SPORE GERMINATION IN THE FUNGUS

Syncephalastrum racemosum

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DEDICATION

This thesis is dedicated to my parents, who have constantly encouraged and helped me throughout my studies not only at Kent but also from the first day I attended school. They have repeatedly stressed the important role that education plays in life and how valuable knowledge is to Man. The thesis is not my achievement after many years of study, but an achievement that I share gladly and thankfully with my parents.

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SUMMARY

The asexual part of the life cycle of the fungus Syncephalastrum racemosum was investigated using light microscopy, transmission and scanning electron microscopy. The structure and organisation of the vegetative mycelium and spore producing hyphae was studied in detail.

Freeze-fracturing, surface replica, metal shadowing and thin sectioning techniques were used to investigate the ultrastructure of dormant and germinated spore walls subjected to various sequential chemical treatments. The spore wall had a highly complex organisation, consisting of four, major ultrastructurally definable layers. (1) A surface layer consisting of a cross-patched network of rodlets, having a 25nm periodicity. (2) A plate-like layer covered by amorphous material. (3) A layer of thick 17.5nm diameter microfibrils. (4) An innermost layer of thin 8nm diameter microfibrils. The emergent germ tube has a smooth outer surface and an inner layer of thin microfibrils. The breakpoint between spore and germ tube is clearly delimited at various levels within the spore wall.

Tentative identification of the wall layers showed: (1) the rodlet layer was mainly protein; (2) the thick microfibrils were probably a β-glucan; (3) the thin microfibrils were chitin.

Freeze-fracturing of dormant and germinated spores revealed differences in plasmamembrane ultrastructure. Large particles (34nm) with complimentary large depressions (33nm) were present only in fractures of the plasmamembrane of dormant spores. During germination there is an increase in the numbers of small particles (8.4nm) in the membrane.

The physiology of spore germination was studied in shake-flask and batch fermenter cultures. In the presence of glucose, spore

swelling preceded outgrowth of germ tubes. Glucose was essential for initiation of these events. Swelling ceased quickly when glucose was removed. Analysis of size distributions within spore populations indicated that the larger spores germinated first.

A self-inhibitor of germination was extracted from culture and spore washings, and was identified as nonanoic acid.

CONTENTS

GENERAL INTRODUCTION

INTRODUCTION

The fungal spore can be looked upon as being the beginning and end of the life-cycle of a fungus. It is usually a resting structure, and the resumption of development with its transformation to a morphologically different structure is termed GERMINATION. The restoration of vegetative metabolic and physiological activity, after a period in which these processes had been checked or changed, provides an excellent model system for studying biochemical and structural events which control cellular differentiation.

The nature of the morphological changes varies considerably among different resting structures. It usually involves the transformation from a non-polar growth form to a polar germ tube growing by extension only at the tip (Bartnicki-Garcia & Lippman, 1969). Germination can be divided into the four following stages based on morphological and biochemical events:

(a) the breaking of the dormant state,

(b) swelling of the spore,

(c) germ tube emergence,

(d) the early development of the outgrowing germ tube.

DORMANCY AND ACTIVATION OF SPORES.

A resting or dormant spore usually has a low level of metabolic activity. This state is termed DORMANCY, which can be defined as any rest period or reversible interruption of the phenotypic development of an organism (Sussman & Douthit, 1973). Sussman and Douthit (1973) subdivided dormancy into two kinds:-

(a) Constitutive (endogenous) dormancy - a condition wherein development is delayed due to an innate property of the dormant stage, such as a barrier to the penetration of nutrients, a metabolic block

or the production of a self-inhibitor.

(b) Exogenous dormancy - a condition wherein development is delayed because of unfavourable chemical or physical conditions of the environment.

From these two definitions, dormancy is imposed upon the spore either by the organism itself or by the conditions of the external environment. If environmental conditions are adverse to the germination of the spore, they are most probably disadvantageous for vegetative growth as well. It would seem therefore, that exogenous dormancy depends upon the spore receiving a signal that conditions are either favourable or not for germination. If conditions are not suitable, the survival of the spore will depend upon whether the particular fungus in question has the ability to produce long-term resting structures resistant to unfavourable climes. Constitutive dormancy is due to the action of the fungus itself. Limitations to spore germination are imposed on the spores, probably during their development and maturation. Upon dispersal, these limitations are either lifted, perhaps immediately (for example, spore crowding) or are removed after the spores have been stimulated or activated in some way.

A treatment, which would stimulate the spores out of their low level of metabolic activity and lead to an increase in germination is termed ACTIVATION (Sussman, 1976). The treatment of spores can be of various forms:- (a) removal of a self-inhibitor, (b) heat stimulation, (c) chemical treatments, for example the addition of a specific chemical such as detergents or organic solvents.

It has been observed that spores of many fungi germinate poorly when they are crowded. That is, the number of spores producing germ tubes decreased as the spore density increased (Allen, 1965). This phenomenon was termed CROWDING. Prior washing of the spores before placing them in germination medium had the effect of overcoming crowding. This was thought to be indicative of the removal of a self-inhibitor of germination (Allen, 1965). Lingappa, Lingappa & Bell (1973) observed that washed conidia of Glomerella cingulata were more permeable to amino acids and incorporated six times more precursor (C^{14} algal protein hydrolysate) than unwashed conidia. By shaking the water washings of the conidia with chloroform, Lingappa and his coworkers (1973) obtained an extract of G. cingulata self-inhibitor. An assay disc containing the chloroform-extract was placed on a nutrient agar plate seeded with conidia of G. cingulata. Upon incubation, a clear zone in which germination was inhibited, was observed around the disc. In the control, outgrowth of germ tubes and mycelial growth occurred around the disc, which was impregnated only with solvent. Further, uptake of labelled amino acids and their incorporation into protein was reduced either by the inhibitor (as chloroform-extract) or by crowding. Lingappa et al (1973) suggested that the self-inhibitor of G. cingulata could act as a regulator of protein synthesis. However, what form this regulation takes, or what the actual inhibitor was, the authors did not know.

An inhibitor of spore germination has been isolated and identified from culture filtrates of Fusarium oxysporum by Garrett and

Robinson (1969). The authors found that culture filtrates of F. oxysporum inhibited germination of sporangiospores of Cunninghamella elegans. Extraction of the F. oxysporum culture filtrates with petroleum ether gave an extract which was identified as nonanoic acid by gas chromatography, using known fatty acids as standards. Nonanoic acid, or even its potassium salt, retarded spore germination in C. elegans. Outgrowth of germ tubes was delayed. A concentration of potassium nonanoate at 250 μM delayed germination for 18h in spores of C.elegans, F.oxysporum, Mucor plumbeus, Rhizopus stolonifer, Aspergillus niger, Penicillium expansum, and Gestrichum candidum. Nonanoic acid was also detected in cultures of these fungi (Garrett & Robinson, 1969). It could be possible that nonanoic acid inhibition may be linked to the β-oxidation pathway by which fatty acids are broken down to acetyl-CoA. Lewis and Johnson (1966) had demonstrated that fatty acids were degraded by β-oxidation in Cunninghamella echinulata. They also postulated that C_8 - C_{14} saturated fatty acids inhibit respiration in C.echinulata by competing with the intermediates of the β-oxidation pathway for Co-A. Addition of Co-A or of pantothenic acid (a precursor in Co-A synthesis) overcame the inhibitory effect (Lewis & Johnson, 1968).

Spores of Dictyostelium discoideum were suspended in 80% ethanol and stirred to obtain a crude extract of a self-inhibitor (Tanaka, Yanagisawa, Hashimoto & Yamaguchi, 1974). The authors identified this self-inhibitor only partially (Tanaka, Hashimoto, Yanagisawa, Abe & Uchiyama, 1975). Part of its structure was

identified as a N-(3-methyl-2-butenyl) adenine moiety, which is known also as a plant growth hormone, cytokinin. This moiety did not of itself have a meaningful inhibitory potential, although at concentrations of 5, 10, 20 and 40nm.ml^{-1} the inhibitor showed 28%, 51%, 95% and 100% inhibition respectively. At 40nm.ml^{-1}, spore swelling was even inhibited. The nature of the inhibition remains unknown.

Germination inhibitors have been detected in Aspergillus nidulans, where the sensitivity of an aqueous suspension of A.nidulans conidia to a fixed dose of radiation is due to the presence of varying amounts of a germination inhibitor (Scott, Alderson & Papworth, 1972). Washing of the conidia with diethylether probably removed the inhibitor so making the spores more sensitive to radiation (U.V. irradiation) (Scott & Alderson, 1974). Sensitivity to irradiation treatment was checked by subsequent plating out of the conidia onto a minimal medium (Scott et al, 1972). In this study, and in the previous ones, the self-inhibitors have been detected in the spores. Garrett and Robinson (1969) however, extracted nonanoic acid from culture filtrates of the fungi concerned. Carlile and Sellin (1963) showed that mycelium of Botrytis cinerea produced a factor which inhibited the germination of its own spores. Cellophane strips were placed across circular growing colonies of B.cinerea and seeded with spores. Only those spores over fresh medium germinated. Those located over the colony did not germinate, arguing for the production of an inhibitory substance by the mycelium.

Removal of the self-inhibitor lifts the block imposed upon the spore and allows it to germinate. Removal of this block (though probably not caused by an inhibitor) can also be produced by heat treatment. This involves incubating the spores at some high temperature for a short period prior to germination. The treatment of heat shock has been best studied in Neurospora crassa (Smith & Berry, 1974), where a temperature of 50-60°C for 20 minutes will ensure an end of the dormancy period. Mills and Eilers (1973) found that a combination of both heat and chemical treatments simultaneously gave the best activation of spores of Coprinus radiatus. Heating at 45°C for 4h with various heterocyclic and aromatic compounds (1×10^{-3} M), followed by cooling to room temperature and checking germ tube outgrowth with time, was found to give the best results. Of the chemicals used, furfural, 2-furfuryl alcohol and 5-hydroxymethyl furfural gave germination values of 88%, 80% and 66% respectively. Spores of C.radiatus in its natural life-cycle pass through the digestive tracts of ruminants, where they are subjected to elevated temperatures. Later, in decomposing manure, additional heat is provided as well as furans. Furfural is one of the most widely occurring organic compounds, as it is a breakdown product of pentosans which are components of all higher plant cells; 5-hydroxymethyl furfural is readily formed from decomposing hexoses (Mills & Eilers, 1973). Hence a link between spore activation and the natural life-cycle of the fungus. Cochrane (1974) also suggested that some fungi, which

are more abundant after vegetation is burned, may be activated by suitable conditions being provided by some combination of temperature and chemicals from decomposing vegetation.

However, some fungi can also provide substances which stimulate germination. Germination of spores of Agaricus bisporus is promoted by A.bisporus hyphae growing in their neighbourhood (Smith & Berry, 1974). Rast and Stauble (1970) showed that one of the peaks on a gas chromatogram of the volatile fraction from A.bisporus mycelium (this volatile fraction being involved in stimulation of spore germination) corresponded to isovaleric acid. The spores readily incorporated labelled isovalerate into succinate, fumarate, malate and citrate (12%); 85% of the incorporation was found in free amino acids and organic acid fractions. Also, addition of isovaleric acid to the incubation medium stimulated carbon dioxide fixation. Air passing directly from A.bisporus cultures over spores inhibited their germination, while carbon dioxide-free air from the same cultures had a stimulatory effect. From these findings Rast and Stauble (1970) suggested that isovalerate "triggers" germination by removing a carbon dioxide self-inhibitor, for isovalerate is an immediate precursor of a carbon dioxide-acceptor (β-methylcrotonyl CoA).

SPORE SWELLING.

Spore swelling is easily observed under the light microscope and occurs in most fungi prior to germ tube formation. Van Etten, Bulla and St. Julian (1974) divided germination into four morphological stages of spore development:- (1) unswollen spores, (2) swollen,

(3) beginning of germ tube formation, (4) fully extended germ tube. They based this division upon a study of germinating sporangiospores of Rhizopus stolonifer. Ekundayo and Carlile (1964) observed that the presence of glucose was necessary for spore swelling in Rhizopus arrhizus. Maximal swelling however required the additional presence of a nitrogen source together with phosphate and either sodium or potassium ions. Transferring the spores to a glucose-free medium caused the cessation of swelling. Sporangiospores of Syncephalastrum racemosum were also found to require the presence of glucose throughout the germination process (Hobot & Gull, 1977). Marchant and White (1966) observed that spores of Fusarium culmorum required both a carbon and nitrogen source for swelling to occur. Using a Warburg apparatus, Marchant and White (1966) demonstrated that during the first hour of the germination process, oxygen uptake increased gradually until linearity was reached. The rate of oxygen uptake increased approximately 50% in the first 30 minutes of germination in Rhizopus stolonifer spores (Van Etten et al, 1974) and thereafter continued to increase linearly. It had increased about 10 to 20-fold at 4h (percentage germination was around 18%). Such findings that swelling was dependant upon an external nutrient supply and that oxygen uptake occurred soon after the initiation of the germination process, suggested that spore swelling was an active process and not just simply due to water-uptake (Ekundayo & Carlile, 1964). Ekundayo and Carlile (1964) had also found that spores of Rhizopus arrhizus germinated better under aerobic conditions. Anaerobic liquid cultures of spores (nitrogen was

bubbled through the medium) gave after 8h only about 10% of spores with germ tubes and swelling was to about 8 μm (spore diameter). Aerobic cultures after 8h had 90% of spores with germ tubes and swelling was to about 13 μm. A similar observation was made with germinating spores of Penicillium atrovenetum by Gottlieb and Tripathi (1968). In glucose Czapek-Dox medium under aerobic conditions, 85% of the spores had swollen within 8h. When spores were incubated in shake-flasks under anaerobic conditions, only 12% of the spores had swollen. Later observations up to 14h revealed that no further increase in swelling occurred and that only those spores that had originally swollen produced germ tubes. Also, the addition of respiratory inhibitors showed a marked effect on spore swelling. Sodium azide, which inhibits cytochrome oxidase activity in the terminal electron transport system, reduced respiration by 83% and swelling by 94% in P.atrovenetum. 2, 4-dinitro phenol, an uncoupler of oxidative phosphorylation, reduced respiration by 87% and swelling by 100%. Therefore, swelling of spores appears to be an energy requiring process. In the case of P.atrovenetum, energy is obtained by the catabolism of glucose, as the presence of glucose was necessary for swelling (Gottlieb & Tripathi, 1968). These results, in conjunction with other work, show that swelling is a complex process requiring the assimilation of glucose, the uptake of oxygen, and the possible involvement of respiration (oxidative phosphorylation). Recent work has also shown that swelling is an active process involving cell wall growth, together with RNA, DNA and

protein synthesis (Schmit & Brody, 1976; Van Etten et al, 1974).

ORGANELLE CHANGES DURING GERMINATION.

Many reports describe organelle changes during germination, with recently the publication of a review article by Smith, Gull, Anderson and Deans (1976). During the germination process, the morphological features of the spore reflect the change of the spore from a non-polar spherical growth form to a polarised tip growing organism. Growth is localised at the apical tips of the emergent germ tubes and involves apical vesicles (Bartnicki-Garcia, 1973). The reason why earlier workers had not observed vesicles was due to the various pre-treatments used. These usually had a deleterious effect on the internal spore structure, even prior to fixation of the material for electron microscopic observation (Bracker, 1971). Bracker (1971) put forward four possible explanations as to why micrographs of apical vesicles were absent from published reports:- (1) the vesicles were lost during fixation, or the fixation procedure was insufficient to resolve vesicles; (2) the vesicles were lost prior to fixation during collection and/or preparation of cells for fixation; (3) the plane of the section(s) did not intercept the vesicles in the germ tube apices; (4) vesicles were actually lacking in the growing cells under study. Bracker (1971) thought that the last possibility seemed unlikely in view of the numerous examples of vesicles in hyphal growth. In his study however, Bracker (1971) found that the treatment of living hyphae prior to fixation had a profound effect on the structures in the germ tube apices.

Germinating spores, washed off agar plate cultures and centrifuged three times in cold 0.2M phosphate buffer (pH 7.0) prior to fixation, lacked apical vesicles. A common phenomenon in these cells was a divergence of the plasmamembrane from its normal position against the wall. This occurred at the germ tube apex as well as in other regions around the cell periphery. It seldom occurred in control spores, which had been fixed immediately by flooding plate cultures directly with fixative. By this process of direct fixation, Bracker (1971) observed that germinating spores of Gilbertella persicaria had few vesicles at the apex of the germ tube, whereas they occurred abundantly in hyphal tips of established mycelia of G. persicaria. Grove and Bracker (1970a) found that hyphae had many more vesicles at their tips than germ tubes, similar observations having been made in Rhizopus arrhizus (Hess & Weber, 1973) and Geotrichum candidum (Steele & Fraser, 1973b).

Nuclear division occurs in the spores either prior to or during germ tube emergence (Steele & Fraser, 1973b). Most fungal spores are multinucleate, such as Rhizopus (Buckley, Sommer & Matsumato, 1968; Hawker & Abbot, 1963a; Necas, Havelkora & Souchek, 1963) or Botrytis cinerea which was reported to contain three to six nuclei per spore (Gull & Trinci, 1971). A similar number was also reported by Buckley, Sjaholm and Sommer (1966). After about 4h the spores of B.cinerea had swollen and often the nuclei were associated with endoplasmic reticulum. This association became more noticeable during germ tube outgrowth (Gull & Trinci, 1971). Nucleoli were observed as more electron dense inclusions in the nucleoplasm in G. candidum (Steele & Fraser, 1973b) and B.cinerea (Gull & Trinci, 1971).

Generally, endoplasmic reticulum increased during germination (Cochliobolus carbonus, Murray & Maxwell, 1974; Penicillium notatum, Martin, Urubura & Villanueva, 1973b). It has, however, been reported as being sparse in some spores and more plentiful in others (Smith et al, 1976). In G. candidum (Steele & Fraser, 1973b), the endoplasmic reticulum apparently lacks a true golgi apparatus (like in many other fungal species). Steele and Fraser (1973b) suggested that this role is probably assumed by part of the endoplasmic reticulum system.

Mitochondria are present in dormant spores, increasing in shape and size during germination. In dormant spores of Rhizopus arrhizus, Hess and Weber (1973) described that the few mitochondria present were spherical in shape and contained few cristae. During germination, there was an increase in their size and the cristae became more abundant (Hawker & Abbot, 1963a; Buckley et al, 1968). In Botrytis cinerea (Gull & Trinci, 1971), although the mitochondria increased in size, the ratio of the areas occupied by mitochondria and cytoplasm remained constant. Gull and Trinci (1971) calculated the cross-sectional area occupied by mitochondria as a percentage of the total cytoplasmic area including walls and vacuoles. Mitochondria occupied 10.9% of the cytoplasmic area in the dormant spores, and 11.6% in the germinated spores.

The mitochondria of dormant spores of Cochliobolus carbonus (Murray & Maxwell, 1974) are spherical and contain a few loosely arranged cristae. On germ tube emergence, mitochondria become elongated with closely packed cristae. It is therefore probable

that the change in shape and increase in cristae is related to changes in metabolism linked to respiratory activity.

Lipid bodies are found in dormant spores and usually disappear during germination. They probably serve as a food reserve or extra source of energy for the spore (Martin, Nicolas & Villanueva, 1973a; Martin et al, 1973b). Vacuoles increase in size during germination of R. arrhizus (Hess & Weber, 1973), but decrease in Penicillium notatum (Martin et al, 1973b). As the germ tube extends, increased vacuolisation in the conidium of B.cinerea was observed (Gull & Trinci, 1971). The vacuoles are delimited by a single membrane and contain deposits of electron dense material.

Ribosomes are few in number in dormant spores, but their numbers increase during germination. This undoubtably reflects the increase in metabolic activity associated with the outgrowth of the germ tube.

MACROMOLECULAR SYNTHESIS DURING GERMINATION.

The synthesis of protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) is an interrelated series of metabolic events. Their synthesis has important consequences upon the development of the fungal spore. However, controversy has reigned over the years as to how macromolecular synthesis controls morphogenesis, and to what extent it occurs in germinating spores.

The sequence set out for macromolecular synthesis is first RNA synthesis (transcription), then protein synthesis (translation), and finally, DNA replication, occurs in some fungi. In Fusarium solani

spores, initiation of RNA synthesis occurred first followed by protein synthesis prior to outgrowth of the germ tubes. DNA synthesis during germination began only at 8h when the level of spores with germ tubes was 81.5% (Cochrane, Rado & Cochrane, 1971). Van Etten and his coworkers (1974) looked at the incorporation of labelled leucine into protein and labelled uracil into RNA in germinating spores of Rhizopus stolonifer. They found that both protein and RNA synthesis began within the first 30 minutes and rapidly increased throughout the germination process. A later study by Roheim, Knight and Van Etten (1974) demonstrated that synthesis of all classes of RNA began within 15 minutes after placing R. stolonifer spores in a germination medium. Spores were pulsed with labelled uracil for 15 minutes, after which they were harvested and quickly frozen in liquid nitrogen. After storing at -80°C, the RNA was extracted and analysed either by polyacrylamide gel electrophoresis or on sucrose density gradient columns (Roheim et al, 1974). However, after the dormancy of Saccharomyces cerevisiae ascospores had been broken, protein synthesis occurred first, followed shortly afterwards by RNA synthesis. DNA synthesis occurred 2h later, three hours after initiation of the germination process (Rousseau & Halvorson, 1973a). Proflavine, an inhibitor of RNA synthesis, was found to inhibit RNA synthesis and germ tube production in Neurospora crassa and Aspergillus nidulans (Holloman, 1970). However, outgrowth of germ tubes was not affected in both Peronospora tabacina and Alternaria solani at concentration ranges of 0-30 $\mu g.ml^{-1}$ of the proflavine. These concentrations were inhibitory to the former two fungi. At higher concentrations than this, proflavine inhibited protein synthesis as

well, and only then adversely affected germ tube production in P. tabacina and A. solani (Hollman, 1970). This result, coupled with Rousseau and Halvorson's (1973a) finding that protein synthesis occurred first in germinating ascospores of S.cerevisiae, suggests that pre-formed mRNA may be present in dormant spores of some fungi.

There is little information about fungal mRNA, because it is heterogenous in size and only comprises about 5% of the total RNA in the cell (Van Etten, Dunkle & Knight, 1976). It is thought that mRNA is derived by post-transcriptional modification of large precursor RNA's, which are termed heterogenous nuclear RNA. After the heterogenous RNA is synthesised in the nucleus, adenine residues are added to the 3'-hydroxy end to give a polyadenylate (poly-A) segment consisting of 150-200 nucleotides. Perhaps around 90% of the heterogenous RNA may stay in the nucleus, the rest migrating to the cytoplasm including the Poly-A segment and serves as mRNA (Van Etten et al, 1976). Roheim and his coworkers (1974), working with Rhizopus stolonifer sporangiospores, detected a heterogenously sedimenting fraction comprising about 5% of the total cellular RNA. With it was associated a polyadenylate containing RNA. A heterogenously sedimenting RNA (i.e. presumptive mRNA) was detected in germinating conidia of Neurospora crassa after 5 minutes into germination (Mirkes, 1974). Sucrose gradients were used to isolate and characterise the RNA fractions. Mirkes(1974) also observed that polyribosomes were present in dormant spores prior to placing them in germination medium. Spores harvested and exposed to hydration contained 30% of their ribosomes as polysomes, whereas those not exposed to hydration contained only 3% of their ribosomes as polysomes (Mirkes, 1974). The presence of

polyribosomes in dormant spores could possibly be indicative of pre-formed (probably stable) mRNA. Mirkes (1974) harvested conidia in either of two ways:- (1) washing the culture flasks with sterile water followed by filtration of the conidia through glass wool (hydrated spores); (2) by inverting a flask containing conidia over a membrane filter chimney equipped with a membrane filter. Then with gentle tapping of the flask and with suction applied to the filter apparatus, conidia (dry) were collected on the membrane filter. Brambl (1975) recognised the problem of whether the presence of polyribosomes (and thus whether pre-formed mRNA exists or not) is influenced by the rehydration of spores during harvesting and prior to placing in a germination medium. He therefore harvested spores of Botryodiplodia theobromae with water and also with non-aqueous fluids (fluorocarbon fluid, FC-43 or paraffin oil). Ribosomal fractions were examined by density gradient centrifugation. Peaks corresponding to polyribosome fractions were detected in both water and non-aqueous fluid harvested spores, and were, as Brambl (1975) pointed out, very similar.

DNA synthesis occurs usually around the time of germ tube emergence or later as already mentioned for Fusarium solani (Cochrane et al, 1971). In R.stolonifer sporangiospores, the DNA content started to increase around 1-2h after initiation of the germination process (0% germ tube emergence), and increased during the remainder of the germination process (Van Etten et al, 1974). The first germ tube appeared 4h after the initiation of germination in Fusarium oxysporum (Kumari, Decallonne & Meyer, 1975). The DNA content of the spores increased noticeably after 5h, reaching a

constant value of DNA per nucleus at 6h. This corresponded with the time of the first nuclear division.

Enzymes involved in both DNA and RNA synthesis have been detected. A DNA-dependant DNA polymerase has been isolated from both germinated and dormant spores of Rhizopus stolonifer (Gong, Dunkle & Van Etten, 1973). Manocha (1973) has isolated a DNA-dependant RNA polymerase in germinating bean rust uredospores. Further, Hollomon (1973) demonstrated that in germinating conidia of Peronospora tabacina, fractions containing particulate-bound ribosomes were involved in transferring amino acids from aminoacyl-tRNA into protein. Endogenous pools of amino acids were found in conidia both before and during germination in Neurospora crassa (Schmit & Brody, 1975). The detection, identification and characterisation of the various enzymes involved in RNA, DNA and protein synthesis, is important in obtaining a clearer understanding of the mechanisms which control macromolecular synthesis in relation to spore germination (Van Etten, 1969).

Protein synthesis is essential for spores to germinate. Cyclohexamide when added arrested morphological development almost immediately in spores of Phycomyces blakesleeanus (Van Laere, Carlier & Van Assche, 1976). 5-fluorouracil (an inhibitor of RNA synthesis) had no effect until the emergence of the germ tube. This suggested that protein synthesis or some other cyclohexamide dependant process, but not RNA synthesis, was required for the first developmental stages (Van Laere et al, 1976). Hollomon (1969) demonstrated that protein synthesis was an essential requirement for germ tube differentiation in conidia of Peronospora tabacina, but that RNA synthesis was not. Inhibitors of RNA synthesis (ethidium

bromide and 5-fluorouracil) did not alter the percentage of spores forming germ tubes. Hollomon (1969) proposed that protein necessary for germ tube development was synthesised on a template of stable mRNA already present in dormant conidia.

The requirements for RNA and protein synthesis vary among different organisms. Therefore a better understanding of the various requirements for synthesis among fungi will enable a more composite picture of macromolecular synthesis and cellular control of germination to be built up.

OTHER BIOCHEMICAL EVENTS

Carbohydrate metabolism of fungal spores has not been widely studied, although it plays an important role as a source of energy, and can supply or be a source of metabolic intermediates involved in synthetic processes. The enzyme components of the Embden-Meyerhof-Parnas (EMP) pathway, the hexose monophosphate shunt, Tricarboxylic Acid (TCA) Cycle and terminal electron transport system have been detected in fungal spores studied so far (Gottlieb, 1976).

Potassium fluoride, an inhibitor of enolase in the EMP pathway, decreased respiration by 66% and swelling by 85% in germinating spores of Penicillium atrovenetum (Gottlieb & Tripathi, 1968). Sodium azide, an inhibitor of cytochrome oxidase activity in the terminal electron transport system, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, also reduced respiration and swelling in P.atrovenetum. Subikova and Subik (1974) observed that azide and cyanide (an uncoupler of oxidative phosphorylation) inhibited the germination and swelling of spores in Aspergillus niger, Penicillium italicum and Rhizopus oryzae.

When 30mM fluoride was added in the first 3h of germination of Saccharomyces cerevisiae ascospores, carbon dioxide production was completely inhibited, whereas oxygen uptake was 10% of the control value (Rousseau & Halvorson, 1973b). After 24h, only few outgrowing spores were observed. Rousseau and Halvorson, (1973b) suggested that in the presence of fluoride, hexose metabolism was shifted from its normal dependence upon glycolysis to the hexose monophosphate shunt, as germination had occurred in some spores. Azide, cyanide and dinitrophenol inhibited oxygen uptake 96%, 52% and 96% respectively, and carbon dioxide production by 80%, 66% and 94% respectively (Rousseau & Halvorson, 1973b).

These results all show that germinating spores are capable of metabolising sugars via the EMP pathway in order to produce the necessary energy for development through the TCA cycle and terminal electron transport chain. Any interference with this metabolic activity adversely affects the process of germination which can be expressed as a cessation of swelling and germ tube outgrowth.

Lipids are broken down during germination and used as an endogenous supply of energy. Total lipid content decreased during germination of Penicillium notatum spores (Martin, Liras & Villnueva, 1974). In the life-cycle, the germinated spores contained the lowest level of total lipid, whilst the resting spores had the highest content (13% of spore dry weight in resting spores, 6% in germinated spores). Marouf and Malhotra (1976) revealed differences in the fatty acid composition of three different stages of the life-cycle of Phycomyces blakesleeanus. In spores, the total unsaturated fatty acid accounted for more than 82% of the total lipid, while it was less than 66% in mycelia and less than 65% in

sporangiophores. The authors suggested that it would be of interest to find out if these changes in the fatty acids occur in the cellular membranes during germination of P.blakesleeanus. This was because earlier Malhotra and Tewari (1973) had detected ultrastructural changes in the plasmamembrane of germinating spores of P.blakesleeanus. Therefore changes in lipid content of germinating spores may be due not only to their utilization as a possible energy source, but also perhaps reflecting changes in the morphological development of cellular membranes.

It has been suggested that lipids can be oxidised to acetyl-CoA which enters the TCA cycle via the Glyoxylate Shunt, so providing energy to cells of plant tissues which are synthesising carbohydrates (Richardson, 1974). A similar observation was made with motile zoospores of Phytophthora palmivora where lipids were the main source of energy (Bimpong, 1975). They were broken down via the glyoxylate cycle to provide energy indirectly through the TCA cycle. Bimpong (1975) showed that the enzyme isocitrate lyase (responsible for entry of substrates into the glyoxylate shunt) was most active in freshly liberated zoospores, although its activity decreased with time. However, she observed that the activities of some TCA cycle enzymes were always very low. Leighton, Stock and Kelln (1970) detected isocitrate lyase activity in macroconidial germination of Microsporum gypseum, this being evidence of the presence of the glyoxylate cycle. Respiratory quotient (RQ) values for ungerminated spores of Puccinia graminis tritici, Puccinia rubigo-vera, Neurospora sitophila and Fusarium solani have been reported to be all in the range associated with the oxidation of lipids. In F.solani, the RQ changed

during germination to a value corresponding to a shift from lipid to carbohydrate metabolism (Gottlieb, 1976). Lipid metabolism may therefore provide an extra source of energy for the spore, especially at the time of germ tube emergence. At this stage of development, the spore may perhaps need some of its endogenous carbohydrate (or an exogenous supply if no internal reserves are present) for new cell wall synthesis. A possible way of lipids being converted into energy is breakdown to acetyl-CoA via β-oxidation (Lewis & Johnson, 1966) and subsequent entry to the TCA cycle via the glyoxylate shunt (Bimpong, 1975; Leighton et al, 1970).

