)	6.0	5.0	80.0 (1.1)	5.7	3.0	115.3 (1.4)	6.0	5.8
GALAC-TOSE	3	68.2 (2.0)	6.8		30.1 (1.5)	7.0		48.3 (1.7)	7.0		36.5 (1.6)	6.8	
	6	90.8 (1.2)	6.8		44.4 (1.2)	7.0		61.3 (1.7)	6.5		49.0 (1.1)	6.2	
	9	100.8 (1.7)	6.5		52.8 (1.2)	6.8		62.0 (1.7)	6.5		54.4 (1.0)	6.0	
	12	70.5 (1.3)	6.5	40	57.0 (1.2)	6.8	50.1	54.4 (1.2)	6.5	45.2	57.4 (1.2)	6.0	52.1
MAN-NOSE	3	70.5 (1.1)	6.6		38.9 (1.2)	6.8		82.5 (1.3)	6.8		47.1 (1.7)	6.8	
	6	100.2 (1.0)	6.6		60.3 (1.5)	6.6		105.2 (1.1)	6.6		65.5 (1.5)	6.6	
	9	120.7 (1.1)	6.5		101.5 (1.5)	6.2		110.3 (1.2)	6.1		115.7 (1.2)	6.2	
	12	98.2 (1.2)	6.5	10.5	103.4 (1.5)	6.2	20. 6	90.6 (2.0)	6.0	12.1	114.5 (1.3)	6.2	23.0
FRUC-TOSE	3	77.7 (1.3)	6.5		40.4 (1.7)	7.0		66.2 (1.2)	6.7		43.5 (1.4)	6.8	
	6	104.2 (1.5)	6.4		55.9 (6.0)	6.8		88.8 (2.5)	6.7		55.7 (1.7)	6.6	
	9	118.6 (1.2)	6.4		90.7 (2.0)	6.6		100.5 (2.0)	6.2		90.7 (1.8)	6.4	
	12	92.5 (1.5)	6.3	10.1	91.3 (2.0)	6.6	17.5	75.5 (1.1)	6.0	14.7	87.9 (2.5)	6.0	15.2
LACTOSE	3	55.8 (1.7)	6.8		25.7 (1.1)	7.0		38.5 (1.7)	7.0		30.1 (2.5)	7.0	
	6	110.5 (1.8)	6.8		50.9 (1.1)	7.0		70.3 (1.9)	6.8		50.8 (2.1)	7.0	
	9	130.2 (2.0)	6.4		75.5 (1.2)	6.6		89.2 (1.2)	6.4		70.8 (2.2)	6.6	
	12	126.5 (1.2)	6.4	20.3	80.8 (1.2)	6.4	27.8	90.5 (2.0)	6.0	23.9	82.7 (2.3)	6.3	30.1
SUCROSE	3	58.1 (1.1)	6.8		27.9 (1.2)	7.0		43.5 (2.0)	7.0		30.6 (2.0)	7.0	
	6	112.1 (1.5)	6.8		53.7 (1.3)	7.0		73.6 (2.5)	6.7		55.5 (1.1)	7.0	
	9	125.5 (1.7)	6.5		72.6 (1.4)	6.5		95.6 (2.5)	6.3		73.5 (1.1)	6.6	
	12	120.6 (1.7)	6.4	14.5	82.6 (1.1)	6.5	20.2	90.1 (2.3)	6.2	18.2	80.9 (2.1)	6.1	20.8
GLUCOSE + GALACTOSE	3	114.7 (1.8)	6.6		40.7 (1.1)	6.6		67.1 (2.1)	6.7		50.2 (2.2)	6.7	
	6	125.2 (1.5)	6.6		80.2 (1.1)	6.6		101.5 (2.1)	6.4		65.1 (2.0)	6.5	
	9	125.0 (1.2)	6.4		112.4 (1.1)	6.4		110.9 (1.5)	6.0		113.7 (2.0)	6.0	
	12	100.5 (1.5)	6.4	15.2	105.5 (1.5)	6.3	20.1	108.4 (1.7)	6.0	19.9	110.5 (1.7)	6.0	20.4
GLUCOSE + FRUCTOSE	3	117.3 (1.3)	6.6		42.5 (1.4)	6.6		83.5 (1.6)	6.7		50.7 (1.6)	6.6	
	6	149.5 (1.2)	6.4		70.5 (1.5)	6.4		120.7 (1.9)	6.3		90.2 (1.4)	6.6	
	9	150.2 (1.2)	6.3		115.2 (1.1)	6.0		127.5 (1.9)	6.3		100.5 (1.0)	6.0	
	12	120.7 (1.2)	6.3	10.1	114.7 (1.1)	6.0	18.1	120.6 (2.0)	6.0	14.7	100.0 (1.5)	6.0	17.5

The Table shows the rate of Utilization, Final pH and Residual Sugars.
Each value is a mean of 6 replicates. Standard errors are shown in brackets.

*Percentage of original concentration.

TABLE: 4

EFFECT OF CARBON SOURCES ON SPORULATION AND GERMINATION. A30 USED.

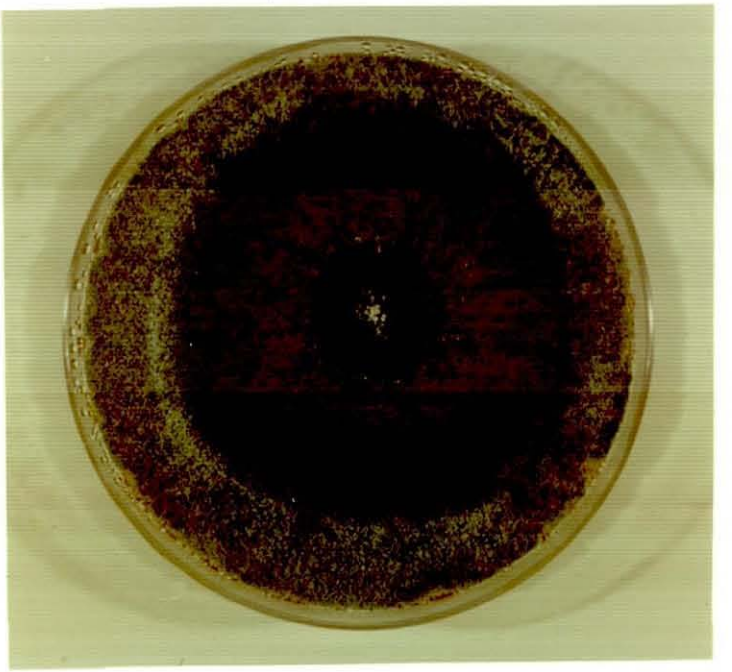
COMPOUNDS	SPORES X $10^6/cm^3$	GERMINATION (%)
GLUCOSE	180 ± 2.5	100
GALACTOSE	75 ± 1.7	50
SUCROSE	160 ± 2.2	80
LACTOSE	100 ± 1.5	70
MEDIUM WITHOUT CARBON SOURCE	3.8 ± 2.0	NIL

EACH VALUE IS A MEAN OF 6 REPLICATES.

STANDARD ERROR SHOWN.

Plate 10. - Effect of continuous light on zonation.
A30 on SAB after 7 days incubation.

Plate 11. - Zonation in alternate light and darkness
(3 h in light and 21 h in darkness).
A30 on SAB after 7 days incubation showing
concentric zones of spores.



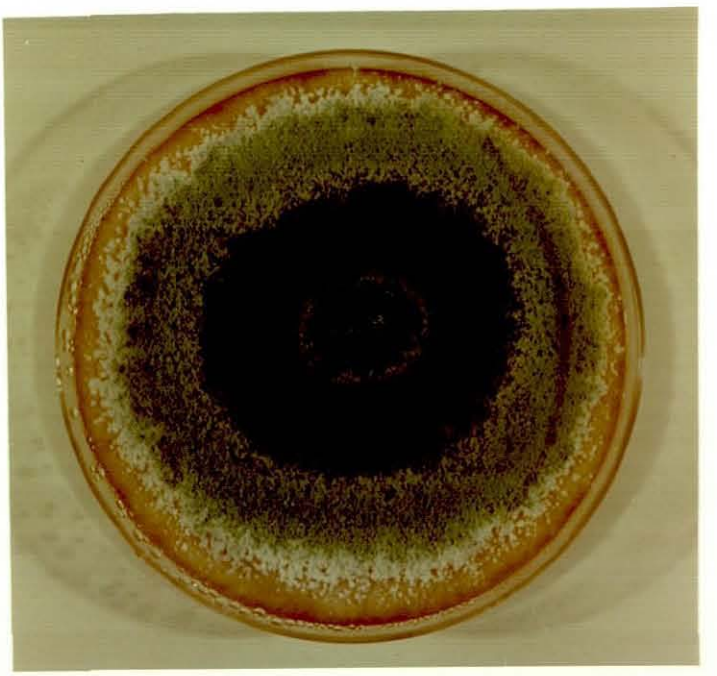
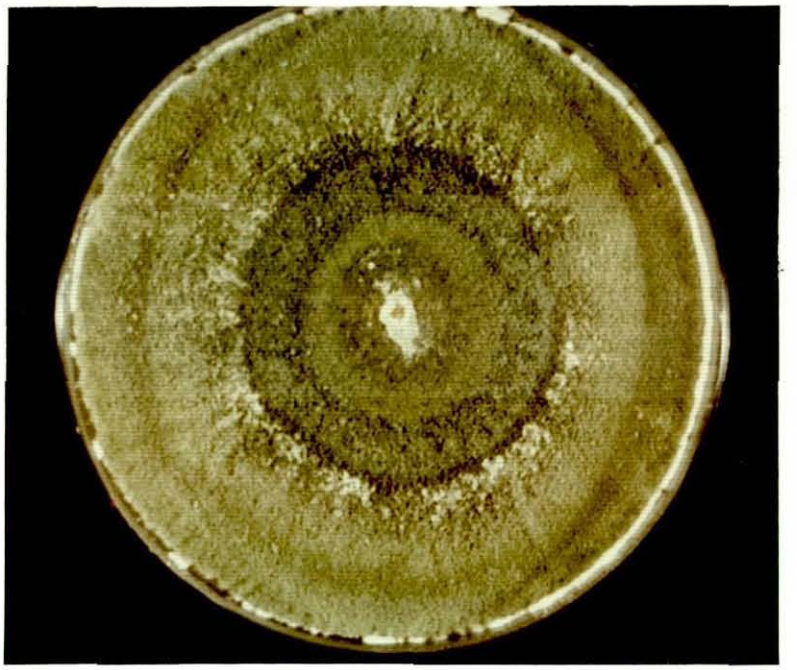
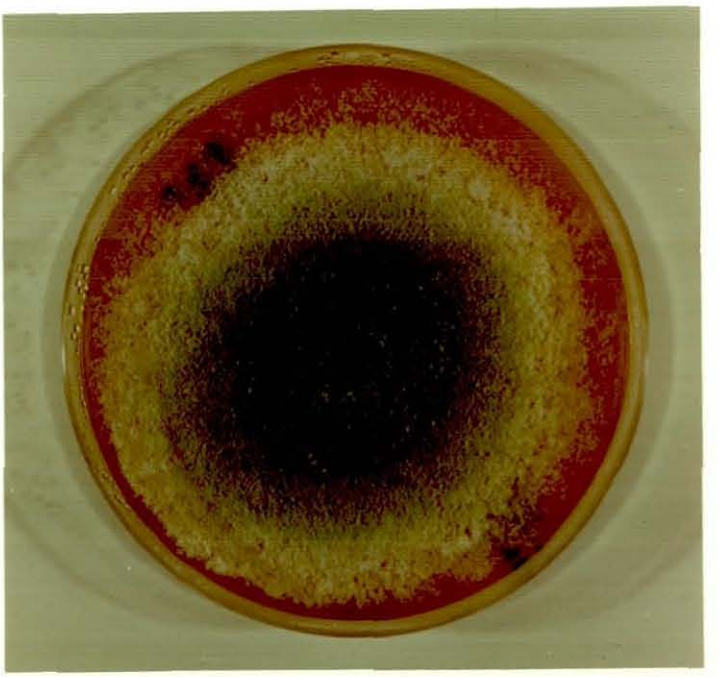


Plate 12. - Zonation on window ledge. A30 on SAB after 7 days. Zones were formed but not clearly defined.

Plate 13. - Zonation: effect of glucose concentration above 4%. No well defined zone.





The utilization of nitrogen sources and the effect of their concentration on growth were also investigated (Fig 16) along with the effect of these sources on sporulation and germination (Table 5). Again the residual nitrogen was determined (Table 6).

The experiment on vitamin requirement showed that A30 required no vitamins whilst the other strains needed either biotin or ascorbic acid for better growth (Table 7).

The results on hydrolysis of organic compounds and their utilization as sole carbon and energy sources are presented in Tables 8-10.

THE PROTEIN PROFILE AND ZYMOGRAM:

Electrophoretic patterns of distinct protein fractions were obtained from all four strains. Fig 17 shows the densitometer tracing of the patterns. These patterns were found to be reproducible and identical. The growth medium was examined electrophoretically and this gave no protein bands.

In order to locate and designate the position of the peaks, the running distance of each peak was measured relative to the tracker dye. A similarity chart (Fig 18) was then drawn. Fractions with mobilities within the Rf range of 0.01 were considered homologous, unless there was a very distinct peak. Esterase and amidase zymograms are presented in Fig 19.

MICROSCOPICAL STUDIES:

The scanning electron microscopy (Plate 14) confirmed the glabrous nature and ovoid shape of the spores. Wall collapse is evident which could be due to either the fixation process or to the vacuum evaporation during drying. Attempts to eliminate it failed. The hyphal swellings seen in the light microscopy of A31 and A33 showed quite distinctly (Plates 16 and 17). The hyphae were apparently separated into shorter fragments and extra cellular material may be present. Figs 15 and 18 show the mycelial growth of A30 and A32. Emerging conidia with short conidiophores can be seen (Plate 17).

TABLE: 5

EFFECT OF NITROGEN SOURCES ON SPORULATION AND GERMINATION. A30 USED.

COMPOUNDS	SPORES X $10^6/cm^3$	GERMINATION (%)
POTASSIUM NITRATE	160 ± 2.5	97
AMMONIUM NITRATE	170 ± 2.0	98
ASPARAGINE	180 ± 1.1	100
ARGININE	175 ± 1.5	100
MEDIUM WITHOUT NITROGEN SOURCE	6.5 ± 2.1	NIL

EACH VALUE IS A MEAN OF 6 REPLICATES.

STANDARD ERRORS SHOWN.

TABLE: 6 SHOWING RESIDUAL NITROGEN AT DIFFERENT LEVELS WITH FINAL pH READINGS.

NITROGEN SOURCE	SUPPLY LEVEL (g/L)	A30		A31		A32		A33	
		RESIDUAL N_2 (%)*	FINAL pH	RESIDUAL N_2 (%)	FINAL pH	RESIDUAL N_2(%)	FINAL pH	RESIDUAL N_2 (%)	FINAL pH
POTASSIUM NITRATE	0.2	2.1 (1.5)	6.5	3.1 (1.8)	6.8	4.2(1.1)	6.8	4.5(2.1)	6.8
	0.4	2.2 (1.7)	6.5	4.2 (2.0)	6.5	4.8 (1.2)	6.8	4.9(2.2)	6.8
	0.8	2.0 (1.5)	6.5	4.6 (2.0)	6.5	10.2(1.5)	6.8	7.7(1.5)	6.5
	1.8	8.0 (1.1)	6.5	10.1 (2.5)	6.5	10.7(1.9)	6.5	11.2 (2.0)	6.5
SODIUM NITRITE	0.2	3.1 (1.2)	7.0	4.5 (2.5)	6.8	4.7(1.4)	6.8	4.5 (1.7)	6.8
	0.4	3.2 (1.7)	7.2	4.7 (1.5)	6.8	4.9(1.3)	7.0	4.9 (1.2)	6.8
	0.8	10.1 (1.9)	7.2	1.2 (1.7)	7.3	7.1(1.2)	7.2	8.1 (1.3)	7.0
	1.6	15.2 (1.3)	7.5	15.5 (1.7)	7.5	13.2(1.1)	7.2	15.7 (1.4)	7.0
AMMONIUM SULPHATE	0.2	3.1 (2.1)	5.5	4.1 (1.3)	5.3	3.7(1.7)	6.2	4.7 (1.5)	6.0
	0.4	3.5 (2.2)	4.5	4.4 (1.4)	4.3	4.5(1.5)	5.5	5.9 (1.2)	4.9
	0.8	10.2 (1.7)	4.0	8.2 (1.5)	4.0	4.9(1.4)	4.0	10.1 (1.3)	4.0
	1.6	15.2 (1.5)	3.5	15.7 (1.5)	4.0	8.5(1.5)	4.0	12.2 (1.7)	4.0
AMMONIUM NITRATE	0.2	2.3 (1.2)	6.3	3.5 (1.1)	6.0	3.9(1.7)	6.0	3.3 (1.5)	6.5
	0.4	2.5 (1.1)	4.0	4.0 (1.1)	6.0	4.3(1.8)	6.0	3.9 (1.9)	6.0
	0.8	7.1 (1.2)	3.5	4.9 (1.0)	4.0	8.2(1.3)	4.0	7.3 (1.6)	5.5
	1.6	10.2 (1.7)	3.5	10.1 (2.0)	4.0	11.1(1.5)	4.0	9.2 (1.3)	4.5
ARGININE	0.2	2.4 (2.0)	6.8	3.1 (1.5)	6.8	3.3(2.0)	6.8	3.4 (1.4)	6.8
	0.4	2.7 (2.0)	7.0	3.5 (1.7)	6.8	3.7(2.1)	6.8	3.9 (1.0)	6.8
	0.8	5.0 (2.0)	7.0	7.2 (1.5)	7.5	6.8(1.1)	7.0	7.1 (1.5)	7.0
	1.6	7.0 (1.5)	7.5	9.1 (1.8)	7.5	10.1(1.5)	7.5	10.5 (1.4)	7.5
ASPARAGINE	0.2	1.2 (1.1)	7.0	2.1 (1.9)	6.8	2.7(1.5)	6.8	2.4 (1.2)	6.8
	0.4	2.0 (1.3)	7.0	2.4 (2.0)	6.8	2.5(1.2)	6.8	2.7 (1.1)	6.8
	0.8	3.5 (1.2)	7.5	2.9 (2.1)	7.0	5.5(1.3)	7.0	5.9 (1.1)	7.0
	1.6	14.2 (1.1)	8.0	12.4 (2.3)	7.0	10.7(1.9)	7.5	11.2 (1.2)	7.0
CYSTEINE	0.2	40.5 (1.4)	6.8	50.5 (2.4)	6.8	50.2(2.0)	7.0	50.7 (1.2)	6.8
	0.4	60.5 (1.5)	6.8	50.9 (2.1)	6.8	50.8(2.1)	7.0	61.5 (1.7)	6.8
	0.8	65.5 (1.8)	6.8	70.0 (2.0)	7.0	70.9(2.0)	7.2	73.5 (1.5)	7.0
	1.6	70.8 (2.0)	7.0	80.5 (2.1)	7.3	75.5(1.5)	7.5	80.9 (1.2)	7.0
METHIONINE	0.2	50.5 (1.1)	6.8	40.2 (1.7)	6.8	40.5(1.1)	6.8	45.2 (1.4)	6.8
	0.4	40.9 (1.0)	6.8	50.5 (1.1)	6.8	50.2(1.7)	7.0	52.5 (1.3)	6.8
	0.8	50.1 (1.4)	6.8	56.5 (1.2)	6.8	54.2(1.8)	7.0	58.9 (1.7)	7.0
	1.6	65.5 (1.1)	7.0	70.5 (1.3)	7.2	67.7(2.0)	7.2	70.2 (1.5)	7.0
UREA	0.2	1.5 (1.7)	7.2	2.0 (1.7)	7.0	1.7(1.7)	7.0	1.9 (2.1)	7.0
	0.4	1.7 (1.1)	7.2	2.2 (1.7)	7.6	2.0(1.6)	7.5	2.0 (1.5)	7.0
	0.8	60.5 (1.1)	8.5	2.5 (1.5)	7.6	50.22(1.5)	8.5	2.4 (1.2)	8.6
	1.6	70.7 (1.7)	9.5	6.0 (1.5)	8.5	70.3 (2.5)	9.0	65.1 (2.5)	9.5

*Percentage of original concentrations

Each value is the mean of 3 replicates. Standard errors are in brackets.

TABLE: 7

VITAMIN REQUIREMENT: DRY WEIGHT AFTER 7 DAYS.

VITAMINS	DRY WEIGHT YIELD (mg)			
	A30	A31	A32	A33
ALL VITAMINS	130.5 ± 1.5	80.5 ± 2.5	100.1 ± 2.1	82.5 ± 1.2
NO VITAMINS	126.5 ± 1.5	15.5 ± 2.5	30.1 ± 2.5	19.9 ± 1.7
L. ASCORBIC ACID	127.7 ± 1.1	75.9 ± 1.5	98.9 ± 2.0	80.5 ± 1.5
d-BIOTIN	128.7 ± 1.2	78.7 ± 2.1	96.9 ± 1.5	79.9 ± 1.7
FOLIC ACID	121.7 ± 1.5	17.1 ± 2.0	25.5 ± 1.9	20.9 ± 1.2
INOSITOL	121.3 ± 1.9	19.5 ± 1.7	27.2 ± 1.1	17.5 ± 1.7
NICOTINAMIDE	120.5 ± 1.1	20.2 ± 2.1	25.9 ± 1.0	19.2 ± 1.9
P-AMINO-BENZOIC ACID	119.9 ± 1.5	15.2 ± 1.1	30.7 ± 2.7	22.5 ± 1.1
PYRIDOXINE	120.1 ± 1.7	19.7 ± 2.0	35.1 ± 2.1	25.1 ± 1.9
RIBOFLAVIN	120.4 ± 1.9	16.7 ± 2.0	29.5 ± 2.1	21.9 ± 2.6
THIAMINE	120.9 ± 1.3	20.1 ± 2.2	37.0 ± 2.1	25.9 ± 2.7
PANTOTHENIC ACID	117.5 ± 2.0	25.2 ± 1.9	33.1 ± 2.5	24.3 ± 2.1

EACH VALUE IS MEAN OF 6 REPLICATES

STANDARD ERROR SHOWN.

TABLE: 8 Showing Range of Hydrolytic Enzyme Produced.

SUBSTRATES	STRAINS			
	A30	A31	A32	A33
STARCH	+	+	+	+
CASEIN	+	+	+	+
GELATIN	+	+	+	+
UREA	+	+	+	+
TWEEN 20	+	+	+	+
" 40	+	+	+	+
" 60	+	+	+	+
" 80	+	+	+	+
TRIBUTYRIN	+	-	-	-
TRIOLEIN	+	-	-	-
CLOVE OIL	-	-	-	-
OLIVE OIL	-	-	-	-
PALM OIL	-	-	-	-
RAPE OIL	-	-	-	-
XANTHINE	-	-	-	-
TYROSINE	-	-	-	-
ADENINE	-	-	-	-
GUANINE	-	-	-	-
DEOXYRIBONUCLEIC ACID	+	+	+	+
RIBONUCLEIC ACID	+	+	+	+
AESCULIN	+	+	+	+
FIBRIN	+	+	+	+
COLLAGEN	-	-	-	-
LECITHIN	+	+	+	+

Key : + - Positive
- - Negative
* - Positive within 3 days
● - " " 6 days
⊕ - " " 7 days
▣ - " " 9 days
⊕ - " " 12 days
◇ - " " 14 days

TABLE: 9 GROWTH AND ENZYME ACTIVITY ON PECTIN AND CELLULOSE.

	A30		A31		A32		A33	
MEASUREMENT	PECTIN	CELLULOSE	PECTIN	CELLULOSE	PECTIN	CELLULOSE	PECTIN	CELLULOSE
DRY WEIGHT (mg)	100.5 ± 1.5	116.7 ± 1.1	60.1 ± 1.0	70.1 ± 2.0	80.5 ± 2.5	84.7 ± 1.3	63.1 ± 1.7	69.5 ± 1.2
PROTEIN ($\mu g/cm^3$)	200.1 ± 1.2	140.2 ± 1.1	180.7 ± 1.5	140.9 ± 2.0	170.5 ± 2.1	130.7 ± 1.0	185.7 ± 1.7	145.2 ± 1.2
REDUCING SUGARS (mg/cm^3)	140.3 ± 1.3	150.9 ± 1.3	130.1 ± 1.2	141.5 ± 1.0	133.9 ± 1.7	137.9 ± 1.9	125.7 ± 2.0	132.9 ± 1.1

VALUES ARE MEAN OF 3 REPLICATES.

STANDARD ERRORS ARE SHOWN.

TABLE: 10 UTILIZATION OF ORGANIC COMPOUNDS AS SOLE CARBON AND ENERGY SOURCES.

