MULTICELLULAR DEVELOPMENT IN THE ASCOMYCETE FUNGUS SORDARIA BREVICOLLIS

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I hereby declare that this thesis was composed by myself, and that the work described herein is my own, with the following exception: the maximum likelihood analysis, and the calculation of the relative likelihood of two models for homothallic development, both described in Chapter Four, were carried out by Professor W. G. Hill.

Susan J. Robertson
September 1993

How wisely Nature did decree,
With the same eyes to weep and see!
That, having viewed the object vain,
We might be ready to complain.

Andrew Marvell

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ABSTRACT

This thesis describes the experimental analysis of several aspects of multicellular development in *Sordaria brevicollis*, a filamentous ascomycete which has previously been considered to be exclusively heterothallic. Sexual reproduction in *S. brevicollis* requires the formation of protoperithecia, which must usually be crossed by fertilisation with spermatia of the opposite mating type before perithecial development can take place. The morphology of protoperithecia and crossed perithecia has been examined using a variety of microscopical techniques. Also described is the formation of two additional types of multicellular structure, which have not been characterised previously, uncrossed perithecia, and vegetative hyphal aggregates (VHAs).

Uncrossed perithecia are produced by homokaryons of both mating types, although the phenomenon is more commonly observed in strains of *mtA*. The term is used to cover a number of developmental stages, which range from enlarged protoperithecia, that form ostioles but exhibit no further differentiation, to perithecia which develop (at least) rudimentary necks. Although the majority of uncrossed perithecia are empty, a proportion of the fruitbodies which develop on *mtA* homokaryons have been found to contain a few ascospores, arranged in linear, 8-spored asci. Homokaryotic spore production has never been observed in strains of *mta*.

The genetic mechanisms underlying the development of ascospores in uncrossed perithecia have been examined using heterokaryon analysis. Heterokaryons constructed using spore colour mutants were used to show that meiosis and recombination occurred during the formation of ascospores in uncrossed perithecia, but that the recombination frequency (calculated from the percentage of symmetrical M II asci) was lower than that for a comparable heterothallic cross. It was also shown that the frequency of third division overlap was significantly higher in the asci from uncrossed perithecia than in those from crossed perithecia. Observations based on homothallic and heterothallic development have been used to construct and evaluate two models for nuclear behaviour during the formation of protoperithecia. The evolution of homothallism and heterothallism in *Sordaria* and *Neurospora* has also been considered in the light of the facultative homothallism seen in *S. brevicollis*.

VHA formation occurs in strains of both mating types. VHAs produced in 4 day old cultures are typically of irregular shape and form, lack the internal differentiation of protoperithecia, and have never been observed to develop ascogenous hyphae, asci or ascospores. No differentiation of a rind layer has ever been observed in VHAs, and no accumulation of storage products has been detected. VHAs lack the dark brown pigmentation characteristic of perithecia, and typically exhibit a pinkish-orange colouration, caused by the presence of a carotenoid-like pigment. At high density, VHAs often merge, to form a continuous layer of tissue over the surface of the medium.

Development of the various multicellular structures produced by *S. brevicollis* is regulated by a number of environmental factors. Of particular importance is the rôle of light: production of protoperithecia and perithecia is inhibited by light, while VHA formation is light-stimulated. Detailed examination of the effect of light at different developmental stages suggests, however, that VHA formation does not inhibit protoperithecial production *per se*. Although perithecial maturation may be adversely affected by the subsequent diversion of nutrients into developing VHAs, inhibition of protoperithecial formation appears to be a direct effect of exposure to light.

Various nutritional effects on multicellular development in *S. brevicollis* have also been examined. It has been shown that while perithecial frequency is influenced by the concentration of glucose in the growth medium, sucrose is not utilised effectively by this fungus. Substitution of acetate for the sugars normally provided in the growth medium, resulted in accelerated mycelial growth, accompanied by complete female sterility. VHA formation was also inhibited by acetate. Media containing xylose supported the development of abundant protoperithecia and perithecia, but not the production of VHAs.

The temperatures which are optimal for mycelial growth and perithecial production in *S. brevicollis* have been shown to be different: protoperithecial formation and perithecial maturation took place over a more narrow temperature range than that which facilitated mycelial growth and VHA formation.

The possible ecological consequences of the observations made in the laboratory have been considered, in the light of the coprophilous life-cycle of this fungus.

CHAPTER ONE

INTRODUCTION

1.1 Aims of this thesis.

The work described in this thesis concerns various aspects of multicellular development in the coprophilous ascomycete *Sordaria brevicollis* (Olive and Fantini, 1961), a species readily cultured in the laboratory and previously the subject of many genetic studies. Several aspects of development have not been characterised before and are addressed here, namely (i) the formation of a number of multicellular structures which are previously unrecorded in the genus; (ii) homokaryotic ascospore production by certain strains of a species which is traditionally regarded as being heterothallic; and (iii) the effects of various environmental factors in determining which of the alternative developmental pathways is followed.

The introduction falls into three major parts. Sections 1.2 and 1.3 outline the types of multicellular structures found in higher fungi. It is intended that this should act as background information with which the structures found in *S. brevicollis* can be compared. Sections 1.4-1.6 relate to the reproductive system in the Sordariaceae, the family to which *S. brevicollis* belongs, and cover details of the morphology of sexual structures, the development of ascospores, genetic analysis of asci, and the breeding systems which determine compatibility at the population level. These subjects are described in considerable detail, being fundamental to an appreciation of the unusual behaviour exhibited by *S. brevicollis*. Section 1.7 deals briefly with some of the environmental variables which affect multicellular development in ascomycete genera, focusing in particular on the rôle of light.

1.2 Fungal nutrition and the mycelial habit.

Fungi differ from green plants in their mode of nutrition; lacking the photosynthetic apparatus which allows plants to harness solar radiation and generate carbohydrate, fungi are heterotrophic. Enzymes secreted into the substrate by fungal hyphae digest complex carbohydrates such as lignin and cellulose, producing simple sugars which can be absorbed across the fungal cell wall and plasma membrane. The rate of absorption of carbon, and other important nutrients such as nitrogen, is related to the surface area over which

absorption takes place. Hence, the ratio between surface area and volume is maximised by the production of narrow hyphae, often only a few microns in diameter. The hyphae of higher fungi are usually septate, cross walls being laid down at intervals to divide a filament into individual cells. In many ascomycetes, septa are perforated by a central pore which allows the migration of organelles and translocation of nutrients throughout the mycelium. The pores may become blocked however, isolating localised portions of hyphae, usually in response to physical damage or nutrient depletion (Gull, 1978). Septation is also thought to be of importance in determining the position of hyphal branches, and thus determining the shape of the mycelium (Collinge and Trinci, 1974).

The development of the fungal mycelium, a branched, interconnecting network of hyphae, can be divided into a number of distinct stages (Gregory, 1984). After spore germination, early growth is divergent, extension from the hyphal tip allowing rapid colonisation of a new substrate. During this phase, any hyphae which meet grow over one another, but do not fuse. As the mycelium matures, interhyphal fusions arise, creating protoplasmic continuity between adjacent filaments and allowing cytoplasmic streaming and nuclear migration to occur over considerable distances.

Whilst the mycelium is well adapted to exploitation of the substrate and the capture of resources, it is less suited to long distance dispersal or the toleration of adverse conditions. Environmental factors such as desiccation, sub-optimal temperature and nutrient depletion may require the fungus to differentiate alternative growth forms. That septation is of importance in facilitating such differentiation is suggested by a correlation between the complexity of septal pore structure in various species, and the degree of tissue specialisation which can be achieved (Gull, 1978). Ultrastructural studies have revealed that septal walls and pores can undergo a series of changes during morphogenesis, and that the structure of the pore may vary on opposite sides of an individual septum producing a relatively sophisticated control mechanism (Heath and Heath, 1975; Beckett, 1981c). Faced with adverse conditions, fungal hyphae may respond simply by blocking septal pores and thickening cell walls to protect small units of cytoplasm within cyst-like structures. Alternatively, hyphae may aggregate to produce a range of multicellular structures, adapted to survival or dispersal. In contrast to the outward, divergent growth of the mycelium, hyphal aggregation may be described as coherent development (Gregory, 1984).

1.3 Hyphal aggregates

Hyphal aggregates range in complexity from relatively simple cords and rhizomorphs, through sclerotia and related stromatic structures, to sporocarps necessary for sexual reproduction (Butler, 1966). The essential features of some of these structures are outlined briefly below.

1.3.1 Rhizomorphs and hyphal cords

Rhizomorphs and other cord-like structures are characterised by their linear growth form: all are aggregations of longitudinally aligned hyphae. Variation in morphology, from the tightly bound rhizomorphs of various *Armillaria* species to the relatively diffuse fans of *Serpula lacrymans*, is determined by variation in the degree of apical dominance expressed (Rayner *et al.*, 1985). Rhizomorphs often facilitate aggressive colonisation by root-infecting fungal parasites (Garrett, 1970), and in species of *Armillaria*, have been shown to possess an apical centre just behind the growing tip which behaves in a manner analogous to histogens of apical meristems in higher plants (Motta, 1971). Three distinct zones are differentiated: cortex, medulla and a central airspace or lacuna. In contrast, hyphal cords and strands exhibit a lesser degree of organisation, and are believed to function in recycling nitrogen in an ageing mycelium (Watkinson, 1978). Neither hyphal cords nor rhizomorphs however exhibit the degree of differentiation found in stromata.

1.3.2 Stromata

Stromata are defined by Kohn and Grenville (1989) as compact hyphal aggregates, usually enveloped by a rind (a zone of cells with thickened, melanised walls). The term thus covers a range of structures from sclerotia (which are usually of determinate morphology) to diffuse, apparently indeterminate networks of hyphae, which envelop the substrate and have therefore been called substratal stromata (Whetzel, 1945). Stromata may also provide a determinate matrix within which the fruitbodies of genera such as *Xylaria* are embedded.

1.3.2.1 Sclerotia

Sclerotia have been defined in functional terms, as aggregations of hyphae which are able to survive conditions too severe for the vegetative mycelium (Willetts, 1972). Hence, structures exhibiting a considerable range of anatomical variation have been called sclerotia (Waters *et al.*, 1975; Hereward

and Moore, 1979; Gaudet and Kokko, 1986). Sclerotia serve as resting structures for many species of plant pathogen, and their longevity (some have been found to remain viable in the soil for 10 years (Coley-Smith, 1979)), coupled with resistance to extremes of temperature, desiccation, radiation and attack by fungicides, can make erradication very difficult. Sclerotium formation is therefore an extremely successful survival mechanism.

Sclerotia have been divided into three categories according to their mode of initiation, and are known as loose, terminal or lateral types (Willetts, 1978). Loose sclerotia have no definite initial, and irregular branching is followed by intercalary septation and hyphal swelling. In contrast, terminal sclerotia exhibit a well defined pattern of branching, producing a knot of hyphae held together by anastomoses. Lateral sclerotia have initials which form by the interweaving of numerous side branches. Some arise from a single hypha (simple type), and others by the branching of several hyphae, grouped to form a strand (strand type). After initiation, substantial translocation of nutrients into the developing sclerotium occurs, sustaining rapid growth of new hyphae, and facilitating the accumulation of stored reserves. In *Sclerotinia sclerotiorum* for example, 88 % of the dry weight of mature sclerotia was found to accumulate within three days of initiation (Cooke, 1971). When this phase of development is complete, the protective rind is formed. The hyphae making up the outer layer of the sclerotial wall become thickened, and in most species, dark brown, melanin-like pigments accumulate. Once the rind is fully developed, all connection between the inside of the sclerotium and the parent mycelium is lost. Within the rind, the tissue may be relatively homogeneous, or may show differentiation into an outer cortex and inner medulla, the medullary hyphae often being embedded in a mucilaginous matrix (Willetts, 1971, 1978).

Sclerotial morphogenesis has been studied both as a simple model for fungal development as a whole, and more specifically, with a view to the control of a number of serious plant diseases. Amongst sclerotial genera are *Coprinus* (Henderson *et al.*, 1983), and a number of commercially important plant pathogens such as *Verticillium dahliae* (Perry and Evert, 1984), various species of *Sclerotinia* (Bullock *et al.*, 1980; Huang, 1983), *Sclerotium* (Backhouse and Stewart, 1987), *Botrytis* (Willetts and Bullock, 1982), *Aspergillus* (Jarvis and Traquair, 1985) and *Rhizoctonia* (Yang *et al.*, 1989). Although many sclerotial fungi have deleterious effects, a few are beneficial. Sclerotium-like structures have been found in several species of ectomycorrhizal fungi (Fox, 1986), and

these may provide suitable inocula for the establishment of mutualistic associations with forest trees (Marx, 1980).

The evolutionary origins of sclerotia are not clear. It has been suggested that they have arisen independently in a number of groups as the result of convergent evolution, and may, in some cases represent degenerate fruitbodies (Willetts, 1972). Whilst sclerotia are typically of determinate character, they lack the structural differentiation and complexity of sexual structures such as sporocarps.

1.3.3 Sporocarps

Fungi often respond to deteriorating conditions by producing spores. Sexual spores are typically generated within relatively complex fruitbodies, whilst many asexual spores, such as conidia, are produced on specialised aerial hyphae. Although conidia are simple to produce, require limited investment of energy and are effective as short-lived propagules, they are genetically identical to the parental mycelium. Deuteromycete fungi (those which lack any known sexual stage) are exclusively conidial, and these species can adapt to environmental change by utilising low levels of mitotic recombination (the parasexual cycle described by Pontecorvo (1956)). Most fungi however, produce sexual spores at some stage in their life cycles. In basidiomycetes, only sexual spores are developed, within complex basidiocarps, while both conidia and sexual spores are produced in many ascomycete genera.

The following sections outline the essential characteristics of basidiomycete and ascomycete sporocarps.

1.3.3.1 Basidiocarps

Basidiomycete fruitbodies exhibit a great variety of form, from toadstools and puff balls to bracket fungi, and often comprise several different tissue types, developed by a combination of septation, hyphal adhesion and cellular specialisation. Basidiocarps are characterised by the production of the basidium, a club-shaped structure inside which meiosis occurs. Post-meiotic nuclei migrate into short projections from the surface of the basidium, known as sterigmata, which are then enlarged to become the basidiospores (Moore-Landecker, 1982). The basidiocarp of mushroom-forming genera is divided into a stipe (composed entirely of sterile tissue), and a cap (from the underside of which the basidiospores are released when mature).

1.3.3.2 Ascocarps

Within the ascocarp (or ascoma) of ascomycete species, spores are produced by free cell formation inside large, sac-like cells known as asci (sing. ascus). The ascus is the site of nuclear fusion, meiotic and mitotic nuclear divisions, and ultimately, the delimitation of sexual spores. Asci can be divided into two basic types, unitunicate (with one layer to the ascus wall) or bitunicate (in which an outer rigid layer encloses an inner more flexible one), although a range of intermediate forms can also be distinguished (Beckett, 1981a ; N. D. Read and A. Beckett, in preparation). Characters such as ascus type, and the form of the ascocarp are central to Ascomycete taxonomy (see Appendix 1). Three basic types of ascocarp, the cleistothecium, the perithecium, and the apothecium, are illustrated in Fig. 1.1, and described briefly below. For a more comprehensive review of ascomatal morphology the reader is directed to Moore-Landecker (1982), from whose text the following outline is derived, unless otherwise indicated.

1.3.3.2.1 *Cleistothecia*

Cleistothecia are entirely closed ascocarps, typically spherical in shape, and of only a few millimetres diameter. Within the cleistothecium, globose asci are distributed throughout the central tissue and are typically evanescent (break down readily), releasing spores into the mature fruitbody. As the wall lacks an ostiole or any other opening, spore discharge is effected only when it is ruptured. In species such as *Chaetomium arxii* this rupture is facilitated by angular plates (sometimes called cephalothecoid plates) which make up the wall and break apart as the fruitbody matures (Benny *et al.*, 1980). Although the presence or absence of an ostiole was formerly considered a taxonomic criterion for separation at the rank of order and above (see Appendix 1), it is now believed that in many cases, the non-ostiolate ascocarp is secondarily derived (Hawksworth, 1987), and both ostiolate and non-ostiolate fruitbodies have been found within single species (von Arx, 1973; Fukuda *et al*, 1971). The latter study on *Gelasinospora longispora*, demonstrated that the type of fruitbody produced was dependent upon the age of the mycelium and the illumination conditions, and was thus a variable characteristic.

Cleistothecia are found in the perfect (sexually reproducing) forms of several Deuteromycete species of *Aspergillus* and *Penicillium*, and are also common in soil and root-inhabiting genera (Fennel, 1973).

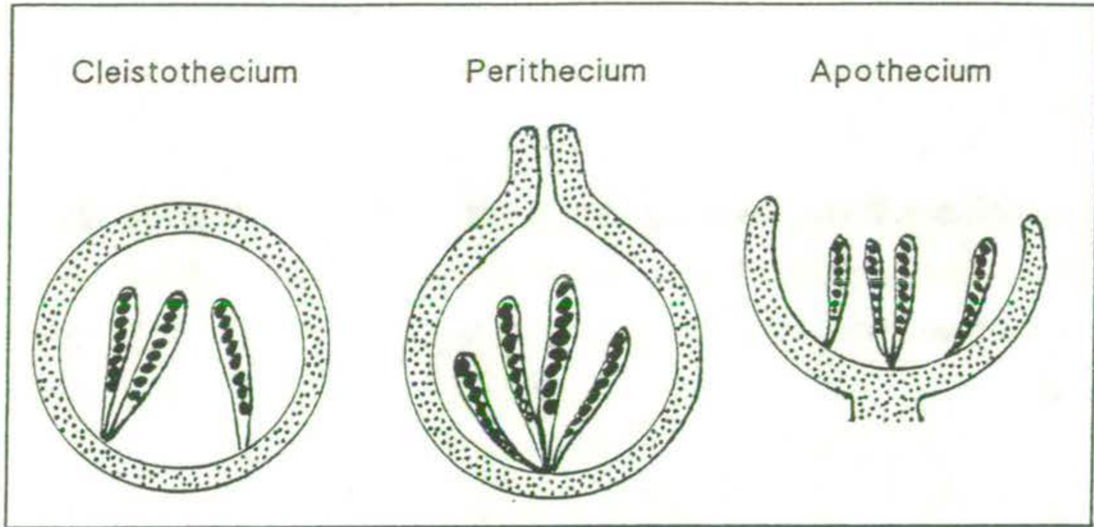


Figure 1.1: Basic ascocarp types.

Cleistothecium: Approximately spherical structure of only a few millimetres diameter, with no ostiole or other opening. Spore liberation is achieved only when the cleistothecial wall breaks down. Typical of many plectomycete species (e.g. *Aspergillus nidulans*). *Perithecium*: Pear-shaped (pyriform) fruitbody, typically produced by pyrenomycete genera, within which asci are arranged in a basal layer. Spore discharge occurs via an apical pore or ostiole. *Apothecium*: Essentially a cup-shaped fruitbody, of variable size. May form mushroom-like structures in genera such as *Morchella*. The spore-bearing hymenium is exposed to the outside when mature, and spores are dispersed by rain and other environmental forces.

1.3.3.2.2 *Perithecia*

The perithecium is an ostiolate, pear-shaped fruitbody, within whose wall, asci are arranged in a basal layer, interspersed with sterile hyphae (or paraphyses). Mature ascospores are discharged through a pore (or ostiole) located at the tip of the neck. In some genera (e.g. *Neurospora*) solitary perithecia are produced on the mycelium (Dodge, 1935), whilst in others they are grouped together, embedded in a layer of stromatic tissue (eg. *Xylaria*) (Rogers, 1975).

The Sordariales, an order comprising over seventy mainly saprotrophic genera, is characterised by the production of perithecia containing unitunicate asci (Hawksworth *et al*, 1983). One family, the Sordariaceae, has been studied particularly extensively, as it includes one of the best characterised of all filamentous fungal species, *Neurospora crassa. Neurospora*, and the closely related genera *Gelasinospora* and *Sordaria*, are not generally considered to include species which produce stromatal structures (Lundqvist, 1972), and in these genera, the perithecium is the principal multicellular structure.

1.3.3.2.3 *Apothecia*

The apothecium is an open, cup-shaped ascocarp. Although apothecia vary in structure and complexity, the hymenial layer, within which the asci develop on the inner surface of the cup, is always exposed at maturity. Often brightly coloured and fleshy in appearance, apothecia range in size from microscopic structures embedded in host tissue, to cup fungi of several centimetres in diameter. Many apothecial species also produce sclerotia or other stromatal structures, and these can give rise directly to apothecia under appropriate conditions. Amongst apothecial genera are serious plant pathogens such as *Sclerotinia* and *Monilinia*, and highly prized edible fungi such as *Morchella* (morels) and *Tuber* (truffles).

A number of variations on the basic form of the ascocarp are illustrated by Hawksworth (1987), who shows that it is possible to distinguish a continuous spectrum of inter-related ascocarp morphologies, from simple cleistothecia to relatively complex apothecia.

The work described in this thesis is concerned with several aspects of multicellular development in *Sordaria brevicollis*, and the following sections examine in detail the processes which culminate in the production of the perithecium.

1.4 Sexual reproduction in the Sordariaceae

Mutant analysis has been used to demonstrate that initiation and maturation of perithecia requires the coordinated interaction of genes at many different loci (Esser and Straub, 1958; Srb and Basl, 1969; Weijer and Vigfusson, 1972; Mylyk and Threlkeld, 1974; Jha and Olive, 1975). It has been estimated that between 200-400 genes may be required for perithecial formation in *N. crassa* (Johnson, 1978), a figure supported by the current tally of over 200 sexual development mutants isolated in this species (Raju, 1992b).

Although fruitbody formation involves many complex interactions, analysis of perithecial formation can be simplified by considering sexual differentiation as a combination of two parallel sequences of structural and genetic development. The formation of the protoperithecium, and the expansion of the sterile parts of the perithecium can thus be distinguished from the processes of nuclear division, nuclear migration and ascospore formation.

1.4.1 Structural development

1.4.1.1 Protoperithecia

The first indication that sexual development has been initiated is provided by the growth of a coiled lateral branch from an intercalary cell of a vegetative hypha (Carr and Olive, 1958; Mirza and Khatoon, 1973; Mai, 1976, 1977; Uecker, 1976; Jensen, 1982; Read, 1983). Asymmetrical hyphal growth results in the development of a compact spiral of several turns, which is isolated from the main hypha by the formation of a septum. Further septa may subsequently be laid down, dividing the ascogonial coil into several multinucleate compartments (Carr and Olive, 1958), each filled with a dense cytoplasm (Viswanath-Reddy and Turian, 1975). The ascogonial coil (sometimes called a protoperithecial initial (Elliot, 1960) or an ascogonium (Mai, 1977)) is rapidly enveloped by fine hyphae which arise from at or near its base (Read, 1983; Sanni, 1984), and the subsequent adhesion of adjacent hyphae results in the formation of a pseudoparenchymatous layer around the central coil. The completed structure constitutes the female reproductive organ, the protoperithecium. When the protoperithecium is viewed in section, the ascogonium is quite distinctive, being of greater diameter and having different staining properties to the hyphae which make up the pseudoparenchymatous layer (Turian, 1978).

It is necessary at this point, to digress briefly from the details of morphological differentiation, in order to outline the nature of the two breeding systems found within the Sordariaceae. This subject will be addressed more fully

in section 1.6, but is of relevance here because the breeding system has a bearing upon development. In *homothallic* species there is no functional mating type, and each individual is self-fertile. In contrast, *heterothallic* individuals can produce spores only when fertilised by a strain of opposite mating type. Although both mating types of a heterothallic species are hermaphrodite, producing male and female reproductive structures on the same mycelium, sexual reproduction normally proceeds beyond the protoperithecial stage only when nuclei of opposite mating type are brought together within the ascogonium. In order to facilitate this event, protoperithecia of heterothallic species produce trichogynes, fine hyphae which arise from the ascogonium and pass between the cells of the protoperithecial wall to the outside (Dodge, 1935). The tip of the trichogyne fuses with a male gamete of opposite mating type (usually a conidium functioning as a spermatium), and subsequently provides a passage for the male nucleus through the protoperithecial wall to the ascogonium. Homothallic species rarely possess trichogynes, and where present these are not thought to be functional (Meyer, 1957; Jensen, 1982).

Protoperithecium formation has been shown to be influenced by a large number of genes and it thus particularly sensitive to mutational disruption. When protoperithecial development is either inhibited or interrupted, female sterility may result (e.g. Westergaard and Hirsch, 1954; Esser and Straub, 1958; Fitzgerald, 1963; Tan and Ho, 1970; Itoh and Morishita, 1971; Ho, 1972; Weijer and Vigfusson, 1972; Bond and MacDonald, 1976; Johnson, 1978; DeLange and Griffiths, 1980a,b ; Perkins *et al.*, 1982).

1.4.1.2 Conidia

Conidia, which often function as male gametes in heterothallic species, may be either multinucleate macroconidia, or uninucleate microconidia. Mycelial fragments may also act as donors of male nuclei (Dodge, 1932; Backus, 1939). All the known heterothallic species of *Neurospora* are characterised by the production of macroconidia (Perkins and Turner, 1988), and there is a general (although not universal) correlation between heterothallism and conidiogenesis in ascomycete species. Within macroconidia (which can function either as fertilising elements or as asexual propagules), the number of nuclei is variable, and can be influenced by the nutritional status of the mycelium (Huebschman, 1952). In the majority of *Neurospora* species, macroconidia are produced in a holoblastic fashion (Springer and Yanofsky, 1989). Conidiation is preceded by the growth of aerial hyphae, which subsequently differentiate chains of

protoconidia by successive apical budding. This is followed by septation and disarticulation to produce free conidia.

Heterothallic species of *Neurospora* also develop smaller, uninucleate microconidia, although these are typically generated in lesser numbers and are comparatively inconspicuous. In heterothallic species of *Sordaria* and *Gelasinospora*, macroconidia are absent and only microconidia are differentiated. In *S. brevicollis*, microconidia develop in an enteroblastic fashion on narrow, highly septate conidiophores (N. D. Read, in preparation), and appear to function primarily as gametes (Olive and Fantini, 1961). Sanni (1984) showed that although 90% of microconidia could be induced to germinate under conditions of high humidity, subsequent hyphal growth was arrested at the germ tube stage.

The conidia of *N. crassa* have been shown to secrete a pheromone-like substance, to which the tips of trichogynes belonging to protoperithecia of the opposite mating type are attracted (Bistis, 1981; 1983). Amongst the mutations which confer male sterility (e.g. Vigfusson and Weijer, 1972) are those in which conidia lose the ability to produce this trichogyne attractant (Griffiths and DeLange, 1978). A wide range of other mutations have been recorded in which conidial development is abnormal, although the conidia which are produced are viable (Springer and Yanofsky, 1989). Since relatively little cytoplasm is enclosed in conidia, little if any of the male cytoplasm is likely to be transported into the ascogonium along with the fertilising nucleus. Hence traits which are cytoplasmically inherited (those carried in the mitochondrial DNA) are typically transmitted by the maternal parent only.

1.4.1.3 Perithecia

In heterothallic species, successful fertilisation of a protoperithecium by a male nucleus results in the formation of a heterokaryon within the ascogonium. Such heterokaryon formation (in this case the presence of non-homologous nuclei in the common cytoplasm of the ascogonium (Caten and Jinks, 1966), and more specifically, the organisation of a dikaryon (pairing of unlike nuclei), appears to trigger further differentiation of the sterile tissues of the perithecium (Ross, 1979). In homothallic species such as *N. terricola* and *S. macrospora*, the development from protoperithecium to perithecium is constitutive and proceeds without interruption.

The onset of the perithecial phase is marked by rapid expansion of the protoperithecium. Uecker (1976) observed that a two fold increase in perithecial

diameter required an eight fold increase in volume in order to maintain the approximately spherical shape, and attributed this expansion to a combination of cell division and cell enlargement. Before expansion of the perithecium is complete, polarity is established and neck formation commences (N. D. Read, in preparation). The structure of the perithecium is illustrated in diagrammatic form in Fig. 1.2.

1.4.1.3.1 *Periphyses and neck development*

The processes associated with neck formation have been studied in some detail in *S. humana*, where the important rôle of periphyses has been demonstrated using scanning electron microscopy (Read, 1983; Read and Beckett, 1985). The typical pyriform shape of the perithecium is largely achieved by the growth of periphyses, specialised hyphae which not only line the neck canal, but also contribute to the tissue of the neck wall. Periphyses originate in the upper part of the perithecium, in a region where increased cell number is achieved by septation and subsequent cell expansion (N. D. Read, in preparation). Periphyses develop to surround a mucilage-filled canal which terminates in an ostiole. Neck extension proceeds by growth from the apex, and as periphyses grow over the edge of the pore, their adhesion and differentiation gives rise to the cells of the neck wall. These cells expand, develop thickened and pigmented walls, and become increasingly vacuolate. The neck is thus largely internally derived. That neck tissues are derived from the periphyses is further suggested by a mutant (*m*) of *S. macrospora* which lacks periphyses and develops only short, non-ostiolate necks (N. D. Read and K. M. Lord, in preparation). The periphyses which line the neck canal have been shown to be rich in ribosomes, and are presumably a site of protein synthesis, possibly contributing to the formation of the extracellular mucilage which fills the neck canal prior to spore discharge (N. D. Read, in preparation). The precise function of this mucilage is unknown but it may act as a lubricant, allowing the extension of asci into the neck canal (Read and Beckett, 1985).

Further evidence for the internal origin of the perithecial neck has been gained from morphological studies on species of *Gelasinospora* (Ellis, 1960; Mai, 1977; Jensen, 1982), *Neurospora* (Nelson and Backus, 1968), *Sordaria* (Mirza and Khatoon, 1973; Uecker, 1976, Sanni, 1984), and *Triangularia* (Moreau and Moreau, 1950), amongst others. Such an origin was also indicated by the construction of "mosaic" perithecia in *N. crassa* (Johnson, 1976). A mutant (*per-1*) in which the perithecial wall lacked the normal pigmentation, and a wild type

Figure 1.2

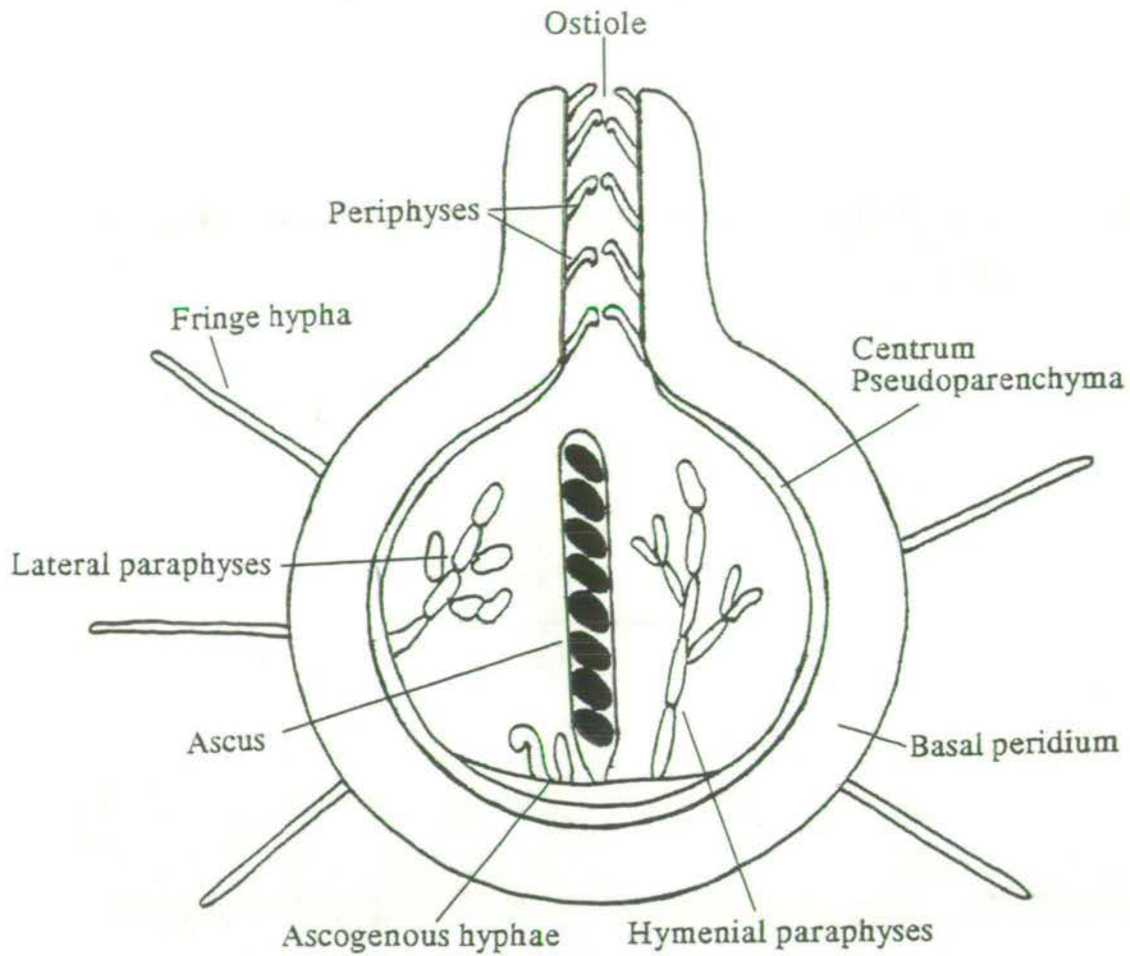


Figure 1.2: Diagrammatic structure of the mature perithecium in longitudinal section. (Redrawn from Read and Beckett, 1985)

strain (*per-1*$^{+}$) were used to construct a single mating type heterokaryon. This heterokaryon was then used as a protoperithecial parent and fertilised with wild type conidia. In the perithecia which formed, some cells exhibited the dark pigmentation characteristic of wild type strains, whilst others where pale in colour, giving a mosaic effect. By scoring the frequency of pigmented and unpigmented cells in various tissues of the mature perithecium, it was possible to construct a model for the origin each tissue (Fig. 1.3). Amongst the conclusions drawn from the experiment were (i) that an initial population of 100-300 nuclei founded each perithecium, and (ii) that a small sub-population from this larger pool gave rise to all of the internal tissues and to the neck. The tissues which comprise the neck are therefore of different origin to those which constitute the remainder of the outer wall.

In many perithecia, a single ostiole is differentiated, and only one neck forms, although this is not always the case. Perithecia with several necks have been observed in *Gelasinospora longispora*, where two types of multi-ostiolate perithecia were described by Sanni (1982). The first type termed "fusion perithecia" were believed to have arisen from the fusion of adjacent protoperithecia. These perithecia had two distinct central cavities, and were linked only by a shared portion of the perithecial wall. A second group of perithecia were apparently derived from single protoperithecia, and had simply differentiated more than one ostiole. Although it is possible that perithecia of the second type arose from the fusion of several ascogonia, it seems more likely that the development of more than one neck was a consequence of an abnormality in the polarity mechanism which controls the site of neck initiation.

1.4.1.3.2 *Paraphyses and the centrum*

Paraphyses are sterile hyphae, which are typically slender, thin walled, septate, branched, and multinucleate (Read and Beckett, 1985). They are predominantly (if not completely) derived from the centrum pseudoparenchyma. This tissue forms the inner-most layer of the perithecial wall and is alternatively interpreted as part of the wall, or as part of the centrum itself. Its assignment to wall or centrum is dependent upon the definition of the latter, but as this was originally described as "the totality of structures within the perithecial wall" (Wehmeyer, 1926), either conclusion can reasonably be drawn. In this thesis the centrum pseudoparenchyma will be considered as part of the centrum, on the basis of the work of Johnson (1977), discussed previously. Johnson demonstrated that the inner parts of the perithecial wall were derived from a small sub-

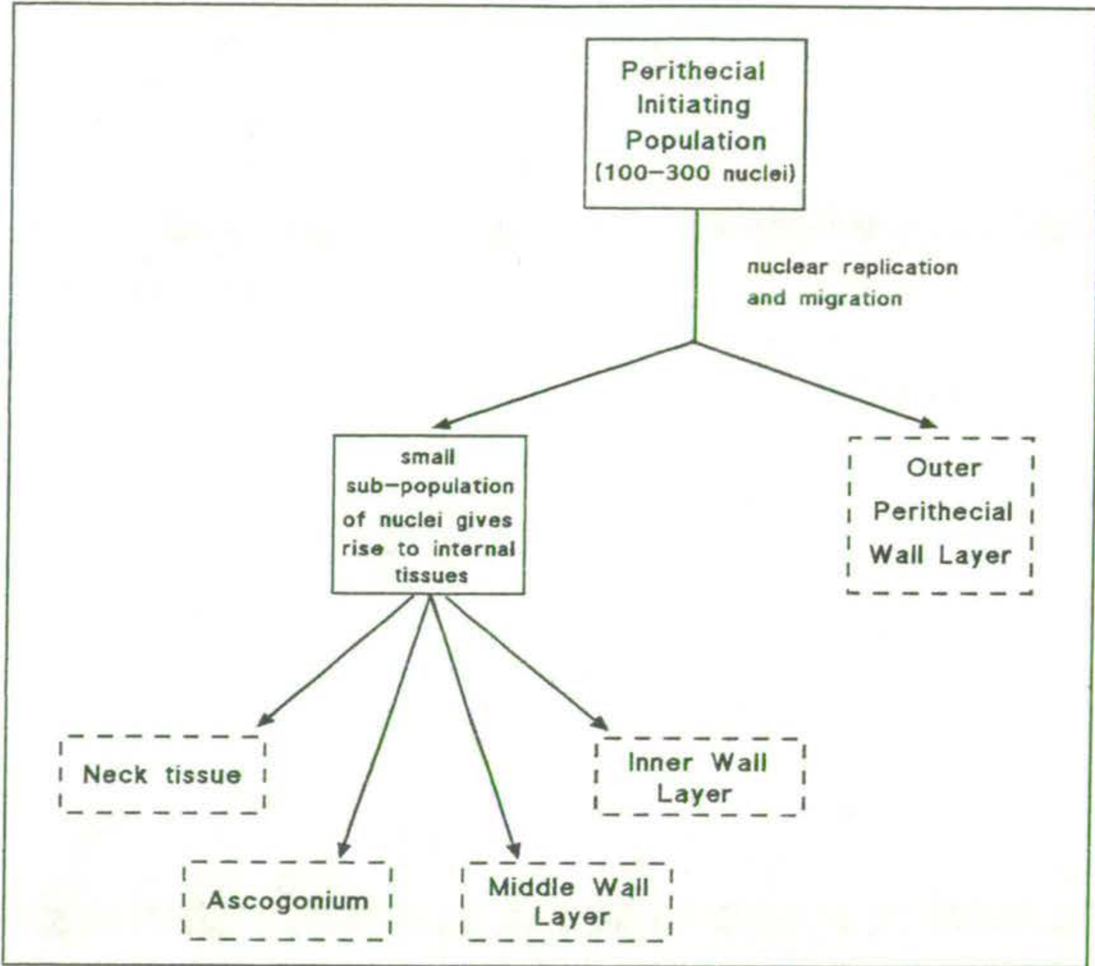


Figure 1.3: Origins of various cell types in the perithecium of *Neurospora crassa*. (Redrawn from Johnson, 1976). The perithecium initiating population (PIP) of 100-300 nuclei gives rise directly to the outer layer of the perithecial wall, and to a small, sub-population of only a few nuclei, which in turn gives rise to all of the internal tissues, including the ascogonium and paraphyses, the neck tissue, and the inner part of the perithecial wall. The inner and outer parts of the perithecial wall are therefore derived from different pools of nuclei, and the inner wall is more closely related to the internal tissues.

population of nuclei which also gave rise to the ascogenous system. They were thus derived from a different pool of nuclei to the outer wall layer (Fig. 1.3). The primary function of the centrum pseudoparenchyma is to give rise to the paraphyses: those which arise from the base of the perithecium, in the region of the ascogenous hyphae are known as hymenial paraphyses, whilst those which have their origins in the pseudoparenchyma lining the side walls are lateral paraphyses (Jensen, 1982). It is possible that some of the hymenial paraphyses may be derived from the ascogenous hyphae, rather than the underlying centrum pseudoparenchyma (Read and Beckett, 1985; N. D. Read, in preparation). Both origins have been demonstrated for paraphyses in *Diaporthe phaseolum* var. *sojae* (Jensen, 1983).