Uredospores of bean rust fungus, Uromyces phaseoli incorporated labelled carbon dioxide into the intermediates of the TCA cycle (Staples & Weinstein, 1959). In this way, carbon dioxide is fixed in some fungal spores in order to probably provide extra carbon skeletons for the TCA cycle intermediates. Yanagita (1963) found that germinating conidia of Aspergillus niger, when cultured in the presence of radioactivity labelled carbon dioxide, became labelled in various cellular fractions:- (1) nucleic acids, (2) lipid, (3) acid-soluble fraction, which was found to include AMP and ATP. Carbon dioxide can, it seems, serve as an extra source of carbon for some germinating spores, although its effect can vary from microorganism to microorganism (Nyiri, 1967).

WALL ULTRASTRUCTURE DURING GERMINATION

The most obvious difference between fungal spores and vegetative hyphae is the cell wall. Whereas in spores, the wall can vary in size, the number of layers and external ornamentation,

vegetative hyphae have thinner walls but also possess a complex architecture (Hunsley & Burnett, 1970). The cell wall protects the intracellular organelles, provides rigidity and form to the cell, and is involved in morphological differentiation during growth. Because of these properties plus differences in ultrastructure and chemical composition, a clearer understanding of both spore and hyphal walls is necessary to provide a better basis for interpreting fungal development.

The ultrastructure of fungal spore walls varies widely, and has been the subject of many thin section studies (reviewed recently by Akai, Fukutomi, Kunoh and Shiraishi, 1976). During germination, the spores swell and a period of spherical wall growth occurs. This was demonstrated by feeding germinating spores of Mucor rouxii with labelled N-acetyl-D-glucosamine and using autoradiography to detect incorporation. The disperse pattern of photographic grains over the entire surface of the swollen spores indicated that spherical wall growth was occurring (Bartnicki-Garcia & Lippman, 1969). At around the time of germ tube emergence, there is a switch from a non-polar to a polar type of growth. The grains become more and more aggregated to one point on the spore, the point where eventually the young germ tube emerges. The label is then found only at the tips of the germ tube (Bartnicki-Garcia, 1973).

Bartnicki-Garcia (1968a) proposed three different types of wall development involved in germ tube outgrowth:-

(a) the new germ tube wall is a direct extension of the spore wall or one of its innermost layers;

(b) de novo formation of a cell wall on a naked protoplast, such as occurs in the encystment of aquatic zoospores of Phycomycetes;

(c) de novo formation of a germ tube wall under the existing spore wall.

One important point to bear in mind with regard to Bartnicki-Garcia's classification, is that it is based on thin section studies using various fixatives. Hawker and Hendy (1963b) studied spore germination in Botrytis cinerea. They observed that dormant conidia had a two-layered wall, a thin electron dense outer layer, and a thicker electron transparent inner one. Upon germination, the outer wall ruptured and the wall of the emergent tube was continuous with the inner one. Gull and Trinci (1971) also observed that dormant conidia of B.cinerea were two-layered. However, they used three different fixation methods for their material:- (1) potassium permanganate (as used also by Hawker and Hendy, 1963b), (2) glutaraldehyde/osmium tetroxide, (3) osmium tetroxide in Kellenberger's buffer. The results of Gull and Trinci (1971) showed that upon germination, the new wall layers were laid down between the plasmamembrane and the inner dormant conidium wall. A third and innermost new wall layer was formed only around the point of germ tube emergence. This wall layer was continuous with the wall of the outgrowing germ tube. These three new wall layers were observed with either glutaraldehyde/osmium or with Kellenberger fixations. Gull and Trinci (1971) therefore suggested that studies of spore germination using thin section techniques should employ the use of the above two fixation procedures. It could also mean, that fungi previously placed in Bartnicki-Garcia's group (a) would have to be transferred to group (c).

The work of Gull and Trinci (1971) together with Bracker's (1971) findings on how apical vesicles can easily be lost due to pre-fixation treatments, stresses the dangers that can be met in preparing material for electron microscopy. The interpretation of thin section micrographs must therefore also take into account the procedures used in preparing the material under discussion. Most ultrastructural studies, by dividing spore wall layers into either electron translucent or electron opaque areas, give little idea of the actual structure of these walls (Hobot & Gull, 1976). Electron microscopical studies employing metal shadowing, freeze-fracture and surface replica techniques, are to be welcomed as being able to provide more information about the architecture of the wall. These techniques, coupled with chemical (Hobot & Gull, 1976) and enzymic (Tokunaga & Bartnicki-Garcia, 1971; Hunsley & Burnett, 1970) treatments of spore and hyphal walls, not only provide chemical and ultrastructural information, but also give a much better overall picture of the wall.

Metal shadowing of cell wall preparations of cysts of Phytophthora palmivora showed that both the inner and outer walls were microfibrillar (Tokunaga & Bartnicki-Garcia, 1971). Total hydrolysis products of the cyst walls (as also of wall extracts of hyphae) revealed that 90% or more of the walls were composed of hexose sugars. Partial acid hydrolysis products suggested that glucan was of three types in the walls with β-1,3, β-1,4 and β-1,6 linkages. Germ tube walls had an outer amorphous layer which could be removed by exo- β -1,3 glucanase enzyme. An inner microfibrillar layer was exposed by the enzyme treatments, which was continuous with the layer of microfibrils of the cyst. These fibrils

were also analysed as being glucans (Tokunaga & Bartnicki-Garcia, 1971). Hegnauer and Hohl (1973) subjected P.palmivora cysts and germ tube walls to an electron microscopical analysis. They employed freeze-etching, thin sectioning and replica techniques. Their ultrastructural results corroborated the findings of Tokunaga and Bartnicki-Garcia (1971). Studies such as these show the advantages of using several techniques coupled to chemical analysis of the wall layers. A much more detailed picture of the wall is built up, so providing a basis for studying the involvement of cell wall development with respect to cellular morphogenesis.

Thin section studies provide information as to whether new wall layer(s) are synthesised and also as to their relative thickness. In Penicillium notatum, germ tube formation is by extension of the inner layers of the spore wall (Martin et al, 1973b). In Alternaria brassicola, the wall of the germ tube is an extension of the inner spore wall (Campbell, 1970). Bussel and Sommer (1973) reported that in Rhizopus stolonifer, a new inner spore wall develops during germination, such that it becomes continuous with the germ tube. This also appears to be the case for Rhizopus arrhizus (Ekundayo, 1966; Hess & Weber, 1973). A new germ tube is also synthesised within the existing spore wall of Aspergillus nidulans (Border & Trinci, 1970). Richmond and Pring (1971) observed that in Botrytis fabae the wall of the germ tube is continuous with a new inner wall layer formed in the conidium. A new vegetative wall was synthesised de novo between the plasmamembrane and existing spore wall in swollen spores of Mucor rouxii (Bartnicki-Garcia, Nelson & Cota-Robles, 1968b). The new wall layer became eventually the wall of the young, emergent germ tube.

CHEMICAL COMPOSITION OF SPORE AND HYPHAL WALLS.

Fungal spore walls, like those of vegetative hyphae, contain polysaccarides, protein, melanin and lipids. The polysaccarides are the largest component of the wall, and can constitute up to 80-90% of the dry weight (Smith & Berry, 1974). However, the comparison of spore and hyphal walls is difficult. Although many studies have been conducted with hyphae, very little work exists on spore wall composition. In fact, it could safely be said that no real in depth work on spore wall analysis has been done. This was one of the disadvantages felt in preparing this thesis - the comparison of results obtained with investigating the spore walls of Syncephalastrum racemosum to other work was problematical. There are reports on spore wall structure using thin section techniques. Some studies also present data on the total macromolecular composition of spores, but no real detailed analysis of spore wall ultrastructure using various electron microscopical techniques or of the spore wall polymers.

Bartnicki-Garcia and Reyes (1964) noted differences between the cell wall of spores and hyphae in Mucor rouxii. The main polymer of the spores was glucan, and they also had some mannan and a melanin-glucosamine-protein complex. In hyphae, the main polymer was chitosan with smaller amounts of phosphate, chitin, protein, mannan, galactose and fucose. Bartnicki-Garcia (1968a) pointed out that whereas in spores of the Zygomycetes glucose was probably the main monosaccaride (indicative of glucan in the walls), the hyphal walls were of the chitosan-chitin type. Even though chitin is probably present in most Zygomycete spore walls, the

differentiation of spore wall into hyphal wall therefore presents changes in wall polymers. An understanding of these changes is important, as cell walls play a major role in maintaining the shape of the organism and thus its growth and development (Bartnicki-Garcia, 1973). A clear reason as to why there should be more detailed studies made of fungal spore walls.

Vegetative hyphal walls are composed of a variety of polymers. Chitin (a polymer of N-acetyl-D-glucosamine) is very common in hyphal walls (Bartnicki-Garcia, 1968a). Chitosan (a polymer of non-acetylated glucosamine) is found in some fungi, especially the Zygomycetes. Although galactose, fucose, xylose and glucuronic acid are found in walls, the most common monosaccaride constituent is glucose. Polymers of glucose are termed GLUCANS. There are different structural compositions of glucan, depending upon the nature of the linkages between the monosaccaride residues. Linkages can be either of the α - or β-form in the 1,2; 1,3; 1,4 or 1,6 carbon atom positions. For example, cellulose is a β-1,4-glucose polymer, whilst another common glucan found in fungal cell walls is β-1,3 linked. This glucan is usually found to have various amounts of β-1,4 and β-1,6 linkages in different fungi. Mannan (a polymer of mannose) is a common component of yeast cell walls, and glycoproteins (polysaccaride-protein complexes) are also present (Gander, 1974).

ARCHITECTURE OF HYPHAL WALLS

By employing the techniques of sequential enzyme or chemical treatments, the complex architecture of hyphal walls has been

revealed. Hunsley and Burnett (1970) subjected whole hyphae of Neurospora crassa and Schizophyllum commune to a sequential treatment with laminarinase, pronase and chitinase. Hyphae of Phytophthora parasitica were treated with laminarinase, pronase and cellulase. The enzyme treatments revealed that both the outer amorphous wall layers of N.crassa and S.commune (here only after KOH treatment had removed the outermost wall layer) were degraded by laminarinase, showing them to be probably of glucan composition. (Probably this glucan has β-1,3 and β-1,6 linkages). The outer layer of P.parasitica was also susceptible to laminarinase. In N.crassa, a network of coarse strands was now observed. Hunsley and Burnett (1970) suggested that this could be a glycoprotein. However, pronase treatment in all three fungi revealed an inner wall layer consisting of a network of randomly orientated microfibrils. Chitinase degraded the fibrils in N.crassa and S.commune, showing them to be chitin. The fibrils in P.parasitica could only be degraded by cellulase, indicating that they were cellulose microfibrils. Protein was present throughout most of the walls, a similar finding being noted in Agaricus bisporus mycelium (Michalenko, Hohl & Rast, 1976). Michalenko and his coworkers (1976) worked with isolated cell walls. It should be pointed out here that Hunsley and Burnett's (1970) work is one of the few studies based upon observations of entire hyphae. Most work is performed with isolated cell wall fractions. Michalenko et al (1976) stained A.bisporus walls with silver hexamide, which has specificity for cystine-containing proteins, and the stain was found deposited over the entire wall. This suggested that protein is present throughout the whole wall. Walls of A.bisporus were found to have an outer KOH-soluble layer (as in

S.commune, Hunsley and Burnett, 1970 - an α-1,3 glucan, Wessels, 1965) and an inner layer of chitin embedded in an amorphous β-1,3 glucan matrix. After KOH extraction of the outer layer, the chitin microfibrils were more clearly observed following β-glucanase digestion (Michalenko et al, 1976). These results of both Michalenko et al (1976) and Hunsley and Burnett (1970) suggest that the hyphal wall is not composed of discrete layers of separate components, but more likely one layer merges into another with no clearly definable interface between them.

Hyphal walls of Pythium acanthicum had an amorphous outer layer and a microfibrillar inner one (Sietsma, Child, Nesbitt & Haskins, 1975). The amorphous layer was soluble in KOH and could be precipitated out of alkaline solution by addition of ethanol. Chemical analysis of the two wall fractions (layers) showed that the amorphous matrix was a β-glucan with 1,3 and 1,6 branches, whilst the fibrils were a β-glucan with 1,4 and 1,3 linkages (Sietsma et al, 1975). Hyphal germ tubes of Phytophthora palmivora were also amorphous in appearance on the outside with an inner layer of microfibrils (Tokunaga & Bartnicki-Garcia, 1971). The outer layer was susceptible to β-1,3 exoglucanase, whilst the microfibrils were most susceptible to a mixture of β-1,3 and β-1,4 endoglucanases. Therefore a β-1,3 glucan on the outer surface covering a more cellulosic-type wall on the inside.

The interesting observation in these studies on hyphal wall architecture shows that the innermost wall layer is usually of a microfibrillar form. It is chitin in N.crassa and S.commune

(Hunsley & Burnett, 1970), as also in A.bisporus (Michalenko et al, 1976). Or it is glucan (probably cellulose, i.e. β-1,4 glucan) in P.acanthicum (Sietsma et al, 1975) and P.palmivora (Tokunaga & Bartnicki-Garcia, 1971). Cellulose microfibrils form the inner wall layer in P.parasitica (Hunsley & Burnett, 1970). Hunsley & Burnett (1970) suggested that chitin plus protein forms a mechanically intact but non-rigid wall at hyphal apices. Further back from the tips, outer wall glucan (maybe also the network of coarse strands in N.crassa) imposes an increasing degree of rigidification. The role of chitin is most probably played by cellulose microfibrils in the other fungi. Therefore, the wall is very important in governing the overall shape of the organism, so influencing its cellular development.

FUNGAL GROWTH

Vegetative hyphae grow by extension at the tip. Grove and Bracker (1970a) observed that hyphae could be divided into three ultrastructurally definable zones:- (1) an apical zone, in which only cytoplasmic vesicles were present to the exclusion of all other cellular organelles; (2) in the subapical zone, endomembrane systems associated with vesicles were found, as were other protoplasmic components:- nuclei, mitochondria, ribosomes, endoplasmic reticulum, dictyosomes, vesicles; (3) the third zone was a zone of vacuolation, the number of vacuoles increasing as the distance from the apex increased. In some cases, Grove and Bracker (1970a) observed apical vesicles whose membranes were continuous with the plasmamembrane. This led them to suggest that the vesicles were involved in wall extension, and to this end they put forward the following model for tip growth (Grove, Bracker & Morre, 1970b). Material is transferred from the endoplasmic

reticulum to the proximal pole of the dictyosome by vesicles blebbing off the endoplasmic reticulum and fusing with the cisternae of the dictyosome. The contents and membranes of the cisternae become transformed as they are displaced towards the distal pole of the dictyosome (Grove, Bracker & Morre, 1968). Vesicles are formed at the distal pole which migrate to the hyphal apex. Here they fuse with the plasmamembrane and discharge their contents into the growing wall region (Grove, Bracker & Morre, 1970b).

Bartnicki-Garcia (1973) put forward a hypothetical model to explain how the hyphal wall extends at the apex by the discharging of material into this region by the apical vesicles. Initially, lytic enzymes from a cytoplasmic vesicle are secreted into the wall. These enzymes attack the microfibrillar inner layer of the wall by splitting either inter- or intra-molecular bonds. The high turgor pressure of the hyphal tube stretches and separates the fibrils so that there is an increase in wall surface area. The amorphous outer layer may also be attacked in the same way. Microfibril synthesising enzymes either in the wall itself or on the outer surface of newly formed plasmamembranes rebuild the microfibrils by synthesising new chains or by extending old ones. Material for this rebuilding of the wall comes in the apical vesicles, which discharge their contents by fusion with the plasmamembrane. The vesicles also contain new amorphous wall material, which are forced into the outer layers of the wall by the high turgor pressure of the cell. In this scheme, a delicate balance between wall synthesising and wall lytic enzymes is maintained in order to control apical wall growth (Bartnicki-Garcia, 1973).

CONCLUSION

The development of a spore into a vegetative structure provides an excellent model system for studying morphogenesis and its cellular control mechanisms. Spores offer various approaches to the problem:- (1) Ultrastructural changes involving (a) organelles, (b) cell walls; (2) changes in macromolecular synthesis; (3) changes in metabolism and respiration (energy processes); (4) chemistry of cell walls; (5) growth - the change from non-polar to polarised development. Only by a clearer understanding of these various processes will more be learnt about how the fungi adapt and grow in various environments.

CHAPTER ONE

ULTRASTRUCTURE OF THE LIFE-CYCLE

INTRODUCTION

The fungus chosen for this study was *Syncephalastrum racemosum* Cohn ex Schroeter. It is a member of the Zygomycete group, growing saprophytically on soil and on dung. Asexual development and growth is similar to the more studied *Mucor*, in that it forms aerial hyphae terminating in a spherical swelling at the tip, the SPORANGIUM or AMPULLA. Its asexual spores are not formed by delimitation of the cytoplasm within the spherical swelling as in *Mucor*. They are produced, however, by a similar cleavage process occurring within sac-like protrusions (MEROSPORANGIA) growing out of the ampulla. This type of spore formation bears a resemblance to what occurs in Ascomycete sporulation, and *Syncephalastrum* could possibly represent an evolutionary link between the Phycomycetes and the Ascomycetes.

The only detailed cytological study of *S.racemosum* to date has been an electron microscopic investigation by Fletcher (1972) of asexual spore formation. Thin sections of elongating merosporangia revealed that two new internal wall layers were formed, both becoming continuous with the ampulla wall, which initially had been single layered. These became more pronounced as cleavage furrows cut off the merosporangial protoplasm to form spore initials. An outer membrane surface layer also developed and it apparently became detached from the two internal wall layers to give a blistered appearance. New spore walls were laid down between the spore initial plasmamembrane and an investing layer. This investing wall layer delimited one spore initial from the next after cleavage of the protoplasm. The spore walls were not associated with the merosporangial wall. The two outer wall layers of the merosporangium

were continuous with the ampulla wall. The spores developed as chains within the merosporangia, appearing mainly rectangular in section.

Fletcher's (1972) thin section study on sporulation gives a lot of information on the changes associated with spore development. The scanning electron micrographs presented in this chapter reveal a more complete picture of the sporulating structures. The chapter also deals with the life-cycle of S.racemosum and the ultrastructural appearance of various stages.

MATERIALS AND METHODS

ORGANISM

The laboratory strain of Syncephalastrum racemosum Cohn ex Schroeter used throughout this study was kindly donated by Dr. Jane North.

GROWTH MEDIUM, GERMINATION STUDIES AND SPORE PRODUCTION

These techniques were as outlined in the materials and methods section of Chapter Two. Spores for surface replicas were grown on cellophane cultures (cf. materials and methods, Chapter Four).

ELECTRON MICROSCOPE TECHNIQUES

(1) Preparation of hyphal tips, aerial hyphae and spores for surface replica examination:

S.racemosum was grown on Millipore filters (pore size 0.45 μm; diameter 47mm) placed on solid defined medium at 37°C. Small areas of the colony at the tip region were cut out of the millipore filters, transferred quickly to the clean surfaces of split mica, and coated in an Edwards 306 Coater with carbon-platinum electrodes at an angle of 45° and carbon-carbon electrodes at 90°. Aerial hyphae were collected by gently brushing the tops of colonies of the fungus with the clean surface of a piece of split mica prior to coating. Suspensions of spores were spread onto clean mica surfaces before coating.

After coating, the replicas were floated off onto distilled water, transferred to concentrated sodium hypochlorite solution, and cleaned of any adhering biological material in several changes of this solution by gentle agitation with a pasteur pipette for 30 minutes. The replicas were then washed in several changes of

distilled water for 30 mins. and mounted onto grids.

(2) Freeze-fracture:

The freeze-fracture procedure was carried out using the Bullivant and Ames (1966) method. The material (very dense spore suspension) was drawn into a thin plastic tube and immersed in Freon 12 which had just thawed out, having been previously placed in liquid nitrogen. Upon freezing, the sample tube was quickly transferred to liquid nitrogen, allowed to cool to the temperature of the nitrogen, and then placed in the central hole of a brass block. The brass block, tweezers, and razor blades had all been pre-cooled in liquid nitrogen. The specimen was then fractured under liquid nitrogen with a sharp razor blade, the brass block transferred quickly to the Edwards 306 Coater, and the fractured surface was coated as per the surface replica technique. Cleaning, washing and mounting of the replica (but on formvar coated grids) was as per the surface replica method.

All replicas were viewed with an AE1 801A transmission electron microscope.

(3) Scanning Electron Microscopy:

Colonies growing on defined medium were flooded with 2.5% glutaraldehyde (pH7.4) and fixed for 4h. Small blocks of agar of various regions of the mycelium were cut out and dehydrated through a graded series of acetone, the final stage being in 100% acetone (two changes) for 24h. The samples were then placed in a Polaron E3000 Critical Point Drying apparatus and dried using liquid carbon dioxide. They were then mounted on scanning microscope stubs by means of double-sided Sellotape, coated in a Polaron E5000

Sputter Coater, and examined in a Cambridge Stereoscan S600 operated at 25Kv.

RESULTS AND DISCUSSION

Syncephalastrum racemosum can be classified as follows (Webster, 1970):

DIVISION	-	EUMYCOTA
SUB-DIVISION	-	ZYGOMYCOTINA
CLASS	-	ZYGOMYCETES
ORDER	-	MUCORALES
FAMILY	-	PIPTOCEPHALIDACEAE
GENERA	-	SYNCEPHALASTRUM
SPECIES	-	Syncephalastrum racemosum

Filamentous fungi, including S.racemosum, grow by hyphal tip extension. Branching occurs in growing hyphae (Fig.1) and leads to the formation of a mycelial mat of interlaced hyphae, having smooth outer walls (Fig.2). Branching does not occur regularly at the tips, a situation which would lead to a dichotomous type of growth. In colonies of S.racemosum, the mycelium is an interwoven net of criss-crossing hyphae (Figs.1-3). New hyphae are formed by lateral branching, where the growing hyphae give rise to side-branches at some distance back from the tip. This new hypha can have branches developing from it, these in turn having side branches as well (Fig.1).

Closer examination of the surface replicas of hyphal tips revealed that just at the apex, small pock marks were discernable (Fig.3). These were more clearly seen in higher magnification as being small depressions or craters just around the tip (Fig.4). It is possible that these depressions are external indications of the sites of vesicle addition, which have been shown by Grove, Bracker and Morre (1970b) to be involved

in hyphal tip extension. In their model, material from the endoplasmic reticulum is transferred to the dictyosome or its equivalent by blebbing of the reticulum to form vesicles. These vesicles fuse with the proximal pole of the dictyosome cisternae, where the material is probably biochemically modified. The transformed lumen material passes to the distal pole by the continued formation of new cisternae. Then, new vesicles are formed, variable in size, for they may fuse with each other. These secretory vesicles move to the hyphal tip where fusion occurs with the plasmamembrane and the vesicle contents are liberated into the growing wall region. The aggregation of vesicles is revealed by thin sections of hyphal tips, three types of vesicle organisation having been identified (Grove & Bracker, 1970a). Investigation of tip growth was not the aim of this thesis. It would, however, be interesting to determine the vesicle distribution in S.racemosum hyphal apices. Also, it may be possible that their number could be correlated with the number of pock marks seen in surface replicas.

A possible way of examining the pock marks further would be by critically point drying hyphal material prior to surface replicating. This should be done, for the pock marks may be due to material drying out in the coating chamber and collapsing, the tips being the most obvious candidates for where this could occur. Bracker (1971) pointed out that the preparation of hyphal tips for electron microscopy must be done carefully, because growing hyphae were very easily disturbed and their ultrastructure disrupted by pre-treatments. It does seem possible however, that these tip depressions are real indications of the external appearance of hyphal apices.

As hyphal tip extension proceeds, another form of development is initiated a little way back. This is the process of asexual spore formation (SPORULATION). Hyphae grow upwards from the mycelial mat. These aerial hyphae (SPORANGIOPHORES) elongate and form a swelling at the tip, the sporangium or ampulla. Figure 5 shows the swollen sporangium, and the sac-like protrusions or merosporangial initials can be seen. The merosporangia develop further by becoming longer (Fig.6). In Figure 7, further extension of the sacs is seen. Also, on some of the merosporangia the base of the sac becomes smaller in diameter than the rest of the structure (Fig.7). This correlates with the observations of Fletcher (1972) that the merosporangia appeared as wide tubular structures connected to the ampulla by a narrow neck. The tubular structure of the merosporangia is evident in Figure 8 and also in Figure 9, where the sacs are fully developed. It is within these merosporangia that the spores are formed as observed by Fletcher (1972).

In Figures 5-7 and 9, the aerial hyphae and the ampulla appear to be covered by crystal-like deposits. Surface replicas of the sporangium show these structures more clearly (Fig.10). Higher magnifications of part of the ampulla reveal crystal-like deposits of a rectangular shape (Fig.11). Small circular structures are also noticeable in these micrographs. These are probably the sites of attachment of the merosporangia to the ampulla prior to dispersal of the spores. These small openings are in keeping with Fletcher's (1972) finding of the sacs being attached to the sporangium by a narrow neck (cf. Fig.7). Crystal-like deposits are also seen in surface replicas of the sporangiophores (Fig.12).

These are still rectangular in shape, though smaller than the structures found on the sporangium surface (Fig.11). The arrangement of these crystals on the surface of sporulating structures does not seem to follow any set pattern.

Jones, McHardy and Wilson (1976) stated that sporangia, and sometimes the hyphae and sporangiospores, of many species in the Mucorales have spines on their outer surfaces. They studied the chemical composition of the spines of Mucor sporangiospores. There were no earlier reports of detailed analysis of these structures, except indications that they might be calcium oxalate. Therefore, Jones and his colleagues (1976) collected the spines from ultrasonically treated spore suspensions and subjected them to micro-analytical examination. X-ray spectrometer results showed that calcium was the main constituent in the spines of Mucor plumbeus, Mucor hiemalis and Cunninghamella echinulata. X-ray powder diagrams of isolated spines of M.plumbeus detected the presence of calcium oxalate dihydrate. These workers did not speculate on the function of these spines or crystals.

Young (1970b) also noted that spicules of varying form are of common occurrence on the walls of sporangiophores of the Mucorales. In Linderina pennispora, the upper part of the outer wall layer of the sporangiophore was covered with spicules, being circular or rectangular in shape and varying in size. These could be removed partly by beating with glass beads (Young, 1970b). The long thin spines on the spores of Mucor plumbeus were also seen

by Young (1968a), with similar structures on Cunninghamella elegans conidia. Spirodactylon aureum and Spiromyces minutus, both members of the Kickxellaceae family, had spores heavily covered with prominent spines. No regular arrangement of the spines was noticeable in S.minutus. In S.aureum, the spines were regularly arranged forming helices on the spore wall (Young, 1968b). Other members of the Kickxellaceae also showed patterns of surface projections on their spores. Small nipple-like protuberances, running as bands roughly transverse to the long axis of the spores, could be seen on the spore surfaces of Coemansia spirila, Coemansia mojavensis, Linderina pennispora, Linderina macrospora and Martensiomyces pterosporus (Young, 1968b). Similar structures were observable on the spore surface of Coemansia aciculifera(Young, 1973), with the transverse bands becoming less regular in appearance towards the proximal region (the point of spore attachment). Young (1969) observed that spores of Piptocephalis benjaminimni were covered with numerous circular spines with blunt heads. In Piptocephalis virginiana the spines also appeared flat-topped and were randomly arranged, but looked more like wart-like projections or ridges. This was also the case in Piptocephalis unispora, where numerous wart-like projections were roughly spherical in surface view, some being rectangular. In side view, the wall spines tended to have flat, capitate spines (Young, 1969), a similar picture having been drawn up by Jeffries and Young (1975). The crystal-like deposits of sporangiophores of S.racemosum, although appearing rectangular in surface view, could be seen as small bumps

with fairly rounded tops in side view (Fig.12). The structures on the sporangium were flat on top (Fig.11). The exact nature and role of these spines/bumps/projections is not known, nor are they restricted to just the Mucorales. Corlett (1970) showed that urediniospores of Puccinia coronata, when viewed with the scanning electron microscope, revealed spines of about 0.75 μm in height on the spore surface. The tips of the spines, although tapering to a point, were rounded off. The author put forward the view that such surface ornamentation as spines or warts on urediniospores or teliospores could possibly be of taxonomic value in identification of rust fungi. It would be interesting to see if this principle could be applied to other fungal groups, such as, for example, the Mucorales.

Fletcher (1972) noted that the spores were formed as chains within the merosporangial swelling. The spores were mainly rectangular in shape, although the tip spore, furthest from the point of attachment to the ampulla, was rounded at one end. Figure 13 presents a surface replica micrograph of mature spores. This rectangular shape except for the end spore is clearly seen. The terminal spore has one rounded end (Fig. 13). A further micrograph of this rarer shaped spore is presented in Figure 14. The appearance of the spore is probably governed by the way in which the cleavage furrows delimit the merosporangial protoplasm into spore initials (Fletcher, 1972). A greater diversity of spore shape is seen in Figure 15. Not only are rectangular spores seen in the micrograph, but also spherical spores with or without depressions in the centre. This central depression can be

observed more clearly in Figure 16. The two rectangular spores on the right are partnered by a circular spore on the left. It has a depression which seems to end in a small area devoid of any of the surface ornamentation seen on the neighbouring spores. Young (1968a) had earlier reported that replicas of S.racemosum spores showed that their surfaces were delicately wrinkled. Figure 16 indicates that this is not so. A distinct surface ornamentation is visible. The spore on the right of the picture (Fig.16) has a circular end with a central depression, similar to that of the circular spores. It indicates that S.racemosum spores are rather cylindrical in shape, the depression probably being the point at which the spore was delimited from another during cleavage in the merosporangia.