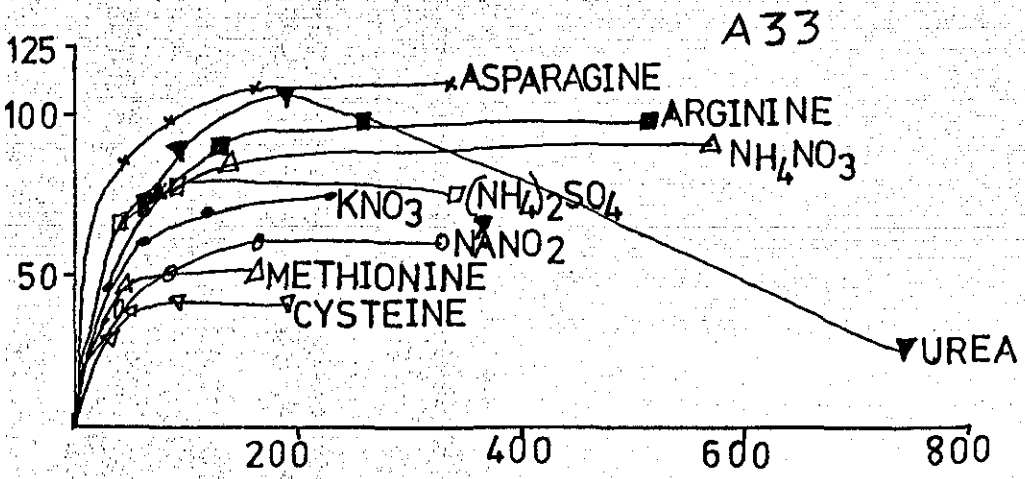
ORGANIC COMPOUNDS		STRAINS			
GENERAL NAME	SINGLE COMPOUNDS	A30	A31	A32	A33
DICARBOXYLIC ACIDS (0.1% w/v)	OXALATE	+ ●	+ ⊕	+ ⊕	+ ⊕
	MALONATE	+ ●	+ ⊕	+ ⊕	+ ⊕
	SUCCINATE	+ ■	+ ■	+ ■	+ ■
	FUMARATE	+ ■	NG	NG	NG
HYDROXY ACIDS (0.1% w/v)	CITRATE	+ *	+ ⊕	+ ⊕	+ ⊕
	LACTATE	+ *	+ ⊕	+ ⊕	+ ⊕
FATTY ACIDS (0.1% w/v)	FORMATE	+ *	+ ■	+ ●	+ ■
	MALATE	+ ●	+ ■	+ ■	+ ■
	PROPIONATE	+ ■	+ ⊕	+ ■	+ ⊕
PHENOL AND DERIVATIVES	PHENOL (0.1%)	NG ?	NG ?	NG ?	NG ?
	PHENOL (0.05%)	+ ⊕ ?	NG ?	NG ?	NG ?
	PHENOL (0.025%)	+ ⊕ ?	NG ?	NG ?	NG ?
	M-CRESOL (0.05%)	+ ⊕ ?	NG ?	NG ?	NG ?
	M-CRESOL (0.025%)	+ ⊕ ?	NG ?	NG ?	NG ?
	P-CRESOL (0.05%)	+ ⊕ ?	NG ?	NG ?	NG ?
	P-CRESOL (0.025%)	+ ⊕ ?	NG ?	NG ?	NG ?
KETO ACID (0.1% w/v)	PYRUVATE	+ ●	+ ⊕	+ ⊕	+ ⊕
AROMATIC COMPOUNDS (0.1% w/v)	MANDELIC ACID	+ ■	NG	NG	NG
	ACETAMIDE	+ *	+ ■	+ ●	+ ■
	BENZAMIDE	+ *	NG	NG	NG
	BENZOATE	+ *	+ ■	+ ●	+ ■
POLYPHENOLICS	CATECHOL	+ ● BP	+ ■ BP	+ ■ BP	+ ■ BP
	GALLIC ACID	+ ● BP	+ ■ BP	+ ■ BP	+ ■ BP
	TANNIC ACID	± BP	NG. BP	NG, BP	NG, BP

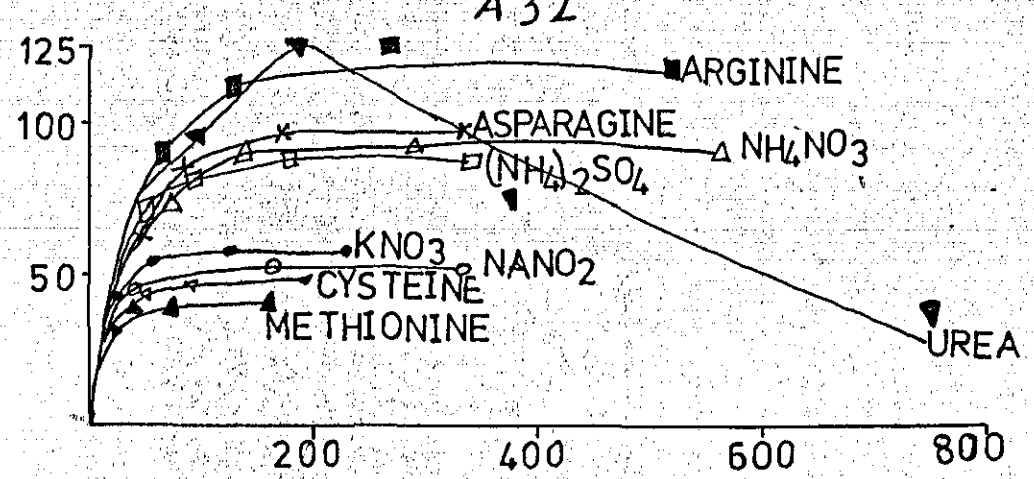
KEY:

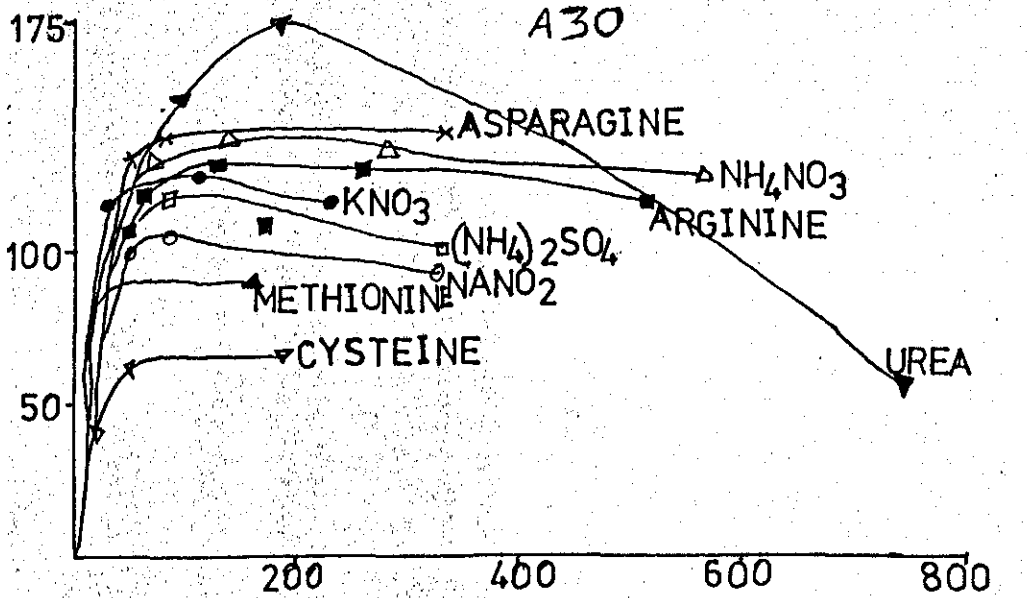
\+ - Growth with colour change (Green to Blue)
± - Scanty growth
NG - No growth
* - Positive within 3 days
● - Positive within 6 days
◈ - Positive within 7 days
■ - Positive within 9 days
⊕ - Positive within 12 days
◇ - Positive within 14 days
? - Medium turned yellow
BP - Brown pigment

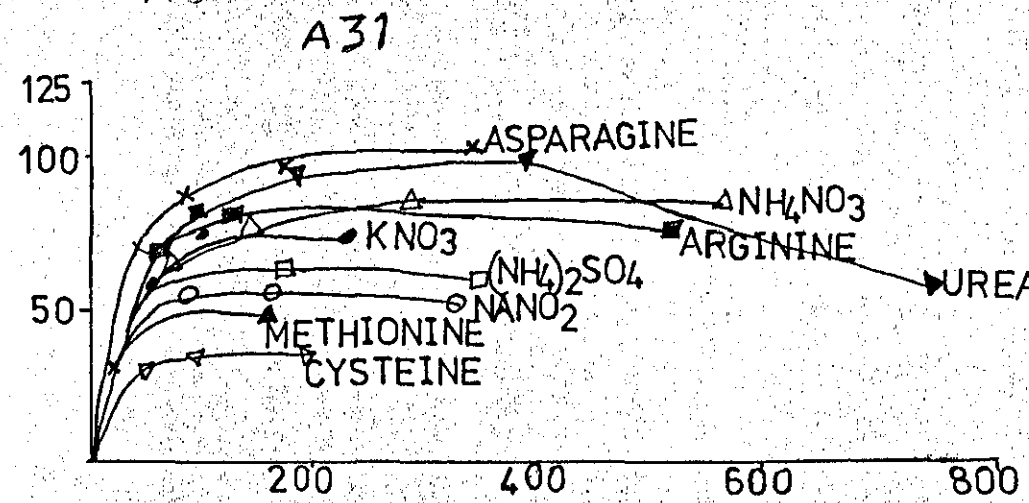
EFFECT OF NITROGEN SOURCES AND CONCENTRATION ON GROWTH

FIG 16









MG N/LITRE →

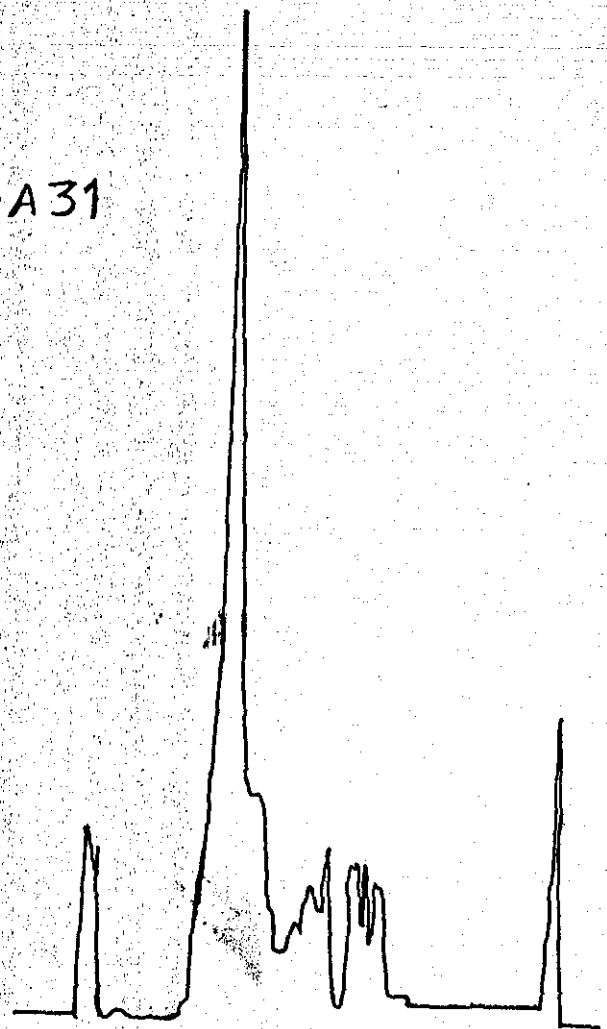
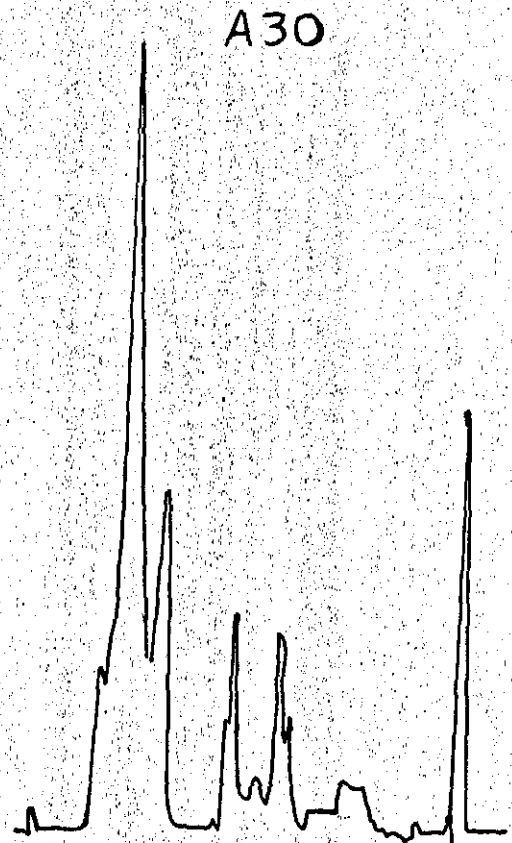


FIG 17(a)



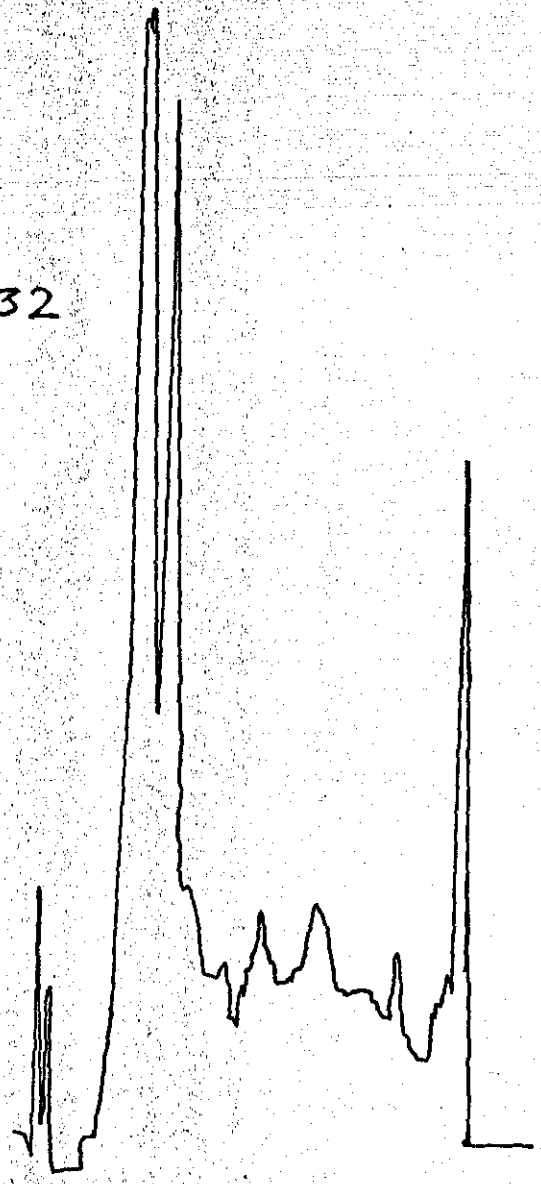


FIG 17(b)

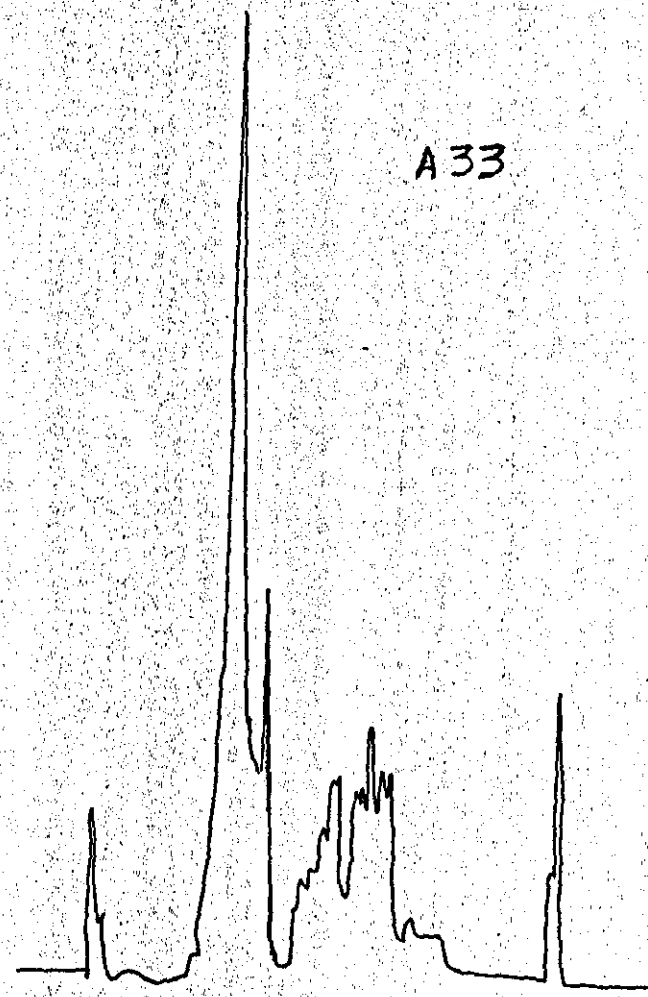
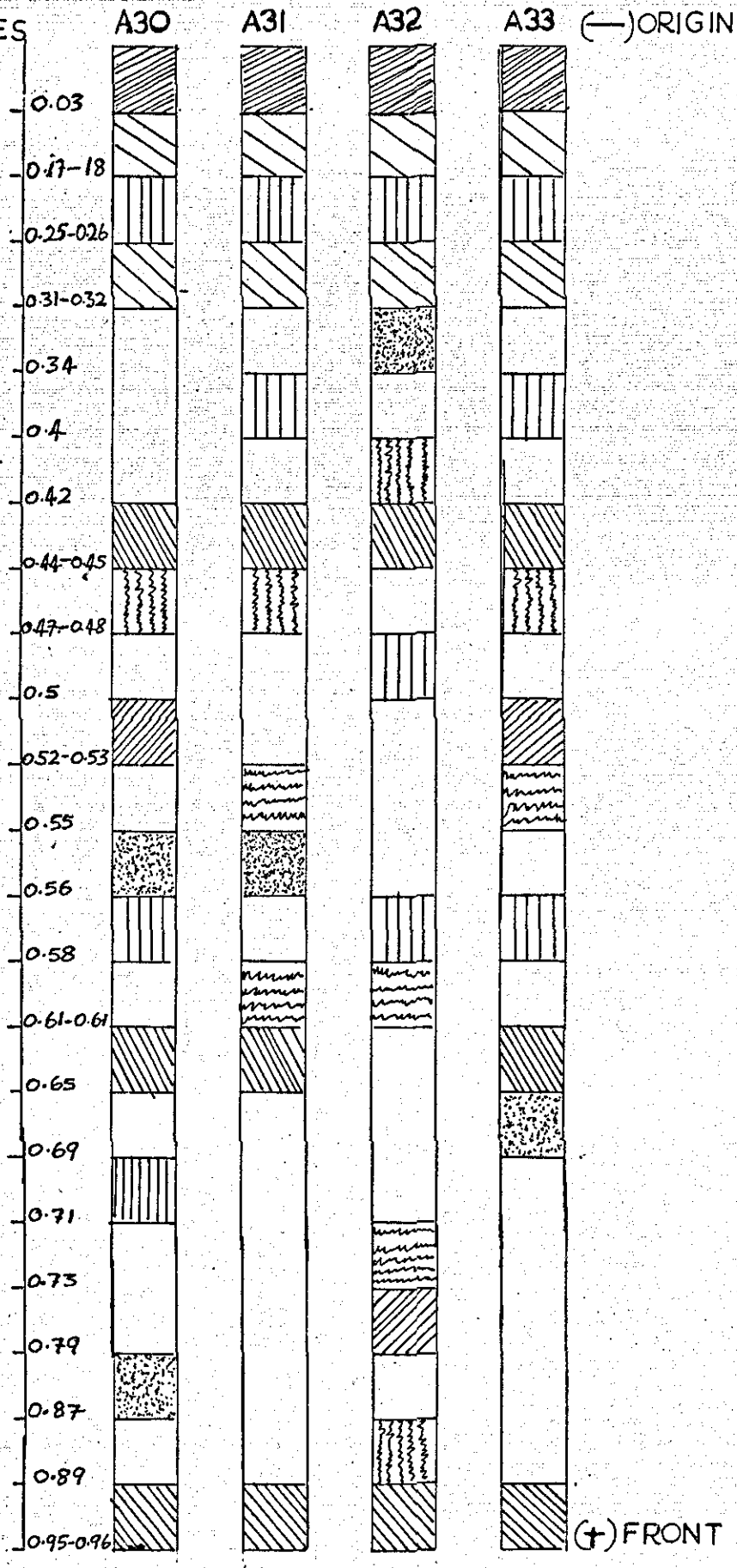


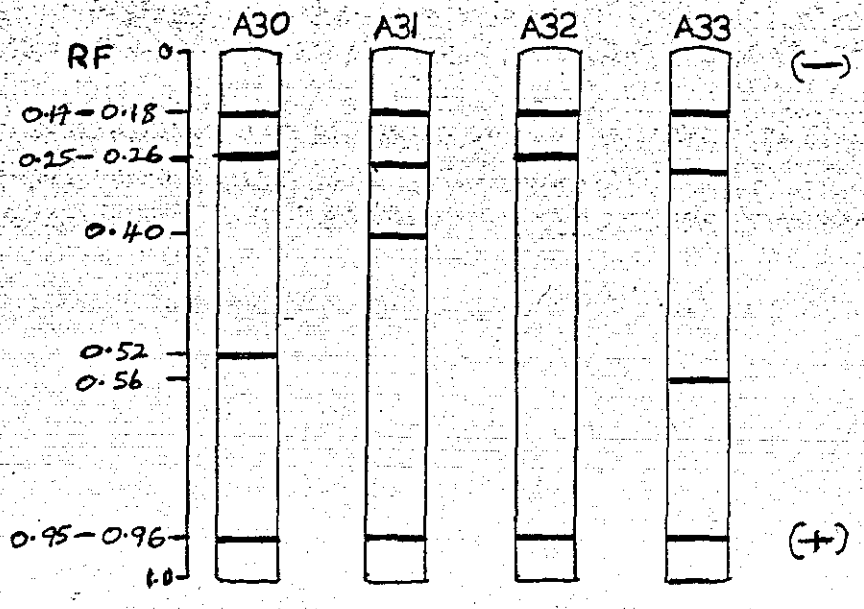
FIG 21

FIG 18 SIMILARITY CHART

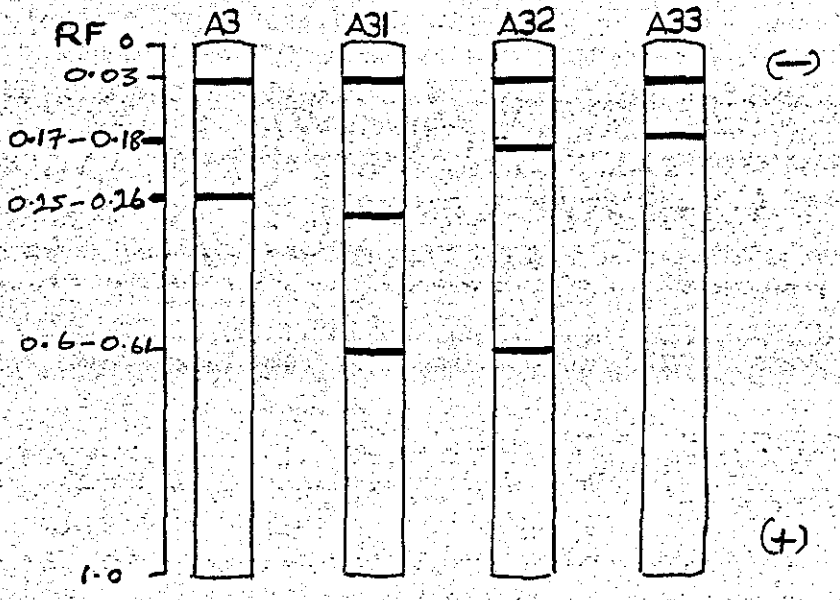


DIAGRAMATIC COMPARISON OF FRACTIONS

FIG 19 SCHEMATIC REPRESENTATION OF ZYMOGRAMS



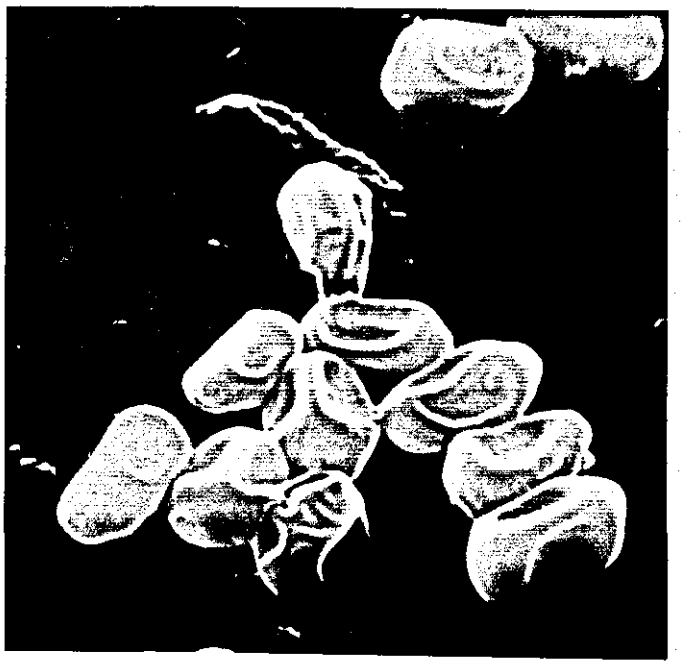
(a) GELS TREATED WITH α-NAPHTHYL ACETATE FOR ESTERASE ACTIVITY



(b) GELS TREATED WITH L-LEUCYL β-NAPHTHYLAMIDE HCL

Plate 14. - SEM of spores of A30 cultured on SAB. Note glabrous nature of spore surface and wall collapse. x 10,000

Plate 15. - SEM of mycelium of A30 cultured in SAB. Liquid medium. The hyphal walls are fairly smooth. x 5,000.



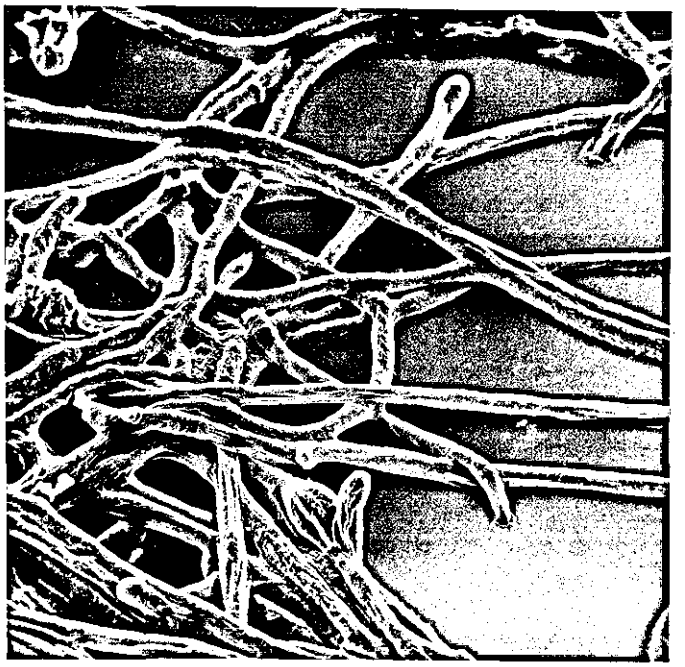
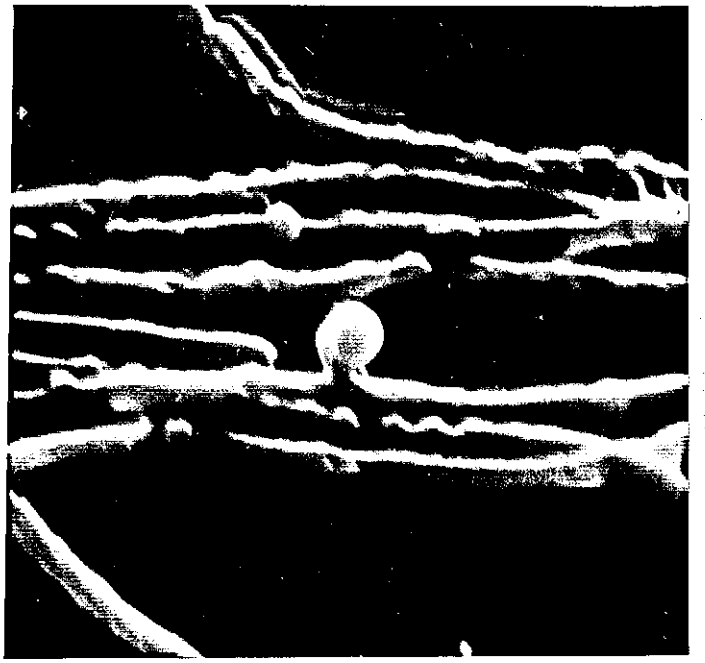
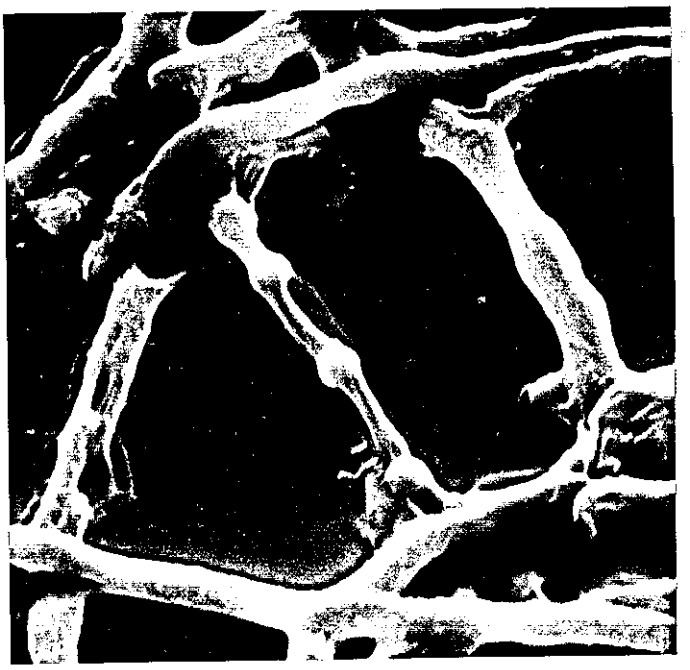


Plate 16. - SEM of mycelium of A31 cultures in SAB. Hyphal swellings and conidium borne on short conidiophore are present. x 5,000.

Plate 17. - SEM of mycelium of A33 cultured in SAB. also showing hyphal swellings. x 5,000.

Plate 18. - SEM of mycelium of A32 grown in SAB showing regular branching with no special feature. x 5,000.