Several functions have been suggested for the paraphyses, including aiding perithecial expansion (Lutrell, 1951) and filling space within the perithecial wall (Jensen, 1981). As the maturing asci enlarge and occupy more of the perithecial volume, both the centrum pseudoparenchyma and the paraphyses are compressed and the latter may disintegrate (Uecker, 1976). It has been found however, that the seemingly degenerate nature of paraphyses observed in much sectioned material may result from the preparative techniques employed and that apparent deterioration may be artifactual (K. M. Lord and N. D. Read, unpublished observations). Less harsh techniques, which have been developed to preserve intact paraphyses, have also demonstrated that the "sub-apical cavity" reported by many authors (e.g. Uecker, 1976; Huang, 1976; Sanni, 1982) is not strictly a cavity, but is filled with extracellular material (Read and Beckett, 1985). This material may be derived from the paraphyses.

1.4.1.3.3 *Lower perithecial wall*

The remainder the perithecial wall protects developing asci from environmental stress such as desiccation, and also from physical damage. The wall of the mature perithecium is a relatively rigid structure within which several distinct layers have been distinguished (Sanni, 1984), although these often include a layer which corresponds to the centrum pseudoparenchyma (Mirza and Khatoon, 1973). The outer part of the perithecial wall (excluding the centrum pseudoparenchyma), is often referred to as the basal peridium. In *S. humana* the perithecial wall has been estimated to be 5-10 cells thick (N. D. Read, in preparation) of which 2-6 cell layers constitute basal peridium (Read and Beckett, 1985). The outer cells of the basal peridium are often thick walled, and show varying degrees of autolysis (Froeyen, 1980). This autolysis was attributed

to nutrient starvation by Froeyen (1980), who proposed that the nutrients supplied to the developing perithecium by the parent mycelium are channelled through the central region of the fruitbody, so that the internal tissues and those which develop to form the neck, continue to be supplied with nutrients after the supply to the outer parts of the perithecial wall has been cut off. Fine fringe hyphae arise from the perithecial wall in species such as *S. brevicollis* (Olive and Fantini, 1961), *S. humana* (Read, 1983; Read and Beckett, 1985), and *N. discreta* (Perkins and Raju, 1986), although these may collapse as the perithecium matures.

During the development of perithecia in wild type strains of genera such as *Sordaria* and *Neurospora*, melanic pigments accumulate in the perithecial wall so that mature perithecia are dark-brown in colour. Within genera such as *Hypomyces* and *Nectria*, however, the perithecial wall is typically red, and in *N. haematococca*, perithecial colour mutants may be grey, pink, white or beige (Babai-Ahary *et al.*, 1982).

1.4.2 Development of fertile tissues

1.4.2.1 Plasmogamy and heterokaryon formation.

Plasmogamy (cell fusion) and karyogamy (nuclear fusion) are separated both temporally and spatially in the heterothallic Sordariaceae. The former normally occurs when trichogyne and conidium fuse, and is followed by the migration of the male nucleus to the ascogonium. A heterokaryon is thus established, but cytological observations indicate that karyogamy is delayed (Raju, 1980). Instead, nuclei multiply within the coenocytic ascogonium, and at some point, non-homologous pairs are organised. Little is known about the behaviour of nuclei within the ascogonium; the traditional belief, that nuclear pairing occurs soon after heterokaryon formation and is followed by several conjugate nuclear divisions (e.g. Fincham *et al.*, 1979), was questioned by Raju (1980), who suggested that the nuclei simply proliferate in a coenocytic condition during most of the phase prior to ascus formation, only pairing when they migrate into the ascogenous hyphae. In either event there is a delay between the establishment of the heterokaryon and the fusion of haploid nuclei.

1.4.2.2 Ascogenous hyphae and crozier formation.

The ascogenous hyphae develop as outgrowths from the ascogonium and form a hymenial layer at the base of the young perithecium. The tip of each ascogenous hypha becomes folded back to give a characteristic hook-shaped

crozier. A pair of non-sister nuclei migrates into each crozier (thus establishing a dikaryon) and divides in its tip (Fig. 1.4 (a,b)). This mitotic division is followed by the deposition of two septa, which divide the crozier into three compartments; a uninucleate apical cell, a binucleate penultimate cell, and a uninucleate stalk cell (Fig. 1.4 (c)). The two nuclei in the penultimate cell enter pre-meiotic interphase and DNA replication occurs (Bell and Therrien, 1977; Iyengar *et al.*, 1977), rapidly followed by nuclear fusion, thus establishing the diploid state for the only time in the life cycle. Karyogamy is accompanied by the enlargement of the penultimate cell which becomes the young ascus (Fig. 1.4 (d)). At the same time the apical and stalk cells fuse, restoring the dikaryon in the stalk cell, which may subsequently gives rise to new croziers (Singleton, 1953).

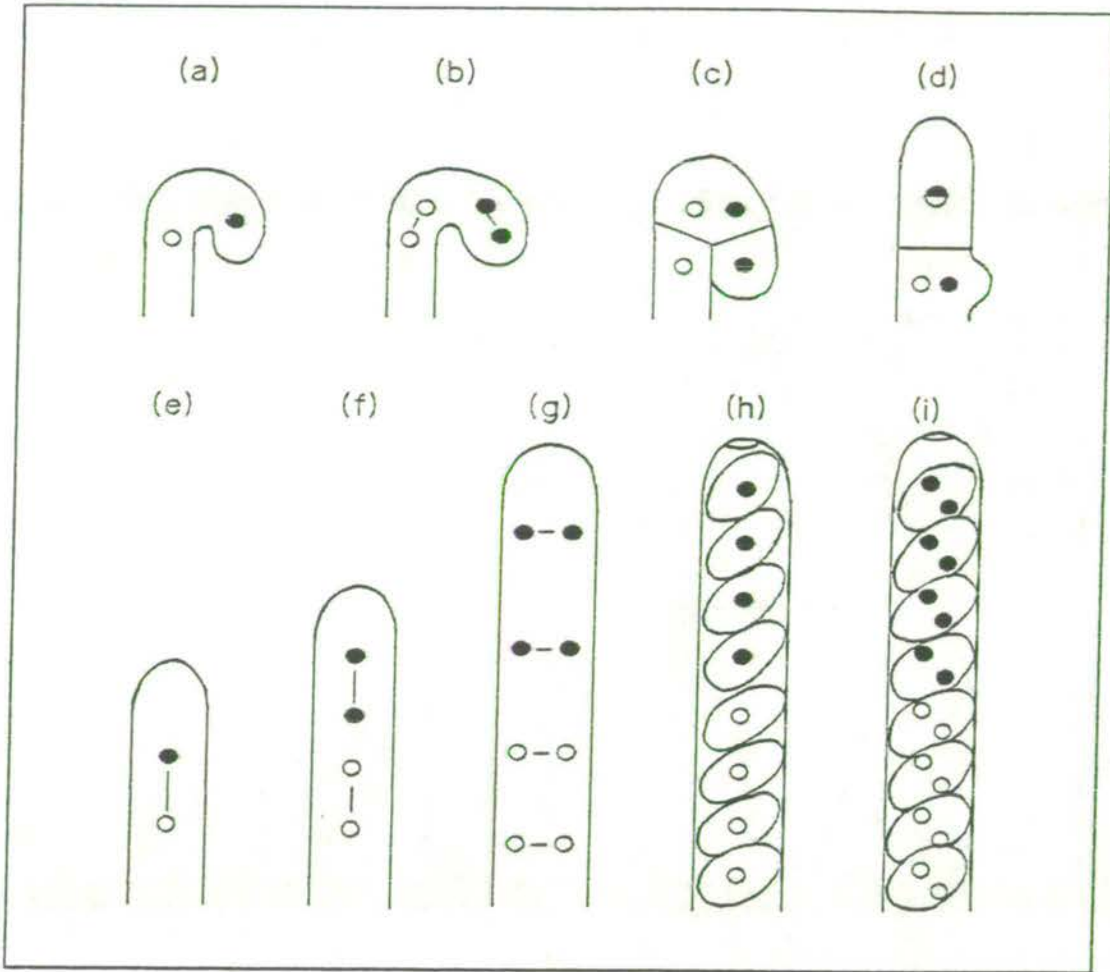
1.4.2.3 Meiosis

Meiosis has been characterised in great detail in *N. crassa* (e.g. Barry, 1969; Gillies, 1972), a species in which many meiotic mutants are available (Smith, 1975; Raju, 1978; Lu and Galeazzi, 1978). Meiosis and subsequent spore formation have been found to be identical in all known 8-spored species of *Neurospora*, whether heterothallic or homothallic (Perkins *et al.*, 1976; Raju, 1978). For a comprehensive review of nuclear behaviour during meiosis and spore formation, the reader is referred to Raju (1980), and for ultrastructural details to Beckett (1981b): only an outline of the various stages will be given here. A convention adopted by Singleton (1953) and followed by most subsequent authors will be observed in description of the various nuclear divisions which occur between karyogamy and spore maturation. The first meiotic division, resulting in the formation of two daughter nuclei is known as Division I, the second meiotic division, yielding four daughter nuclei, as Division II, and a subsequent mitosis prior to spore delimitation as Division III. A second mitosis, which takes place within the maturing spore, is referred to as Division IV.

Division I: After nuclear fusion, the diploid nucleus migrates to the mid-point of the young ascus. During meiotic prophase the chromosomes elongate, reaching their maximum length during zygotene and pachytene (Singleton, 1953). During zygotene, the chromosomes pair while still relatively contracted and synaptonemal complexes are formed. In early pachytene, the bivalents can be seen to be composed of two homologous chromosomes, and by late pachytene the chromosomes, which have been elongating throughout zygotene and pachytene, reach their maximum length of 10-20μm (Raju, 1980). Crossing over

Figure 1.4: Crozier formation and nuclear division during ascospore genesis in *Neurospora crassa*. (Redrawn from Raju, 1980). Not to scale. (a) A pair of non-sister nuclei migrates into a crozier (the curved tip of an ascogenous hypha). The crozier is therefore dikaryotic. (b) A conjugate nuclear division occurs in the crozier. (c) two septa are laid down, dividing the crozier into three cells, a uninucleate stalk cell, a binucleate penultimate cell, and a uninucleate terminal cell. The penultimate cell is the ascus initial. (d) The nuclei in the penultimate cell fuse, establishing the short-lived diploid phase in the young ascus. The stalk cell and terminal cells fuse, restoring the dikaryon. (e) Soon after fusion, the diploid nucleus enters meiosis and the haploid condition is restored in the developing ascus. The spindle which forms at anaphase I is orientated parallel to the long ascus of the ascus, and the daughter nuclei are well separated prior to division II.(f) The two spindles which form at anaphase II are similarly orientated, so that at the end of division II a column of four haploid nuclei (an ordered tetrad) results. (g) Each nucleus then undergoes a mitotic division (Division III), whose spindles are arranged transversely, or occasionally tangentially in the elongating ascus. Two columns of four nuclei are produced, one on either side of the ascus. (h) The nuclei are realigned in a single column of eight, so that the position of each spore pair reflects the position of the parent nucleus in the tetrad. An ascospore wall is laid down around each nucleus. (i) Prior to the development of pigmentation, a second mitotic division (Division IV), occurs within each young ascospore, so that each spore is binucleate.

Figure 1.4



and recombination occurs. At metaphase I, highly contracted chromatids are grouped at the equator of the meiotic spindle, and as the nucleus proceeds into anaphase I, the chromatids migrate towards the spindle poles. During anaphase the spindle elongates, so that the resulting chromosome groups are further apart than the length of the metaphase nucleus (Singleton, 1953) (Fig. 1.4 (e)). At this stage the chromatids are highly contracted and are visible only as dots or short rods (Furtado, 1970). During telophase and the following interphase, chromosomes elongate, but no DNA replication takes place.

Formation of the spindle is regulated in part by structures known as centriolar bodies (Furtado, 1970) or spindle pole bodies (SPBs)(Raju, 1980). SPBs are associated with the outside of the nuclear envelope, and act as microtubule-organising centres, a function which may also be fulfilled by the nuclear envelope itself, and by a third, cortical organising centre situated near the apical tip of the young ascus (Thompson-Coffe and Zickler, 1992). The microtubules of the ascus cytoskeleton appear to function not only in controlling spindle orientation, but also in maintaining the shape of the developing ascus. In *S. macrospora*, the composition of the ascus wall has been shown to be different to that of hyphal walls, resembling instead the non-fibrillar, rather plastic wall of the growing hyphal tip (Sengbusch *et al.*, 1983). It has been suggested that, like the thin walls of hyphal tips, the ascus wall would have insufficient strength to withstand osmotic pressure exerted by the cytoplasm without the mechanical support provided by the cortical cytoskeleton (Wessels, 1986; Thompson-Coffe and Zickler, 1992). The theory is supported by observations of mutants in which ascus morphology is defective: such strains were found to have abnormal cortical organising centres, or to lack such centres altogether (Thompson-Coffe and Zickler, 1992). The central rôle of microtubules in fungal morphogenesis is reflected by the action of griseofulvin, which interferes with the activity of microtubules, and disrupts not only nuclear division, but also ascospore formation (Ott and Ardizzi, 1989) and germ tube development (Barja *et al.*, 1992).

Division II: Second division spindles, like those of division I, are orientated parallel to the ascus wall, and in most *Neurospora* species, nuclei are well separated. The 4 daughter nuclei which are the product of division II are arranged linearly in the elongating ascus (Fig. 1.4 (f)), and constitute an ordered tetrad (section 1.5.1). Although the development of asci is asynchronous, and a perithecium from an eight day old culture typically contains a range of structures

from croziers to maturing spores, relatively few Division II asci are observed, suggesting that this phase is comparatively short-lived.

The length of the ascus provides a useful index for the stage of nuclear division. During early prophase I in *N. crassa*, the young ascus is of around 30μm in length, increasing to 60μm as the chromosomes elongate at zygotene. At pachytene the ascus reaches 75-100μm, and between diplotene and division III extends to 150-170μm (Singleton, 1953). Ascus length has also been correlated with development in *S. fimicola* (Carr and Olive, 1958).

1.4.2.4 Mitosis (Division III)

Meiosis yields four haploid daughter nuclei, each of which undergoes a mitotic division, to give the eight nuclei around which ascospores are delimited in most ascomycetes. During division III, spindles are orientated either obliquely or transversely, so that the resultant nuclei are arranged in files of four on either side of the ascus (Fig. 1.4 (g)). Prior to the delimitation of ascospores, the nuclei are realigned to form a single column of eight (Raju, 1980). The order of nuclei in the tetrad is thus preserved in the 8-spored ascus.

1.4.2.5 Ascospore formation

Spore delimitation by free cell formation (the formation of new cells within an existing cell wall, in this case the ascus wall), is initiated by the alignment of segments of endoplasmic reticulum to form an ascus vesicle (Hohl and Streit, 1975). This membranous envelope encloses all of the ascus cytoplasm and pinches inwards from the ascus wall to cut off units of cytoplasm around individual nuclei. The membranes which form the ascus vesicle have been reported to have their origin in the nuclear envelopes of the prospective spores (Carroll, 1969) or in the ascus plasmalemma (Syrop and Beckett, 1972).

The precise details of spore formation vary between species. In *S. brevicollis*, each nucleus is enclosed by a double membrane, isolating a roughly elliptical portion of sporeplasm from the remaining ascus cytoplasm (Fig. 1.4 (h)), as the latter becomes increasingly vacuolate (Hackett and Chen, 1976). Division IV occurs inside the delimited spore, with the mitotic spindle orientated across the shorter axis (Fig. 1.4 (i)). Between the two delimiting membranes an electron-translucent zone is formed. Proliferation of this layer, with the deposition of an electron dense region on either side, results in the formation of the delimitation ascosporium. To its interior the subascosporium is differentiated, becoming progressively thicker, more dense, and of greater

structural complexity as the spore develops. The subascosporium is the site of deposition of the melanic pigments which give the mature spore its dark pigmentation. The sequence of colour changes observed in the developing ascospore, from hyaline to yellow, beige, grey-brown and dark-brown, reflects the increasing complexity of the subascosporium, and the interruption of this sequence at various points results in the formation of a range of spore-colour mutants (Chen, 1965). Several different mutations can result in the formation of the same spore colour, however. There are for example, at least six loci, situated on several chromosomes, at which the presence of a mutant allele results in the formation of yellow spores (Chen, 1965). Spores with hyaline walls may lack any subascosporium, and their germination is typically poor. In contrast, grey-brown spores, whose development is blocked only when approaching maturity, show high germination frequencies (Chen, 1965).

Observation of spore colour mutants in *N. crassa* has revealed that a number of nuclear divisions occur within the ascospore after wall formation is complete, so that the mature spore is highly multinucleate when discharged (Byrne, 1975; Raju, 1992b). In *Sordaria* species, each mature ascospore has a single germ pore, located at one end of the spore, through which hyphal growth recommences upon germination (Olive and Fantini, 1961). In most species of *Neurospora* there are two such pores, one at either end of the spore (Perkins *et al.*, 1976).

The linear asci and ordered tetrads produced in genera such as *Neurospora* and *Sordaria*, have made these groups particularly useful for various forms of genetic analysis, and the basic principles underlying this type of analysis are outlined in the following sections.

1.5. Genetic Analysis

1.5.1 The significance of ordered tetrads

In the majority of asci, Mendelian laws are obeyed, resulting in a 2:2 segregation with respect to every parental difference in each tetrad (Fincham *et al.*, 1979). As a consequence of the tightly regulated orientation of meiotic spindles during ascospore formation, the position of spores in the mature ascus reflects the segregation of genetic markers at the first or second division. When studying segregation patterns, crosses involving isolates bearing spore colour mutations are particularly useful, as these can be rapidly scored visually, whilst use of nutritional markers requires more time consuming dissection of asci, germination of spores, and testing of the resulting mycelia.

1.5.1.1 Segregation patterns for a single gene

Fig. 1.5 illustrates the segregation patterns obtained from a cross between a wild type isolate and a spore colour mutant. (It is assumed that only a single crossing over event may take place between a particular gene and its centromere.) The segregation of genetic markers at division I indicates that no recombination has occurred and results in the formation of asci with a [4+4] wild type/mutant spore sequence (known as M I type). In contrast, division II segregation is indicative that recombination has take place during meiosis. The asci produced as a result have either [2+2+2+2] or [2+4+2] spore sequences, and are known respectively as asymmetrical and symmetrical M II types. The frequencies of asymmetrical and symmetrical M II type asci are expected to be similar, as they are determined by random migration of chromatids. The combined frequency of symmetrical and asymmetrical M II class asci (the recombination frequency) is equal to twice the map distance between the locus under study and its centromere. Hence the frequency of M II class asci can be used to estimate the position of the locus under study. (Each class of asci can be sub-divided according to the position of spores relative to the ostiole. For example, the frequency of M I asci in which four wild-type spores are located at the tip should be equal to that in which four mutant spores are at the tip.)

1.5.1.2 Segregation patterns for two unlinked loci

In a cross between two isolates bearing different, non-allelic mutations, three types of tetrad can also be obtained (Burnett, 1975). Parental ditype (PD) asci are those in which two of the meiotic products have the genotype of one parent, and the other two that of the other parent. Non-parental ditype (NPD) asci also show a 1:1 segregation, but the two genotypes differ from both parental genotypes. Tetratype (T) asci contain four different genotypes, two of which are parental and two non-parental. The genotypes of the various tetrad types derived from the cross $a^+ b^+$ x $a\ b$ are illustrated below:-

PD	NPD	T
$a^+ b^+$	$a^+ b$	$a^+ b^+$
$a^+ b^+$	$a^+ b$	$a\ b$
$a\ b$	$a\ b^+$	$a^+ b$
$a\ b$	$a\ b^+$	$a\ b^+$

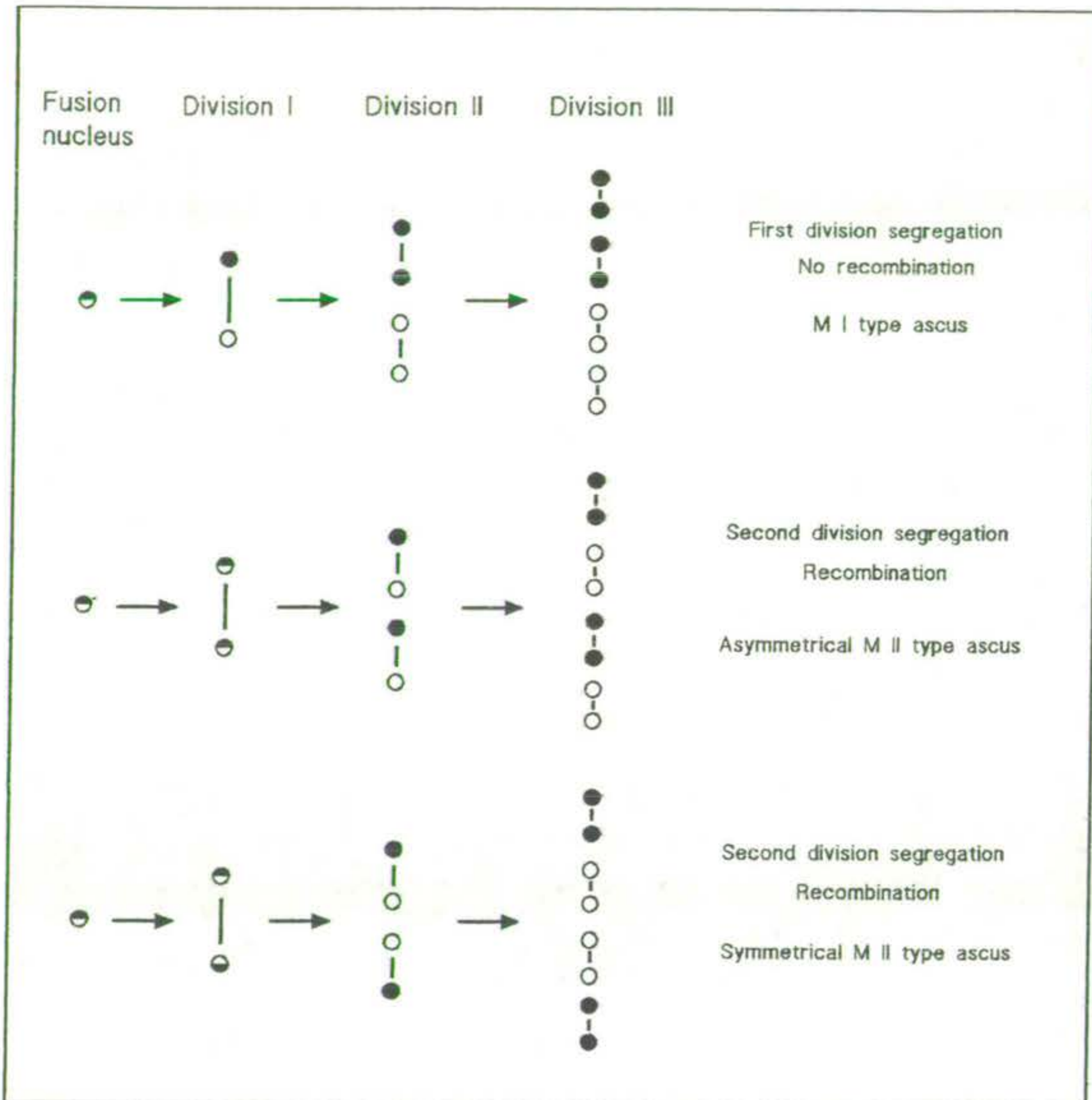


Figure 1.5: Sequence of nuclear divisions during ascus development. (The precise alignment of nuclear spindles within the ascus is shown in Fig. 1.4, Division III spindles are shown here with longitudinal orientation for simplicity only.) Genetic markers segregate either at Division I or Division II. Division I segregation occurs in the absence of recombination, and produces an M I type ascus with a [4+4] wild type/mutant spore sequence. Division II segregation occurs following recombination, and may result in either of two M II class asci. Asymmetrical and symmetrical M II asci are produced at approximately equal frequencies, determined by the random migration of chromosomes at Division II. The combined frequency of asymmetrical and symmetrical M II type asci is the recombination frequency.

When loci are unlinked, PD and NPD tetrads arise from first division segregation, and tetratype tetrads from second division segregation of at least one locus. The presence of a tetratype ascus is thus a reliable indicator that recombination has taken place.

1.5.2 Deviations from expected tetrad frequencies

1.5.2.1 Spindle overlap

During early study of the genetics of *S. brevicollis*, an anomaly was noted in the relative frequencies of asymmetrical and symmetrical M II type asci (Shaw, 1962; Chen, 1965). An unexpected excess of asci in the asymmetrical M II class was observed, in proportions which varied both according to the isolates mated in any particular cross, and to the direction of reciprocal crosses (Chen and Olive, 1965). Biased segregation had previously been reported in *Neurospora* (Whitehouse and Haldane, 1946) *Ascobolus* (Bistis, 1956) and *Venturia* (Boone and Keitt, 1956), but always at a much lower frequency than that found in *S. brevicollis.* Cytological observations indicated that the excess of asymmetrical M II asci observed in *S. brevicollis* was generated by partial spindle overlap at division II. In *N. crassa*, the overlap of division II spindles is rare (Singleton, 1953) although such events have occasionally been reported, at frequencies of up to 0.5 % (Howe, 1956). In *S. brevicollis*, however, partial spindle overlap was observed to generate an excess of 78 % of asymmetrical M II asci in certain crosses (Chen and Olive, 1965). Fig. 1.6 illustrates the effect of such spindle overlap on the three classes of asci described in Fig. 1.5. It can be seen that M I asci are converted to the asymmetrical M II class, and asymmetrical M II asci are modified to resemble the M I class, whilst the symmetrical M II class remains unaltered. The numerical imbalance generated between the symmetrical and asymmetrical M II classes is a consequence of the different sizes of the M I and M II classes before overlap. This is in turn dependent upon the recombination frequency.

The precise frequency of spindle overlap is believed to be a consequence of the genetic background of a cross (Chen and Olive, 1965). Aceto-orcein staining of meiotic chromosomes in *S. brevicollis* showed that the separation of anaphase groups at the end of divisions I and II was at least three times the length of the original nucleus, greater than that previously observed by Singleton (1953) for *N. crassa* (W. Haut, addendum to Shaw (1962)), and it may be that the high frequency of spindle overlap in *S. brevicollis* is, in part, related to the greater length of meiotic spindles. It has also been suggested that spindle overlap

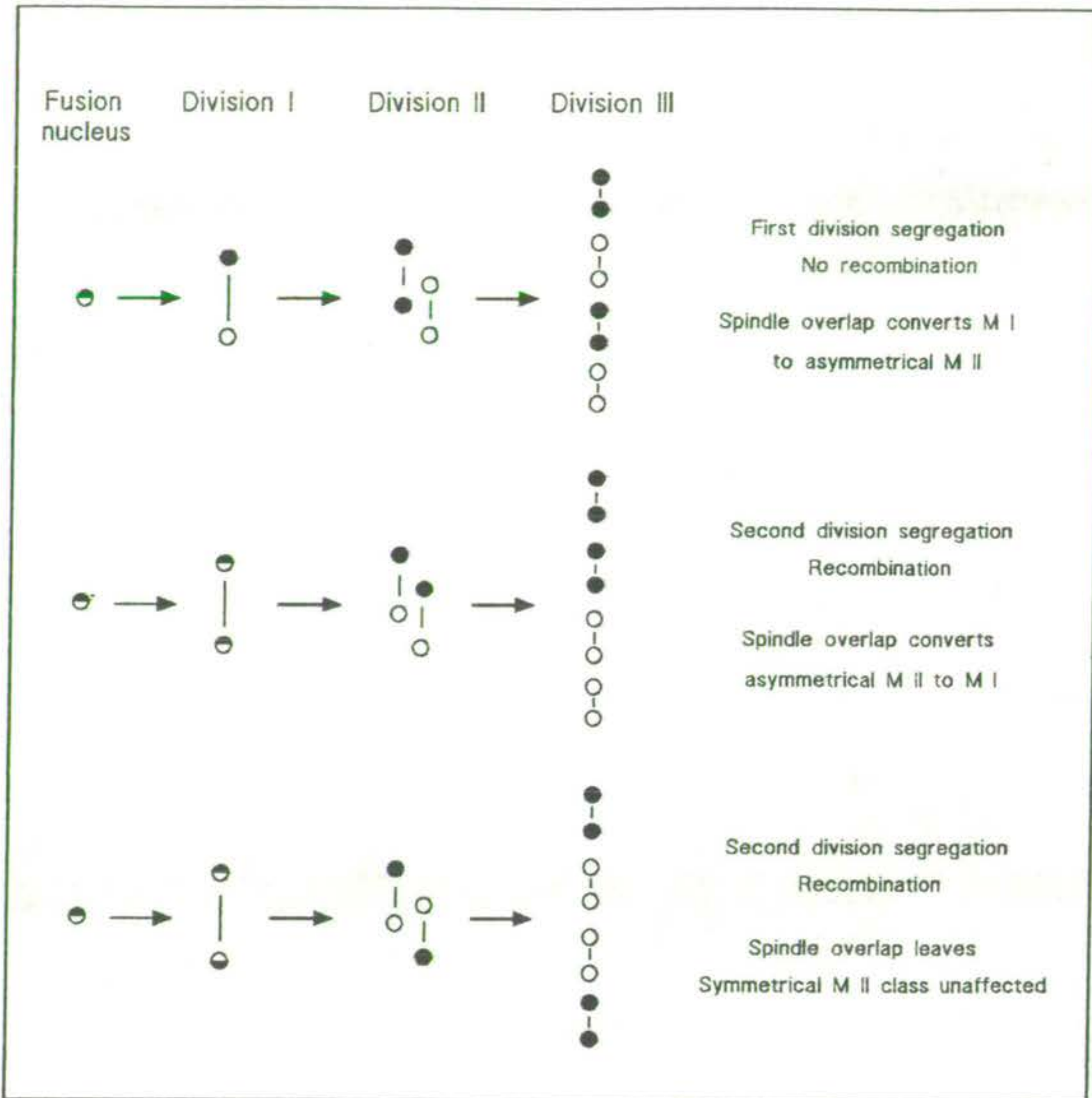


Figure 1.6: Effect of partial spindle overlap at Division II.

The three classes of asci illustrated in Fig. 1.5 may be modified by partial spindle overlap at Division II. M I asci are converted into the asymmetrical M II class, and *vice versa*, whilst the symmetrical M II class remains unaffected. Since the M I class before overlap is typically much larger than either of the M II classes, an excess of asymmetrical M II over symmetrical M II asci is generated.

frequency may have an environmental component, as considerable variation has been obtained between genetically identical replicates (Berg, 1966). Whatever its cause, partial spindle overlap in *S. brevicollis* necessitates the calculation of recombination frequencies and map distances from the frequency of asci in the symmetrical M II class (Shaw, 1962).

The foregoing explanation relates to partial spindle overlap. Although complete spindle overlap is also possible, it is believed to occur only at a very low frequency (Shaw, 1962; Bond, 1969). Support for this view has been obtained by observation of crosses involving the yellow spore colour mutant, *S229*. *S229* is an allele of the y^4 locus on linkage group V (Chen, 1965), a locus which is tightly linked to its centromere and does not recombine. In a cross between *S229* and wild type, any symmetrical M II asci recorded would therefore result from complete spindle overlap at division II: in practice no such asci were observed (Chen and Olive, 1965). The theoretical effects of complete overlap are illustrated in Fig. 1.7. In a cross involving two unlinked loci, spindle overlap will have no effect on the genotypes produced. The presence of a tetratype ascus in such a cross is therefore very reliable evidence that recombination has taken place.

Raju (1992a) uses the term *nuclear passing* to include both the effects of spindle overlap, and those of nuclear migration and repositioning following division. Nuclear migration may occur after divisions II and III, but is more likely to take place after division III, when two columns of four nuclei are realigned into a single file of eight.

1.5.2.2 Polarised segregation

Polarised segregation, the non-random migration of chromosomes to the poles of anaphase spindles, has been described in a number of ascomycetes (Catcheside, 1944; Mathieson, 1956; Nakamura and Egashira, 1961), and used to explain apparent anomalies in the frequencies of various ascus types. The phenomenon can be detected by examination of M I class asci: the frequency with which four wild type or four mutant spores are located at the tip of the ascus should be equal, and significant deviation may be indicative of polarised segregation. Studies carried out on *S. brevicollis* have failed to produce any evidence for polarised segregation however (Shaw, 1962; Berg, 1966), and it is not believed to be a phenomenon of great significance in most species (Raju, 1980).

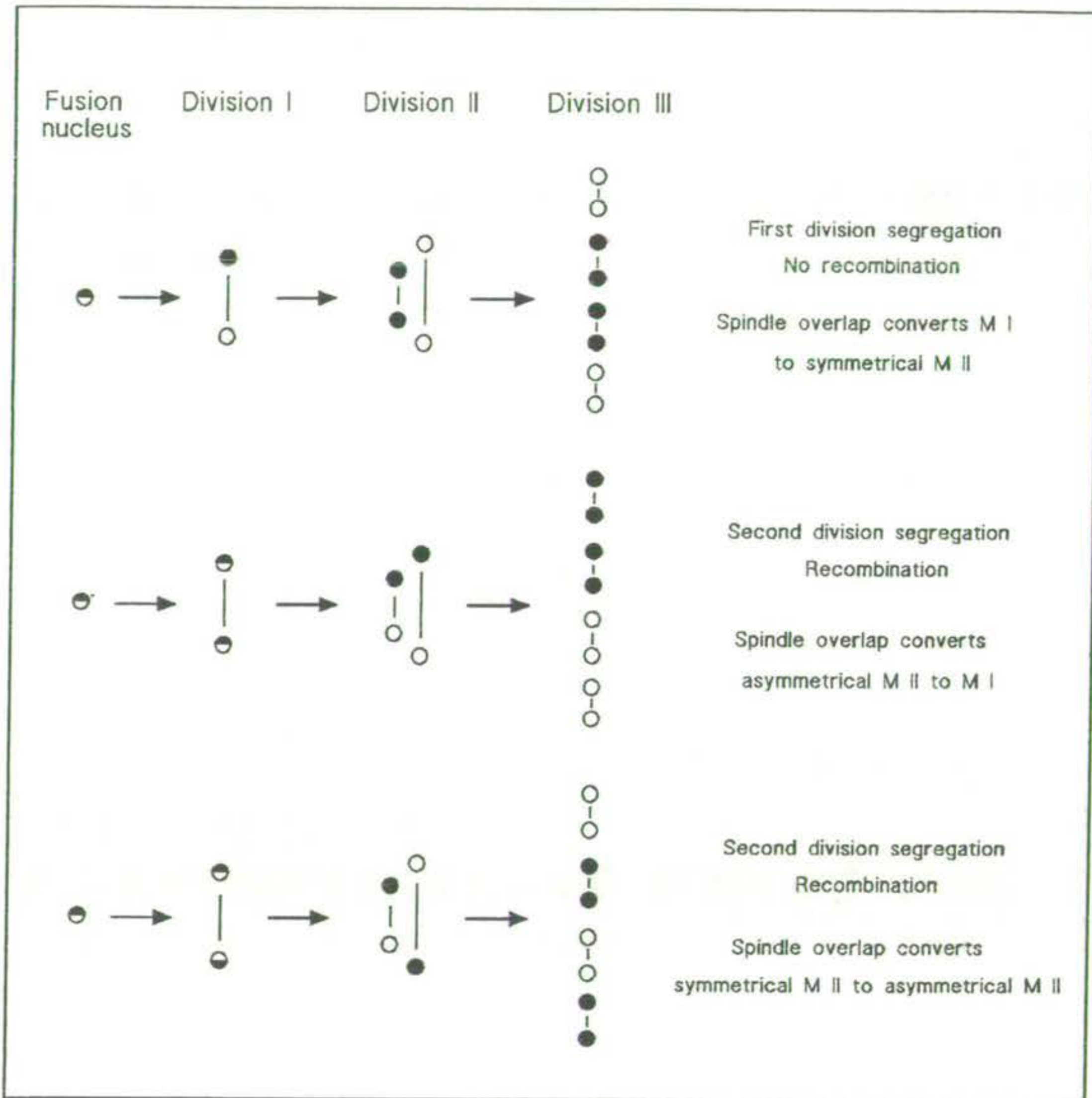


Figure 1.7: Effect of complete spindle overlap at Division II.
M I class asci are converted to symmetrical M II type, asymmetrical M II type are converted to M I type, and symmetrical M II to asymmetrical M II.

1.5.2.3. Preferential maturation and discharge of spores

Apparent deviation from the predicted frequencies of various types of ascus may also be caused by non-random maturation of spores (Shaw, 1962), and consequently, the non-random discharge of asci (Lamb, 1966). It has been demonstrated that the proportions of the various classes of asci within a perithecium may vary during the period of spore discharge (MacDonald and Bond, 1974).

1.6 Breeding systems in the Sordariaceae

Two fundamentally different breeding systems are known in ascomycete species, homothallism and bipolar heterothallism. In both cases, genes acting at the population level determine the individuals between which mating can occur, and thus regulate the amount of heterogeneity which will be present in the next generation (Stamberg and Koltin, 1981).

1.6.1 Homothallism

All individuals of a homothallic species are self-fertile and can complete their sexual cycle in isolation (Fincham *et al.*, 1979). In such species there is no functional mating type, and although meiosis occurs during ascospore formation, in a homozygous strain, all ascospores are genetically identical to the parent. These fungi are not necessarily homozygous however, since heterokaryosis is relatively widespread (Burnett, 1975). In a heterokaryon, two nuclei which fuse at karyogamy may be genetically dissimilar, producing hybrid perithecia. Hybridisation experiments carried out on the homothallic *Sordaria fimicola* revealed that perithecia produced on a heterokaryon were of three types: pure wild type, pure mutant, and hybrids (containing both wild type and mutant spores) (Olive, 1954). Within hybrid perithecia, homozygous asci were observed only rarely, suggesting either (i) a greater attraction between nuclei of different genotypes during dikaryon formation in ascogenous hyphae, or (ii) that in the majority of perithecia, all asci were derived from two founder nuclei which divided in pairs. A third explanation is that nuclear fusion is random, but that some sort of advantage is conferred by the heterokaryotic nature of the young ascus (Burnett, 1975). A similar phenomenon has also been noted in *Aspergillus*, suggesting that, in some cases, heterozygosity is favoured in asci of homothallic species (Pontecorvo *et al.*, 1953), thus maintaining genetic variation where inbreeding would appear to promote homozygosity.

1.6.2 Heterothallism

Two forms of outbreeding have been identified in fungal species. The first, and least well understood system, relies upon sexual dimorphism or dioecy, in which male and female organs are produced on different mycelia (Fincham *et al.*, 1979). Although common in algae, such dimorphism is largely limited to a group of aquatic fungi which includes several species of *Achlya* (Raper, 1960).