As in Figure 16, the spore surface is seen to have a rodlet pattern (Fig.17) which is more clearly seen in Figure 18. Figure 18 is a freeze-fracture of the spore revealing a surface layer arranged in a criss-cross pattern of small groups of rodlets having a periodicity of 25nm. Rodlets have also been observed on the surfaces of conidia of various Aspergillus and Penicillium species (Hess, Sassen & Remsen, 1968; Hess & Stocks, 1969). The pattern and number of rodlets varied from species to species. Penicillium megasporum spores were covered with small rodlets of varying length (5 - 500nm), but with no real structural organisation (Sassen, Remsen & Hess, 1967). Conidia of Aspergillus fumigatus, however, had a criss-cross pattern of surface rodlets of diameter of approximately 10nm (Ghiorse & Edwards, 1973). The conidia in

Aspergilli are attached to phialides (the mother cell at the base of the spore chain). The phialides also have surface rodlets of the same diameter, but their pattern of distribution was different. The criss-cross patterned network was not so ordered or sharply delimited as on the spore surfaces. Ghiorse and Edwards (1973) reported that similar rodlets as on the phialides were found on the conidiophores (aerial hyphae), which is very interesting in relation to Figures 11 and 12. These figures show a layer of surface rodlets on both the sporangiophore (Fig. 12) and sporangium (Fig.11). The criss-cross pattern seen on spore surfaces is here less distinctive. Also the rodlets appear longer in length. Aerial hyphae have a totally different surface to that of the vegetative hyphae of the mycelial mat (Figs. 2-4). No crystal-like deposits are present and surface rodlets are absent in vegetative hyphae (Figs. 19 and 20). Observing these differences in outer wall structure of the hyphae raises the question as to whether such differences exist in other fungi. This has further relevance in studies of the gross chemical composition of hyphal walls, for these studies often include both vegetative and aerial hyphae of differing ages. Bartnicki-Garcia and Reyes (1964) noted differences in gross chemical composition between the walls of sporangiospores, hyphae and the yeast-like phase of Mucor rouxii. The spores had as their main component glucan, together with melanin, glucosamine, protein and mannan. Both the hyphae and the yeast-like phase had chitosan as their main component, but differed

from each other in the amount of the remaining constituents:- phosphate, chitin, protein, mannan, galactose and fucose. Thus differences in gross chemical composition can be detected in the various stages of the M.rouxii life-cycle (Bartnicki-Garcia & Reyes, 1964). It is probable, therefore, that two similar cell types, serving different aspects of development, will possess different wall polymers. These findings on the pronounced differences in surface structure between vegetative hyphae and sporangiophores of S.racemosum emphasises the ultrastructural and biochemical variations that can occur between cells of apparently similar appearance. Gross chemical analysis of populations of varying cell type and age may well represent a simplified view of fungal cell differentiation.

S.racemosum spores are dispersed when the merosporangium becomes detached from the ampulla by wind (Webster, 1970). Afterwards, all that is left is an empty bag (Fig. 21). The outer wall of the merosporangial sac is smooth, although closer examination reveals the possible presence of some rodlets on the surface (Fig.21). This is quite probable, for Fletcher (1972) observed that as merosporangial development continued, an outer membranous surface layer, continuous with the outer ampulla wall, was laid down. The ampulla surface has a rodlet layer (Fig.11), and it is postulated in Chapter Five that this outer surface rodlet layer is the same as the outer (membranous) electron opaque layer of thin sectioned dormant spores.

Under favourable conditions when nutrients are available, the spores germinate. At first, they swell (Fig.22), and finally a

new germ tube emerges (Fig. 23). Bartnicki-Garcia (1968a) had put forward the thesis that upon germination a new wall was synthesised within the existing spore walls, this becoming the new germ tube wall. Alternately, the germ tube wall was a continuation of one or more of the existing spore wall layers. In Figure 23 a possible break-point between spore wall and germ tube wall can be observed. The break-point may represent a splitting or breaking up of the surface layer, perhaps together with one or more underlying wall layers. As the germ tube grows longer by hyphal tip extension, lateral branching occurs again, the mycelial mat is formed and the life-cycle progresses.

CHAPTER TWO

THE PHYSIOLOGY AND INFLUENCE OF GLUCOSE AVAILABILITY ON EVENTS DURING GERMINATION.

INTRODUCTION

Light microscopy of germinating spores usually reveals two gross morphological processes: spore swelling and germ tube emergence. Recent research has shown that a complex series of biochemical and ultrastructural events occur throughout germination. Cell wall growth, RNA, DNA and protein synthesis are initiated or continue during the swelling phase (Weber & Hess, 1976). Van Etten, Bulla and St. Julian (1974) divided the germination process in Rhizopus stolonifer into four morphologically definable stages:- (1) unswollen spores, (2) swollen spores, (3) the beginning of germ tube formation, (4) fully extended germ tube. Germ tube outgrowth began about 3.5h after inoculation of the R.stolonifer spores into germination medium. Dry weight began to increase noticeably around this time. However, oxygen uptake increased approximately 50% in the first 30 minutes, and thereafter continued to increase linearly until it had increased 10 to 20-fold after 4h. Labelled leucine and uracil were incorporated into protein and RNA within the first 30 minutes of germination (Van Etten et al, 1974). Therefore, in R.stolonifer, the swelling phase of the spores is accompanied by various metabolic activities involving oxygen uptake, protein and RNA synthesis. Sporangiospores of Rhizopus arrhizus required the presence of glucose together with a nitrogen source, phosphate, and either sodium or potassum ions for maximal swelling (Ekundayo & Carlile, 1964). If spores were transferred to a glucose-free medium, swelling soon ceased. Marchant and White (1966) demonstrated that the presence of both

carbon and nitrogen sources was necessary for swelling of Fusarium culmorum macroconidia. A similar requirement was observed in spores of Penicillium atrovenetum (Gottlieb & Tripathi, 1968). If glucose was removed from the medium, only 2% of the population swelled. Gottlieb and Tripathi (1966) also found that oxygen was essential for swelling of P.atrovenetum spores as well as for the formation of germ tubes. Under aerobic conditions in glucose Czapek-Dox medium, 85% of the spores had swollen after 8h. When spores were incubated under anaerobic conditions, only 12% had swollen. Observations after 14h revealed that no further increase in swelling had occurred, and that only swollen spores produced germ tubes. Ekundayo & Carlile (1964) reported that in Rhizopus arrhizus, spores under aerobic conditions swelled to about 13μm. in diameter and about 90% of them had produced germ tubes. Under anaerobic conditions, spore swelling was to about 8 μm and only about 10% formed germ tubes.

Respiratory inhibitors showed a marked effect on spore swelling. Gottlieb and Tripathi (1968) treated swelling spores of Penicillium atrovenetum with various metablic inhibitors. Potassium fluoride decreased respiration by 66% and swelling by 85%. Sodium malonate inhibited respiration by 73% and swelling by 82%. This inhibition could be reversed by adding an excess of sodium succinate, thus indicating the need for the TCA cycle during spore swelling. Sodium azide, an inhibitor of cytochrome oxidase activity in the terminal electron transport system, reduced respiration by 83% and swelling by 94%. 2-4, dinitrophenol, an uncoupler of oxidative phosphorylation, reduced respiration by 87% and swelling by 100%.

Protein synthesis is closely related to swelling. Inhibitors of protein synthesis, (cyclohexamide, puromycin, blasticidin S), prevented swelling of P.atrovenetum spores (Gottlieb & Tripathi, 1968). Trichoderma lignorum was more sensitive to cyclohexamide inhibition than Penicillium notatum spores (Martin & Nicolas, 1970). 50 $\mu g.ml^{-1}$ completely inhibited swelling and germ tube formation in T.lignorum, whilst in P.notatum swelling was inhibited by only 30%. There was, however, no germ tube formation. Sodium azide inhibited swelling and germ tube outgrowth in both fungi. Carbon and nitrogen sources were also necessary for spore swelling and outgrowth of germ tubes in T.lignorum and P.notatum (Martin & Nicolas, 1970).

Swelling is dependant upon the presence of both carbon and nitrogen sources. If one or the other or both are absent, swelling is markedly reduced (Ekundayo & Carlile, 1964; Marchant & White, 1966; Gottlieb & Tripathi, 1968; Martin & Nicolas, 1970). Oxygen is necessary for swelling (Ekundayo & Carlile, 1964; Gottlieb & Tripathi, 1968), and this points to respiration being important to the spore at this stage of its development. This is borne out by the effect of respiration inhibitors. Spore swelling and germ tube outgrowth is reduced by TCA cycle, electron transport and oxidative phosphorylation inhibitors (Gottlieb & Tripathi, 1968; Martin & Nicolas, 1970). The normal functioning of the EMP pathway is also necessary (Gottlieb & Tripathi, 1968). It is therefore most probable that for spore swelling to proceed normally, there is a requirement for the production of energy (via the TCA

cycle) by the catabolism of glucose (via EMP) (Gottlieb & Tripathi, 1968). Swelling, which also involves the uptake of water, is an active process involving metabolic activities important for germination to occur.

MATERIALS AND METHODS

GROWTH MEDIUM

The laboratory strain of Syncephalastrum racemosum was grown on the following defined medium ($g.L^{-1}$): glucose, 10; $(NH_4)_2 SO_4$, 6; $Na_2 HPO_4 . 2H_2O$, 11.5; $KH_2 PO_4$, 9.3; EDTA, 0.6; $Mg SO_4 . 7H_2O$, 0.25; $CaCl_2$, 0.05; $Zn SO_4 . 7H_2O$, 0.02; $Cu SO_4 . 5H_2O$, 0.005; $Fe SO_4 . 7H_2O$, 0.1; $Na_2 SO_4$, 0.5; $Na_2 MoO_4 . 2H_2O$, 0.005. The phosphates with ammonium sulphate and the chelated trace elements were autoclaved separately at 121°C for 15 mins. Glucose was autoclaved at 115°C for 10 mins. The alternative carbon sources were prepared in the same manner as glucose. Solid medium was produced by adding 15g of agar to 1L of medium.

SPORE PRODUCTION

The fungus was grown on 100ml. solid defined medium in Roux bottles at 37°C for 7 days and then stored at 25°C until required.

GERMINATION STUDIES

Spores were approximately 3 weeks old when harvested. They were washed out of two Roux bottles, containing sporulated mycelium, with sterile distilled water and filtered through four layers of sterile muslin to remove hyphal debris. The spore concentration of the filtrate was determined using a Fuchs-Rosenthal haemocytometer. Spores were then centrifuged at 2800g for 2 mins, washed once with distilled water, centrifuged again and resuspended to a known concentration in sterile distilled

water. Germination experiments were done using 25ml. defined medium in 250ml. Erlenmeyer flasks on an orbital shaker at 185 rev. $min.^{-1}$. Unless otherwise stated, the final spore concentration was 1.26×10^6 spores. ml^{-1} culture medium. Some germination experiments were carried out in a batch fermentor consisting of a 1L Quickfit pot containing 500ml. medium (Fig. 24). The pot was fitted with inoculation and sampling ports, sterile air inlet and outlet ports with air flow control. The aeration rate was $300ml.min^{-1}$. The medium was agitated by a propeller shaft connected to an overhead electric motor. Samples (0.4ml. from the flasks; 5.0ml. from the fermentor) were examined microscopically to assess germination. Spores were considered germinated if the length of their germ tube was at least half their diameter; percentage germination counts were calculated from observations of 200 spores. Spore diameters were measured on the screen of a Reichert Visopan microscope (Reichert-Jung, Slough, Buckinghamshire) using a calibrated ruler. Average spore diameters were calculated from measurements of 50 spores. Calculations of size distributions were also made from this group of 50 spores.

SHIFT EXPERIMENTS

To transfer spores from glucose medium to glucose-free medium, shake flask cultures were filtered through Millipore filters (pore size, 0.45μm; diameter, 47mm), washed twice with 12.5ml. sterile glucose-free medium and resuspended in the appropriate volume

of fresh glucose-free medium. For transfers from glucose-free medium to glucose medium, spores were filtered, washed twice with 12.5ml. sterile glucose medium and resuspended in the appropriate volume of fresh glucose medium. The final resuspension medium and washing medium were prewarmed to 37°C and the whole procedure was carried out as swiftly as possible in an incubation room maintained at 37°C.

GERMINATION EVENTS

Freshly harvested spores of S.racemosum had a yellow refractile appearance when viewed by phase contrast microscopy. This refractility was lost and phase darkening occurred upon swelling of the spores. After germ tube formation, spores started to clump forming aggregates which gradually became visible to the naked eye. This aggregation made counting of germ tubes more difficult at later stages. It was found that brief sonication in a Millipore ultrasonic cleaning bath disaggregated spore clumps without breaking the cells. At about 12h, clumping of spores with long germ tubes became so extensive that counting was no longer feasible. In this study, the term "germination" is used to describe all the events from initiation to outgrowth of the germ tube.

PHASE CONTRAST MICROSCOPY

Dormant, swollen and germinated spores were observed in a Zeiss Universal microscope, photographs being taken with a C35M camera.

RESULTS

LIGHT MICROSCOPIC OBSERVATIONS OF GERMINATION

When spores of Syncephalastrum racemosum were placed in defined medium, the dormant spores (Fig.25) at first began to swell (Fig.26). During this time, the refractility of the spores was lost and phase darkening occurred. Eventually, a switch was made from a non-polar to a polarised mode of growth with the subsequent emergence of a young germ tube (Fig. 27). The germ tube developed by polarised tip growth (Fig. 28) to give rise to the new vegetative mycelium.

COLONY RADIAL GROWTH RATE

Agar plates, inoculated with a small centrally placed drop of spore suspension, were incubated at various temperatures (19°C, 25°C, 30°C, 37°C, 44°C) in order to determine the best temperature for growth of S.racemosum. As can be seen from Figure 29, the best growth($530\,\mu m.h^{-1}$) was at 37°C. This temperature was used for all subsequent germination studies.

VARIATION OF CARBON SOURCE

The carbon source of the defined medium was varied to determine the range of compounds that would support outgrowth of the germ tube. Succinate and citrate did so very poorly (Fig.30), whilst sucrose and fructose gave higher rates of outgrowth (16% and 46% of spores having germ tubes at 11h respectively). The best rates obtained in this experiment were with galactose and glucose (Fig.30). Glucose was chosen as the carbon source for all subsequent experiments.

Although succinate and citrate were very poor carbon sources, glucose-containing defined medium supplemented with either succinate or citrate (at concentrations of 0.01M) had significantly higher rates of outgrowth at 12h (Fig.31); glucose containing medium, 76%; supplemented with succinate, 90%; with citrate, 88%.

GERMINATION SEQUENCE

Spore swelling, percentage germ tube formation and dry weight data for germination in a batch fermenter system are presented in Figure 32. Spores began to swell soon after inoculation into the defined medium and the increase in spore diameter was linear for the first 6.5h of germination, the overall increase in size being from 3.4μm to 7.6μm. Outgrowth of germ tubes was first noticed at 3.5h and reached a maximum (92%) by 10h. Dry weight of the spores increased noticeably just after the emergence of the first germ tubes.

The range of spore diameters within this population is presented in Figure 33 and indicates that all the spores within the culture increase their size during germination. There is no overlap in the size distribution of the spore population at 0h and at 11.25h.

GLUCOSE DEPRIVATION

If glucose was omitted from the medium, outgrowth of germ tubes did not occur (Fig. 34). Spores deprived of the carbon source also did not swell (Fig. 34) or undergo phase darkening, indicating that glucose was required even in those early pre-outgrowth stages of germination.

GLUCOSE CONCENTRATION

Varying the final glucose concentration of the defined medium affected the rate of germ tube outgrowth and consequently the percentage of the population with germ tubes at 12h (Fig. 35). Increasing the glucose concentration to around 1% (W/V) also caused an increase in the numbers of spores with germ tubes in the population. Further increases in the glucose concentration above 1% (W/V) had little effect on the level of germ tube outgrowth.

SHIFT-DOWN TO NON-PERMISSIVE CONDITIONS

Knowing that glucose was essential to ensure normal development of germinating spores (Fig.34), the effects of shifting a culture from glucose medium to glucose-free medium at various points in the germination sequence was studied. Spore were transferred to glucose-free medium at hourly intervals after inoculation into glucose medium. Average spore diameters and germ tube production were monitored before and after the shift. Figure 36 shows the effect of the shift down to non-permissive conditions on spore swelling. In this experiment, cultures were transferred at hourly intervals up to 6h after inoculation. In all these cases the shift-down caused a rapid cessation in swelling. Figure 36 shows that the average spore diameter of the population one hour after transfer was significantly lower than that expected if glucose had not been removed.

A compilation of the results of these experiments is given in Figure 37, where the average spore diameter of the population at 12h is plotted against the time spent in the

glucose medium. It can be seen that the longer the period spent in the glucose medium before shift-down, the larger the final average spore diameter. This curve parallels the data for the increase in spore diameter in a normal control culture (Fig.37). Similar results were obtained for the percentage of the population with germ tubes at 12h (Fig.38). The longer the period spent in glucose medium, the higher the number of spores with germ tubes in the population at 12h. Spore populations which had been shifted down after 1, 2, or 3h, times at which germ tube outgrowth had not started in the control cultures, did produce some germ tubes (Fig.38) even though glucose had been removed and swelling had ceased prematurely (Fig. 36).

Figure 39 shows the range of spore diameters within a population of germinating spores at different times. It shows, as in Figure 33, that all the spores within the culture increase their size during germination. As germ tube formation begins it is the larger members of the spore population which produce the first germ tubes (Fig. 39B). The histogram for the spores with germ tubes in Figure 39B (4.75h after inoculation) occupies the larger diameter classes of the histogram of the total spore population. Eventually, as spore swelling and germ tube outgrowth increase and reach their maximum values, these two size distributions merge (Fig. 39C).

Size distributions were assessed at 12h for spore populations which had been in glucose medium and then shifted down at various times to glucose-free medium. These analyses showed that a population contained more spores in the larger diameter classes the longer the

initial period spent in glucose medium (Fig. 40). Again, the spores with germ tubes were the larger spores of the population (Figs. 40A,B). Spores which had been in glucose medium for only 1h and then shifted down still managed to produce some germ tubes (8.5% at 12h). Figure 40A shows that in this extreme population it was the larger spores which tended to produce the germ tubes.

SHIFT-DOWN/SHIFT-UP

The effect on spores of a shift-down to non-permissive germination conditions followed, some time later, by a shift-up back to glucose medium was examined. Spores were inoculated into glucose medium and then shifted down to glucose-free medium after 3 or 6h; 24h after the shift-down cultures were transferred back to glucose medium. The number of spores producing germ tubes was monitored throughout the experiment and the results are presented in Figure 41. Removal of glucose at 3 or 6h stopped germ tube development. The shift-up to glucose medium 24h later resulted in an increase in germ tube outgrowth after a lag period. This lag was about 4h for the culture which had been shifted down at 3h and about 3h for the culture shifted down at 6h. Spores which had been incubated in glucose-free medium for 24h and were then shifted up to glucose medium, showed a lag of about 3.5 to 4h before a near normal sequence of germ tube outgrowth started in the population. Microscopic examination of the cultures undergoing the shift-down/shift-up protocol suggested that spores which had already formed germ tubes before glucose deprivation did not develop further when glucose was again available after the shift-up.

DISCUSSION

Spore germination in Syncephalastrum racemosum follows a developmental sequence of spherical spore swelling, localised outgrowth of the germ tube, and the subsequent polarised growth of the new hypha. Germ tube outgrowth is first observed in some spores around 3 to 4h after inoculation into the nutrient medium. The number of spores with germ tubes then increases to a plateau of around 90% at 10 to 12h. Aggregation of germinated spores makes estimations of the number of spores with germ tubes rather difficult in these later stages. Spore swelling precedes the emergence of germ tubes, whilst the dry weight appears to increase markedly only at the time of germ tube emergence. This pattern of germination and dry weight increase is similar to that observed by Van Etten, Bulla and St. Julian (1974) in sporangiospores of Rhizopus stolonifer. In this fungus, germ tube outgrowth was first observed after 3 to 3.5h. It reached a value of over 80% by 6h. A much quicker rate of germination then in S.racemosum. Dry weight also began to increase noticeably around the time of germ tube emergence (Van Etten et al, 1974).

The results show that the presence of glucose is essential both for spore swelling and germ tube emergence in S.racemosum sporangiospores. If glucose is absent from the incubation medium neither of these two morphological processes is initiated (Fig.34). Incubation in a glucose-free medium does not appear to affect viability and spores will germinate almost as normal if glucose is added subsequently (Fig.41). The fact that the normal lag period of 3 to 4h is present before such spores produce germ tubes implies that glucose is required at the very start of the germination

sequence. The shift-down experiments from a glucose-containing medium to a glucose-free medium in the first few hours of incubation, show that swelling is particularly glucose dependant (Fig. 36). In fact, glucose is essential throughout the first 10h for the spore population to achieve the normal size before germ tube formation.

The shape of the normal germination curve and of the shift experiments show that there is a variation in the time taken for an individual spore within the population to produce a germ tube (Figs. 37 and 38). Brief exposure to glucose during the first few hours of incubation followed by removal of the glucose did result in some germ tube outgrowth at 12h. It is, however, the larger members of these populations which produce the germ tubes even though they are smaller than the normal size at germ tube emergence (Figs. 37 and 40). The results with S.racemosum indicate that complete spore swelling is not a prerequisite for outgrowth of the germ tubes. Similar results were obtained with another Phycomycete, Rhizopus arrhizus (Ekundayo & Carlile, 1964). Sporangiospores of R.arrhizus, after inoculation onto solid defined medium containing glucose, were transferred at hourly intervals to water agar. The spores had initially been plated on-to cellophane covering the medium, hence making transfer a simple procedure. Normal germination on glucose medium occurred after about 4h. Spores transferred after only 1, 2, or 3h on glucose medium still managed to germinate (80%, 100% and 100% respectively at 8h). However, Ekundayo and Carlile (1964) found that swelling in these cases had only increased by about another 1μm by 8h. Normally, it would have increased another 6.5 to 8μm.

Varying the concentration of glucose in the incubation medium produced variations in the rates of germ tube emergence (Fig. 35). Gottlieb and Tripathi (1968) obtained a similar graph with germinating spores of Penicillium atrovenetum. Increasing the glucose concentration not only increased the percentage of spores within the population with germ tubes, but also increased the swelling (spore diameter) of the spores. The optimum glucose concentration for maximal swelling and germ tube production in P.atrovenetum was between 4 and 5mg.ml^{-1}.

Analysis of size distributions of partially germinated spore populations, scoring sizes of all spores and sizes of only those with germ tubes, indicated that the fully germinated spores occupy the larger size classes in the histograms (Fig. 39). Such a variability within the population is analogous to that found for Schizosaccharomyces pombe (Padilla, Carter & Mitchison, 1975). Spores of this fission yeast were separated into distinct size classes by zonal centrifugation and it was found that the duration of the pre-germination interval was size dependant. Large spores (approximately 50 μm^3) germinated up to 6h before the smaller ones (approximately 11μm^3). In future studies, where synchrony of germ tube emergence is a priority, it may be useful to separate size classes of fungal spores before inoculation into nutrient medium.

CHAPTER THREE

IDENTIFICATION OF A SELF-INHIBITOR OF GERMINATION

INTRODUCTION

The preceding Chapter dealt with some basic aspects of spore germination in Syncephalastrum racemosum. In all experiments the same concentration of spores was used throughout (1.26×10^6 spores. ml^{-1}). However, it was noticed that higher spore concentrations gave lower percentages of spores with germ tubes. Many fungi germinate poorly or not at all at high spore inocula concentrations (Cochrane, 1974; Macko, Staples, Yaniv and Granados, 1976). This effect is known as CROWDING and is suggestive of the presence of an inhibitory substance which is interfering with the normal germination sequence. A dilution of the spore suspension or washing of the spores removes the inhibitor and allows germination to proceed (Cochrane, 1974). These inhibitory substances are thought to be formed by the fungus at around the time of sporulation (Macko et al, 1976). The phenomenon of fungi preventing their own germination by the production of inhibitory substances is termed SELF-INHIBITION. Self-inhibition has been reported to occur in many fungi and has been the subject of several reviews by Allen (1965), Cochrane (1974), Sussman and Douthit (1973), and recently by Macko et al (1976).

Nevertheless, the crowding effect alone is not sufficient evidence that self-inhibition is occurring (Macko et al, 1976). The inhibitory substance may well be present, but other factors may influence the reluctance of the spore to germinate. An increase in the nutrient concentration or in the concentrations of oxygen or carbon dioxide may be required at higher spore concentrations, because of competition for important substances among the crowded spores. Arthrospores of Geotrichum candidum germinate in the absence

of exogenous nutrients at low spore concentrations, but at high spore concentrations show self-inhibition (Park & Robinson, 1970). Spores at low concentrations germinated in distilled water just as well as similar populations did in the nutrient growth medium. Additions of nutrients to distilled water cultures with high spore concentrations increased germination showing that the nutrients were able to overcome the block imposed by the presence of a self-inhibitor (Park & Robinson, 1970). Self-inhibition was also demonstrated in Aspergillus nidulans (Trinci & Whittaker, 1968). As the spore concentration was increased, so the percentage of conidial germination dropped off (1.57×10^5 conidia.ml^{-1} = 84% germination; 1×10^7 conidia.ml^{-1} = 44% germination). On aerating spore suspensions with air containing carbon dioxide, the germination of a culture containing 1×10^7 conidia.ml^{-1} had increased to 75%. The result suggested that self-inhibition at higher concentrations may be due to an insufficient supply of carbon dioxide during the early stages of germination (Trinci & Whittaker, 1968). Therefore, as in G. candidum, an increase in nutrient supply can overcome the block to germination.

Nutrient addition, however, does not always overcome the block to germination imposed by the self-inhibitor. Lingappa and Lingappa (1966), working with conidia of Glomerella cingulata, observed that at a concentration of 1×10^9 spores.ml^{-1} less than 1% of the conidia developed germ tubes. At concentrations of 1×10^5 spores.ml^{-1}, 85% of the spore population had germ tubes, even though the same nutrient conditions were present. Thus, the

inhibition of germination is due solely to the presence of some inhibitory substance. Subsequent experiments showed that protein synthesis (measured by noting the incorporation of radioactive reconstituted algal protein hydrolysate into germinating conidia) was 5 - 6 times greater in cultures with concentrations of 7.2×10^6 spores.ml^{-1} than in cultures where the density was 7.2×10^7 spores.ml^{-1}. Protein synthesis was essential for germination, for addition of cyclohexamide (5 μg.ml^{-1}), an inhibitor of protein synthesis, prevented germination of conidia (Lingappa, Lingappa & Bell, 1973). These authors found that an assay disc containing a chloroform extract of the water washing of crowded cultures of G. cingulata, when placed in the centre of a petri dish seeded with conidia, had a clear zone around it in which no germination took place. In the control, mycelial growth occurred around the central disc. Therefore the inhibitor could be isolated from the water washings of the culture by shaking with chloroform. However, no attempt was made to identify the inhibitor. Germination was found to be a two-step process. Cultures with high spore concentrations were diluted at regular intervals with nutrient medium and the time taken for them to germinate noted. The longer the time spent at higher densities, the shorter the time taken for the spores to produce germ tubes after dilution. After 5h incubation at high concentrations, all subsequent dilution cultures took 2h to germinate. This was interpreted as suggesting that the two-step process had an initial step lasting 5h which was relatively unaffected by crowding. The terminal step of 2h was only entered if the conidia were diluted.

It was at this point that the inhibitor probably blocked protein synthesis, so affecting the germination sequence adversely (Lingappa et al, 1973). It should be pointed out that the only parameter these authors measured was that of protein synthesis. Therefore their observed effect could possibly not be a direct result of inhibition but just associated with it indirectly.

Self-inhibitors have been found in many fungi (Macko et al, 1976), one of which was Aspergillus nidulans. Scott, Alderson and Papworth (1972) observed that the resistance of A.nidulans conidia to U.V. irradiation during incubation in buffer decreased with time. They linked this with the diffusion out of the spores of a germination inhibitor. Scott and Alderson (1974) discovered later that conidia extracted from mycelium with ether water were sensitive to U.V. irradiation immediately, unlike spores extracted with Tween 80. Tween 80 extracted spores decreased in their resistance to U.V. irradiation until a plateau of surviving conidia (able to germinate upon plating on nutrient medium) was reached after 5h. With ether water extraction, the plateau was reached after 5 minutes of incubation in phosphate buffer. Therefore a better extraction of the self-inhibitor was achieved using a solution of diethylether (1% v/v) in distilled water.

The self-inhibitors have generally been suggested to be present in the spores. Nevertheless, the water washing of the mycelium to remove the spores and subsequent washings of the spores followed by extraction of the inhibitor from the washings, could indicate three points:- (1) the inhibitor is present in the spores, (2) the inhibitor is present in the mycelium or (3) in both. The inhibitor can be synthesised in the spore, or synthesised in the

mycelium and subsequently deposited in the spore. Carlile and Sellin (1963) observed that mycelial cultures of Botrytis cinerea overlaid with a thin strip of cellophane seeded with B.cinerea spores gave interesting results. Those spores on the piece of cellophane overlaid on the mycelium failed to germinate. Those overlaid on the medium did. The authors suggested that the mycelium of B.cinera produced a factor which inhibited the germination of its own conidia.

Garrett and Robinson (1969) found that culture filtrates of 11 day old cultures of Fusarium oxysporum inhibited spore germination not only in F.oxysporum but also in Cunninghamella elegans. Petroleum ether extracts of the culture filtrate revealed the presence of several fatty acids (or their methyl esters):- among them were octanoic, nonanoic, decanoic, tetradecanoic acids. In subsequent tests, only nonanoic acid (and also its potassium salt) was found to be inhibitory to spore germination in C.elegans. Garrett and Robinson (1969) also detected nonanoic acid in cultures of several fungi:- Mucor plumbeus, Rhizopus stolonifer, C.elegans, Chaetomium globosum, Aspergillus niger, Penicillium expansium and Geotrichum candidum.

For this reason, water washings of dormant spores of Syncephalastrum racemosum were subjected to extraction with diethylether and tested to see whether fatty acids, particularly nonanoic acid, were present. Chapter Three presents the results of this extraction, the discovery of nonanoic acid in the washings, and its effect on spore germination in S.racemosum.

MATERIALS AND METHODS

ORGANISM

The laboratory strain of Syncephalastrum racemosum.

SPORE PRODUCTION, SPORE HARVESTING, GERMINATION CONDITIONS AND EVENTS

As per materials and methods of Chapter Two.

PREPARATION OF NONANOIC ACID SOLUTIONS

Nonanoic Acid (Sigma Ltd.) was dissolved in acetone to give a standard solution of $1mg.ml^{-1}$. Appropriate serial dilutions were made with acetone to give stock solutions of which when 0.25ml. was placed in 25ml. of medium it would give the final required concentration of nonanoic acid in the culture. All controls had 0.25ml. of acetone added to them. The solutions were all prepared 1h prior to the requisite experiment being carried out.

EXTRACTION OF NONANOIC ACID

Dormant spores were washed 6 times prior to inoculation for two of the fermenter runs which provided the data for Figure 42. These water washings (266ml.) were acidified to pH2.0 with concentrated HCl (Garrett & Robinson, 1969; Shepherd & Mandryk,1962) and extracted five times (shaking for 4 min.each time) with diethylether. The ether extract (200ml.) was reduced by rotary evaporation to a volume of 1-2ml. The temperature of the water bath was $42^{\circ}C$. The solution left was yellowish-brown in colour and oily in appearance.