Transmission Electron Microscopy:

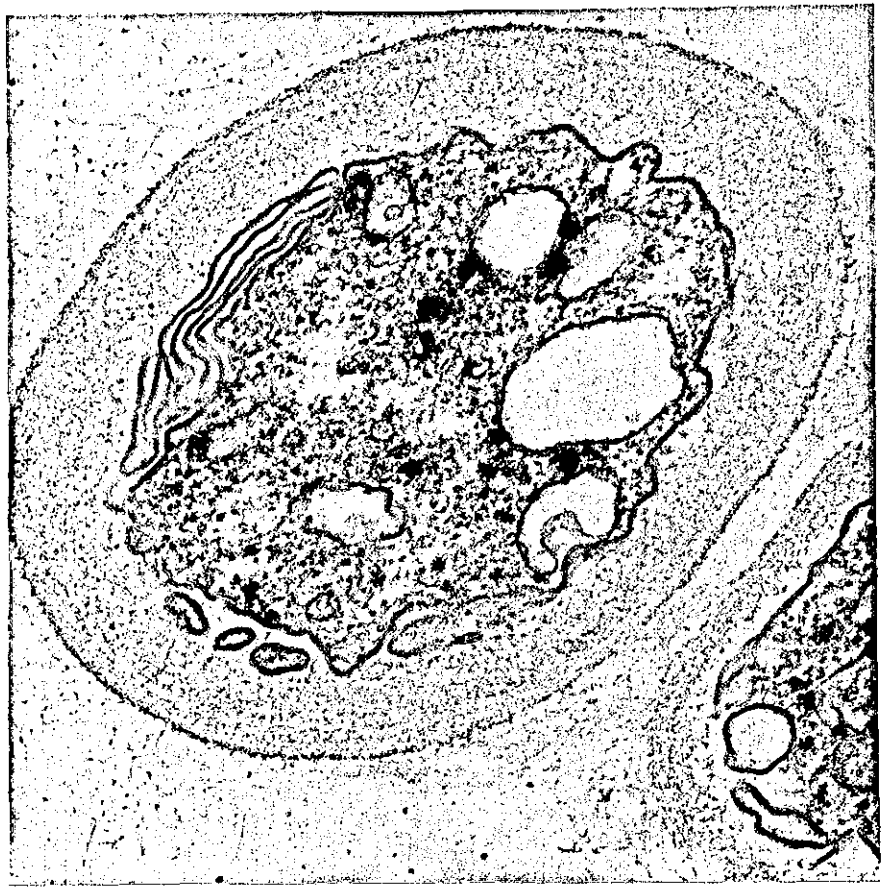
Plate 19 shows the typical appearance of a resting fungal spore. The nucleus was well defined and surrounded by a well marked endoplasmic reticulum. Mitochondria with their regular ovoid shape and contour were present. The absence of large vacuoles and relatively small number of vacuoles was characteristic of low metabolic activity at this stage.

During the process of germination the spores were swollen with the walls becoming thicker. This can be seen in Plate 20. The increased metabolic activity, at this stage was characterised by the presence of more and larger vacuoles, more mitochondria with enlarged and irregular shapes. The plasmalemma is folded in upon itself, probably the start of plasmalemmasomes. Lomasomes can also be seen. These structures did not develop from any particular sites but were randomly situated. The germ tube appeared to originate from the inner wall (Plate 21).

The hyphae are characterised by the concentration of metabolic activity at the tip, where the endoplasmic reticulum, cytoplasmic vesicles and mitochondria are well defined and the plasmalemma are thrown into series of invaginations (Plate 23). A close association seemed to exist amongst the endoplasmic reticulum, plasmalemma and mitochondria (Plate 24). The hyphal wall may be a 3-layered structure (Plate 25) although the demarcation is not quite distinct. The separation of hyphae into cells by the formation of septa occurs in the mature hyphae with a central disc-like pore remaining (Plate 25). Lomasomes, as well as myelin-like structures were seen in the hyphae using the two fixing techniques (Plates 20, 26 and 27).

Plate 19. - TEM section of A33 grown in SAB showing the usual features of a fungal cell. Fixation by OsO_4. x 45,000. Resting spore.

Plate 20. - TEM section of A31 cultures in SAB. The wall is greatly increased; the plasmalemma can be seen folded on itself. Lomasomes are also present. Fixation by OsO_4 . x 35,000. Pre-germination spore.



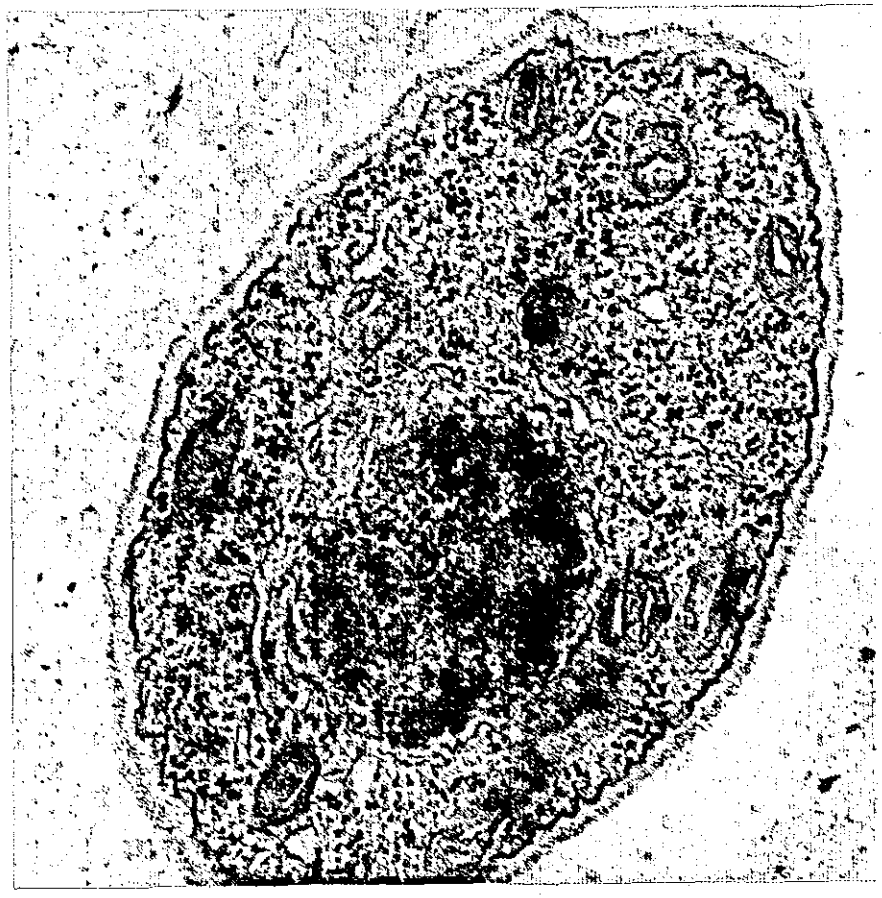
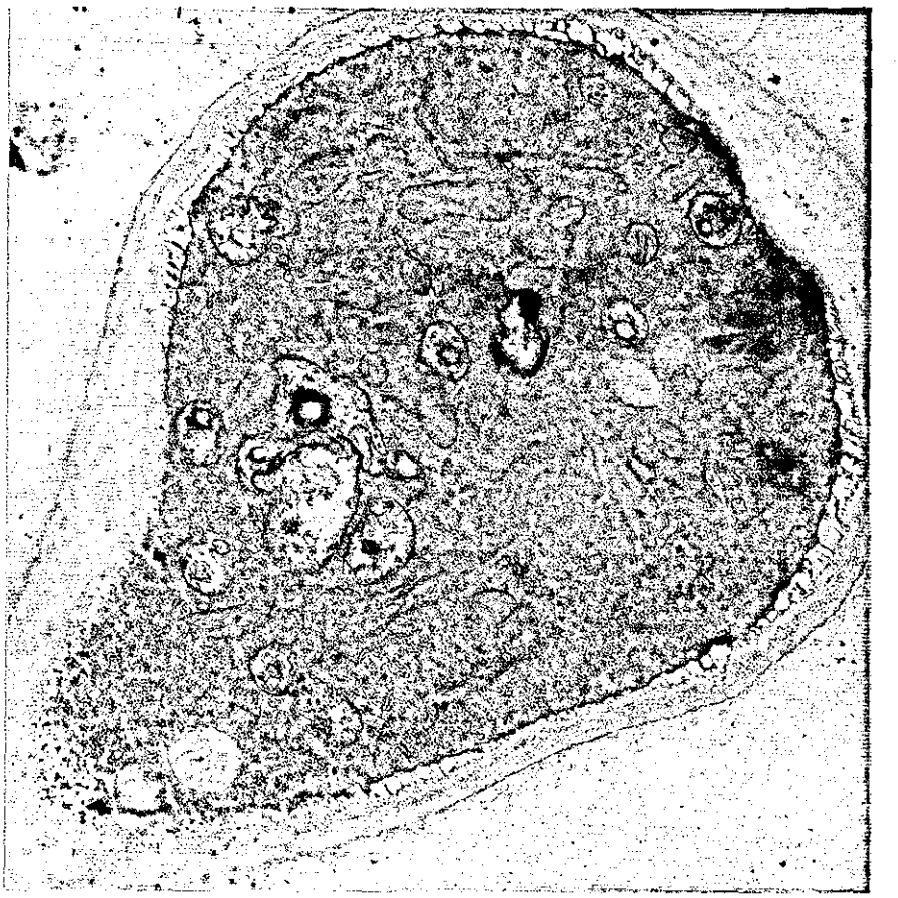


Plate 21. - A30; TEM of spore with an emerging germ tube. Fixation by OsO_4. x 30,000

Plate 22. - A30; TEM of a germinating spore with an elongated germ tube. Fixation by OsO_4. x 32,000.



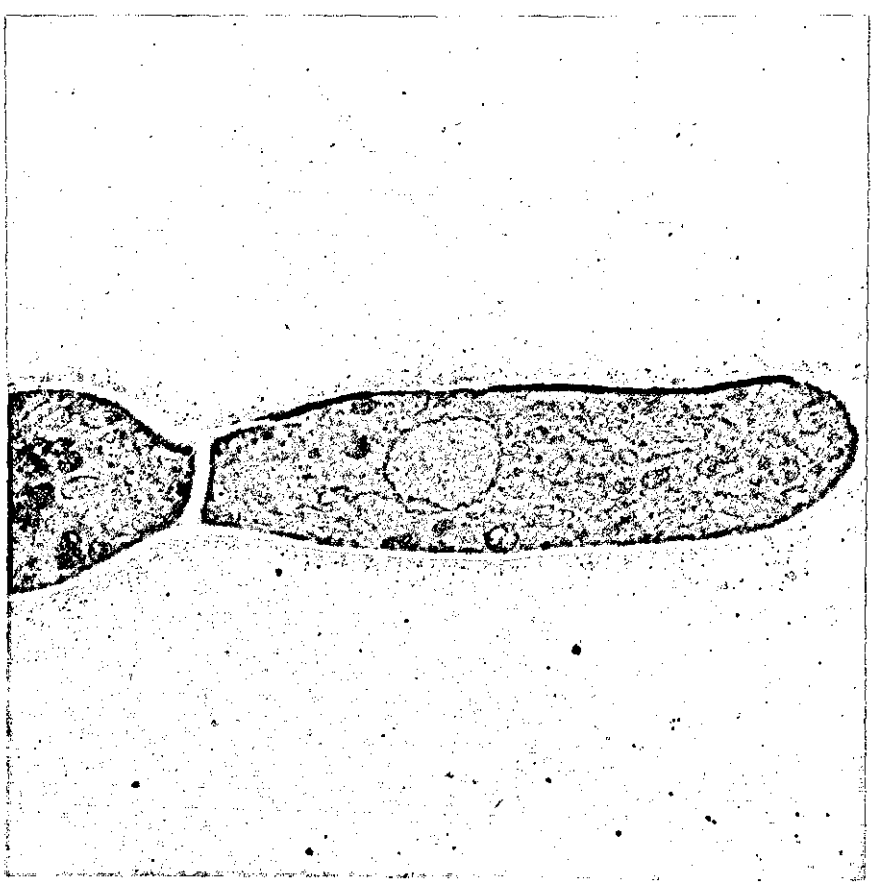
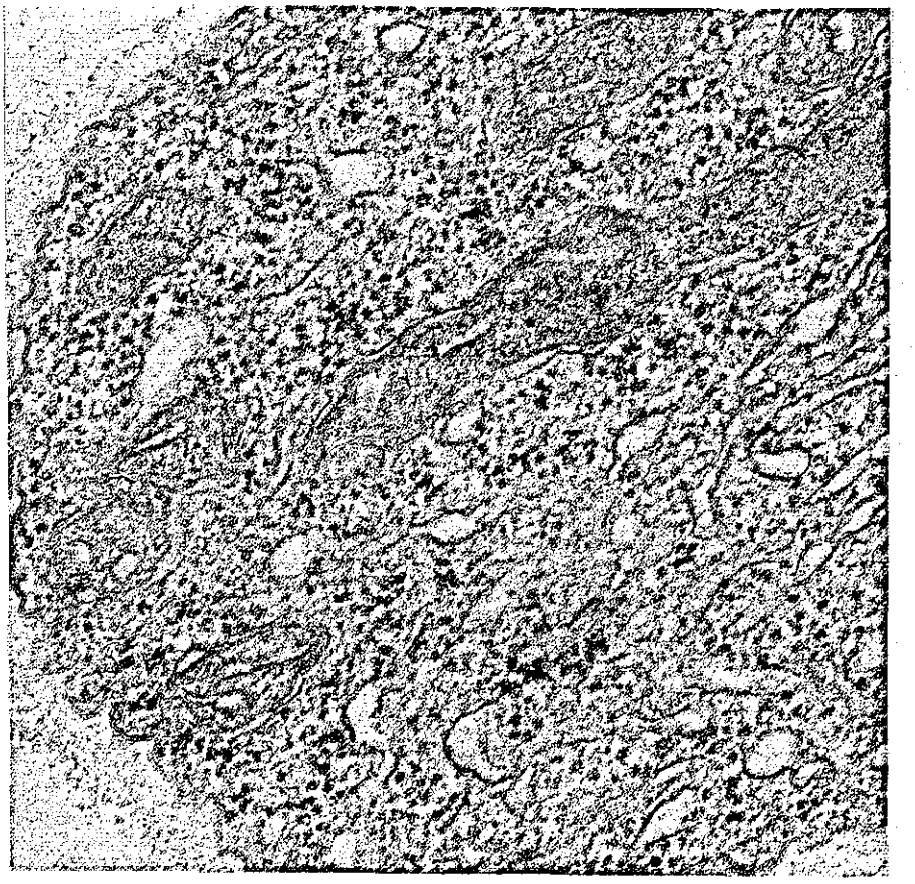


Plate 23. - A33; TEM of a longitudinal section of hyphal tip with numerous cytoplasmic vesicles, mitochondria and endoplasmic reticulum. O_S O_4 fixed. x 75,000.

Plate 24. - A32; TEM of a longitudinal section of hyphal tip showing close association of endoplasmic reticulum, mitochondria and the plasmalemma. O_S O_4 fixed. x 70,000.



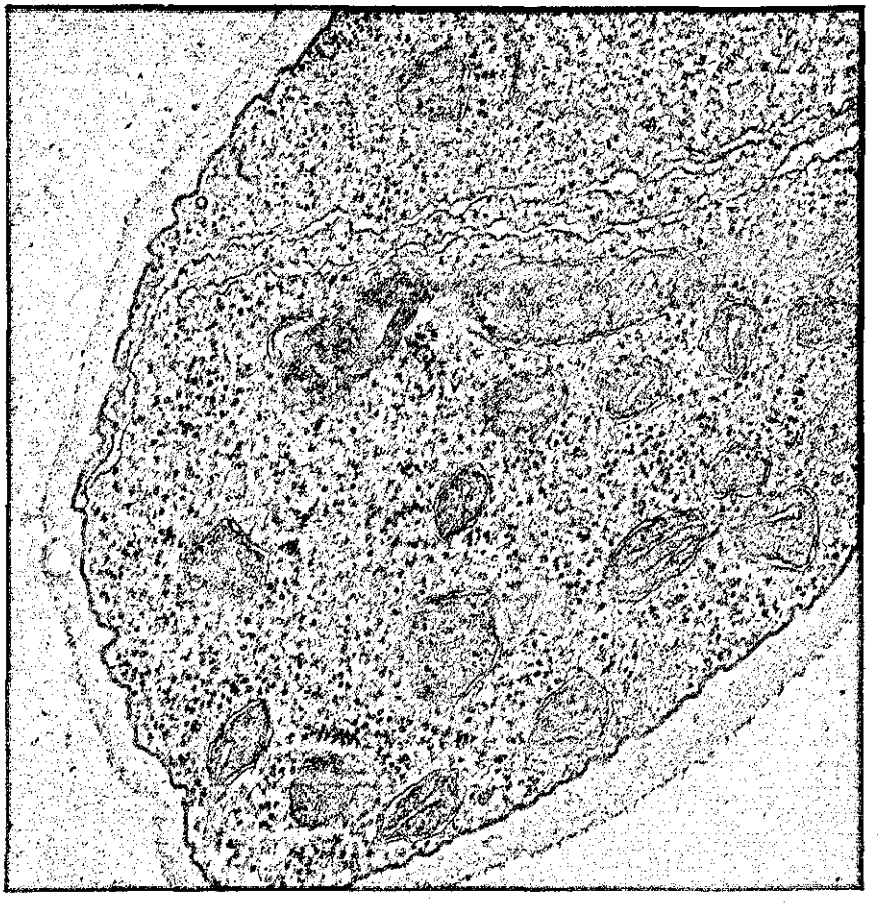
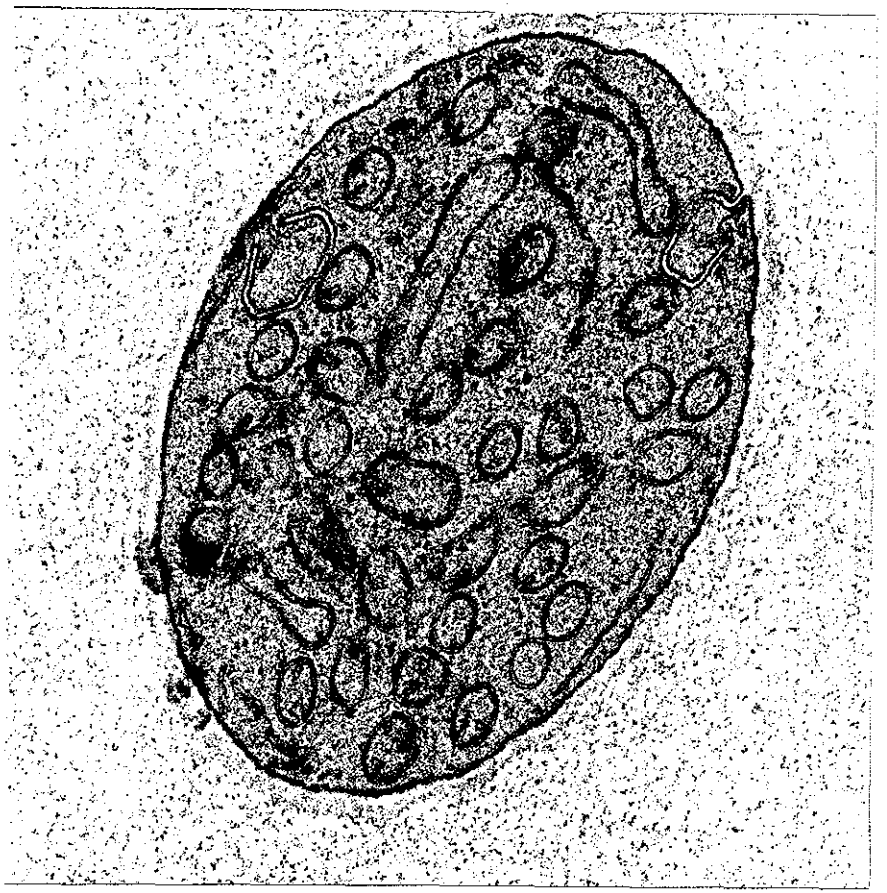


Plate 25. - A30; TEM section showing septa with a disc-like pore. A developing lomasome can be seen. The wall is possibly 3-layered. Fixation by OsO_4. x 40,000.

Plate 26. - A33; TEM section showing lomasomes and lightly stained cell wall. $KMnO_4$ fixed. x 32,000.



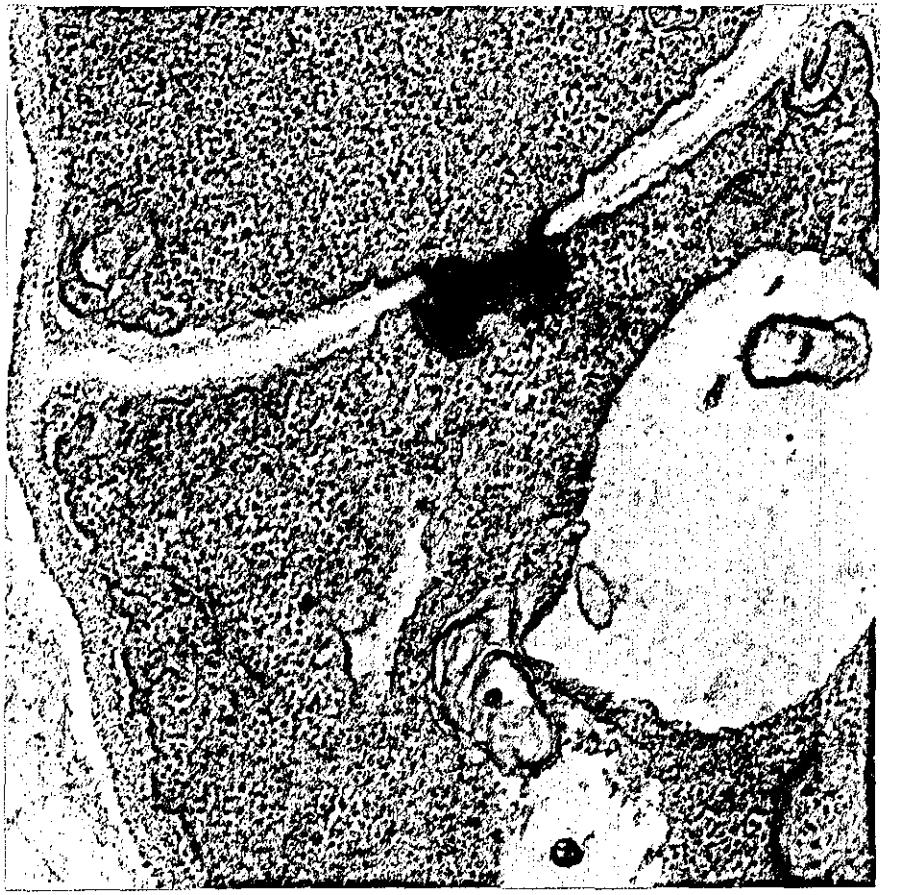
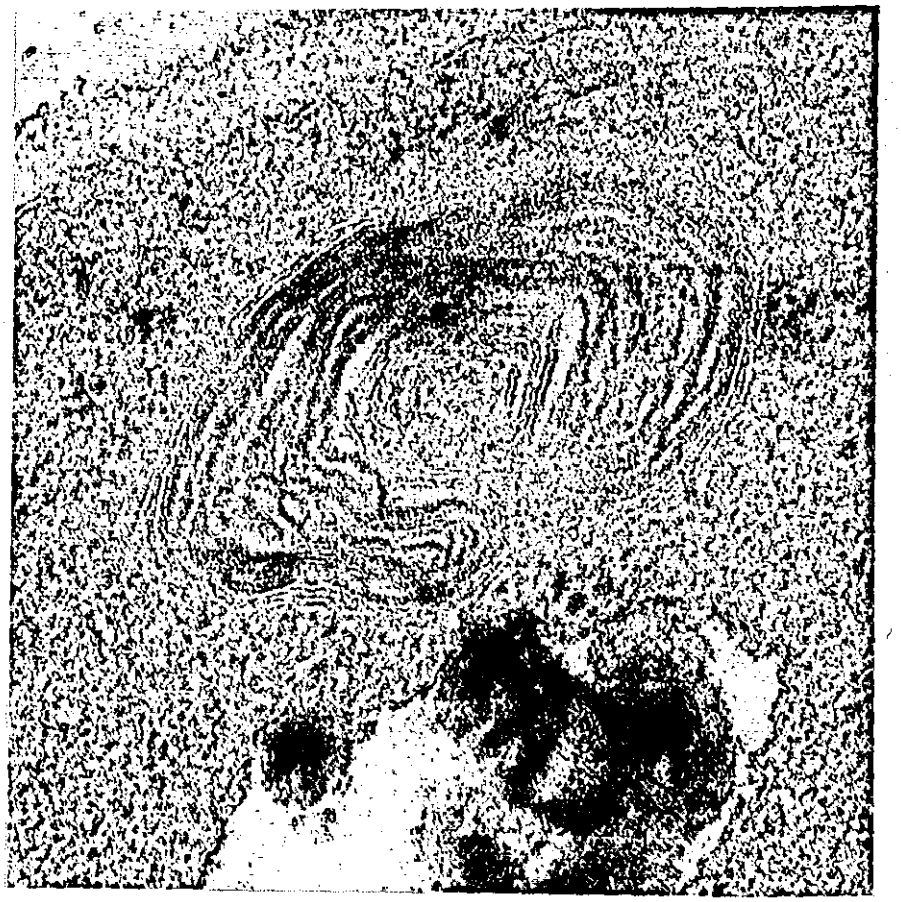
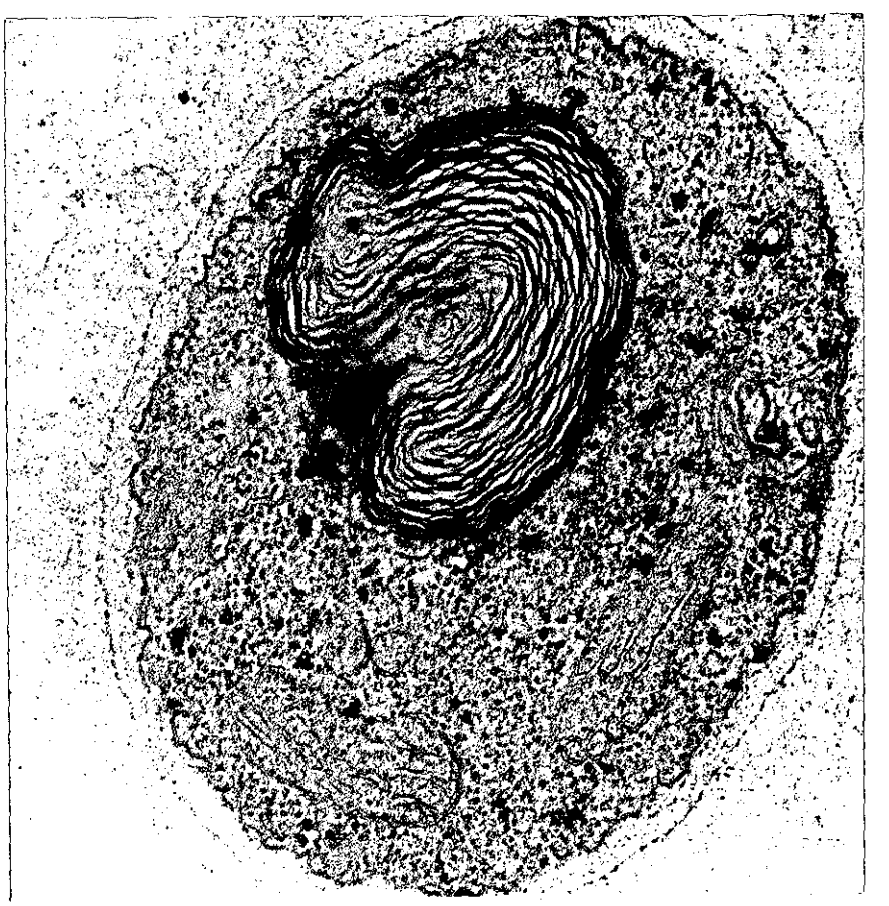


Plate 27. - A32; TEM section showing myelin-type structure, cytoplasmic whorl. O_sO_4 fixed. x 150,000.

Plate 28. - A30; TEM section showing a highly convoluted structure. $KMnO_4$ fixed. x 60,000.





AMINO ACID ANALYSIS:

Table 12 presents a quantitative result of the amino acids obtained from the strains. Similar results were achieved with paper chromatography. All amino acids were present except tryptophan which is always completely destroyed on acid hydrolysis and hydroxyline. In the table, methionine was absent in strains A30 and A31 but it was detected by the paper chromatography. Methionine is known to oxidise during hydrolysis. This can be minimised under anaerobic condition, which was applied during the hydrolysis for the paper chromatography.