In most heterothallic ascomycete species, all isolates are morphologically identical, although mating can only take place between individuals of different mating type. Mating type is determined by the presence of two alternative alleles at the mating type locus. In filamentous ascomycetes such as *Neurospora* and *Sordaria* these alleles are designated *mtA* and *mta*, although no dominance relationship is implied. In other ascomycete genera the mating type genes are denoted by symbols such as + and -, *a* and α, *mat+* and *mat-*.

1.6.2.1 Molecular nature of heterothallism

Recent isolation of genomic clones containing the mating type alleles of *N. crassa* has revealed that the *mtA* and *mta* sequences are highly dissimilar (Vollmer and Yanofsky, 1986; Glass *et al.*, 1988). The *mtA* allele contains a 5301 base pair region which has no corresponding sequence in *mta* strains (Glass *et al.*, 1990a). Within this region, 928 base pairs encode an *mtA* function which is constitutively expressed. The *mta* allele also contains a unique region, in this case 3325 base pairs, containing a 1260 base pair sequence which encodes the *mta* function (Staben and Yanofsky, 1990). Because the *mtA* and *mta* sequences are so dissimilar, it has been proposed that the term *idiomorph* should be used in preference to *allele* (Glass *et al.*, 1990a). Investigation of other heterothallic species of *Neurospora*, *Sordaria* and *Gelasinospora* has revealed that the molecular basis of heterothallism is similar in all cases. Each mating type contains a single copy sequence which hybridises to one of the mating type probes from *N. crassa* (Glass *et al.*, 1990b; D. J. Bond and A. J. Leigh-Brown, unpublished).

1.6.2.2 Functions of the mating type genes

The mating type genes are believed to function as "master switches", triggering a sequence of morphological development which culminates in fertilisation (Raju, 1992b). These events are described in some detail in section 1.4, and include processes such as the production and reception of sexual pheromones (Bistis, 1981; 1983).

In species such as *N. crassa*, the mating type genes also function as heterokaryon incompatibility (*het*) genes in the vegetative phase (Perkins *et al.*, 1982). Stable heterokaryons will only form reliably between mycelia of the same mating type, which also carry the same allele at a number of incompatibility loci distributed throughout the genome (Garnjobst, 1955; Perkins *et al.*, 1976). If paired mycelia differ at one or more of these loci, a lethal cytoplasmic incompatibility reaction follows hyphal fusion, and heterokaryon formation is prevented (Garnjobst and Wilson, 1956). Molecular analysis of *N. crassa* has showed that both the sexual and heterokaryon incompatibility functions of the mating type idiomorphs are localised to single open reading frames, although these sequences are quite dissimilar in each mating type (Glass *et al.*, 1990a; Staben and Yanofsky, 1990). However, a similar examination of the mating type genes of *Podospora anserina* showed that in this species, no incompatibility function is associated with mating type (Picard *et al.*, 1991).

1.6.3 Distribution of homothallism and heterothallism

Whilst homothallism predominates amongst the Zygomycotina, and both homothallism and heterothallism are encountered in Ascomycotina, the most complex of fungi, the basidiomycetes, are exclusively heterothallic. It is thus tempting to assume that heterothallism, accompanying the highest degree of structural complexity, is an advanced condition, and homothallism is consequently a more primitive state. The probable evolution of homothallism and heterothallism in members of the Sordariaceae will be discussed later (section 4.3.4), but it is worth noting at this point that each system has its merits. Homothallism facilitates inbreeding, and thus retains successful combinations of genes, which have allowed the colonisation of a stable environment. Superficially, it would appear that this function could be fulfilled with a greater degree of efficiency by the production of asexual spores, since these are typically less costly to produce in terms of energy expenditure. It has been suggested therefore that the retention of the sexual system in homothallic species such as *Sordaria macrospora* or *Neurospora africana*, reflects not only the possibility for DNA repair facilitated by meiosis, but also the greater longevity of ascospores relative to conidia (Metzenberg and Glass, 1990). In contrast, heterothallism promotes outbreeding and generates novel genetic combinations which may allow the fungus to exploit new environments. However, a heterothallic individual in isolation is infertile, and thus unable to produce long-lived sexual spores.

It is clear that numerous genetic factors may influence the development of multicellular structures during sexual reproduction in the Sordariaceae. Many additional effects are exerted by environmental variables, such as light, temperature, nutrient availability, and the carbon:nitrogen ratio in the substrate.

1.7 Environmental factors affecting multicellular development

1.7.1 Light

The effects of light on fungal development can be divided into two categories, morphogenetic effects (in which the formation of a structure is induced or inhibited) and non-morphogenetic effects (where light influences the rate or direction of growth, or the synthesis of a compound) (Page, 1965). Amongst examples of morphogenetic effects are the formation of sclerotia and various reproductive structures, whilst non-morphogenetic effects include phototropism, spore discharge, and carotenogenesis (Tan, 1978). The majority of light responses are mediated by wavelengths at the blue/ultraviolet (UV) end of the spectrum, although occasional responses to green, yellow and red light have been reported (reviewed by Moore-Landecker, 1992). Blue/near UV responses are frequently referred to as blue light effects.

1.7.1.1 Blue light effects

1.7.1.1.1 *Photoreceptors*

Characteristic blue light effects, such as perithecial formation in *Nectria haematococca* (Curtis, 1972), have action spectra which show main peaks at around 450 nm, and little or no activity above 520 nm. Two groups of fungal pigments have absorption spectra of very similar form, namely flavins and carotenoids. Since the primary requirement for a photoreceptor is that it should absorb the wavelengths which cause the response, both flavins and carotenoids have attracted considerable attention as potential mediators of blue light effects. As the spectral characteristics of either pigment may be modified by the local environment *in vivo*, the action spectra for the various responses cannot be used to differentiate conclusively between the two pigments. Mutant analysis, however, yields evidence that carotenoids are not the blue light receptor for responses such as protoperithecial induction in *N. crassa* (Degli-Innocenti *et al.*, 1983; Russo, 1986), or phototropic neck curvature in the same species (Harding and Melles, 1983). In several albino (*al*) mutants of *N. crassa* the levels of measurable carotenoids are very much lower than in wild type strains, but photoresponses are not reduced in magnitude as might be expected. For a variety

of reasons (of which the observed photoresponses of albino mutants is only one), current opinion favours a flavin as the major photoreceptor for blue light effects, via post reduction of cytochrome b (Muñoz and Butler, 1975; Inoue, 1984; Song, 1984).

1.7.1.1.2 *Photoresponses*

The literature concerning responses to blue light is vast and cannot be covered in detail here. (For a list of reviews see Tan, 1978, and for other review articles see Senger, 1984). This brief overview will concentrate upon aspects of ascocarp induction and maturation, and will focus upon three species which have been studied in some detail, *Pyronema domesticum*, *Gelasinospora reticulispora*, and *N. crassa.* These examples are chosen to illustrate some typical responses to blue light, but it is important to bear in mind that fungal light requirements are frequently very precise, and that the observation of a particular phenomenon in one species does not mean that a similar effect can necessarily be inferred in even the most closely related group.

The light requirements for fruitbody production in the discomycete *P. domesticum* have been investigated in a series of experiments (Moore-Landecker, 1975; 1979a; 1979b). In this apothecial species, exposure to light for 3-6 hours is normally required for the initiation of gametangia, and further light is needed for the maturation of ascospores. Two distinct methods have been discovered however, whereby this light requirement can be by-passed. Placing dark-grown cultures in circulating air currents resulted in the formation of apothecia, although these were typically poorly developed, and seldom produced mature ascospores (Moore-Landecker and Shropshire, 1982). More successful in terms of spore development, was the addition of potassium permanganate (an oxidising agent) to dark-grown cultures, stimulating the development of mature apothecia (Moore-Landecker, 1983), although these lacked the normal pinkish pigmentation. The latter effect supports the case of a flavin as the receptor, since the artificial addition of an oxidising agent effectively by-passed the light requirement.

Light has also been found to play an important rôle in regulating perithecial formation in *G. reticulispora*, exerting either promotive or inhibitory effects at different stages of development. During early mycelial growth, before the hyphal front reached the end of the growth tube, neither continuous light nor continuous darkness caused the initiation of perithecial production. However, an inductive dark period, followed by exposure to light greatly increased perithecial

production (Inoue and Furuya, 1970). Once the growing mycelium reached the edge of the containing vessel, perithecia were produced irrespective of the light environment. A minimum dark inductive period of 30 hours was required to produce the photoresponse (Inoue and Furuya, 1974a), but a short light break during the dark period could remove the stimulatory effect (Inoue and Furuya, 1974b). After exposure to inductive blue light, irradiation with near ultraviolet light prevented perithecial formation (Inoue and Watanabe, 1984). The action spectrum for the photoresponse was found to shift slightly according to the length of the dark induction period, peaking between 450 and 460 nm, and showing no effect beyond 520 nm (Inoue and Furuya, 1975a). Microscopic observations of photoinduced mycelia revealed that the fine hyphae which gave rise to protoperithecia were already present at the end of the dark induction period (Inoue and Furuya, 1975b).

Photoinduction of protoperithecia in *N. crassa* is a blue light effect with a threshold of about 4 Jm^{-2} (Degli-Innocenti *et al.*, 1983). Although a low level of constitutive protoperithecial production occurs in darkness, a far greater number of protoperithecia are developed when a period of darkness is followed by irradiation with blue light. Such photoinduction is only one of several blue light effects recorded in this fungus. Amongst others are various effects on circadian rhythms (Lakin-Thomas *et al.*, 1990), phototropic curvature of perithecial necks (Harding and Melles, 1983) and the induction of carotenoid synthesis (Harding and Shropshire, 1980; Rau, 1980). Analysis of these responses has been facilitated by the isolation of a number of mutants which are "blind" to the effects of blue light. White collar (*wc*) mutants exhibit normal (constitutive) carotenoid production in the conidia, but lack the ability to synthesise photoinduced carotenoids in the mycelium (Perkins *et al.*, 1982). All the *wc* mutations isolated to date, map to one of two loci, designated *wc*-1 and *wc*-2 (Degli-Innocenti and Russo, 1984 a,b), and it is possible that one of these loci may code for the blue light receptor (Russo, 1988). Although the mutants exhibit normal linear growth, protoperithecial induction by blue light is impaired, and it would appear that the *wc* strains are regulatory mutants, blocked in the light induction pathway. Within 30 minutes of the exposure of dark grown mycelia of *N. crassa* to light, increases in the levels of 13 translatable mRNA species were detected (Nawrath and Russo, 1990). These mRNAs, which could be divided into four distinct classes according to the time of their appearance, were absent from *wc*-1 and *wc*-2 mutants. In contrast, the albino (*al*) mutants mentioned previously are defective

for enzymes in the carotenoid biosynthesis pathway, and exhibit normal phototropisms (Harding and Melles, 1983).

Advances have also been made in the understanding of the molecular nature of the light response in *N. crassa*. The cDNAs from four light regulated genes were cloned by Sommer *et al.* (1989), who found that two of these genes, named *bli*-3 and *bli*-4 (blue light induced), were induced within two minutes of illumination. The speed of this response suggested that a regulatory factor was already present before light treatment, but may have only been able to bind to the promoters of the genes after a light-induced modification. None of the four *bli* genes were induced in *wc* mutants. Other studies have indicated that protein dephosphorylation constitutes a necessary step in light transduction (Lauter and Russo, 1990).

1.7.1.2 Red light effects

Although the majority of fungal responses are mediated by light at the blue/UV end of the spectrum, a few effects are generated by red or far-red light. Amongst these are perithecial production in *Mycosphaerella ligulicola* (McCoy *et al.*, 1975) and conidiation in *Aspergillus nidulans* (Mooney and Yager, 1990). In the latter study, light-dependent conidiation was shown to be determined by the *velvet* gene, whose inductive effect could be reversed by an immediate shift to far-red light, in a manner similar to phytochrome-mediated responses in plants, and it has been suggested that phytochrome may be the red light receptor in fungi.

1.7.2 Nutrient Sources

The effects of nutrient sources upon fungal development have been documented extensively (Lilly and Barnett, 1947; Bretzloff, 1954; Hawker, 1966; Hodgkiss, 1969) and have been reviewed recently (Moore-Landecker, 1992). The availability of particular nutrient sources and, specifically, the precise ratio of carbon:nitrogen in the substrate, may be of great importance in some fungal species, whilst others have relatively non-specific requirements. Fungi generally require a higher concentration of carbon than nitrogen: in *S. fimicola*, concentrations of carbon:nitrogen between 5:1 and 10:1 promote production of perithecia (Hall, 1971). Glucose, sucrose and a number of other polysaccharides such as starch are widely utilised as carbon sources, whilst common nitrogen sources include various nitrates and ammonium acetate (Moore-Landecker, 1992). Some fungi also require particular vitamins or trace elements for fruiting,

and may be influenced by the presence of certain fatty acids. Linoleic acid, for example, has been demonstrated to stimulate perithecial production in *Nectria haematococca* (Dyer *et al.*, 1993).

The fruitbodies of *Sordaria* species often appear in the early stages of fungal succession on animal dung, together with *Mucor* and *Pilobolus*, but before basidiomycete genera such as *Coprinus* (Harper and Webster, 1964; Morinaga *et al.*, 1980). This succession reflects the greater size of basidiocarps, and the longer period of mycelial growth required for accumulation of the reserves which are necessary to support fruitbody formation (Harper and Webster, 1964). Amongst other environmental factors which may affect this succession are the pH of the substrate, and the relative humidity.

1.7.3 Temperature

Temperature affects a number of developmental processes in fungi, ranging from spore activation and germination (Anderson, 1978), to control of hyphal branching (Trinci, 1974), and the formation of ascocarps (Moore-Landecker, 1992). The temperature range conducive to fruiting in fungi is typically narrower than that which permits vegetative growth: in *S. macrospora* for example, the production of perithecia was inhibited above 30°C although mycelial growth was possible at higher temperatures (Esser, 1980). Similarly in *N. crassa*, where a certain amount of variation was found between strains, the optimal temperature for protoperithecial production was typically in the range 25-30°C (McNelly-Ingle and Frost, 1965). When cultures of *N. crassa*, previously maintained at 35-37°C, were transferred to incubators at 25°C, ascogonia and perithecia were differentiated, suggesting that production of the compound(s) required for ascogonial morphogenesis was blocked at the higher temperatures (Viswanath-Reddy and Turian, 1972). Further experiments showed that a substance which induced ascogonial formation was produced 36-48 hours after inoculation, at 25°C, and that subsequent transfer of cultures to a higher temperature did not inhibit ascogonial production. Available nutrient sources may also have an effect upon the response to temperature. When grown on a medium containing a polyol as a carbon source (instead of the usual sucrose) *Neurospora* mycelia were able to differentiate protoperithecia at 37°C (Viswanath-Reddy and Turian, 1975).

In addition to affecting the initiation of fruitbodies, temperature may also determine their fertility: in *Ophiobolus graminis*, sub-optimal temperature

resulted in the formation of abnormal ascogenous hyphae and sterile perithecia (Weste, 1970).

1.8 *Sordaria brevicollis*

S. brevicollis, originally isolated from zebra dung (Olive and Fantini, 1961), has been identified more recently on buffalo dung in Africa (Khan and Krug, 1989), and on opossum dung in New Zealand (D. P. Mahoney, pers. comm.). When first isolated, *S. brevicollis* was the only known heterothallic species in an otherwise homothallic genus. Homothallism is common amongst coprophilous fungi, and although several other heterothallic species of *Sordaria* have since been identified (Fields and Maniotis, 1963; Fields, 1968; Cailleux, 1971), homothallic species such as *S. humana* and *S. fimicola* are much more widely reported. The "traditional" life cycle of *S. brevicollis* is illustrated in Fig. 1.8. Several structures produced by this fungus under laboratory conditions are omitted from the diagram, however, and are described briefly below.

Structures which were tentatively called "sclerotia" by Broxholme (1988), are described in this thesis as vegetative hyphal aggregates (VHAs) - a term chosen to avoid implying any particular function. The characterisation of VHAs is described Chapter Three, as part of an examination of all of the multicellular structures produced by *S. brevicollis* in the laboratory. Also described in the same chapter is the formation of perithecia on uncrossed homokaryons. Although the majority of homokaryotic perithecia are empty, some contain a few viable ascospores, which when germinated, give rise to mycelia of parental mating type. Cultures of *S. brevicollis* may therefore produce at least four different multicellular structures - protoperithecia, crossed perithecia, uncrossed perithecia, and VHAs.

Figure 1.8

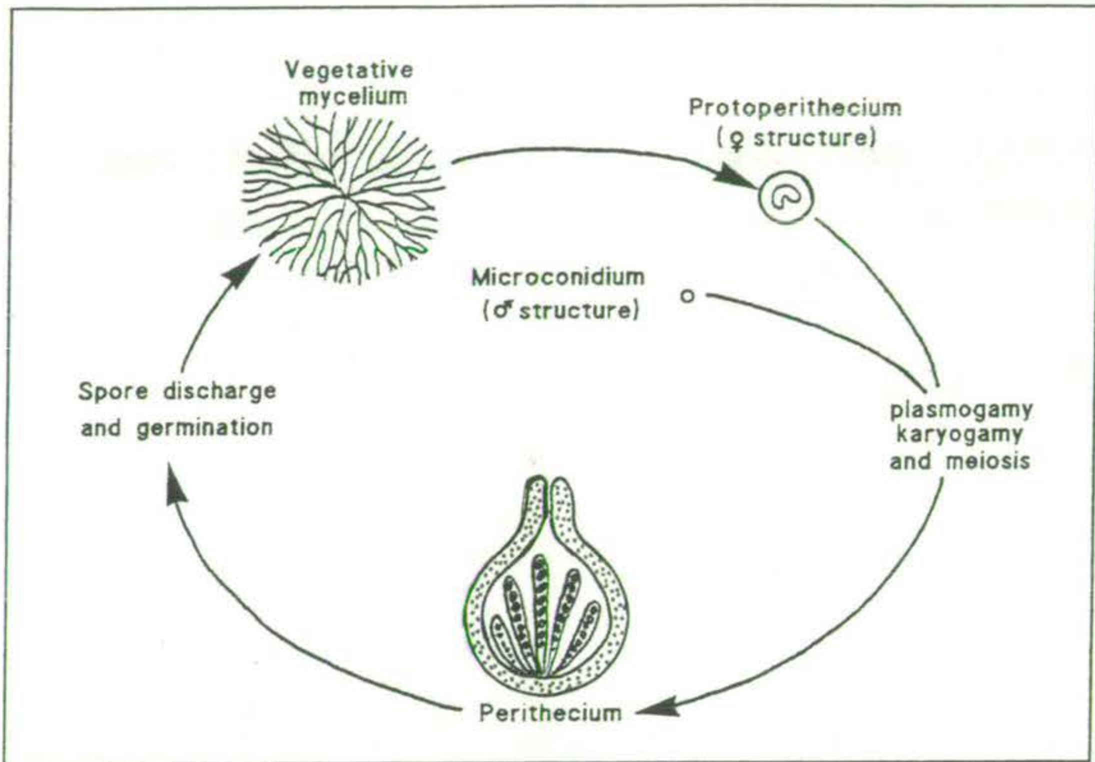


Figure 1.8: Life cycle of *Sordaria brevicollis*.

Protoperithecia are produced on 2-4 day old mycelia of both mating types, but typically develop further only when fertilised by microconidia of the opposite mating type. After plasmogamy (cell fusion), protoperithecia expand rapidly into young perithecia, within which karyogamy and meiosis take place. At maturity, a single perithecium may contain several hundred asci, each with four spores of *mtA* and four of *mta*. Spores are discharged 8-10 days after inoculation, and germinate immediately to produce new mycelia.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Fungal strains

Wild type and mutant strains of *Sordaria brevicollis* were obtained by germinating ascospores from stock crosses supplied by D. J. Bond, and were direct descendants of the original isolates of this species (Olive and Fantini, 1961). Wild type isolates were used in all experiments unless otherwise stated.

A number of spore colour mutants employed in genetic analysis of homokaryotic fruiting (Chapter Four), are detailed below:-

S187 is an allele of the y^9 locus on linkage group II (approximately 16 map units from the centromere), and results in the production of yellow spores (MacDonald and Bond, 1974). *S229*, an allele of the y^4 locus on linkage group V (tightly linked to the centromere), also produces yellow spores (Chen, 1965). *c70*, an allele of the b^1 locus on linkage group II, produces buff spores and reddish-brown protoperithecia (Chen, 1965). A hyaline strain, produced by a mutation of the h^3 locus on linkage group VI, forms both perithecia and ascospores which lack the dark pigmentation of wild type strains (Sang, 1978).

A second heterothallic Sordaria species, *S. sclerogenia* (Fields and Grear, 1966), recently isolated in New Zealand, was obtained from D. P. Mahoney (Wellington, New Zealand). This is the only known sclerotial species within the genus. Cultures of *Neurospora crassa* (St Lawrence wild type strain) were supplied by N. D. Read.

2.2 Chemicals

Unless otherwise indicated, chemicals were obtained from British Drug Houses (BDH) (Poole, Dorset), and were of the highest quality available.

2.3 Media and Growth Conditions

Corn meal agar (CMA) (Oxoid, Basingstoke) was prepared according to the manufacturer's instructions, and supplemented with 3 g sucrose, 2 g glucose, and 1 g yeast extract per litre of distilled water (MacDonald and Bond, 1974). The pH of all growth media was adjusted to 6.8 $\pm$ 0.1. Sterile disposable Petri plates of 8.5 cm diameter (Sterilin, Hounslow) were each poured with 20.0 ml of agar using an automatic plate pourer (Petrimat, Denmark). Although poured

plates could be kept for long periods at 4°C, freshly prepared plates were used whenever possible (particularly during time-course experiments) as the initial growth of mycelia on older plates was sometimes delayed by several hours.

For some experiments, the surface of the agar was overlaid with a cellophane membrane (525 gauge, uncoated "rayophane", AA Packaging, Walmer Bridge, Lancs.). Discs were cut from dry cellophane, washed in distilled water, and then interleaved with a hard filter paper (Whatman No. 50). After autoclaving, membranes were transferred onto the agar surface.

Two types of rabbit dung agar were prepared. For the first, 8 freshly collected rabbit pellets were sterilised, and evenly spaced around the perimeter of a Petri plate, which has just been poured with 20.0 ml CMA. For the second type, pellets were air-dried for 24 hours, ground to a coarse powder, and added to the media (at the equivalent of 8 pellets /20.0 ml) prior to autoclaving. Before and during plate pouring, the media was agitated gently to ensure mixing of the faecal material. Plates containing powdered cellophane membranes (ground in liquid nitrogen) or cellulose powder, were prepared in a similar manner. In both cases, the amount of added material was equivalent to the weight of an 8.5 cm diameter cellophane disc.

Cultures were initiated by placing a small inoculum of vegetative mycelium in the centre of a Petri plate. After inoculation, plates were sealed with Parafilm (American National Can, Greenwich, Connecticut) and incubated at 25°C unless otherwise stated. White light was supplied by Thorn Cool White fluorescent tubes at an intensity of approximately 136 μmol quanta $m^{-2}s^{-1}$.

2.4 Mycelial storage and spore germination

Over a period of several months, vegetatively propagated strains of *S. brevicollis* exhibited progressive loss of fertility and reduced mycelial growth rate, often necessitating reisolation of new material from stock crosses. Air-dried mycelial cultures, which exhibited prolonged viability and fertility, were prepared as follows. The parafilm sealing Petri plates containing 4 day old cultures was removed, and plates were left unsealed for approximately 20 days, until the growth media and fungal mycelium dried to a thin, brittle disc. The disc was cut into 5 mm squares using sterile scissors, and the pieces stored in a sterile culture bottle until required for use as an inoculum. In this semi-dormant state, isolates could be stored for over twelve months without marked deterioration, although the ageing process often proceeded rapidly once growth recommenced.

Germination of spores from stock crosses was enhanced by the addition of 0.7% (w/v) sodium acetate to the growth medium (MacDonald and Bond, 1974). Recently mature spores usually germinated within 6 hours at 25°C, and spores which had not germinated after 24 hours were discarded. After germination, the young mycelium, in a small block of agar, was transferred to the standard growth medium. The growth rate and fertility of new isolates were monitored for several days before new experimental strains were selected. Vigorous, fertile strains were used whenever possible, on the premise that experimentally induced variation in perithecial number would be more easily detected if overall numbers were high. Mating type was established by crossing each new isolate with a test strain of known mating type.

2.5 Crossing techniques

The crossing of opposite mating types and the subsequent production of fertile perithecia could be achieved in either of two ways.

2.5.1 Confrontation cross

Inocula from two different mycelia were placed at opposite sides of the same Petri plate and incubated in darkness for 8-9 days. Where sexually compatible mycelia met, two lines of perithecia developed, often separated by a narrow zone of vegetative tissue. This technique was used for mating type testing, as hyphal interaction between opposite mating types could be detected only 3-4 days after inoculation.

2.5.2 Spermatisation

Spermatisation was carried out in a laminar flow cabinet in order to reduce contamination. Microconidia were routinely harvested from 4 day old mycelia by flooding cultures with approximately 10 ml sterile distilled water, and then stirring gently with a sterile glass rod. The resulting suspension was filtered through two layers of sterile muslin to remove any large mycelial fragments, diluted as required with distilled water, and added to 4 day old cultures of opposite mating type. After 5 minutes, excess water was poured away, plates were resealed, and incubated for 6 days to allow perithecial maturation.

This technique was used for quantitative experiments as the even distribution of fruitbodies which resulted allowed accurate measurements of perithecial number to be made. For experiments requiring incubation in the dark, spermatisation was carried out in a darkroom with a red safe-light. Brief

exposure of protoperithecial cultures to dim red light had no effect on the subsequent formation of perithecia compared to cultures fertilised in complete darkness (data not shown).

2.5.2.1 Spermatial concentration

The concentration of microconidia in spermatial suspensions was determined using a haemocytometer (Modified Fuchs Rosental, BS 748). The spermatial suspension from 10 Petri plates, produced as described above, was concentrated by centrifugation at 1250 g for 10 minutes, and then the pellet was resuspended in 5.0 ml distilled water (containing a drop of lactophenol cotton blue to stain fungal material). Haemocytometer counts indicated that a typical 4 day old, dark-grown culture produced $2.0 - 2.5 \times 10^5$ microconidia, and that this figure remained relatively constant in successive subcultures until after an isolate started to exhibit the first signs of ageing. At the concentration routinely used in experiments (the microconidia from one "male" plate being used to fertilise the protoperithecia of four "female" plates), it was estimated that approximately 6×10^4 microconidia were supplied to each protoperithecial culture, and that these were not limiting to the number of perithecia which developed.

2.6 Light Filters

The following polyester filters (Lee Colortran International, Andover, Hampshire) were used to manipulate culture illumination: "Moonlight Blue" (product no. 183), "Fire" (019), and Neutral Density (210 and 211). The blue and red filters used had approximately equal transmission. The manufacturer's transmission spectrum for each filter was verified using a Beckman DU64 spectrophotometer (Fig. 2.1).

Filters were used to construct rectangular packets, each holding four Petri plates. The edges and corners of these packets were carefully sealed to prevent the penetration of white light, and the temperature within each packet was monitored using a maximum/minimum thermometer. Since the temperature within the packet made from the red filter rose to 3°C above ambient, experiments using this filter were carried out with the incubator temperature set to 22°C.

Figure 2.1

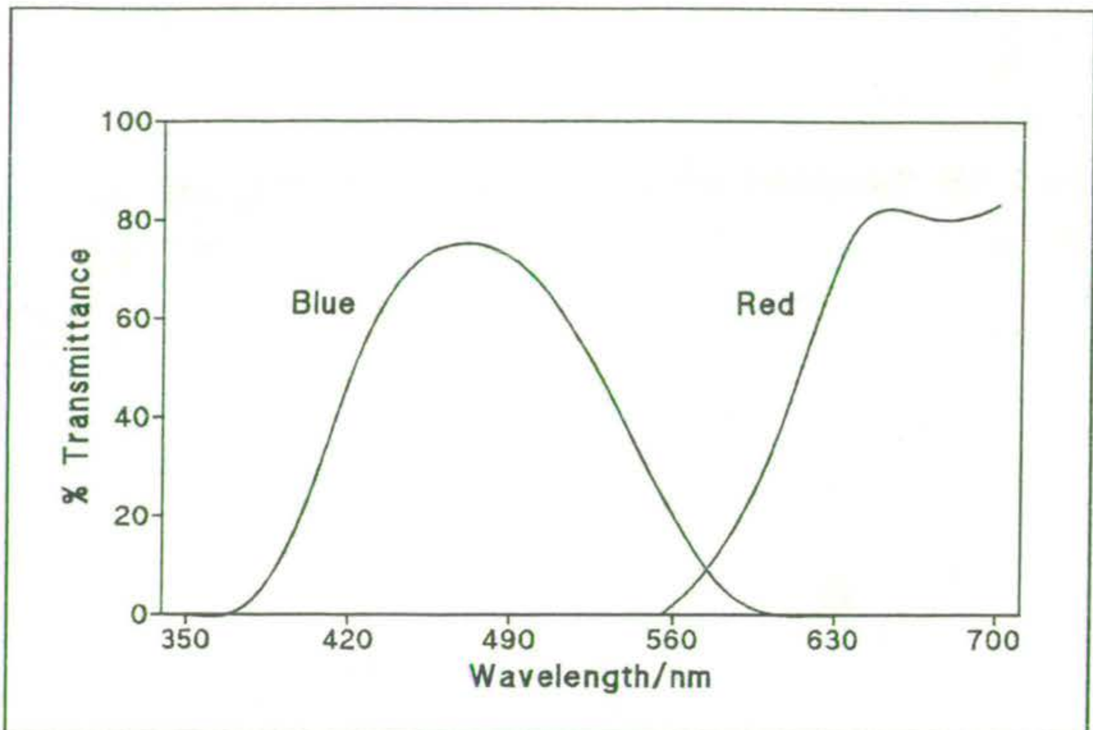


Figure 2.1: Transmission spectra for blue ("Moonlight Blue") and red ("Fire") filters.

2.7 Microscopy

2.7.1 Light and fluorescence microscopy

Whole tissues and prepared slides were examined using a Reichert-Jung Polyvar photomicroscope, fitted with fluorescence equipment. The following filter blocks were used for fluorescence microscopy: U1 (330-380 nm excitation filter; 420 nm dichroic mirror; 418 nm long pass filter); and B1 (450-495 nm excitation filter; 510 nm dichroic mirror; 520 nm long pass filter). For low power observations and photography, a Zeiss stereomicroscope was used. Black and white photographs were recorded on either Kodak Technical Pan or Kodak T max 100, 35 mm film. Colour images were recorded on Kodak Ektachrome 400, 35 mm slide film.

2.7.2 Low temperature scanning electron microscopy (SEM)

Specimens on 5 mm^3 blocks of agar cut from the mycelium were prepared using the EMscope SP2000 cryopreparation system. Material was cryofixed in slushed nitrogen, partially freeze-dried at -65°C and sputter-coated with gold according to the technique described by Read *et al.*, (1983). Specimens were examined below -155°C on a temperature controlled stage in a Cambridge Instruments S250 scanning electron microscope, at an accelerating voltage of 5 kV. Micrographs were recorded on Ilford FP4 120 film.

2.8 Preparation of material for light microscopy

2.8.1 Infiltration and embedding

Throughout fixation, dehydration and infiltration, specimens were rotated continuously on a clinostat to ensure thorough mixing. Such mixing, particularly of the resin components, was essential for successful infiltration of the tissue, and poor mixing resulted in inferior quality sections. Material was fixed overnight in 5% (v/v) glutaraldehyde (Agar Scientific, Stansted, Essex) in a 0.1 M potassium orthophosphate buffer (pH 6.8), rinsed three times (10 minutes each) in fresh buffer solution, then post-fixed for two hours in 1% (w/v) osmium tetroxide (Agar Scientific). After three washes in distilled water (10 minutes each) specimens were dehydrated by passage through an ethanol series (10%, 20%, 30%, 40%, 50%, 70%, 90%, 100% (v/v) ethanol), allowing 10 minutes at each concentration, and then left overnight in 100% ethanol at 4°C. Material was then transferred from ethanol to propylene oxide (PPO) through a series of ethanol/PPO mixtures in the following proportions by volume:- 3:1, 2:1, 1:1, 1:2, 1:3, PPO (10 minutes each in the first two mixtures, and 30 minutes each

thereafter). A similar procedure was followed to transfer material from PPO into Spurr's Resin (Agar Scientific), prepared according to the manufacturer's instructions. The resin had a pot-life of 3-4 days when stored at 4°C. Pure Spurr's resin was changed three times daily for 7-10 days according to the nature of the material, denser specimens being given longer infiltration time. Material in which complete infiltration proved difficult to achieve was maintained under a vacuum during infiltration. When infiltration was complete, specimens were transferred into freshly prepared resin (either in rubber moulds (Agar Scientific), or in plastic moulds made from disposable beakers), and polymerised overnight at 70°C. The hardness of the resin was adjusted by variation of the volume of one component (DER 736) in the resin mixture.

Protoperithecia, which were unpigmented and of only 25-50 μm diameter, proved difficult to handle individually, and were often lost during infiltration. When the mycelial surface was scraped gently with a coverslip, small clusters of protoperithecia could be removed, and if handled carefully, these remained loosely connected throughout the fixation and infiltration processes. Being of larger size than individual protoperithecia, such clusters were easier to manipulate, and although some protoperithecia were inevitably damaged by the procedure, groups of several protoperithecia could be sectioned quickly in the same resin block.

2.8.2 Sectioning and staining

Resin blocks containing specimens were trimmed and mounted on a Reichert-Jung Ultracut microtome for sectioning. Glass knives were prepared as described by O'Brien and McCully (1981) using an LKB Type 7801A knife maker (Pharmacia LKB, Uppsala, Sweden). Sections of 0.5-1.0 μm thickness were floated onto a drop of filtered distilled water on a microscope slide (Gold Star) (Chance-Propper Ltd, Warley), and stretched slightly to remove wrinkles by passing a cotton bud soaked in chloroform over the slide several times, taking care not to touch the water surface. All microscope slides were cleaned with 60% ethanol prior to use. Slides were dried on a hot plate at 70°C for at least 30 minutes to allow the sections to become securely attached, and then stained with 1% toluidine blue in 1% borax solution. A large drop of filtered stain was placed over the sections and allowed to dry slightly, before excess stain was rinsed off with distilled water. The staining period required varied according to the nature of the specimen and the hardness of the resin. Over-staining could be corrected by careful rinsing in 60% ethanol, although this procedure also increased the

tendency of sections to come off the slides. Stained sections were stored in a slide box until required; no decolourisation of the stain was observed in unmounted sections.

2.8.3 Mounting of sections

Several different mountants were used, although none proved entirely satisfactory. When sections were mounted in distilled water, small areas of many sections became progressively less well attached to the slide, and it became impossible to focus whole sections in the same plane. Alternative mounting media employed were 25% (v/v) glycerol, and high quality immersion oil, suitable for fluorescence microscopy (Cambridge Instruments Ltd, Cambridge). Both mountants reduced the tendency of sections to lift from the slide, but proved difficult to remove after observation. Although slides could be stored in their mounted state, the stain became decolourised over a period of a few days.

2.9 Cytological staining

Unless specified to the contrary, staining procedures were carried out on intact perithecia, which were cracked open to release asci prior to mounting. To reduce adhesion to the glass, and aid the uniform spreading of rosettes of asci, microscope slides were rinsed in 5% (v/v) Ilfotol wetting agent (Ilford) and then air dried prior to use.

2.9.1 Feulgen

Feulgen staining was carried out according to McLeish and Sunderland (1961) with modifications (R. F. Lyndon, pers. comm.). To prepare Feulgen reagent, 1 g basic fuchsin (Sigma Chemical Company, Poole, Dorset) was dissolved in 200 ml boiling distilled water, shaken well, and cooled to 50°C, before filtration through Whatman No. 1 filter paper in a Buchner funnel. The filtrate was transferred to a clean bottle, and 30 ml 1 M HCl added, followed by 3 g potassium metabisulphite ($K_2S_2O_5$). The solution was allowed to bleach for 24 hours in the dark, shaken with activated charcoal, and filtered rapidly (Whatman No. 1 filter paper). The reagent was stored in the dark at 4°C. SO_2 water was produced by adding 5 ml 1 M HCl and 5 g $K_2S_2O_5$ to 100 ml distilled water.

Perithecia were fixed for 1 hour in absolute ethanol-glacial acetic acid (3:1 v:v), washed for 15 minutes each in 3 changes of absolute ethanol, and then passed through an ethanol series into water (90%, 80%, 70%, 50%, 30%, 10%

v/v). After hydrolysis in 5 M HCl for 25 minutes at 25°C, perithecia were rinsed in distilled water and stained for at least 2 hours in Feulgen reagent. After staining, perithecia were washed for 15 minutes each in 3 changes of SO_2 water (pH 1.7), and then twice in distilled water.

2.9.2 DAPI

DAPI (4', 6-diamidino-2-phenylindole) (Sigma Chemical Company, Poole, Dorset) was dissolved in distilled water (100 $\mu g\ ml^{-1}$) and kept in the dark at 4°C. This stock solution was diluted to a working strength of 5 $\mu g\ ml^{-1}$ (Butt *et al.*, 1989) as required. Perithecia were stained for approximately 30 seconds, then washed 3 times in distilled water before splitting open to release the asci. Slides were examined under a Polyvar microscope, using the U1 filter block.

2.9.3 Acriflavin

Staining with acriflavin (Sigma Chemical Company, Poole, Dorset) was carried out according to Raju (1986). Unfixed perithecia were removed from the culture surface, hydrolysed in 4 M HCl for 30 minutes at 25°C, rinsed in distilled water, and then stained for 30 minutes in a solution of acriflavin (200 $\mu g\ ml^{-1}$) in potassium metabisulphite (5 $mg\ ml^{-1}$ in 0.1 M HCl). After three washes of 5 minutes each in a 10 M HCl-70% ethanol mixture (2:98 v:v), and then three washes in distilled water, stained perithecia could be stored in distilled water at 4°C for up to 24 hours. Thereafter the stain tended to spread from the nuclei into the ascus cytoplasm. Perithecia were split open in 25% (v/v) glycerol, and isolated centra transferred to a microscope slide for mounting and examination. Over-staining resulted in a general yellow-gold fluorescence of the cytoplasm making successful observation of chromosomes very difficult. Slides were examined under a Polyvar microscope, using the B1 filter block.

When staining crossed perithecia, a modified technique was occasionally used to produce higher quality preparations: perithecia were dissected before fixation, and the centra stained in isolation. When the whole perithecium was stained, cracking the fruitbody open inevitably damaged wall cells, releasing any residual stain into the mounting medium, and reducing contrast. Dissection of perithecia prior to staining avoided background fluorescence, but made the whole process more time consuming. This technique was not suitable for uncrossed perithecia, where only a small proportion of the specimens examined contained spores, and centra were too small to handle in isolation.

2.10 Other staining procedures

2.10.1 Laccase localisation using ADBP

Histological staining for laccase activity was carried out using a modified version of the method developed by Hermann *et al.* (1983). The staining reaction is based on the oxidation of 4-amino-2,6-dibromophenol (ADBP), and the coupling of the colourless reaction product to 3,5-dimethylaniline (DMA), to produce intense blue-green staining in laccase-positive tissues.