GAS CHROMATOGRAPHY

The yellowish-brown liquid after methylation was passed through a Pye Unicam Series 104 gas chromatograph using a

2.1mm. x 4mm . internal diameter coiled glass column packed with 3% (w/w) SE30 (methyl silicone gum) on 100-120 mesh Gas Chrom Q. The column and injection temperature were both 150°C. The nitrogen flow rate was 40ml.min^{-1}. The chart speed 1cm.min^{-1}. Nonanoic acid (Sigma Ltd.) was used as a standard.

RESULTS

CROWDING EFFECT

Figure 42 shows the results obtained from several fermenter pot runs with different spore concentrations. The lower the spore concentration, the higher the final percentage of spores with germ tubes. The germ tube percentage was taken 11h after inoculation, and the data is presented in Table 1. With a concentration of 8×10^5 spores.ml^{-1}, the percentage of spores with germ tubes was 94%; with a concentration of 2.2×10^6 spores.ml^{-1}, the percentage was 76%. However, spores that were washed, not twice, but six times with distilled water prior to inoculation (fermeter runs 6 and 7) had percentage values of 92% and 93% respectively (spore concentration = 1.5×10^6 spores.ml^{-1}). Twice washed spores of run 4 (1.46×10^6 spores.ml^{-1}) had a percentage germ tube emergence of 78%. A difference of 14-15% between washing the spores four times more in runs 6 and 7.

IDENTIFICATION OF NONANOIC ACID

The water washings of runs 6 and 7 were subjected to ether extraction and to Gas Liquid Chromatography (GLC) analysis to look for the presence of nonanoic acid. A standard solution of nonanoic acid was also passed through the GLC in the same manner as the water washings extract. Figure 43 presents the scan obtained. A standard of diethylether had a retention time (R_t), of 4.45 minutes (sample trace 3). The nonanoic acid standard had an R_t of 1.9 minutes (sample trace 2). The water washings sample (trace 1) had three peaks. One of R_t value of 4.45 minutes corresponding to diethylether, one unknown peak of R_t value 0.95

minutes, and one peak of R_t value 1.9 minutes. The last peak coincides exactly with the standard peak of nonanoic acid (R_t = 1.9 minutes). Therefore good evidence for the existence of nonanoic acid in cultures of Syncephalastrum racemosum.

EFFECT OF NONANOIC ACID UPON SPORE GERMINATION

Spores of S.racemosum were inoculated into flasks containing growth medium with different concentrations of nonanoic acid. The nonanoic acid concentrations ranged from 10-200$\mu g.ml^{-1}$. The control contained acetone (0.25ml.). The percentage of germ tube emergence and average spore diameters at various times was noted and the results are presented in Figures 44 and 45. Figure 44 shows that as the nonanoic acid concentration is increased, so the time at which germ tube emergence starts is delayed. That is, the lag period (the time from inoculation till the start of germ tube emergence) is lengthened by increasing concentrations of nonanoic acid. The percentage germ tube emergence after 13.5h decreases with increasing nonanoic acid concentration. Figure 46 shows that as the nonanoic acid concentration increases so the percentage of germ tube emergence decreases till a value of 0% is obtained for the nonanoic acid concentration of 200$\mu g.ml^{-1}$. This decrease in percentage germ tube emergence is a similar observation to that made in Figure 42, with the percentage decrease paralleling the increase, not in nonanoic acid concentrations, but spore density. A closer examination of Figure 44, however, suggests that the percentages at

13.5h, or even those at 16h are not the final values obtained by the various flask cultures containing nonanoic acid at different concentrations. It could be that the final values will all be higher, but whether they all reach the same level is not shown. Cultures with a concentration of nonanoic acid of 200$\mu g.ml^{-1}$ failed to produce germ tubes even after 36h incubation, suggesting that cultures with different nonanoic acid concentrations may reach different final values of percentage of germ tube emergence.

Figure 45 shows that nonanoic acid affects not only the time of appearance of the germ tube but also spore swelling. There is a lag or delay in the initial swelling of the spores with concentrations of nonanoic acid of 50 and 150$\mu g.ml^{-1}$. At 12h, the control culture and the culture with a concentration of nonanoic acid of 50$\mu g.ml^{-1}$ had average diameters of 7.5μm. Their respective percentage germ tube values were however, 75% for the control and 38% for the nonanoic acid (50$\mu g.ml^{-1}$) culture. The other nonanoic acid culture (150$\mu g.ml^{-1}$) had at 12h, an average diameter of 5.5μm and a percentage of germ tube emergence of 1%. The control culture had this average diameter of 5.5μm at 5.5h together with 6% germ tube emergence. At 16h, the 50$\mu g.ml^{-1}$ culture had spores of average diameter 8.0μm, larger than those obtained by the control after 13.5h (7.0μm) - at 16h clumping in the control made accurate counting of germ tube number impossible. The 150$\mu g.ml^{-1}$ culture at 16h had spores with an average diameter of 6.4μm and a percentage germ tube emergence of 26%. When the control had an average spore diameter of 6.4μm (at 7.75h), the percentage of

germ tube emergence was 24%. These results suggest that nonanoic acid slows down or retards germination and hence the effect on delayed swelling. It probably is involved with events leading to germ tube emergence as the time of germ tube emergence is delayed with increasing nonanoic acid concentrations.

STABILITY OF NONANOIC ACID IN CULTURE MEDIUM

Although nonanoic acid seems to affect the germination sequence in S.racemosum spores, its effect is overcome as germ tube emergence occurs after a lengthened lag period. This could be due to the spores slowly overcoming with time the inhibitory effect of nonanoic acid, or that nonanoic acid is broken down in the medium. To test this possibility, six flasks with culture medium were prepared. Numbers 1 - 4 were set up on Day 1 with either nonanoic acid ($200\mu g.ml^{-1}$) or acetone (control flasks 1 and 3). Flasks 1 and 2 were inoculated with spores on Day 1 and the percentage germ tube emergence followed. On Day 2, flasks 3 and 4 were inoculated. Fresh nonanoic acid and acetone were added to flasks 5 and 6, and these were inoculated as well. All three flasks with nonanoic acid did not produce germ tubes (Fig.47). Control flaks with acetone all produced germ tubes, albeit flask 5 had a delayed time of initial germ tube emergence. The result showed that nonanoic acid was not chemically broken down in the medium over at least a period of 24 - 36h, the duration of the previous experiment being up to 16h.

DISCUSSION

n-Nonanoic acid (sometimes called pelargonic acid) is a saturated fatty acid of formula $CH_3 (CH_2)_7 . COOH$. It is, like other saturated fatty acids of chain length below 10 carbon atoms, an oil at room temperature (Stanier, 1968). The yellowish-brown oil extracted by diethylether from water washings of both Syncephalastrum racemosum mycelia and dormant spores was tentatively identified as containing nonanoic acid by GLC analysis. Washing of the spores several times is sufficient to remove much of the inhibitor activity (Allen, 1965). In S.racemosum, six washes of the dormant spores with distilled water had the effect of increasing the percentage of germ tube spores in the population (Fig.42; Table 1). Scott, Alderson & Papworth (1972) mentioned that growth of conidial samples of Aspergillus niger depended upon whether the spores were collected dry or by flooding slopes, and how much washing the suspension received. Lingappa, Lingappa and Bell (1973) observed that washing spores of Glomerella cingulata four times prior to inoculation into germination medium made the conidia more active regarding protein synthesis than once washed conidia. They correlated protein synthesis in spores to the presence of some factor which was associated with the cell surface and remained at a high concentration when cells were kept at a high density. Probably a similar phenomenon occurs in spores of S.racemosum, in that, at high spore concentrations, the diffusion of nonanoic acid into the medium is possibly either fully or partially blocked. Therefore, either the spores do not enter the germination sequence (i.e. swelling and germ tube emergence), or if they do, then at a slower rate. This latter

point seems to be the case if nonanoic acid is added to the growth medium. For then the time of germ tube emergence is delayed (Fig.44) and the swelling rate reduced (Fig.45).

Germ tube emergence was also retarded in cultures of Cunninghamella elegans by the presence of volatile metabolites of Fusarium oxysporum (Robinson & Garrett, 1969). Germinating cultures contained either acetaldehyde or n-propanol. In acetaldehyde cultures, germ tube emergence was 3% after 3h, 25% after 12h and 58% after 24h. In n-propanol cultures, germ tube emergence was 0% after 3h, 12% after 12h and 54% after 12h. Control cultures had germ tube emergence values of 96% at 3h, 99% at 6h, and 100% at 12h. Further, the germination of sporangiospores of C.elegans was delayed for at least 24h in a solution of potassium nonanoate at a concentration equivalent to a 6ppm (v/v) solution of nonanoic acid.

If self-inhibitors are present in fungi, the question as to their location and mode of action must be raised. Lingappa et al (1973) suggested that as washing of the spores prior to inoculation increased the number of spores producing germ tubes, then the inhibitor may be associated with the cell surface of the spore. Carlile and Sellin (1963) demonstrated that the mycelium of Botrytis cinerea produced a factor which inhibited spore germination. Culture filtrates of Fusarium oxysporum were found to contain nonanoic acid, as were cultures of several other fungi (Garrett & Robinson, 1969). These studies do not really give any clues as to the location of the self-inhibitors. Are they in the spores or in the mycelium? The problem is that water washings of the culture may remove the inhibitor from

either mycelium or spores or both. A critical study would have to involve an effective separation of spores and mycelium without the use of water. Mirkes (1974) harvested dry conidia of Neurospora crassa by inverting a flask containing sporulating mycelium over a membrane filter chimney equipped with a membrane filter. Then gentle tapping of the flask and with suction applied to the filter apparatus, dry conidia were collected on the membrane filter. The cellular location of the inhibitor could then perhaps be ascertained as to whether it is on the outside of the cell, in the wall, the periplasmic space, or inside the spore cytoplasm.

Exogenously added nonanoic acid affects S.racemosum spores by delaying the time of germ tube emergence. It appears therefore to be taken up by the spores and it is possible that nonanoic acid, an intermediate chain length fatty acid, may cause inhibition of β-oxidation. Lewis and Johnson (1966) isolated some of the intermediates of the β-oxidation pathway in Cunninghamella echinulata. Further work showed that short-chain fatty acids (C_2 - C_8) were inhibitory at acidic pH's of 5.5 and 6.5 (Lewis & Johnson, 1967). The pH of S.racemosum growth medium was approximately 6.2 throughout germination. However, Lewis and Johnson (1967) found that oxygen uptake was reduced in the presence of these fatty acids. The degree of inhibition increased with increasing chain length, although with longer chained compounds (C_{10} - C_{18}), the degree of inhibition decreased as the chain length increased. The inhibition of endogenous oxygen uptake in C.echinulata could be overcome by the addition of either Coenzyme-A or pantothenic acid (a precursor in the biosynthesis

of Co-A). Lewis and Johnson (1968) suggested, because of this reversal of inhibition by Co-A or pantothenic acid, that the inhibition of respiration was at an enzymic level.

The inhibition of respiration (oxygen uptake) was observed by Steele (1973a) working with germinating arthrospores of Geotrichum candidum. Increasing the spore concentration of the inoculum had the effect of reducing the rate of oxygen uptake in germinating cultures. It may therefore be probable that nonanoic acid acts as an inhibitor of lipid breakdown in spores of S.racemosum. Respiration of the spores, which may occur straight away or soon after inoculation into growth medium, is either completely blocked or reduced to a level at which the spores develop at a slower rate. Park and Robinson (1970) observed oxygen uptake 45 minutes after inoculation of Geotrichum candidum spores into germination medium. Therefore respiration may occur early on in the germination sequence. Hence any inhibition of respiration would seriously affect the development of the spores.

Glucose was shown to be necessary for spore swelling and germ tube emergence in S.racemosum (Hobot & Gull, 1977; Chapter Two). If the glucose concentration of the medium was increased, then the extra supply of carbon for both respiration and metabolic intermediates could possibly overcome the block of lipid breakdown caused by the self-inhibitor. An experiment in which the glucose concentration was varied (from 0.1% through to 1%, 2%, 5% and 10%) with the nonanoic acid concentration constant in all cases ($200\mu g.ml^{-1}$) was performed. With all the cultures, only swelling of the spores was observed, but no germ tube emergence even after 36h. An increased supply of glucose appears therefore not to be able to overcome the inhibitory effect.

Possibly the concentration of nonanoic acid was so high as to make any increase in glucose concentration non-effective. The concentration of nonanoic acid may have been much higher than that normally found in spores.

The concentration of nonanoic acid in the spores is not known, although culture filtrates of Fusarium oxysporum were found to contain 6ppm (v/v) of nonanoic acid (Garrett & Robinson, 1969). Recently nonanoic acid and two other inhibitory substances (acetaldehyde, n-propanol) have been found to be accumulated in the soil after inoculation with 16 species of fungi (Garrett, 1972). Germination of spores of Cunninghamella elegans was retarded (in comparison with controls) in either soil that had previously been inoculated with the 16 species of fungi or in soil supplemented with the inhibitory substances. Accumulation of inhibitory substances in the soil may cause the phenomenon of soil fungistasis. This adds to the competitive nature of the natural environment, perhaps by preventing the germination of other fungal spores within the vicinity of a growing mycelium. However, the concentrations at which these inhibitors are found in the soil still remains to be elucidated.

In conclusion, the well known phenomenon of self-inhibition is found to operate in the germination of spores of S.racemosum. The preliminary experiments reported in this Chapter together with findings from other authors, appear to indicate that fatty acids, particularly nonanoic acid, are involved in self-inhibition. Self-inhibition may therefore not just be an obstacle to spore germination found under laboratory conditions, but possibly have ecological significance. As such, it is worthy of a more intensive study.

CHAPTER FOUR

THE ULTRASTRUCTURE AND CHEMICAL ANALYSIS OF THE SURFACE RODLET LAYER OF SPORANGIOSPORE WALLS

INTRODUCTION

Chapter One (Fig.18) showed that the outer wall of Syncephalastrum racemosum dormant spores was covered with a criss-cross network of rodlets. Rodlets were also observed on the surfaces of sporangiophores (Fig.12, Chapter One). Ghiorse and Edwards (1973) revealed the presence of rodlets arranged in intricate textile-like patterns on the surfaces of Aspergillus fumigatus conidia. Similarrodlets were seen on the phialide surface. Hess and Stocks (1969) investigating various Aspergillus conidial surfaces using freeze-etching techniques (as had Ghiorse and Edwards, 1973), found surface rodlets on all the organisms they looked at. Generally, these consisted of linear arrays of particles approximately 5nm. in diameter which formed bands with a centre to centre spacing of approximately 10nm between the rodlets. The conidia of ten species of Aspergillus were studied. Hess and Stocks (1969) found that there was a very prominent interlacing pattern of rodlets in Aspergillus aculeatus, Aspergillus awamori, Aspergillus fischeri, Aspergillus fumigatus, Aspergillus mellus, Aspergillus niger, Aspergillus ustus and Aspergillus wentii. However, in Aspergillus nidulans and Aspergillus ochraceus, although rodlets were present, there was only a slight interlacing pattern evident.

The individual rodlets of Aspergillus were identical to those observed on the surfaces of Penicillium conidia by freeze-etching (Hess, Sassen & Remsen, 1968). Ten species of Penicillium conidia were investigated and the rodlets were also found to consist of linear arrays of particles approximately 5nm in diameter which

formed bands with a centre to centre spacing of approximately 10nm between the rodlets. A prominent interlacing pattern of rodlets was seen in Penicillium camemberti, Penicillium cyclopium, Penicillium cylindrosporum, Penicillium digitatum, Penicillium megasporum and Penicillium rugulosum. A slight interlacing pattern of rodlets was observed on the surfaces of Penicillium brevi-compactum, Penicillium claviforme and Penicillium herquei conidia. On Penicillium chrysogenum spore surfaces, the interlacing pattern was absent. The groupings of rodlets were small, with most groups containing five rodlets or less. These groups were also widely dispersed revealing large areas of the underlying surface, which had a smooth, amorphous appearance (Hess et al, 1968).

Freeze-etching of basidiospores of Lycoperdon pyriforme and Lycoperdon decipiens revealed a surface layer of rodlets similar in appearance to those of Penicillium and Aspergillus (Bronchart & Demoulin, 1971). Bronchart and Demoulin (1971) also observed rodlets on the surfaces of other Gasteromycete fungal spores, namely Tulostoma brumale, Geastrum fimbriatum and Podexis pistillaris. Psathyrella velutina, a member of the Agaricales, also possessed rodlets on its spore surface.

Hess et al (1968) found that the rodlets of Penicillium were removed or possibly dissolved by the action of KOH. No attempt at isolating or characterising this rodlet layer was attempted. Recently, Hashimoto, Wu-Yuan and Blumenthal (1976) isolated and partially characterised a rodlet layer of the microconidial wall

of Trichophyton mentagrophytes. Electron micrographs of the freeze-etched surface of the isolated T.mentagrophytes microconidial wall showed groups of rodlets with a slight interlaced pattern being noticeable. The purified microconidial walls were first extracted with sodium phosphate buffer (pH 6.5) containing 8M urea, 1% (v/v) mercaptoethanol and 1% sodium dodecyl sulphate. This was followed by incubating the walls with glusulase (snail intestinal enzymes) and purified β-1,3-Dglucanase and chitinase. The remaining purified rodlet layer was found to be around 15 - 30nm thick and accounted for approximately 10% of the original wall weight. Freeze-etching of the isolated rodlet layer still revealed the presence of the rodlets. The rodlets were found to be resistant not only to the chemicals and enzymes used in the extraction procedure but also to trypsin, pepsin, protease and lysozyme enzymes. Hot alkali (1N NaOH at 100^{o}C) was the only treatment that caused rapid and complete disintegration of the rodlet layers. Hashimoto et al (1976) subjected the isolated rodlet layer to chemical analysis. They found that the major constituent of the rodlets was protein(s), which accounted for about 80-85% of the dry weight. An amino acid analysis revealed a relatively high content of glycine, lysine, aspartic acid and glutamic acid. Carbohydrates accounted for 7-10% of the dry weight, hydrolysis yielding a ratio of mannose to glucose of 1:1. These two sugars accounted for more than 95% of the total carbohydrate of the rodlet layer. No detectable amounts of lipids or phosphorous were found. An interesting feature of this layer was that although it was composed mainly of proteins, it was totally resistant to various protein degrading enzymes.

It was suggested that the high content of glycine, lysine, aspartic acid and glutamic acid in the rodlet protein may imply the presence of cross-linkages between amino acids, melanin (this pigment was found to be released during the hot alkali-treatment) or other structural subunits. Such cross-linkages may partially account for the resistance of the rodlets to proteolytic enzyme attack (Hashimoto et al, 1976). Bull (1970a) had previously demonstrated that melanised cell walls of Aspergillus nidulans were resistant to attack by β-1,3 glucanase and chitinase. The rates of activity of these two enzymes were found to decrease in the presence of melanin.

Kitajima and Nozawa (1975) isolated pure walls of Epidermophyton floccosum and subjected them to digestion with a snail gut enzyme preparation for 12h. The remaining fraction was the exo-layer. Negatively stained preparations showed a thin, stranded network 10-20nm thick. Chemical analysis of this layer showed that its main components were protein (63%), mannose (10%) and glucosamine (17%). Like the rodlet layer of T.mentagrophytes, the exo-layer of the dermatophyte E.floccosum was resistant to snail enzyme and amino acid analysis also revealed high amounts of glycine, aspartic and glutamic acid. However, alanine, threonine and serine were also present in high amounts (Kitajima & Nozawa,1975). The authors suggested that the high proportions of these amino acids may be due to their contribution to linkages between carbohydrates and protein moieties. The exo-layer was also soluble in a solution of 10mM Tris-HCl buffer (pH8.0) containing 1% sodium dodecyl sulphate,

10% sucrose, 1mM ethylenediaminetetraacetic acid (EDTA) and 40mM dithiotreitol at 37°C. (The rodlet layer of T.mentagrophytes was not soluble in an urea-mercaptoethanol-sodium dodecyl sulphate solution, but the pH of that solution was 6.5 (Hashimoto et al, 1976). It is probable that alkali conditions are necessary for dissolving the rodlet protein layer.) Polyacrylamide gel electrophoresis was performed on the dissolved samples of E.floccosum exo-layer. Five bands were observable after staining with comassie blue (a stain specific for proteins). These bands corresponded to molecular weights (MW) of 150000, 84000, 74000, 52000 and 42000. Staining for carbohydrates using periodate-Schiff stain revealed one band corresponding to a MW of 154000. Kitajima and Nozawa (1975) suggested that the exo-layer is probably composed of a glycoprotein with the smaller MW bands possibly being polypeptide bands of the 154000 MW protein.

Freeze-etched studies of the bacterial spores of Bacillus cereus have revealed a cross-patched array of fibres on the outer surface of the spore coat (Aronson & Fitz-James, 1975). These fibres were similar to the rodlets observed on Penicillium, Aspergillus and Trichophyton spore surfaces. It raises the possibility that the surface layers of bacterial and fungal spores are similar chemically. Watabe, Kakiuchi and Kondo (1975) solubilised coat proteins of Bacillus thiaminolyticus with either 5% sodium dodecyl sulphate (SDS) or 5% SDS plus 8M urea and/or 0.06N NaOH. Both solutions had a final pH of 10.5. Polyacrylamide gel electrophoresis revealed an SDS-soluble

band of MW 15000. However, SDS-urea extracted fractions showed two bands of 14500 and 32000 MW. When alkali was added to this extraction mixture, the two bands appearing had MW's of 12000 and 25000. Watabe et al (1975) conjectured that possibly the coat protein was composed of two subunits, a monomer of 12000-15000 MW and a dimer.

A low molecular weight spore coat protein (14000 MW) was found in Bacillus subtilis (Mitani & Kadota, 1976). Spore coat protein was extracted from a purified spore coat fraction with 1% SDS plus 0.1M dithiothreitol in either 0.1M sodium borate buffer (pH 10.0) or in 0.08N NaOH. Upon polyacrylamide gel electrophoresis, a single distinct band was given which corresponded to a MW of approximately 14000. However, the coat protein dissolved in 0.08N NaOH solution also gave several other diffused bands. Amino acid analysis of the spore coat fractions from B. subtilis showed high amounts of aspartic acid, glycine and glutamic acid. (These amino acids were also present in high amounts in the protein fractions of T.mentagrophytes (Hashimoto et al, 1976) and E.floccosum (Kitajima & Nozawa, 1975).). Lysine, serine and alanine were also present in high proportions. The two extracted fractions of B.subtilis were extremely soluble at high pH values (higher than pH 11) and extremely low at pH6-8. However, if 1% SDS was added, the precipitation of protein hardly occurred at all the pH values tested (range 2.5 - 12) (Mitani and Kadota, 1976). A similar phenomenon was observed with crystal protein fractions of

Bacillus thuringiensis which had been extracted from fractionated spores with either 0.1N NaOH or 8M urea plus 10% β-mercaptoethanol (pH 8.5) (Delafield, Somerville & Rittenberg, 1968). Maximum precipitation of protein occurred at pH values of approximately 3.5 - 6.5. However, no data on the effect of SDS on solubility was included.

Delafield et al (1968) obtained solutions of spore protein from Bacillus thuringiensis by treating isolated broken spores with either alkali (0.01N NaOH) or a mixture of 8M urea and 10% β-mercaptoethanol. Antiserum was prepared against alkali crystal solution by immunising rabbits with purified whole crystals or with alkali crystal solution. Rabbits were also immunised with whole vegetative cells. Lines of identity between spore and crystal precipitates were observed using the Ouchterlony double-diffusion technique. Anti-whole crystals and anti-whole cell antisera precipitated against alkali or urea extracted spore protein, but only the anti-whole crystal serum precipitated against the urea or alkali extracted crystal protein. This showed that crystal protein was relatively free of cellular components. However, antiserum prepared from immunising rabbits with alkali-soluble crystal only precipitated against alkali/urea extracted spore protein and alkali/urea extracted crystal protein. The result therefore suggested that the crystal and spore coat fractions contained one or more common proteins (Delafield et al, 1968).

Amino acid analysis of both crystal and spore coat protein fractions revealed a striking similarity (Somerville, Delafield &

Rittenberg, 1968). The composition of the whole spores was very close to that of the crystal protein, with high amounts of aspartic acid, glutamic acid, glycine, valine, leucine and threonine in both fractions. Although much fainter bands were formed, the major bands of the two fractions (crystal and spore coat proteins) were indistinguishable on polyacrylamide gel electrophoresis. As the crystal protein comprised a large fraction of the total protein of the spore, and since it was absent from the vegetative cell, Somerville and his coworkers (1968) assumed that it contributed significantly to the unique spore characteristics.

Purified spores of some Bacillus species were treated with 8M urea plus 10% β-mercaptoethanol (pH8.5) (Somerville et al, 1968). The organisms tested were Bacillus thuringiensis, Bacillus cereus, Bacillus megaterium, Bacillus subtilis and Clostridium roseum. Their amino acid analyses were remarkably similar although not identical. High amounts of aspartic acid, glutamic acid, glycine, alanine, threonine, serine and leucine were found. A similar high content of these amino acids was found in extracts of the outermost cell wall layer of Clostridium thermosaccharolyticum and Clostridium thermohydrasulfuricum (Sleytr & Thorne, 1976). Isolated cell walls of both these two species of Clostridium when negatively stained had an ordered array of subunits-hexagonal in C.thermohydrosulfuricum and tetragonal in C.thermosaccharolyticum. The authors also noted that the protein was predominantly acidic, having up to 20-25% acidic amino acid residues as compared with 8-9% basic residues.

Similarities therefore exist between the amino acid compositions

of fungal and bacterial spore wall proteins. This Chapter presents the results of investigating the nature of the surface rodlet layer of Syncephalastrum racemosum spores coupled with a comparison of other fungal and bacterial spore walls.

MATERIALS AND METHODS

GROWTH MEDIUM

Cultures of Syncephalastrum racemosum were grown on Sabouraud's medium in order to produce a high number of spores. The composition of the medium was ($g.L^{-1}$):- glucose,40; mycological peptone,10; agar (minimal),15. The medium was autoclaved at 115°C for 10 minutes.

SPORE PRODUCTION

S.racemosum was either grown in Roux bottles or on cellophane covered media at 25°C. It was found necessary to grow S.racemosum on cellophane because replicas of dormant spores observed in the electron microscope had a layer of fibrillar agar covering their surfaces. This made observation of surface detail impossible. The agar film was present, because when spores were harvested off the Roux bottles with distilled water and filtered through sterile muslin to remove hyphal debris and large pieces of agar, fine particles of agar were still left in the resultant spore suspension. To overcome this problem, media was poured into square sterile petri dishes and allowed to solidify. Cellophane sheets, cut previously to the size of the petri dishes were autoclaved at 121°C for 60 minutes. In a sterile inoculation cabinet, the sheets of cellophane were carefully placed on to the medium surface. Flamed forceps were used. The petri dishes were turned upside down and left overnight at 37°C. This had the effect of drying off any excess water on top or beneath the cellophane sheet which might interfere with the growth of the fungus. The next day, the cellophane surface was exposed for 10 mins. to ultraviolet light within the sterile inoculation chamber to further minimise the risk of contamination by other organisms.

Next, a drop of spore suspension was placed on the cellophane and spread over the entire surface by a sterile glass spreader. Incubation was at 25°C.

SPORE HARVESTING

Spores were harvested as per materials and methods of Chapter Two.

CHEMICAL TREATMENTS OF THE SPORES

Table 2 shows the sequential treatments the spores were subjected to. After harvesting, some of the spore pellet was removed for electron microscopical analysis. The rest of the spore pellet was incubated with 5% KOH on an orbital shaker for 6h at 37°C. After centrifugation at 2500rpm for 30 mins., the alkaline supernatant (A) (brown colour) was centrifuged at 12000rpm for 20 mins. The pellet was discarded, and 0.1ml. of 8% Na_2SO_4 solution added for every 1ml. of supernatant (A). (The Na_2SO_4 acts as a primer for the precipitation of glucon material when ethanol is added to the solution - Dr. C. Pogson and Dr. K. Elliot, private communication). To (A), cold absolute ethanol (-30°C) was added to give a final concentration of 70% alcohol. The solution was stored overnight at 4°C. The next day it was centrifuged at 12000rpm for 20 mins., and the pellet (1) was washed six times with sterile distilled water (this solution was brown coloured and was stored in the deep-freeze for melanin/pigment extraction). The pellet (1) was then washed three times with 100% ethanol, three times with diethylether, and stored in a dessicator at room temperature to dry. The ethanol supernatant (B) was light green in colour and was dialysed against six changes of distilled water for 48h. It was then freeze-dried on an Edwards Freeze Drier (Model EF3) at 0.05 torr, -40°C, and the pellet (B) stored in a dessicator at room

temperature to dry.

The spore material after the initial 6h in 5% KOH was washed three times in dstilled water, and then incubated a further 18h in 5% KOH at 37°C. Prior to this treatment, some material was taken for E.M. observations. (Material for E.M. observations had been grown previously on cellophane sheets. Material for chemical analysis was grown in Roux bottles). The alkaline extract of the 18h treatment was treated as the previous extract until separation of the ethanol supernatant and pellet (2) was reached. Pellet (2) was treated exactly like pellet (1).

MELANIN/PIGMENT EXTRACTION

Water washings of pellet (1) (pH9.6; cf.Table 2) were treated with glacial acetic acid till the pH fell to 2.5 (Ellis & Griffiths, 1974). The precipitated material was isolated by centrifuging at 4000rpm for 20 mins., washed three times in distilled water and dried in a dessicator at room temperature (Pellet (3)). After drying, some of the material was dissolved in 5% KOH by heating in a boiling water bath for U.V. analysis. Standard samples of Sepia melanin and Aspergillus nidulans melanin were dissolved in the same way.

MACROMOLECULAR ESTIMATIONS

Estimations were carried out on Pellet (1). Carbohydrate estimations were by the phenol-H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers and Smith (1951). Protein estimations were by the method of Lowry, Rosenbrough, Farr and Randall (1951).

Absorbances were measured on a Pye-Unicam SP500 Series 2 Spectrophotometer.

TOTAL HYDROLYSIS OF PELLET (1)

0.2mg. of pellet (1) was placed in an empty, dry Quickfit MF24/0 glass tube. 0.2ml. of 1M H_2SO_4 was added and the tubes heated for 6h at 96-97°C in a boiling water bath. After 6h the tubes were allowed to cool, 0.8ml. of distilled water added plus 0.4g $BaCO_3$ to neutralize the solution to a pH of approximately 6.0 - 6.2. The suspension was shaken thoroughly and centrifuged at 12000rpm for 20 mins. The supernatant was collected, transferred into freeze-drying ampules and freeze-dried overnight in an Edwards Freeze Drier (Model B5A) at 0.05 torr.

Next day, the dried material was resuspended in 0.2ml. of distilled water and spotted on to Whatman No.1 Chromatography paper. Chromatograms were run for 48h using the descending paper chromatography technique with a solvent system of ethyl acetate/pyridine/water (8/2/1). Sugar spots were developed with alkaline silver nitrate.