CARBOHYDRATE CONTENT:

Initial lack of good separation of the sugars on thin layer chromatography (TLC) led to the use of several systems. The TLC result is presented in Table 13 and this was confirmed by the gas liquid chromatography (See Appendix).

AMINO SUGARS:

These were detected by paper chromatography and estimated (Table 11).

CHITIN:

Applegarth's[170] method was applied to detect chitin. It also indicated the absence of chitosan. The quantity was determined by the fractionation method of Mahadevan and Tatum[171].

The main cell wall components are presented in Table 11.

ENZYMIC TREATMENT:

Treated cells were examined under the electron microscope(EM) by the shadow casting technique.

The controls (Plates 29-32) presented granular surfaces with amorphous appearance. Cellulase and chitinase treatments were quite comparable to the controls (Plate 33). Pronase treatment, after 48 h incubation, affected cells slightly but a prolonged treatment showed some microfibrillar structures (Plate 34). The snail juice treatment

TABLE: 11

PRINCIPAL COMPONENTS OF CELL WALL PREPARATIONS FROM THE TEST STRAINS.

COMPONENTS	A30	A31	A32	A33
TOTAL CARBOHY-DRATES	90.2	88.3	85.9	83.9
PROTEIN (HARTREE)	6.3	8.1	10.2	10.5
PROTEIN (AMINO ACID ANALYSER)	4.3	6.3	9.6	8.7
LIPID, FREE	1.2	1.4	1.1	2.0
LIPID, BOUND	2.0	2.5	2.2	2.7
GALACTOSAMINE	1.4	2.4	1.7	1.3
GLUCOSAMINE	1.9	2.3	2.5	3.2
CHITIN	2.1	4.0	3.0	4.4

VALUES EXPRESSED AS PERCENTAGE OF CELL WALL.

TABLE: 12

AMINO ACID COMPOSITION OF CELL WALLS.

EXPRESSED AS g /100 g SAMPLE

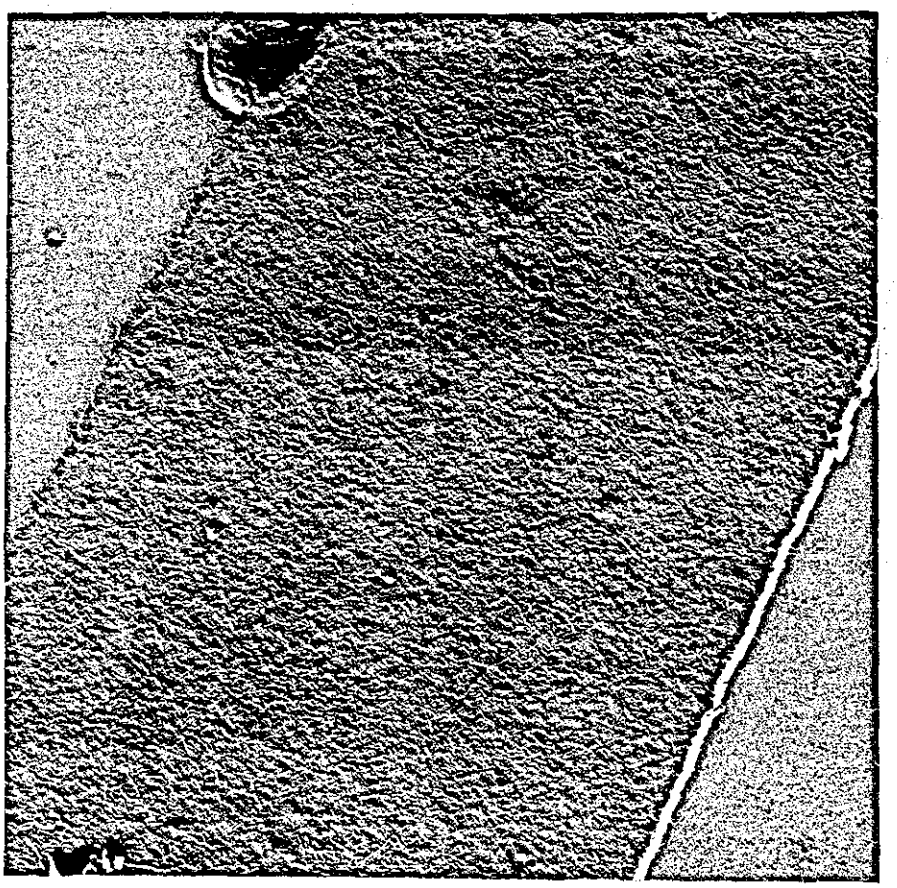
AMINOACID	STRAINS			
	A30	A31	A32	A33
ASPARTIC ACID	0.160	0.190	0.530	0.300
THREONINE	0.200	0.290	0.400	0.350
SERINE	0.130	0.220	0.410	0.320
GLUTAMIC ACID	0.140	0.190	0.710	1.030
PROLINE	0.130	0.230	0.240	0.360
GLYCINE	0.090	0.150	0.280	0.200
ALANINE	0.130	0.190	0.590	0.620
CYSTINE	0.040	0.040	0.090	0.100
VALINE	0.140	0.250	0.500	0.250
METHIONINE	0.000	0.000	0.080	0.010
ISOLEUCINE	0.080	0.150	0.260	0.090
LEUCINE	0.120	0.260	0.440	0.170
TYROSINE	0.060	0.150	0.310	0.140
PHENYLALANINE	0.110	0.160	0.510	0.150
HISTIDINE	2.370	3.120	3.460	4.160
LYSINE	0.090	0.150	0.250	0.060
AMMONIA	0.280	0.370	0.310	0.290
ARGININE	0.040	0.170	0.240	0.080

TABLE: 13

PERCENTAGE OF NEUTRAL SUGARS TO TOTAL SUGAR CONTENT.

	A30	A31	A32	A33
GLUCOSE	50.9	48.6	46.1	64.9
GALACTOSE	12.3	21.1	13.8	8.9
MANNOSE	23.0	20.1	25.1	17.7
RHAMNOSE	13.8	10.2	15.0	8.5

Plates 29 and 30. - Shadow-cast preparations of strains A30 and A31 in buffer. Note the amorphous nature of walls.
A30 x 20,000.
A31 x 25,000.



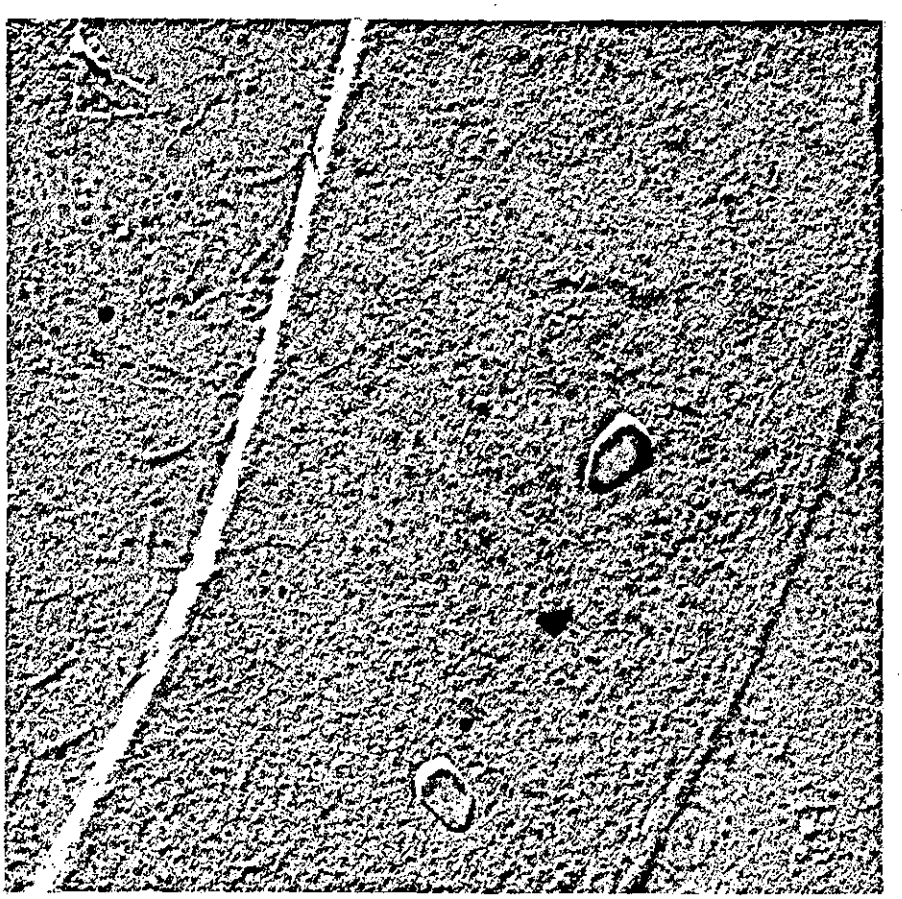
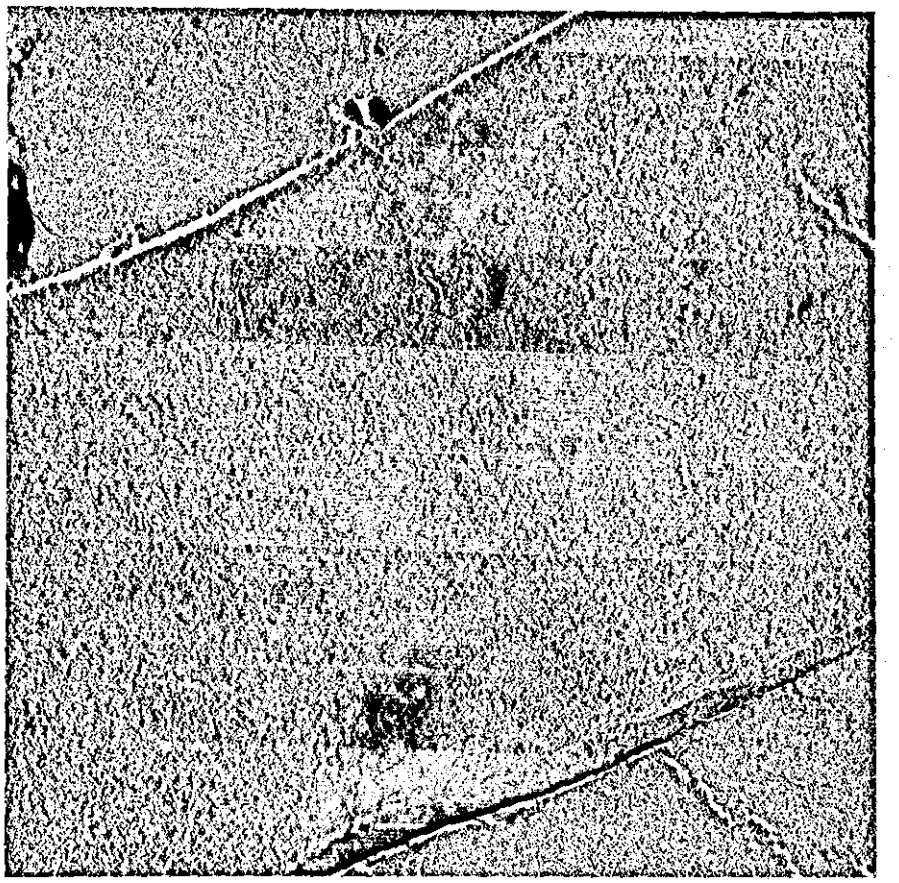


Plate 31 and 32. Shadow-cast preparations of strains A32 and A33 in buffer. Note the amorphous nature of the walls.
A32 x 25,000.
A33 x 20,000.



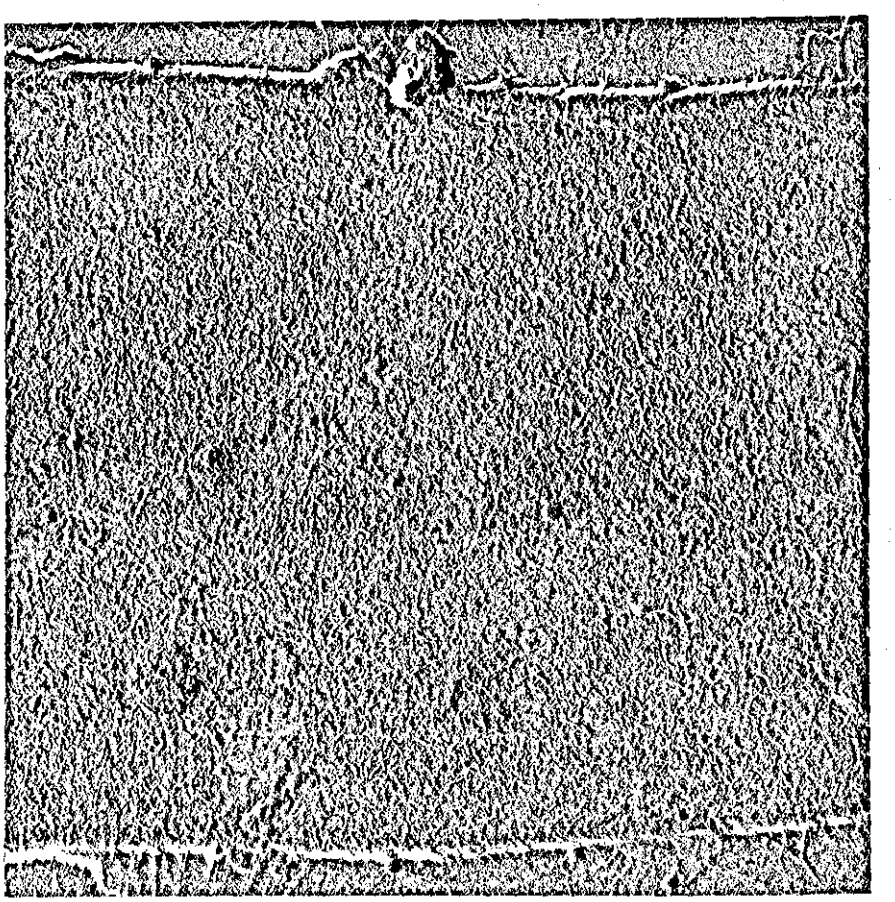
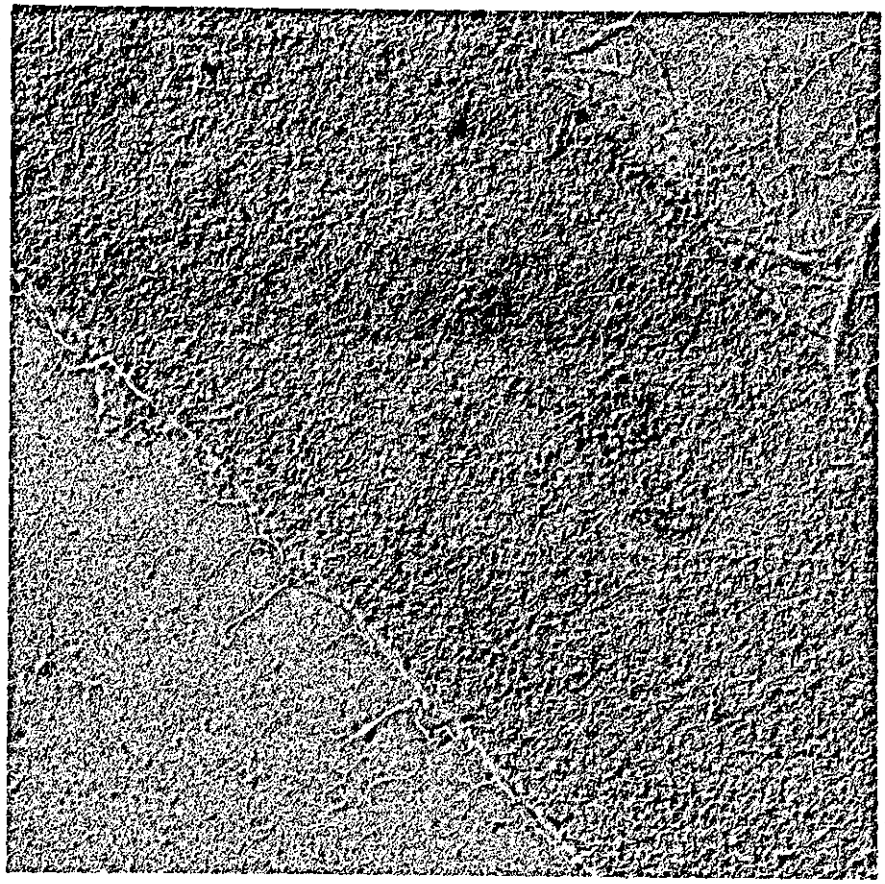
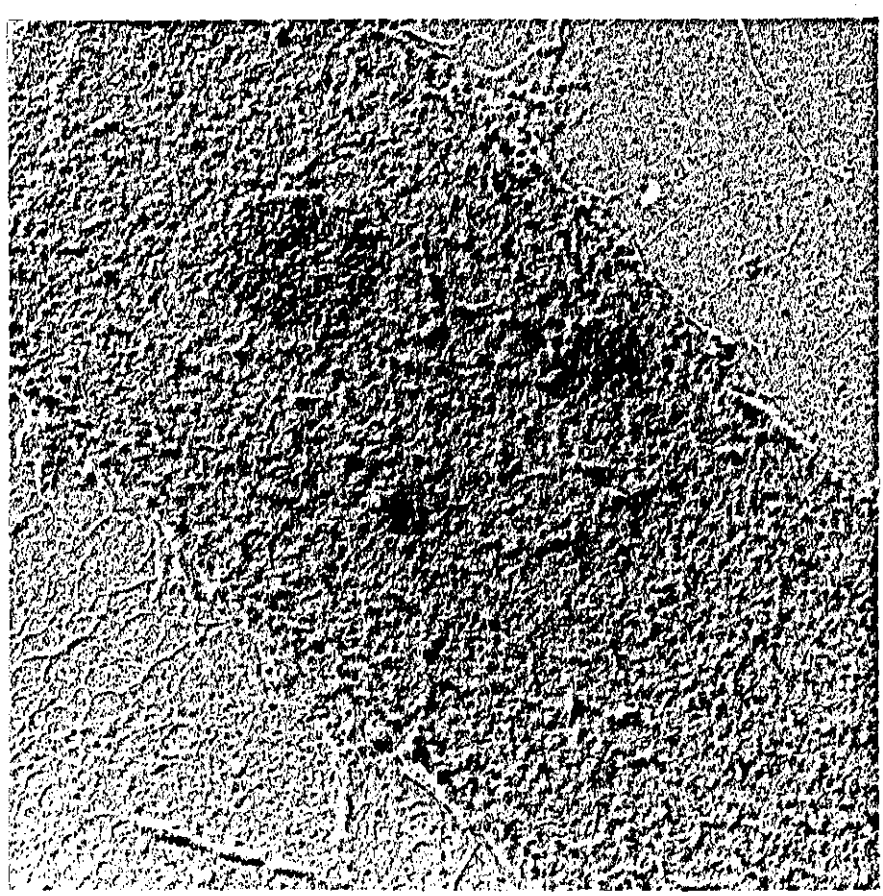


Plate 33. - A30; Shadowed section of chitinase treated walls. Note little change of appearance. x 25,000.

Plate 34. - A30; shadowed section of cell treated with pronase for 72h. Note surface distortion and still masked microfibrils. x 25,000.





showed a strong effect on cells (Plate 35). All strains developed a series of ditches, some increasing to crater-forms; a few course scattered microfibrils were noticeable. A snail juice/pronase treatment (Plates 36 and 37), showed distinct view of the course undirectional and non-uniform microfibril indicating the removal of the amorphous matrix- from the interstices. Chitinase/pronase treatment showed some few scattered microfibrils as in pronase treatment. Snail juice/pronase/chitinase showed degeneration as well as disappearance of microfibrils (Plate 38).

ANTIMICROBIAL ACTIVITY:

Only A30 was used for these tests. The minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC) of various agents were determined (Table 14). Sub-lethal doses of specific agents were then used in morphogenetic studies and examined by light microscopy SEM and TEM.

The polyenes, pyrimidine derivatives, and actidione caused lateral and terminal branching. Sporulation was inhibited at higher concentration. Griseofulvin induced a curling effect.

Plate 39 showed a typical growth of the organism in the antibiotic medium. Septation was regular and conidiation low. A mature and a few developing conidia can be seen and hyphal branching was quite regular. The SEM and TEM were similar with earlier microscopic studies.

Ramihyphin: used at 100 $\mu g/cm^3$ induced a highly ramified growth (Plates 40 and 41). "Blastospore-like" structures were formed and the cells were frequently vacuolated. The SEM presented cells with very undulating surfaces without a definite shape.

Rapamycin: At 10 $\mu g/cm^3$ slender hyphae were produced. Conidiation increased and spores appeared like chlamydospores. In the SEM, cell collapse and possibly leakage were evident; also the conidia have lost their smooth surfaces (Plates 42 and 43).

TABLE: 14

MIC AND MCC OF VARIOUS ANTIMICROBIAL AGENTS - AFTER 24 H INCUBATION AT 30°C

ANTIMICROBIAL AGENTS		MIC (µg/ml)	MCC (µg/ml)
AMPHOTERICIN B	POLYENES	1.56	25
NATAMYCIN		0.78	7.03
NYSTATIN		1.56	5.27
TRICHOMYCIN		0.78	7.03
5-FLUOROCYTOSINE	PYRIMIDINE	100	>200
5-FLUOROURACIL	BASE	50	>200
CLOTRIMAZOLE	IMIDAZOLE	0.39	0.88
MICONAZOLE	DERIVATIVES	0.05	0.18
GRISEOFULVIN	OTHERS ANTIFUNGALS	50	>200
RAPAMYCIN		12.5	75.5
RAMIHYPHIN		150	>200
ACTIDIONE		1.56	150
ACULEACIN A		25	>200
POLYOXIN D		100	>200
CYTOCHALASIN A		100	>200
GENTAMICIN	ANTIBACTERIAL	>200	ND
RIFAMPICIN		50	112.5
TETRACYCLINE		>200	ND
CHLORAMPHENICOL		>200	ND
2,4-DINITROPHENOL	UNCLASSIFIED	200	>400
ETHIDIUM BROMIDE		3.12	200
PHANQUONE		>200	>400

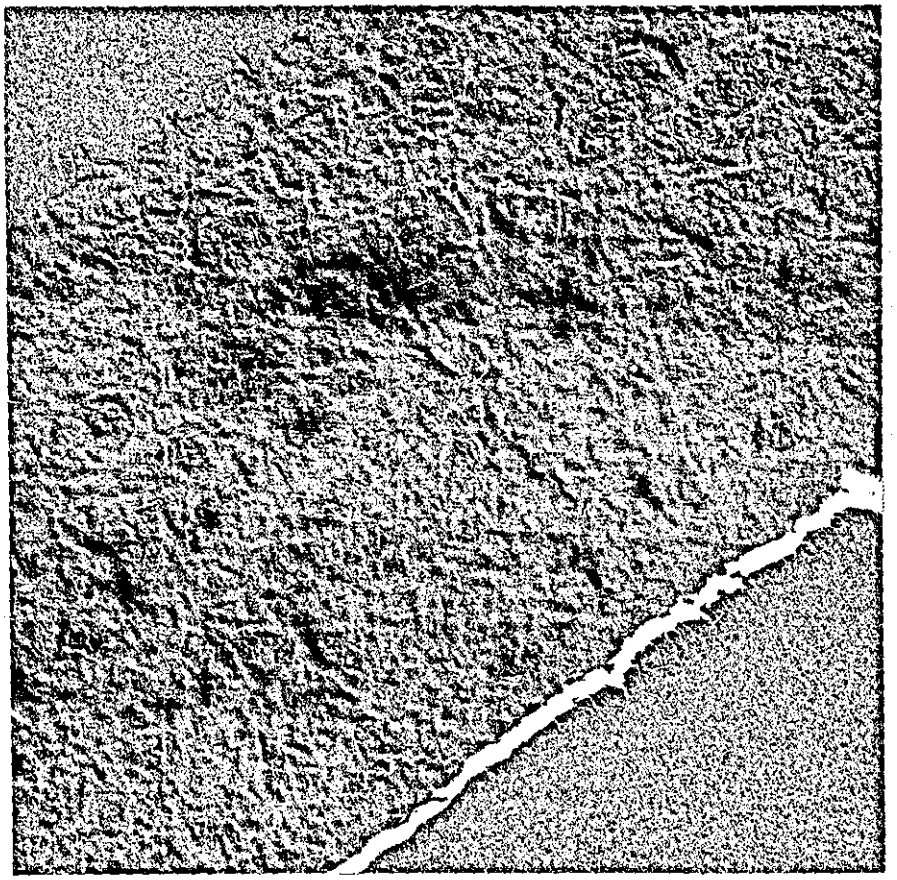
MIC: MINIMUM INHIBITORY CONCENTRATION

MCC: MINIMUM CIDAL CONCENTRATION

ND: NOT DONE

Plate 35. - A32; shadowed section of β-glucanase treated walls. Note some coarse strands and cratered appearance. x 30,000.

Plate 36. - A30; shadowed sections of walls treated sequentially through β-glucanase and pronase. Note distinct undirectional microfibrils. x,30,000.



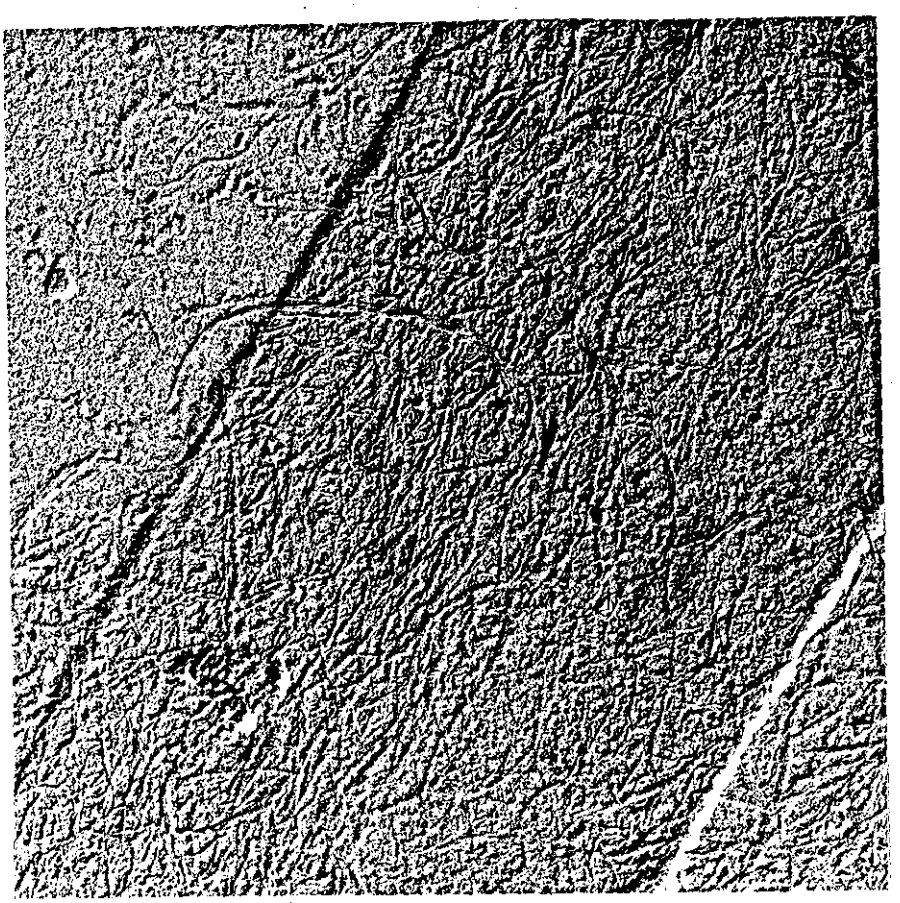
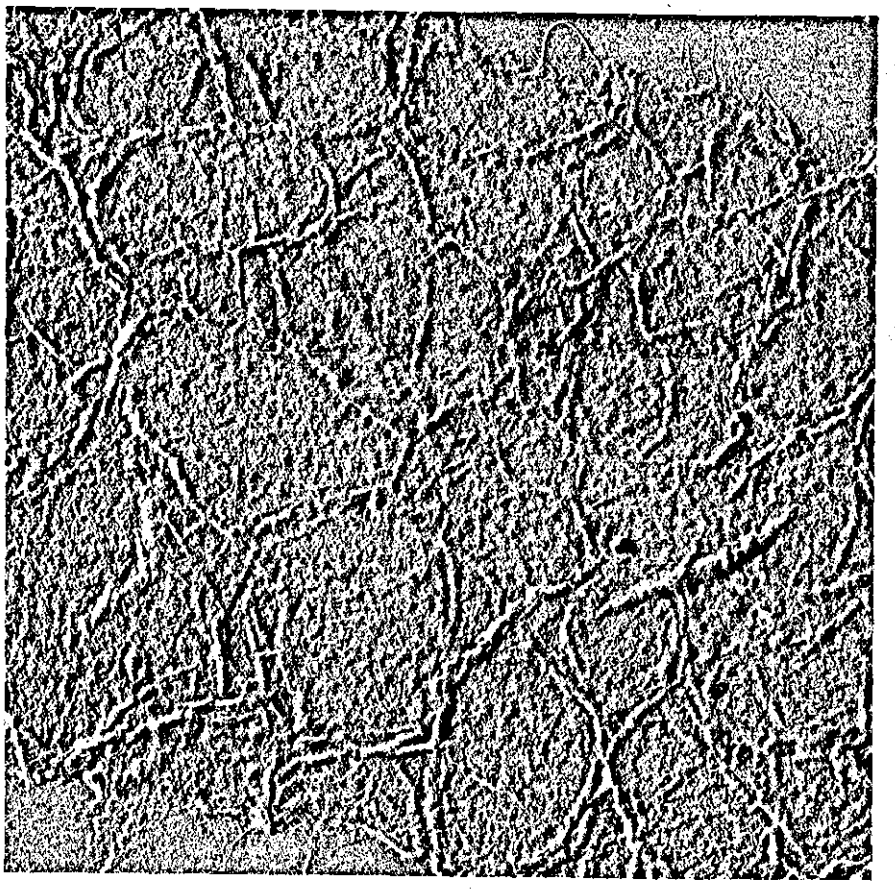

b

Plate 37. - A31; shadowed section of walls treated sequentially through β-gluconase and pronase. Note coarse distinct microfibrils. x 32,000.