The reagent was prepared by dissolving 4 g ADBP (Aldrich Chemical Company, Gillingham, Dorset) in 100 ml absolute ethanol, and then adding 10 ml DMA (Sigma Chemical Company, Poole, Dorset). This reagent was diluted with an equal volume of buffer solution (37 mM citric acid, 126 mM Na_2HPO_4, pH 6.0). Strips of mycelium were cut from cultures grown on CMA overlaid with cellophane membranes, and flooded with the reagent for 5 minutes. The liquid was decanted and the mycelial strip incubated at 20°C for 30 minutes. After thorough washing in distilled water, material was transferred to a microscope slide and examined immediately, as the intensity of the blue-green colouration in laccase positive tissue faded after a few hours.

2.10.2 Lipid localisation using Nile red

The distribution of lipids was examined using the lipid-specific, fluorescent dye Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) (Greenspan *et al.*, 1985). A stock solution of 8 mg ml^{-1} Nile red (Eastman Kodak, New York) in acetone was stable for several months in the dark at 4°C. This solution was diluted with acetone to a working strength of 100-200 μg ml^{-1} as required. Strips of mycelium cut from cultures grown on CMA overlaid with cellophane membranes were immersed in stain in a glass Petri plate for 5 seconds, and then washed in distilled water. The mycelium was separated from the underlying cellophane and mounted in distilled water. Slides were examined under the Polyvar microscope using the B1 filter block.

2.10.3 Vital staining using fluorescein diacetate

Vital staining of microconidia was carried out using fluorescein diacetate (FDA). After the non-polar FDA molecule crosses the plasma membrane, ester bonds are hydrolysed, releasing polar fluorescein, which accumulates in the cytoplasm (Butt *et al.*, 1989). Living cells may then be identified by the yellow-green fluorescence of fluorescein under blue light. A stock solution of 5 mg ml^{-1} FDA in acetone was diluted with distilled water to a working strength of 0.01 %

w/v as required. A few drops of the dilute solution were added to an Eppendorf tube containing a concentrated spermatial suspension. After mixing carefully, a drop of suspension was placed on a clean microscope slide, covered, and examined under the Polyvar microscope using the B1 filter block.

2.10.4 General staining of fungal material

2.10.4.1 Cotton blue

Cotton blue in lactophenol (1.0% w/v) was used as a convenient, stain for fungal cell walls, particularly in microconidia. Lactophenol Cotton blue was also used to stain trichogynes according to Backus (1939): the stain was added to protoperithecial cultures using a pipette, and after a few seconds excess stain was removed by washing with clear lactophenol. Further washes in lactophenol were used to correct over-staining.

2.10.4.2 Trypan blue

Trypan blue in lactophenol (0.05% w/v) was used to increase the contrast between VHAs and the underlying mycelium in light-grown cultures. Cultures were flooded with approximately 2 ml of the stain, left to stand for 30 seconds, then washed with clear lactophenol.

2.11 Extraction of carotenoid pigments

Carotenoid pigments were extracted according to Davies and Grierson (1989). Cultures grown on CMA overlaid with cellophane membranes were scraped from the membranes using a coverslip, and 4 g (fresh weight) of fungal tissue was ground with powdered glass in 10 ml hexane-acetone (3:2, v:v). This mixture was shaken with an equal volume of distilled water, the organic phase was removed, and the water phase re-extracted. Organic phases were pooled and evaporated to dryness in a rotary evaporator at 35°C, then redissolved in 2 ml hexane. The absorption spectrum of the concentrated pigment was determined using a Beckman DU64 spectrophotometer.

2.12 Genetic analysis of homothallic fruiting

2.12.1 Analysis of uncrossed perithecia from wild type isolates

Uncrossed perithecia from 10 day old, wild type isolates were split open, and the presence or absence of ascospores was recorded. Intact asci were dissected in a drop of 10% (w/v) sucrose solution on a microscope slide, spores were germinated on CMA containing 0.7% (w/v) sodium acetate, and the mating

type of each new mycelium was determined. Ascospores were manipulated using a fine tungsten needle, sharpened by dipping its tip in boiling sodium nitrite.

2.12.2 Analysis of homothallic fruiting using heterokaryons

2.12.2.1 Establishment of heterokaryons

Heterokaryons between wild type and y^9, and between y^4 and y^9 were established on CMA by superimposing equal sized inocula of *mtA* isolates in the centre of a Petri plate. These inocula were cut from immediately behind the mycelial front of 48 hour old cultures (just before the hyphal front reached the edge of the Petri plate) using the wider end of a sterile Pasteur pipette. Recently germinated isolates were used whenever possible, as these formed heterokaryons more reliably than older isolates.

2.12.2.2 Estimation of nuclear ratios

The uniformity of heterokaryon formation and the nuclear ratio for each heterokaryon were estimated in two ways. Pieces of agar bearing mycelium were cut from the heterokaryon and scraped over the surface of a 4 day old culture of a b^1 mutant to fertilise its protoperithecia. When mature, the resulting perithecia were examined individually to determine whether they resulted from crossing with a microconidium carrying a yellow or a wild type nucleus. The nuclear ratio was also estimated using the heterokaryon as a female: after uncrossed perithecia had been removed from the mycelium, the heterokaryon was fertilised using a suspension of b^1 microconidia. Mature perithecia were examined as described above, allowing a direct estimate of the nuclear ratio to be made.

Experiments showed that nuclear ratios varied between heterokaryons, and also when a heterokaryon was subcultured. In consequence, all analyses were carried out using single Petri plates in which the heterokaryon had been created directly from its constituent homokaryons. Any plates in which sectoring occurred (growth of individual homokaryons without heterokaryon formation), or in which only a few uncrossed perithecia developed on the heterokaryon, were discarded.

2.12.2.3 Analysis of uncrossed perithecia from heterokaryons

Each of the perithecia which formed on an unmated heterokaryon was removed using a sterile needle, split open on a microscope slide in a drop of 10% sucrose solution, and its contents recorded. For asci containing spores of more

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than one colour (segregating asci), the exact sequence of black and yellow spores was noted.

2.13 Extraction of fertility-inducing substances from mated cultures

A modified version of the method of Islam and Weijer (1969) was used to extract fertility-inducing substances from mated cultures of *S. brevicollis*. All filter papers used were Whatman No. 1. Five sterile filter paper discs (8 cm diameter) were placed in each of 50 Petri plates (250 filter papers in all), and 20.0 ml Vogel's liquid medium (Vogel, 1956) was added to each plate. Inocula from strains of opposite mating type were placed on the uppermost filter paper in each plate, plates were sealed with Parafilm, and were incubated in the dark for 10 days, to allow perithecial production. At the end of this period, all filter papers, fungal mycelia and any residual media, were transferred to a beaker and extracted twice in 500 ml aliquots of sterile distilled water, for 2 hours at 25°C. The pooled extracts were filtered through three layers of muslin, followed by two layers of filter paper, in order to remove any mycelial fragments. Activated charcoal (10 g) was added to the filtrate, the mixture was shaken vigorously for 2 minutes, and allowed to stand at room temperature for 3 hours. The charcoal was filtered through two layers of filter paper, and dried at room temperature overnight. When completely dry, 100 ml chloroform was added to the charcoal, the mixture was shaken well, left to stand for 3 hours at room temperature and then filtered through 3 layers of filter paper. The charcoal was re-extracted in chloroform, producing a total of approximately 180 ml chloroform extract, which was evaporated to dryness in a rotary evaporator at 35°C. The residue was redissolved in 5 ml chloroform and stored at 4°C until required.

To test for biological activity, 10 μl of extract was added to 5 mm diameter discs of sterile filter paper (cut using a hole punch). These discs were placed on the surface of single mating type cultures, and the production of perithecia was monitored in the region of each disc. Filter paper discs soaked in chloroform, and dry discs were used as controls. Discs were placed directly onto mycelia of various ages between 48-96 hours after inoculation, and were also placed on the growth medium in advance of the hyphal front.

2.14 Other methods

2.14.1 Determination of fresh and dry weights

Cultures inoculated on CMA overlaid with cellophane membranes were sampled at 12 hour intervals, with 5 replicate Petri plates at each time point. The

mycelium scraped from each plate was weighed, oven dried at 120°C for at least 12 hours, and reweighed. Thereafter, mycelia were returned to the drying oven for a further 12 hours, and then weighed again, to ensure that complete dryness had been achieved.

2.14.2 Determination of perithecial frequency and distribution

For uncrossed perithecia, and crossed perithecia when present at low frequency (<1000 fruitbodies per 8.5 cm diameter Petri plate), all the perithecia on each culture were counted, while at higher frequencies, a sampling technique was developed as follows. In preliminary experiments, each culture was divided into 16 sectors (each approximately 23° of arc), the frequency of perithecia in 4 sectors from each culture was determined, and used to calculate an expected frequency for the whole Petri plate. The total perithecial frequency for each plate was also determined, and compared with the expected frequency. The results indicated that where perithecial distribution was uniform (as judged by visual inspection of cultures), the differences between calculated and observed frequencies were not significant (data not shown). Where there were marked differences in perithecial distribution, however, the sampling technique did not provide an accurate assessment of total frequency, and any cultures in which perithecial distribution was clearly non-uniform (usually a result of contamination or uneven spermatisation) were discarded.

Perithecial distribution was assessed by dividing each of the sectors described above into a number of smaller portions. Initially, sectors were divided into 5 smaller areas, although in later experiments this was increased to 8.

CHAPTER THREE

PATHWAYS OF MULTICELLULAR DEVELOPMENT IN SORDARIA BREVICOLLIS

3.1 Introduction: Aspects of multicellular development

Although many of the genetic and environmental factors which influence the development of multicellular structures in fungi have been reviewed previously (see Chapter One), it will be useful to consider two further aspects here. The first concerns the rôle of the containing vessel in stimulating multicellular development of fungi in culture, the so-called "edge effect", and the second, the involvement of a group of copper-containing enzymes, polyphenol oxidases, in hyphal adhesion during fruitbody formation.

3.1.1 The "edge effect"

Fruitbody formation in many fungi is initiated when the mycelium reaches the edge of the containing vessel, or encounters some other obstacle. First noted in *Pyronema confluens* (Robinson, 1926), the phenomenon has since been observed in a range of ascomycetes including *Chaetomium globosum* (Buston and Rickard, 1956), *Gelasinospora reticulispora* (Inoue and Furuya, 1970), *Podospora anserina* (Lysek, 1976), *Sordaria fimicola* (Pollock, 1973, 1975), *S. macrospora* (Bahn and Hock, 1973; Hock *et al.*, 1978) and *S. brevicollis* (MacDonald and Bond, 1976). Several hypotheses have been advanced to account for the stimulation of reproduction by a barrier, namely: (i) a build up of inducing substances (such as sugar phosphates) at the edge of a colony (Buston and Rickard (1956); (ii) the inactivation of an inhibitor by the accumulation of metabolites at the colony margin (Chet and Henis, 1968); and (iii) increased development of side branches after the growth of leading hyphae is checked at the edge of the Petri plate (Hock *et al.*, 1978). MacDonald and Bond (1976) demonstrated that the edge effect which they observed in *S. brevicollis* was independent of the size and shape of the containing vessel, and of the volume of media used. They failed, however, to find evidence that accumulation or exhaustion of particular metabolites, or the production of extracellular inducing substances, could trigger protoperithecial production, and concluded instead that *perturbation* in metabolism was the stimulus for fruiting. This perturbation could be caused not only by a physical barrier, but also by disturbing linear growth of

the mycelium. Until the hyphal front reached the edge of the Petri plate, cultures of *S. brevicollis* were "sterile", and no protoperithecia were produced.

3.1.2 Phenol oxidase activity

Phenol oxidase activity is widespread in fungi, and has been linked with pathogenicity (Bar-Nun *et al.*, 1988; Rigling *et al.*, 1989), degradation of lignin (Ander and Erikkson, 1976), cell lysis (Boucherie *et al.*, 1981) and pigmentation (Clutterbuck, 1972), as well as the formation of multicellular structures such as rhizomorphs (Worrall *et al.*, 1986), sclerotia (Chet and Hütterman, 1982), and fruitbodies (Leatham and Stahmann, 1981). (For reviews of phenol oxidase activity in plants and fungi see Mayer and Harel (1979) and Mayer (1987).) Phenol oxidases may be involved in several different processes in the same fungus: in *Podospora anserina* for example, phenol oxidases are associated not only with fruitbody formation (Esser and Minuth, 1970) but also with vegetative incompatibility reactions and cell lysis. Cytoplasmic incompatibility in this species is due to the interaction of non-allelic genes at 9 or more different loci, and results in cessation of growth followed by cell disintegration (Labarère and Bernet, 1978). Among the catabolic enzymes identified in lysing cells by Boucherie *et al.* (1976) were several phenol oxidases not normally produced in vegetative hyphae.

Amongst a number of different phenol oxidases which have been characterised in fungi are laccases (*p*-diphenol oxygen oxidoreductases). Many fungi produce several laccases, often with differing structure, molecular weight and function. In *Aspergillus nidulans* for example, laccase I was shown to participate in the synthesis of a conidial pigment, while laccase II was localised in hülle cells and cleistothecial primordia (Hermann *et al.*, 1983). The involvement of a second type of phenol oxidase, tyrosinase, was studied during early sexual development in *N. crassa* (Hirsch, 1954; Barbesgaard and Wagner, 1959), where tyrosinase activity was positively correlated with the formation of sexual primordia. Like laccases, tyrosinases have been found to exhibit a degree of structural variability, and Horowitz *et al.* (1961) were able to identify four different forms of tyrosinase amongst a dozen wild type strains of *N. crassa.* There is evidence however, that although necessary for sexual differentiation, the presence of tyrosinase is not sufficient for protoperithecial formation. In *N. crassa*, certain female sterile mutants which lacked the ability to produce tyrosinase, still failed to develop protoperithecia when tyrosinase synthesis was induced artificially (Horowitz *et al.*, 1970). Metzenberg (1972) concluded that

laccase and tyrosinase in *N. crassa* were "hard times" enzymes, produced not only as a result of nutritional stress, but also as a response to a number of inhibitors of protein synthesis.

Although phenol oxidases appear to fulfil a number of different rôles in fungi, those enzymes involved in multicellular development are thought to catalyse the formation of phenolic polymers between adjacent fungal cell walls, thereby increasing the cell-cell adhesion involved in fungal morphogenesis (Bu'lock, 1967). Prillinger and Esser (1977) showed that mutants of *P. anserina* which were deficient in laccase activity, produced reduced aerial mycelium and developed aberrant sexual primordia, and phenol oxidase activity has also been correlated with fruiting ability in basidiomycete species such as *Schizophyllum commune* (Leonard and Phillips, 1973; Leslie and Leonard, 1979a). Fruiting in *S. commune*, which normally occurs in the dikaryotic state, has also been observed in some isolated monokaryons, although the structures produced are often poorly developed (see Chapter Four). Laccase activity, closely associated with the development of dikaryotic basidiocarps, was detectable at much lower levels in monokaryotic isolates which possessed the ability to fruit, but was absent (or at least not detected) in those monokaryons which did not fruit at all (Leonard and Phillips, 1973).

Laccase activity has also been shown to be developmentally regulated in *S. brevicollis* (Broxholme *et al.*, 1991). Cytochemical staining of a hyaline mutant was used to demonstrate that laccase activity was present in developing perithecia two days after spermatisation, but was absent from protoperithecia of a similar age which had ceased to enlarge.

3.1.3 Multicellular structures in *S. brevicollis*

Various aspects of sexual development in *S. brevicollis* have been described previously (e.g. Olive and Fantini, 1961; Sanni, 1984) and may be briefly summarised as follows: protoperithecia are formed on four day old mycelia (which have reached the edge of the Petri plate), and after fertilisation by microconidia of opposite mating type, develop into perithecia, each containing several hundred asci. The work presented in this chapter involved characterisation of the multicellular structures associated with conventional sexual development in S. *brevicollis*, i.e. protoperithecia and perithecia. Also described and illustrated are several additional multicellular structures which are produced by this species under laboratory conditions, namely vegetative hyphal aggregates (VHAs), and uncrossed perithecia. The latter develop in unmated

cultures of certain *mtA* strains, and occasionally contain a few viable ascospores. The "sclerotia" produced by *Sordaria sclerogenia* (Fields and Grear, 1966) and colourless perithecia formed in a hyaline mutant of *S. brevicollis* are also described for comparative purposes.

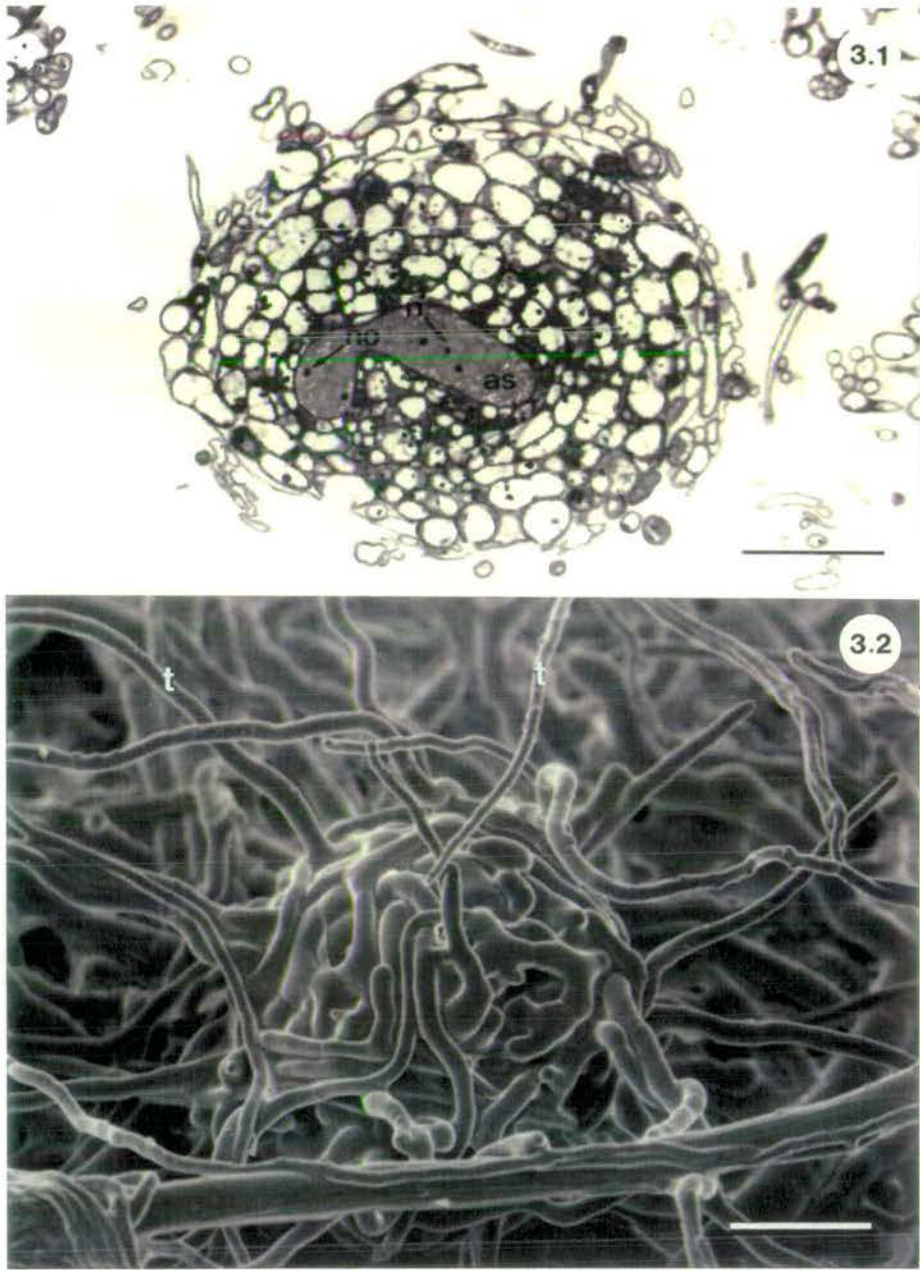
3.2 Results

3.2.1 Characterisation of multicellular structures in *S. brevicollis*

3.2.1.1 Protoperithecia

General observations: Protoperithecial formation commenced only after the mycelial front had reached the edge of the Petri plate, an event which occurred approximately 60 hours after inoculation. There was no light requirement for the initiation of sexual development, and maximum numbers of protoperithecia were produced by incubation in the dark. In 4 day old, wild type cultures, protoperithecia were typically hyaline, and visible to the naked eye, at approximately 50 μm diameter. Under the dissecting microscope, numerous fine hyphae could be seen to arise from the protoperithecial surface, and these often extended over distances of up to 500 μm. Such hyphae frequently appeared to grow towards other protoperithecia in uncrossed cultures. Protoperithecial numbers were enhanced by growing cultures on media overlaid with cellophane membranes, a technique which also permitted easier observation of protoperithecia by restricting their development to a single plane. In cultures inoculated directly onto the growth medium, a proportion of protoperithecia formed beneath the surface of the agar. Protoperithecial numbers were typically highest in newly germinated isolates, and declined as isolates aged. This decline was gradual at first, but often accelerated rapidly when isolates reached 2-3 months old, eventually resulting in female sterility. The distribution of protoperithecia varied between isolates, and was also modified by light exposure during development (see Chapter Five).

Protoperithecial structure: Protoperithecia from 4 day old cultures were sectioned after being embedded in Spurr's resin. Fig. 3.1 illustrates many features of protoperithecial structure. Protoperithecia were essentially spherical, with a wall of 5-6 cells in thickness enclosing a large multinucleate ascogonium. The size of individual cells in the protoperithecial wall varied considerably, but no discrete layers could be identified. The ascogonium, typically of much greater diameter than the surrounding hyphae, was easily distinguished by the dense, granular nature of the cytoplasm. No clear septation of the ascogonium was ever



Figures 3.1 and 3.2: ***Sordaria brevicollis*. Protoperithecia.**

Figure 3.1: Thin section through 4 day old protoperithecium. Within the multinucleate ascogonium (as), several large nuclei (n) with prominent dark-staining nucleoli (no) are visible. The protoperithecial wall is typically 5-6 cells thick, although no distinct layers can be identified. The cells immediately surrounding the ascogonium are frequently smaller in diameter, and more densely cytoplasmic than those in the outer part of the wall. Bar = 15 μm. **Figure 3.2**: Scanning electron micrograph of 4 day old protoperithecium. Some of the hyphae arising from the surface of the protoperithecium are probably trichogynes (t). Bar = 20 μm.

detected in sectioned material. Numerous large nuclei were frequently observed within the ascogonium, often with prominent dark-staining nucleoli, and occasionally with visible chromosomal material. No trichogynes were conclusively identified: the fine hyphae often seen to emanate from the surface of the protoperithecia were only ever observed to arise from the cells of the outer layer of the protoperithecial wall. Further attempts to demonstrate continuity between the ascogonium and potentially receptive hyphae outside the protoperithecium were made using lactophenol Cotton blue to stain fresh material, as described by Backus (1939) for protoperithecia of *Neurospora sitophila*, but although the ascogonial coil could readily be observed, no differential staining of trichogynes was noted.

The external features of protoperithecial morphology were examined by low-temperature SEM, as described in Chapter Two. Fig. 3.2 illustrates the appearance of a typical protoperithecium, with several fine, trichogyne-like hyphae arising from the surface. The protoperithecial wall (which appeared in section to be composed of individual cells) could be seen under the scanning electron microscope, to comprise many tightly knitted filaments, with no inter-hyphal spaces.

3.2.1.2 Crossed perithecia

3.2.1.2.1 *Wild type*

General observations: When four day old, dark-grown cultures were crossed with microconidia of opposite mating type, enlarged protoperithecia were clearly visible to the naked eye within 24 hours of spermatisation. Slight pigmentation and ostiole formation were evident in young perithecia 1-2 days after crossing, although neck development was not pronounced until day 3. Thereafter, perithecial pigmentation and neck elongation continued, with mature perithecia beginning to discharge ascospores on days 5 - 6. The dimensions of the mature perithecium were 300 - 350 x 250 - 300 μm, with the smallest perithecia typically being produced in areas of greatest density.

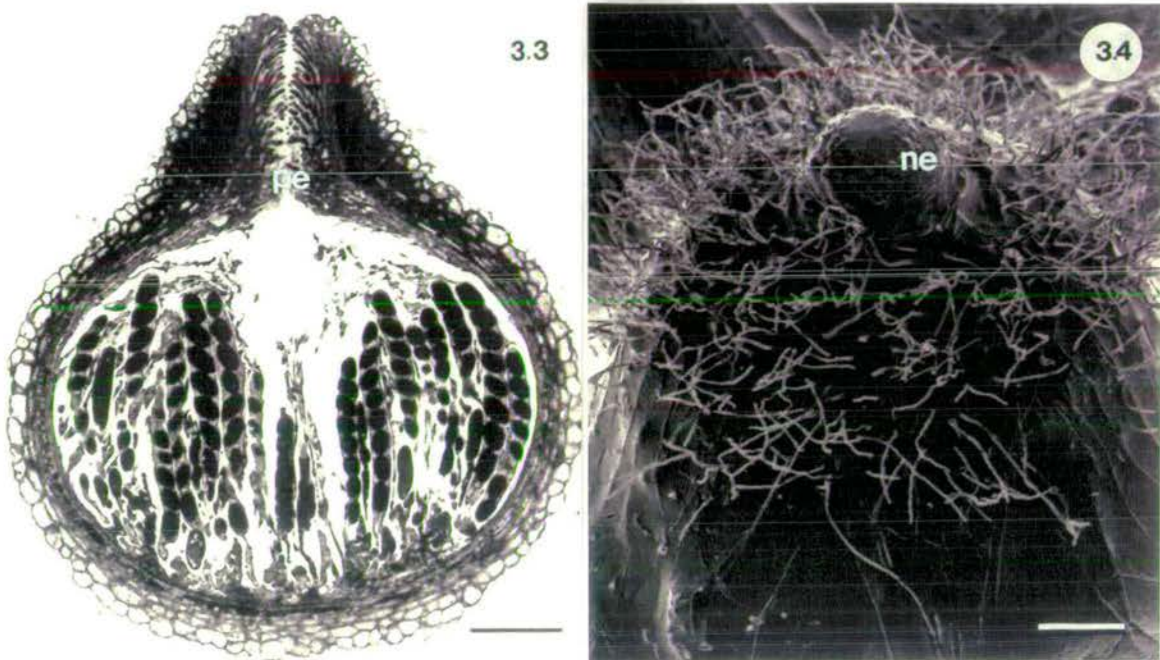
Wild type perithecia exhibited both phototropic neck curvature, and light-stimulated spore discharge. When 8 - 9 day old, dark-grown cultures (in which no spore discharge had taken place), were transferred to the light, spore discharge commenced within 10 - 15 minutes. In 10 day old cultures, spore discharge took place in complete darkness.

Perithecial structure: Perithecia for sectioning were removed from 8 day old, dark grown cultures, and were fixed immediately, before light-stimulated spore discharge, or phototropic neck curvature took place. In 8 day old perithecia (four days after spermatisation), a range of ascus development from ascogenous hyphae to mature spores could be observed.

As the specific epithet "brevicollis" suggests, the perithecial neck was typically rather short, although neck development may not have been fully complete in 8 day old cultures (Fig. 3.3). Although rather wider at the base of the neck, the perithecial wall was typically around 20 μm in width, and was approximately 6 cells thick, with no intercellular spaces. In much of the neck region, however, individual wall cells could not be distinguished, as considerable thickening and pigmentation had occurred. The centrum pseudoparenchyma remained relatively intact only at the base of the neck, where the continuity of the periphyses which lined the neck canal with the centrum pseudoparenchyma at its base was evident. The paraphyses, also derived from the centrum pseudoparenchyma, had disintegrated in perithecia from 8 day old cultures, leaving a quantity of cellular debris in the upper part of the central cavity. The external appearance of a typical 10 day old, crossed perithecium is illustrated in Fig. 3.4. Six days after crossing, neck development was complete, and the cells in the neck region could be clearly distinguished from the remainder of the perithecial wall. Numerous fine hyphae which arose from the perithecial wall were preserved using low-temperature SEM.

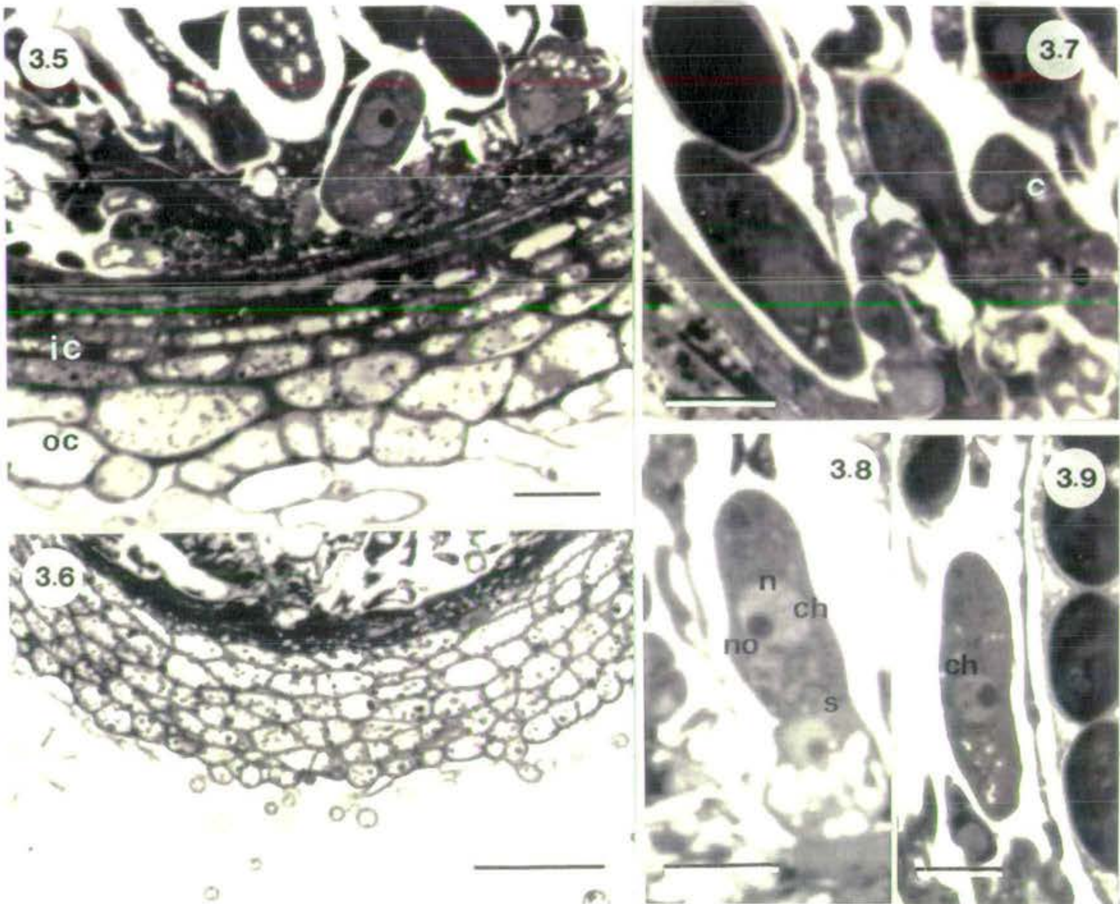
In most fruitbodies, the perithecial wall appeared to be made up of two layers. Inner cells were elongated in longitudinal section (probably having been crushed by the expanding centrum), and had a very dense appearance, whilst the outer 1-2 cells retained a slightly globose profile (Fig. 3.5)). In some perithecia of similar size and age, however, wall cells were of more uniform dimensions, and the wall appeared wider as a result, with no distinct layers (Fig. 3.6).

Asci arose from a region at the base of the centrum, and in an 8 day old perithecium, a range of developmental stages from croziers to pigmented ascospores was present (Figs. 3.5, 3.7-3.9). Large nuclei with dark staining nucleoli were evident in many young asci, and occasionally pairs of small, dark structures could also be observed (Fig. 3.9). These may have been clumps of chromosomal material.



Figures 3.3 and 3.4: ***Sordaria brevicollis*****. Crossed perithecia.**

Figure 3.3: Thin section through perithecium from an 8 day old, dark-grown culture. The neck canal, lined with numerous inward-curving periphyses (pe), terminates in an ostiole, through which mature spores are eventually ejected. In the specimen illustrated, the paraphyses have largely collapsed, leaving cellular debris in the upper part of the perithecial cavity. The sequential nature of ascus formation is illustrated by the range of developmental stages present in this fruitbody: while the most mature asci contain pigmented spores approaching maturity, crozier formation and ascus initiation continue at the base of the centrum. The perithecial wall, typically around 20 μm thick, is of greatest width at the base of the neck, where thickening and pigmentation have occurred to such an extent that few individual cells can be distinguished. Bar = 40 μm. **Figure 3.4:** Scanning electron micrograph of a 10 day old perithecium. Cells of the neck region (ne) are clearly distinct from the remainder of the perithecial wall, and typically lack fringe hyphae. Bar = 50 μm.



Figures 3.5 - 3.9: *Sordaria brevicollis*. Crossed perithecia.

Figure 3.5: Lower perithecial wall and young ascus. The cells making up the inner part of the perithecial wall (ic) have become flattened by expansion of the centrum, and little detail of their contents can be observed. The cytoplasm of many of the outer cells (oc), which retain a more globose appearance, can be seen to contain numerous dark-staining granules. Several of the outermost cells appear to be vacuolate. Bar = 10 μm. **Figure 3.6**: Lower perithecial wall from second specimen, in which little flattening of the inner wall cells has taken place, so that the wall appears to be made up of a single layer approximately 5-6 cells thick. Bar = 20 μm. **Figure 3.7**: Croziers (c) and very young asci continue to develop in 8 day old perithecia. Bar = 10 μm. **Figures 3.8 and 3.9**: Young asci with large fusion nuclei (n), prominent nucleoli (no), and chromosomal material (ch). The position of the septum (s) at the base of the ascus, is often accompanied by a slight constriction in the hyphal wall. Bars = 10 μm.

3.2.1.2.2 *Hyaline mutant*

General observations: Four day old protoperithecia produced by a hyaline mutant were indistinguishable from wild type, and after fertilisation, young perithecia exhibited a similar pattern of expansion and neck initiation to the wild type perithecia described previously. Perithecial pigmentation was almost entirely absent, however, being confined to very pale brown colouration in the neck region. When spermatised with wild type microconidia, the mutant produced hyaline perithecia containing numerous asci, in which black and hyaline spores were produced in a 1:1 ratio. In neither mating type of the hyaline mutant were perithecia ever observed in uncrossed culture.

Perithecial structure: In thin section, the hyaline perithecium was quite distinct from wild type, particularly with respect to the shape of the neck, and the structure of the perithecial wall (Figs. 3.10 and 3.11). The necks of hyaline perithecia differed from wild type, being much wider at the tip, and less distinct from the remainder of the perithecial wall (c.f. Fig. 3.3). They were functionally equivalent to wild type, however, exhibiting phototropic curvature, and hyaline perithecia also showed light-stimulated spore discharge. The pseudoparenchymatous structure of the wall of a wild type perithecium was largely absent, and individual hyphae could be distinguished, separated by material which stained very poorly with toluidine blue (Fig 3.11). Many cells appeared more densely cytoplasmic than those in the wall of a wild type perithecium, a feature which may be directly associated with the lack of pigmentation, or may reflect superior preservation of cell contents during the preparation of hyaline material for sectioning. The centrum pseudoparenchyma, which lined the inside of the perithecial wall, often appeared to be partially detached from the wall (Fig. 3.10), although this may have been a result of poor sectioning technique. As in wild type, the centrum pseudoparenchyma was continuous with the base of the periphyses lining the neck (Fig. 3.10). Many spores had already been discharged from the hyaline perithecium illustrated in Fig. 3.10, leaving crushed paraphyses and the remains of ascus walls in the upper part of the central cavity, although young asci were still visible in the lower region.

3.2.1.3 Uncrossed perithecia - wild type

General observations: In certain 10 day old, uncrossed cultures, four types of multicellular structure could be distinguished: (1) unpigmented protoperithecia

Figures 3.10 and 3.11

Figures 3.10 and 3.11: *S. brevicollis.* Crossed perithecium (hyaline mutant). Figure 3.10: Thin section through perithecium from 11 day old, dark-grown culture of a hyaline mutant (*hya*). The neck region of the mutant perithecium lacks the clearly defined structure of the wild type fruitbody (c.f. Fig. 3.3). Many asci have already been ejected from the specimen illustrated, leaving a central cavity containing cellular debris from collapsed paraphyses and ascus walls, and young asci in varying stages of development. Bar = 40 μm. **Figure 3.11**: The wall of the hyaline perithecium differs from wild type, lacking the pseudoparenchymatous structure seen in Figs. 3.5 and 3.6. Individual hyphae (h) retain a rounded outline and often appear to contain more dense cytoplasm than the cells of the wild type perithecial wall. Interhyphal spaces are filled with material which stains poorly with toluidine blue. Bar = 15 μm.

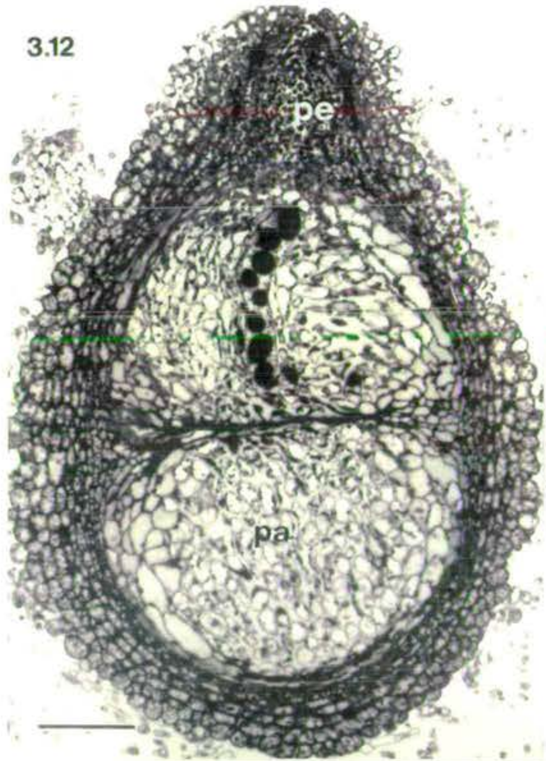
of approximately 50 μm diameter; (2) enlarged, pigmented protoperithecia of 50 - 100 μm diameter; (3) pigmented, ostiolate perithecia, of 100 - 200 μm diameter, either without necks, or with only rudimentary necks; and (4) pigmented, ostiolate perithecia of 150 - 250 μm, with at least partially developed necks. A variable proportion (typically around 10%) of type 4 perithecia were found to contain a few asci. Although perithecia of types 1 - 3 were seen in unmated cultures of both mating types, the phenomenon was much more pronounced in *mtA* strains, and *only* in *mtA* strains were spore-bearing (type 4) perithecia ever observed. It proved impossible to distinguish spore-bearing perithecia reliably on the basis of their external appearance, although darker pigmentation and more pronounced neck development indicated a higher probability that a perithecium contained spores. Essentially, those uncrossed perithecia which most closely resembled typical crossed perithecia were most likely to be fertile. The term *fertile* is used here as an indication of the presence of delimited ascospores in an uncrossed perithecium. That a perithecium did not contain spores did not necessarily mean that it had not developed ascogenous hyphae, or even young asci, merely that they had not developed further.