GEL ELECTROPHORESIS

Samples for gel electrophoresis were dissolved in 0.01M NaOH + 0.05M KCl buffer (pH 12). The gels were prepared with a final concentration of acrylamide of 7.5%. The gels were polymerised in 0.375M Tris-HCl buffer (pH 8.3), and equilabrated with the NaOH + KCl (pH 12) buffer for 60 mins. prior to loading of the gels. The current for equilibration was 3mA per tube. After loading of material on to the gels, electrophoresis was carried out at room temperature with a current of 8mA per tube (Somerville, Delafield & Rittenburg, 1968). Gels were stained with Comassie Blue.

INFRA-RED SPECTROSCOPY

Material for I.R. spectroscopy was prepared by the pressed KBr disc method. (Approximately 4-5mg. of sample to 0.5g KBr). I.R. scans were obtained with a Perkin-Elmer 457 Grating Infrared Spectrophotometer.

ULTRA-VIOLET SPECTROSCOPY

U.V. extinction (range 350-650nm) was recorded on an Unicam SP8000 Ultraviolet Recording Spectrophotometer.

ELECTRON MICROSCOPE PROCEDURES

(a) Surface replica : as per materials and methods of Chapter One.

(b) Freeze-fracture : as per materials and method of Chapter One.

GLUCAN STANDARDS

α-1,3 glucan of *Aspergillus nidulans* was a gift from Dr. B.J.M.Zonneveld (Genetisch Laboratorium, Kaiserstraat 63, Leiden, The Netherlands). S-glucan of *Schizophyllum commune* was a gift from Prof. J.G.H.Wessels (Biological Centre, University of Groningen, Haren, The Netherlands).

AMINO ACID ANALYSIS

Amino acid analysis was performed by Dr. C.F.Thurston (Microbiology Dept., Queen Elizabeth College, London).

RESULTS

ULTRASTRUCTURAL OBSERVATIONS

(1) Untreated Spores

Freeze-fracturing of dormant spores of *Syncephalastrum racemosum* revealed a surface layer consisting of a criss-cross network of rodlets (Figs. 48 and 49). The patch-like nature of this outermost wall layer is very striking and can also be clearly seen in surface replicas of dormant spores (Figs. 50 - 53). The surface rodlets have a mean periodicity of 25nm. Higher magnifications of the rodlets show their regular patterned appearance (Figs. 54 and 55). It is also seen that the rodlets of *S.racemosum* can be detected using two different electron microscopical techniques, whereas the rodlets of the *Penicillium* conidia (Hess, Sassen & Remsen, 1968), *Aspergillus* conidia (Hess & Stocks, 1969), and *Trichophyton mentagrophytes* spores (Hashimoto, Wu-Yuan & Blumenthal, 1976) were only demonstrable after freeze-etching. Hess *et al* (1968) commented that in using carbon replica techniques, they found that these techniques resulted in poor resolution and had not provided detailed information concerning surface patterns. Figures 50 to 53 are surface replicas of *S.racemosum* dormant spores.

(2) Alkali treated spores

Incubation of the spores with 5% KOH for 6h removed the surface rodlet layer completely (Fig. 56). The KOH treatment revealed an underlying amorphous layer with no rodlets present (Figs. 57 and 58). The removal of the rodlet layer (solubilised by the alkali) was most effective (Figs. 59-63), and was used subsequently as the first step in isolating the rodlet layer from intact spores.

CHEMICAL ANALYSIS

(1) Pellet (B)

Pellet (B) was obtained by freeze-drying of the ethanol supernatant (B) (Table 2). It is one of the two fractions obtained from the initial 6h 5% KOH treatment. The other fraction is pellet (1). As bacterial proteins had been observed to have a rodlet or fibrillar appearance (Aronson & Fitz-James, 1975), pellet (B) was investigated to see if any protein material was present. It was dissolved in 0.01M NaOH + 0.05M KCl (pH 12) buffer and run on 7.5% polyacrylamide gels (Somerville, Delafield & Rittenburg, 1968). The result is seen in Figure 64A. After comassie blue staining, one single intense band is noticeable. It runs very close to the end of the gel and suggests that pellet (B) probably contains protein.

The results of an amino acid analysis of pellet (B) is presented in Table 3. High amounts of aspartate (+ asparagine), glutamate (+ glutamine), glycine, serine and alanine are present. Threonine and proline are also present in larger amounts than the other amino acids. Acidic amino acids are the most common (aspartate + asparagine and glutamate + glutamine: 19.2%), as are the amino acids having a small R-group (glycine, serine and alanine: 31%). (R = side chain of the amino acids). Basic amino acids form only a small percentage of the total (lysine, histidine and arginine: 11.6%).

(2) Pellet (1)

A total of 1.75mg of pellet (1) material was obtained after initially harvesting spores off 72 Roux bottles. These low yields of material limited the number of chemical/biochemical analyses that could be done on this and other surface rodlet fractions. For example,

for a satisfactory I.R. scan, 5mg of material was required. However, protein/carbohydrate estimations of pellet (1) showed that 60% of the material was protein, whilst 40% was carbohydrate. In this analysis, 72% of the original material was accounted for in the final estimations. Total hydrolysis products of pellet (1) revealed two spots (Fig.65). One ran like glucose having a R_G value of 1.0, the other having a R_G value of 0.74 and not corresponding to any of the other sugars run as standards (Table 4). The nature of this sugar spot remained unresolved. The result pointed to the possibility of a glucan being one of the main carbohydrate components of the pellet (1) fraction. Although protein was the greater component of this fraction (as it also most probably is in pellet (B)), the carbohydrate component of the fraction was investigated by means of infra-red spectroscopy.

Barker, Bourne, Stacey and Whiffen (1954) had shown that the absorbance range of 760-930cm^{-1} could form a basis for the classification of the principal glucosidic linkages in a polyglucosan. A peak in the range 891 $\pm$ 7cm^{-1} was indicative of β-linkage, whilst a peak in the range 844 $\pm$ 8cm^{-1} was indicative of α-linkage. In addition, from a consideration of peaks in the range 914 $\pm$ 13cm^{-1} and 766 $\pm$ 10cm^{-1}, it was possible to obtain information about the position of such linkages in α-polyglucosans. Unfortunately, no such correlation was possible with the β-anomer.

I.R. spectra of pellet (1) (Fig.66) reveal peaks at 930, 890, 875, 770 and 740cm^{-1}. The peak at 890cm^{-1} is indicative

of β-linkage, whilst the two peaks at 930 and 770cm^{-1} suggest α-linkages as well. It is possible that the glucan component of the pellet (1) fraction has both α- and β-linkages. The small peak at 875cm^{-1} is difficult to interpret. A corresponding peak has not been found in the literature. Maybe the peak is due to the unknown sugar (R_G = 0.74, Table 4) detected in the total hydrolysis analysis.

(3) Pellet (2)

The I.R. scan of pellet (2) is similar to that of pellet (1) (Fig.66). It has peaks at 930, 890, 770 and 740cm^{-1}, but only a shoulder at 875cm^{-1}. Pellet (2) was isolated in the same way as pellet (1), except it came from the alkaline solution of the second extraction (18h, 5% KOH at 37°C; Table 2). No rodlets are present after the initial 6h KOH treatment (Figs. 56-63) only the underlying amorphous material. The amorphous material removed by this second alkaline treatment reveals a more rugose type of surface (Fig.76, Chapter Five). This raises the possibility that the amorphous material may be similar to the surface rodlet layer, except that it could differ in its protein to carbohydrate ratio. The ratio is important as to the structural integrity of the layer, more protein perhaps dictating the appearance of the rodlets. Alternatively, the initial alkaline extraction could pick up some of the amorphous material as well.

Further characterization of the carbohydrate component was not possible, as yields of material were always low. Periodate oxidation and methylation analysis of the sugars would provide useful

information as to the nature of the linkages between the sugar moieties.

(4) Pellet (3)

The alkaline supernatant (A) (Table 2) was brown in colour, suggesting that some pigment(s) may be removed by alkali treatment. Subsequently, the ethanol supernatant (B) was light green in colour and the precipitate grey (pellet (1)). Upon washing of this pellet (1), the distilled water used in this procedure turned brown, again indicating that some pigment(s) had been separated during ethanol precipitation into the pellet (1) fraction. Treating the water washings (pH 9.6) with glacial acetic acid to give a final pH of 2.5 yielded a dark brown precipitate. This fraction was pellet (3).

Pellet (3) was soluble in hot 5% KOH, as was Sepia melanin and melanin from Aspergillus nidulans. The absorbance of these three samples over the ultraviolet range 650-350nm was measured. The three scans are presented in Figure 67. No absorbance peaks are seen (as is expected of melanins: Bull, 1970b; Ellis & Griffiths, 1974), and the plots of log absorbance against wavelength are presented in Figure 68. From these graphs with negative slopes, the gradient for each sample was calculated and compared with values for other known melanins from fungal sources (Table 5). The values for the various fungi range from -0.0015 to -0.0030. The gradient of the extracted sample (pellet (3)) of S.racemosum is -0.0030, the same value as that for Humicola grisea melanin (Ellis & Griffiths, 1974). A.nidulans melanin gave a value of -0.0027. The S.racemosum fraction agrees well with other known values for melanin,

and is itself probably a melanin pigment.

Melanins do not give characteristic I.R. spectra and are of no diagnostic value (Bull, 1970b). However, the I.R. scan of pellet (3) (Fig.66) gave peaks at 930 and 890cm^{-1} with shoulders at 770 and 740cm^{-1}. This is very similar to the I.R. scans of pellets (1) and (2) (Fig.66).

(5) Glucans of other fungi

When the investigation of identifying the rodlets of S.racemosum was initially undertaken, it was suspected that they may be mainly of carbohydrate origin. Therefore the accent of the study was on I.R. spectra together with the use of α-1,3 glucan from Aspergillus nidulans (Zonneveld, 1971) and S-glucan (an α-1,3, glucan) from Schizophyllum commune (Wessels, 1965; Wessels, Kreger, Marchant, Regensburg and De Vries, 1972). Both glucans had been extracted from hyphal walls. Furthermore, freeze-etched studies of both intact and isolated S-glucan of S.commune showed it to be made up of small parallel arrays of short rodlets randomly orientated with respect to each other. The periodicity of these rodlets was 10nm (Wessels et al, 1972). However, the studies with S.racemosum suggest that the rodlets seen on the spores are mainly protein, with perhaps the carbohydrate being a minor component. For this reason the question was raised as to whether the rodlets of S.racemosum S-glucan were due to co-purifying protein rather than to carbohydrate (glucan). Pure samples of S-glucan and A.nidulans α-1,3 glucan were dissolved in the pH12 buffer (0.01N NaOH + 0.05M KCl) and run on polyacrylamide gels as described in the materials and methods

section of this Chapter. The results are presented in Figures 64B and 64C for α-1,3 glucan and S-glucan respectively. A single, distinct band is seen on both gels, suggesting that protein is present in both glucans.

DISCUSSION

ULTRASTRUCTURE OF THE RODLET LAYER

The rodlet layer is a constant feature of Syncephalastrum racemosum dormant spore surfaces. It can be observed using either the techniques of freeze-fracturing or surface replicas. Similar rodlet patterns have been seen on the surfaces of Penicillium and Aspergillus conidia (Hess, Sassen & Remsen, 1968; Hess & Stocks, 1969). Some Gasteromycete fungi also exhibit rodlets on their spore surfaces (Bronchart & Demoulin, 1971). Recently, Hashimoto, Wu-Yuan and Blumenthal, (1976) showed that freeze-etched spore surfaces of the dermatophyte Trichophyton mentagrophytes had a rodlet pattern as well. This report was one of the first to isolate and partially characterize the rodlet layer in fungi. Similar rodlet patterns have been observed in the bacteria such as in Bacillus cereus (Aronson & Fitz-James, 1975), and work has been done on characterising the spore coat fractions probably consisting of these rodlets. Therefore a comparison between bacterial and fungal rodlets is possible.

AMINO ACID DATA AND POLYACRYLAMIDE GEL ELECTROPHORESIS

Gel electrophoresis of pellet (B) revealed a single band suggesting that protein material was present. Subsequently, amino acid analysis data revealed a high amount of acidic amino acids together with the lower molecular weight amino acids (where the R-side group is small; cf. Table 3). A comparison of the amino acid analysis of various bacterial and fungal wall fractions is presented in Table 6. Firstly, the rodlets of Trichophyton

mentagrophytes spores have a high acidic amino acid composition (23.93%) as does the S.racemosum sample (19.2%). The amount of low MW amino acids is also high in both; 19.98% in T.mentagrophytes, 31% in S.racemosum. Both were low in basic amino acids. Secondly, this trend is also seen in the exo-layer fraction of Epidermophyton floccosum (also a dermatophyte). The acidic amino acid composition is 23.8%, and the low MW amino acid composition is 35.1%. Thirdly, the high proportion of both acidic and low MW amino acids is also very evident in the bacterial examples quoted in Table 6. For example, for Bacillus cereus, the acidic amino acid composition is 19.2% and the low MW amino acid composition is 27.6%. For Clostridium thermosulfuricum, the acidic composition is 20.35%, the low MW composition is 23.58%. Fourthly, glycoproteins extracted from isolated spore walls of Microsporum gypseum by Page and Stock (1974) had a high proportion of both acidic amino acids (19.5%) and low MW amino acids (23.9%). Basic amino acids only accounted for 1.7% of the total amino acid composition. A peptide-glucan fraction from Armillaria mellea fruit bodies also had high proportions of glutamic and aspartic acids (Amar, Delaumeny & Vilkas, 1976). Low MW amino acids were also present in high amounts. The peptide-glucan fraction had been extracted from fruit bodies by disintegrating them in a Waring Blendor and boiling with water. Material was precipitated out by the addition of ethanol. Amino acid analysis revealed the above mentioned information. However, alkali treated material had 80% less threonine. Amar and his coworkers (1976) suggested that the destruction of threonine may cause the separation

of the peptide and carbohydrate moieties. Hashimoto et al (1976) had thought that the presence of a high content of glycine, lysine, aspartic acid and glutamic acid in the rodlet protein of T.mentagrophytes may imply the presence of cross-linking between amino acids, melanin or other structural subunits.

Therefore, the acidic amino acids together with the low MW amino acids of pellet (B) (as also in the E.floccosum exo-layer and T.mentagrophytes rodlets) may contribute to cross-linkages between peptide (protein) and carbohydrate/melanin moieties.

PELLETS (1), (2) AND (3)

Pellet (1) was found to be composed of 60% protein and 40% carbohydrate, this being glucose and some other unknown sugar (R_G value 0.74, Table 4). Pellet (1) came from the initial alkali extraction (cf. Table 2) and was precipitated out when cold ethanol was added. The ethanol supernatant (B) upon freeze-drying yielded pellet (B). This could imply that the alkali treatment somehow separates the protein-carbohydrate of pellets (B) and (1) in a manner similar to cleavage of the peptide-glucan fraction of Armillaria mellea (Amar et al, 1976). Further, the rodlet layer of Trichophyton mentagrophytes was found on analysis to contain 7-10% carbohydrate (Hashimoto et al, 1976). Mannose and glucose in a 1:1 ratio accounted for more than 95% of the total carbohydrate. The exo-layer of Epidermophyton floccosum had 10% mannose and 17% glucosamine in addition to 63% protein (Kitajima & Nozawa, 1975).

The last paragraph in the previous section suggested linkage between the peptide and carbohydrate fractions via acidic and low MW amino acids. Both peptide and carbohydrate fractions are present in T.mentagrophytes (Hashimoto et al, 1976) and S.racemosum rodlets, and in the exo-layer of E.floccosum (Kitajima & Nozawa, 1975). Therefore such linkage appears possible. It is most probable, however, that the protein (the major constituent in the rodlets) dictates the rodlet structure in both fungal and bacterial cell walls.

Hashimoto et al (1976) noted that the rodlets of T.mentagrophytes were resistant to attack by proteolytic enzymes such as trypsin, pepsin, protease, lysozyme and glusulase. They suggested that cross-linkages between the protein-carbohydrate-melanin subunits may account partially for this resistance. The peptide-glucan fraction of A.mellea was resistant to attack by pronase (Amar et al, 1976), and the exo-layer of E.floccosum was resistant to commercial snail gut enzyme (Kitajima & Nozawa, 1975). Amar et al (1976) suggested that either the peptide had an unusual structure or that the polysaccaride chain shielded it from attack. Such resistance is probably present in S.racemosum rodlets. Various proteolytic and carbohydrate degrading enzyme treatments of S.racemosum spores had no effect on the rodlet surface.

However, the involvement of melanin in this resistance to enzymic attack must also be borne in mind. Chet and Henis (1969) observed that melanin-less sclerotial walls of Sclerotium rolfsii were less susceptible to attack by either chitinase or β-1,3 glucanase than

melanin-containing sclerotia. Melanin-free sclerotia were obtained by growing the fungus in the presence of EDTA. Bull (1970a) showed that soluble fungal melanin inhibited exo-β-1,3 glucanase activity by 76%, whilst chitinase was completely inhibited.

The release of melanin or melanin-like pigment(s) was observed when the isolated rodlet layer of T.mentagrophytes was treated with hot (100°C) 1N NaOH. The ultraviolet light absorption spectra were found to be essentially identical to melanin isolated from Aspergillus nidulans (Hashimoto et al, 1976). Melanin pigment was extracted from Sclerotium rolfsii sclerotia with 1N KOH at 100°C (Chet & Henis, 1969). 5% KOH treatment of S.racemosum spores also released a dark brown pigment(s). Table 2 shows that water washings of pellet (1) became brown coloured and pellet (3) was obtained by adding glacial acetic acid to the water washings to give a final pH of 2.5 (Ellis & Griffiths, 1974). Table 5 details a comparison of UV absorbtion data of various fungal melanins. Pellet (3) compares favourably with the other melanins, and is probably a melanin or melanin-type pigment itself. The possible association of melanin with the rodlet layer in both T.mentagrophytes and S.racemosum probably helps in conferring resistance of this layer to enzymic attack.

Furthermore, the I.R. scans of pellet (3) (possibly melanin) and pellet (2) (extracted from the second alkaline treatment, cf. Table 2) bear remarkable similarity to the scan of pellet (1). Both pellets (1) and (2) have absorbance peaks at 930, 890, 770, 740cm^{-1}, with pellet (2) having a shoulder at 875cm^{-1} where pellet (1)

has a peak. Pellet (3) has peaks at 930 and 890cm^{-1} and shoulders at 770 and 740cm^{-1} (Fig.66). The peak at 890cm^{-1} is indicative of β-linkage, whilst peaks 930, 770 and 740cm^{-1} are suggestive of α-linkage (Barker, Bourne, Stacey & Whiffen, 1954). An alkali soluble glucan extracted from the walls of the mycelial form of Paracoccidioides braziliensis had absorbtion peaks at 812, 840 and 890cm^{-1}, suggesting the existence of both α- and β-glycosidic linkages (Kanetsuna & Carbonell, 1970). Maybe an analogous situation exists in the S.racemosum fraction

ASSOCIATION OF THE VARIOUS SURFACE LAYER COMPONENTS

This Chapter has shown that there is evidence suggesting the presence of a protein-carbohydrate-melanin complex in the spore walls of S.racemosum. The alkali extraction procedure may probably cause the destruction of the linkages between the various constituents of the complex (Amar et al, 1976). A result of this could be observable differences in the protein/carbohydrate content of the various isolated fractions. It is also possible that the ultra-structural differences between the rodlet and underlying amorphous region arise from variations in the protein/carbohydrate/melanin content of the complex. The rodlet structure is probably due largely to protein being the major component.

Hashimoto et al (1976) analysed the rodlet layer of T.mentagrophytes spores to be composed of protein (80-85% dry weight of the wall) linked to carbohydrate (7 - 10%) and melanin (2 - 3%). The presence of high amounts of aspartic and glutamic acids together with glycine and lysine suggested the presence of

such cross-linkages. These amino acids are also found abundantly in S.racemosum (Table 6).

A melanin complex was isolated from spore walls of Mucor rouxii (Bartnicki-Garcia & Reyes, 1964). Isolated spore walls were extracted five times for 30 minutes each with 1N HCl at 100°C under nitrogen. A deep, dark brown solution was obtained with very little insoluble pigment. The pigment could be extracted by precipitation by adjusting the pH to 6.3. The pigment was alkali soluble.

Bartnicki-Garcia and Reyes (1964) showed that the pigment had UV absorbtion spectra characteristic of melanin. Furthermore, 6N HCl hydrolysates revealed that 25.7% of the purified pigment was glucosamine and 14.8% protein. The melanin content was 59.5%. An interesting feature of this complex was that the amino acid composition of the protein was similar to that of spore walls, aspartic acid, glutamic acid and alanine being the most abundant. The data for M.rouxii compares favourably with the high acidic amino acid contents of both fungal and bacterial spore walls (Table 6). The above amino acids may therefore play a role in cross-linkage between the protein and glucosamine/melanin, as was suggested by Hashimoto and his colleagues (1976).

The results of both Hashimoto et al (1976) and Bartnicki-Garcia and Reyes (1964) show the presence of a protein-carbohydrate-melanin complex in spore walls. Moreover, the ultrastructure of the complex in T.mentagrophytes is similar to the rodlets observed on spores of

S.racemosum. Therefore, it is quite conceivable that a similar complex may exist in S.racemosum spore walls.

Although the nature and extent of the cross-linkages between the various components of the complex is not clear, such complexes may play an important ecological role. The peptide-glucan fraction of Armillaria mellea was resistant to attack by proteolytic enzymes (Amar et al, 1976), as was the exo-layer of Epidermophyton floccosum (Kitajima & Nozawa, 1975). Such a resistance to attack from proteolytic enzymes was also observed in T.mentagrophytes spores (Hashimoto et al, 1976). The protein-carbohydrate linkage may confer some resistance to attack, as does the presence of melanin (Bull, 1970a; Chet & Henis, 1969). Also, the location of melanin in the spore wall may serve to protect the spore cytoplasm and nucleus against ultraviolet and visible radiation (Bartnicki-Garcia & Reyes, 1964). The spore wall could well play a significant role in the resistance of the spore to adverse conditions.

GLUCANS OF OTHER FUNGI

Gel electrophoresis of samples of S-glucan from Schizophyllum commune (Wessels, 1965) and α-1,3 glucan from Aspergillus nidulans (Zonneveld, 1972) revealed the presence of a single major band on both gels. This suggests that protein is present in both preparations and co-purifies with these alkali soluble glucans.

The extraction procedure of S-glucan included treatments with pepsin and trypsin before extraction of the glucan from the hyphal walls with alkali (Wessels, 1965; Wessels, Kreger, Marchant, Regensburg & De Vries, 1972). However, it has been shown that spore wall proteins

can be resistant to attack from proteolytic enzymes (Hashimoto et al, 1976; Amar et al, 1976; Kitajima & Nozawa, 1975).

The α-1,3 glucan of A.nidulans is also alkali soluble and comprises 22% of the dry weight of the complete hyphal wall (Zonneveld, 1972). Neutral carbohydrates are by far the largest constituent of the hyphal walls, being 57.6% of the dry weight of complete cell wall. Amino sugars comprise 19.1%, proteins 10.5% and lipids 4.6% of the dry weight of the walls. The involvement of the protein (10.5%) in the wall was not investigated by Zonneveld (1972). As with Wessels and his co-workers (1972), emphasis is placed on the analysis of carbohydrates.

The role of protein in fungal walls is little understood and often not studied. Therefore future studies on fungal walls should take into account that proteins may be associated with other wall constituents. Analytical techniques will have to be adapted to accommodate lines of investigation which will throw light upon this area of wall structure.

CONCLUSIONS

A continuation of this work by Dr. K. Gull has shown that the outer wall rodlets (pellet (B)) stain with periodic acid-Schiff reagent on polyacrylamide gels indicating a glycoprotein nature, and have a MW of around 12000 (estimated from SDS-polyacrylamide gels).

CHAPTER FIVE

THE ULTRASTRUCTURE AND CHEMICAL ANALYSIS OF DORMANT SPORANGIOSPORE WALLS

INTRODUCTION

Although a lot of information exists on both the ultrastructure and chemistry of hyphal walls (Bartnicki-Garcia, 1968a), little is known about fungal spore walls. A good knowledge of spore walls is necessary, because spores are involved in the dispersal of the fungus from one nutritionally favourable area to another. Also, some spores are able to survive through adverse conditions thus maintaining the spores by providing a dormant stage in the life cycle.

These properties of the spores are reflected in structural differences between them and the hyphae, not least in the ultrastructural and chemical form of the cell wall. Generally, upon germination, the wall of the new outgrowing germ tube is not the same as that of the spore (Bartnicki-Garcia, 1968a). Therefore, studies of hyphal and spore walls are important in relation to obtaining a clearer understanding of the cellular control of morphogenesis. This is linked closely to the growth of the organism, especially at the hyphal tips, for vegetative hyphae develop by extension at the hyphal apex.

HYPHAL TIP GROWTH

Grove, Bracker and Morre (1970b) investigating the ultrastructure of hyphal tips of Pythium ultimum, an Oomycete fungus, found that young hyphae were divided into three zones. An apical zone, which was composed of many cytoplasmic vesicles with other cellular organelles generally absent. A subapical zone, having various organelles such

as nuclei, dictyosomes, mitochondria, endoplasmic reticulum, ribosomes. The dictyosomes were found positioned close to endoplasmic reticulum or nuclei, with vesicles adjacent to the dictyosomes. The third zone, a zone of vacuolation, had more and more vacuoles the further the distance from the hyphal tip. An increase in lipid content was also observable. Because some of the apical vesicles were noticed to be continuous with the plasmamembrane, Grove and his coworkers (1970b) hypothesized that they may be involved in tip growth. The vesicles could contain wall precursors or enzymes involved in wall construction. They proposed a model in which vesicles were released from the endoplasmic reticulum and passed to the dictyosomes, where they became associated with the proximal pole. The contents and membranes of the dictyosome cisternae are transformed as they become displaced to the distal pole (Grove, Bracker & Morre, 1968). The displacement was due to the formation of new cisternae. At the distal pole, new vesicles were formed, these migrating to the hyphal apex. Fusion occurred with the plasmamembrane, the vesicle contents being released into the wall region. Cytoplasmic vesicles were found at hyphal apices of fungi representing the Oomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes by Grove and Bracker (1970a). This apical zone was rich in vesicles, but generally lacking other cell organelles. Some vesicles were found to be continuous with the plasmamembrane at the tips. In the subapical region, endomembrane systems associated with vesicles were observed. Grove and Bracker (1970a) concluded that these observations in the fungi supported the

hypothesis of cytoplasmic vesicle involvement in hyphal tip growth.

Autoradiography provides evidence that growth does occur at the tips. Gooday (1971) studied the incorporation of radioactively labelled glucose into Phytophthora parasitica, and labelled glucose or N-acetylglucosamine into Neurospora crassa and Schizophyllum commune. N-acetylglucosamine is a precursor of chitin. Gooday (1971) observed that even with a short pulse of one minute all three fungi became labelled exclusively at the tips. Also, the incorporation of label fell off sharply the further the distance from the apex. These results complimented those of Bartnicki-Garcia and Lippman (1969) who showed that labelled N-acetyglucosamine was taken up exclusively by hyphal tips of Mucor rouxii. Cell wall synthesis occurred at the highest rate at or within 1μm of the apex; further from the apex, about 3μm, cell wall synthesis decreased abruptly by about 50 per cent per micron. However, Katz and Rosenberger (1971) observed that the pattern of labelling changed when hyphae were treated with cyclohexamide. Cyclohexamide is an inhibitor of cytoplasmic protein synthesis. In the absence of the inhibitor, incorporation of labelled N-acetylglucosamine occurred only at the hyphal apex. This result was in complete agreement with those of Gooday (1971) and Bartnicki-Garcia and Lippman (1969). But when cyclohexamide ($10\mu g.ml^{-1}$) was present, incorporation of the labelled precursor occurred along the length of the Aspergillus nidulans hyphae. The rate of uptake of N-acetyglucosamine was the same in both

control cultures and cultures treated with inhibitor. Also, such treatments increased the number of branches and septa formed. Since cyclohexamide is an inhibitor of protein synthesis, this new pattern of precursor incorporation and formation of more branches could not have been due to the action of newly formed enzymes. The authors thought that the activation of pre-existing enzymes, located along the length of the wall, and constituting one of the factors involved in wall synthesis, could explain the changed labelling pattern. Also, if cyclohexamide was removed, normal apical incorporation was re-introduced. This was taken as supporting the view of an enzyme activation mechanism, the cyclohexamide having somehow interfered with this system. Katz and Rosenberger (1971) postulated that this activation, although remaining undefined, could be one of several possibilities. The exposure of a primer, the increased supply of substrate, the accumulation of activators or removal of inhibitors.

Such experiments as have just been discussed show that cytoplasmic vesicles are intimately involved in fungal growth, which occurs at the hyphal tip. Wall growth has also been postulated to involve the concerted action of hydrolytic and biosynthetic enzymes (Barras, 1972). The hydrolytic enzymes create regions within the existing wall polymers for new cell wall material to be inserted by biosynthetic enzymes. A delicate balance between these two types of enzyme exist, therefore, at the growth points of fungal cells (Bartnicki-Garcia, 1973). Chitin synthetase has been found in Aspergillus flavus (Moore & Peberdy, 1976) and in Cunninghamella

elegans (Moore & Peberdy, 1975), and in many other fungi. However, there is little direct evidence for the involvement of lytic enzymes in fungal growth (Smith & Berry, 1974), although circumstantial evidence demonstrating their active presence can be put forward. Smith and Berry (1974) listed some of this circumstantial evidence as:- (a) there was a positive correlation between the level of cell wall lytic enzymes and apical growth; (b) the ability of hyphae to fuse or anastomose by self-dissolution; (c) spore germination involving wall softening prior to germ tube emergence; (d) autolytic breakdown in many of the Basidiomycete sporophores. Lytic enzymes may be present in the vesicles, although in what manner, and if associated with wall synthesising enzymes remains to be elucidated.

Bartnicki-Garcia (1973) integrated these various findings concerning tip growth in his model for explaining cell wall growth. His model, for convenience, considered a wall composed of only two wall layers: an outer amorphous substance and an inner microfibrillar skeleton. Lytic enzymes from cytoplasmic vesicles were secreted into the wall region. This occurred after fusion of the vesicles with the plasmamembrane (Grove et al, 1970b). These enzymes attacked the microfibrillar skeleton by splitting either inter- or intra-molecular bonds. These dissociated fibrils (thinned out or broken) could no longer withstand the high turgor pressure of the cell and so became stretched out or separated from one another. This gave, consequently, an increase in surface area of the wall. A similar

attack may also be necessary on the amorphous matrix of the wall. Next, microfibril synthesising enzymes, operating either in the wall itself or on the outer surface of newly formed plasmamembrane, rebuild the microfibrils. The synthesising enzymes may of course have been brought to the wall region via the vesicles. However, the vesicles do bring the precursors necessary for microfibril rebuilding. They also deposit amorphous material into the wall region, the turgor pressure of the hyphae forcing the material through the microfibrils to the outer surfaces of the wall. In this way the wall has expanded one unit area without losing any of its overall properties, although how the polar growth of the hyphal tube is maintained is as yet unknown.(Bartnicki-Garcia, 1973).