Plate 38. - A33; shadowed section of walls treated sequentially through β-gluconase/pronase/chitinase. Note, degenerative effect and disappearance of microfibrils. x 30,000.



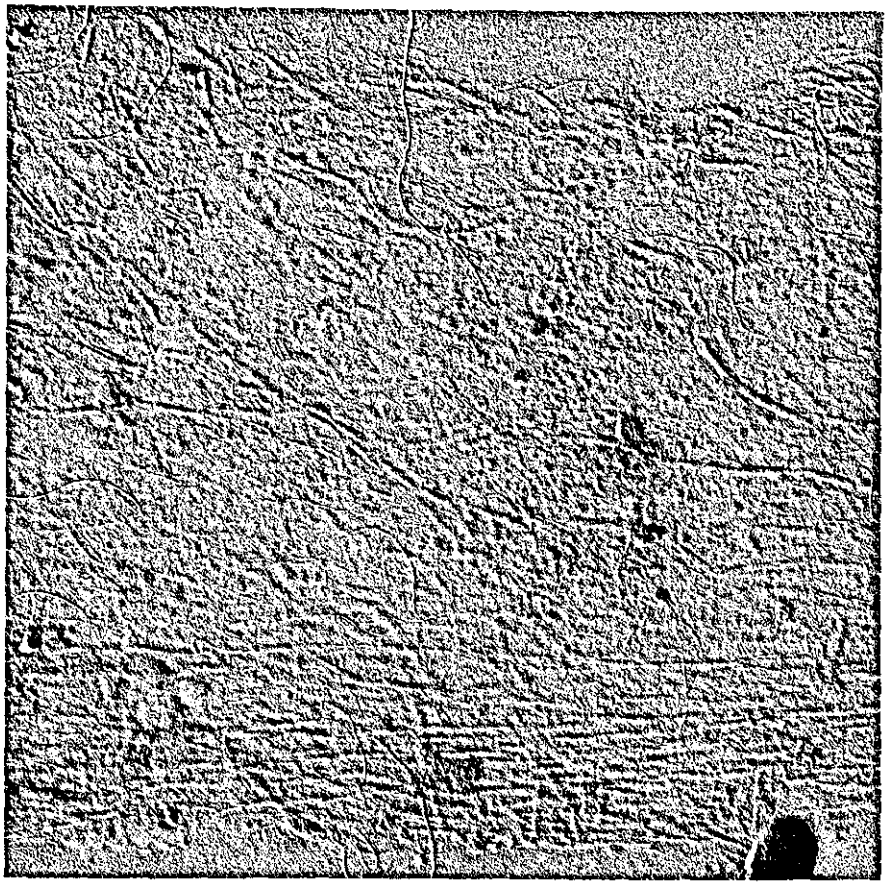
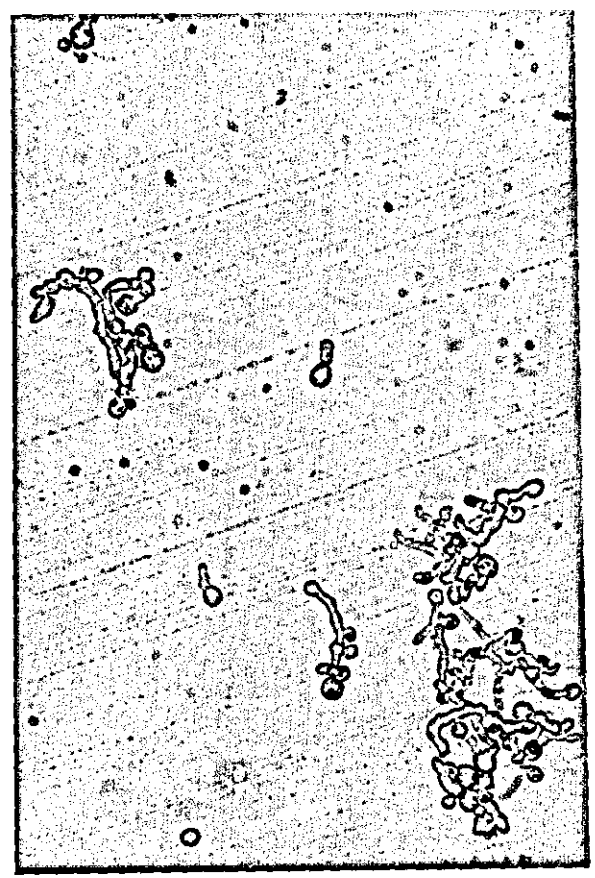


Plate 39. - A30; Light micrograph of growth in Shadowmy's medium for antibiotics testing. Note regular branching- a mature phialide and absence of conidia. x 400.

Plate 40. - Light micrograph of A30 grown in the presence of 100 $\mu g/cm^3$ of Ramihyphin. Note greatly distorted with blastospore-like cells. x 400.

Plate 41. - SEM of A30 grown in the presence of 100 $\mu g/cm^3$ of Ramihyphin. Note depth of ramification; cells have no definite shapes. x 10,000.



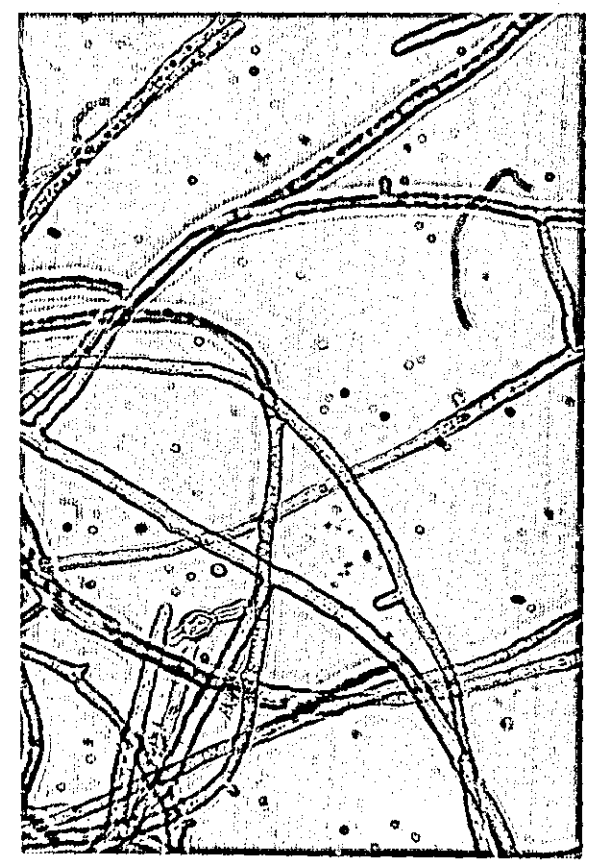
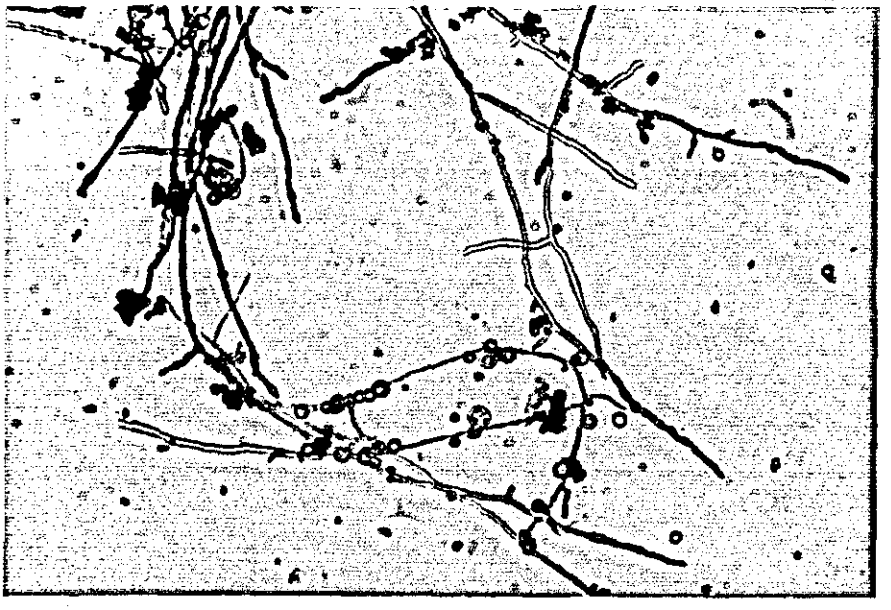
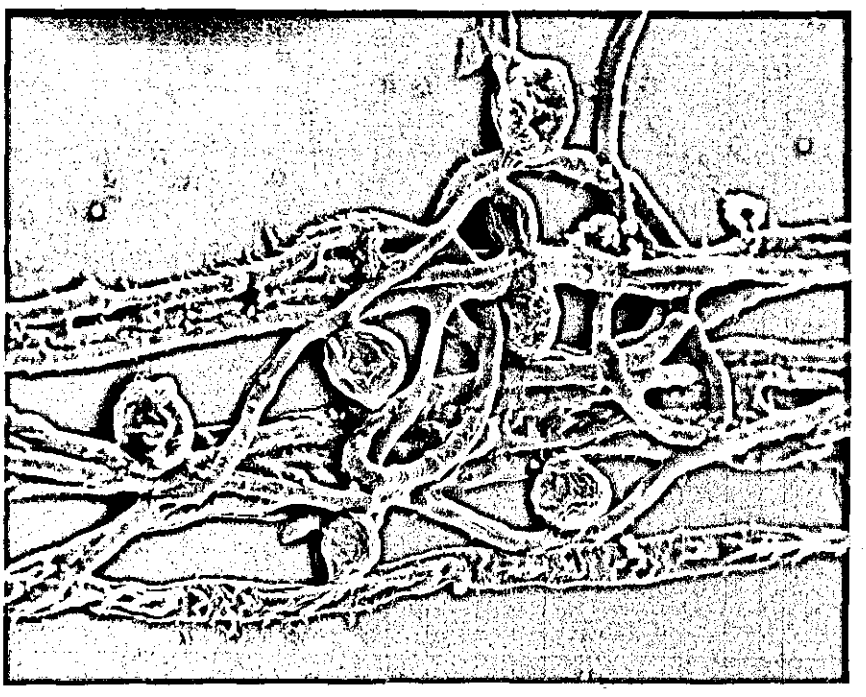


Plate 42. - Light micrograph of A30 grown in the presence of 10 $\mu g/cm^3$ of Rapamycin. Note reduced hyphae and presence of spores looking like chlamydospores. x 400.

Plate 43. - SEM of A30 grown in the presence of 10 $\mu g/cm^3$ of Rapamycin. Note presence of distorted spores, degenerating hyphae and possibly leakage. x 10,000.





Cytochalasin A: At 70 $\mu g/cm^3$, the hyphae were enlarged and showed high vacuolation. They were segmented into beady forms or a series of hyphal swellings. At 95 $\mu g/cm^3$, the segments broke off into rounded forms and different hyphal shapes developed (Plates 44 and 45). The SEM showed the depth of fragmentation. Some giant cells were also produced (Plates 46 and 47). The TEM (Plate 48) shows a hyphae undergoing segmentation with faint cell wall and lipid deposition. No cytoplasmic distortion was observed.

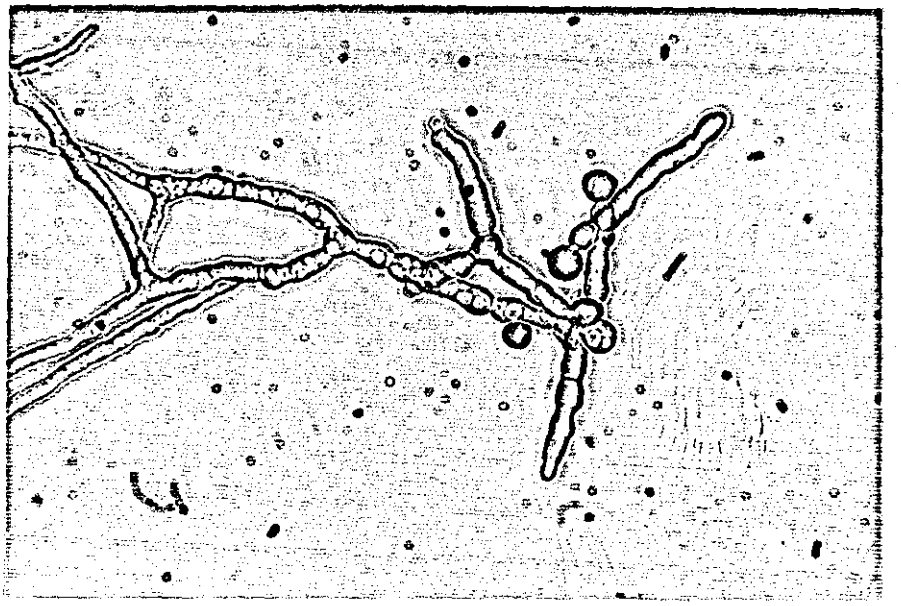
Polyoxin D: This agent, used at 75 $\mu g/cm^3$, induced excessive ballooning of cells which tended to clump together or in chains (Plate: 49). Blastospore-like cells were again produced. Giant cells at hyphal tips and cell collapse could be seen (Plates 50 and 51). The TEM showed severe cytoplasmic distortion. Swollen mitochondria; mitochondria undergoing autolysis and also granular nuclei were present. Cytoplasm was much less dense and cell wall was not very recognisable (Plates 52 and 53). The effect of Polyoxin D on chitin was examined by the shadow-casting technique. Plate 54 showed some loose isolated microfibrils which are similar to those encountered in the enzymatic studies. Some protoplasts were produced by a prolonged treatment. It was observed that these protoplasts failed to revert to the hyphal growth.

Aculeacin A: This drug produced excessive branching and some hyphal swellings. Segmentation and surface distortion was present (Plates 55 and 56) surface distortion too. The TEM shows a dense and severely distorted cytoplasm (Plate 57).

Plate 44. - Light micrograph of A30 grown in the presence of 75 $\mu g/cm^3$ of cytochalasin A. Note vacuolation and hyphal beading. x 400.

Plate 45. - SEM of A30 in the presence of 75 $\mu g/cm^3$ of cytochalasin A. Note depth of hyphal beading. x 10,000.

Plate 46. - Light micrograph of A30 in the presence of 95 $\mu g/cm^3$ of cytochalasin A. Note extensive hyphal beading and presence of various shaped cells. x 400.



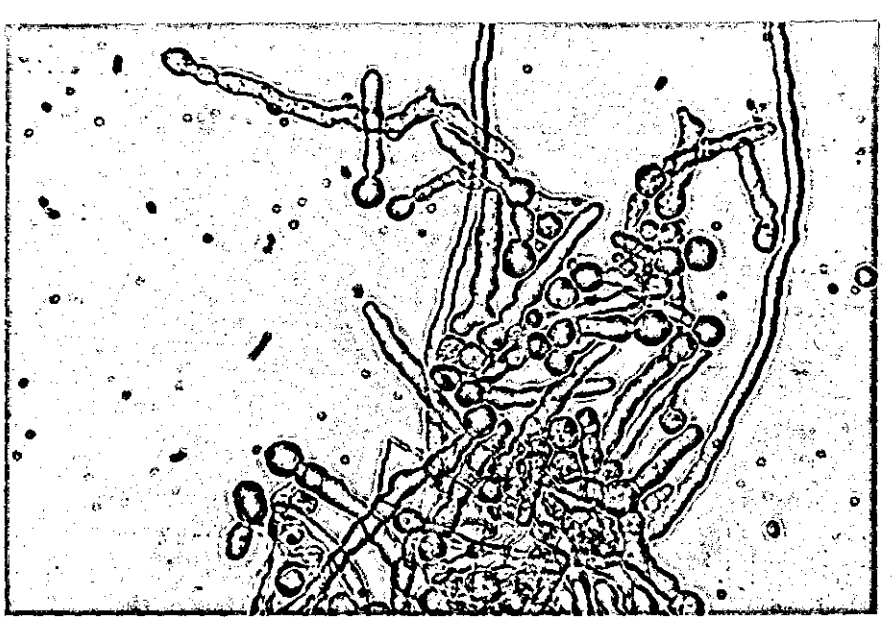


Plate 47. - SEM of A30 grown in the presence 95 $\mu g/cm^3$ of Cytochalasin A. Note presence of hyphal beading and balloon cells. x 10,000.

Plate 48. - TEM of A30 in the presence of 95 $\mu g/cm^3$ of Cytochalesin A. There is no significant cytoplasmic distortion. x 20,000.

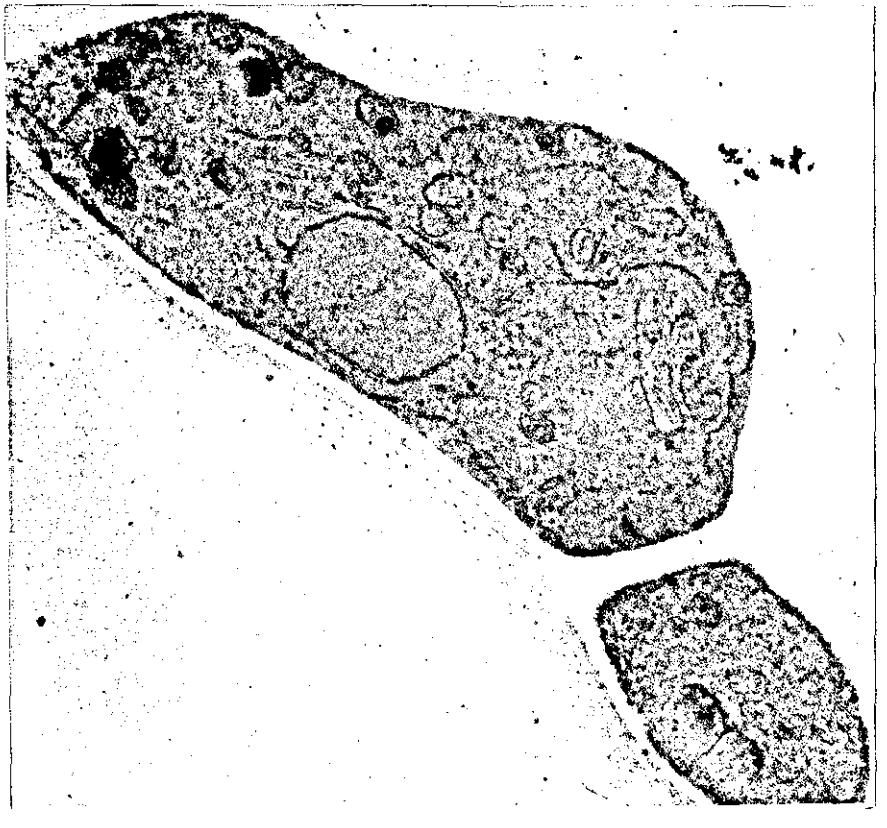
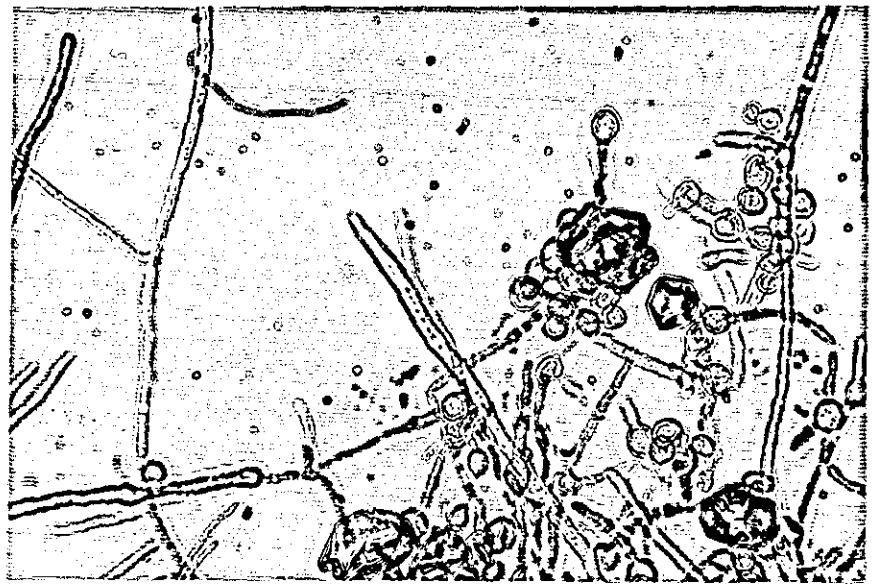
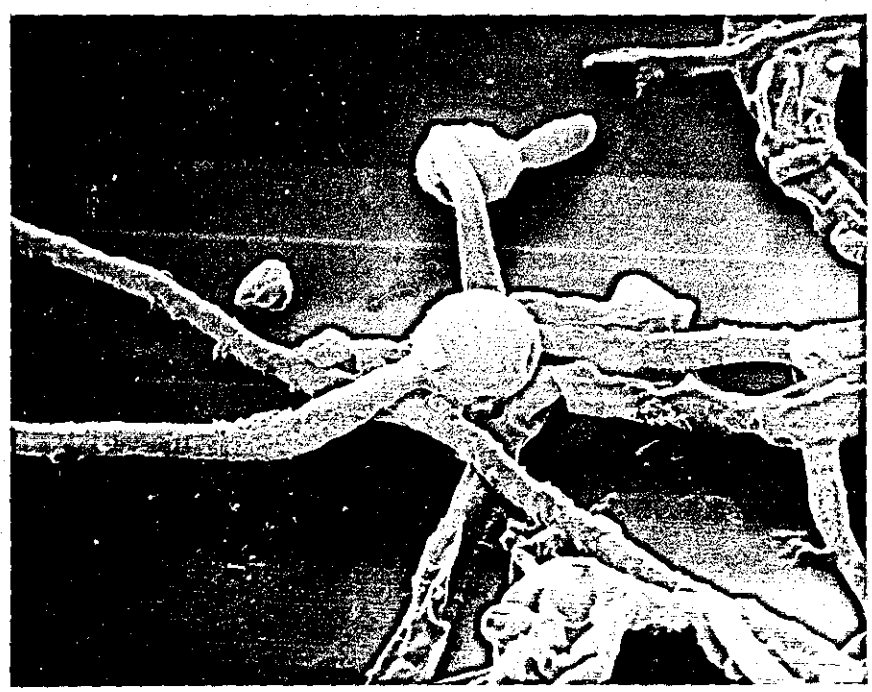


Plate 49. - Light micrograph of A30 in the presence of 75 $\mu g/cm^3$ of Polyoxin D. Note excessive ballooning of cells. x 400.

Plate 50. - SEM of A30 in the presence of 75 $\mu g/cm^3$ of Polyoxin D - showing balloon cells. x 10,000.

Plate 51. - SEM of A30 in the presence of 75 $\mu g/cm^3$ of Polyoxin D showing distortion of cellular surface. x 10,000.





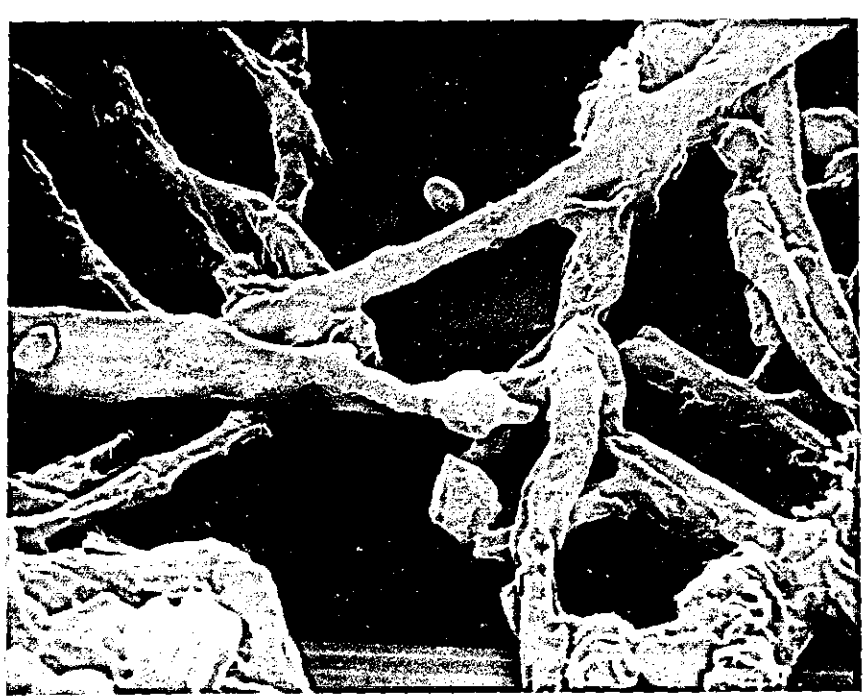
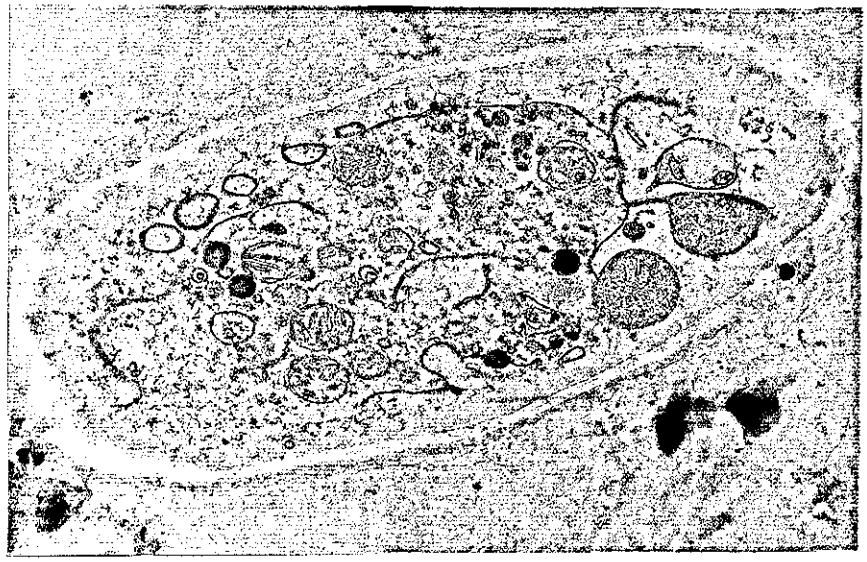
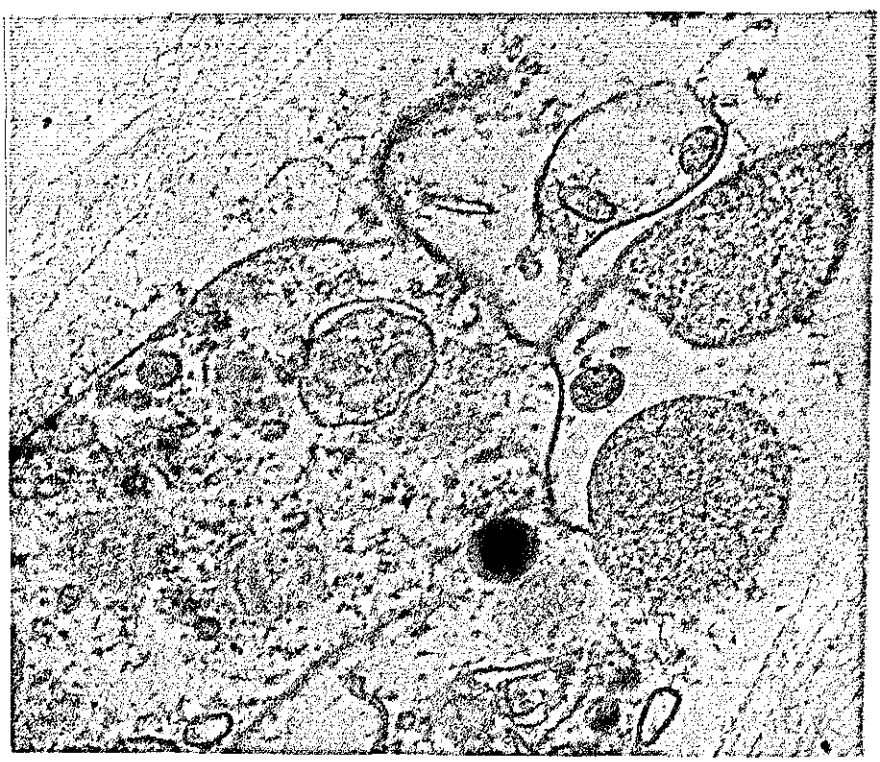


Plate 52. - TEM of A30 in the presence of 75 $\mu g/cm^3$ of Polyoxin D. Note cytoplasmic distortion. x 30,000.