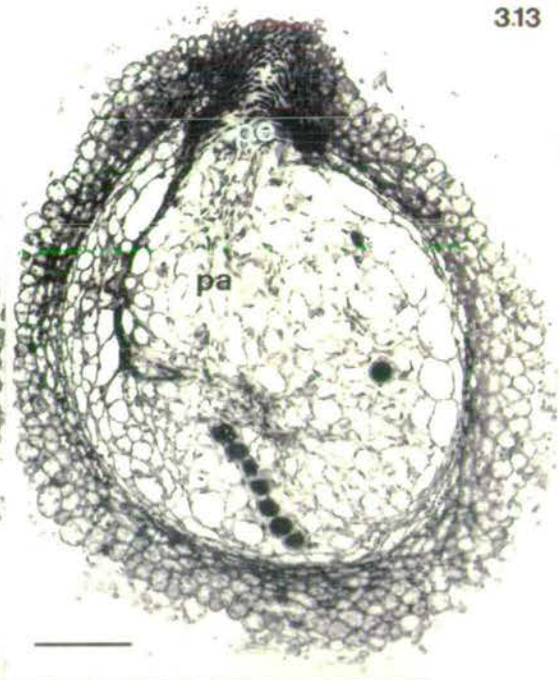
Uncrossed perithecia typically developed rather more slowly than crossed fruitbodies, and pigmented ascospores were very seldom observed in specimens of less than 9 - 10 days old. The discharge of spores from uncrossed perithecia was a rare but regular event, and groups of eight spores could occasionally be observed on the Petri plate lid.

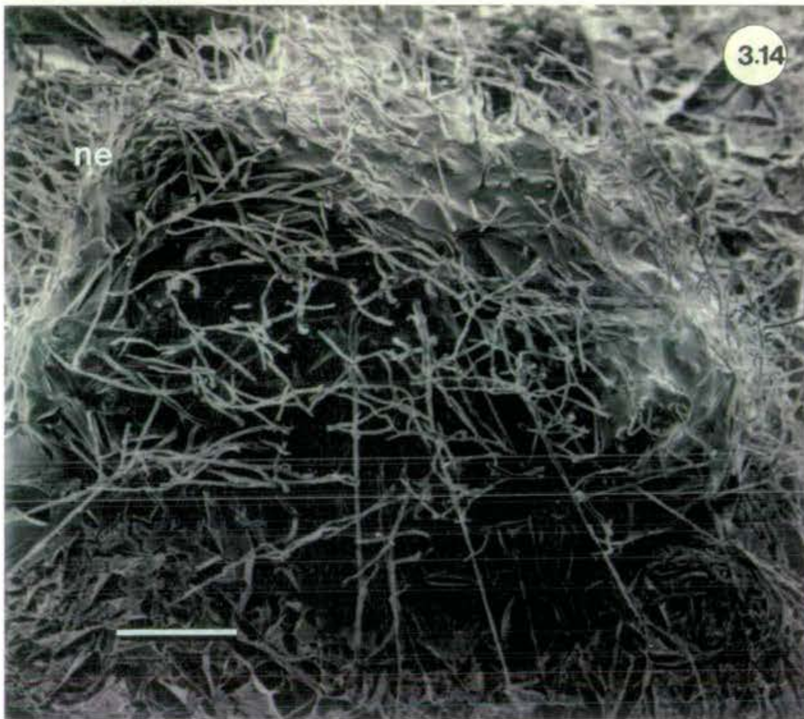
Uncrossed perithecial structure: When perithecia from 10 - 12 day old, uncrossed cultures were sectioned, it was found that variation in external appearance was reflected by a diversity of internal structure. To describe an uncrossed perithecium as "typical" would be something of a misnomer, as no two specimens examined were identical. Nevertheless, several common characteristics were determined, and are illustrated in Figs. 3.12 and 3.13. Much of the centrum pseudoparenchyma and the paraphyses which were derived from it remained intact in uncrossed perithecia. Large, thin-walled pseudoparenchymatous cells were particularly evident around the edge of the centrum, with paraphyses filling the central space. The walls of uncrossed perithecia, like those of crossed perithecia (Fig. 3.5), were approximately 6 cells thick. However, since the cells of the inner part of the wall of a typical crossed perithecium were elongated in profile (having been crushed by the expanding centrum), the wall of the uncrossed perithecium (where no such flattening took place), often appeared to

Figures 3.12 - 3.14: ***Sordaria brevicollis.*** **Uncrossed perithecia (wild type). Figures 3.12 and 3.13:** Thin sections through fruitbodies from 10-12 day old cultures. Both examples contain two asci, although in each case only one is fully visible. Neck development is rudimentary, although periphyses (pe) are visible. Paraphyses (pa) remain intact, so there is no perithecial "cavity" as such. The cells of the inner part of the perithecial wall are not flattened to in the manner often seen in crossed perithecia (c.f. Fig. 3.5), so that the uncrossed perithecial wall appears to be of greater thickness (c.f. Fig 3.3). Bars = 50 μm. **Figure 3.14:** Scanning electron micrograph of uncrossed perithecium from 12 day old, dark grown culture. The perithecial neck (ne) is often poorly differentiated and only a few slightly globose cells are visible at its tip. The perithecial wall is covered with fringe hyphae. Bar = 50 μm.

Figures 3.12 - 3.14







be rather wider than that of the crossed fruitbody. In fact the number of cell layers was similar in both cases. Neck development in uncrossed perithecia was often incomplete, even in specimens which contained spores, and although the short neck canal was lined with periphyses, a clear passage to the outside was not always evident. The region of thickened, heavily pigmented cells found at the base of the neck in crossed specimens (Fig. 3.3) was largely absent.

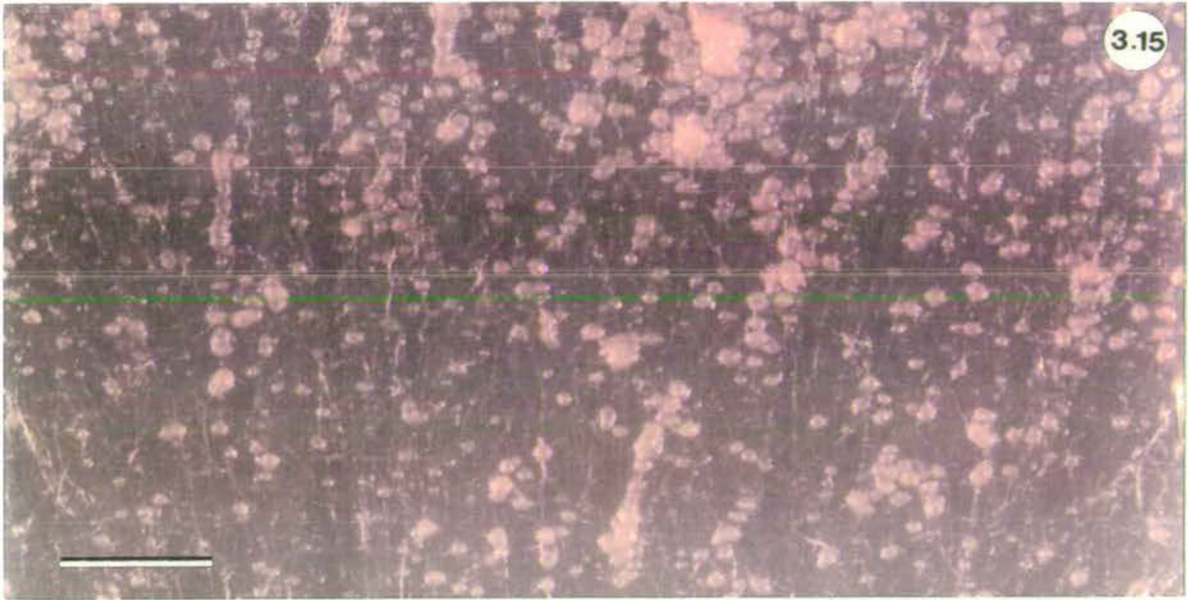
In observations of fresh material, it was found that one or more asci were present in up to 30% of uncrossed perithecia, although the figure was often much lower (see Chapter Four). In the majority of the spore-bearing perithecia sectioned, only one or two asci were detected, and sections through whole asci were seldom achieved because asci were often irregularly orientated and slightly curved. The ascospores were of equal size to those from crossed perithecia, however, and eight spores were produced in all those asci observed in uncrossed cultures. It was not clear whether the areas of dark staining material evident within the centra of the perithecia illustrated (Figs. 3.12 and 3.13), represented a region of hymenial cells from which asci were derived. In crossed perithecia, ascogenous hyphae typically arose from a region at the base of the central cavity, above which neck formation took place, facilitating eventual spore discharge. In uncrossed perithecia, however, the orientation of asci was often irregular, and this may have reduced their capacity to eject mature spores.

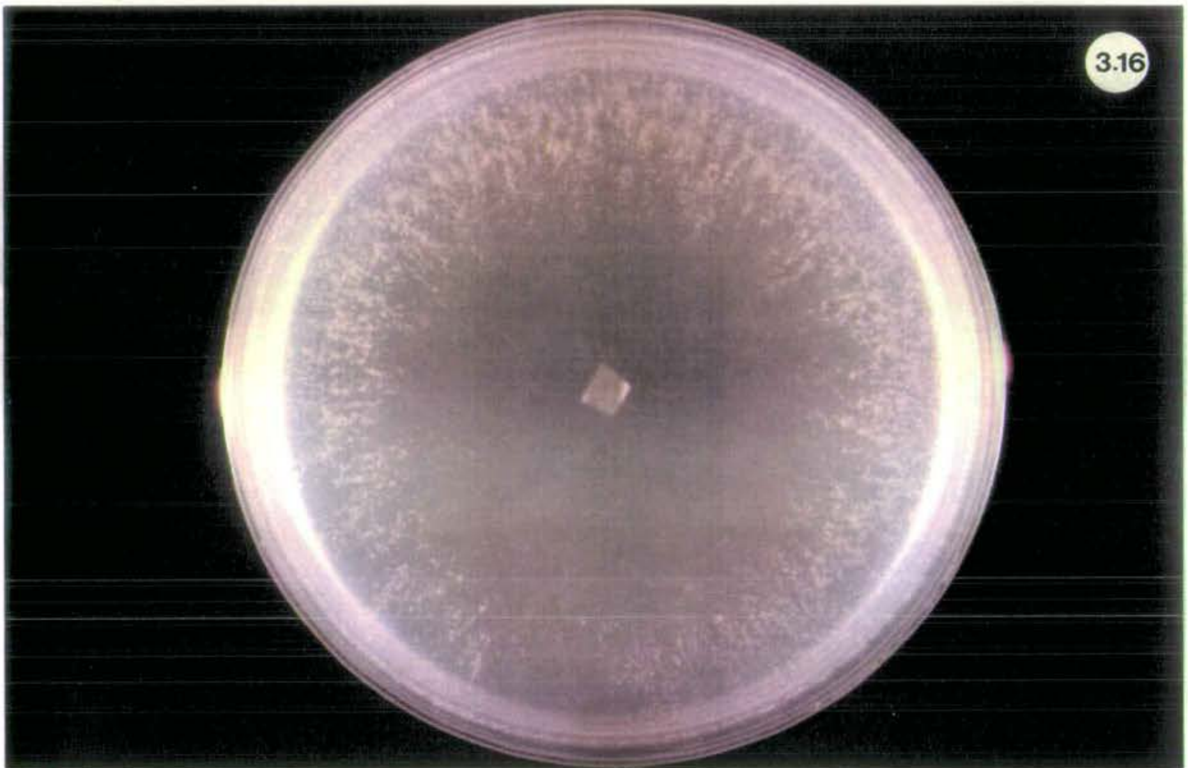
Under the scanning electron microscope, numerous fine hyphae were observed to arise from the surface of uncrossed perithecia. Perithecial necks were not easily distinguished, often being marked only by a small area where the swollen neck cells were visible (Fig. 3.14).

3.2.1.4 Vegetative hyphal aggregates (VHAs)

3.2.1.4.1 *Individual VHAs*

General observations: VHAs formed on the surface of light-grown cultures, often beginning to develop *before* the mycelial front reached the edge of the Petri plate. VHAs, which were never seen to form beneath the surface of the growth medium, accumulated a pinkish pigment also produced in light-grown mycelia. Viewed under a dissecting microscope, VHAs were of *very* roughly semi-spherical shape, and in 4 day old cultures, reached approximately 250 μm diameter (Fig. 3.15). Little discernible development occurred after 4 days, apart from a slight increase in size. Although their distribution was variable, VHA density generally increased in the outer part of the Petri plate (Fig. 3.16). VHA production was enhanced by growth on cellophane membranes, often reaching





Figures 3.15 and 3.16: ***Sordaria brevicollis.*** **VHAs.**

Figure 3.15: Typical appearance of VHAs in 4 day old, light-grown culture. Note pinkish colouration, irregular size and shape of VHAs, and their tendency to merge together. **Figure 3.16:** Light-grown culture, with VHAs at greatest density in the outer part of the Petri plate.

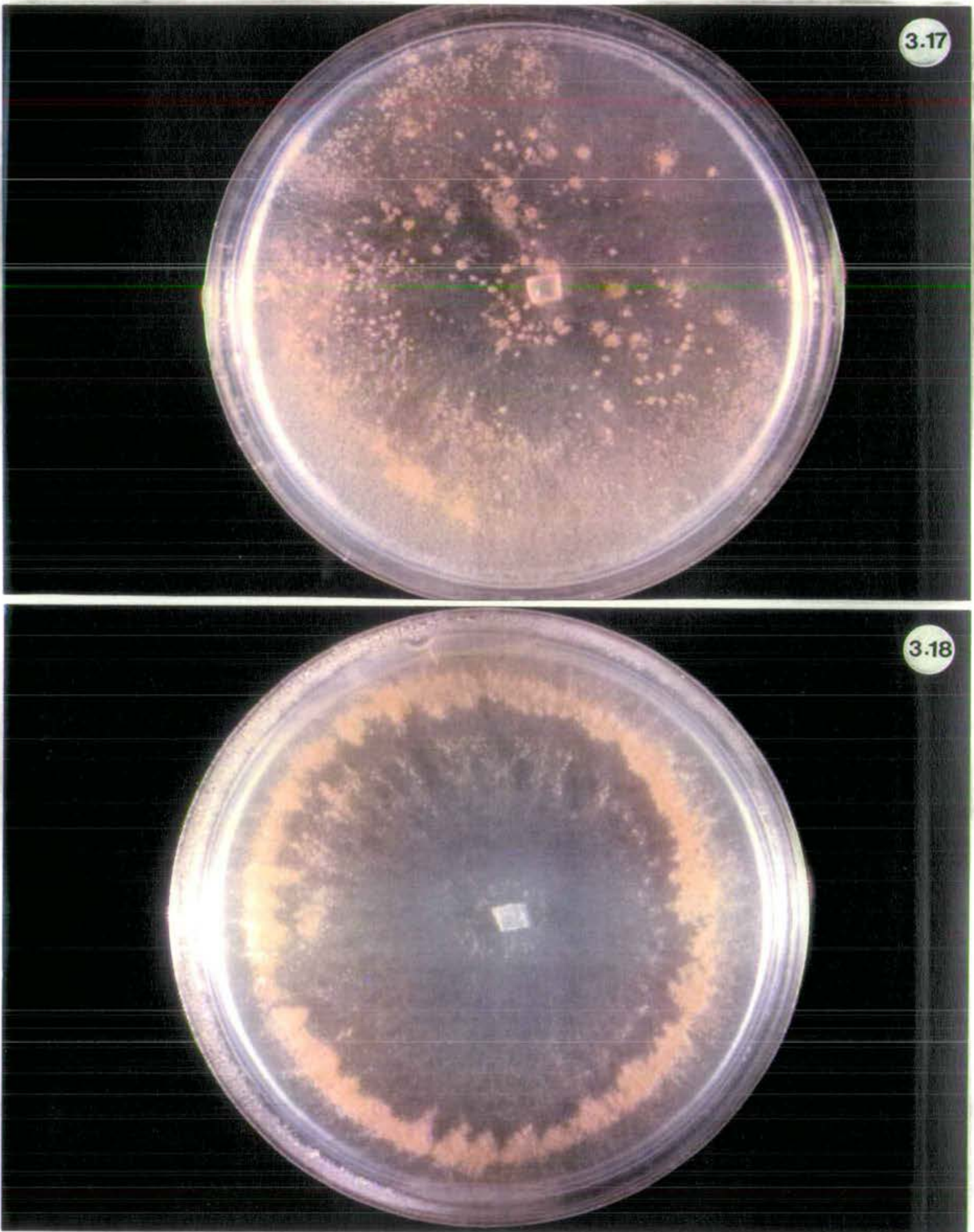
such high density that a zone of continuous tissue was formed (Figs. 3.17 and 3.18).

VHA structure: VHAs from 4 day old, light-grown cultures of *S. brevicollis,* were prepared and sectioned as described previously. Fig. 3.19 illustrates the lack of internal differentiation seen in VHAs. Thin-walled hyphae, of a wide range of diameters, were only loosely grouped together, and no differentiation into discrete layers could be distinguished. Individual hyphae often appeared vacuolate. No evidence from sectioned material suggested that VHAs were abortive protoperithecia: they lacked the typical compact, spherical shape of protoperithecia, and no traces of ascogonial structures were ever detected.

When examined under the scanning electron microscope, VHAs appeared as mounds of hyphae, poorly differentiated from the surrounding mycelium (Fig. 3.20). The trichogyne-like hyphae which were seem to arise from the surface of protoperithecia were absent.

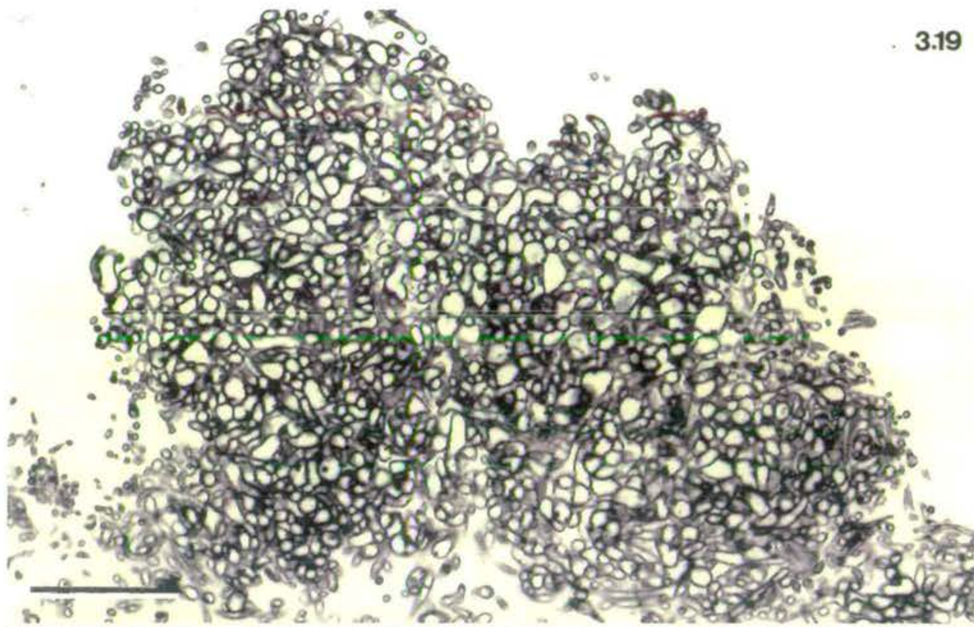
3.2.1.4.2 *Continuous VHA tissue*

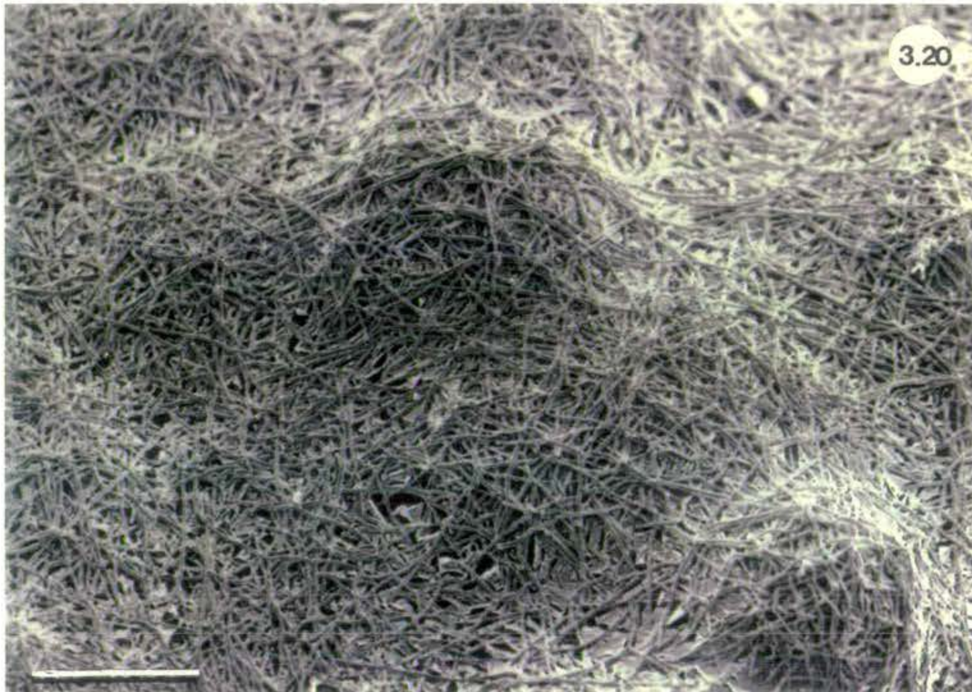
General observations: Unless specified to the contrary, the term "VHA" is used to denote the individual structures described above, but although initially formed as more-or-less discrete structures, VHAs often merged to form a thin layer of tissue over the surface of the culture media, particularly when grown on CMA overlaid with cellophane (Fig. 3.18). The rôle of the substrate in stimulating this intense VHA production was examined on a variety of media containing powdered cellophane membranes, pure cellulose, and rabbit pellets. Uncrossed, light-grown cultures were examined over a 10 day period. VHA density and distribution on plates containing powdered cellophane membranes, pure cellulose, and powdered rabbit pellets showed no significant deviation from control plates of standard growth media (CMA), with relatively uniform production of individual VHAs occurring on all plates. Plates containing whole rabbit pellets showed a different distribution pattern: 5-6 days after inoculation, each rabbit pellet was covered in a thick layer of continuous VHA tissue of approximately 1 mm in thickness, while the production of VHAs on the surface of the growth media was sparse. Occasionally, small perithecia were found to be embedded within the layer of VHA tissue, although these perithecia were never seen to contain ascospores. Unfortunately, it was not possible to cut satisfactory thin sections of this material, as sand grains from the surface of the rabbit pellets rapidly destroyed knife blades. Microscopic observation of fresh material



Figures 3.17 and 3.18: ***Sordaria brevicollis.*** **VHAs.**

Variable appearance of light-grown cultures on CMA overlaid with cellophane membranes. At high density VHAs coalesce to form a zone of continuous tissue.





Figures 3.19 and 3.20: ***Sordaria brevicollis.*** **VHAs.**

Figure 3.19: Thin section through VHA from 4 day old, light-grown culture Note (i) the irregular outline; (ii) the numerous fine hyphae, many of which appear to be vacuolate; and (iii) the presence of many inter-hyphal spaces. Bar = 50 μm.

Figure 3.20: Scanning electron micrograph of VHAs in 4 day old, light-grown culture. VHAs are poorly differentiated from the underlying mycelium, appearing as irregular masses of hyphae. The hyphae which arise from the surface of protoperithecia and perithecia are absent from VHAs. Bar = 200 μm.

however, suggested that the VHA tissue was of similarly homogeneous nature to small VHAs, and no evidence of a distinct surface layer was found. Although several attempts were made to reproduce the balance of nutrients and substrate texture provided by rabbit pellets, and thus to establish the factors of importance in stimulating such intense VHA production, this was never achieved.

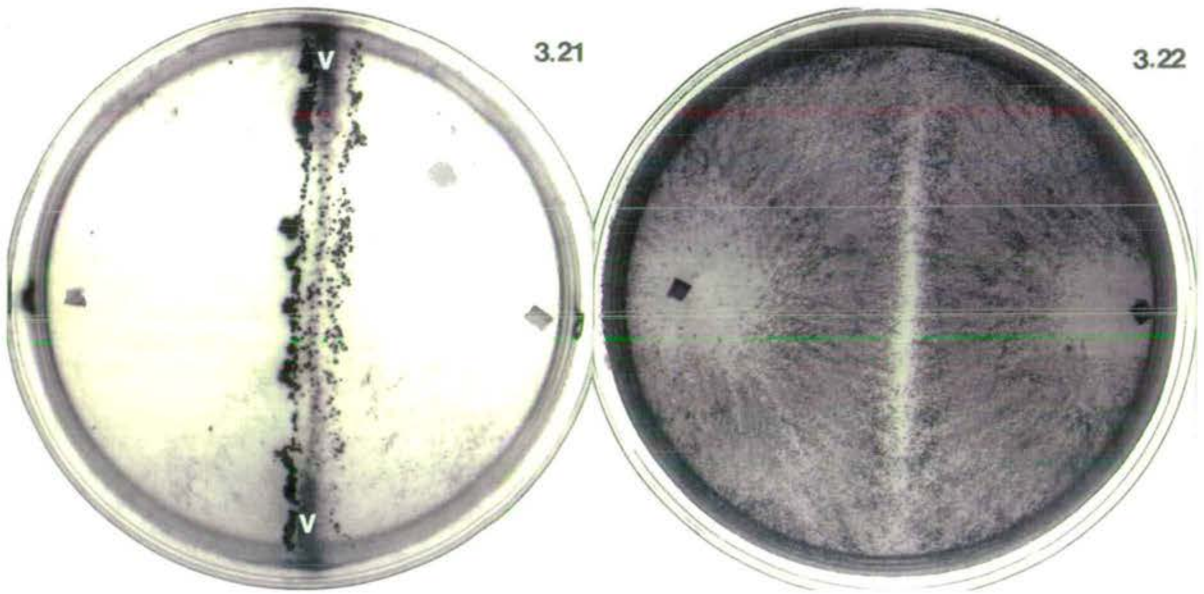
A second type of continuous VHA-like material was frequently formed at the junction of two mycelia of opposite mating type, producing a ridge of tissue of up to 5 mm in width, and 2-3 mm millimetres in height, on either side of which perithecia subsequently formed (Fig. 3.21). The reaction was not uniform however: VHA tissue often petered out towards the centre of the Petri plate, resulting in the formation of two separate regions of VHA material, and certain combinations of sexually compatible strains exhibited either much weaker VHA production, or none at all (although such strains produced individual VHAs in a normal manner). At the junction of two mycelia of the same mating type, no region of VHA tissue was formed, although in light-grown cultures, VHAs were produced over the remainder of the Petri plate (Fig. 3.22).

The VHA-type tissue formed at the junction of sexually compatible mycelia differed from all VHA material described previously in one important respect, being produced either in the light or in the dark. When formed in the dark, the pink pigmentation characteristic of VHA tissue was absent, but this developed rapidly upon exposure to light.

3.2.1.4.3 *Large VHAs*

General observations: A third type of VHA formation was observed on (at least) 15-20 day old cultures, which had been grown in the light during the first 4 days after inoculation. These structures, illustrated in Fig. 3.23, were of similar colour and texture to small VHAs, but of much greater size (up to 2 mm diameter), and of more regular shape (being approximately spherical). Large VHAs were typically formed in clusters, on ageing mycelia which were starting to accumulate dark brown pigments.

The precise cultural conditions required for optimal production of these structures were never established. Large VHAs often developed on relatively old plates which had been left on the laboratory bench, and which had therefore been subjected to variation in light regime and temperature. They were almost exclusively formed in cultures grown on cellophane membranes, but were never observed on plates containing rabbit pellets, powdered membranes, or cellulose

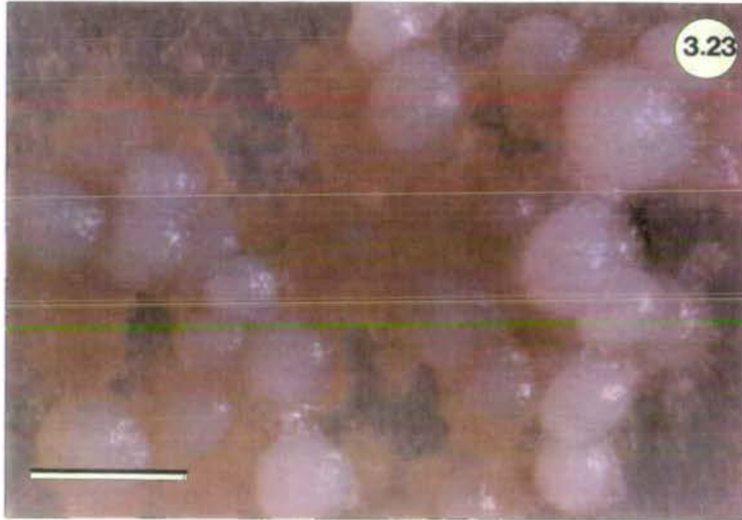


Figures 3.21 and 3.22: *Sordaria brevicollis*. Confrontation "crosses".

Figure 3.21: Typical appearance of a confrontation cross, with development of VHA-like tissue (v) at the junction of mycelia of opposite mating type. Perithecia are produced on either side of a ridge of VHA-like material, with the greatest perithecial frequency on the *mtA* side, in this case. In a dark-grown culture, there are no VHAs elsewhere on the plate. **Figure 3.22:** A light-grown "cross" between two strains of the same mating type (*mtA* in this instance). The culture is stained with trypan blue in lactophenol to illustrate the distribution of VHAs. No VHA-type material develops at the junction between the mycelia, although VHAs are formed over much of the remainder of the culture.

Figures 3.23 - 3.25: ***Sordaria brevicollis.*** **Large VHAs.**

Figure 3.23: Typical appearance of large VHA structures on 20 day old cultures, grown on CMA overlaid with cellophane membranes. Note the same pinkish colouration as seen in small VHAs (c.f. Fig. 3.15), but far greater size, and more regular shape. Large VHAs were typically produced in small clusters. Bar = 2 mm. **Figure 3.24:** Large VHA in thin section, showing two regions of differing hyphal density. Hyphae in the central region are relatively loosely packed, with many inter-hyphal spaces, whilst towards the outside, hyphal density and wall thickness increase. No region corresponding to a rind layer can be identified. Bar = 200 μm. **Figure 3.25:** Scanning electron micrograph of large VHAs. Note the greater differentiation from the underlying mycelium than seen in smaller VHAs (c.f. Fig. 3.20). Bar = 200 μm.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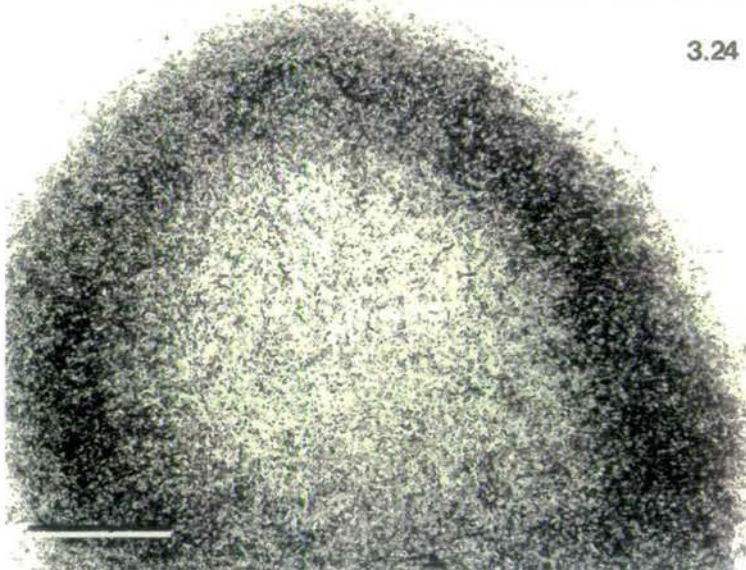
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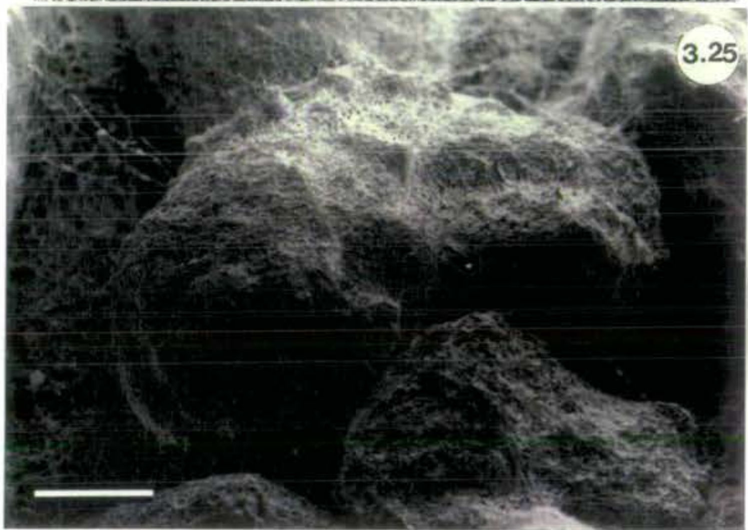
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powder. Crossed cultures never developed large VHAs, nor did uncrossed cultures which were incubated in constant darkness.

Large VHA structure: In thin section, large VHAs from 20 day old cultures were seen to possess a loosely woven texture, essentially similar to that of the smaller VHAs illustrated previously. Although they lacked clearly defined layers, hyphal density and wall thickness was observed to increase towards the surface, producing more intense staining in this region, whilst the central area showed numerous inter-hyphal spaces (Fig. 3.24). No rind layer was present, however, nor were sexual structures of any kind ever detected. No further development of large VHAs was ever observed, although cultures were monitored over a period of several months. Little additional detail was provided by examination of large VHAs under the scanning electron microscope, although it was evident that these structures were more clearly differentiated from the underlying mycelium than were the smaller VHAs described previously (Fig. 3.25).

3.2.2 Characterisation of sclerotia from *S. sclerogenia*

General observations: Cultures of *S. sclerogenia* were examined in order to establish the form that sclerotia might be expected to take if they were present in *S. brevicollis*. *S. sclerogenia* is the only known sclerotial species in the genus, and sclerotia do not appear to be common in coprophilous fungi. Numerous sclerotia developed on either light or dark grown cultures, 3-4 days after inoculation, and superficially resembled individual VHAs in their size and rather irregular form. Sclerotia were initiated as short side branches, and formed throughout the growth media, either individually or in small clusters. Under the dissecting microscope it was evident that sclerotia were composed of relatively few large cells, and that although the inner cells were darkly pigmented, the outer cells were hyaline. By 24 hours after intiation, sclerotia had hardened and were very difficult to squash.

Sclerotial structure: Small sclerotia from 4 day old cultures were sectioned as described previously. Two distinct zones could be distinguished within each sclerotium, an inner region of large, thick walled cells whose contents stained very intensely with toluidine blue, and an outer region of apparently vacuolate cells which were unpigmented (Fig. 3.26). These unpigmented cells occasionally formed short, branched projections from the surface of the sclerotium. Within the central region, two cell types were identified, although they may have simply

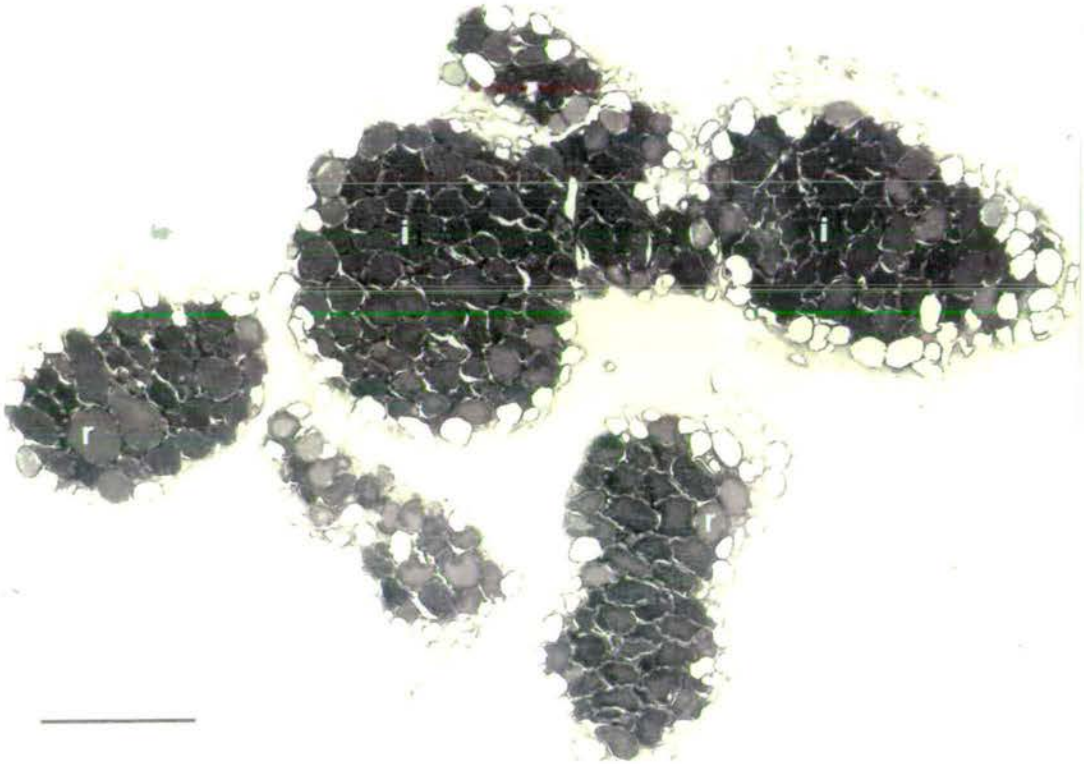


Figure 3.26: ***Sordaria sclerogenia*: Sclerotia.**

Thin section through cluster of sclerotia from 4 day old, dark-grown culture. Each sclerotium is made up of two different regions: in the central area, cells are typically large, thick-walled and stain intensely with toluidine blue, whilst cells at the edge of each sclerotium are smaller, have thinner walls, do not stain readily, and appear to be vacuolate. Amongst the inner cells, a further division can be made into cells of relatively rounded outline (r), which often contain small, light-staining spherical structures (which may be nuclei), and cells of more irregular outline (i), within which very little detail can be observed. These "sclerotia" clearly do not conform to the structure of typical sclerotia. Bar = 100 μm.

have been at different developmental stages. Some cells, generally those which stained less deeply, were of relatively smooth outline, and could be seen to contain small spherical structures (possibly nuclei). The remaining cells were of irregular shape and were tightly fitted together, possibly contributing to the physical strength of the sclerotium.