YEAST-MOULD DIMOPHISM

It has been found however that if the environmental conditions are altered, then in some fungi a change from polar to non-polar growth (or vice-versa) can occur. Such a change or DIMOPHISM from a mycelial (M-form, polar growth) to a yeast-like (Y-form, non polar, spherical growth) development is often dependent upon several factors, these being either (a) temperature, (b) temperature and nutrient conditions (this phenomenon having been little studied), (c) nutrient conditions. Bartnicki-Garcia and Nickerson (1962a) found that if sporangiospores of Mucor rouxii were allowed to germinate under air or under nitrogen gas, outgrowth of germ tubes occurred followed by a development of vegetative,

filamentous hyphae. Introduction of carbon dioxide into an anaerobic atmosphere, however, incuded the development of spherical, budding yeast-like cells. Linked to the effect carbon dioxide had on development in M.rouxii, Bartnicki-Garcia and Nickerson (1962b) postulated that these changes influenced the cell wall polymers of the fungus. The cell wall is the structure which ultimately is responsible for cellular morphology, so any alteration in its structure would affect the form the fungus developed. Upon isolation of cell walls from both filamentous and yeast-like forms, it was found that the yeast-form had significantly more mannose and protein. Chitosan was the main component in both types of wall, with chitin present in smaller quantities (Bartnicki-Garcia and Nickerson, 1962c). Also, electron microscopy of thin sectioned material revealed that filamentous-type walls were only one tenth as thick as yeast cells.

The difference in wall chemistry between the Y- and M-forms has also been observed in some pathogenic fungi. These fungi develop in their pathogenic stage by a yeast-like growth, but when not in their host they often have a mycelial form of growth. If the temperature under which they are being cultivated in vitro is lowered from 37°C to 20°C, then their development changes from the Y-form to the M-form. The Y-forms of Paracoccidiodes braziliensis (Kanetsuna & Carbonell, 1970), Blastomyces dermatitidis

(Kanetsuna & Carbonell, 1971), Histoplasma capsulatum (Kanetsuna & Carbonell, 1974) and Histoplasma farciminosum (San-Blas & Carbonell, 1974) have an alkali soluble glucan (α-1,3 glucan) as their main glucose polymer in the walls. A β-1,3 glucan is the lesser component in all except in P.braziliensis, where it is absent. However, in the M-forms, β-1,3 glucan is the main carbohydrate polymer in P.braziliensis, H.capsulatum and H.farciminosum, and no α-glucan is found. In B.dermatitidis, the M-form has 60% α-glucan, 40% β-glucan, as opposed to 95% and 5% repsectively in the Y-form. Chitin was present in both Y- and M-forms.

It has been suggested that the α-glucan is a polymer more flexible in property, thus more amenable to a budding-type of growth involving cell swelling. The β-1,3 glucan is a more rigid component of the cell wall helping to maintain the tubular shape of the hyphae. Manners, Masson and Patterson (1974) working with various yeast glucan preparations, postulated that one possible function of β-1,6 glucan could be to serve as a filling material or plasticiser within the relatively rigid wall framework provided by the β-1,3 glucan. Its function could be physically to prevent excessive hydrogen bonding or other means of aggregation of essentially linear segments of chains of β-1,3 linked D-glucose residues. This would mean that the wall retained some measure of flexibility, as is required for example, for wall expansion during growth. The β-glucan fibres, by end to end linkage, would be able to form a cylindrical hyphal tube, with polarised tip growth being

a consequence of this type of wall structure.

Such a type of development was thought by Kanetsuna, Carbonell, Azuma & Yamamura (1972) to be helped by proteins present in the cell wall. The proteins may have some degree of rigidity because of the high amount of disulphide linkages present in M-form walls, and also the low activity of protein disulphide reductase which could possibly prevent the balloon like growth of β-glucan. They found that cell-free extracts of the whole cells of the Y-form of P.braziliensis had five times more protein disulphide reductase activity than the M-form. The M-form cell wall contained twelve times more disulphide linkage than the Y-form. These findings, together with a theory put forward earlier where protein disulphide reductase played an important role as a division enzyme Candida albicans (Nickerson & Falcome, 1956) (where a mutant lacking this enzyme was filamentous in form), directed Kanetsuna and his coworkers (1972) to suggest the involvement of this enzyme in dimorphism. They also regarded chitin as playing a role in the M-form development. This was because β-glucan could only be extracted by alkali after prior hydrolysis of chitin by chitinase enzyme. In other words, fibres of protein and chitin may be interwoven with fibres of β-glucan in the M-form, giving the wall greater mechanical integrity for maintaining its shape. By way of contrast, in the Y-form, the α-glucan and chitin form two separate layers. That such a close

relationship between glucan and chitin is possible is supported by the work of Stagg and Feather (1973). Working with Aspergillus niger hyphal walls, they found that up to 50% of the wall was alkali-resistant. And the alkali-insoluble fraction was found to contain a substantial amount of glucan, the rest being chitin. The glucan was separated from the chitin (with which it was closely associated) by acetylation. This allowed for the partial separation of the two components, the glucan acetate being soluble in chloroform.

THE CELL WALL IN DEVELOPMENT - MICROCYCLE CONIDIATION AND FRUCTIFICATION

Sporangiospores of Mucor rouxii do form germ tubes under anaerobic conditions in the presence of carbon dioxide (Bartnicki-Garcia and Nickerson, 1962a). They develop by spherical swelling, that is, a yeast-like growth. A similar phenomenon has been observed in germinating conidia of Aspergillus niger by Anderson and Smith (1972). They found, however, that germ tube outgrowth did not occur at a temperature of 44°C. All conidia produced germ tubes at 30°C, with a decrease in germ tube production occurring as the temperature was raised (38°C to 43°C). At 44°C, the conidia continued swelling till giant cells were formed. By incubating the conidia initially at 44°C for 48 hours and then shifting the temperature to 30°C, an interesting development took place. The large, swollen conidia produced conidiophores directly without the normal intervening mycelial phase which happens with conidia cultivated solely at 30°C (Anderson & Smith, 1971). These conidiophores were similar to, but smaller, than normal conidiophores, and produced viable conidia. The authors termed this form of development, MICROCYCLE CONIDIATION. Just as Bartnicki-Garcia and Nickerson (1962b) postulated that the

change to a spherical yeast-like growth in M. rouxii influenced the differences in the cell wall between the hyphae and yeast cells, it would be interesting to see what changes, if any, occurred in the walls of giant swollen cells of A.niger.

Fructification, which starts in fungi after the carbon source in the medium has been depleted, has been linked to the presence of a wall polymer in Aspergillus nidulans (Zonneveld, 1972). Analysis of cell walls of A.nidulans revealed that an α-1,3 glucan was present (22% dry weight of complete two day old cell walls) (Zonneveld, 1971). It was noticed, that if α-1,3 glucan synthesis was inhibited by 2-deoxy-glucose, then no cleistothecia were formed (Zonneveld, 1973). Development in A.nidulans could be divided into three main stages (Zonneveld, 1972):- (1) hyphal growth, 0-3 days; (2) conidiospore formation, 0.5 - 3 days; (3) cleistothecium formation, 3 - 7 days. If glucose was the carbon source, then it was mainly depleted by the third day. Coupled with this was an increase in α-1,3 glucan content, starting on the first day, reaching a peak by the third day, nearly all depleted by the seventh day. Activity of α-1,3 glucanase enzyme was evident by the third day, reaching a maximum on the fifth and being reduced to nearly zero by the ninth day. Linked with practically the total depletion of glucose and the maximum content of α-1,3 glucan on the third day, was the achievement of the maximal dry weight. From these findings, Zonneveld (1972) deducted that as no carbon source was available to the fungus after the third day for cleistothecium development, then the role was filled by α-1,3 glucan. The glucan was therefore stored up in the

walls mainly for use as a reserve polymer. Thus as glucan is depleted, the α-1,3 glucanase activity increases to breakdown the α-1,3 glucan in the walls. In this way, the fungus ensures its further development, even though the growth associated carbon source has been used up completely.

WALL POLYMERS

Carbohydrates are the largest components of fungal cell walls, approximately 80 - 90% of the wall dry weight. Proteins, together with lipids and melanins are also present. The most common carbohydrates are polymers of glucose (glucans), mannose (mannans), galactose (galactans) plus amino sugars such as N-acetylglucosamine (chitin), whilst chitosan is a poorly or non-acetylated polyglucosamine. The various linkages encountered most often in glucans are presented in Table 7. Gorin and Spencer (1968) have written a review discussing the various polysaccarides, and the nature of their linkages, which are found in fungal walls. Chitin is found in many fungi (Bartnicki-Garcia, 1968a), as are β-1,3 glucan and β-1,6 glucan. These glucans are found, for example, in Neurospora crassa, Schizophyllum commune, Phytophthora parasitica (Hunsley & Burnett, 1970); in Aspergillus nidulans (Bull, 1970b); Trichoderma viride (Benites, Villa & Acha, 1975); Tremella mesenterica (Reid & Bartnicki-Garcia, 1976). A glycoprotein has been detected in N.crassa (Hunsley & Burnett, 1970); α-1,3 glucan in S.commune (Wessels, 1965), in A.nidulans (Bull, 1970b; Zonneveld, 1971), in Cryptococcus species, a soil yeast (Bacon, Jones, Farmer & Webley, 1968). Mannans have been detected in many yeasts, for example, in species of Candida (Ballou, Bishop, Cooper, Hasenclever & Blank, 1967) and Saccharomyces (Sandula, Sikl & Bauer, 1973).

WALL ARCHITECTURE

Much of this analytical work has been coupled with ultrastructural investigations. Hunsley and Burnett (1970) investigated the structure of hyphal walls of Neurospora crassa, Schizophyllum commune and Phytophthora parasitica. They examined samples under the electron microscope using either the techniques of metal shadowing or thin sectioning. Electron microscopy was used after subjecting hyphae to sequential treatments with various enzymes. Chitinase, laminarinase and pronase were used on Neurospora and Schizophyllum; cellulase, pronase and laminarinase on Phytophthora. The treatment helped to reveal not only the hyphal wall architecture, but also provide information as to the nature of the wall polymers. Hunsley and Burnett (1970) were able to build up a good picture of the walls of these three fungi.

In shadow cast preparations, N.crassa hyphae had a somewhat granular appearance on the outer surface. Chitinase and pronase were without effect on this outer layer. But laminarinase removed it to reveal a network of coarse strands. The interstices were filled with amorphous material, which could be removed by pronase treatment. This gave a clearer picture of the reticulum layer. The fact that it was a true wall layer was shown by treating intact hyphae with brief (5 mins.) ultrsonication, upon which, the reticulum was revealed. Continued treatment with a laminarinase/pronase treatment had the effect of removing the reticulum by sliding it off the underlying wall layers, or by degrading it. An amorphous region of low electron density showed

up, but with further enzyme treatment a layer of microfibils was exposed. The network of microfibrils was degraded by a sequential treatment of laminarinase/pronase/chitinase. In thin sections, three wall layers could be seen, the central layer being electron opaque and much thinner than the other two. Hunsley and Burnett (1970) suggested that in N.crassa the outer layer was probably an amorphous glucan containing β-1,3 - and β-1,6 - linkages. The reticulum could possibly be a glycoprotein embedded in amorphous protein material. The protein increased in concentration into the wall, so forming a discrete layer under the reticulum. The layer of microfibrils was chitin.

S.commune, like N.crassa, also had an amprphous appearance on its outer surface, but was less coarsely granular. Laminarinase, pronase or chitinase had no effect however on this layer. Wessels (1965) had earlier shown that S.commune has an alkali soluble α-1,3 glucan in its walls. After KOH treatment, Hunsley and Burnett (1970) found that the wall was now irregularly roughened in appearance in the sub-apical region, but less so at the apex. In thin sections, the thickness of the four layered wall had been reduced from 210nm to 120nm coupled with a loss of electron density, especially in the outer region. They presumed that the α-1,3 glucan had been removed. Further treatment with laminarinase revealed a more finely granular amorphous layer. Microfibrils were just visible beneath this material. Pronase treatment at this stage revealed the randomly arranged network of microfibrils. Chitinase treatment virtually caused their total dissolution. From these findings, the

authors suggested that the outer wall of S.commune is an amorphous layer of α-1,3 glucan. Beneath it is another amorphous layer, but this time a mixture of β-1,3 and β-1,6 glucans. They cover a discrete layer of protein which when removed reveals a layer of chitin microfibrils.

Both pronase and cellulase treatments had little effect on the finely granular amorphous outer wall layer of P.parasitica. After treatment with laminarinase, microfibrils became distinguishable underneath the amorphous material. If a laminarinase/pronase treatment was employed, then the microfibrils appeared much sharper and clearer. Treatment with laminarinase/cellulase caused the almost total digestion of the firbils. With this data, Hunsley and Burnett (1970) proposed that the outer amorphous layer was a glucan containing β-1,3- and β-1,6-linkages. The inner layer was that of cellulose microfibrils embedded in protein material.

On the basis of their observations, Hunsley and Burnett (1970) suggested that the innermost area of protein plus chitin (or protein plus cellulose in P.parasitica) together with a little outer glucan form a mechanically intact, but non-rigid wall at the apex. As development proceeds away from the apex, an increasing degree of rigidification is applied. The additional strengthening is probably caused either, (a) by reticulum development plus outer glucan in N.crassa;(b) by the α-1,3 glucan outer layer in S.commune; (c) by outer glucan in P.parasitica. Manners and his colleagues (1974) had suggested that β-1,6 glucan could serve as a filling material or plasticiser within the relatively rigid wall framework provided by β-1,3 glucan. It would be interesting to see whether at the hyphal apex there is more β-1,6 glucan than in the subapical region.

The β-1,3 glucan is moved involved in helping to give the wall the rigidity it needs to maintain its shape.

Cyst walls of Phytophthora palmivora are distinctly microfibrillar on both their outer and inner surfaces (Tokunaga & Bartnicki-Garcia, 1971). The isolated walls appeared as a thin fabric of long, tightly interwoven, randomly orientated network of fibrils. Upon germination, however, the emergent germ tube had a smooth outer wall. The cyst walls (and sporangial walls) were completely solubilised by a mixture of β-1,3- and β-1,4- endoglucanases. Exo-β-1,3-glucanases had little effect on the structure of the cyst microfibrils. Working with isolated walls, Tokunaga and Bartnicki-Garcia (1971) were able to subject the walls to chemical analysis. Glucose was the main sugar found to be liberated from cyst and hyphal walls upon either acid hydrolysis or enzymic degradation, together with some laminaribiose, cellobiose and gentiobiose on partial acid hydrolysis of walls. From this, Tokunaga and Bartnicki-Garcia (1971) deducted that the walls were composed of a glucan with β-1,3, β-1,4, and β1,6 linkages. Treatment of the hyphal walls of the emerging germ tube with β-1,3 - exoglucanase revealed a layer of microfibrils continuous with those of the cyst walls. Therefore, the germ tube wall had an outer layer of β-1,3 glucan. The cyst microfibrils were predominantly a β-1,3 glucan with varying amounts of the other linkage (β-1,4; β-1,6). The differences in the proportion of the linkages probably reflected the structural variations seens in the different wall layers (Tokunaga and Bartnicki-Garcia, 1971). Enzyme treatments of the walls as carried out by Hunsley and Burnett (1970) and by Tokunaga and Bartnicki-Garcia (1971), when coupled to electron

microscopic observation, reveal a great deal about fungal wall structure and its possible functions.

However, certain problems do exist in the use of enzymes for the detection of wall layers. The preparation must be pure, or at least have a known spectrum of hydrolytic activity. Other problems that must be faced are:- (1) is the substrate still susceptible to attack? (2) is it accessible or is it masked by thin layers of encrusting material, and (3) are there inhibitors of the enzymes present in the wall or incubation medium? Enzyme treatments, although very useful, should not be used alone. Chemical techniques are also available to provide information about wall structure and composition. Kreger and Kopecka (1975) showed that protoplasts of the yeast Saccharomyces cerevisiae were able to produce fibrillar nets. These nets were also visible in normal walls of the yeast, forming an entire inner layer. The nets were alkali soluble and upon neutralisation of the alkali solution the microfibrils were precipitated out. Their fibrillar organisation could be seen in metal shadowed preparations under the electron microscope. By subjecting the precipitated material to X-ray diffraction analysis, and comparing with known standards of glucans, the material was identified as a β-1,3 glucan. In a similar way, chitin was also identified in the yeast wall. Wang and Bartnicki-Garcia (1970) also subjected the glucan and chitin wall components of the yeast-form of the dimorphic fungus, Verticillium albo-atrum to X-ray diffraction analysis. They showed that the walls contained an alkali insoluble microfibrillar network. This was composed mainly of β-1,3 glucan and

was soluble in hot acid. After such treatment, the acid insoluble glucan contained β-1,6- linkages, together with chitin. These various types of glucans were demonstrated in the X-ray diagrams when compared with known glucans from other organisms.

Infra-red spectroscopy is another useful tool in analysing cell walls. It is especially helpful in determining whether α- or β-glucans are present in the carbohydrate preparation. Barker, Bourne, Stacey and Whiffen (1954) investigated the infra-red spectra of a very wide range of carbohydrates, and came to the following conclusion. The technique offered a powerful means for the comparison of supposedly identical samples of carbohydrates. The spectra over the frequency range 730-960cm^{-1} enabled derivatives of D-glucopyranose to be assigned to either the α- or β-series. The α-anomers absorbed at 844 $\pm$ 8cm^{-1}, and the β-anomers at 891 $\pm$ 7cm^{-1}. In addition, with the -anomer, it is possible from a consideration of absorbances in the bands at either 917 $\pm$ 13cm^{-1} or 766 $\pm$ 10cm^{-1} to obtain information about the position of such α-linkages. Unfortunately, no such correlation is possible with β-anomers.

Carbonell, Kanetsuna and Gil (1970) had observed that in the yeast-form of Paracoccidiodes braziliensis the outer wall was composed of short fibres. These were alkali soluble, and could be precipitated out of alkali solution by neutralisation with glacial acetic acid. The material was subjected to infra-red spectroscopic analysis, and revealed absorbtion peaks at 820, 845 and 925cm^{-1} (Kanetsuna & Carbonell, 1970), peaks which were consistent with those obtained for α-1,3 glucan by Zonneveld (1971) in Aspergillus nidulans, in Schizophyllum commune by Wessels, Kreger, Marchant, Regensberg and

De Vries (1972), and by Bacon et al (1968) in Cryptococcus species.

Most of the work with cell walls has been carried out with isolated wall fractions. The work of Hunsley and Burnett (1970) with intact mycelium is a notable exception. However, as Taylor and Cameron (1973) pointed out in their review, dealing with the problems and pitfalls concerning fungal cell wall analysis, it remains unlikely that the wall preparation can be sensibly equated with the functional cell wall of the organism from which it was isolated. It was with this problem in mind why whole fungal spores of Syncephalastrum racemosum were subjected to sequential chemical treatments. Therefore, not only was it possible to recover the various wall fractions from the various stages of the treatments, but also to observe microscopically the new wall polymers revealed at each stage. These observable wall layers probably resemble more closely the true appearance of the polymers in the wall than those seen in isolated wall preparations. Even so, it must always be borne in mind that whether working with intact hyphae and spores or with isolated walls, the chemical treatments may affect the structure of the wall polymer under observation.

A wide variety of techniques now exist to help in the analysis of fungal walls. Such techniques reveal the type of carbohydrate present, the nature of the linkages, the quantitative analysis, the ultrastructure. Some of these methods are listed in Table 8. They can also include the study of cell wall regeneration in protoplasts (De Vries, 1974; Kreger & Kopecka, 1975). Gel electrophoresis, coupled with amino acid analysis, can be exploited for the investigation

of proteins and glycoproteins (cf. Chapter Four).

This chapter will deal with a study of the ultrastructure and chemistry of the inner wall layers of S.racemosum dormant spores.

MATERIALS AND METHODS

ORGANISM, GROWTH CONDITIONS, SPORE PRODUCTION AND HARVESTING

The laboratory strain of Syncephalastrum racemosum was grown and harvested as set out in the materials and methods section of Chapter Four.

CHEMICAL TREATMENTS AND EXTRACTION OF WALL LAYERS

The sequential chemical treatments with the points at which samples were taken for electron microscopic (EM) observation are shown in Table 9. After harvesting of the spores, they were incubated on an orbital shaker with 5% KOH for 6h at 37°C. After three washes in sterile distilled water, a small aliquot of the sample was removed for EM observation. The rest of the sample was subjected to further incubation with 5% KOH at 37°C for 18h, washed three times in sterile distilled water, and a small aliquot removed for EM observation. The remaining sample was autoclaved in a small screw cap bottle with a 1:1 (v/v) mixture of hydrogen peroxide (H_2O_2) and glacial acetic acid (CH_3COOH) at 121°C for 10 mins. After three washes in sterile distilled water, some of the sample was removed for EM observation. The sample was subjected to treatment with 5% KOH at 100°C for 1h. After centrifuging (all centrifuging was at 4000rpm for 2 mins.) the alkali supernatant (A) was kept and treated as per Table 10. The remaining pellet was washed three times with sterile distilled water and observed in the electron microscope. This was a layer of thin microfibrils, the only material now left from the entire spores. It was dried and stored in a dessicator at room temperature for chemical characterisation.

Supernatant (A) was centrifuged at 12000rpm for 20 mins. to remove any contaminating material (Table 10). To (A) was added

cold absolute ethanol (-30°C) to give a final concentration of ethanol in solution of 70%. The solution plus its precipitate was stored overnight at 4°C. The next morning, the solution was centrifuged at 12000rpm for 20 mins., the supernatant discarded, the pellet washed three times with distilled water, and stored in a dessicator at room temperature to dry. The pellet corresponded to the layer of thick microfibrils observed in the results section.

TOTAL HYDROLYSIS

(a) Thick Microfibrils

A small aliquot of the sample was placed in an empty, dry, clean Quickfit MF24/0 glass tube; the sample was incubated with 0.2ml. 1M H_2SO_4 overnight at 37°C. It was then placed in a water bath (96- 97°C) for 7.5h, cooled, 0.8ml. distilled water added and neutralised with 0.4g $BaCO_3$. The solution was thoroughly shaken and centrifuged at 12000rpm for 20 mins. The pellet was discarded and the supernatant freeze-dried overnight in an Edwards Freeze Drier (Model B5A) at 0.05 torr.

(b) Thin Microfibrils

A small aliquot of the sample (in a Quickfit MF24/0 glass tube) was incubated overnight with 0.1ml. of 70% H_2SO_4 at room temperature. Next day, 1.2ml. of distilled water was added to give a concentration of 1M H_2SO_4, and the solution heated in a water bath (96 - 97°C) for 6h. The tube was cooled, 5.2ml. of distilled water added, and the solution neutralised to around pH6.0 - 6.2 with 2.6g $BaCO_3$. The solution was shaken thoroughly, centrifuged at

12000rpm for 20 mins., and the supernatant freeze-dried overnight in the same way as the thick microfibril sample.

CHROMATOGRAPHY

After freeze-drying, both samples were resuspended in 0.1ml. distilled water, spotted onto Whatman No.1 chromatography paper, and the chromatograms were run by the descending technique for 48h. The solvent system used was ethyl acetate/pyridine/water in a ratio of 8/2/1. Spots were developed with alkaline silver nitrate. R-glucose values (R_G) were all calculated from the central glucose spot run together with two other standard sugars.

INFRA-RED SPECTROSCOPY

I.R. spectroscopy was performed by the pressed KBr disc method using a small amount of the appropriate dried wall material and 0.5g KBr. I.R. spectra were obtained on a Perkin-Elmer 457 Grating Infrared Spectrophotometer.

SUGAR STANDARDS

Purified crustacean chitin for I.R. spectroscopy was obtained from B.D.H. Ltd. Sugars for chromatography (glucosamine-HCl, galactosamine-HCl, mannose, galactose) were obtained from Koch-Light Ltd. Glucose was from B.D.H. Ltd.

ELECTRON MICROSCOPE OBSERVATIONS

Surface replica and freeze-fracturing techniques on spore samples were carried out as described in the materials and methods section of Chapter One.

Metal shadowing was carried out using only carbon-platinum electrodes at an angle of 45° (cf. materials and methods, Chapter

One) on material dried down onto formvar coated grids.

Preparation of material for thin sectioning

Samples were fixed in 2.5% glutaraldehyde in cacodylate buffer solution (pH7.4) overnight. Washed for 30 mins. with cacodylate buffer (pH7.4) - two changes. They were then post-fixed in 1% osmium tetroxide for 2h and washed several times with distilled water. The material was dehydrated through a graded series of alcohol and embedded in Spurr's resin (Spurr, 1969). Thin sections were cut on a LKB Ultratome III using glass knives. Sections were stained in 5% uranyl acetate for 10min. and then post-stained in Reynold's lead citrate (1%) for 5mins.

All samples were observed in an AEl 801A transmission electron microscope.

RESULTS

ULTRASTRUCTURAL STUDIES

(1) Initial alkaline treatment

After 6h in 5% KOH at 37°C, the surface rodlet layer had been removed revealing a smooth appearance to the spore wall (Figs. 69 and 70). Further treatment of the spores with 5% KOH at 37°C for 18h gave them a more roughened appearance, the smoothness of the wall as seen in Figure 70 having disappeared (Fig. 71). In Figure 71 a general view of the spores is seen, the treatment being very effective in that all the spores have a rough surface. Microfibrils seeming to be located beneath this rugose layer can also be observed at the edge of certain spores (Fig.71). Higher magnifications of this new wall layer confirmed its rough nature (Figs. 72 - 76). Also, the wall layer is seen as having many wrinkled bumps covering it. These bumps have been uncovered by the prolonged 5% KOH treatment for 18h, so that beforehand they were probably embedded in an amorphous layer which had been initially observable after 6h 5% KOH treatment (Fig.70). The structural integrity of the spores is still maintained as evidenced by the micrographs (Figs. 72-75).

(2) Acetic acid/peroxide treatment

As further KOH treatment had no effect on the rugose wall layer, the spores were subjected to autoclaving with a 1:1 (v/v) mixture of H_2O_2 and CH_3COOH. This process had very interesting results, for it gave spores a characteristic "fried-egg" appearance, together with revealing a layer of thick microfibrils embedded in an

amorphous matrix (Figs. 77-79). These micrographs show the reproducibility in obtaining the "fried-egg" appearance after autoclaving. In Figure 77, the layer of thick microfibrils can clearly be seen as forming a cross-patch network of tightly-knit fibrils. These thick microfibrils have an average diameter of 17.5nm. In Figs. 78 and 79, the microfibrils are again visible but are less distinct because of being embedded in amorphous material. Figure 80 shows two spores side by side, with the one on the right showing the layer of thick microfibrils very clearly, the one on the left less so, the fibrils being embedded in the amorphous matrix. Figure 81 shows a similar situation. The closely interwoven microfibrils are a very repetitive feature of this layer, and close examination of the fibrils has failed to produce any hints of whether the 17.5nm diameter microfibrils are composed of smaller units (Figs. 82 - 84). The amorphous matrix, although obsuring the thick microfibrils in some cases (Figs. 77 - 81), is present among the fibrils (Figs. 82 - 84). Higher magnifications reveal in more detail the criss-cross network of thick microfibrils (Figs. 85 and 86).

Observable in the series of Figures 77 - 84 is material which could either be considered contaminating aggregates resulting from the autoclaving, or an actual wall layer which had been removed, but was not soluble in the H_2O_2/CH_3COOH solution. Sheets of this material are seen in the "fried-egg" preparations (Fig. 79). The sheets on closer examination (Fig. 87) look similar to the rugose layer revealed after the initial 18h KOH treatment (Fig. 71 - 76). Although the plate-like layer in some cases covers partially or totally the "fried-egg"

spores, this could just be caused by the sheet material settling down on the spores after removal by autoclaving and prior to taking samples for EM observations (Fig. 88). This plate-like layer also has wrinkled bumps on it (Figs. 87 and 88), similar to those seen in earlier preparations of the rugose wall layer (Figs. 72 - 74).

(3) Second alkaline treatment

The layer of thick microfibrils was removable with 5% KOH treatment at 100°C for 1h. Examination by metal shadowing of the residue revealed a layer of thin microfibrils of an average diameter of 8nm (Figs. 89 and 90). The thin microfibrillar net was composed of long fibrils, criss-crossing an underlying fibrillar mesh (Figs. 89 and 90). In figure 91, the criss-cross network is seen in greater detail, as are the longer, overlying microfibrils. Higher magnifications of these longer microfibrils revealed that they are probably formed of aggregates of several fibrils (Fig. 92). Figure 93 also shows these fibrillar aggregates of diameters ranging from 16 to 26nm. As EM observations revealed that the residue after the chemical treatments was just a layer of thin microfibrils, extraction of this wall layer was not essential, so it was simply washed in water and dried in a dessicator for chemical analysis studies (cf. materials and methods section).

(4) Thin sectioning

These sequential chemical treatments have revealed the appearance of various wall layers, but have not given any idea of how thick these layers are, or even how thick the spore wall is. Figure 94 shows a thin sectioned spore. The fixation is not very

good because of the thick and complex nature of the wall, as seen in the preceding micrographs. The spores not only fixed badly, but also took up resin very poorly because of insufficient dehydration. Because of these fixation problems, numerous attempts with varying conditions were tried, but no effective fixation procedure was found. However, the thin section of the spore wall under higher magnification (Fig. 95) revealed the wall as having three distinct layers:- (1) an outer layer, A; (2) a middle electron opaque layer, B; and (3) an inner electron translucent layer, C. Layer A looks like being both electron translucent and opaque, and not even surrounding the spore (Fig. 94). This could be due to the stages of preparation of the material for EM observation, so causing some of the outer layer to peel away from the spore. The plasmamembrane is also visible in both micrographs. (Figs. 94 and 95).

The electron micrographs reveal that with the successive chemical treatments employed, there is loss of cytoplasmic contents from the spores. The gradual loss in shape of the spores suggests this point. Autoclaving produces the most pronounced change in shape, with probably the majority of the cytoplasmic contents being soluble in the H_2O_2/CH_3COOH mixture. Cellular debris appears to be absent from the micrographs of the fibrillar layers.

CHEMICAL ANALYSIS

(1) Layer of thick microfibrils

The layer of thick microfibrils was easily soluble in 100°C 5% KOH and precipitated well out of alkaline solution by addition

of cold ethanol. However, yields of this material were very low. This was so even if the spores were harvested from many Roux bottles. Material from several extractions (one extraction was from 80-100 Roux bottles) was pooled together to give enough of the sample for infra-red spectroscopic analysis. Around two extractions gave enough for total hydrolysis procedures.