Plate 53. - Higher magnification of section on Plate 52 to show extent of cytoplasmic distortion. Note granular nucleic, swollen mitochondria and ill-defined cell wall. Fixed in Kmn 04 x 65,000.

Plate 54. - Shadowed wall section of A30 in the presence of 75 $\mu g/cm^3$ of Polyoxin D, showing disappearing microfibrils. x 20,000.





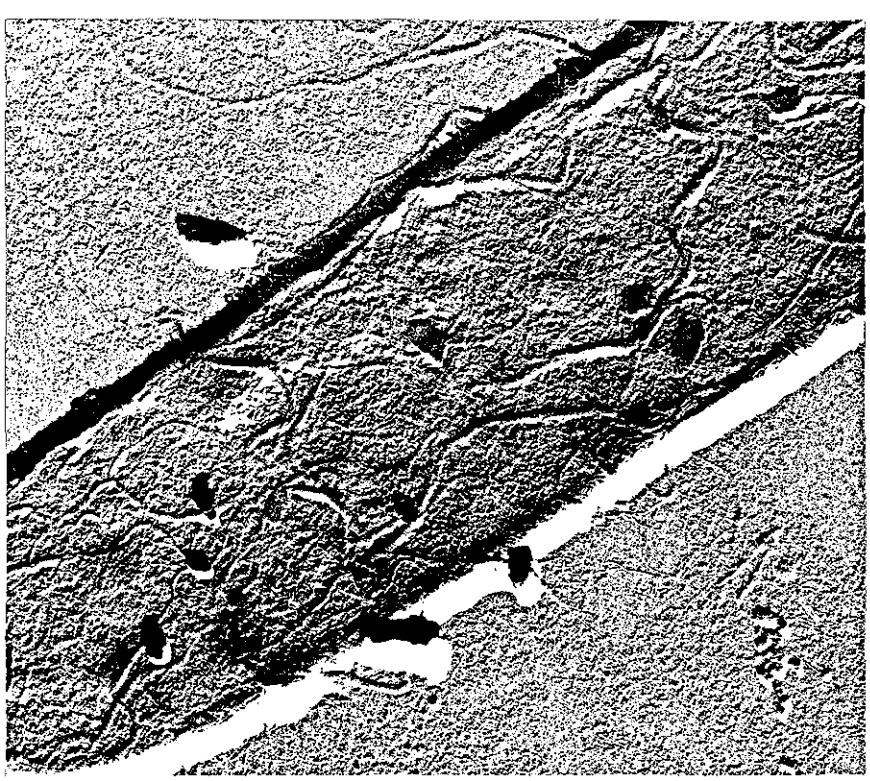
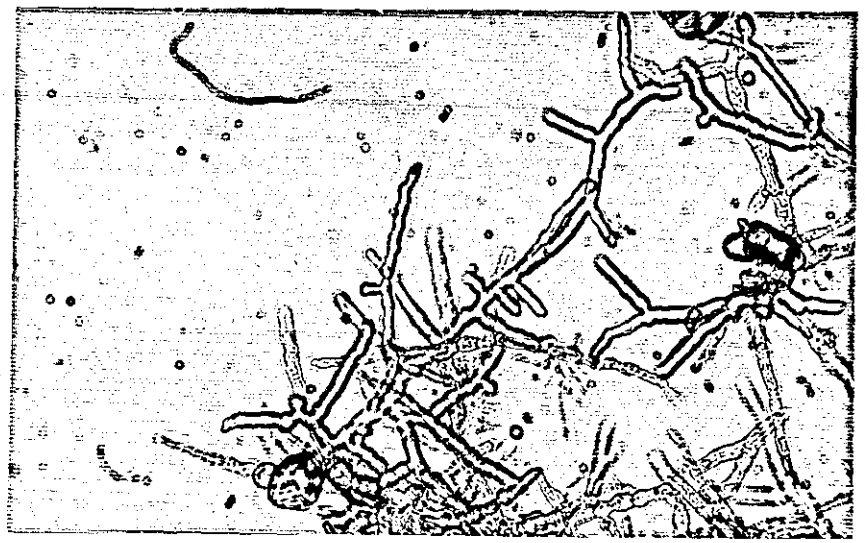
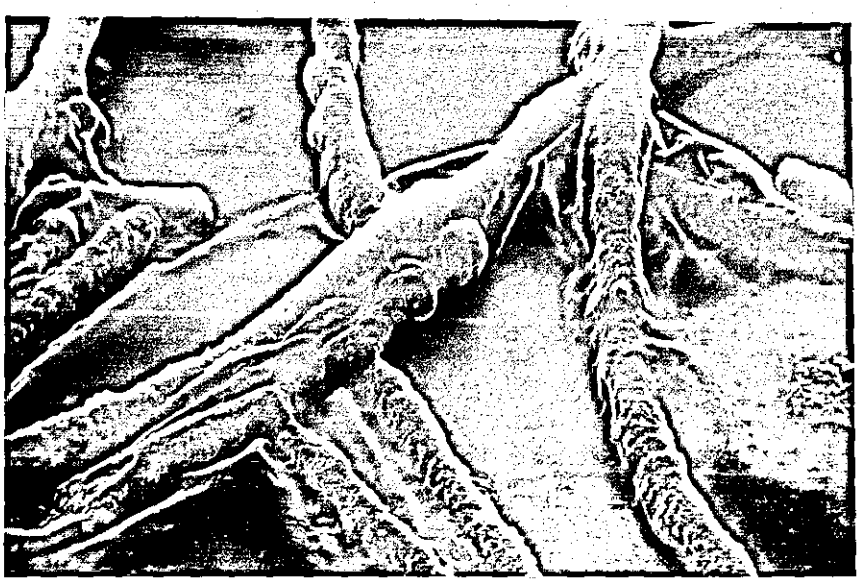


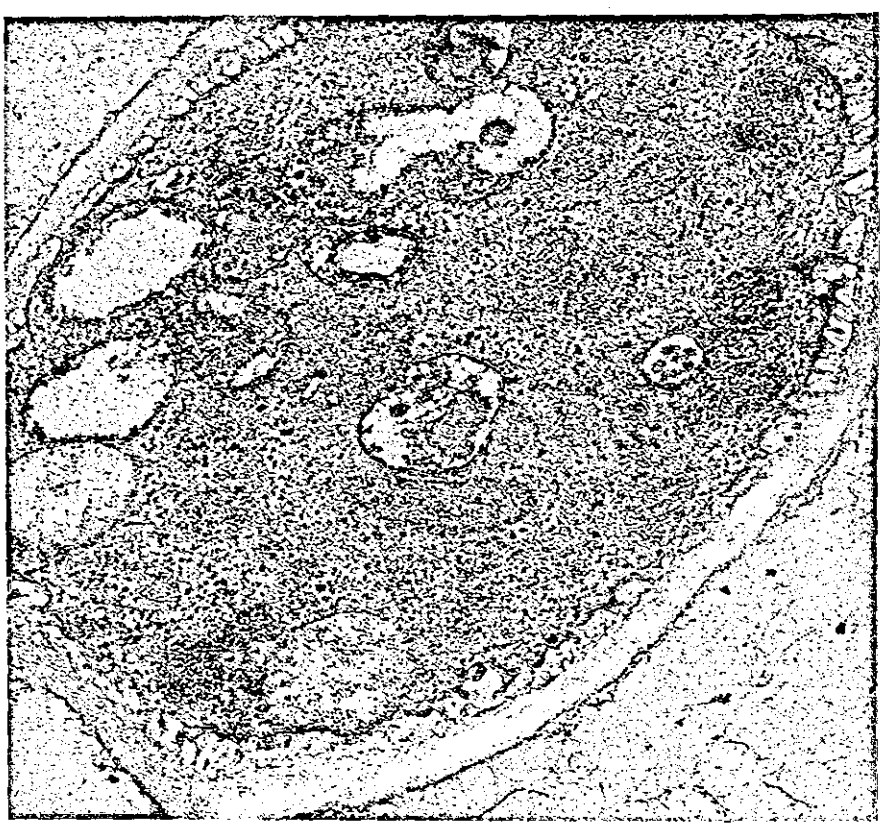
Plate 55. - Light micrograph of A30 in the presence of 20 $\mu g/cm^3$ of Aculeacin A. Note excessive and irregular branching. x 400.

Plate 56. - SEM of A30 in the presence of 20 $\mu g/cm^3$ of Aculeacin A. Note degenerating hyphae and hyphal beading. x 10,000.

Plate 57. - TEM of A30 in the presence of 20 $\mu g/cm^3$ of Aculeacin A. Note cytoplasmic distortion. x 50,000.







Release and Regeneration of Protoplasts:

First examination of mycelial preparation after 3 h incubation in snail juice showed slight degeneration and no production of protoplasts. At 10 h degeneration had gone far accompanied with liberation of protoplasts. The mixture of snail juice and chitinase had a greater degenerative effect with a high release of protoplasts after 3 h incubation. Chitinase on its own was ineffective. It was observed that the age of cultures played a part; the older the culture, the longer the release of protoplasts.

The protoplasts were basically spheroid but varied in size. They measured an average of 2 x 2.3μ (Plate 58).

Regeneration was observed after 3 h incubation. Direct production of germ tubes was the system observed (Plate 60).

PLANT INOCULATION:

Sectioned plants showed some wood staining around the point of inoculum. The staining, though not quite extensive, could be due to either the fungus infectivity or the incissus made or could even be a combination of both.

The fungus was reisolated pure from all the sections and it exhibited all the usual features. 25% of the plants died. Thin sections were cut and stained to demonstrate the presence of the fungus (Plate 62). Phytoalexin was not detected in any of the cases.

CELL CULTURE:

Sycamore cells were grown in the cell culture medium. The growth was monitored by PCV and dry weight (Fig 20).

The strain, A30, was grown in the cell culture medium to examine;

1) the growth rate in the medium and

2) to check for production of degrading enzymes. The fungus produced the poorest growth (Fig 21) it had done on any tested medium.

The cell-free extract did not degrade pectin (Table 15).

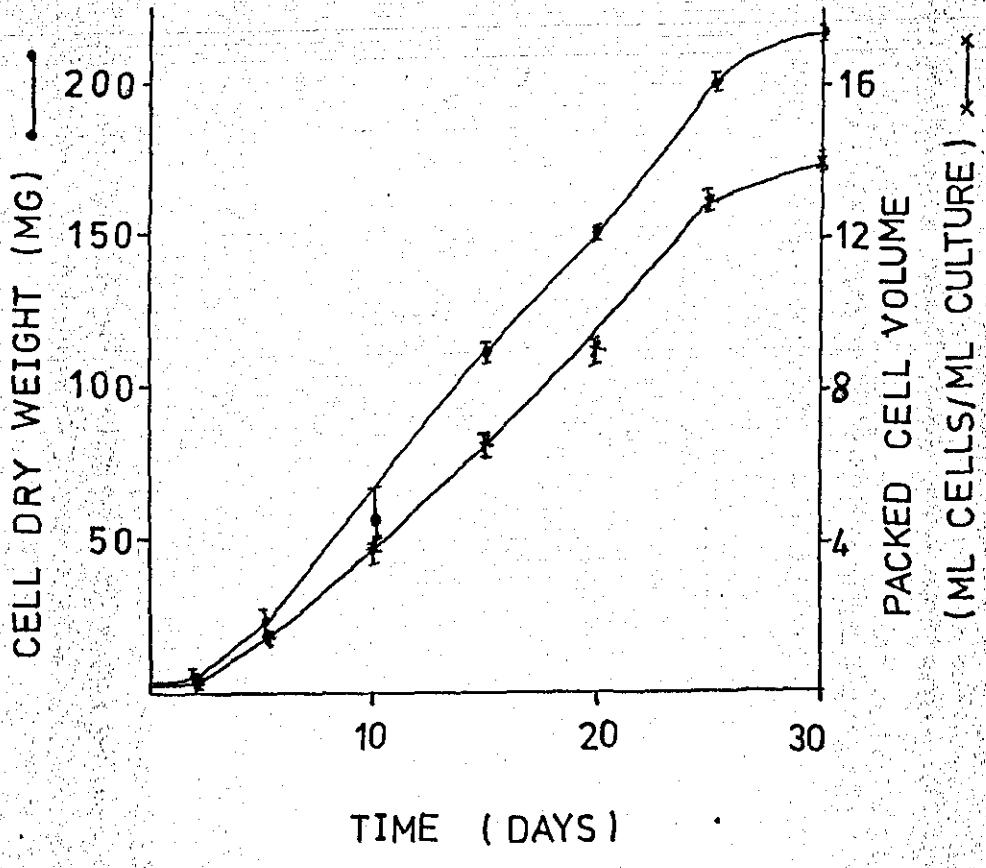
TABLE: 15

ENZYMATIC ACTIVITY PRODUCED IN CELL CULTURE MEDIUM WITH AND WITHOUT SYCAMORE CELLS.

ENZYMES TESTED	ENZYME ACTIVITY WITHOUT CELLS	ENZYME ACTIVITY WITH CELLS
PECTINASE	NIL	200 $\mu g/m^3$
CELLULASE	NOT MEASURABLE	250 $\mu g/m^3$
α AMYLASE	131 $\mu g/m^3$	350 $\mu g/m^3$
PRONASE	900 $\mu g/m^3$	300 $\mu g/m^3$
LIPASE	NOT DETECTED	+ *

Test performed in triplicates.

* Difficult to quantify owing to precipitation; Tween 20 used as substrate.



GROWTH CURVES FOR SYCAMORE CELLS
(EACH POINT IS A MEAN OF SIX REPLICATES)

FIG 20

GROWTH CURVE OF A30 IN CELL CULTURE MEDIUM.

(EACH POINT IS A MEAN OF SIX REPLICATES)

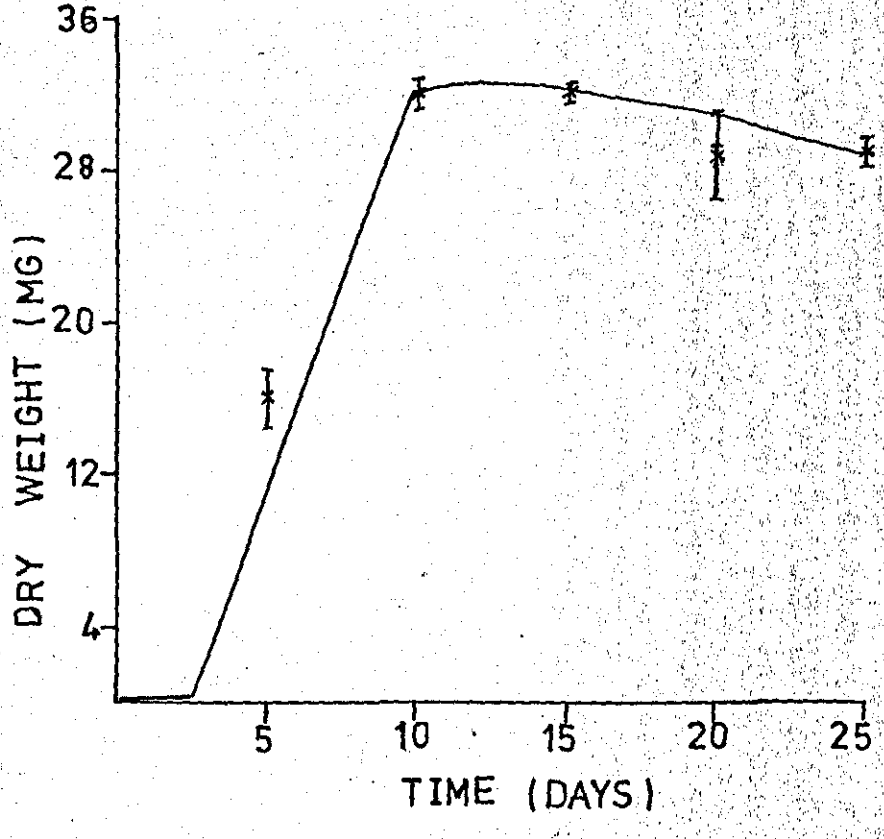
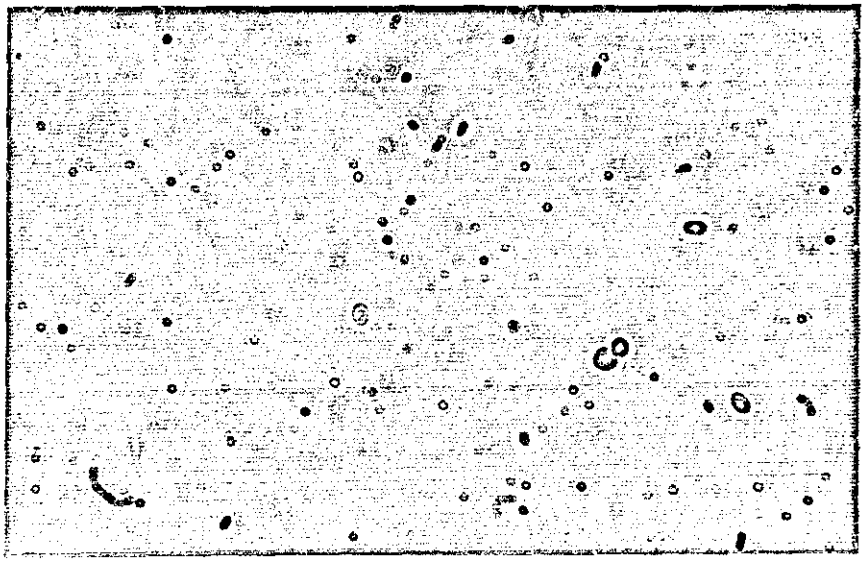
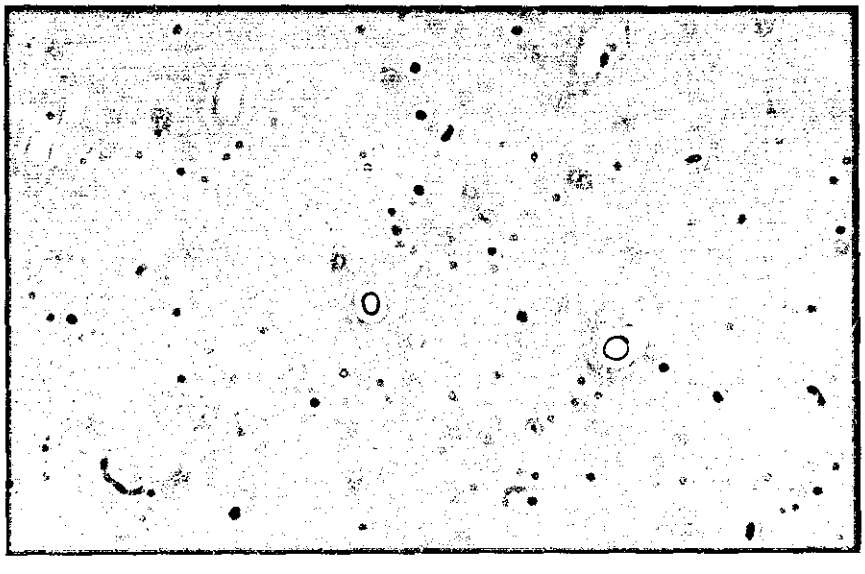


FIG 21

Plate 58. - A30; Light micrograph of production of protoplasts. x 400.

Plate 59. - A30; Phase contrast micrograph of protoplast production. x 400.





Plates 60 and 61. - A30; Phase contrast micrograph of protoplast regeneration. x 400.

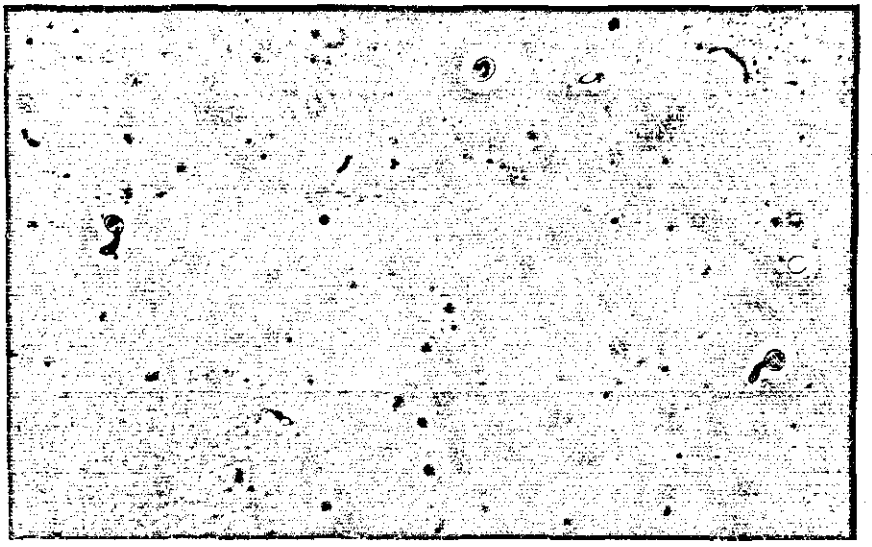
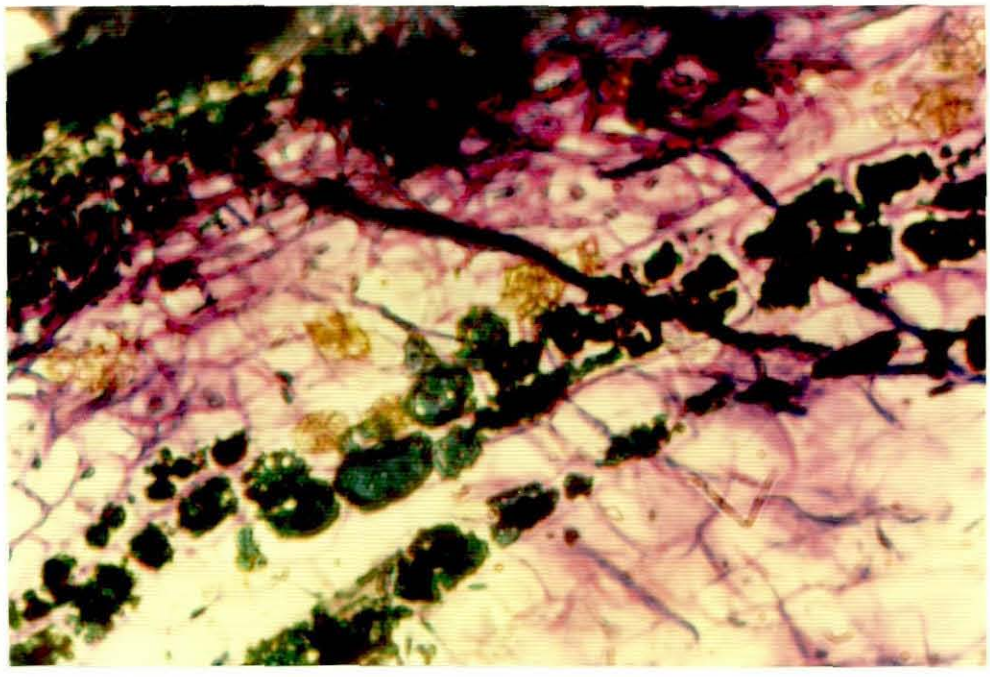
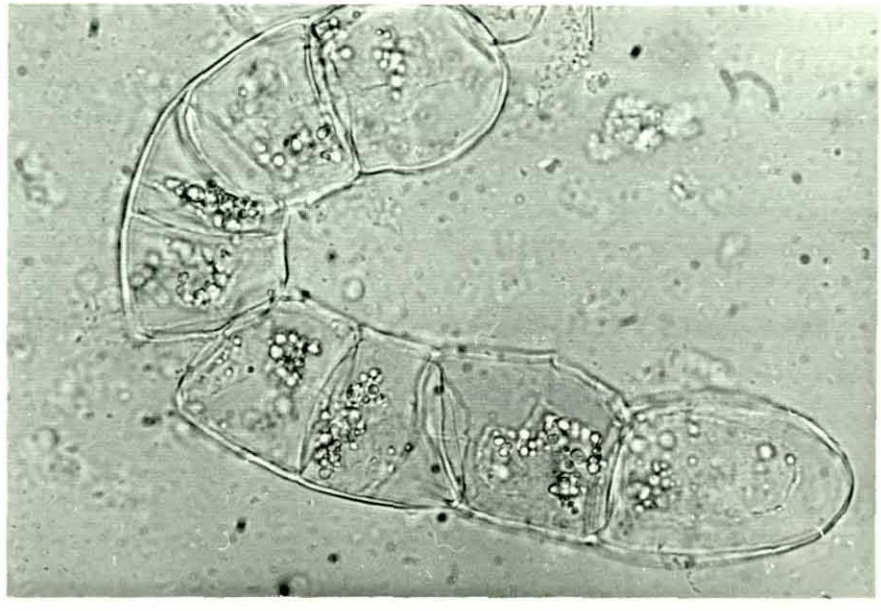
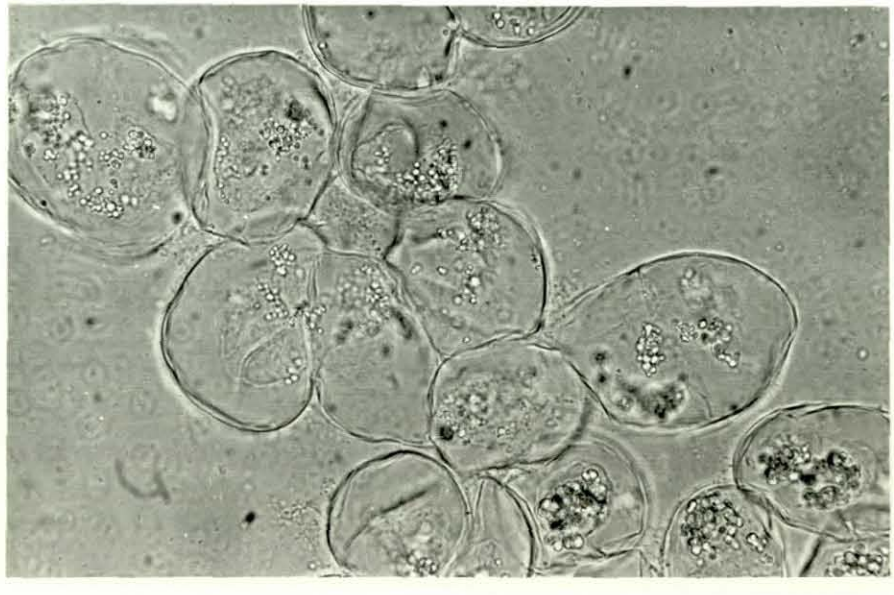


Plate 62. - Stained section of sycamore saplings. Note growing hyphae, strain A30 used. x 400.