3.2.3 Investigation of laccase activity during multicellular development

3.2.3.1 Laccase activity in *S. brevicollis*

3.2.3.1.1 *Uncrossed cultures - 4 days old*

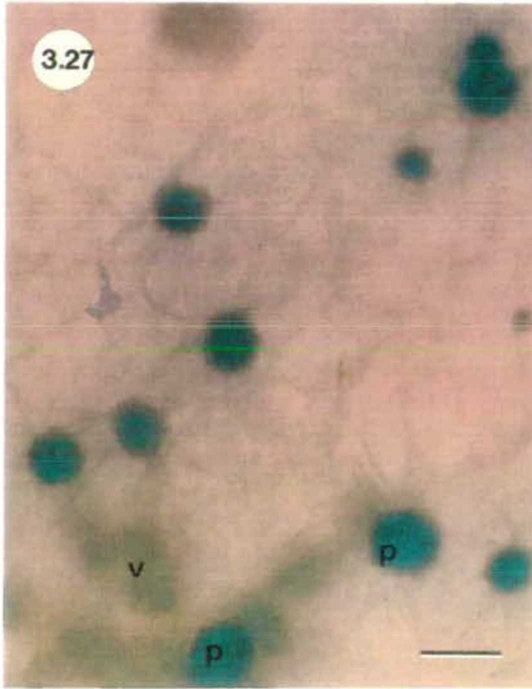
Laccase activity was assayed using a modified version of the method of Hermann *et al.* (1983). Both light- and dark-grown cultures of *S. brevicollis* were examined at four days old, when protoperithecia were present in dark grown cultures, and both VHAs and occasional protoperithecia had developed in the light. Protoperithecia from light- and dark-grown cultures stained an intense blue-green, indicating localised laccase activity, whilst VHAs stained very weakly or not at all (Fig. 3.27). Deposits of a very dark precipitate which formed on the surface of some protoperithecia, were seen at high magnification to be intracellular, and were largely confined to the bases of hyphae which arose from the protoperithecial wall (Fig. 3.28). There was no evidence of any laccase activity in the surrounding medium. Laccase activity was examined in protoperithecia and VHAs from strains of both mating types, although no variation was observed.

3.2.3.1.2 *Crossed cultures 5-10 days old*

Laccase activity in crossed, wild type cultures was examined for 48 hours after spermatisation: thereafter the staining reaction was masked by development of melanic pigments in the perithecial wall. Later stages in perithecial formation were monitored in a hyaline mutant (whose protoperithecia stained in a manner identical to those of wild type strains). Examination of crossed wild type cultures 24 hours after fertilisation, revealed the presence of two distinct populations of protoperithecia: those protoperithecia which had become enlarged continued to give a strong, laccase-positive staining reaction, whilst those protoperithecia which had not enlarged showed a much weaker response. Two days after fertilisation, developing wild type perithecia continued to stain positively, whilst those protoperithecia which had not enlarged showed no laccase activity. Later stages in perithecial development, monitored in the hyaline mutant, showed that although the whole of the perithecial wall

Figures 3.27 - 3.30: *Sordaria brevicollis*. Laccase localisation.

Figure 3.27: Protoperithecia and VHAs from a 4 day old, light-grown, wild type culture. Protoperithecia (p) show an intense blue-green colouration, indicative of localised laccase activity, whilst VHAs (v) stain weakly or not at all. Bar = 50 μm. **Figure 3.28**: A dark precipitate on the surface of certain protoperithecia is intracellular, and largely confined to the bases of hyphae which arise from the protoperithecial wall. Bar = 40 μm. **Figure 3.29**: Crossed perithecia from 10 day old, dark-grown cultures of a hyaline mutant (*hya*). Although the whole of the perithecial wall stains positively, the strongest laccase activity is localised in the neck region (arrow). Bar = 150 μm. **Figure 3.30**: Uncrossed protoperithecia/perithecia from an 8 day old, dark-grown culture. Only those structures which have become enlarged continue to exhibit laccase activity. Bar = 75 μm.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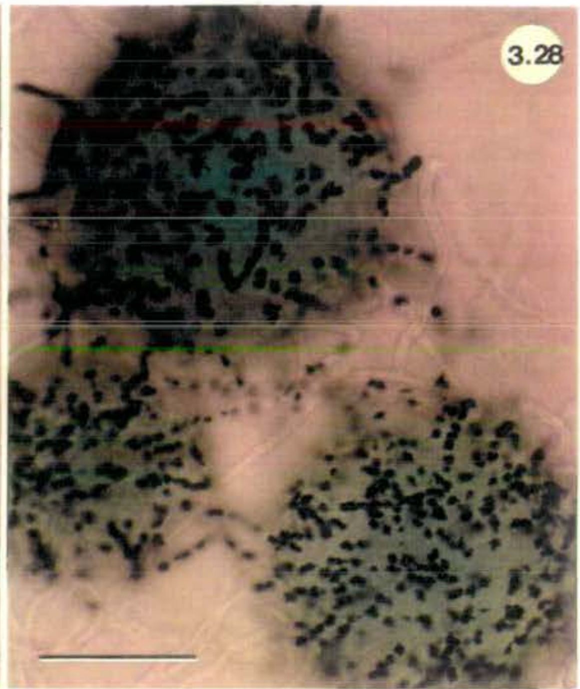
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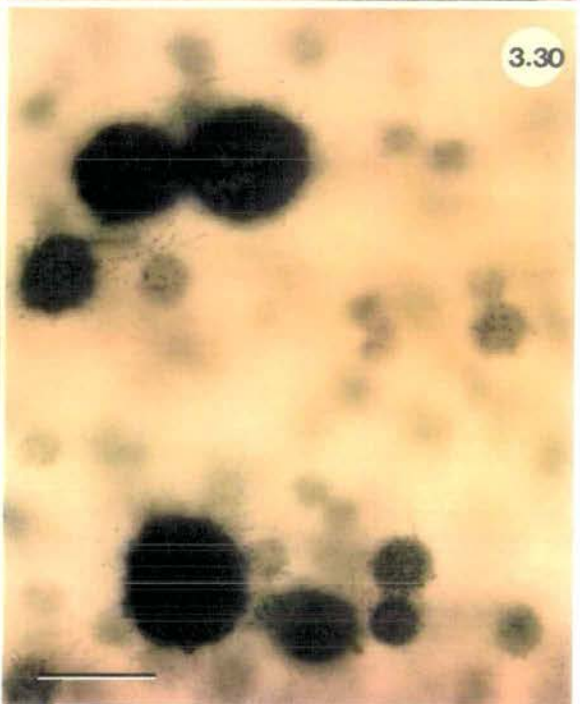
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continued to stain positively, the strongest laccase activity was localised in the neck region (Fig. 3.29).

3.2.3.1.3 *Uncrossed cultures, 5-10 days old*

Unfertilised wild type cultures of both mating types were examined between 5 and 10 days old, and the laccase activity in uncrossed perithecia was followed. (After fertilisation of wild type protoperithecia, increasing pigmentation of the perithecial wall soon obscured the staining reaction, but in uncrossed protoperithecia, perithecial expansion was typically less rapid and pigmentation less intense, allowing the blue-green pigment produced in laccase-positive tissues to be distinguished.) Initial observations indicated that the few uncrossed perithecia produced in *mta* cultures stained in a similar manner to those from *mtA* cultures, and as the latter were typically far more numerous, subsequent detailed observations were confined to *mtA*.

In 6 day old, uncrossed cultures of *mtA*, two populations of protoperithecia could be distinguished: those which continued to exhibit laccase activity, and those which did not. There was no pronounced difference in size between staining and non-staining protoperithecia. In 8 day old cultures, laccase activity was largely restricted to protoperithecia which had become enlarged (Fig. 3.30), and any staining in protoperithecia which had not enlarged was confined to the formation of crystals of very dark pigment, in hyphae on the protoperithecial surface, similar to that illustrated in Fig. 3.28, but without the blue-green staining of the protoperithecial wall.

3.2.3.2 Laccase activity in *S. sclerogenia*

A brief investigation of laccase activity was also carried out during sclerotial development in *S. sclerogenia* . Laccase activity was examined in 3 day old cultures, a stage at which sclerotia had begun to differentiate, but no protoperithecia had formed. The central part of very young sclerotia stained the same blue-green colour as the protoperithecia of *S. brevicollis*. This reaction was soon masked by the development of black pigmentation however, and no laccase activity was detected in the hyaline cells on the surface of older sclerotia.

3.2.4 Investigation of lipid localisation during multicellular development

In an effort to develop a rapid method for distinguishing protoperithecia and VHAs in culture, 4 day old material from wild type strains was stained with the lipid-specific dye, Nile red. Lipid localisation was also examined in mature

perithecia from a hyaline mutant (hya). (In wild type perithecia, positive staining was obscured by the melanic pigments which developed in the perithecial wall.)

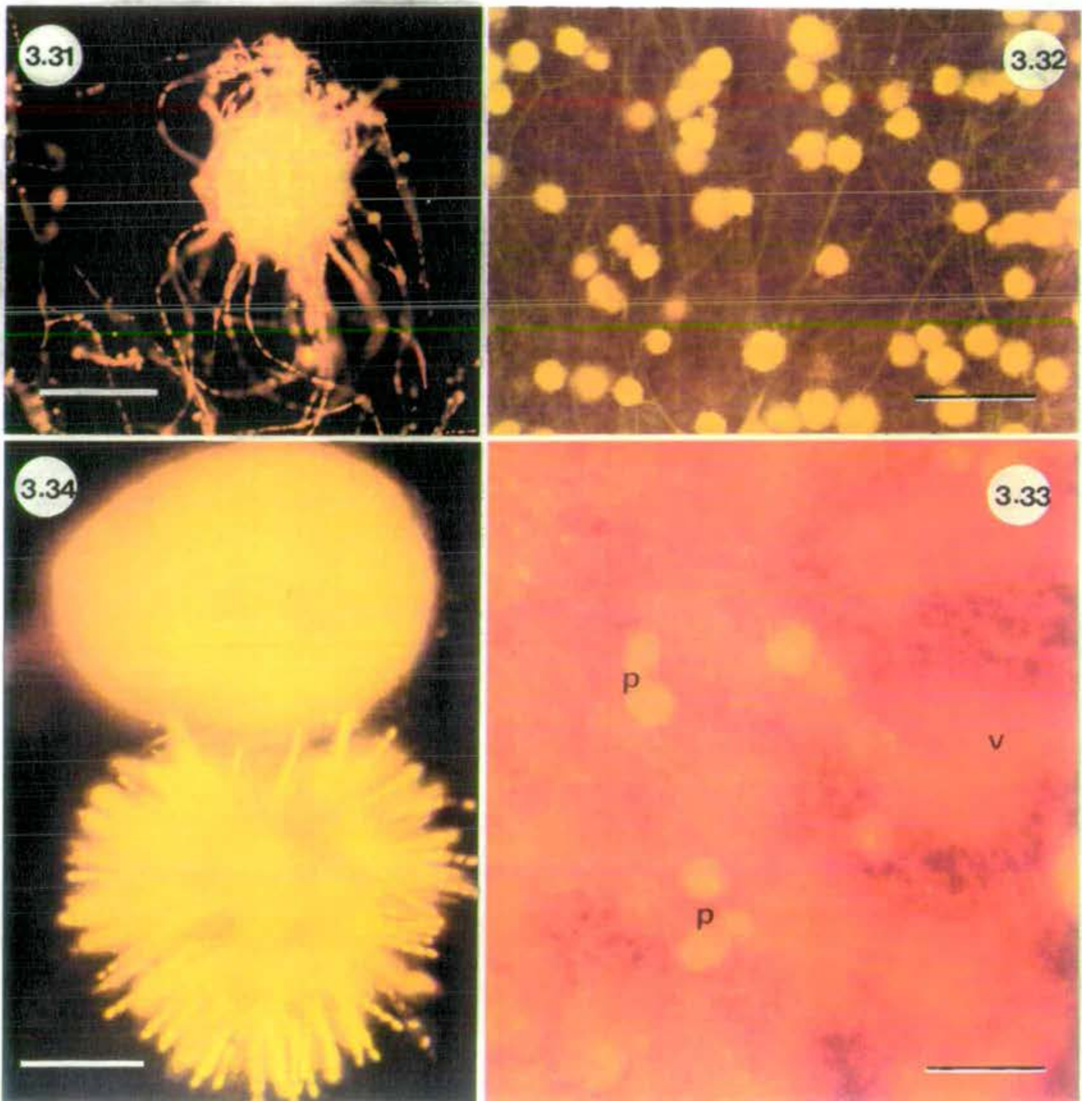
When excited with blue light, stained protoperithecia emitted a strong yellow-gold fluorescence, characteristic of lipid-rich tissue (Figs. 3.31 and 3.32). Small lipid droplets were also detected in the mycelium, and particularly in the hyphae which arose from the protoperithecial wall (Fig. 3.31). In contrast, VHAs stained very weakly if at all (Fig. 3.33), and light-grown mycelia generally appeared less lipid-rich than cultures grown in the dark. Large quantities of lipid were also detected in maturing perithecia, particularly within the developing ascospores (Fig. 3.34).

3.2.5 Neck number in crossed perithecia

During the examination of perithecial cultures it was noted that a substantial minority of fruitbodies developed more than one neck. This phenomenon occurred in both crossed and uncrossed cultures. In some instances perithecia had clearly become fused together relatively late in development, being linked only by a shared portion of the peridial wall. In many other cases however, the perithecial wall appeared continuous, although as many as 5 or 6 necks were formed. Potential explanations for the development of the latter type of perithecium were (i) that they had arisen from clusters of protoperithecia which fused during very early development, and thereafter developed a continuous wall around several centra; or (ii) that the polarity which normally governed neck formation had broken down for some reason.

Although occasional perithecia with 5 or 6 necks were observed, the formation of 2 or 3 necks was much more typical. In contrast with single-necked perithecia, whose necks invariably differentiated on the surface furthest away from the growth medium, multiple necks were seen in a variety of orientations, often pointing directly into the agar. In crossed cultures, spores were frequently discharged from more than one neck per perithecium, and were often discharged into the medium.

Initial attempts to section crossed perithecia with multiple necks (with a view to observing any internal division within the centrum), proved rather inconclusive: in some cases centra appeared to be divided (at least in the plane of section), whilst in other instances, internal development was identical to that of single-necked specimens. An experimental approach using spore colour mutants was therefore adopted.



Figures 3.31 - 3.34: ***Sordaria brevicollis*****. Localisation of lipids.**

Figures 3.31 and 3.32: Protoperithecia from 4 day old, dark-grown cultures. Protoperithecia emit a strong yellow-gold fluorescence, characteristic of lipid-rich tissues. Bars = 50 μm (Fig. 3.31) and 150 μm (Fig. 3.32). **Figure 3.33:** VHAs and protoperithecia in 4 day old, light-grown cultures. The yellow-gold fluorescence of the protoperithecia (p), is absent from VHAs (v). Bar = 150 μm. **Figure 3.34:** Crossed perithecium from 10 day old, dark-grown culture, exhibiting intense fluorescence of both the perithecial wall, and the ascospores. Bar = 100μm.

It is shown in Chapter Four, that in 10-30% of perithecia, more than one "female" nucleus is involved in the formation ascospores within a single perithecium, resulting in the production of a mixed centrum. If perithecia with two or more necks resulted from the fusion of several protoperithecia, it might reasonably be expected that perithecia with multiple necks would possess a higher frequency of mixed centra than those with a single neck, as the pool of available female nuclei would be larger. Heterokaryons were established between *mtA* isolates of wild type and *S187* (yellow-spored) strains, as described in Materials and Methods. The uniformity of heterokaryon formation was monitored by determination of the nuclear ratio. This was achieved by using samples of the four day old heterokaryon as a microconidial parent in a cross with buff (*c70*) protoperithecia. Cultures were incubated in darkness for four days to allow protoperithecial formation, and then fertilised with a suspension of *c70* (buff) microconidia. After a further six days, perithecia were removed from the mycelium and sorted according to neck number. Four hundred individual perithecia, removed from a single culture (200 with a single neck, and 200 with two or more necks) were crushed open on clean microscope slides in 10% sucrose solution.

Examination of perithecial contents showed that each perithecium was derived from a cross between: (i) black and buff nuclei; (ii) yellow and buff nuclei; or (iii) black, yellow and buff (mixed) nuclei. The frequency of each class is shown in Table 3.1.

Table 3.1: Frequencies of different perithecial types from a cross between a wild type/*S187* heterokaryon (female parent), and *c70* (male parent). The nuclear ratio for this heterokaryon was 9:1 in favour of the mutant (yellow) nuclei.

Perithecial type	Parental nuclei involved in cross			Total
	Black x buff	Yellow x buff	Black x buff Yellow x buff	
Single Neck	12	164	24	200
Multiple Necks	10	170	20	200

The number of mixed centra in perithecia with two or more necks was actually slightly lower than that in perithecia with a single neck, although this difference was not significant ($X^2 = 0.20$ - from tables a value of $X^2 > 3.84$

indicates significance at p = 0.05, with one degree of freedom). The results suggested that few of the perithecia with multiple necks resulted from the fusion of a number of adjacent protoperithecia, or at least that if fusion had occurred, only one centrum had developed further.

3.3 Discussion

3.3.1 Protoperithecia

Protoperithecial structure in *S. brevicollis* conformed in most respects with the pattern described in other members of the Sordariales (Harris *et al.*, 1975; Huang, 1976; Uecker, 1976; Mai, 1976, 1977; Read, 1983). However, two features recorded in related species could not be confirmed: neither hyphal continuity between trichogyne and ascogonium, nor septation of the ascogonium were ever observed during the present study.

The fusion of trichogyne and conidium, and the subsequent passage of the fertilising nucleus through the trichogyne to the ascogonium, is an accepted method of fertilisation in heterothallic pyrenomycetes. Conidial fertilisation in *Neurospora sitophila* was first illustrated by Dodge (1935) and Backus (1939), who showed trichogynes "burrowing" between the cells of the protoperithecial wall from the ascogonium to the outside, and demonstrated that the tips of such hyphae were receptive to spermatia of opposite mating type. In Dodge's preparations, trichogynes exhibited intense protoplasmic staining, and were quite easily distinguished from the surrounding wall cells, which stained only poorly. Unfortunately, the precise staining procedure used was not recorded, and none of the more recent studies so clearly illustrate a direct connection between ascogonium and trichogyne. Dodge also noted that the majority of the fine hyphae which arose from the protoperithecium of *N. sitophila* had no connection with the ascogonium, and concluded that these "pseudotrichogynes" were sterile. The difficulty in distinguishing trichogynes from sterile hyphae was acknowledged by Backus (1939) and more recently by Bistis (1981), who recognised receptive hyphae in *N. crassa* only by their "serpentine appearance, and .... sharply tapered tips". Bistis (1981,1983) demonstrated that trichogynes were attracted by pheromone-like substances produced by conidia of opposite mating type, and that fertilisation followed shortly after the fusion of trichogyne and conidium. He did not establish, however, that receptive hyphae were direct extensions of the ascogonium as in *N. sitophila.*

The fine hyphae which arose from the outer cells of the protoperithecial wall in *S. brevicollis*, appear to correspond to Dodge's "pseudotrichogynes". The

failure to observe true trichogynes during the present study may have been a consequence of: (i) the relatively small number of protoperithecia sectioned; (ii) the multidirectional growth of trichogynes required to pass between wall cells; or (iii) the staining protocols used. It should be noted, however, that attempts to use lactophenol cotton blue to produce the differential staining of trichogynes reported by Backus (1939) were equally unsuccessful. Since protoperithecial cultures of *S. brevicollis* were readily fertilised by microconidia of opposite mating type, it must be assumed that a functional mechanism for the transport of male nuclei to the ascogonium was present. Even so, a *direct* connection between the tip of a receptive hypha outside the protoperithecium and the ascogonium at its centre remains to be demonstrated in *S. brevicollis*, and it is not inconceivable that fertilising nuclei could be transported to the ascogonium via a temporary passage, created by the unblocking of septa between cells of the protoperithecial wall. It is rather doubtful, however, whether the time required for transport via such a route is reconcilable with the rapidity of protoperithecial expansion after crossing.

Septation of the ascogonium, a phenomenon previously reported in species of *Neurospora*, *Gelasinospora* and *Sordaria* (Dodge, 1935; Jensen, 1982; Read, 1983), was not observed in protoperithecia of *S. brevicollis*. Septation of the ascogonial coil in *S. humana* was observed under by SEM, before the protoperithecial wall was formed (Read, 1983), and has also seen in young ascogonial coils of *S. brevicollis* during the course of this study, but never observed in sectioned, 4 day old protoperithecia. One of relatively few really conclusive demonstrations of ascogonial septation was that provided by Dodge (1935) in *N. sitophila*, where the ascogonium exhibited intense protoplasmic staining, and unstained cross-walls were clearly visible. The hypha of the ascogonial coil was of a similar width to those making up the protoperithecial wall, and very much narrower than the ascogonia seen in thin sections of *S. brevicollis* during this study (Fig. 3.1). The failure to observe ascogonial septation in *S. brevicollis* may simply indicate that too few protoperithecia were sectioned to detect cross walls, and it is also possible that the staining techniques used in previous studies more clearly emphasised the presence of septa. Apparent variation in ascogonial size between *S. brevicollis* and *N. sitophila* may be the result of differential shrinkage caused by the alternative preparative techniques used, although it should be observed that the cells forming the protoperithecial wall in the two species are of very similar size.

3.3.2 Crossed perithecia

Wild type crossed perithecia conformed in most respects to the structure described previously in *S. humana* (Uecker, 1976; Read, 1983; Read and Beckett, 1985). Slender hyphae which arose from the outside of the perithecial wall in *S. brevicollis* corresponded to "perithecial hyphae" in *N. crassa* (Johnson, 1976) and "fringe hyphae" in *S. humana* (Read, 1983), although it was not clear whether these were related to the hyphae which arose from the cells of the protoperithecial wall or had developed separately during perithecial expansion. The thickness of the perithecial wall, and the number of layers which could be distinguished varied between perithecia, and was probably a consequence of varying expansion of the centrum. The material which lined the inside of the wall constituted the remains of the centrum pseudoparenchyma and was not part of the wall itself. The perithecial wall in *S. brevicollis* was reported by Sanni (1984) to be made up of three layers, although the inner layer was probably centrum pseudoparenchyma. Other *Sordaria* species have been described as having perithecial walls made up of either two or three layers (two in *S. macrospora* (Walkey and Harvey, 1967) and three in *S. fimicola* (Ingold and Hadland, 1959a)), although wall structure may in fact vary between perithecia, as has been seen in *S. brevicollis*.

The cause of multiple neck development in *S. brevicollis* is unknown, although the results obtained from heterokaryon analysis suggest that such fruitbodies do not arise from the fusion of adjacent protoperithecia. The development of perithecia with several necks was previously noted in *S. brevicollis* by Sanni (1984), and has also been observed in other *Sordaria* species by Moreau (1953).

Hyaline perithecia differed from wild type in several respects, in addition to their lack of pigmentation. Both in external appearance and internal structure, the necks of hyaline perithecia were distinct from wild type, although the shape of the hyaline neck closely resembled that of perithecia from the buff spore colour mutant, *c70* (not illustrated). In the latter, buff spore colour was accompanied by abnormal perithecial pigmentation: protoperithecia and young perithecia were of a reddish-brown colour, and although the colour deepened as perithecia developed, mature perithecia were easily distinguished from wild type. Thus two mutants with abnormal pigmentation of ascospores and the perithecial wall, exhibited shorter, wider necks than those typical of wild type fruitbodies, suggesting that pigmentation and perithecial morphology may be linked in some way. The structure of the hyaline perithecial wall also differed from wild type,

with loosely arranged hyphae, which lacked the pseudoparenchymatous structure of the wild perithecial wall. Nevertheless, the walls of hyaline perithecia exhibited strong laccase activity, which, it has previously been suggested, is associated with hyphal adhesion (Broxholme *et al.*, 1991). In view of the structure of the hyaline perithecial wall, however, this interpretation should perhaps be questioned, although no other function for laccase (clearly not associated with pigmentation in this case) is obvious.

3.3.3 Uncrossed perithecia

Perithecia which contain few or no ascospores have been reported in several ascomycete species, either as the product of interspecific crosses (Olive and Fantini, 1961; Perkins and Raju, 1986) or as the result of meiotic mutations in homothallic, pseudohomothallic, and heterothallic species (El-Ani and Olive, 1962; Simonet and Zickler, 1972; Raju and Perkins, 1978). Although these fruitbodies are variously described as *sterile* perithecia, *pseudo*perithecia, or *barren* perithecia, they typically exhibit arrested sexual development, rather than a complete lack of fertile tissues. Sterile and barren (terms with unfortunate anthropomorphic connotations) are often qualified with *partially* or *completely*, and it is not always evident whether a sterile or barren cross produces no perithecia, no ascospores, or unviable ascospores. Further confusion is generated by a tendency to use the same terms to describe a mycelium before protoperithecial formation has commenced (MacDonald and Bond, 1976), and also by the use of "sterile" to describe a range of mutants which either produce no sexual structures or structures which are defective (section 1.4.1.1). In this thesis, both terms will be avoided whenever possible: spore-bearing perithecia are clearly fertile, but it is not possible to state unequivocally that perithecia without spores are sterile.

In their original description of *S. brevicollis*, Olive and Fantini (1961) observed that "Most of the protoperithecia, if unfertilised, remain small and undeveloped. A few scattered ones, however, may enlarge to the size of mature perithecia, but these never contain asci." A similar phenomenon was reported in another newly-isolated heterothallic *Sordaria*, *S. heterothallis* by Fields and Maniotis (1963), and was again noted in *S. brevicollis* by Sanni (1984). Siddiq (1989) recorded the presence of "immature, sterile perithecia" on *mtA* cultures of *S. brevicollis*, but did not state whether or not such structures possessed ostioles or necks. The observation that there is a differential ability to produce uncrossed perithecia between the two mating types, is supported by the present study.

Although both mating types have been found to produce uncrossed perithecia, far greater numbers of perithecia are produced by *mtA* strains, and only on *mtA* cultures have spore-bearing uncrossed perithecia ever been found. Perkins and Raju (1986) recorded the presence of abundant "barren pseudoperithecia" or "false perithecia" in isolates of a relatively rare heterothallic species, *Neurospora discreta*, from a single site in Texas. These perithecia, which grew to a diameter of about 200 μm, did not develop necks, and were never seen to contain asci. Clearly, the production of enlarged, pigmented protoperithecia in an unmated isolate of a heterothallic species is not unique to *S. brevicollis*. Unreported elsewhere, however, is the development of uncrossed perithecia with necks or the production of viable ascospores.

Ascospore liberation has been investigated in several *Sordaria* species (Ingold, 1953; Ingold and Dring, 1957; Walkey and Harvey, 1967). In *S. fimicola*, as in many other pyrenomycete species, the asci remain attached to the base of the perithecium during spore discharge. Asci elongate one at a time, so that the tip is forced up the neck canal and through the ostiole. Although the ascus wall is highly extensible, a point is reached when the internal hydrostatic pressures cause the wall to rupture, and the spores are ejected. The elongation of asci prior to spore liberation is apparently achieved by their own growth, and by the pressure of the surrounding asci. Although very few asci were formed in the majority of the uncrossed perithecia produced by *S. brevicollis*, spore discharge was occasionally observed. Spores were ejected with sufficient force to reach the lid of the Petri plate, but whether this was with equal vigour to the discharge of spores from crossed perithecia was not determined.

Several factors may be cited to explain why only a proportion of uncrossed perithecia were seen to discharge their spores:- observations on the internal structure of uncrossed perithecia suggested that paraphyses sometimes persisted until spores were mature, possibly restricting ascus elongation; the necks of uncrossed perithecia were not always fully developed, and may on occasion have been occluded; and the pressure from surrounding asci, which may be instrumental in the discharge of spores from crossed perithecia, was clearly absent in uncrossed fruitbodies. Spore discharge is clearly important to coprophilous species, which must complete their life cycle and liberate spores before their substrate is broken down and incorporated into the soil, and although spores which remain inside the perithecium may eventually be liberated when the perithecial wall is decomposed, they may also be lost completely. The

discharge of even a few spores from uncrossed perithecia could therefore be of considerable importance in an ecological context.

3.3.4 VHAs

Although the origin of VHAs is unclear, there are two obvious possibilities: VHAs may be degenerate sexual structures of some kind, or may be of a sclerotial character. These alternatives are evaluated in the following pages. Examination of the internal structure of VHAs revealed no evidence that they were defective protoperithecia: no structures resembling ascogonia were ever observed, and the compact organisation of typical protoperithecia was absent. Although there are detailed records of the gross morphology of a great many developmental mutants, some producing defective protoperithecia (e.g. Garnjobst and Tatum, 1967), there are relatively few descriptions of the internal structure of mutant strains. One study (N. D. Read and K. M. Lord, in preparation) used light and electron microscopy to characterise a number of developmental mutants of *S. macrospora*, which had originally been isolated and analysed by Esser and Straub (1958). Three mutants were defective in various aspects of protoperithecial development: *spd* produced aberrant ascogonia and protoperithecia; f_1 produced ascogonia and large protoperithecia of irregular form, distorted by uncontrolled internal tissue differentiation; and *pl* developed ascogonia and small clumps of protoperithecia which lacked paraphyses. In each case, the structures formed were essentially spherical, and of relatively compact form, and bore no resemblance to the VHAs produced in *S. brevicollis*.

The likelihood that VHAs are abortive protoperithecia is further reduced by the observation that they were not subject to the "edge effect" which stimulated protoperithecial formation, nor did they form in the dark where protoperithecial development was optimal. VHAs and protoperithecia also exhibited differential laccase activities. Although only one laccase localisation technique was used (and VHAs may contain other phenol oxidases, such as tyrosinase), the results obtained are consistent with the hypothesis that laccase is involved in hyphal adhesion during multicellular development (Bu'lock, 1967), since VHAs, in which hyphae were loosely arranged, exhibited very little laccase activity.

While VHAs were tentatively described by Broxholme (1988) as "sclerotia", they clearly lack many sclerotial characteristics, bearing little resemblance to the "typical" sclerotia of species such as *Sclerotinia* (Huang, 1983). Neither do VHAs resemble the sclerotia of *Sordaria sclerogenia* (Fields

and Grear, 1966). The external appearance of the sclerotial structures produced by the latter species most closely resembles that of papulaspores described by Weresub and LeClair (1971) in the hyphomycete genus *Papulaspora.* Previously classed as bulbils (Hotson, 1942; Barron, 1968), these structures were reported to have a central core of enlarged, thickened and pigmented cells, which were "germinable", surrounded by a sheath of smaller, thinner walled cells which were sterile, and would thus appear to correspond anatomically to the sclerotia of *S. sclerogenia.* Whether or not the latter are properly termed "sclerotia" is open to question, as is the closeness of the relationship between *S. sclerogenia* and *S. brevicollis.* Fields and Grear (1966) paired strains of *S. sclerogenia* with *S. brevicollis* and a third heterothallic species, *S. heterothallis* (Fields and Maniotis, 1963) but failed to observe any interspecific reaction, and a similar result was obtained with *S. brevicollis* during the present study.

Although in many respects *S. brevicollis* closely resembles *N. crassa*, the two species differ in one important detail, namely that wild type strains of *N. crassa* produce copious numbers of macroconidia. These function not only as fertilising elements, but also as effective asexual propagules, and typically contain carotenoid pigments which are thought to protect against the harmful combination of light and oxygen (Ruddat and Garber, 1983). The formation of conidia is stimulated by several environmental factors including light, and is preceded by a period of aerial growth of conidiophores. Amongst numerous conidial mutants are several in which the generation of chains of conidia, and the disarticulation of individual spores is blocked at various stages of development (Springer and Yanofsky, 1989). It has been suggested that in certain fungal species, sclerotia may have developed from interwoven conidiophores which have not differentiated chains of conidia because of unsuitable conditions (Townsend and Willetts, 1954; Willetts and Bullock, 1992), and it might be envisaged that VHAs represent an intermediate stage in the evolution of conidiophores to sclerotia. A mutation at a relatively early stage in conidiogenesis in *S. brevicollis*, which caused irregular growth and branching of conidiophores, without the formation of conidia, might conceivably have resulted in the formation of structures resembling VHAs. Composed of very fine, loosely interwoven hyphae, VHAs formed only at the surface of the medium, were stimulated by light, and developed carotenoid pigments, all characteristics of macroconidial formation.

The possible evolutionary origins of VHAs are of rather less relevance in an ecological context than their present function - if they have one. It may be

argued that many organisms possess vestigial structures, and that VHAs have no significant rôle in the life cycle of this fungus under natural conditions. It is also possible that VHA production is a mutation acquired in culture. If VHAs are produced in the wild, however, the reduction in reproductive capability which accompanies VHA development, would appear to require some compensating positive feature if not to be acted against by selective pressures.

The continuous layer of VHA tissue formed on rabbit pellets suggests certain functional parallels with the stromatic tissue found in members of the Xylariaceae and Hypocreaceae, where sterile material provides both support and protection for the sexual fruitbodies. The development of stromatic tissue, is not typical of the Sordariaceae, however (Lundqvist, 1972; Müller and von Arx, 1973). Jong and Davis (1973) proposed the establishment of a new genus, *Stromatoneurospora*, within the Xylariaceae, for the stromatic *Neurospora*-like species which had previously been assigned to genera such as *Sphaeria*, *Chromocreopis*, and *Xylocrea*. The stroma which they described was bounded by a distinct surface layer, however, of a type never seen in the VHAs produced by *S. brevicollis*. Amongst a number of xylariaceous genera which produce perithecia associated with thin, superficial stromata is *Hypocopra* (Krug and Cain, 1974) where variable amounts of stromatic tissue develop around the ostiole, although again, a surface layer is present.

The relatively diffuse nature of VHA tissue, and the absence of a rind of any sort, also precludes close comparison with the stromatal anamorphs in the Sclerotiniaceae, described by Kohn and Grenville (1989). These authors highlighted a difficulty encountered when examining stromata produced *in vitro*, namely that they may differ considerably from those developed on natural hosts or substrates. It may be the case in *S. brevicollis* that the conditions required to stimulate full development of VHA tissue have yet to be achieved in the laboratory.

Only the large VHAs, produced occasionally on older cultures, showed any evidence of differentiation into two layers, but there was no evidence to suggest the development of a rind. Again it may be argued, however, that development in the laboratory was not complete. It seems probable that large VHAs arose from small VHAs when suitable conditions were created, although it is possible that they may have been initiated from distinct primordia. Their formation may have been triggered by exhaustion of particular nutrients in the growth medium, by the build up of staling products, or by particular combinations of light, temperature and humidity. The formation of large VHAs

on older mycelia suggested certain developmental parallels with sclerotial development in response to starvation, but no survival value could be inferred from their morphology.

It seems likely, in view of similarities in texture and pigmentation, that individual small VHAs, continuous layers of VHA tissue, and large VHAs formed on older cultures are fundamentally similar in nature, although differing in growth form. The character of the tissue formed at the junction of mycelia of opposite mating type is more doubtful however, particularly as this material formed in the absence of light. The formation of a zone of VHA tissue at the junction of mycelia of opposite mating type, corresponds, superficially at least, to a similar phenomenon observed in *N. crassa* by Griffiths and Rieck (1981). These authors studied crosses involving the aconidial mutant *fl* (fluffy), and reported the development of a "clear" zone between the lines of perithecia, occupied by a dense mycelium. Within the zone, hyphae were often "intertwined and braided to form rope-like structures", and on the *mtA* side of the zone, a band of orange pigment (presumed to be a carotenoid) was formed. In *S. brevicollis,* this "clear zone" more closely resembles a ridge, which may in places reach several millimetres in height, on either side of which perithecia develop (Fig. 3.21).

Extension of the same study to include the mating type mutant a^m (33-1), isolated by Griffiths and DeLange (1978), produced interesting results which also have a bearing upon VHA development in *S. brevicollis.* The a^m (33-1) allele has the effect of removing the heterokaryon incompatibility function which is normally associated with mating type in *N. crassa*, whilst retaining crossing ability (Griffiths and DeLange, 1978). It was therefore supposed that, if the clear zone described above was associated with the incompatibility function, its presence would be removed by the a^m (33-1) allele (Griffiths and Rieck, 1981). When 12 double mutants ($fl;a^m$) were backcrossed to a *fl;A* parent, in six cases the standard mating reaction was observed (two parallel lines of perithecia separated by a clear zone), whilst in the other six, a single line of perithecia was produced. This result demonstrated not only that the incompatibility function of the mating type locus did *not* control the presence of the clear zone, but also that the segregation of some other genetic factor, unlinked to the mating type locus, *could* eliminate the zone. Although the standard mating reaction must have required the direct or indirect action of the mating type genes (since the reaction was only observed in *A* x *a* crosses), other genes must also have been involved. A simple model which proposed the presence of a single gene, whose action

inhibited the formation of the clear zone, accounted for most, but not all of the results obtained from a further set of backcrosses, and it was concluded that the control must be more complex. The mating reactions in *N. crassa* and *S. brevicollis* are apparently similar, and the formation of VHA tissue in crosses of *S. brevicollis* may be under similar control to that described in *N. crassa*.

It is possible that the morphological similarities between VHA tissue in the clear zone and other VHA types produced by *S. brevicollis* are coincidental, and that the genetic mechanisms which regulate their formation are quite separate. It is believed however, that the distribution of individual VHAs is also controlled by more than one gene, since four different VHA distribution phenotypes have been identified (D. J. Bond, pers. comm.)

Although "barrage" is often used as a convenient term to describe the clear zone between lines of perithecia (e.g. Raju, 1992), it is worth highlighting the distinction between the zone of VHAs produced in *S. brevicollis* crosses and the classic barrage phenomenon found in *Podospora anserina*. As noted in the introduction to this chapter, cytoplasmic incompatibility in the latter species is a result of the interaction of non-allelic genes at number of unlinked loci (*c, d, e, r,* and *v*) with further complexity added by the presence of allelic series at several loci (Boucherie *et al.*, 1976). When incompatible mycelia meet, RNA and protein synthesis is reduced, and several proteases and phenol oxidases are produced, causing cell lysis (Boucherie and Bernet, 1978). Similar incompatibility is caused by *S* and *s* alleles, although these are not involved in the mating reaction. When incompatible homokaryons meet, the hyphal tips which anastomose die back to a septum, where numerous side branches develop (Blaich and Esser, 1971). In sexually compatible strains, mating occurs across the barrage, producing a line of perithecia on one or both sides of a clear zone between the mycelia. Whilst producing a similar visual effect, the reaction between opposite mating types in *S. brevicollis* resulted in the formation of a ridge of interwoven hyphae, and no hyphal lysis was observed.

The formation of VHAs in *S. brevicollis* was light-stimulated, and almost completely absent in darkness (the exception being the tissue produced between lines of perithecia in crossed cultures). The exposure of young cultures to light, not only initiated the formation of VHAs, but also had an inhibitory effect upon sexual reproduction, reducing the overall number of perithecia formed. Although light plays no clear rôle in perithecial formation in some species, *S. fimicola* for example (Ingold and Dring, 1957), illumination is of primary importance in the control of sexual reproduction in many ascomycetes (section 1.7.1.1.2). In most

documented cases, light appears to initiate or stimulate such development. In *S. brevicollis*, however, perithecial development is optimal in complete darkness, and although the magnitude of the inhibitory effect of light is strain-specific, some degree of inhibition is always observed when cultures are grown in continuous light for the first 4 days after inoculation.

The pathways of multicellular development *S. brevicollis* which have been characterised in this chapter are summarised in Fig. 3.35. The work described in the following chapter, concerns a detailed examination of the pathway which culminates in the formation of spore-bearing perithecia in uncrossed cultures.