Total hydrolysis gave only one sugar spot which ran like glucose (Fig. 96) on paper chromatograms. The R_G value was 1.02, exactly the same as a test glucose spot (Table 11, position F; spot F, Fig. 96). Glucose fraction F was treated in the same way as the wall sample for the total hydrolysis procedure. No galactose or mannose was detected nor any other sugar spot. The result showed that the monosaccaride content of the thick microfibrils was solely glucose, indicating that this layer is probably a glucan.

Descending paper chromatography was used, as it was found that this method applied for 48h gave the best separation of glucose, galactose and mannose. It was important to distinguish between these three sugars because of their preponderance in fungal cell wall polymers. Asoending thin layer chromatography using silica gel or cellulose plates was also tried, but failed to give a good separation of these three sugars.

The infra-red (I.R.) spectra of the thick microfibrils revealed a peak at $880cm^{-1}$ (Fig. 97) which is indicative of β-linkage between glucose units (Barker *et al*, 1954). Compared to the I.R. scans of laminarin (a β-1,3 glucan) from *Laminaria hyperborea* (Fig. 97), this sample has an absorbance peak at $885cm^{-1}$ indicative of β-linkage;

and both samples have absorbance peaks at 1620 - 1640cm^{-1}. However, the scans are not similar in the range 1000-1600cm^{-1}. It is suggested here that the thick microfibril preparation is contaminated with some other material such as protein. This may either be some of the plate-like layer, or perhaps some of the amorphous material in which the layer of thick microfibrils is embedded, or contaminating cytoplasmic protein. If the laminarin of L.hyperborea is mixed 1:1 (w/w) with a standard protein (bovine serum albumin), the peak at 885cm^{-1} is still obtained, but the scan in the range 1000-1700cm^{-1} is changed, such that the absorbance peaks normally detected in pure laminarin have been masked (Fig. 97). Therefore, it is possible that proteins may mask the normal I.R. scan of glucans, a possible occurrence in the I.R. scan of the thick microfibrils (Fig. 97).

(2) Layer of thin microfibrils

The residue after hot 5% KOH treatment was found to be just a layer of thin microfibrils (Table 9). Total hydrolysis of this layer revealed two spots; one ran like glucose, the other like glucosamine (Fig. 98). The R_G values were identical to glucose (glucose = 1.0 (E), thin microfibrils = 1.0; Table 12) and to glucosamine (glucosamine = 0.36 (E), glucosamine = 0.35 (G), thin microfibrils = 0.35; Table 12). The result indicated that the layer of thin microfibrils was mainly composed of a polymer of glucosamine, presumably chitin/chitosan. The glucose spot on the chromatogram was of interest, in that it possibly indicates the carry over of glucan from earlier fractions or it may represent a hydrolysis product from a glucan specifically associated with the

thin microfibrils. Stagg and Feather (1973) characterised a chitin-associated glucan from the hyphal walls of Aspergillus niger, this glucan having a ratio of α- to β-linkages of 4:1. They separated the glucan from the chitin by acetylation of their alkali-resistant material and found it contained 85-90% 1,3-glucosidic linkages and 10-15% 1,4-linkages. Their glucan could therefore well be a mixture of α-1,3 glucan and β-1,4 glucan, the latter providing a point of bonding to chitin. Perhaps an analogous situation exists between the thick and thin layers of microfibrils.

Infra-red spectroscopy of the thin microfibrils revealed absorbance peaks which were typical for chitin (Fig.99) - peaks at 2900, 1645, 1555, 1425, 1375, 1315, 1265, 1200, 1155, 1065, 1025, 960 (a shoulder), 900cm.$^{-1}$ The scan closely resembled that of commercial chitin (B.D.H. Ltd) (Fig.99) which had absorbance peaks at 3100, 2930, 2880, 1630, 1550, 1415, 1375, 1310, 1260, 1205, 1155, 1110, 1065, 1025, 950 and 895cm^{-1}. A comparison of I.R. scans of S.racemosum chitin and chitins from other organisms (together with commercial chitin) is presented in Table 13. The S.racemosum sample does not have as many absorption maxima as the other chitin samples (S.racemosum has 12 peaks, commercial chitin 16; Table 13). Also, it does not have the peak characteristic for chitosan at 1590cm^{-1}. Chitosan differs from chitin in the range 800-2000cm^{-1}, especially in having weaker bands near 1640cm^{-1} and 1550cm^{-1} (Michell & Scurfield, 1967). Table 13 clearly shows that the thin microfibrils of S.racemosum have 12 absorbance peaks common to chitin I.R. scans, and in this respect compares favourably with chitin samples from

other fungi. It has 12 peaks in common with the B.D.H. Ltd. commercial chitin and 11 peaks in common with commercial standard crab chitin obtained by Michell and Scurfield (1967).

DISCUSSION

In the introduction to this chapter, mention was made of some of the problems that might be associated with sequential enzymic treatments to obtain a model of fungal wall architecture. Enzymes were not used in this study for some rather specific and compelling reasons. Firstly, Syncephalastrum racemosum spores contain melanin pigement, and it has been very clearly shown that such melanins can effectively inhibit the functioning of carbohydrate degrading enzymes (Bull, 1970a; Chet & Henis, 1969). Secondly, there is evidence from fungal spore systems that proteins found in spore walls can be resistant to proteolytic attack (Hashimoto, Wu-Yuan & Blumenthal, 1976; Kitajima & Nozawa, 1975; Amar, Delaumeny & Vilkas, 1976). For these reasons, chemical treatments were employed in this study, many of which have been used previously in studies of gross chemical compositions of fungal walls (Aronson & Preston, 1960).

One of the questions posed by the sequential chemical treatments of S.racemosum spores is what effects, if any, do they have on the various wall layers. Are the micrographs of these wall regions true representations of how they look in the spore wall, or have they been altered by the various procedures? This is in fact a question all electron microscopists face. In defence of the treatments employed here, it is pointed out that the initial treatment is 6h plus 18h in 5% KOH at 37°C. A total of 24h in KOH (Table 9). The alkali removes the surface rodlet layer, the underlying amorphous material and reveals a rugose or plate-like

layer. Autoclaving removes the plate-like layer, and reveals spores having a "fried-egg" appearance. (Fig. 79). In these preparations, two observations can be made. Firstly, the sheets of material seen in the micrographs (Figs. 79 and 87) are similar to the rugose layer revealed after the initial 24h 5% KOH treatment (Fig. 76). Therefore, the autoclaving treatment, although sounding rather harsh, has had little effect on this plate-like layer. Secondly, the layer of thick microfibrils are subsequently removed by 100°C 5% KOH treatment. Although being alkali soluble, the thick microfibrils are still present even after the intial 24h 5% KOH treatment. No deleterious effects appear to be seen in micrographs of the thick fibrils (Figs. 85 and 86), indicating that the initial 24h 5% KOH treatment did not affect them. This was probably due to the rugose or plate-like layer being impermeable to KOH, so perhaps also setting up a barrier to the entry of fixatives and resin into the spore. It would therefore make it difficult to prepare satisfactory enough samples for thin sectioning.

However, it is probable that some ultrastructural alteration must occur, but the evidence presented here points to the extraction procedure being selective for the various wall layers (Table 9). The chemical treatments therefore allowed for the extraction of various wall layers and their subsequent chemical analysis. The analysis were more concerned with a general identification of the wall layers rather than their total macromolecular characterisation.

The electron microscopic observations utilized mainly surface replica techniques which show the actual structure exposed by chemical treatment. Thin section micrographs, although helpful, are not on their own sufficient to provide information on wall architecture, for they only divide the wall into electron translucent and electron opaque areas. The micrographs presented in this chapter show the advantages of the surface replica technique.

With either a chemical or enzymic approach to wall analysis, little information exists on fungal spore wall structure. Papers have been published on the surface structures of spores, and thin section studies are also available. However a paucity of knowledge surrounds the actual configuration and chemical composition of the spore wall, making it difficult to relate the results obtained with S.racemosum to other organisms.

PLATE-LIKE/RUGOSE LAYER

The sheets of material observed in the "fried-egg" preparations after autoclaving treatment (Figs. 79 and 87) are similar to the rugose layer revealed by 24h incubation with 5% KOH (Figs. 72-74). The plate-like layer appears to be an impermeable barrier to the initial KOH treatment and possibly to fixatives and resin pretreatments in thin section material preparation. It is also fairly insoluble in the autoclave mixture of H_2O_2 and CH_3COOH, showing how resistant it is to various reagents. Its removal from the spores has the interesting property of causing the spores to lose their relatively spherical shape and become analogous to "fried-eggs" in appearance. The autoclave treatment appears to peel off the plate-like layer by splitting or cracking it, with the subsequent loss of shape incurred by the spores. This suggests that the rugose layer may be

partly responsible for maintaining the structural integrity of the spores.

As this layer was insoluble in the various reagents used, no chemical analysis was attempted, although a pronase or protease enzyme treatment would have been interesting. For it is possible that this layer could be a glycoprotein complex conferring mechanical strengthening to the spore, as does the glycoprotein reticulum in hyphae of Neurospora crassa (Hunsley & Burnett, 1970). Hunsley and Burnett (1970) using enzyme dissection techniques revealed a glycoprotein layer in hyphae of N.crassa as consisting of a network of coarse strands. They postulated that reticulum development, coupled with outer glucan deposition, formed a mechanically intact but non-rigid wall. This situation in N.crassa hyphae may be present in S.racemosum spore walls, the rugose layer linking with the surface layer of rodlets plus its underlying amorphous material (Figs. 69 and 70) to form a similar mechanically intact but non-rigid wall layer. Non-rigid, as evidenced by spore swelling during germination.

Further, the glycoprotein reticulum of N.crassa has been found to be a highly ordered structure of peptides linked to carbohydrate moieties, but not linked to other major wall components (Wrathall & Tatum, 1973). The ease with which the plate-like layer peels off during the autoclaving treatment revealing the layer of thick microfibrils is consistent with a theory of it not being too tightly bound to the underlying thick microfibrils, analogous to the situation in N.crassa (Wrathall & Tatum, 1973).

LAYER OF THICK MICROFIBRILS

The sole sugar spot detected after total hydrolysis of this wall component was glucose, indicating that the layer of thick microfibrils is probably a glucan. Infra-red spectroscopy revealed a peak at 880cm^{-1}, characteristic of β-linkage (Barker et al, 1954). The chemical analyses thus point to the thick microfibrils being a β-glucan, although because of difficulty in obtaining enough material analyses leading to deductions concerning the nature of the linkages was not possible. However, this layer is probably not cellulose (β-1,4 linkages), for few fungi have been found to contain both cellulose and chitin (the layer of thin microfibrils in S.racemosum) in walls. This combination has been only found in about one fungus of the Rhizidiomyces species (a Hyphochytridiomycete) but not in the higher fungi (Bartnicki-Garcia, 1968a).

It is possible that the S.racemosum β-glucan is a β-1,3 glucan with possibly β-1,4 and β-1,6 linkages included in the polymeric structure as well. This hypothesis is put forward because this seems to be the general β-glucan structure found in fungal walls. Cysts of Phytophthora palmivora are composed of two microfibrillar glucan layers, being predominantly β-1,3 linked, with different proportions of β-1,6 and β-1,4 linkages (Tokunaga & Bartnicki-Garcia, 1971). In Saccharomyces cerevisiae, β-1,3 glucan with β-1,6 branches is present (Manners, Masson & Patterson, 1973), this glucan having a microfibrillar structure, (Kreger & Kopecka, 1975). Hyphal walls of Pythium acanthicum have a microfibrillar layer of β-1,3 glucan with β-1,4 linkages, this layer being covered by an amorphous matrix of glucan material containing β-1,3 and β-1,6 linkages (Sietsma, Child, Nesbitt & Haskins, 1975). A microfibrillar layer

of β-1,3 glucan has been observed and analysed in Armillaria mellea (Jelsma & Kreger, 1975). Regeneration of new walls on protoplasts of Trichoderma viride showed a fibrillar network consisting of both β-1,3 and β-1,6 glucans (Benitez, Villa & Acha, 1975). Isolated vegetative yeast cells of the basidiomycete Tremella mesenterica contained β-1,3 and β-1,6 linked glucans (amorphous in appearance), besides also α-1,3 glucan (Reid & Bartnicki-Garcia, 1976). Amorphous layers of glucan containing β-1,3 and β-1,6 linkages were observed and identified in hyphal walls of Neurospora crassa, Schizophyllum commune and Phytophthora parasitica (Hunsley & Burnett, 1970).

The composition of the β-glucan wall components vary in the type of linkage present, these differences in the number of each linkage reflected in changes of the structural form of the glucan. The outward expression of these variations in linkages being visible as either a microfibrillar or an amorphous layer (Tokunaga & Bartnicki-Garcia, 1971). Manners, Masson and Patterson (1974) had postulated that β-1,3 glucan adds rigidity to the cell wall, the β-1,6 glucan acting as filling material or plasticiser to give the wall some measure of flexibility. This could mean that the fungus controls its morphological development by the ratio of β-1,3 to β-1,6 linkages in its wall polymers, not forgetting that the outer wall components may also play a part in this development.

The ultrastructural form of the thick microfibrils of S.racemosum is strikingly similar to the form of isolated and well

characterised β-glucan fibrils from Armillaria mellea. The β-1,3 glucan fibrils in A.mellea were found to be 7.5 - 15nm in diameter (Jelsma & Kreger, 1975), whilst those in Phytophthora palmivora were 10 - 15 nm wide (Tokunaga & Bartnicki-Garcia, 1971). The thick microfibrils of S.racemosum had an average diameter of 17.5nm. Kreger and Kopecka (1975) noted that in Saccharomyces cerevisiae, metal shadowed material had fibrils of 20-25nm diameter, whilst the glucan fibrils when negatively stained had diameters of 12-20nm. This suggests that various EM preparations can give different ideas of the sample under observation. Thus the importance of using several EM techniques to analyse the microfibril structure. Glucan microfibrils of S.cerevisiae obtained from regenerated walls of the yeast's protoplast (unlike old walls as above) had diameters of about 20nm (Kreger & Kopecka, 1975).

The evidence of both total hydrolysis, infra-red spectroscopy and ultrastructure together with the information gathered from work done on other fungal β-glucans, suggests that the layer of thick microfibrils in S.racemosum spore walls is composed of a β-glucan.

LAYER OF THIN MICROFIBRILS

Total hydrolysis products revealing a spot comparing favourably with glucosamine (Fig. 98, Table 12) plus the infra-red scan (Table 13), point conclusively to the layer of thin microfibrils being composed of chitin. S.racemosum chitin has an average diameter of 8nm. The diameter of chitin fibres in Histoplasma farciminosum yeast and mycelial forms is 4-6nm (San-Blas & Carbonell, 1974), these fibres forming bundles ± 50nm in diameter. The chitin microfibrils of

hyphal walls of Polyporus myllitae had diameters varying between 10 and 22nm (Scurfield, 1967). These wall fibrils had been exposed after a sequential chemical treatment involving extraction with hot 2% KOH, three extractions with a 1:1 (v/v) mixture of glacial acetic acid and hydrogen peroxide at 70°C for 1h, finally followed by boiling in 2% H_2SO_4 for 6h. The chitin appeared as an interwoven network of microfibrils. Their orientation changed in passing across the hyphal wall towards the inside of a hypha, by becoming much more strongly transverse to the longitudinal axis. They appeared much more densely packed together in the centre of the hyphal tube (Scurfield, 1967). Hunsley and Burnett (1970) noted chitin microfibrils in Neurospora crassa and Schizophyllum commune as being a randomly arranged network of criss-crossing microfibrils. Some of the fibrils appeared longer in length than others. Isolated walls of the vegetative yeast phase of Tremella mesenterica also had a randomly arranged network of chitin microfibrils on the surface (Reid & Bartnicki-Garcia, 1976). These observations are similar to those of S.racemosum chitin microfibrils, where the micrographs (Figs. 92 and 93) show a randomly orientated network of fibrils. Longer fibrils are visible on the surface. Some of these longer microfibrils are aggregated into bundles of about 16-26nm in diameter. They could probably give a greater mechanical strength or structural integrity to this layer.

During germination new chitin is probably synthesised. It may then play an important role in giving the new hyphal tube the mechanical strength and integrity it requires. Bartnicki-Garcia and Lippman (1969) observed wall growth during the swelling phase

of Mucor rouxii sporangiospores. An anaerobically germinating culture of M.rouxii spores (under nitrogen) was exposed to radioactively labelled N-acetyl-D-glucosamine. Autoradiographs were taken at various stages during swelling and germ tube emergence. At first, the label was incorporated uniformally over the whole spore. It showed that new wall material, probably chitin, was being synthesised. As swelling proceded, the label became noticeably aggregated to one part of the spore. This was the beginning of the transition from a non-polar, spherical growth to a polarised growth by tip extension. When the germ tube emerged, nearly all the label was found in the hyphal apex (Bartnicki-Garcia & Lippman, 1969). If the hyphal wall differs in structure from the spore wall, then probably only chitin of the spore polymers is synthesised during swelling. Therefore, the other wall layers are progressively weakened, either mechanically by the expanding spore, or by dissolution due to lytic enzymes. It could be a combination of both factors. The gradual weakening of the spore wall would allow the germ tube to emerge (cf. Chapter Six).

GENERAL PICTURE OF THE SPORE WALL

The sequential chemical treatments have revealed the spore wall of S.racemosum as having several complex layers:- (1) a surface rodlet layer; (2) an underlying amorphous layer; (3) a rugose or plate-like layer; (4) a layer of thick microfibrils; (5) a layer of thin microfibrils. Therefore essentially five wall layers are present, although the thin section micrograph of the spore reveals only three layers (Fig. 94). Three distinct wall layers were observed in conidia of Aspergillus nidulans in thin section

preparations, the total thickness of the wall varying from 246-515nm (Florance, Denison and Allen Jr, 1972). Spore walls of S.racemosum had a thickness of 140-160nm. Layer A was 36-56nm thick, layer B 20nm, and layer C 84nm. Two wall layers were observed in thin sectioned conidia of Collectrotichum legenarium (Akai & Ishida, 1968). Comparing the pictures of fungal walls obtained with thin sectioning and with either freeze-fracturing or surface replica techniques, it can be concluded that the three dimensional view of the wall observed with the latter two methods gives a great deal more information on wall architecture. However, few such studies have been linked to chemical analysis. Hunsley and Burnett (1970), Tokunaga and Bartnicki-Garcia (1971), Reid and Bartnicki-Garcia (1976), Kreger and Kopecka (1975) and Wang and Bartnicki-Garcia (1970) are just a few of the authors who have attempted both chemical and ultrastructural analysis. Young (1974), working with sporangiospores of Kickxella alabstrina, subjected them to chemical treatments and observed carbon replicas of the wall both before and after treatments. The outer wall consisting of amorphous material was removed by treatment with boiling 5% KOH to reveal an inner wall of microfibrils. No attempt was made at analysing chemically either the fibrils or even at recovering the outer amorphous layer from the alkaline solution. The work presented in this chapter on S.racemosum spores is therefore difficult to discuss in relation to other work because there is relatively little literature on spore walls. The above authors have all worked mainly with hyphal walls. Thus the results obtained with S.racemosum is probably the first critical attempt to characterise spores both

ultrastructurally and chemically. Problems were met with fixation of the spores for thin sectioning, and chemical analyses were limited by the amount of material that it was possible to extract. For example, after several extractions (one extraction being the total harvesting of spores from 80-100 Roux bottles) around 1-2mg of thick microfibrillar material was obtained. An awareness of the shortcomings of working with whole spores coupled with the lack of enough material to do fine biochemical analyses, meant that the chemical characterisations presented here are only tentative and do not cover the fine details of the wall. However, the results are very interesting, especially those dealing with spore wall architecture (cf. Chapter Four for the surface rodlet layer).

A summary of the results is presented pictorially in Figure 100. The spore wall is presented as being composed of five ultrastructurally identifiable layers. Layer A is postulated as being composed of two layers, an outer surface rodlet layer (the electron opaque area) and an underlying layer of amorphous material (less electron opaque, more electron translucent). This corresponds to what Sassen, Remsen and Hess (1967) observed in Penicillium megasporum. Using not only chemical fixation but also freeze-fracturing techniques, they drew up a picture of the spore wall as having three layers covered by an external surface rodlet layer. The rodlet layer was on top of a base layer, this base layer being observed in thin sections as just below the rodlet layer. It was distinct from the other three wall layers. Surface rodlets have also been observed on the spore surfaces of other Penicillium (Hess, Sassen & Remsen, 1968), and Aspergillus species (Hess & Stocks, 1969). Chemical analyses

of the surface rodlet layer of S.racemosum seem to indicate that it is a protein which may be closely associated with the underlying base layer, forming possibly a protein-glucan-melanin complex (cf. Chapter Four).

The rugose or plate-like layer is unique for it has not been seen in hyphal walls. Hunsley and Burnett (1970) observed a network of coarse strands which they proposed may be a glycoprotein reticulum. It could possibly help in maintaining the structural integrity of the Neurospora crassa hyphal tube by giving additional strength to the structure. Perhaps a similar role is played by the rugose layer in S.racemosum, for when this layer is removed by autoclaving, the spores have their more spherical appearance seen under the microscope and give the "fried-egg" appearance.

The layer of thick microfibrils was identified tentatively as a β-glucan. Again, this is a structure not seen in spores before, but it bore close resemblance to the microfibrils of β-1,3 glucan seen in Armillaria mellea hyphae (Jelsma & Kreger, 1975), in cyst walls of Phytophthora palmivora (Tokunaga & Bartnicki-Garcia, 1971) and in Saccharomyces cerevisiae cell walls (Kreger & Kopecka, 1975). Bartnicki-Garcia and Reyes (1964) had found that spores of Mucor rouxii had a high content of glucose and that this was mainly glucan. Bartnicki-Garcia (1968a) postulated that although hyphal walls of the Zygomycetes were composed of two main polymers, chitin and chitosan, the main component of the spore walls of this group was glucan. Therefore, it is possible that the large glucan component of the spores may be due to the thick microfibrils, at least in S.racemosum, for glucans can be both microfibrillar or amorphous in structural form

(cf. hyphae of Phytophthora palmivora (Tokunaga & Bartnicki-Garcia, 1971) where there is an outer wall of amorphous material and an inner microfibrillar layer - both are composed of β-glucan).

The innermost layer of the spore wall as shown in Figure 100 is the layer of thin microfibrils. They were identified as chitin by the chemical tests of total hydrolysis and infra-red spectroscopy. Chitin, as Bartnicki-Garcia (1968a) pointed out, is one of the two main hyphal wall components of the Zygomycetes. It is also present in spore walls. As in S.racemosum spores, in the hyphae it also forms the innermost wall layer (cf. Chapter Six). This is in keeping with the findings of Hunsley and Burnett (1970) who observed chitin microfibrils as being the innermost of the wall layers in hyphae of N.crassa and S.commune. The chitin in both these two fungi formed a criss-cross network of randomly orientated microfibrils as was observed also in spore walls of S.racemosum.

A model representation of the spore wall of S.racemosum is shown in Figure 101. The five wall layers are clearly shown, but are they really five distinct layers forming discrete layers of single components, or are they more likely to be interlinked so forming a more homogenous structure? Freeze-fracturing of the spores revealed an interesting phenomenon. The fracture, following the line of weakness, either went over the surface of the spores (revealing the surface rodlet layer) or through the plasmamembrane or through the spore (cf. Chapter Seven). Freeze-fracturing of Tilletia caries teliospores revealed three wall layers, as did thin sectioning, but (as in S.racemosum) the fracture did not go between

any of the wall layers so as to reveal their architecture (Allen, Hess & Weber, 1971). This argues for filler material to be present in the spore walls, so linking or merging one layer into the next, such that no real interface exists between the wall layers. Therefore, no distinct lines of weakness would be present in the wall. Support for the theory that the wall is composed of various polymers embedded in filler material, but with no real interface between discrete layers (zones) comes from the work of Michalenko, Hohl and Rast (1976) on Agaricus bisporus mycelium. They demonstrated the presence of cysteine-containing proteins throughout the whole hyphal wall of A.bisporus by using the silver hexamide method. Silver hexamide is a stain specific for cystine. The hyphal wall of A.bisporus had an outer mucilage layer, a KOH-soluble glucan layer, and an inner layer of chitin embedded in an amorphous matrix composed of β-1,3 glucan. Protein was present throughout all these layers, and carbon-platinum replicas of the walls showed that one layer would merge into another, there being no distinct boundary between the layers (Michalenko et al, 1976). This is the reason why the model picture of the spore wall of S.racemosum is lightly shaded over to indicate the probable presence of filler material, which possibly could be protein (Fig. 101). It should also be mentioned here, that although the micrographs show five distinct layers, the layer of thick microfibrils is embedded in an amorphous matrix and the bumps of the plate-like layer are embedded in the amorphous base layer. Therefore, just as Michalenko et al (1976) rejected discrete wall layers of single polymers in A.bisporus walls, so the same is applied to the spore walls of S.racemosum, where one layer probably merges into another.

CHAPTER SIX

THE ULTRASTRUCTURE OF SWOLLEN AND GERMINATED SPORANGIOSPORE WALLS

INTRODUCTION

The germination process in fungi may be divided into two stages: (1) spherical growth, (2) emergence of a germ tube (Bartnicki-Garcia, Nelson & Cota Robles, 1968b). Ultrastructural changes accompany both these stages of development, such changes having been the subject of two recent reviews (Smith, Gull, Anderson & Deans, 1976; Akai, Fukutomi, Kunoh & Shiraishi, 1976). The cell wall is one of the main areas of variation between the two cell types. The wall plays an important role in the morphogenetic development of fungi and is closely associated with growth (Bartnicki-Garcia, 1973; cf. introduction to Chapter Five). Therefore an understanding of the changes associated with the developing germ tube wall is important in helping to provide a better picture of cellular morphogenesis.

Bartnicki-Garcia (1968a) proposed three basically different mechanisms of vegetative hyphal wall formation during spore germination:-

(1) the vegetative wall is derived directly as an extension of the spore wall, or one of its innermost layers;

(2) de novo formation of a cell wall on a naked protoplast;

(3) de novo formation of a vegetative wall under the spore wall. This type of wall formation, Bartnicki-Garcia (1968a) proposed, was a unique mode of germination which may be exclusive of fungi with vegetative walls of the chitosan-chitin composition. These are the Zygomycetes.

DIFFERENCES IN ELECTRON MICROSCOPIC ULTRASTRUCTURAL OBSERVATIONS

Although Bartnicki-Garcia (1968a) tried to show his three groups of wall formation to be related to various groups of the fungi, recent work has tended to question this approach. Botrytis cinerea, a member of the Fungi Imperfecti, was originally placed in group (1). Hawker and Hendy (1963b) had observed that upon germination, the outer spore wall ruptured and the emergent germ tube was surrounded by an extension of the elastic inner one. Gull and Trinci (1971) found that the dormant spore wall was composed of two layers. This was in agreement with the findings of Hawker and Hendy (1963b). However, during germination Gull and Trinci (1971) observed the formation of three new inner wall layers in the spores. Two were continuous around the whole spore. The third, and also the innermost of these three new layers, was formed only near the point of germ tube emergence. This third new wall layer extended to become the new wall of the emergent germ tube. Gull and Trinci (1971) suggested that because the earlier account of Hawker and Hendy (1963b) was based only upon the use of $KMnO_4$ fixed material, their results gave a restricted view of the germination events. Gull and Trinci (1971) had used material fixed by three different preparative methods, so probably overcoming any shortcomings that may have arisen during sample preparations. Their result meant, that B.cinerea had to be transferred from group (1) to group (3) of Bartnicki-Garcia's (1968a) classification. Richmond and Pring (1971) noticed that in Botrytis fabae, a new wall layer is laid down in the centre of the old two layered conidial wall. This new wall later became the new germ tube

wall. Therefore again a result which places an Imperfect Fungus in group (3), a group which had previously been set apart for Zygomycete fungi.

Richmond and Pring (1971), unlike Gull and Trinci (1971), used only a $KMnO_4$ fixation for their spores as had Hawker and Hendy (1963b). This raises the question about how careful various workers should be about preparing fungal material for electron microscope observation. As can be deduced from the preceding paragraphs, various fixation procedures can be used, such that the results obtained with one procedure can vary with those obtained from another. Bracker (1971) emphasised the importance of pre-fixation handling if apical vesicles were to be observed in germ tubes of Gilbertella persicaria. Spores were streaked onto potato dextrose agar plates. At an appropriate time (usually when about 60% of the spores had germ tubes), fixative was added directly to the cultures. In other treatments, the germinated spores were either prewashed in Tween 80 or phosphate buffer (pH7.0) followed by centrifugation for 60-90 seconds. Only those spores which had not been pretreated at all, showed vesicles at the hyphal apices. Pretreatment of the spores had the property of causing a disruption of cellular organisation. No vesicles were observable, and if so, only very rarely. The plasmamembrane was generally found to have separated from the cell wall. This occurred at the germ tube apex, as well as in other regions around the cell periphery. It rarely occurred in spores which had been fixed immediately. Therefore, both hyphal tips and germ tubes are delicate and sensitive, such that both pretreatments and fixative procedures could upset the cellular organisation including

wall structure. Under these conditions interpretation of micrographs is difficult, and could lead to the formation of misleading data. In order to overcome these problems, the electron microscopist must use the various techniques available to present a clearer and more informative picture of fungal development.

WALL ULTRASTRUCTURE IN FUNGI (THIN SECTIONING TECHNIQUES)

Bearing in mind the difficulties of interpreting electron microscope data, an open mind can be kept when reviewing work associated with studying cell wall changes during germination. Penicillium griseofulvum, although not eligible for Bartnicki-Garcia's group (3) as it is not a Zygomycete fungus, does form a new inner wall during germination (Fletcher, 1971). Its spores have a two layered wall, but the germ tube wall is continuous with only the new, inner layer. In this respect, P.griseofulvum differs from both Penicillium megasporum and Penicillium notatum. Remsen, Hess and Sassen (1967) found that no new wall layer was formed during germination of P.megasporum conidia. The conidial wall has an external surface rodlet layer with base layer, plus three inner wall layers. The innermost layer increased in thickness at the point of germ tube emergence. It resulted in a splitting of the other wall layers, allowing for the expansion and growth of the emerging germ tube through this split. The wall of the germ tube was continuous with the innermost wall layer. In P.notatum, the germ tube wall was continuous with the two innermost wall layers of the four layered spore wall (Martin, Urubura & Villanueva, 1973b).

Border and Trinci (1970) observed that two new wall layers are laid down within the existing three spore wall layers during germination of Aspergillus nidulans conidia. Of these, only the innermost layer of the two new layers was continuous with the germ tube. However, Florance, Denison and Allen Jr. (1972) suggested that the two new wall layers were not synthesised during germination, but appeared just after hydration of the spores. Spores had been in water for 10.5h. They concluded that their data did not answer the question of whether the two new layers are synthesised de novo during hydration, or whether it is derived from existing material.