Plates 63 and 64. - Light micrograph of sycamore cells grown in a shake culture for 7 days at 25°C. Note some differentiation within cells. x 400.







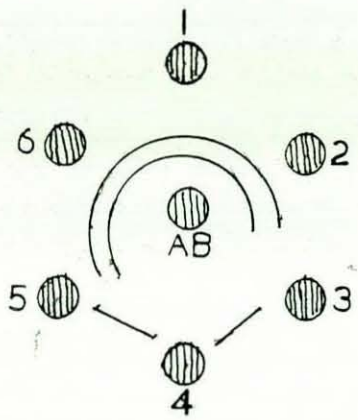
The sycamore cells (Plates 63 and 64) were infected with the fungus. The cells steadily degenerated with proliferation of the fungus (Plates 65 and 66). The cells were fully degraded in two days. Cell free extract from this infected culture showed a very high lytic activity (Table 15) and also solubilised fresh sycamore cell suspension releasing polysaccharides.

IMMUNO-DIFFUSION TEST:

Precipitin lines obtained from the test were not strong enough for photography. Several attempts were then made to stain the lines which only resulted in all the surrounding area being masked by the stain. A schematic diagram is, therefore, presented (Fig 22). Two precipitin lines separated out. There were non-specific lines from saline and Staphyloccocus aureus. The lines from the strains formed arcs (Fig 22) and full circles (Fig 22). Originally, it was thought to be distance effect (i.e. distance between antigen and antibody). This was, therefore, varied several times, still the results remained the same.

FIG 22 DOUBLE-DIFFUSION TEST

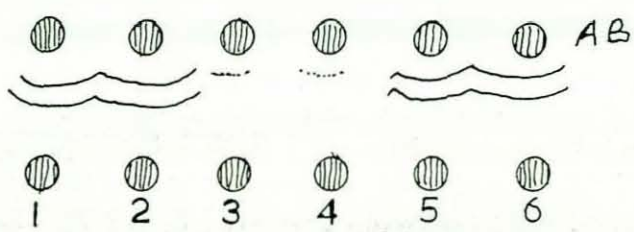
(a)



CENTRAL WELL CONTAINED MYCELIAL ANTISERUM (AB)

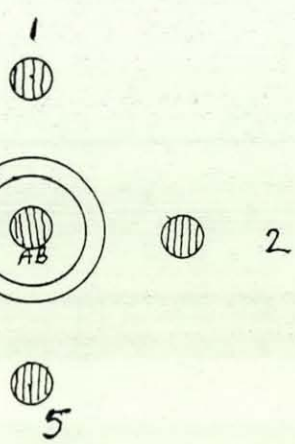
1 — A30 ANTIGEN
2 — A31 "
3 — STAPHYLOCCUS AUREUS ANTIGEN
4 — SALINE
5 — A32
6 — A33

(b)



LINES OF IDENTITY SHOWED AMONGST THE STRAINS

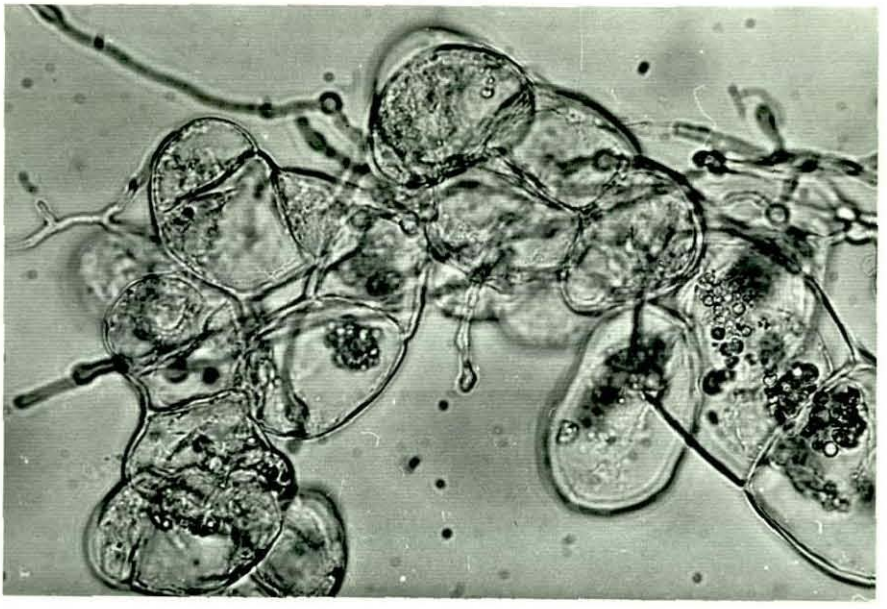
(c)

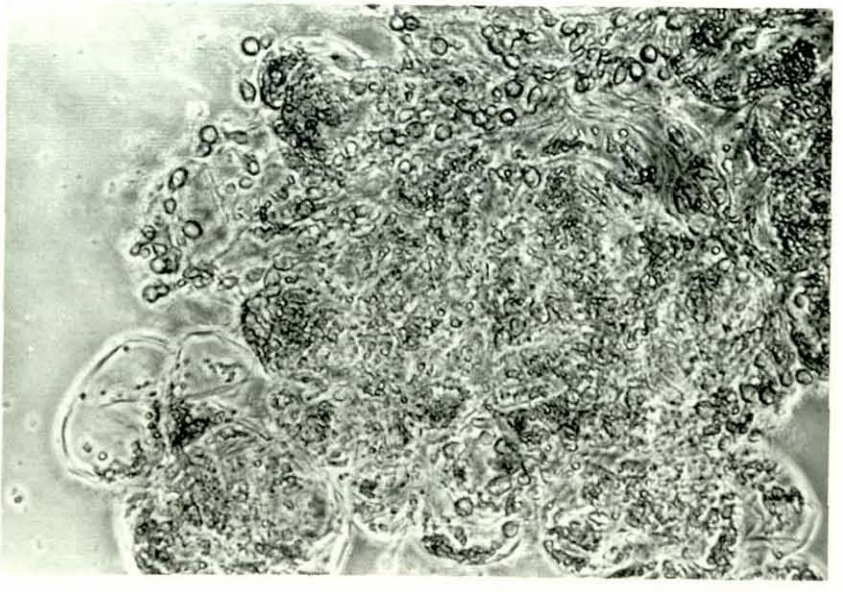


ANTIGEN TREATED WITH PRONASE

Plate 65. - Light micrograph of sycamore cells infected with A30 spores after 24h incubation. Note proliferation of fungus. x 400.

Plate 66. - Light micrograph of Sycamore cells infected with A30 spores after 48 h. Note degeneration of cells and extensive growth of fungus. x 400.





CHAPTER SIX

DISCUSSION

ENVIRONMENTAL FACTORS AFFECTING GROWTH:

The life of every organism is influenced by environmental factors. An understanding of the influence of these factors is important in any work involving the morphology and composition of fungi. The effect of environmental factors on fungal growth is often discussed in isolation and whilst these factors can be studied separately, they are frequently inter-related.

As would be expected, temperature affected the growth of *Cryptostroma corticale*. The strains grew over a wide range of temperature with the minimum for growth being 5°C, although growth was very slow. There was no growth at 0°C but it was not surprising that the cells remained viable. A difference in the optimum temperature was observed amongst the strains. While the optimum for A30 was 30°C, it was 25°C for all the other strains (Fig 8).

Generally, the temperature curve is skewed to the right. The lower the optimum temperature (22°-25°C) the more symmetrical the curve becomes and the higher the temperature the more pronounced the skew (90) which could be due to denaturation of a rate-limiting enzyme. It was found that the skew was less pronounced in the higher optimum range as seen in A30. Though, this is not common (90) it is associated with a broader range of good growth. This trend continued throughout the work. The ability to grow at 37°C may be one reason why this strain has been reported as causing human diseases such as extrinsic alveolitis. It has also induced lesions and survived in experimentally infected animals [25 and 26). However, it should be emphasised that temperature optima and the growth ranges should not be taken as absolute since they are dependent on various factors such as medium composition and the method of measuring growth. Nevertheless, the concept of temperature optima provides a useful basis for physiological studies.

Townrow[184] who worked on a strain of C. corticale isolated from sycamore also recorded the optimum temperature as 25°C whilst the isolate from maple log was found to grow at 37°C [26]. It might seem unlikely that the optimum temperature for sporulation was lower than that for growth (Figs 8 and 9) but this is not unusual as similar results have been obtained with Cephalosporium[185] and Fusarium conglutinons[90]. Mathre and Johnston[186] stated that the ability to sporulate at a temperature lower than that for growth would be an advantage to fungi in general since it must produce spores for infective purposes during the late Autumn to early spring when soil temperatures are low. Whether this proposition is true in nature is hard to say since C. corticale is capable of growth and sporulation at low temperatures in the laboratory studies. Growth at lower temperature is obviously beneficial for survival of the fungus.

Cryptostroma corticale grew and sporulated at low temperatures but outbreaks of sooty bark disease are more prevalent in warm summers. This indicates that less activity occurs during cold weather and infection and spread take place at temperatures around the optima since the most severe outbreaks have been during the hottest summers.

The organisms are mesophilic fungi, and showed a typical heat resistance response (Fig 10). Deverall[187] stated that most fungal spores are killed by brief exposure to temperatures of 60°C. Exceptions to this undoubtedly exist as shown in Byssochlamys nivea[188] which will survive exposure to 88°C for 1 h. However, few conidia resist heat.

pH had a similar effect to temperature. The fungi grew over a range of pH values and growth ceased at the low and high extremes (Fig 11). A30 covered the widest range, pH 2-12. The optima for all the strains were between pH 5 and 6 which conforms with the report that most fungi have pH optima between pH 5 and 7[189].

Generally, fungi tolerate acidic pH, a criterion made use of in selective isolation of fungi from bacterial contaminants. The optima for sporulation and germination also fell within the same pH range(Fig 12).

The effect of pH is not a unitory factor because it is dependent on ionisation of the medium constituents, the cell surface and intracellular and extracellular enzymes. Therefore, the effect of changing pH values should be viewed with some caution since these values can be interpreted in terms of availability of nutrients, growth temperature and time of harvest. However, in a controlled system, it offers a guide in media preparation and also forms a basis for growth studies. Cryptostroma corticale, with the ability to grow in such a wide range of pH, has the advantage of being very competitive in its habitat.

Another environmental factor which affects growth is the level of carbon dioxide in the atmosphere. A graded effect on the growth of C. corticale was observed. Growth was stimulated in all the strains and the highest stimulation effect was observed at 4% concentration of the gas. Cryptostroma corticale can, therefore, be termed a carboxyduric fungus (Fig 13).

Carbon dioxide plays an important role in fungal differentiation which is seen in the retardation of growth of some fungi in the absence of the gas[90]. The stimulatory effect of carbon dioxide on the fungus can be advantageous because the fungus can survive in a polluted environment with higher atmospheric concentrations of the gas. However, high partial pressure of the gas inhibit fungal growth though the level is variable. Macauley and Griffin[190] grouped some soil fungi according to the effect of carbon dioxide on their growth. They showed that Fusarium oxysporum and Chaetomium species were inhibited by concentration above 15%, in Curvularia species it was 6.5% and in Cochliobulus sativus they recorded their lowest concentration of 3.3%.

Burges and Fenton[191] also reported on varied effect of carbon dioxide. An increase beyond 5% inhibited growth of Penicillium nigricans while Zygorrhynchus vuilemini seemed unaffected. The effect of carbon dioxide on the growth of the C. corticale strains was also varied. Concentration of carbon dioxide above 10% was inhibitory to A30, 8% for A32 and 5% for A31 and A33. Growth in Sclerotium rolfsii was enhanced by 1 to 2% concentration of carbon dioxide while 10% was inhibitory[192]. Hartman et al[193] showed that Verticillium albo-atrum required carbon dioxide when glucose or glycerol was the sole carbon source but not when succinate or acetate was supplied. This confirmed the work on S. rolfsii in which growth was enhanced in a system lacking succinate dehydrogenase[192]. In C. corticale aerial mycelial growth increased with increase in carbon dioxide concentration and sporulation was inhibited (Fig 14). This indicates a morphogenetic effect which has also been demonstrated in S. rolfsii[194]. A similar effect has been reported for various fungi (195 and 196).

Glucose at a range of osmolarities in nutrient medium produced a graded effect but this response varied amongst the strains (Fig 15). The strains can therefore, be classed as osmoduric. Strain A30 showed the highest level of tolerance, growing on a glucose concentration of 30%. This strain was isolated from Acer campestre. The osmotic pressure of the plant could be the reason why only this strain infects A. campestre.

The resistance to high osmotic pressure constitutes a distinguishing factor for the various strains as does salt tolerance. Tresner and Hayes[197] grouped fungi by their salt tolerance; the Penicillium and Aspergillus being the high level tolerants, withstanding 20% salt concentration and the Basidiomycetes, the least tolerants, only resisting 2% concentration. Cryptostroma corticale is slightly haloduric. The strain A30 grew at 4% after 14 days while all the other strains grew at 2% concentration.

Kane and Fischer [198] also divided the dermatophytes into five groups on salt tolerance. The most sensitive were inhibited by 5% salt concentration. Trichophyton tonsurans is an example of this group and the most tolerant were inhibited by 15% concentration. T. mentagraphytes belongs to this group.

The investigation on light effect showed that growth was not significantly affected by the varied light treatments. Sporulation was highest in alternating light and darkness. This was comparable to sporulation in darkness while the lowest result was obtained in continuous light. These results were in agreement with other workers. Darbin [199] has reported that light had no effect on the growth of Pellicularua filamentosa and darkness has been shown to induce sporulation in several fungi[90]. Light has also been shown to induce sporulation in Fusarium oxysporum[200] and in Hymenula cerealis[186]. These reports indicate that the effect of light is variable. Leach [201] appreciated these confusing results. He then stated that this situation is brought about by workers ignoring the importance of the quality of light and its intensity and other interacting factors such as light effect on substrates. However, the results from this work seemed consistent with the natural situation. C. corticale being an endogenous parasite, is occluded from continuous light and so carried out its processes either in the dark or with little light present.

Light showed a contributory effect on zonation, which was seen to depend on alternate light and darkness; fluctuation in temperature and availability of carbon source notably glucose.
Several workers have attributed zonation to alternation of light and darkness; in Cephalothecium [202] and in Fusarium [203].
Bisby [204] reported that zonation in F. discolor sulphureum was due to two factors; - alternation of light and darkness and variation in temperatures. Fluctuations in temperature induces two types of zonation. The first of these was seen in Cercospora beticola [205].

where the zones were entirely mycelial and the second, as seen in this work, where growth falls in zones of spores. Hafiz[206] showed similar effects with Ascochyta rabiei as compared to that on C. corticale, however, he failed to specify the nutrient source. Brandt[207] has shown in Neurospora crassa that zonation is dependent on fluctuation in temperature and nutrients which were seen in C. corticale but he also obtained zonation in the dark which is contradictory.

In the nutritional studies, it was found that the fungi utilized all the varied sources of carbon. Glucose was the best carbon source for growth while glucose and sucrose were equally the best for sporulation and germination. This is of some significance in the life of the fungus as glucose is biologically the commonest of the hexoses.

The efficiency of utilization of glucose was also observed in the growth study on various media. The most rapid and best growth was obtained from the media with high content of carbohydrates especially glucose viz SAB, PDA and MEA. Similar results were reported for Aspergillus flavus[208] and Hymenula cerealis[186].

Galactose was the least utilized of all the carbon sources studied. Lilly and Barnet[183] and Lindberg[209] reported on the low number of fungi which can utilize galactose. The inefficient utilization of galactose may be due to the inability of the fungus to convert the sugar to a phosphorylated derivative which is then able to enter the main pathways of respiration and synthesis. Perlman[210] and Lindberg[211] however, showed that though galactose does not make a good source of carbon, its utilization may be improved in a mixture with other utilizable sources.

This is a case of adaptive growth rather than a diauxic growth because the utilizable source can be of a more complex structure as seen in Gliomastix convoluta[183]. In this fungus a mixture of galactose and sucrose produced better growth than sucrose on its own, which was a better utilization source than galactose.

Summary

Four strains of Cryptostroma corticale have been characterised morphologically and physiologically.

The growth, sporulation and germination were affected differentially by environmental factors. A30 grew rapidly and sporulated abundantly. A32 grew well but not quite as rapidly as A30 A31 and A33 were both equally slow with A33 producing spores in low numbers.

The optimal conditions for growth sporulation and germination under laboratory conditions have been established. The strains were mesophilic, osmoduric, carboxyduric and slightly heloduric.

All the strains showed a non-fastidious nutritional character. Of the carbon sources tested glucose was the best for growth; glucose and sucrose provided the best sources for sporulation while galactose was the poorest carbon source for growth, germination and sporulation. All the nitrogenous sources were efficiently utilized except methionine and cysteine. Arginine, asparagine and urea produced the best growth yield.

The strain, A30, was found to be a prototroph while the other strains were partially auxotrophic to either d-biotin or L-ascorbic acid.

The strains exhibited a high heterotrophic characteristic which could be due to the large number of hydrolytic enzymes they produced.

The pattern the organisms showed in the assimilation and degradation tests may provide a means of differentiating them.

When the cellular proteins were subjected to electrophoresis, the strains showed broadly similar and strain specific patterns.

Electron microscopy studies illustrated no difference in morphology amongst the strains and related genera. Using lytic enzymes it was revealed that the cell wall was possibly three layered and made up of mainly glucan and chitin; cellulose was absent. The carbohydrates were the major polymers composing about 84-90% of the cell walls, proteins were between 5-10% and the lipids 1-3%.

Inhibitory and fungicidal levels have been established for the antimicrobial agents.
The imidazole derivatives produced the greatest lytic effect, followed by the polyenes. Morphogenetic studies were carried out.
Ramihyphin, Rapamycin, CytochalasinA, Aculeacin A and Polyoxin D had the greatest morphogenetic effects causing ballooning of cells and cells of varied ramifications. Polyoxin D, owing to its inhibitory effect on wall synthesis, produced protoplasts which failed to revert to the mycelial form unlike the protoplasts produced with lytic enzymes.

The experimental infection investigation revealed germination in the plant tissue and the fungus was reisolated. This characteristic and the ability of the fungus to produce cell wall degrading enzymes within sycamore cells as well as degrading the cells indicate that C. corticale is probably a facultative parasite.

Similar growth was evident from this work; a mixture of glucose and galactose produced better growth in all the strains than galactose alone. This bears a practical implication since the C. corticale strains utilized pectin as a sole carbon source, galactose being a component part of pectin. The ability to utilize mixtures of sugars should be advantageous since in nature, sugars are rarely free.

An obvious point emerged from the investigation on nitrogen sources. The importance to the fungal growth of the nature of the source and the supply levels were revealed. levelled out or dropped.

All the nitrogenous sources were utilized to a varying degree. The ease with which the fungi utilized the inorganic sources is not a common feature amongst the Hyphomycetes. The Saprolegniaceae moulds failed to grow on ammonium sulphate and potassium nitrate[212] while the Achlya species produced poor growth on nitrates[213]. Poor utilization of the ammonium nitrogen was also recorded in Fusarium oxysporum[200].

The poor utilization of ammonium nitrogen in fungi is due to the inhibition of growth by the low pH produced in the medium[214]. The average final pH level recorded in this investigation was pH 4 (Table 6). The strains of C. corticale grew at lower pH levels, therefore, were able to utilize the ammonium nitrogen sources efficiently.

The organic nitrogen sources notably asparagine, arginine and urea were the best sources for growth. Asparagine has been widely reported as the best nitrogen source for growth in several fungi e.g. Coprinus species[215], Fusarium oxysporum[200], Chaetomium species and Myrothecium verrucaria[216].

In general organic nitrogen is better for growth than inorganic nitrogen[90,217] though exceptions exist.

In this study, the organic sources did produce the best growth. On the other hand, the organic sources were the least utilized. Methionine

and cysteine produced the poorest growth. Pateman and Kinghorn[218] also reported methionine and cysteine as poor nitrogen sources in Aspergillus nidulans and Neurospora crassa.

Urea had been reported as a good nitrogen source for several fungi[90, 218]. Urea produced good growth yield in all the strains upto the supply level of 186 mgN/L and thereafter, there was a dramatic drop. This was obviously due to a pH shift as evident in the final pH value of the medium (Table 6). The rise in pH would be due to ammonia, a breakdown product of urea. The ability to utilize urea is an important characteristic in fungi as urease is essential for the oxidative degradation of the purines which are quite toxic even at low concentration[218].

The investigation on the vitamin requirements has shown A30 as prototrophic while the other strains were partially auxotrophic towards biotin and ascorbic acid. This was not a case of double requirement but that either would satisfy the requirement. A few vitamins showed stimulatory effects (Table 7). Biotin requirement amongst the hyphomycetes has also been reported[219] for Volucrispora aurantiaca. Biotin is a coenzyme involved in a wide variety of reactions such as decarboxylation reactions, conversion of ornithine to citrulline and hexokinase system. These activities may be the reason for the partial requirement frequently reported for the vitamin[220]. This partial requirement can be satisfied by the addition of metabolites which constitute products of reactions where biotin takes part. In Candida albicans for instance, biotin can be replaced by glyceryl mono-oleate[221].

The partial requirement of these strains was reflected by growth in the vitamin-free medium where growth did occur but an exogenous supply produced more vigorous growth. A complete requirement for vitamins may be genotypic while a partial requirement can be influenced by environmental factors such as constituents of media, temperature as is shown in Neurospora crassa requiring riboflavin and Saccharomyces cerevisiae for pantothenate[222].

Cryptostroma corticale exhibited a non-fastidious nutritional character. This will no doubt contribute to its success ecologically. Although no qualitative differences were obvious in their nutrition, the strains showed a number of quantitative differences in their growth rate, temperature and pH effects.

TAXONOMIC CHARACTERISTICS:

The organisms demonstrated an ability to utilize a range of complex and simple organic compounds as sole carbon and energy sources- and also, they showed a high hydrolytic capacity. These hydrolytic enzymes including pectinase, cellulase, pronase and α-amylase may have pathogenic implications as they can play a part in invasiveness. Their biochemical characters, can constitute a system for grouping as well as delineating the strains of C. corticale, as has been seen in the investigation, they exhibited several similar and distinguishing features by the amount and rate of enzyme production and also by the various compounds hydrolysed and rate of hydrolysis. The most obvious differences occurred in the utilization of phenols as sole carbon and energy source. Strain A30 failed to grow on 0.1% concentration of phenol and cresol but at lower concentrations growth occurred. All the other strains showed no growth and neither did they grow on mandelic acid, which was utilized by A30. Again tributyrin and triolein were hydrolysed by A30 but not by the other strains. It can be inferred from this investigation that A30 showed a greater heterotrophic character than the other strains. Gordan and Mihm[161] used the decomposition of

organic compounds to characterise the Nocardia; Stockdale[223] and Rosenthal and Sokolsky[224] have used the enzymatic studies in differentiating the dermatophytes and some pathogenic fungi respectively. Murray (225) in his review also stated the usefulness of the assimilation and degradation characters in toxonomy.

Fungi had been characterised by their protein profile and enzyme patterns. This relates to differences or similarities in their genetic information and since one zone makes one enzyme, the system should give stable characters.

Several fungi have been differentiated and grouped into genera by this system for instance the Dermatophytes[228]; Aspergillus species[227] and the Verticillium species[228]. Garber and Rippon[229] expressed the value of this system as a taxonomic tool after viewing the limitations.

There were at least eleven protein fractions in each strain. Six homologous fractions were detected. The esterase zymogram (Fig 19a) showed four bands in all the strains except in A32. The bands at 0.17 - 0.18, 0.25 - 0.26 and 0.95 - 0.96 were common to all the strains. Again in the amidase zymogram (Fig 19 b) the band at 0.03 was common to all the strains. The strains, therefore, passes protein fractions characteristic of all the strains and also strain specific fractions.

Characterisation of the strains was also examined serologically. The usefulness of serology has been demonstrated for the Dematiqceous fungi[230]; Phytophthora infestans[232]. Its aid to taxonomy and identification has been reviewed[233 and 234]. The results obtained demonstrated identity amongst the strains and the presence of two antigens. The circular precipitin lines obtained were peculiar and difficult to explain apart from the obvious case of identity. The antigens resisted treatment with pronase, cellulase and α-amylase but a

sequential treatment through pronase and α-amylase produced no precipitin lines, indicating the destruction of the antigen, thus, suggesting that the antigen was probably a glycoprotein.

Emanuel et al [235] have also shown the antigens in fungi implicated in farmers' lung disease to be glycoprotein. They also remarked on the involvement of more than one antigen.

Similarity has been drawn between the conditions caused by C. corticale in maple bark disease and other fungi associated with farmers lung [21 and 236]. It is therefore likely the antigens are similar. Rankin et al [237] also reported similar results in cases of granulomatous pneumonitis.

STRUCTURAL STUDIES

The strains did not exhibit any differences in morphology but A30 grew more rapidly and produced spores more abundantly than the other strains. The strains were similar in their cytology and showed no major differences in organisation from related fungi such as Penicillium notatum [238] and Aspergillus species [239].

The swelling of the dormant spore well prior to germination seen in C. corticale has also been reported in conidia of Aspergillus nidulans [240] and in several other fungi viz Botrytis cinerea [239]. The invagination of the plasmalemma probably represents the region for germ tube formation and emergence. The germ tube seemed to have originated from the inner wall of the spore. This feature has been reported in Aspergillus oryzae [241] and Botrytis cinerea [241]. This differs from de novo formation of germ tube walls seen in Fusarium culmorum [242] and Rhizopus stolonifer [243]. There was a general increase in the cellular organelles indicative of active respiration and biosynthetic metabolism.

The presence of cytoplasmic vesicles in the apical region of the hyphae is seen in other fungi which are taxonomically diverse [245] and is also found in other growing tip cells like pollen tubes [246], root hairs [247] budding yeast cells [248] and algal rhizoids [249].

Brenner and Carroll[250] also found accumulation of cytoplasmic vesicles at the apex in Ascodesmus species and though they could not state their origin, considered them to be involved in wall formation. Rose[251], again, showed the role of the cytoplasmic vesicles in cell wall envelope biogenesis of the yeast cell.

The origin of the cytoplasmic vesicles is merely speculative; from budding of endoplasmic reticulum[89]. Rosenberger[127] stated they are from endomembrane systems and added that they move towards the apex, fuse with the plasmalemma at the tip and disappear with the cease of growth extension only to reform at the start of growth. This theory fits the structure where the endoplasmic reticulum could be seen in close association with the plasmalemma (Plate 24).

The presence of plasmalemmasomes and lomasomes[252] has been a controversial topic. Bracker[253] reported that they may be artefacts produced by fixatives; Campbell[254] and Cantino and Truesdell[255] also thought they are artefacts. Heath and Greenwood[256] used a variety of fixatives and showed that lomasomes and plasmalemmasomes are constant structures in Saprolegnia ferax and Dictyuchus sterile. Lomasomes and plasmalommasomes were regularly demonstrated in C. corticale and found in mature hyphae as well as regions of high metabolic activity. Beck and Greenwalt[257] described structures they termed " cytoplasmic whorl", as having double stranded convoluted, myelin-type structures. They showed these cytoplasmic whorls as regular features and suggested that they have physiological basis connected with cellular differentiation. These cytoplasmic whorls are reminiscent of lomasomes (Plate 27). Cole and Aldrich[258] showed their presence in fixed and unfixed sections and Kozer and Weijer[259] confirmed this in Neurospora crassa.