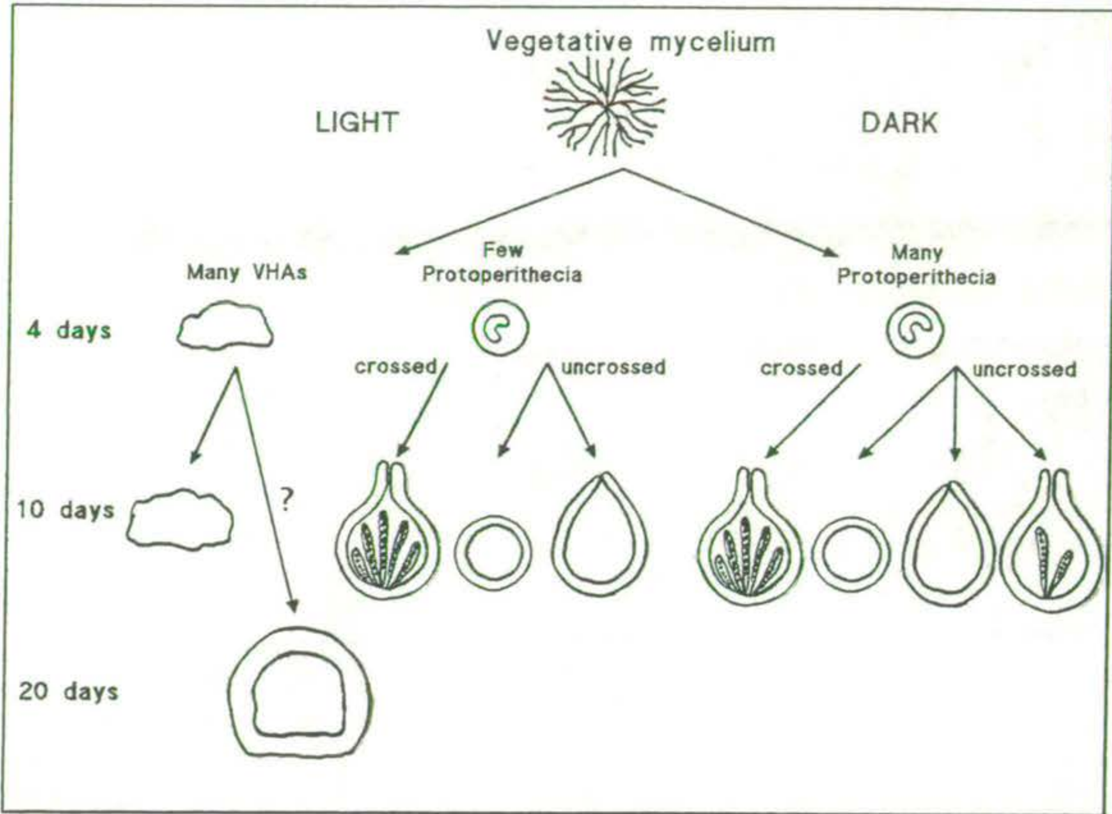


Figure 3.35: Pathways of multicellular development in *Sordaria brevicollis*. The vegetative mycelium of *S. brevicollis* gives rise to VHAs and/or protoperithecia according to the illumination conditions. Under constant white light, 4 day old cultures typically develop numerous VHAs, and relatively small numbers of protoperithecia. In 10 day old, light-grown cultures, VHAs may enlarge slightly, or at high density, may merge to form a continuous layer over the surface of the growth medium. It is not known whether the large, approximately spherical structures which were occasionally observed in 20+ day old, light-grown cultures represent a later stage of development of the small VHAs seen in younger cultures, or whether they arise independently. When fertilised, protoperithecia in light-grown cultures mature into normal crossed perithecia, containing numerous asci. Uncrossed perithecia are comparatively rare in light-grown cultures, and seldom contain ascospores. Cultures incubated in the dark produce numerous protoperithecia but no VHAs. In unfertilised dark-grown cultures, a proportion of protoperithecia develop into uncrossed perithecia, which may contain a few asci.

CHAPTER FOUR

HOMOTHALLISM AND HETEROTHALLISM IN SORDARIA BREVICOLLIS

4.1 Introduction

Although heterothallism in fungi promotes outbreeding and genetic diversity, a great many species are homothallic. Since homothallic individuals can complete their sexual cycle in isolation, the ascospores produced are genetically identical to the parent and probably have greatest value in providing a means of survival or dispersal. Homothallism and heterothallism are not mutually exclusive, however: the homothallic species *S. fimicola* for example, has been shown to develop a form of heterothallism in response to mutations causing self sterility (El-Ani and Olive, 1962). The reverse situation (homokaryotic fruiting in a heterothallic species) is more unusual, but has been recorded in both ascomycete and basidiomycete genera, and is reviewed briefly here as an introduction to homokaryotic fruiting in *S. brevicollis.*

4.1.1 Monokaryotic fruiting in basidiomycetes

Basidiomycetes are predominantly heterothallic and, with the exception of the Uredinales, have no sexual organs. (There are for example, no structures analogous to the protoperithecia found in the Sordariaceae.) Instead, hyphae from compatible monokaryons fuse, allowing nuclear interchange and dikaryon formation. The proliferation of the dikaryon is typically facilitated by the production of clamp connections, which maintain the binucleate state of each hyphal compartment (Casselton, 1978), and provide a useful diagnostic feature for most dikaryotic mycelia. Such mycelia, which may persist for long periods, give rise directly to basidiocarps under favourable conditions.

Typically, the fusion of two compatible monokaryons must precede basidiocarp formation. However, in more than thirty basidiomycete species, including *Polyporus ciliatus* (Stahl and Esser, 1976), *Agrocybe aegerita* (Esser and Meinhardt, 1977) and *Schizophyllum commune* (Esser *et al.*, 1979), isolated monokaryons have been observed to produce basidiocarps. In some cases these fruitbodies have proved to be sterile, but in other instances basidia were found to develop viable spores. Microscopic examination of the basidia from

monokaryotic cultures of *P. ciliatus* revealed that only two spores were differentiated on each basidium (instead of the usual four), and cytological observations showed that fruiting took place in the haploid state, without karyogamy or meiosis (Stahl and Esser, 1976) (Fig. 4.1).

A recent study of homokaryotic fruiting in *A. aegerita* revealed a further degree of complexity (Labarère and Noël, 1992). In this species, three types of homokaryotic fruiting were distinguished: abortive homokaryotic fruiting (AHF), in which basidiocarps never opened or sporulated; true homokaryotic fruiting (THF), in which two-spored basidia were produced (as in *P. ciliatus*); and pseudo-homokaryotic fruiting (PHF), in which four-spored basidia were differentiated. Examination of the mycelia of PHF cultures revealed the presence of clamp connections suggesting dikaryotisation, and normal Mendelian segregation of mating type factors occurred amongst the progeny. These observations were attributed to mating type switching in vegetative nuclei, a process facilitated by the existence of potentially expressible *A* and *B* mating type factors, stored as silent copies in the genome. The model is analogous to that proposed for the interconversion of mating types in certain yeasts (Herskowitz, 1988). Two kinds of spore-bearing basidiocarps have therefore been identified on isolated monokaryons, those with two-spored basidia (both spores being of parental mating type), and those with four-spored basidia (with spores of two different mating types). The two-spored basidia were produced mitotically in the haploid phase, and the four-spored type after karyogamy and meiosis.

4.1.2 Apomictic spore production in ascomycetes

The production of "sexual" spores in the absence of meiosis has also been observed in ascomycetes. Mainwaring and Wilson (1968) studied the cytology of a strain of *Podospora arizonensis* in which the nuclei in the penultimate cell of the crozier did not undergo the normal sequence of fusion, meiosis and mitosis. Instead, the two nuclei remained close together as the young ascus elongated, and only when the ascus had attained its full size did two mitotic divisions take place, giving rise to 8 daughter nuclei (Fig. 4.2). Heslot (1958) observed apomictic behaviour of a similar kind in a mutant of *S. macrospora*. Apomixis has also been reported in two other homothallic *Sordaria* species (Dengler, 1937) and in species of *Ascobolus* (Bjorling, 1944), although in these cases a single nucleus gave rise to all eight spores in each ascus, after three mitoses.

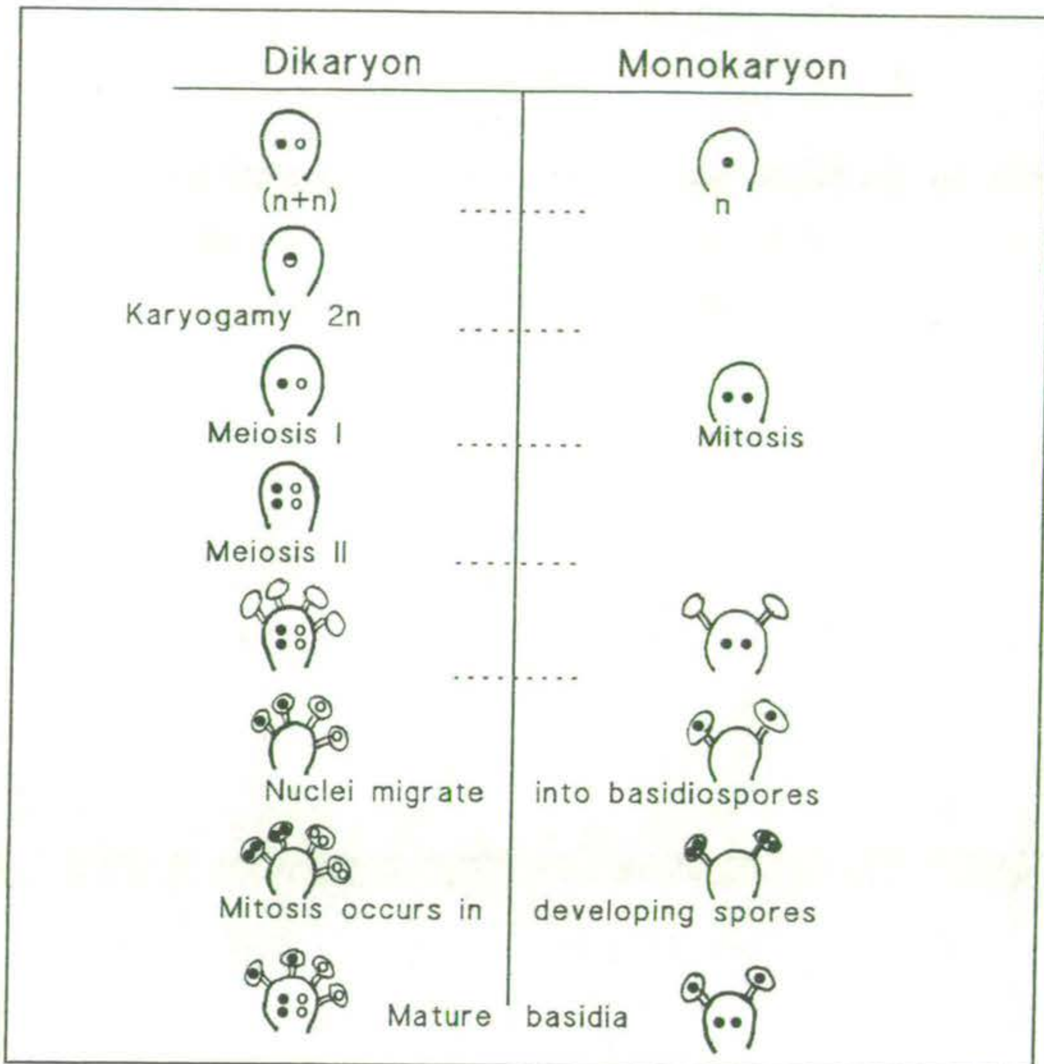


Figure 4.1: Stages of basidial development during dikaryotic and monokaryotic fruiting in a basidiomycete. Redrawn from Stahl and Esser (1976). Basidia formed on monokaryotic mycelia have only two spores, which are produced without karyogamy or meiosis.

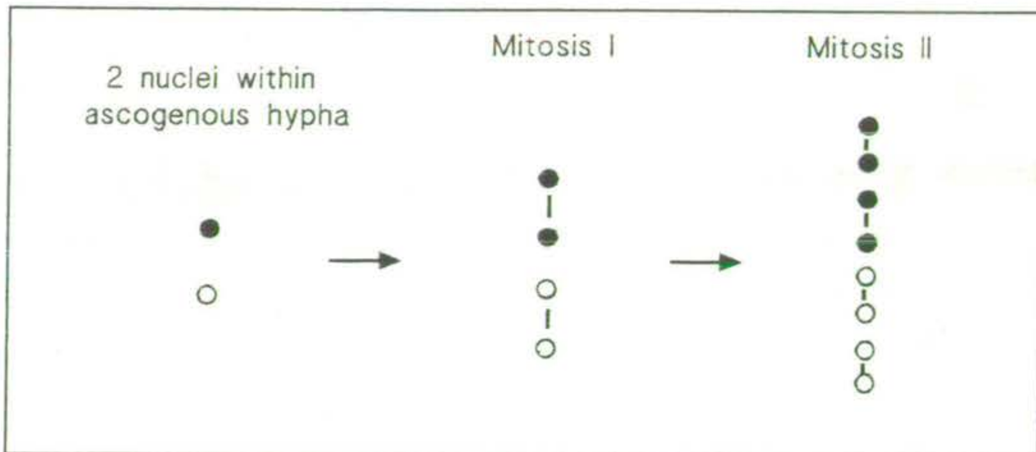


Figure 4.2: Diagrammatic representation of the nuclear divisions which take place during apomictic spore formation in *Podospora arizonensis*. (For original photographs, see Mainwaring and Wilson (1968).) Although each ascus contains 8 spores, these are produced following two mitotic divisions, and neither karyogamy or meiosis take place. (The spindle orientations shown here are simplified: Mainwaring and Wilson illustrated spindles of mitosis I which were parallel to the ascus wall, but overlapping, whilst those of mitosis II were orientated either longitudinally or transversely.)

4.1.3 Induction of fruiting by "sex hormones"

Several authors have reported that sexual morphogenesis in both homothallic and heterothallic fungi may be stimulated by a group of endogenous substances, variously described as hormones, erogens, pheromones, and sex factors (Nelson, 1971; Dyer *et al.*, 1992), which may be involved in the early stages of mating type recognition, or may function in the initiation and development of fruitbodies. In the homothallic species *Aspergillus nidulans* for example, a group of substances termed "precocious sex inducers" (or psi factors) were found to inhibit asexual sporulation and stimulate the development of fertile cleistothecia (Champe *et al.*, 1987; Champe and El-Zyat, 1989). These factors were apparently specific to *A. nidulans* and did not affect closely related species. In *Nectria galligena* the activity of "mycosporines" has been linked to sexual reproduction (Dehorter and Lacoste, 1979; Dehorter and Bernillon, 1983). The formation of mycosporines was light-induced, and cultures maintained in constant darkness neither produced mycosporines nor differentiated protoperithecia. The light requirement for perithecial production in this species was partially by-passed by addition of mycosporin I to dark-grown cultures. Like the psi factors in *A. nidulans*, mycosporines were relatively specific and did not induce similar effects in other species (Arpin and Bouillant, 1981).

The heterothallic species *Pyrenopeziza brassicae* has been shown to produce a "sex factor" (SF) which suppresses asexual sporulation, stimulates the production of immature, sterile apothecia in unmated cultures, and increases the number of apothecia which develop in mated cultures (Ilott *et al.*, 1986; Siddiq, 1989; Siddiq *et al.*, 1989, 1990). Unlike psi factors and mycosporines, however, SF has been reported to influence aspects of vegetative and sexual development in a a range of other ascomycete and basidiomycete species (Siddiq, 1989) although these effects may in part have been due to nutritional substances present in the lipid extract. Partial purification has indicated that SF contains at least 20 components, and it seems that different components may be responsible for different types of activity.

Although in *P. brassicae* only sterile apothecia were produced in response to the addition of SF to unmated cultures (Ilott *et al.*, 1986; Siddiq, 1989), studies on *N. crassa* have recorded the development of fertile perithecia (Islam and Weijer, 1972; Vigfusson and Cano, 1974; Islam, 1981). Islam and Weijer (1972) reported the activity of a chloroform extract from mated cultures which induced the formation of fertile perithecia in cultures of *mtA*. The segregation of mating type observed amongst the spores, was a result attributed at the time to the

possible compound nature of the mating type locus. Subsequent analyses have shown that there are no copies of the *mta* idiomorph within the *mtA* genome, however (Glass *et al.*, 1988), and the segregation for mating type was probably the result of contamination. Vigfusson and Cano (1974) described the activity of a proteinaceous substance which they called erogen B, also extracted from mated cultures of *N. crassa.* When erogen B was added to unmated cultures of either mating type, fertile perithecia were produced, most of whose spores carried genetic markers from only one parent. Occasional perithecia containing asci with a 4:4 segregation of genetic markers were attributed to accidental contamination by wild type conidia. Given the potential significance of these findings the apparent failure of the authors to pursue the results further is regrettable, and there are no published records of these experiments being replicated elsewhere. Whilst homokaryotic fruiting has been stimulated in a number of heterothallic species, the work carried out on *N. crassa* provides the only example in which viable spores have been produced.

4.1.4 Homokaryotic fruiting in *S. brevicollis*

The morphological characteristics of the uncrossed perithecia produced by certain *mtA* cultures of *S. brevicollis* were illustrated in Chapter Three, and this chapter concentrates largely on the processes which lead to spore formation within such fruitbodies. Nuclear behaviour during ascus formation has been studied using both cytological staining techniques and heterokaryons analysis, in order to determine whether any of the forms of self fertility described in the foregoing pages provide an adequate model for homokaryotic fruiting in *S. brevicollis.* In addition, a preliminary investigation of the growth conditions which promote homokaryotic fruiting has been carried out, and attempts have been made to enhance homokaryotic fruiting using extracts from mated cultures.

4.2 Results

Cytological staining and heterokaryon analysis were used to determine whether meiosis took place during spore formation in uncrossed perithecia: if no meiosis occurred, then parallels might be drawn between homokaryotic fruiting in *S. brevicollis* and monokaryotic fruiting in basidiomycetes, or apomictic spore production in other ascomycetes. If normal meiosis took place, then perithecial production in homothallic *Sordaria* species would provide a better model for the homokaryotic fruiting observed in *S. brevicollis.*

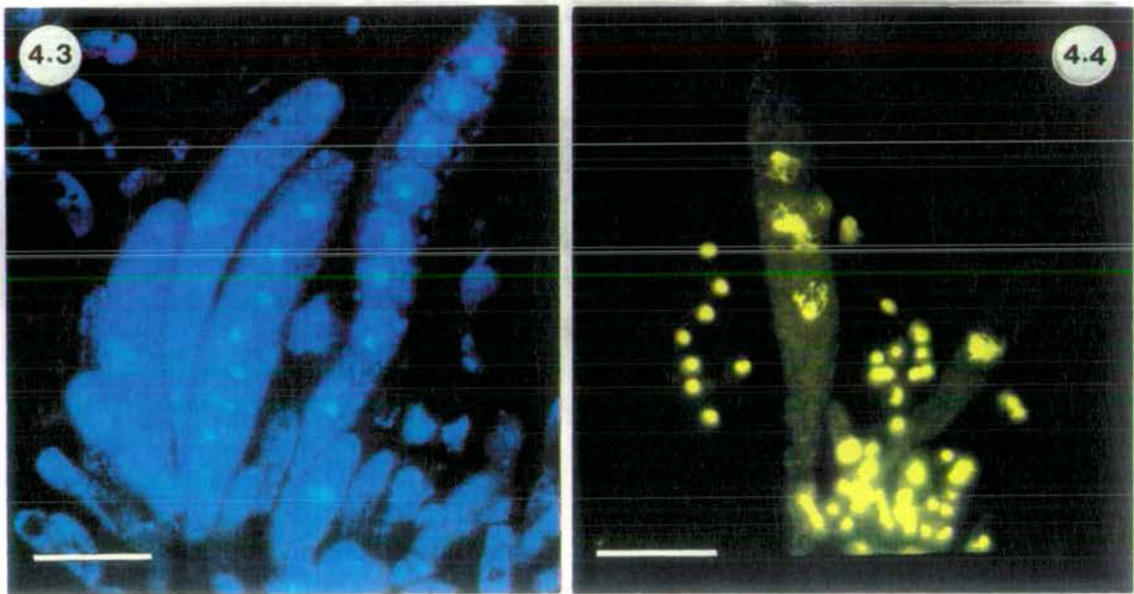
4.2.1 Cytological Staining

4.2.1.1 Choice of stain and staining procedure

Before examining asci from uncrossed perithecia, various stages of ascus development in wild type, crossed perithecia were characterised. Stains prepared as described in Chapter Two, were tested on asci in early meiosis. Two conventional stains, aceto-orcein and Feulgen produced indifferent results, whilst the fluorescent stain, DAPI, produced strong and relatively persistent staining of nuclei, but gave poor resolution of individual chromosomes (Fig. 4.3). Acriflavin-stained material showed better resolution of chromosomes (Fig. 4.4), and although the yellow-gold fluorescence faded under the microscope, acriflavin was selected for subsequent use. Since acriflavin is specific to DNA, the RNA-rich nucleolus did not stain strongly.

4.2.1.2 Ascus cytology: crossed perithecia

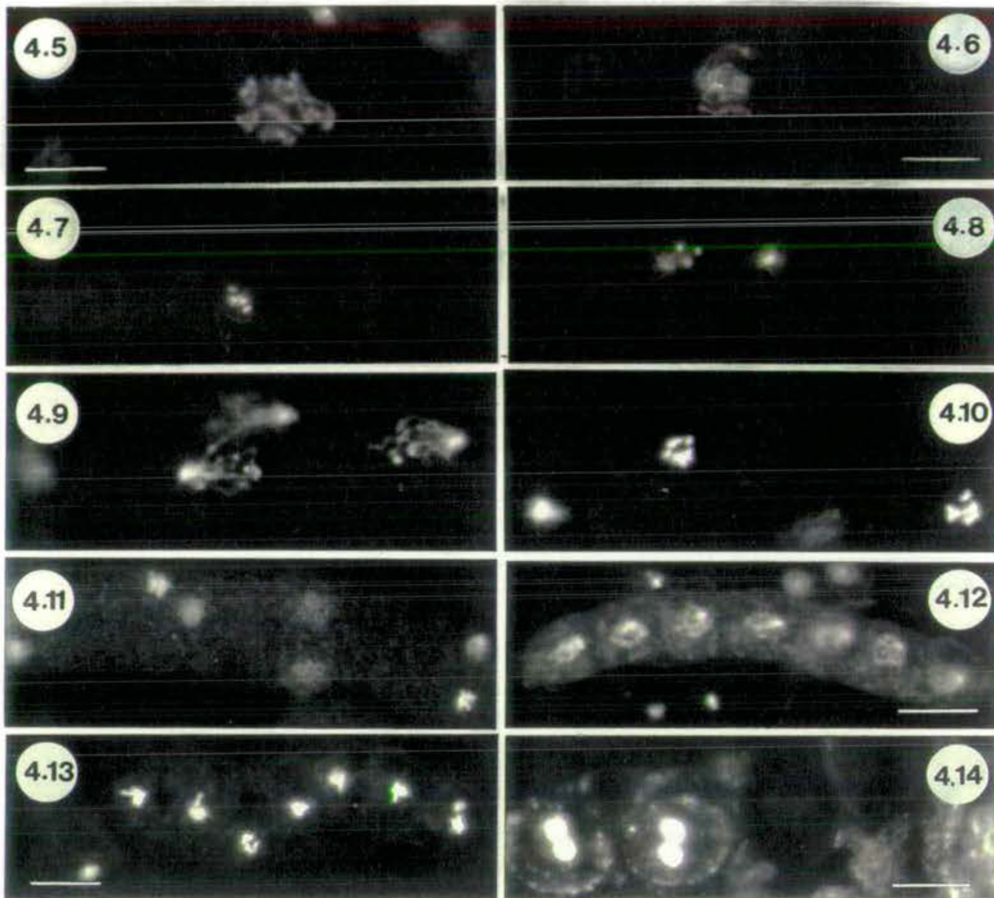
Fixation was carried out 36-48 hours after crossing of dark grown cultures, a period when asci in a range of developmental stages from karyogamy to spore delimitation could be found within a single perithecium. The most commonly observed stages were early prophase I and interphase following division II, suggesting that these phases were of relatively long duration. It was assumed that, as in *N. crassa* (Singleton, 1953) and *S. fimicola* (Carr and Olive, 1958), the length of the ascus in *S. brevicollis* was a good indicator of the stage of meiosis which had been reached. The chromosomes, which gradually elongated during zygotene and pachytene (Fig. 4.5) became rather diffuse and difficult to distinguish at diplotene (Fig. 4.6), then contracted rapidly, so that at the beginning of metaphase I they appeared as tiny dots of highly condensed material (Fig. 4.7). Chromosome groups migrated to opposite spindle poles during anaphase I, and at telophase were typically separated by 10-15 μm (Fig. 4.8), although this distance increased as the ascus elongated prior to the second meiotic division. Partial spindle overlap was common during division II. At telophase II, daughter nuclei were visible as four groups of condensed chromosomes, which subsequently elongated at the onset of interphase (Fig. 4.9). Interphase chromosomes were tightly grouped together on the polar side of the nucleus (attached by their centromeres to spindle pole bodies according to Mu'Azu (1973)), while the free arms extended across the nucleus, so that each group of chromosomes resembled a small jelly fish. At the onset of division III (mitosis) the chromosome arms contracted (Fig. 4.10), and the nuclei divided across the width of the ascus (Fig. 4.11). During interphase following division III,



Figures 4.3 and 4.4: *Sordaria brevicollis*. Fluorescent staining of young asci from crossed perithecia. Figure 4.3: DAPI. Although nuclei in both asci and paraphyses fluoresced strongly, the resolution of individual chromosomes was often poor, and increasing the length of the staining period resulted in background staining of the ascus cytoplasm. Bar = 35 μm. **Figure 4.4:** Acriflavin. Acriflavin provided superior resolution of chromosomal material, with little or no staining of ascus cytoplasm. The longer of the two asci illustrated, is in interphase following division II, while the shorter ascus is still in prophase of division I. Several other large, diploid nuclei can be distinguished amongst the material at the base of the asci. The remaining, haploid nuclei belong to the paraphyses. Bar = 30 μm.

Figures 4.5 - 4.14: Nuclear behaviour during ascus development in *Sordaria brevicollis*. Various stages of meiotic and mitotic division are illustrated with acriflavin-stained material from young perithecia (36-48 hours after crossing). (For a diagrammatic representation of spindle orientation during ascus formation see Fig. 1.4.) At various stages of nuclear division, interpretation of acriflavin-stained material is made difficult by the fact that nucleoli and spindle plaques do not stain effectively. In these instances, where the length of the ascus has been used as a diagnostic tool, the alternative interpretation is noted in brackets. **Figure 4.5**: Pachytene. Bar = 10 μm. **Figure 4.6**: Diplotene. Chromosomes become increasingly diffuse during the latter stages of prophase I, so that individual structures are difficult to distinguish. Bar = 15 μm. Figs. 4.7 - 4.11 are at similar magnification, and scale bars are omitted. **Figure 4.7**: Metaphase I. At the end of prophase, the chromosomes contract rapidly, so that at the onset of metaphase I, only tiny dots of highly condensed material are visible. **Figure 4.8**: Telophase I (possibly metaphase II, though the chromosome groups are not well separated). Although stages of anaphase I were rarely observed (indicating that this phase was of short duration), asci in telophase were more frequent, with groups of condensed chromosomes separated by the length of the division I spindle. **Figure 4.9**: Interphase II. The ascus illustrated has undergone partial spindle overlap during division II. Elongated chromosomes are tightly grouped together at their centromeres, with free arms extending across the nucleus. Interphase II is a commonly observed stage, and is probably of relatively long duration. **Figure 4.10**: Metaphase III (possibly telophase II). At the end of meiosis the chromosome arms contract, and the four daughter nuclei enter mitosis. **Figure 4.11**: Telophase III. Mitotic spindles are orientated transversely in the ascus, resulting in the formation of two columns of four nuclei. In this case one of the spindles has been displaced slightly. **Figure 4.12**: Interphase III. All 8 nuclei are realigned into a single column, and spore delimitation commences. Bar = 20 μm. **Figure 4.13**: Metaphase-Anaphase IV. At the end of interphase the chromosomes contract and nuclei undergo a second mitosis within the delimited spore. Bar = 20 μm. **Figure 4.14**: Binucleate ascospores. Bar = 10 μm.

Figures 4.5 - 4.14



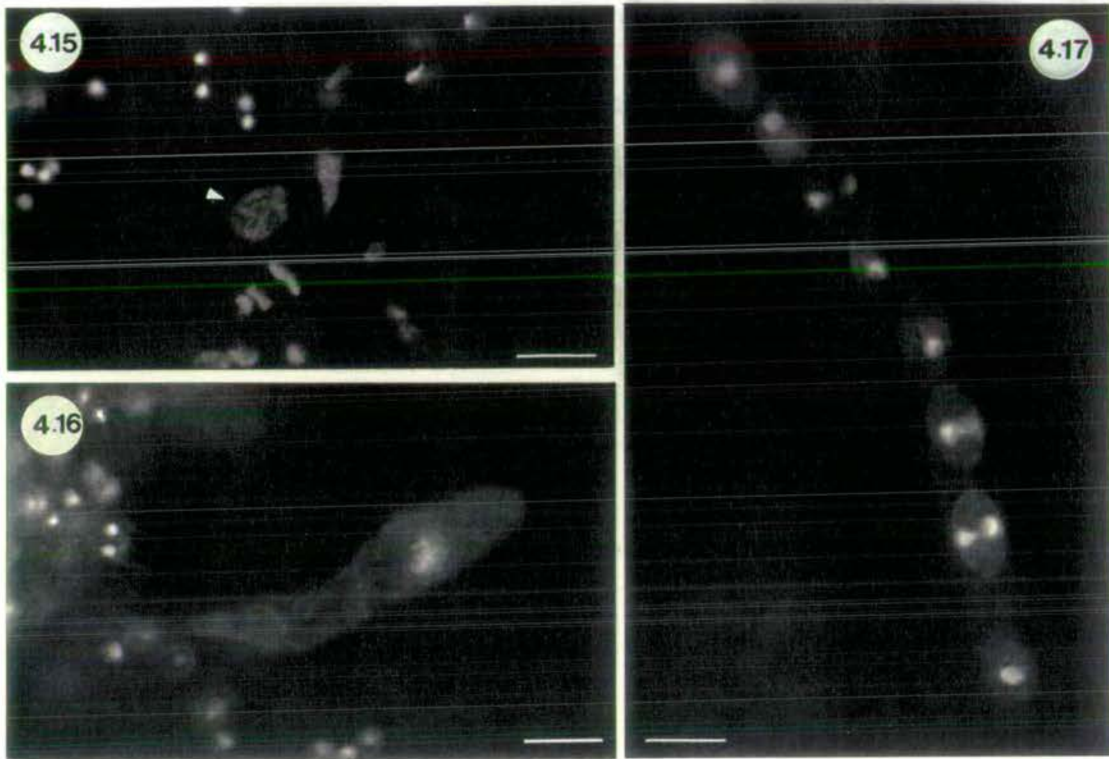
the daughter nuclei were realigned into a single column of eight, and spore delimitation commenced (Fig. 4.12). At the end of interphase, the chromosomes contracted once again (Fig. 4.13), and underwent a second mitotic division, across the width of the spore (Fig. 4.14)), so that spores were binucleate at the stage when pigmentation of the ascospore wall prevented any further observations.

4.2.1.3 Ascus cytology: uncrossed perithecia

Selecting the optimal time for staining proved rather difficult when dealing with uncrossed perithecia. After protoperithecial cultures were crossed, the range of developmental stages within each young perithecium could be predicted fairly accurately on the basis of the time which had elapsed since spermatisation. By 48-56 hours after crossing, some asci had already attained their full length and contained delimited spores, whilst numerous younger asci were also present. The rate of development of uncrossed perithecia proved less easy to judge, and as only one or two asci generally formed within each spore-bearing perithecium, the precise timing of staining was critical. The optimal time for examination of uncrossed cultures was typically 8 days after inoculation, but this could vary by more than 24 hours in either direction. In addition, spore-bearing perithecia typically accounted for around 10% of uncrossed perithecia in the wild type strains used for cytological analysis, so the great majority of the fruitbodies examined contained only multinucleate paraphyses.

As a consequence of these difficulties, the progress made with cytological observations of uncrossed perithecia was rather limited. Occasional nuclei which were far larger than those in the surrounding paraphyses were observed in uncrossed cultures (Fig. 4.15), and in young asci, single large nuclei were visible (Fig. 4.16). Intermediate developmental stages from early prophase to spore delimitation proved illusive, however, and where dividing nuclei were observed, they were often obscured by the strongly staining nuclei in the surrounding paraphyses. It was, however, possible to observe the later stages of ascospore formation, and to confirm that, as in crossed perithecia, spores were uninucleate when delimited, and that a second mitotic division took place across the shorter axis of the young spore, before pigmentation of the spore wall occurred (Fig. 4.17).

Observation of single large nuclei in young asci from uncrossed perithecia provided some evidence that nuclear fusion and meiosis had taken place during spore formation. As only a relatively small number of asci were examined,



Figures 4.15 - 4.17: *Sordaria brevicollis*. Ascus development in uncrossed perithecia. Figure 4.15: The nucleus in the centre of the frame (arrow) is larger than those in the surrounding paraphyses, and may be a diploid post-fusion nucleus. Bar = 15 μm. **Figure 4.16:** Young ascus from 10 day old, uncrossed perithecium. When compared with those from crossed perithecia, the length of this ascus suggests that the nucleus is probably in pachytene of prophase I. Only one nucleus is present within the ascus, indicating that if two nuclei contribute to the ascus, karyogamy has taken place. Bar = 15 μm. **Figure 4.17:** Division IV occurs in young ascospores, as in asci from crossed perithecia. Bar = 20 μm.

however, this finding could not be regarded as conclusive. Nor could any judgements be made regarding the nature of the nuclear divisions which took place prior to spore delimitation. Given adequate time, and the optimisation of additional staining procedures (in particular to allow visualisation of nuclear membranes, nucleoli, and spindle pole bodies), cytological observations might have provided further useful information concerning fruiting in unmated cultures. In the time available, however, heterokaryon analysis proved more productive.

4.2.2 Genetic analysis of homokaryotic fruiting using heterokaryons.

In crossed perithecia, asci normally show 2:2 segregation of all markers for which the parents differed, and in species such as *S. brevicollis* where spores are produced in ordered tetrads, certain aspects of nuclear division in the young ascus can be inferred from the final sequence of spores (section 1.5.1). Of particular interest is the presence of symmetrical M II asci (or tetratype asci when more than one gene is segregating) which are indicative that recombination, and therefore karyogamy and meiosis have taken place prior to spore formation. One of the first objectives of heterokaryon analysis was to establish whether similar spore sequences could be identified in asci from uncrossed perithecia.

In initial experiments carried out by D. J. Bond (pers. comm.), auxotrophic markers were used to force heterokaryons on minimal medium. No homokaryotic fruiting was observed in the isolated homokaryons, and although heterokaryons were subsequently transferred to corn meal agar to encourage fruiting, no uncrossed perithecia were observed.

In a second set of experiments, homokaryotic fruiting was monitored in *mtA* strains of a range of autonomous spore colour mutants. In buff (*c70*) and hyaline mutants, perithecia were formed in uncrossed cultures, but were never found to contain spores. In both mutants, the spore colour mutation was accompanied by abnormal pigmentation of the perithecial wall, and by atypical neck development (the necks being of similar shape to those of the hyaline mutant whose structure was illustrated in Chapter Three.) A yellow spore colour mutant (*S229*) produced abundant protoperithecia, but these did not develop into perithecia in unmated cultures. Although *S229* protoperithecia often became slightly enlarged, pigmented and sometimes ostiolate, they never differentiated necks and were never found to contain spores. A second yellow spore colour mutant (*S187*), however, exhibited homokaryotic spore production

at a frequency comparable to wild type isolates, and this mutant (in which perithecial pigmentation and neck formation were as wild type) was selected for further study.

4.2.2.1 Analysis of perithecia from *S187*/wild type heterokaryons

Heterokaryons between *S187* and wild type strains were established on CMA, without the use of forcing markers. Preliminary experiments indicated that the optimal time for perithecial analysis was 9-10 days after inoculation. In younger perithecia, many spores were immature and their colour could not be scored with conviction, whilst in older specimens, asci showed an increased tendency to breakdown, releasing individual spores. It is not known whether the latter phenomenon occurred inside intact perithecia or was simply a consequence of damage to the ascus wall during dissection. In addition, older uncrossed perithecia occasionally discharged a few spores, either onto the surrounding growth medium, or onto the Petri plate lid. Six heterokaryons which produced relatively numerous uncrossed perithecia were examined in detail, and these are documented in the following pages. The number attached to each heterokaryon remains constant throughout.

4.2.2.1.1 *Frequency of spore production in uncrossed perithecia*

All of the ostiolate perithecia were removed from each uncrossed heterokaryon, and the contents of each fruit body recorded. To allow rapid assessment of relatively large numbers of perithecia, this analysis was carried out at low magnification using a dissecting microscope, and in consequence it is possible that some young asci may have been overlooked. Estimates of the frequency of spore production may therefore be too low. Nevertheless, the percentage of spore-bearing perithecia in uncrossed heterokaryons was rather higher than that typical of the wild type homokaryons previously used for cytological analysis, and ranged from 15% (heterokaryon 2) to 29% (heterokaryon 6) (Table 4.1). None of the perithecia examined contained more than 9 asci, and fruitbodies containing 4 or more asci accounted on average for less than 2% of perithecia. The number of asci produced in uncrossed perithecia clearly did not conform to a normal distribution, but approximated more readily to a Poisson series, calculated from the formula $pN = \frac{e^{-m} m^N}{N!}$

where m is the mean number of items per sample, and N the number of samples examined. In this case m = 0.3627 (the total number of asci (1507 - calculated from the data in Table 4.1) divided by the total number of perithecia (4155)).

Table 4.1: Frequency of spore production in uncrossed perithecia. Perithecia are classified according to the number of asci which they contain: %[a] = percentage of all uncrossed perithecia for heterokaryon, %[b] = percentage of spore-bearing perithecia for heterokaryon (including those perithecia which contained only aborted asci). In each case, the distribution of spore-bearing perithecia shows that the great majority of fruitbodies contained only one or two asci. The distribution of spore-bearing perithecia for the combined heterokaryons is plotted in Fig. 4.18 and compared with a Poisson distribution calculated for the same data set.

Table 4.1

Heterokaryon	Perithecia examined	Perithecia with spores	Asci per perithecium									
			0	1	2	3	4	5	6	7	8	9
1	166	45	121	26	8	6	2	0	1	1	1	0
%[a]		27.11	72.89	15.66	4.82	3.61	1.20	0.00	0.60	0.60	0.60	0.00
%[b]				**57.78**	**17.78**	**13.33**	**4.44**	**0.00**	**2.22**	**2.22**	**2.22**	**0.00**
2	525	79	446	40	32	5	2	0	0	0	0	0
%[a]		15.05	84.95	7.62	6.10	0.95	0.38	0.00	0.00	0.00	0.00	0.00
%[b]				**50.63**	**40.51**	**6.33**	**2.53**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
3	911	149	762	81	41	19	4	2	1	0	0	1
%[a]		16.36	83.64	8.89	4.50	2.09	0.44	0.22	0.11	0.00	0.00	0.11
%[b]				**54.36**	**27.52**	**12.75**	**2.68**	**1.34**	**0.67**	**0.00**	**0.00**	**0.67**
4	695	110	585	54	29	15	5	4	1	0	1	1
%[a]		15.83	84.17	7.77	4.17	2.16	0.72	0.58	0.14	0.00	0.14	0.14
%[b]				**49.09**	**26.36**	**13.64**	**4.55**	**3.64**	**0.91**	**0.00**	**0.91**	**0.91**
5	1131	196	935	92	58	25	8	4	4	2	2	1
%[a]		17.33	82.67	8.13	5.13	2.21	0.71	0.35	0.35	0.18	0.18	0.09
%[b]				**46.94**	**29.59**	**12.76**	**4.08**	**2.04**	**2.04**	**1.02**	**1.02**	**0.51**
6	727	210	517	112	53	19	10	6	4	5	1	0
%[a]		28.89	71.11	15.41	7.29	2.61	1.38	0.83	0.56	0.69	0.14	0.00
%[b]				**53.33**	**25.24**	**9.05**	**4.76**	**2.86**	**1.90**	**2.38**	**0.48**	**0.00**
Total	4155	789	3366	405	221	89	31	16	11	8	5	3
%[a]		18.99	81.01	9.75	5.32	2.14	0.75	0.39	0.26	0.19	0.12	0.07
%[b]				**52.02**	**27.83**	**11.31**	**3.84**	**1.65**	**1.29**	**0.94**	**0.77**	**0.35**

The observed frequencies of perithecia containing between 0 and 5 asci are compared with the calculated Poisson series in Fig. 4.18. X^2 tests (for the method see Appendix 2) indicated that only the calculated and observed frequencies of perithecia containing one ascus were significantly different.