A new wall layer is formed in germinating arthrospores of Geotrichum candidum (Steele & Fraser, 1973b). It develops between the original spore wall and plasmamembrane, its formation being confined to the region where germ tube emergence occurs. The new wall layer is continuous with the germ tube wall. This is an analogous situation to Botrytis cinerea (Gull & Trinci, 1971), where two new inner wall layers are formed around the entire spore. However, the third and innermost layer develops only at the point of germ tube emergence. It is also continuous with the germ tube wall.

During the swelling phase of spores of Piptocephalis unispora (Jeffries & Young, 1976), a new inner wall layer is formed in the two wall-layered spores. Then, upon germ tube emergence, the inner wall of the spore appears to be continuous with the outer layer of the germ tube. The newly synthesised wall within the germinating spore is continuous with the inner wall layer of the germ tube.

Germinating sporangiospores of Rhizopus stolonifer, Rhizopus arrhizus (Buckley, Sommer & Matsumato, 1968), Rhizopus nigricans and Rhizopus sexualis (Hawker & Abbot, 1963a) produce germ tubes whose walls are continuous with a new inner wall layer of the spore.

OTHER APPROACHES/TECHNIQUES FOR INVESTIGATING WALL STRUCTURE

All the previously mentioned authors relied upon thin sectioned material to detect cell wall changes during germination. Thin sectioning delimits the wall into two areas - (a) an electron opaque area, and (b) an electron translucent area. The major portion of fungal walls always appears electron transparent. It seems very probable that two adjacent wall layers or two adjacent walls (the overlap region between spore and germ tube wall) may differ only very slightly in electron density, but may have large and important variations in chemical and biophysical compsotion (cf. Fig.100, layer C - Chapter Five). The thin sectioning technique therefore has major drawbacks when used as the main tool to study cell walls. Hunsley and Burnett (1970) showed that hyphal walls have a complex architecture (cf. introduction to Chapter Five). Therefore, wall ultrastructural changes should be observed with as wide a variety of electron microscope techniques as possible.

Hegnauer and Hohl (1973) subjected germinating cysts of Phytophthora palmivora to an electron microscopic analysis involving freeze-etching, thin sectioning and replica techniques. They found that in P. palmivora there is no new wall synthesis during germination. The cyst wall is totally fibrillar. The fibrils are tightly interwoven

and embedded in an amorphous matrix. The germ tube wall has a fibrillar inner layer and an amorphous outer layer. Thin sections showed a line of demarcation at around the area of germ tube emergence. At this point, the cyst fibrils ran into and under the amorphous material of the germ tube. Tokunaga and Bartnicki-Garcia (1971) treated germinating spores of P.palmivora with a β-1,3 glucanase. Such treatment removed the amorphous material of the germ tube, revealing a continuous network of fibrils from cyst to emergent hypha. A result also obtained by Hegnauer and Hohl (1973), where the cyst fibrils are embedded in an amorphous matrix and are continuous with the germ tube fibrils. The hyphal fibrils are covered by a layer of amorphous material, this being probably a β-1,3 glucan (Tokunaga & Bartnicki-Garcia, 1971).

A study like this presents a much clearer view of wall changes during germination. It is for these reasons that in this study spores of Syncephalastrum racemosum were not only fixed for thin sectioning, but were also subjected to the same chemical treatments as the dormant spores in the previous chapter (Chapter Five). The results of such treatments upon the germinated spores of S.racemosum are presented in this chapter.

MATERIALS AND METHODS

GROWTH MEDIUM, SPORE PRODUCTION AND GERMINATION MEDIUM

As outlined in the materials and methods section of Chapter Two.

CHEMICAL TREATMENTS

These were exactly the same as those applied to dormant spores of Syncephalastrum racemosum in Chapter Five, (cf. materials and methods section of Chapter Five and Table 9).

ELECTRON MICROSCOPE OBSERVATIONS

(a) Thin sectioning

Germinating spores were harvested from shake flask cultures 10h after inoculation, centrifuged for 2 minutes at 4000rpm, and the supernatant medium discarded. The pellet (germinating spores) were immediately resuspended in 2.5% glutaraldehyde in cacodylate buffer solution (pH7.4). After 1h, following centrifugation for 2 mins. at 4000rpm, the germinating spores were resuspended in fresh 2.5% glutaraldehyde overnight. The procedure then followed exactly the scheme set out in the materials and methods section of Chapter Five.

(b) Surface replica

The techniques employed were the same as those outlined in the materials and methods section of Chapter One.

All preparations were viewed with an AE1 801A transmission electron microscope.

RESULTS

THIN SECTIONS OF GERMINATING SPORES

As with preparing dormant spores for thin sectioning, so germinating spores presented similar problems over fixation and embedding. This, as was pointed out in Chapter Five, is probably due to the thick and impermeable wall layers (especially the plate-like layer) of the spore wall.

Dormant spores of Syncephalastrum racemosum had three wall layers visible in thin section (Fig.102). An outer fluffy layer (A), a middle electron opaque layer (B), and an inner electron translucent layer (C). During germination, the spores swell, and a young germ tube begins to emerge (Figs. 103 and 104). In swollen spores, at the time when the germ tube makes its first appearance, a new inner wall layer has formed around the whole spore. Figures 103 and 104 show this new wall layer (D) to have developed between the innermost of the dormant spore wall layers (C) and the plasmamembrane (P). Layer D is electron opaque and is continuous with the wall of the young emerging germ tube. The other three wall layers (A, B and C) do not cover the germ tube. A breakpoint between the three dormant spore layers (A, B and C) and the newly formed germ tube wall (D) is seen at the point X in Figure 103. Figure 106 also shows this breakpoint between the spore wall layers and the new emergent germ tube wall. This is more clearly seen in Figure 105. Layer D now appears to be composed of two layers (D_1 and D_2). An inner, electron opaque band (D_2) covered by layer D_1, which

has a banded appearance. At higher magnifications (Fig. 107), the breakpoint (X) between the three dormant spore layers (A, B and C) and the two new layers (D_1 and D_2) is much more pronounced. The D_1 layer is more electron opaque than the D_2 layer, and here appears as a discrete layer.

Mitochondria are present in the germinating spores in fairly high number (Figs. 103 and 104). Also, the cytoplasm contains many ribosomes, this being indicative of active metabolic activity going on in the developing spore. A nucleus (N) with nucleolus (R) and nuclear membrane (T) is visible in both Figures 103 and 104.

CHEMICALLY TREATED SPORES

(1) Surface layer/breakpoint of untreated spores

Surface replicas of untreated spores show a breakpoint (X) between the surface rodlet layer of the spore(S) and the smooth surface of the emergent hyphae (H) (Figs. 108 and 109). Some spores had two emergent germ tubes, the breakpoint being visible between both young hyphae and parent spore (Fig.110). The breakpoint (X) had a V-shape suggestive of the outer layer being partially broken mechanically by the emergent germ tube (Fig.110). These V-shaped breakpoints are shown in higher magnifications in Figures 111 and 112. In Figure 111, the rodlet pattern of the spore wall is clearly seen, as is the smooth outer wall of the germ tube. The rodlet pattern is not so clear in Figure 112, but the contrasting smooth nature of the young hyphal wall is again very apparent. The surface layer of the dormant spores is a cross-patched network of rodlets (Fig. 116). However, on swollen spores, the regular pattern is in disarray (Figs. 113-115). The cross-

patched network is very difficult to make out, the rodlets appearing to be more linearly arranged (Figs. 114 and 115). The break-up of the characteristic cross-patch covering of rodlets is also observable on spores with developing germ tubes (Fig.117). On some swollen spores, the rodlets have been so dispersed and disarranged, that areas free from rodlets have appeared (Figs.118 and 119). The break-up of the rodlet pattern reveals that the rodlets are not all that much longer than seen on dormant spores. The disarranged rodlets appear to be composed of linear segments of fibrils (Fig. 119).

(2) Initial alkali treatment

The initial 6h treatment with 5% KOH at 37°C removed (as with the dormant spores, cf. results of Chapters Four and Five) the surface rodlet layer and revealed the plate-like layer covered with amorphous material. The breakpoint between spore wall and emergent hyphae was still present (Figs. 120 and 121). The emergent young germ tube wall was still smooth when compared to the rugose nature of the spore. Figures 122 and 123 show young germ tubes with smooth walls contrasting with the granular surface of the swollen spore. As the germ tube develops, its smooth surface appearance becomes more apparent. The breakpoint (X) was also visible (Figs. 124-128). As with micrographs depicting the breakpoint between the surface rodlets and the germ tube (Figs. 110-112), so also after the initial 5% KOH treatment, the breakpoint was often V-shaped (Figs. 129-133).

Further treatment with 5% KOH for 18h at 37°C had little

effect. The breakpoint between the plate-like layer of the spore and young hyphae was still present (Figs. 134-136). The surface of the germ tube was still smooth, although some hint of an underlying microfibrillar layer was found (Figs. 134 and 135). The amorphous layer of the germ tube is therefore fairly alkali insoluble.

(3) Autoclaving (CH_3COOH/H_2O_2) treatment

With this treatment it was very difficult to get any micrographs of germinating spores mainly because of clumping of the specimen material. This occurred even with a low number of germ tube spores present. However, the treatment did show that the amorphous material of the germ tubes is soluble in the CH_3COOH/H_2O_2 mixture. When removed, it revealed a layer of thin microfibrils (Fig. 137). On spores with emergent germ tubes, no layer of thick microfibrils was seen. Only a layer of thin microfibrils continuous with the layer of thin microfibrils of the germ tube was revealed (Fig. 138).

(4) Second alkali treatment

On finally treating the sample with hot 5% KOH, the layer of thin microfibrils (as seen in dormant spores, cf. Chapter Five, Fig.89) was clearly revealed in swollen spores (Fig. 139). This was a randomly oriented criss-cross network of thin fibrils. In spores with germ tubes the thin microfibrils were common to both the spore and emergent germ tube (Fig. 140), the random arrangement of the fibrils being noticeable in both. Higher magnification of the point of attachment of the germ tube to the spore, emphasised the continuity of the layer between spore and hypha (Figs. 141 - 143). There is no

straight forward or obvious breakpoint between spore and germ tube at the point of attachment (Y). Microfibrils, either from the spore or hypha, freely criss-cross over each other and run from one area to the other (Fig.144).

DISCUSSION

THIN SECTIONS OF CELL WALL CHANGES IN GERMINATING SPORES OF *Syncephalastrum racemosum*

The thin sections of germinating spores of *S.racemosum* show a new wall layer (D) to be formed. It can be divided into two layers, D_1 and D_2. The D_2 layer is formed around the whole spore. However, the micrographs are not clear as to whether the D_1 layer also develops in this manner, or if its formation is restricted to the area of germ tube emergence. This uncertainty arises from the germinating spores being difficult to fix, as were the dormant spores (cf. Chapter Five). The reason here is probably the same. That is, the relative impermeability of the dormant and swollen spores to fixative and resin.

The D_1 and D_2 layers become the walls of the emerging germ tube. Bartnicki-Garcia (1968a) suggested that the formation of a new cell wall layer during spore germination was a common feature of Zygomycetous fungi. The formation of new wall layers has been observed in such Zygomycetes as *Gilbertella persicaria* (Bracker, 1971) and *Rhizopus* species (Hawker & Abbot, 1963a; Buckley, Sommer & Matsumato, 1968). *Mucor rouxii* spores during their spherical swelling phase develop a vegetative wall between the spore wall and the plasmamembrane (Bartnicki-Garcia, Nelson & Cota Robles, 1968b). It forms around the entire spore. During swelling, the vegetative wall probably grows in a uniform manner underneath the spore wall. At the time of germ tube emergence, there is a reorganisation of cellular morphogenesis from a non-polar

to a polar type of growth. Wall formation becomes restricted to a small area of the spore wall. This results in the formation of an apical dome from which will arise the young germ tube. Further such polarised growth confined to the apical region leads to the development of a tubular hypha (Bartnicki-Garcia et al 1968b). Probably an analogous situation exists in germinating spores of S.racemosum. Here, the D layer is a vegetative wall formed uniformly around the whole spore during swelling. It eventually becomes the germ tube wall when the spore switches from a non-polar type of growth to polarised growth. The two components (D_1 and D_2) of the D layer are probably not initially synthesised at the same time. Possibly the D_2 layer is a reflection of the first type of growth (spherical), whilst D_1 is the result of the start of localised growth.

CHEMICAL TREATMENTS/SURFACE REPLICAS

The chemical treatments revealed that the emergent germ tube was two layered. It had a smooth outer wall composed of amorphous material, and an inner layer consisting of a randomly arranged network of thin microfibrils. This microfibrillar layer was continuous with the network of thin microfibrils of the swollen spore. The fibrils were also very similar to the fibrils observed in the dormant spores. The young, emergent hyphae has microfibrils of diameter 8.3nm; the swollen spores 8.1nm; and the dormant spores 8.0nm. These microfibrils of the germ tube are probably chitin, this being the composition of the dormant spore thin microfibrils (cf. results section of Chapter Five).

Hunsley and Burnett (1970) observed microfibrillar meshes, similar to those seen in young hyphae of S.racemosum, in Neurospora crassa and Schizophyllum commune. They treated the microfibrils with chitinase. It caused the dissolution of the fibrillar network observed just prior to enzymatic treatment, indicating that these microfibrils were probably chitin. By analogy with this work and with the dormant spores of S.racemosum (cf. Chapter Five), the germ tube microfibrils in S.racemosum are probably composed of chitin.

The chemical nature of the amorphous outer layer was not investigated. It is alkali insoluble, but was removed by the CH_3COOH/H_2O_2 autoclaving treatment. Zygomycete fungi have hyphal walls commonly composed of chitosan and chitin (Bartnicki-Garcia, 1968a). Mucor rouxii hyphal walls were composed of 32.7% chitosan (per cent dry weight of the cell wall). This was by far the most common polymer of these walls, chitin being only 9.4%. The chitosan possibly plays a similar role that glucans have in other hyphal walls, namely by being a component adding to the mechanical strength or structural integrity of the wall structure (Bartnicki-Garcia, 1968a). Chitosan could therefore possibly be, together with chitin, one of the main wall components of S.racemosum hyphae.

The vegetative germ tube wall is ultrastructurally different from the spore wall. This difference is probably reflected in the chemical compositions of the two types of wall. Differences in chemical composition between the sporangiospores and hyphae of M.rouxii were observed by Bartnicki-Garcia and Reyes (1964). Glucan was the main component of the spore walls; chitosan in the hyphal walls. The hyphae also lacked any glucan polymers. In spore walls of M.rouxii, glucose

formed 42.6% of the cell wall dry weight. The figure was 0% in hyphae. Chitosan was 32.7% in hyphal walls and only 9.5% in spore walls. Chitin too was more abundant in hyphae, where it formed 9.4% of the total dry weight - it was only 2.1% in the spores. Protein was also found in the spore walls (16.1%), whereas it composed only 6.3% of the hyphal wall (Bartnicki-Garcia, 1968a). Thin sections of germinating spores of M.rouxii showed the formation of a new and ultrastructurally different wall layer - the vegetative germ tube wall (Bartnicki-Garcia et al, 1968b). Although detailed data on the specific wall polymers in S.racemosum spores and hyphae is lacking, it would seem likely that a similar situation exists to that described by Bartnicki-Garcia for Mucor rouxii (Bartnicki-Garcia, 1968a; Bartnicki-Garcia & Reyes, 1964). The plate-like layer, surface rodlets and thick microfibrils (layers characteristic of the spore) do not appear in the germ tube wall. This could fit in with the above work of Bartnicki-Garcia if these wall layers represent protein and glucan polymers of the spore wall.

COMPARISON OF THIN SECTIONS TO SURFACE REPLICA RESULTS

Two different electron microscope techniques were used to investigate changes in cell wall ultrastructure in germinating spores of S.racemosum. Both techniques revealed a clear break point between the spore wall and emergent germ tube wall. Thin sections showed that the three spore wall layers (A, B and C) were not present in the germ tube wall. The surface replica technique coupled with chemical treatments showed the same sharp delimitation between spore and germ tube walls. The surface rodlet layer and base layer (layer A in thin sections, cf. Fig.100 in Chapter Five), the rugose or plate-like layer (layer B), and the layer

of thick microfibrils (layer C) are not part of the new germ tube. Only the layer of thin microfibrils of the spore is continuous with the layer of fibrils of the emergent hypha. However, in the dormant spore layer C of thin sectioned spores (cf. Fig.100, Chapter Five) was proposed to be composed of both the layer of thick microfibrils and the layer of thin microfibrils. The results in this chapter show clearly that the electron translucent layer C is not part of the new germ tube wall (Fig. 107). Surface replicas show that the layer of thin microfibrils is present in the germ tube wall. It is suggested here that the new layers, D_1 and D_2, seen in thin sectioned material correspond to the outer amorphous layer (D_1) and the inner layer of thin microfibrils (D_2). Furthermore, although layer D_2 is electron opaque and layer C is electron translucent, the thin microfibrils may form only a small percentage of layer C in the dormant spore. Therefore, upon germ tube emergence, only one new wall layer is synthesised. This is the outer amorphous layer of the germ tube (D_1). The other layer of the germ tube is an extension of the layer of thin microfibrils of the dormant spore, which are also synthesised during the swelling phase of growth (D_2). Bartnicki-Garcia (1973) exposed germinating spores of Mucor rouxii to labelled N-acetyl-D-glucosamine for 5 minutes. Initially, the deposits of silver grains were found all around the spore, as development was by spherical swelling. As the time of germ tube emergence approached, the grains became more and more grouped around one localised point. This was the switch from a non-polar to a polar type of growth. The emergent germ tube had most of the silver grains at its growing tip. The grains corresponded to chitin

and chitosan polymers (Bartnicki-Garcia, 1973), although probably it is mainly chitin which is being initially synthesised during the swelling phase, with chitosan being possibly formed later around the point of germ tube emergence. Perhaps an analogous situation exists in S.racemosum spores, with the D_2 layer (thin microfibrils, probably chitin) being synthesised initially with the D_1 layer being formed at the point of germ tube emergence.

This section of the discussion shows the relative importance of using a variety of electron microscope techniques to study spore wall changes during germination. Thin sectioned work would have given the information that two new wall layers are formed during germination of S.racemosum spores. However, surface replica techniques coupled with chemical treatments showed, that although the germ tube wall is two layered, it only has one unique wall (D_1). The other (D_2) is continuous with the innermost spore wall layer, the layer of thin microfibrils. Therefore, a more knowledgeable picture of spore wall ultrastructure is built up by combining various techniques available in the laboratory.

MODEL FOR GERM TUBE WALL DEVELOPMENT

Figure 145 shows diagramatrically the overall picture built up from thin sectioned and chemically treated material of changes in wall ultrastructure from dormant to germinated spores of S.racemosum. The dormant spore has three layers which are unique to it and are not present in the germ tube. These are layer A (the surface rodlets), layer B (the plate-like layer) and layer C (but only the layer of thick microfibrils, as the thin microfibrils are present in the germ tube, D_2). In the swollen spores, some disruption of the

surface rodlet area is apparent (Figs. 113-115, 118 and 119). Germ tube spores (after initial 5% KOH treatment for 6h) show a clear breakpoint between the plate-like layer (B) and the emergent germ tube (Figs. 130-133). The V-shape of the broken wall (also seen for the outer surface, Fig.110) together with the disruption of the outer rodlets suggests a mechanical breaking up of the wall through swelling and germ tube development. This is probably linked to enzymic degradation of the wall polymers as well. The D_1 layer is probably formed around the point of germ tube emergence, with the D_2 layer of thin microfibrils being initially synthesised around the whole of the swelling spore.

CHAPTER SEVEN

THE ULTRASTRUCTURE OF THE PLASMAMEMBRANE OF DORMANT AND GERMINATED SPORANGIOSPORES

INTRODUCTION

The ultrastructure of fungal plasmamembranes has been relatively little studied, although with the techniques of freeze-fracturing now availble this field may receive more attention. Malhotra and Tewari (1973) compared membrane structures between dormant and germinated sporangiospores of Phycomyces blakesleeanus. They coupled the technique of freeze-fracturing with that of freeze-etching. Their results showed that the plane of fracture normally went through the interior of the plasmamembrane, splitting it into two possible fractures. The concave fracture represented the fracture face of the outerhalf of the membrane viewed from the cytoplasmic side, whereas the convex fracture represented the fracture face of the inner half of the membrane viewed from the cell wall side.

Malhotra and Tewari (1973) found that the concave fracture revealed the membrane to have three types of particles, namely (1) small (5-8nm); (2) large, homogenous (30-35nm); (3) compound (30-35nm) composed of four subparticles. The convex fractures showed only small particles (5-8nm) plus two types of depressions which corresponded to the homogenous and compound particles of the concave fracture. After activating the spores by heat-shocking, the concave fracture of germinating spores showed the gradual disappearance of the large homogenous particles, till after about 12-20h no such particles were visible. No compound particles are apparent 6h after heat-shocking. Germ tube emergence was evident in most spores by 20h. On both fracture faces, the number of small particles increased markedly. However, the relationship between these particle changes and germination is not known (Malhotra & Tewari, 1973).

MATERIALS AND METHODS

PRODUCTION AND HARVESTING OF SPORES

As per the materials and methods section of Chapter Two. Swollen and germ tube spores were harvested after 8h incubation in the growth medium, (cf. materials and methods section, Chapter Six). The percentage of spores with germ tubes in the population was 15%.

FREEZE-FRACTURE

As per the materials and methods section of Chapter One. The time taken from the start of spore harvesting to placing the samples in Freon 12 was 20-25 minutes. All samples were viewed with an AE1 801A transmission electron microscope.

RESULTS

DORMANT SPORES

In all the micrographs of freeze-fractured spores, the large arrow shows the direction of the shadow, and the symbol whether it is concave (⌣) or a convex (⌢) fracture.

Concave fractures of Syncephalastrum racemosum dormant spores revealed a smooth surface covered with an irregular pattern of large particles (Fig. 146). The large particles, a constant feature of this plane of fracture, have an average diameter of 33nm (Figs. 147 and 148). Although no regular pattern of these large particles is seen, in some instances they are arranged linearly into either groups of four (Fig. 146) or groups of three (Figs. 147 and 148).

Convex fractures revealed a surface covered with numerous small particles and an irregular pattern of large depressions (Figs. 149 and 150). The average diameter of the small particles was 8.4nm, whilst that of the large depressions was 34nm. Some of these large depressions were arranged linearly in groups of three (Fig. 149), and it is probable that they correspond to positions occupied by the large particles seen in the concave fractures. Cross fractures showing the relative thickness of the spore wall can be observed in Figs. 146, 147 and 149.

SWOLLEN SPORES

Concave fractures of swollen spores (8h after inoculation)

showed a relatively smooth surface compared with an irregular pattern of small particles (Figs. 151 and 152). Their average diameter was 10.8nm. No large particles as were observed in the concave fractures of dormant spores were seen.

Convex fractures revealed a surface covered with numerous small particles having an average diameter of 10.7nm (Figs. 153-155). An irregular pattern of intermediate particles (of average diameter 20.8nm) was also present (Figs. 154 and 155). However, the large depressions seen in convex fractures of the dormant spores were absent.

GERM TUBE SPORES

A convex fracture of a germ tube plasmamembrane revealed a surface covered with numerous small particles (Fig. 156). These had an average diameter of 8.2nm. In the fracture of a young, emergent germ tube in Figure 157, besides the small particles, intermediate particles are also seen. These are irregularly arranged like the small particles, but have an average diameter of 21.1nm. Again, no large depressions are seen.

DISCUSSION

Freeze-fracturing of Syncephalastrum racemosum spores revealed two possible fracture faces, concave or convex (Fig. 158). It indicated that the plasmamembrane splits through its interior on fracturing exposing the complimentary faces. The concave fracture revealed the inner face of the plasmamembrane which is backed by the cell wall. The convex fracture revealed the inner face of the plasmamembrane which is backed by the cytoplasm (Fig. 158). Fractures of S.racemosum dormant spores showed the presence of large particles (33nm) on the concave face, whilst large depressions (34nm) were present on the convex side. It is most probable that these two observable structures are complimentary to each other (Fig. 158). Similar complimentary faces between large homogenous particles (30-35nm) or compound particles (30-35nm) on the concave fracture face and large depressions on the convex face were observed in freeze-fractured plasmamembranes of Phycomyces blakesleeanus dormant spores (Malhotra & Tewari, 1973). Numerous small particles (5-8nm) were also present in the convex fracture face of P.blakesleeanus. A similar observation was made on the convex fracture face of S.racemosum, with the small particles having a mean diameter of 8.4nm. Malhotra and Tewari (1973) pointed out that one of the characteristic features of plasmamembranes of most cells studied so far was that the convex fracture face always showed more particles than the concave surface. This is the case for both P.blakesleeanus and S.racemosum.

In swollen spores of S.racemosum, the concave fracture face was

covered only with small particles (10.8nm). None of the large particles, characteristic of dormant spore membrane, were seen. This change was also noticeable on the convex fracture face, where no large depressions were observable. However, the surface was covered with numerous small particles (10.7nm) and a few intermediate particles of an average diameter of 20.8nm (Fig. 158).

Ultrastructural changes also occur in the plasmamembrane of germinating spores of P.blakesleeanus (Malhotra & Tewari, 1973). Upon heat shocking of P.blakesleeanus sporangiospores, the large homogenous particles on the concave fracture face form aggregates, each aggregate being made up of a few particles. The number of homogenous particles forming aggregates is greatly increased by about 12h after heat shocking. However, by 6h after heat shocking, no compound particles are discernable. The convex fracture shows depressions corresponding in size and shape to the aggregates of homogenous particles. Also, areas devoid of homogenous particles/ aggregates and depressions are observable on both fracture faces. Malhotra and Tewari (1973) thought that this indicated the likelihood of these areas arising from the aggregation of large homogenous particles. By about 12-20h after heat shocking (corresponding with the time of germ tube emergence) no large homogenous particles were seen on the concave fracture face. During the time the above changes are taking place, there is an increase in the number of small particles (5-8nm) on both fracture faces (Malhotra & Tewari, 1973). The increase in small particle number is seen especially for the

concave fracture face of S.racemosum swollen spores (Fig. 158). There is also the disappearance of the large particles and depressions 8h after inoculation into growth medium. Some intermediate particles (20.8nm) are observable on the convex fracture face. These may possibly be large particles which have not completely disappeared. It is possible that the large particles could become dissociated to form the intermediate and smaller particles.

MODEL FOR S.racemosum PLASMAMEMBRANE

A diagramatic representation of the plasmamembrane of S.racemosum is presented in Figure 159. A similar picture was shown for the plasmamembrane of P.blakesleeanus by Malhotra and Tewari (1973). The plane of fracture passes through the centre of the plasmamembrane, so revealing the two inner surfaces in either convex or concave fractures. This is the characteristic fracture plane obtained with plasmamembranes (Malhotra & Tewari, 1973; Branton, 1971). It is borne out by the complimentarity of the two faces, namely the large particles of the concave fracture (33nm) and the corresponding large depressions (34nm) on the convex face. With spore swelling, there is a subsequent loss of large particles and an increase in the number of small particles especially on the concave fracture face. Branton (1971) pointed out that a comparison of various biological systems showed that the number of particles associated with the membrane was greatest in physiologically active membranes, such as chloroplast lamellae which perform the light

reactions of photosynthesis, and least in inactive membranes such as the myelin layers which function mainly as metabolically inert insulators around the nerve axon.

Spore swelling is an active process involving a variety of metabolic parameters (cf. Chapter Two). Bartnicki-Garcia (1973) pointed out that during swelling of sporangiospores of Mucor rouxii, labelled N-acetylglucosamine was distributed all over the spore indicating the synthesis of chitin and/or chitosan. Further autoradiographic studies with labelled N-acetylglucosamine have shown it to be incorporated into the apical tips of growing hyphae of M.rouxii. The pattern of incorporation was ascertained as showing the position of chitin synthetase in the hyphal tip (McMurrough, Flores-Carreon & Bartnicki-Garcia, 1971). These authors also found that chitin synthetase total activity was highest in the cell residue fraction (85%) and lowest in the soluble supernatant (1.5%) and mitochondrial fractions (3.3%). The microsomal fraction contained 10.2% of the total activity. Repeated breaking and washing of purified cell walls did not decrease their chitin synthetase activity, a result which suggested that the enzyme was intimately associated with the cell walls (McMurrough et al, 1971).

Cabib and Farkas (1971) isolated a particulate chitin synthetase from Saccharomyces carlsbergensis which was essentially inactive. The enzyme could be activated about 15-fold either by the addition of trypsin or by the addition of an activating factor. The activating factor (AF) was isolated from early stationary phase cells of S.carlsbergensis by precipitation of clear supernatant with ammonium sulphate.

A heat-stable inhibitor protein could also be isolated. When added together with AF to a mixture of particulate chitin synthetase, incorporation of labelled N-acetylglucosamine decreased, showing that the inhibitor prevented the action of AF. Cabib and Farkas (1971) suggested that chitin synthetase, being a particulate fraction, was associated with the cell wall. The inhibitor was present in the cytoplasm to prevent any AF reaching the cell wall. The inhibitor was present in the cytoplasm to prevent any AF reaching the cell wall sites of chitin synthetase, if by some chance the AF was released into the cytoplasm from, perhaps, vesicles. Stimulation of the enzyme could be obtained after mild sonic oscillation treatment of S.carlsbergensis cells, suggesting that the AF factor may be enclosed in vesicles. Vesicles, containing the AF factor, would migrate to the point where new bud formation (and therefore chitin synthesis) was to take place, hence activating the enzyme (Cabib & Farkas, 1971). Bartnicki-Garcia (1973) had proposed in his model of apical tip growth, that vesicles involved in wall synthesis fuse with the plasmamembrane so that their contents may pass to the walls. Malhotra and Tewari (1973) found that pronase treatment of the P.blakesleeanus spores resulted initially in an aggregation of large homogenous particles on the fractured faces of the plasmamembrane with subsequently a reduction in their height. This suggests that these particles are partly composed of protein. Similarly, Branton (1971) observed initially the aggregation of particles with little loss in number on the fractured faces of red blood cell ghosts when these cells were

subjected to proteolytic attack. Subsequently, the loss of particles was simultaneously monitored with the amount of protein in the plasmamembranes. The eventual removal of the particles was accompanied by a loss in protein. The initial resistance of the particles to pronase digestion (as also in P.blakesleeanus)probably may be due to their being buried in the hydrophobic region of the membrane matrix into which pronase diffusion would be greatly retarded. However, the eventual loss of the particles is a good indication of their being protein material (Branton, 1971).

An increase in particle number therefore seems to indicate an increase in physiological activity (Branton, 1971), with the particles probably being protein material (Malhotra & Tewari, 1973; Branton, 1971). The work of Cabib and Farkas (1971), McMurrough et al (1971) and Bartnicki-Garcia (1973) suggest that chitin synthetase is associated with a particulate cell wall fraction. It could be possible that the observed increase and changes in particles on plasmamembranes are related to, or reflect, the increase in cell wall synthesis occurring during swelling.

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