The origin of these structures is doubtful. Suggestions exist that they may be derived either from the passage of vesicles through the

plasmalemma or by budding off or proliferation of vesicular structures [89] or vesicles budding off endoplasmic reticulum passing through plasmalemma [260]. An early initiation of lomasome formation can be seen in Plate 25. This seems rather more connected to the plasmalemma than endoplasmic reticulum. This thought seems more feasible with the proposition that lomasomes are derived from vesicular structures passing through the plasmalemma. In plate 26 the structure appeared continuous with the plasmalemma and a similar structure is present in Plate 20. It could be possible for folded plasmalemma such as that to bud-off to give lomasomes. The convoluted structures in Plate 28 could not be ascribed to a definite origin.

The origin of lomasomes and plasmolemmasomes is still obscure but they appear to be part of the cell. Structures conforming to their description have been reported in other cells namely algae [261, 262] and in higher plants [263, 264]. Of the fixations used O_sO_4 was found better for cell wall demonstration while $KMnO_4$ was superior on membranous structures.

The cell wall, though slightly diffused, appeared to have three layers (Plate 25). Two layered walls have been reported in Mucor rouxii [124] and three layers in Cochliobolus myobeanus [265].

Enzymatic treatment of the walls demonstrated that the wall surface was granular with an amorphous appearance. Chitinase and cellulase had no effect on the surface when used separately and sequentially indicating the absence of chitin and cellulose on the surface. The surface was readily susceptible to β-glucanase from snail gut juice and very slowly to pronase.

A sequential treatment through β-glucqnase and pronase revealed a distinct complexity of interwoven microfibrils which disappeared on prolonged treatment with chitinase while cellulase had no effect. It can, therefore, be inferred that the wall is made of mainly glucan on the surface and the chitinous microfibrils on the inner most surface with the interstices and reticulum-matrix filled with glycoprotein.

The wall, thus, presented a structure of three integrated but co-axially distributed regions, each with a prominent polymer. This conjecture is supported by the three-layered proposal, with glucan and chitin being the predominant polymers. This is consistent with the work of Hunsley and Burnett[125] and Salton[266] also showed in Neurospora crassa that the microfibrils were embedded in a homogenous matrix. Though the glucan in C. corticale was not split, it is now clear that there are two glucans. The R-glucan containing β (1→3) and β- (1→6) linkages and the S-glucan which is a straight chain containing α- (1→3) linkages[127, 267].

The analysis of cell wall components showed that all the wall components found in one strain were present throughout the other strains except that there were quantitative differences. The walls comprised largely of polysaccharides as regularly reported in fungal walls. Minor quantities of other components such as protein and lipids were also present.

Crook and Johnston[268] reported similar results in related genera. They found two hexosamines, galactosamine and glucosamine, in Botrytis cinerea, Aspergillus niger and in Neurospora sitophila. The absence of cellulose in the cell wall was shown beyond doubt. Cellulase had no effect on the cell wall; there was no hydroxyproline, the amino acid which is characteristic of cellulosic cell walls[269] and Applegarth's test proved its absence. Cellulose has never been reported in the Imperfect fungi. The high level of carbohydrates notably glucose is seen in all fungi[89, 270].

These structural and chemical components are useful in taxonomic characterisation. Bartnickin Garcia [118] divided the fungi group into eight classes on the basis of wall chemistry and showed how this fitted with other classifications based on a range of criteria of phylogenetic significance. Dart and Stretton [271] and Stretton and Dart [179] split Aspergillus species and C. corticale by their total fatty acid composition after growth in a defined, fat free medium.

This system of classification by chemical components should, however, be viewed critically as the components and their percentages are likely to vary significantly under several cultural conditions [116, 118]. Moreover, the recovery of the various components is subject to the method of cell wall preparation as well as techniques for extracting the components. In the amino acid analysis, for instance, methionine appeared to be present in all the strains by the paper chromatography while this component was recorded absent in strains A30 and A31 by the amino acid auto-analyser. Background contamination that can cause complication of chromatograms has been reported [272]. However, instruments are now available for accurate analysis of samples. With improved techniques of sample preparations, this system could make a reliable contribution in taxonomy.

Investigations were carried out to establish minimum inhibitory and fungicidal levels of antimicrobial agents on C. corticale. Also the activities of these agents at sub-inhibitory levels on the cellular structure were demonstrated. The imidazole derivatives were the most effective by producing the lowest fungicidal levels on C. corticale. The polyenes were also quite effective but their fungicidal levels were higher than had been obtained in Candida albicans [135] and Torulopsis glabrata [273]. C. corticale, as well as being incriminated in diseases of sycamores and maple trees, also cause ailments amongst the workers on the logs, thus, constituting an economic hazard,

The only treatment applied has been to remove the patients from exposure to the spores of the fungus [13, 24]. Complete recovery took up to a year and fatal cases have even been reported [24]. The reported cases of maple bark diseases have all been allergic conditions as such, these drugs will be ineffective but they could be used in a systemic infection. Aspergillus fumigatus which causes an allergic condition also becomes systemic. The possibility of C. corticale turning systemic cannot be ruled out in the face of indiscriminate usage of antibiotics and immunosuppressive drugs. These factors cause debilitation in the man making him prone to infection.

Brown [11] stated that there is not thought to be a cure for the disease in the plant. So far, all infected trees have been cut down and burnt with every effort being made to prevent the spread of the spores. The new drugs with fairly low MIC such as Rapamycin, Aculeacin A and Polyoxin D could be tried. Indeed, Polyoxin D has been used in plant pathology in Japan [274] and aureofungin, a polyene, is used in agriculture in India [275].

A limitation on the use of drug is likely from the insiduous characters of the fungus in the plant because by the time the symptoms are fully recognised the damage has been done. A possible method of control lies in breeding and using resistant varieties of the plant. This is the method of choice for many diseases such as viral and vascular pathogens for which adequate cure and control are not available [36]. C. corticale has been found in the vascular tissue of sycamore trees [9] as such this system of control may therefore, be adopted.

The antimicrobial agents caused morphogenetic effects to a varying degree. Barathova and Betina [276] have shown that ethidium bromide and 5-fluorouracil produced granulation, vacuolation and undulation on Botrytis cinerea.

In C. corticale, ethidium bromide also caused granulation which was accompanied by slight distortion of the cell shape. 5-fluorouracil only caused a slight granulation. The distortion effect of ethidium bromide must have been due to its strong anti-chelating property. 2.4 dinitrophenol had no significant effect. However griseofulvin produced the curling effect reported [276].

The most striking morphogenetic effects were induced by Cytochalasin A, Ramihyphin, Rapamycin, Polyoxin D and Aculeacin A. In cytochalasin A, the cell surface effect was quite pronounced, though not much cytoplasmic distortion was observed (Plate 48). This indicates that the gross alteration of the wall was due to interactions of the wall and plasmalemma. Wessels et al [272] reported of intra-cellular disorganisation of microfilaments systems. This was not seen in C. corticale and Bradley [278] reported on reversible inhibition of cytoplasmic streaming without disorganisation of microfilaments. Rapamycin produced a reduction in the hyphal width and increased sporulation. The reduction could be due to leakage in the cell wall which could lead to collapse and breakdown of cells as evident in the SEM (Plate 43). Ramihyphin induced the highest ramification. The hyphae were broken into fragments with the surfaces severely distorted, thus portraying its name, ramification of hyphae [279].

Katz *et al* [280] proposed that inhibition of cell wall synthesis could increase the frequency of branching. This could explain the increase in branching in polyoxin D and aculeacin A. As seen in Plate 50 polyoxin D treatment was also accompanied by swelling and bulging of the cells [281 and 282]. Aculeacin A inhibits wall synthesis by inhibiting the synthesis of glucan [151] while Polyoxin D specifically inhibits chitin synthesis [282]. The shadowed sections from polyoxin D treatment (Plate 54) revealed the effect of the drug on chitin with a gradual disappearance of the microfibrils. Protoplasts were eventually produced but these failed to revert to the hyphal growth. DeVries and Wessel [283] reported that polyoxin D also inhibits the synthesis of R-glucan. They suggested that the swelling of the cells could be due to the absence of chitin and R-glucan, thus signifying the importance of these polymers in hyphal morphogenesis.

Gooday [274] stated that the existence of polyoxin D does hold out hope that chitin synthesis can be used as a target for a rational therapy for fungal diseases. He also drew a resemblance between polyoxin D and penicillin by their mode of action.

Reports that protoplasts revert to the original hyphal growth have been made on several fungi; *Aspergillus nidulans* [163]; *Neurospora crassa* [284] and *Fusarium culmorum* [285]. The inability of polyoxin D treated protoplasts reverting to the hyphal growth initiated the investigation for the production and reversion of protoplasts of *C. corticale*. Protoplasts were produced and did revert to the mycelial growth. Chitinase alone was ineffective in producing protoplasts but in conjunction with snail gut juice the productive effect was very rapid. This confirmed the observation on the cell wall,

consisting of mainly glucans and chitin. A similar result was reported by Skujins et al [286] on Fusarium solani and Aspergillus oryzae. Inability of the protoplasts to revert to the original mycelial form has been reported in Fusarium culmorum by Garcia-Acha et al [287] who went on to suggest that this could be due to the location of the original hyphae, absence of nuclei or absence of other organelles. Protoplasts of Penicillium chrysogenum and Cephalosporium acremomium have been used to demonstrate that some metabolic activities takes place in the intact mycelium [288]. Consequently they have a high potential use as a tool for fungal morphogenetic studies in the context of cell wall synthesis.

IMPLICATION IN PATHOGENICITY

Cryptostroma corticale has been implicated as a potential pathogen. Peace [3] doubted its pathogenicity, though, he admitted there is strong circumstantial evidence that C. corticale is the cause of sooty bark disease, he thought there is no proof. His doubts were confirmed by the failure of experimental inoculation of sycamore plants, basing his theory on reduced virulence in the fungus caused by low temperature.

Results from this work have shown the number of cell wall degrading and hydrolytic enzymes produced by C. corticale. The ability to produce an array of enzymes capable of degrading the complex components of the plant cell wall is a characteristic of pathogens [50], and also of saprophytes but it is a prerequisite and also essential for pathogenicity [289]. The molecular basis of pathogenicity still remains illdefined. It is apparent, however, that the ability to cause disease is dependent on a number of molecular factors and in considering these factors the ability to produce cell wall degrading enzymes by an organism should represent one aspect. These enzymes should be produced in the host environment and seen to effect changes.

It is now evident that plant pathogens produced wall degrading enzymes[50,52].

These enzymes were produced by C. corticale in vitro as well as in sycamore tissue culture. The sycamore cells were completely degenerated. An estimate of the cell wall degrading enzymes showed that production in the tissue was twofold higher than in the medium.

Karr and Albersheim[290] showed the necessity of the cell wall degrading enzymes for effective degradation of the substrates in the plant wall. Subsequent studies by English et al[291] and Bashman and Bateman[292] have demonstrated the ability of pectin enzymes in disrupting isolated walls and resulting in solubilization of pectin materials including neutral polymers containing galactose and arabinose. Talmadge et al[293] and Bauer et al[294] further emphasized the importance of pectin enzyme in cell wall degradation. They stated that the release of polysaccharide in sycamore cells by cellulase and pronase was ten-fold higher when pre-treated with pectinase than when acting alone. This was also demonstrated in Rhizoctina solani[295], in Sclerotuim rolfsii[296]. Bateman and Jones[297] and Mullen and Bateman[298] reported the ability of pathogens to excrete the degrading enzymes in infected tissues and their disruption of cell walls during pathogenesis.

Factors from plant cell walls or tissues are known to enhance production of degrading enzymes by pathogens. These have been demonstrated in tomato by Fusarium oxysporum[299] and in green elm by Ceratocystis ulmi[300]. This may account for the increase of enzyme production by C. corticale in the sycamore cells.

The artificial inoculation of sycamore saplings produced only one dead plant out of four. This may seem insignificant but the fungus was demonstrated in the tissues and was also reisolated pure. Robertson[301] has a limited success in the experimental infection work but a more promising result has been achieved (C.W.T. Young personal communication)

The lack of much success could be due to lose of virulence in the organism from the laboratory culturing. It could also be due to host specificity since the strain, A30, used was isolated from maple log. Since this strain destroyed sycamore cells and being the fast growing strain, it was likely to give infection in a reasonable time. However, conditions in the tissue culture differ from those in the plant. There are more nutrients and no antagonistic agents present and so suitable for the strain to flourish. Nevertheless, the presence of hyphae in the tissue signifies that germination occurred since spores were used as the inoculum. This may be significant, as host resistance factors would be present in the plant. This indicated some activity by the organism and points out that the organism might have lost its virulence in the laboratory. The ability to germinate, however, shows that after several passages the organism can gain back its virulence.

Peace (3) indicated that the loss of virulence, he suggested in C. corticale, was due to low temperature. It has been shown in this work and it is generally known that low temperature can only reduce activity. He also pointed out that active infection and spread were possible in hot weather which is also evident from this work. He hypothesised that C. corticale is a saprophyte but at the same time contradicted it by stating that the lesions found on diseased trees were such as one would associate with an active pathogen. In his final remark, he said, the organism is becoming less active and so less important but was hoping the future will show if C. corticale can reappear.

The results obtained from this work, though far from being conclusive, give definite indication of C. corticale being pathogenic. C. corticale, can therefore, be described as a facultative parasite.

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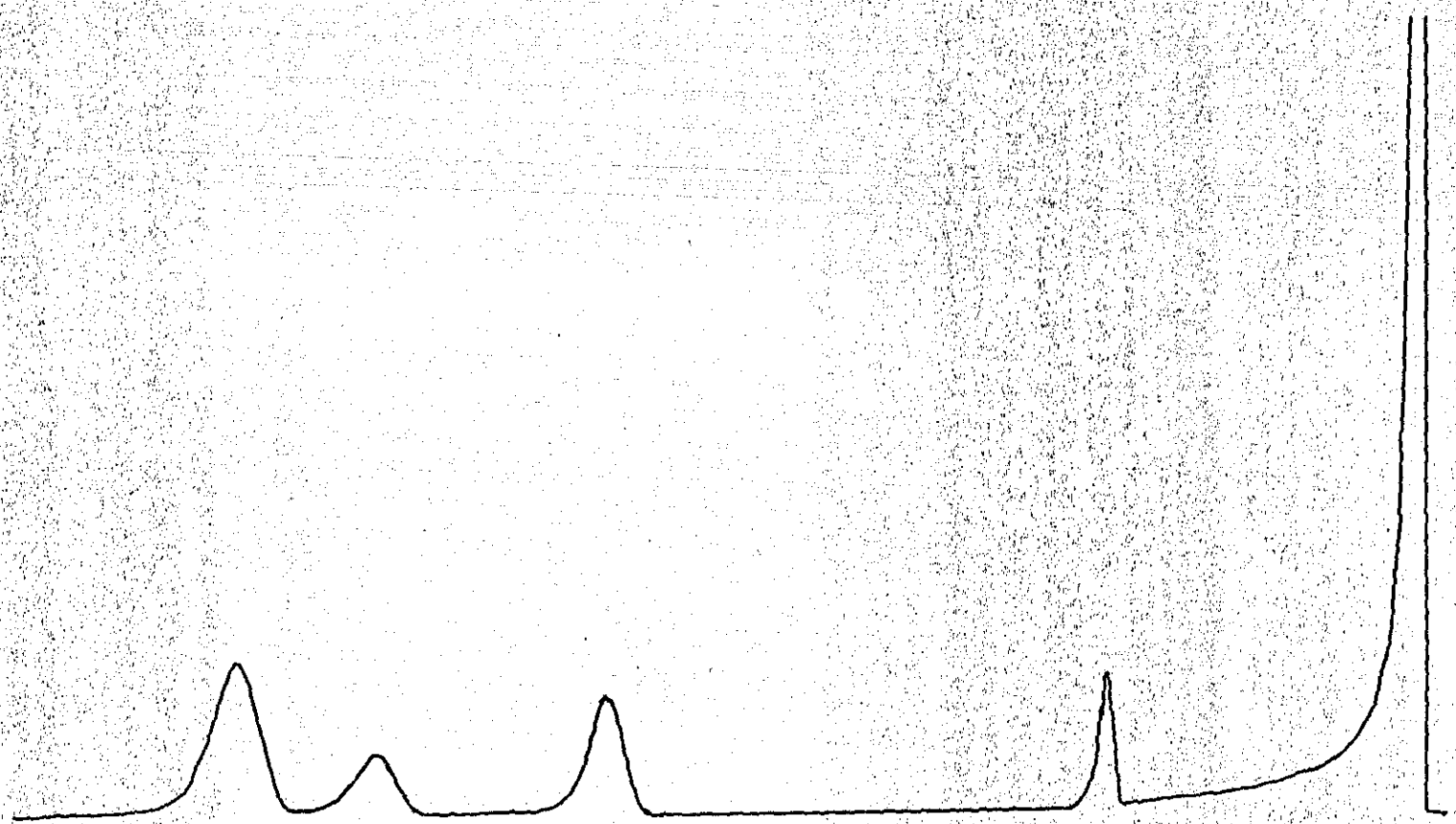

A 30

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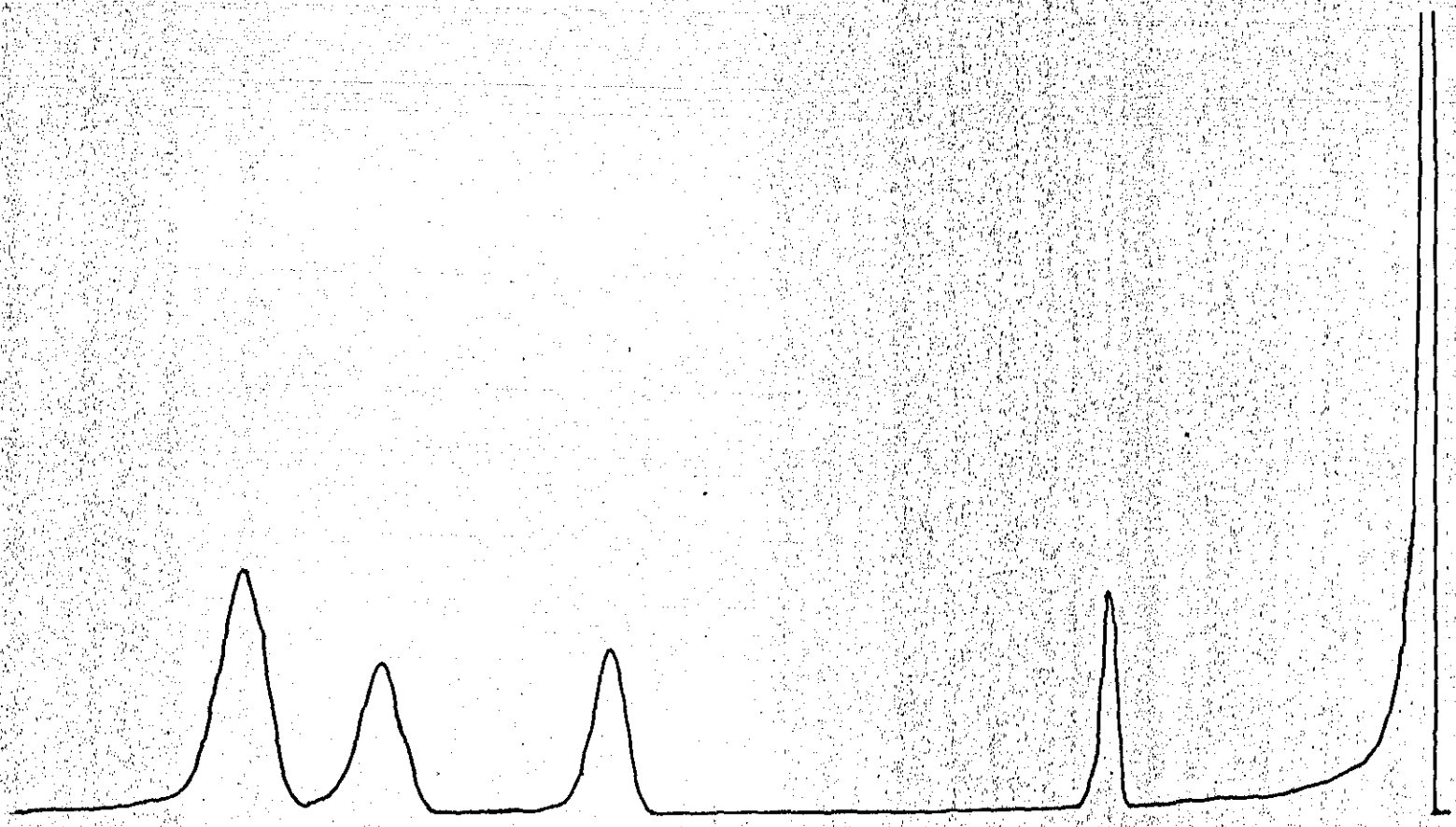

A 31

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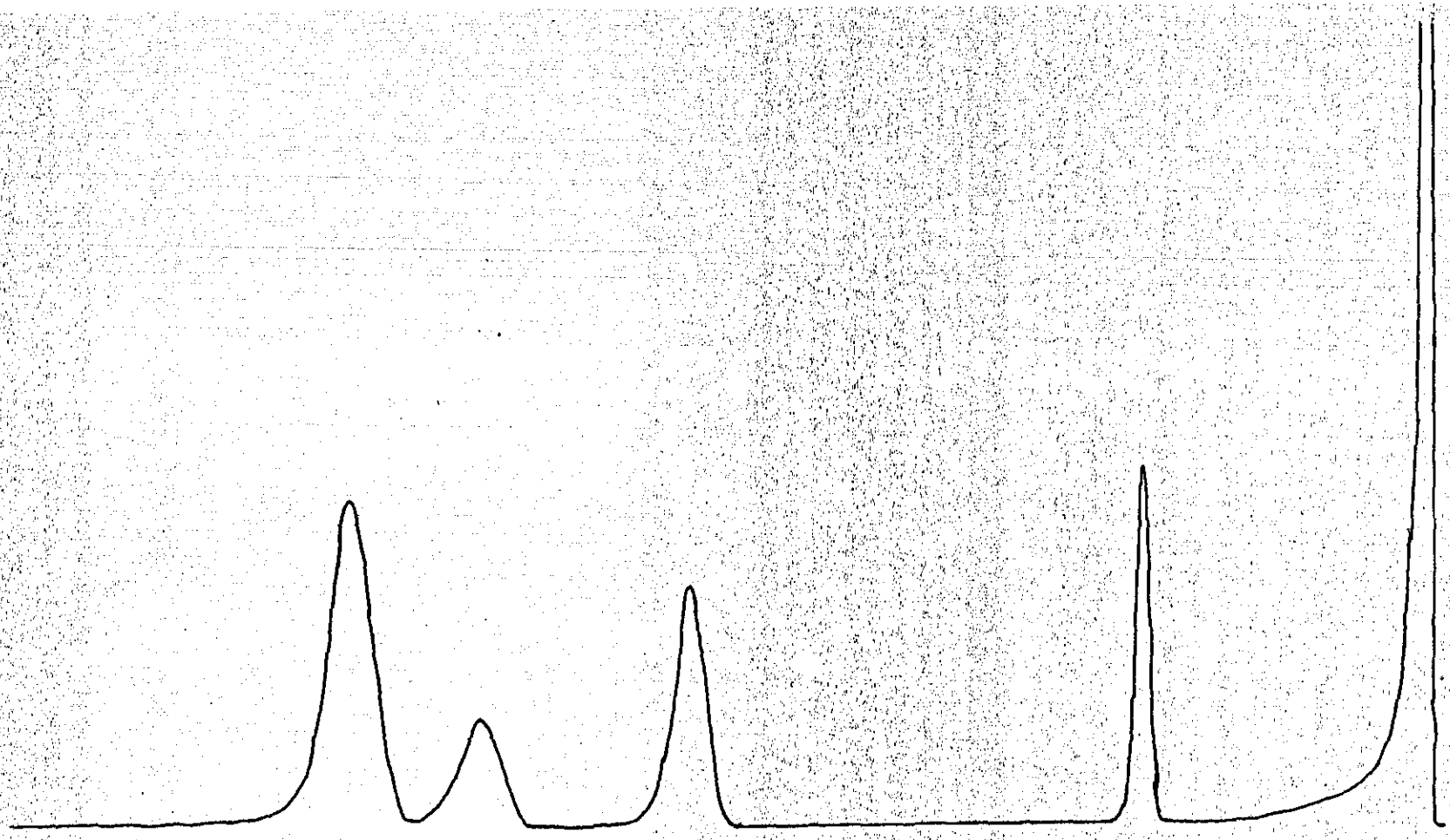
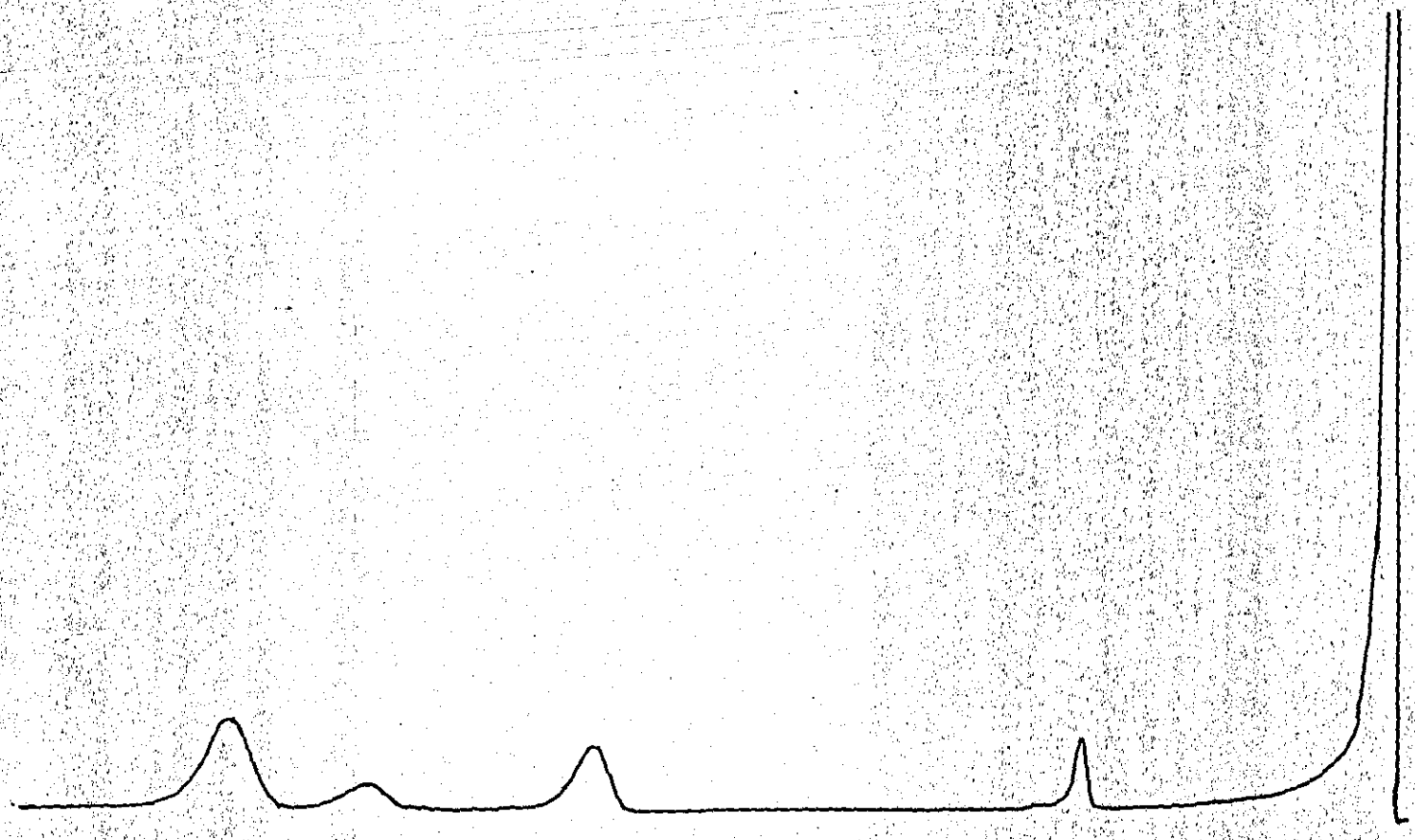


A32

GAS LIQUID CHROMATOGRAM ON 3% SE 30 CQ



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GAS LIQUID CHROMATOGRAM ON 3% SE CQ

APPENDIX

Publication arising from this thesis.

"Electron Microscope Study of Cryptostroma corticale"

Cytobios (in press).