4.2.2.1.2 *Determination of nuclear ratios and observation of nuclear behaviour in crossed heterokaryons*

In order to determine its nuclear ratio, samples from each heterokaryon were used to fertilise buff (*c70*) cultures. When the heterokaryon was used as a male parent, two types of perithecia were produced: those derived from a black x buff cross and those from yellow x buff. In the case of heterokaryon 1 for example, 75/200 perithecia were of the former type and 125/200 of the latter, indicating a nuclear ratio of 37 black:63 yellow (Table 4.2). After the removal of all uncrossed perithecia, each heterokaryon was fertilised with buff microconidia and crossed perithecia were allowed to develop. For heterokaryon 1, 92/200 of these perithecia were found to be derived from a yellow x buff cross, and 67/200 from black x buff, with the remaining 41/200 containing a mixture of asci (both yellow x buff and black x buff)(Fig. 4.19). This illustrated that in at least 20% of perithecia, two ascogonial (female) nuclei contributed to the centrum. In fact the true figure was probably nearer 40%, as only those mixed perithecia where two genetically distinct ascogonial nuclei were involved have been detected by this analysis. If all perithecia had two female founder nuclei, then the nuclear ratio of 63:37 (obtained using the heterokaryon as a male parent) would predict a frequency of 46% mixed perithecia (calculated from the Hardy-Weinberg formula $p^2 + 2pq + q^2 = 1$, where $p = 0.63$, $q = 0.37$, and $2pq$ is the frequency of mixed perithecia). In fact the observed frequency of mixed perithecia was 41/200, less than half of that predicted, suggesting that not all perithecia had two founder nuclei.

Similar calculations for the other heterokaryons showed that the frequency of perithecia with more than one female founder might be expected to be approximately double the number of mixed centra detected where the proportions of black and yellow nuclei were relatively similar, and rather higher when nuclear ratios were more asymmetrical. The range of mixed perithecia detected by the method described varied from 10-30%, suggesting that the true proportion of perithecia with more than one female founder nucleus lay between 20-60%.

Figure 4.18

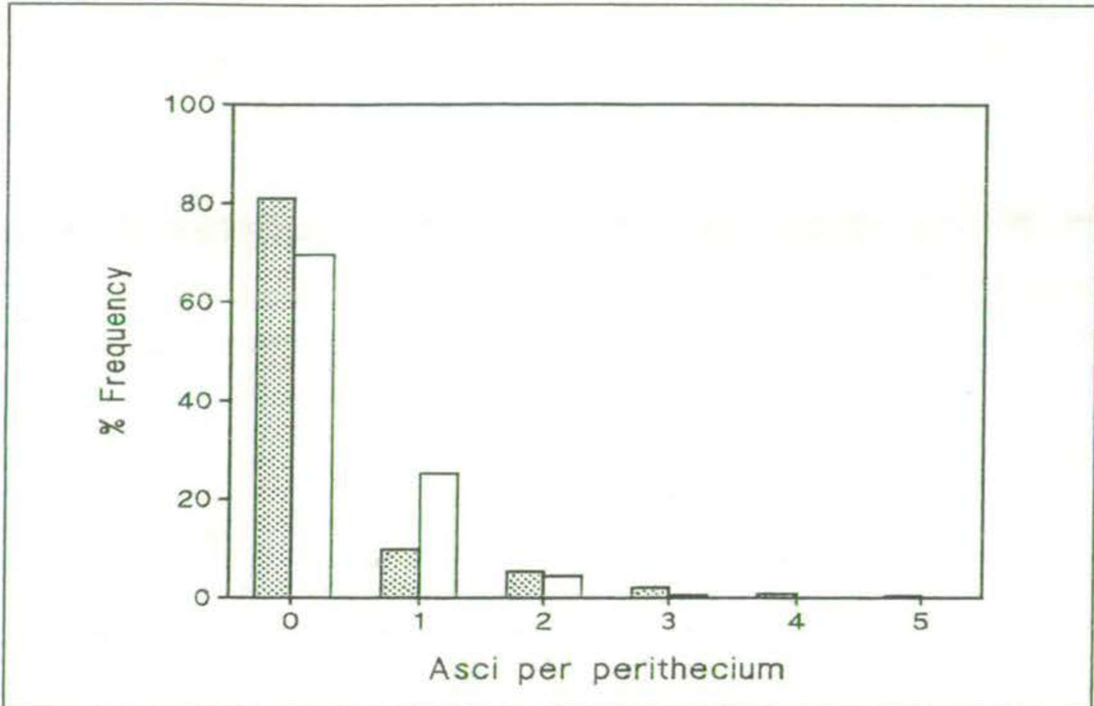


Figure 4.18: Distribution of asci in perithecia from uncrossed heterokaryons. Observed frequencies of uncrossed perithecia containing between 0 and 5 asci per fruitbody (Total %[a] in Table 4.1) are compared with the expected distribution for a Poisson series. Shaded bars are observed values, open bars calculated values. The calculations (see text for details) are based on the total number of asci (1507) and the total number of perithecia (4155) which can both be deduced from Table 4.1.

Het.	N.R.	Spore-bearing perithecia classified according to spore colour			
		Aborted spores	Black spores	Yellow spores	Black and yellow spores
1	37:63	11	4	13	17
%		(24.44)	11.76	38.24	50.00
2	38:62	8	17	18	36
%		(10.13)	23.94	25.35	50.70
3	49:51	9	31	36	73
%		(6.04)	22.14	25.71	52.14
4	43:57	2	22	38	48
%		(1.82)	20.37	35.19	44.44
5	16:84	22	5	131	38
%		(11.24)	2.87	75.29	21.84
6	61:39	6	87	40	77
%		(2.86)	42.65	19.61	37.75

Table 4.2: Spore-bearing uncrossed perithecia classified according to the colour of the spores which they contain. Het. = heterokaryon; N.R. = nuclear ratio (wild type (black) : mutant (yellow)). Aborted spores are included and shown (in brackets) as a percentage of total spore-bearing perithecia. Other percentages refer to spore-bearing perithecia minus those containing only aborted spores, so that % black + % yellow + % black and yellow = 100.

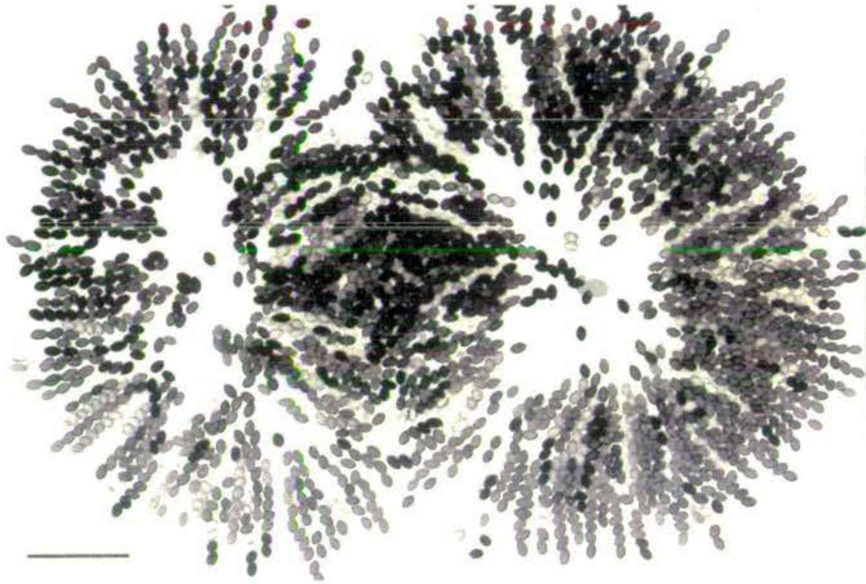


Figure 4.19: ***Sordaria brevicollis.*** **Mixed rosette from *S187*/wild type heterokaryon, crossed with buff (*c70*) microconidia.** The asci in the upper part of the rosette are derived predominantly from a black x buff cross, and those in the lower part predominantly from a yellow x buff cross. Bar = 100 μm.

Figure 4.20: ***Sordaria brevicollis.*** **Centrum from an uncrossed perithecium from an S187/wild type heterokaryon.** Of the 4 mature asci present, 3 contain only black spores, whilst a single ascus segregates for spore colour. This indicates that not all of the asci within each uncrossed perithecium are derived from a single pair of nuclei. Bar = 50 μm.

4.2.2.1.3 *Segregation of spore colour in uncrossed perithecia*

Spore-bearing perithecia from each uncrossed heterokaryon were classified in four groups according to the colour of the spores which they contained: aborted spores only, black only, yellow only, and black and yellow (Table 4.2). Perithecia which contained only aborted spores were scored as spore-bearing and included in Table 4.2, but omitted from subsequent analyses. Also omitted from later evaluation were perithecia which contained broken asci and could not be subject to precise classification. Neither the nuclear ratio nor the combination of strains paired in the heterokaryon appeared to affect the frequency of perithecia containing only aborted spores, which ranged from 2-25% (Table 4.2). It seems likely, however, that in some instances, spores which were scored as aborted in 9-10 day old perithecia may simply have been immature.

The proportion of perithecia containing solely black or yellow spores was clearly related to the nuclear ratio for each heterokaryon: in heterokaryon 5 for example, where the nuclear ratio was 16:84 in favour of the mutant yellow nuclei, less than 3% of scorable spore-bearing perithecia contained only black spores, whilst 75% contained only yellow spores. The proportion of perithecia containing both black and yellow spores was greatest when the nuclear ratio approached 50:50 (52% in heterokaryon 3), and declined with increasing asymmetry in the nuclear ratio (22% in heterokaryon 5). Amongst those perithecia which contained both black and yellow spores, a distinction could be made between perithecia which contained only segregating asci (with four black and four yellow spores) and perithecia containing a mixture of segregating and non-segregating asci (Fig. 4.20).

The total number of segregating asci from each heterokaryon is recorded in Table 4.3. Analysis of the various spore sequences observed in these asci revealed that, in addition to the three basic classes described previously (MI, asymmetrical and symmetrical M II), 3rd division overlap asci were present at frequencies ranging from 10% (heterokaryon 5) to 22% (heterokaryon 2). In asci of this type, the mitotic spindles of division III (usually orientated transversely or obliquely in the developing ascus) become displaced, and may result in the separation of identical spore pairs (Fig. 4.21). Also included in Table 4.3 is a comparable data set for crossed perithecia. Comparison of segregation patterns in crossed and uncrossed perithecia is complicated by an imbalance in the size of the 3rd division overlap category - for the combined heterokaryons this class represented 16% of the total segregating asci, but accounted for only 4% of

Heterokaryon	Segregating asci examined	Segregating asci according to spore sequence			
		MI	Asymmetrical MII	Symmetrical MII	3rd div^n overlap
1	24	8	11	2	3
%	(46.15)	33.33	45.83	8.33	12.50
2	55	25	12	6	12
%	(50.93)	45.45	21.82	10.91	21.82
3	109	63	25	7	14
%	(48.66)	57.80	22.94	6.42	12.84
4	72	33	17	8	14
%	(38.83)	45.83	23.61	11.11	19.44
5	59	28	17	8	6
%	(17.82)	47.46	28.81	13.56	10.17
6	137	55	52	8	22
%	(39.09)	40.15	37.96	5.84	16.06
Total	456	212	134	39	71
%		46.49	29.39	8.55	15.57

Crossed perithecia

Total	570	243	218	87	22
%		42.63	38.25	15.26	3.86

Table 4.3: Classification of segregating asci according to spore sequence. The frequency of segregating asci detected within all of the uncrossed perithecia on each heterokaryon is shown as a percentage (in brackets) of the total scorable asci for that heterokaryon. At the foot of the table, a comparable data set for crossed perithecia (wild type x *S187*) is included. The sizes of the third division overlap classes in crossed and uncrossed perithecia are significantly different (see Appendix 2 for statistical analysis), and since these asci are all derived from one of the other classes, they must be reallocated before the true frequencies of each of the basic ascus types can be determined. The method used for this reallocation is also presented in Appendix 2.

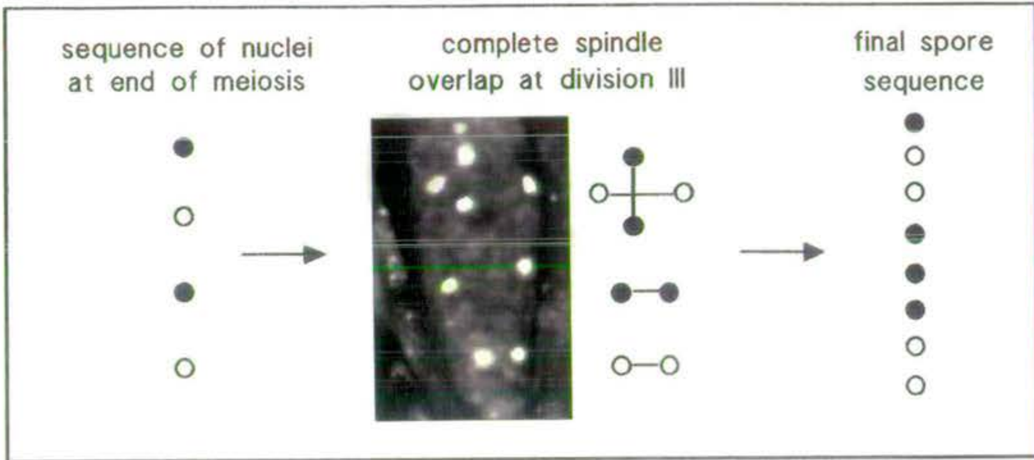


Figure 4.21: Division III spindle overlap in *Sordaria brevicollis*. Possible interpretation of arrangement of nuclei in a telophase III ascus from a crossed perithecium (stained with DAPI). The spindle of the top pair of nuclei is orientated longitudinally rather than transversely, resulting in complete spindle overlap at division III.

Figure 4.22: Tetratype ascus from an uncrossed perithecium from an *S187*/*S229* heterokaryon. Tetratype asci were indicative of crossing over in the y^9-centromere interval. Bar = 40 μm.

segregating asci from crossed perithecia. (A X^2 test indicated that this difference was highly significant - see Appendix 2.) As 3rd division overlap asci were all derived from one of the other three classes, it was necessary to reallocate these asci as accurately as possible before a true assessment of nuclear behaviour during spore development could be made. This reallocation, and the statistical analysis of corrected figures is also detailed in Appendix 2. The analysis showed that in excess of 70% of 3rd division overlap asci from heterokaryons were derived from the asymmetrical M II class, and around 27% from the MI class.

The presence of symmetrical M II asci within uncrossed perithecia from heterokaryons provided evidence that meiosis and recombination had taken place. The mean frequency of recombinant asci was only 9% (± 1), however, which was significantly lower than the 15% observed in crossed perithecia. (The latter figure corresponds well to the value of 16 map units quoted by MacDonald and Bond (1974) as the distance between the y^9 locus and its centromere.) The low recombination frequency apparent in uncrossed perithecia suggested that some aspects of nuclear division in such fruitbodies might be abnormal. As explained previously, complete spindle overlap at division II (which could theoretically give rise to symmetrical M II asci in the absence of recombination) is believed to occur at only a very low frequency in crossed perithecia of *S. brevicollis* (Bond, 1969). In order to eliminate the possibility that such overlap gave rise to all the symmetrical M II asci observed in uncrossed perithecia, two further experiments were carried out, using heterokaryons involving a second yellow spore colour mutant.

4.2.2.2 Analysis of perithecia from *S229*/*S187* heterokaryons

Heterokaryons were established between two yellow spore-colour mutants, *S229* and *S187*. Although *S229* did not exhibit homokaryotic fruiting when in isolation, its nuclei contributed to hybrid perithecia on uncrossed heterokaryons. The y^4 locus, of which *S229* is an allele, is tightly linked to its centromere and does not recombine.

Uncrossed perithecia from heterokaryons were analysed as described previously. Although relatively few spore bearing perithecia were formed, three types of segregating asci were recorded: parental ditype, non parental ditype, and tetratype. Tetratype asci (Fig. 4.22) were indicative of recombination in the y^9 centromere interval and could not have been produced as a result of spindle overlap. Hence the presence of tetratype asci demonstrated that meiosis and recombination occurred during the formation of asci in uncrossed perithecia.

4.2.2.3 Analysis of perithecia from *S229*/wild type heterokaryons

Uncrossed perithecia from *S229*/wild type heterokaryons were found to contain 3 types of asci, MI, asymmetrical M II and 3rd division overlap. Those in the last category were all derived from the asymmetrical M II class: no symmetrical M II type asci were observed. (Any asci of this type produced would have been the result of complete spindle overlap at division II, as no recombination of the y^4 locus occurs.) These results confirmed that meiosis and recombination, not abnormal spindle overlap, were responsible for most, if not all of the symmetrical M II asci detailed in Table 4.2.

4.2.2.4 Mating type of spores from uncrossed perithecia

Asci from wild type, uncrossed perithecia and segregating asci from heterokaryons were dissected, and the spores germinated on acetate agar. Although germination rates were often poor, mating type tests revealed that all viable mycelia were of *mtA*. In 8 asci 100% germination was achieved. When viewed under the microscope, many spores from uncrossed perithecia appeared concavo-convex, and it is possible that the spore wall was not fully developed. Yellow spores were typically more difficult to germinate than wild type, and this may also have reflected the incomplete development of the ascospore wall in these mutants (section 1.4.2.5).

4.2.3 Effect of environmental factors on homothallic fruiting

During examination of the cytology and genetics of homothallic fruiting in *S. brevicollis*, it became obvious that several environmental factors influenced the number of uncrossed perithecia which were produced. It was noted in particular, that unmated cultures grown in constant light produced only a few fruitbodies, and that these very rarely contained ascospores. The effects of various environmental factors on multicellular development are examined in some detail in Chapter Five, but the effect of light on homothallic fruiting will be considered briefly here, as it appears that the development of the fertile tissues within the perithecium may be affected.

Petri plates containing CMA (both with and without cellophane membranes) were inoculated with a strain of mtA, and incubated either in the light or in the dark for 4 days. Four day old plates were crossed, then incubated for a further 6 days to allow perithecial maturation. Perithecial frequency was determined in 10 day old cultures, and fruitbodies were split open to confirm that they contained asci. In a second experiment, the frequency of perithecia in 10 day

old, uncrossed cultures was determined, after incubation under the same light regimes. All uncrossed perithecia were split open and their contents examined under the dissecting microscope. The results presented in Fig. 4.23 show that in most respects the factors which influenced the frequencies of crossed and uncrossed perithecia were similar - the production of both types of fruitbody was optimal when cultures were grown in complete darkness, and was enhanced by growth on cellophane membranes. In one important respect however, the development of uncrossed perithecia was distinctive. Irrespective of their frequency, crossed perithecia always contained numerous asci, whilst in uncrossed cultures, the proportion of perithecia which contained spores varied from 0.0 - 14.0% (Fig. 4.23). It should be noted however, that because the number of uncrossed perithecia on light grown plates was low, the frequency of perithecia containing spores was calculated from a relatively small sample, which may not have been representative.

A second factor observed to influence the frequency of homokaryotic perithecia, namely the concentration of the spermatial suspension used to cross protoperithecial plates, is examined in Chapter Five.

4.2.4 Artificial stimulation of homothallic fruiting

A chloroform extract from mated wild type cultures of *S. brevicollis* was prepared as described in Chapter Two. The extract was tested on several strains of both mating types, over a period 2-8 days after the inoculation of cultures. Eight days after the addition of the extract, the number of uncrossed perithecia per plate was determined, and compared with control cultures. In no instance was any detectable response to the extract observed: homothallic fruiting was not stimulated in *mta* cultures, nor were significant numbers of additional perithecia produced in *mtA* cultures (data not shown). Homothallic perithecia from *mtA* cultures treated with the extract showed no increase in the frequency of spore production. Although cultures which were 2-4 days old at the time the extract was added, showed a very slight increase in perithecial production around the edge of the filter paper disc, this effect was also seen in control plates (to which filter paper discs without extract had been added)(data not shown). The observed response may have been attributable to an additional carbon source provided by the paper disc, or may have resulted from an "edge effect" when growing hyphae encountered an obstacle in their path. No evidence was obtained which suggested that an extract from mated cultures could increase the level of homokaryotic fruiting in *S. brevicollis*.

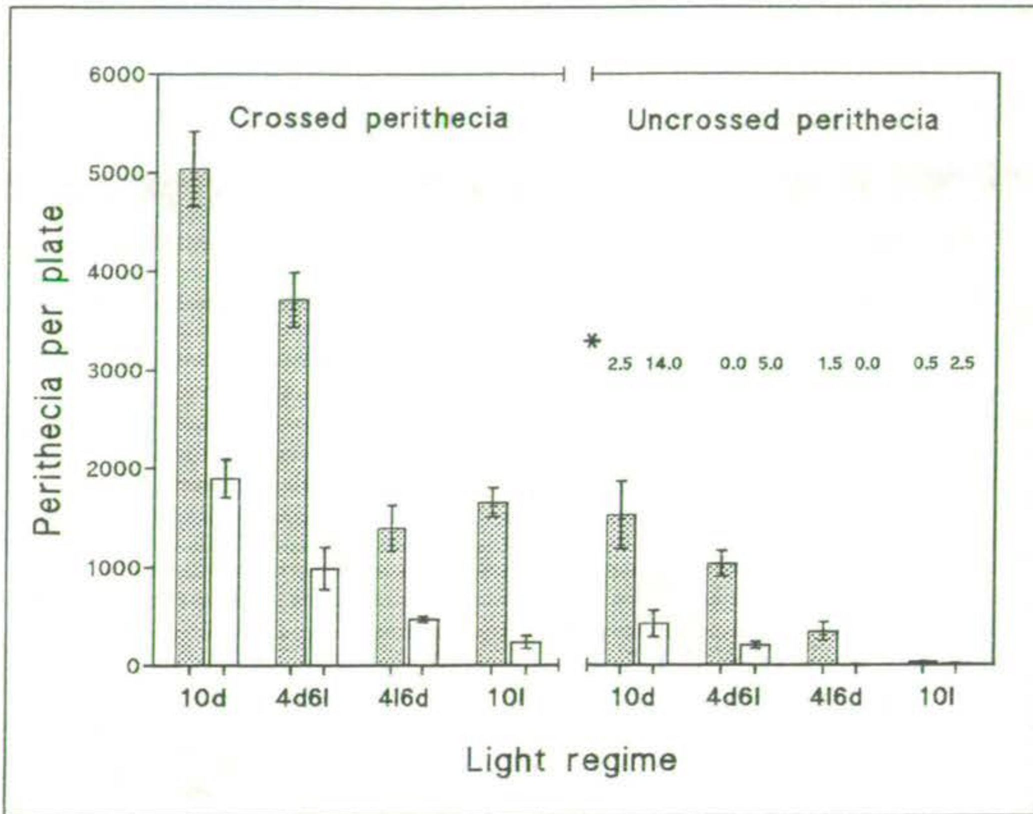


Figure 4.23: Effect of light and cellophane membranes on the frequency of crossed and uncrossed perithecia in *Sordaria brevicollis* (*mtA*). Perithecial numbers were determined in 10 day old cultures, which had been exposed to the light regimes detailed on the X axis: 10d = 10 days dark; 4d6l = 4 days dark, followed by 6 days light, and so on. Protoperithecia were crossed by spermatisation 4 days after inoculation. Shaded bars represent cultures grown on CMA overlaid with cellophane membranes: unshaded bars represent cultures grown directly on CMA. Each bar represents the mean for 8 replicate plates ± standard error. All crossed perithecia contained numerous asci, irrespective of light regime or the presence of cellophane membranes. The percentage of spore-bearing uncrossed perithecia for each treatment is noted on the figure (*).

4.3 Discussion

4.3.1 Observations on homothallic fruiting in *S. brevicollis*

Segregation of spore colour markers in tetratype asci from uncrossed heterokaryons indicated that nuclear fusion and recombination of genetic markers had taken place during spore formation. The possibility that spores were produced after a series of mitotic divisions (as in the apomictic ascomycete *Podospora arizonensis*, or during monokaryotic fruiting in certain basidiomycete species - see section 4.1) was thus eliminated. All of the spores germinated from uncrossed perithecia gave mycelia of *mtA*, indicating that no mating type switching had taken place. Mating type switching has often been observed in yeasts (e.g. Herskowitz, 1988) and has also been reported in several filamentous ascomycetes (see Perkins, 1987 and references therein), although the mating type loci in species such as *N. crassa* and *S. brevicollis* are apparently very stable. Like *N. crassa*, each genome of *S. brevicollis* contains only one copy of the mating type sequence, so there are no silent copies to facilitate switching (Glass *et al*, 1988; D. J. Bond, unpublished; C. Cisar, pers. comm.). Although homothallic spore production was never seen in *mta* strains of *S. brevicollis*, the mutation(s) which allow spore production are not necessarily part of the mating type sequence, as not all *mtA* isolates were found to produce spores. It is also possible that the expression of homothallic fruiting may be inhibited by the presence of other genes: neither the auxotrophic mutants nor spore colour mutants examined (with the exception of *S187*) showed evidence of spore production in uncrossed cultures.

The discovery that only *mtA* spores developed in homothallic perithecia in *S. brevicollis* also eliminated the possibility that self-fertility followed a similar pattern to that observed by Martin (1969) in *N. crassa.* A cross between two auxotrophic mutants of *N. crassa* yielded numerous aborted asci and a small number of viable spores, which germinated to produce self fertile mycelia (Martin, 1969). This phenomenon was attributed to non-disjunction during meiosis, resulting in the formation ascospores which were disomic for the chromosome containing the mating type locus. Spores carrying both *mtA* and *mta* sequences produced mycelia which were temporarily self fertile, but which reverted to the haploid state after several subculturings. Amongst the progeny of self fertile individuals, normal 1:1 segregation of mating type occurred.

Although spore production in uncrossed cultures of *S. brevicollis* apparently took place in a manner similar to that in homothallic species of *Sordaria* or *Neurospora* (e.g. Raju, 1978), there were several notable differences.

Fewer than 30% of uncrossed perithecia in *S. brevicollis* contained ascospores, and those which did develop spores produced relatively few asci. It is not known whether all the uncrossed perithecia contained ascogenous hyphae, and if so, why less than a third of perithecia developed spores. In general, those uncrossed perithecia which most closely resembled crossed perithecia in pigmentation and neck development were most likely to be spore-bearing, suggesting that the development of fertile and sterile parts of the perithecium was connected. Such a link is supported by evidence from examination of a series of "barren" mutants of *N. crassa* (Raju and Perkins, 1978). These mutants resulted from a range of unbalanced duplications, chromosome rearrangements, dominant and recessive genes, and exhibited cytological development which was blocked at various points between crozier formation and spore maturation. Those mutants whose development was blocked at an early stage produced rudimentary necks or no necks at all, and the authors concluded that, either neck development was induced by ascus development, or that both processes shared a common trigger. The correlation between neck development and spore maturation is not universal, however: a mutant (*m*) of *S. macrospora* for example, produced perithecia with rudimentary, non-ostiolate necks, although ascospore formation was normal (N. D. Read and K. M. Lord, in preparation).

The segregation patterns observed in asci from uncrossed heterokaryons deviated somewhat from the frequencies seen in crossed perithecia, and the recombination frequency was significantly lower than expected. This may indicate that the details of chromosome pairing in homothallic perithecia are abnormal in some respects, at least in the case of chromosome II, on which the y^9 locus is situated. Also of note was an unusually high frequency of asci exhibiting 3rd division spindle overlap. This ranged from 10-22% in uncrossed heterokaryons (16% for all heterokaryons combined), compared with 4% in crossed perithecia. The observed frequency of 3rd division overlap is increased when a correction is made to allow for overlaps which passed undetected. Partial overlap in MI asci was detected only when the central pair of nuclei were involved (one in three potential overlaps). In contrast, all partial overlaps in asymmetrical M II asci were detected, and two out of three in the symmetrical M II class. The apparent excess of 3rd division overlap asci which were derived from the asymmetrical M II class in perithecia from uncrossed heterokaryons (section 4.2.2.1.3 and Appendix 2) is therefore explained. For the combined heterokaryons, over 70% of 3rd division overlap asci were traced back to the asymmetrical M II class, and only 27% to the MI class, a ratio of approximately

3:1. After correction for undetected overlap, it is estimated that an average of 25% of the asci from uncrossed heterokaryons undergo 3rd division spindle overlap, compared with 7% from crossed perithecia.

Division III overlap in *S. brevicollis* was observed by Berg (1966) at frequencies from 2-5%, and was attributed in part to the asynchronous nature of the first mitotic division within each tetrad, although no evidence for such asynchrony was observed by Mu'Azu (1973). The same phenomenon has also been reported in crossed perithecia from *N. crassa* on several occasions, at a frequency comparable to that detected during the present study (Howe, 1956; Mitchell, 1959, 1960).

There are several potential explanations for the abnormally high level of 3rd division overlap observed in uncrossed perithecia:-

(i) In crossed perithecia, the shape of young asci is maintained in part by pressure exerted by the developing centrum. In homothallic perithecia the central cavity is largely filled by paraphyses which are unlikely to exert a similar force, and the walls of the young asci may bulge slightly. The ascus might therefore be rather shorter and wider than normal, facilitating spindle overlap. This hypothesis would also predict a similar elevation in the level of 2nd division overlap in uncrossed perithecia however, and this was not observed (see Appendix 2).

(ii) Alternatively, there may be abnormalities in the networks of cortical microtubules, which are involved not only in spindle orientation, but also in the maintenance of cell shape (Thompson-Coffe and Zickler (1992), and references therein). These authors examined the activity of microtubule organising centres (MTOCs) in mutant strains of *S. macrospora* which produced abnormally wide asci. They discovered that such mutants often lacked particular MTOCs, and exhibited abnormal patterns in the microtubule network.

(iii) It is also possible that division III "overlap" results, at least in part, from irregularities in the realignment of nuclei into a single file after mitosis.

(iv) The timing of ascus elongation may also be instrumental in generating 3rd division overlap. Mu'Azu (1973) noted that the tendency for spindle overlap at division II was affected by the degree of separation of nuclei at the end of division I, which itself depended upon the onset of meiosis relative to elongation of the ascus. Abnormally late enlargement of asci in homothallic perithecia might account for the high levels of spindle overlap observed at division III, although again this increase is not observed at division II.

When considering the effects of environmental influences such as light on perithecial development, it is important to appreciate that the formation of the sterile parts of the perithecium and the development of the fertile tissues may be affected independently, or may interact. It is equally important to remember that the timing of exposure may be critical (see Chapter Five). Illumination caused a reduction in the frequencies of both crossed and uncrossed perithecia, with the most pronounced effect caused by exposure to light during the first 4 days of growth (Fig. 4.23). Illumination during perithecial maturation (between 4 and 10 days after inoculation) also reduced the number of perithecia which developed in crossed and uncrossed cultures, although this reduction was less marked. Irrespective of the light regime, crossed perithecia contained numerous asci, and illumination had no apparent inhibitory affect on the development of the fertile tissues. Homokaryotic spore production was more sensitive to light, however. In the dark, on CMA without cellophane membranes, 14% of uncrossed perithecia contained asci (approximately 60 perithecia per 8.5 cm Petri plate): exposure to light during the first 4 days of growth reduced this figure to zero, and exposure during the latter 6 day period resulted in the formation of only 10 spore-bearing perithecia per plate (5%). The interpretation of these results is complicated by the interaction of two factors - the effect of light on the formation of uncrossed perithecia, and the effect of light on the development of the fertile tissues. Growth on cellophane membranes introduces a third factor, and clearly causes an increase in the number of uncrossed fruitbodies, without stimulating spore production. In fact the total number of spore bearing perithecia fell from 60 per plate (10 d without membranes) to 38 per plate (10 d with membranes). Although these figures are significantly different ($p \leq 0.05$), it seems likely that the frequency of ascus production remains relatively constant, irrespective of the number of perithecia formed on the mycelium. The numbers of spore-bearing perithecia formed under other light treatments are too small to draw sensible conclusions, and the experiment would need to be repeated with far greater numbers of replicate plates to produce improved data. Nevertheless, these preliminary results allow the formulation of the following hypothesis. The production of a homokaryotic ascus is determined by a random genetic event, the frequency of which is independent of the frequency of protoperithecial formation. Although the factors which are conducive to such events are unknown, illumination is inhibitory. The expression of homokaryotic fruiting is clearly dependent on the production of perithecia, however, so factors reducing

the frequency protoperithecia and perithecia will also reduce the observed frequency of spore formation.

4.3.2 Nuclear behaviour during protoperithecial formation

4.3.2.1 Observations based on crossed perithecia

When an *S187*/wild type heterokaryon was used as a female parent in a conventional cross with buff (*c70*) microconidia, 10-30% of the perithecia which matured contained more than one type of ascus, indicating that more than one "female" nucleus was involved in the formation of ascogenous hyphae in these perithecia. As explained previously, correction for instances where two founder nuclei were of the same genotype and thus passed undetected, suggested that up to 60% of crossed perithecia may have had at least two "female" contributors. The contribution of more than one female nucleus to a rosette of asci has been observed on several occasions in fertilised heterokaryons of *N. crassa* (Sansome, 1947; Radford and Threlkeld, 1970; Johnson 1977) where between 18% and 50% of perithecia were found to contain more than one female nucleus. Although some of these observations were based on relatively small samples of perithecia, (only eight specimens were examined by Sansome) they indicate that in a variable proportion of crossed perithecia, ascospores are derived from more than one female nucleus. The average number of such "founder" nuclei must be low however, otherwise every perithecium would be expected to contain asci of more than one type (see later).

When heterokaryons were used as male parents, mixed perithecia were much less frequent (under 1%), indicating either that protoperithecia were rarely fertilised by more than one microconidium, or possibly that rare microconidia contained more than one nucleus. Similar studies on *N. crassa* indicated that more than one male nuclear type was present in 2% (Nakamura and Egashira, 1961), 5% (Johnson, 1979) and 22% (Weijer and Dowding, 1960) of perithecia. Fertilisation of protoperithecia by more than one male nucleus has also been reported (at a frequency of 27%) in *Endothia parasitica* by Anagnostakis (1982).

4.3.2.2 Observations based on uncrossed perithecia

The frequencies of perithecia containing only black spores, only yellow spores, or both black and yellow spores were closely related to the nuclear ratio for each heterokaryon (see Table 4.2), and suggested that in general, a relatively small number of nuclei contributed to each perithecium. In most instances, the results approximated to those expected if two nuclei were the parents of all asci

within each centrum (calculated from the Hardy-Weinberg formula). The expected frequencies of each type of perithecium are shown in Table 4.4 and should be compared with the observed frequencies in Table 4.2. If a much larger number of nuclei founded each centrum, the proportion of perithecia containing only a single spore colour would be expected to fall, whilst the number containing both black and yellow spores would rise substantially (Table 4.5)). More detailed analysis of perithecia containing black and yellow spores showed, however, that a simple model with two nuclei founding *each* perithecium could not operate.

In Table 4.6, spore-bearing perithecia from uncrossed heterokaryons are classified according to ascus frequency and spore colour. Those perithecia classed as "mixed" contained asci of two types, both segregating and non-segregating (Fig. 4.20), and their presence showed that two different pairs of nuclei had given rise to asci. Although these perithecia were detected at a relatively low frequency (in only 34/714 scorable, spore-bearing fruitbodies from the combined heterokaryons), it should be remembered that over 50% of perithecia contained only one ascus. When expressed as a proportion of perithecia with two or more asci, mixed perithecia totalled 34/307, approximately 11%.

Two possible models for nuclear behaviour during homothallic development in *S. brevicollis* were considered:-

Model 1: Each rosette of asci was founded by a small number of "female" nuclei and one "male" nucleus from the ascogonium. In this model, one male nucleus is the "father" of all the asci within a perithecium, irrespective of the number of female nuclei (Fig. 4.24 a,b).

Model 2: Each rosette of asci was founded by a small number of nuclear pairs from the ascogonium. In this model, those ascus rosettes which are founded by more than one pair of nuclei have an equal number of male and female nuclei (Fig. 4.25).

The concept of male and female nuclei is not normally applied to homothallic development, and none of the nuclei are "male" in the conventional sense. All of the nuclei involved in homothallic spore production in *S. brevicollis* were of the same mating type, and in an unfertilised homokaryons were genetically identical. Amongst all of the literature concerning sexual development in fungi, there is little information on how nuclear pairing may be

Table 4.4: Expected frequencies of each type of uncrossed perithecium, based on the nuclear ratio for each heterokaryon, assuming 2 "founder" nuclei. Frequencies are calculated using the Hardy-Weinberg formula ($p^2 + 2pq + q^2 = 1$, where p is the proportion of wild type nuclei, and q the proportion of mutant nuclei).

Table 4.5: Expected frequencies of each type of uncrossed perithecium, based on the nuclear ratio for each heterokaryon, with up to 10 "founder" nuclei. The effect of increasing the number of nuclei founding a perithecium is illustrated using heterokaryons 3 and 5. (With 3 founder nuclei for example, the Hardy-Weinberg formula is expanded to $p^3 + 3p^2q + 3pq^2 + q^3 = 1$.) It is evident that if a large number of nuclei founded each perithecium, the chances of obtaining "all black" or "all yellow" centra would be very low.

Table 4.4

Heterokaryon.	Nuclear ratio	Expected frequencies of various perithecial types calculated from nuclear ratios, assuming two "founder"nuclei		
		Black spores	Yellow spores	Black and yellow spores
1	37:63	13.69	39.69	46.62
2	38:62	14.44	38.44	47.12
3	49:51	24.01	26.01	49.98
4	43:57	18.49	32.49	49.09
5	16:84	2.56	70.56	26.88
6	61:39	37.21	15.21	47.58

Table 4.5

	Number of nuclei	Expected frequencies of various perithecial types based on nuclear ratio and number of "founder" nuclei		
		Black spores	Yellow spores	Black and yellow spores
Heterokaryon 3				
	2	24.01	26.01	49.98
	3	11.76	13.27	74.97
	4	5.76	6.77	87.48
	5	2.82	3.45	93.73
	6	1.38	1.76	96.86
	7	0.68	0.90	98.42
	8	0.33	0.46	99.21
	9	0.16	0.23	99.61
	10	0.08	0.12	99.80
Heterokaryon 5				
	2	2.56	70.56	26.88
	3	0.41	59.27	40.32
	4	0.07	49.79	50.14
	5	0.01	41.82	58.17
	6	0.00	35.13	64.87
	7	0.00	29.51	70.49
	8	0.00	24.79	75.21
	9	0.00	20.82	79.18
	10	0.00	17.49	82.51

Table 4.6: Detailed analysis of spore-bearing uncrossed perithecia according to number of asci, and spore colour. Bn = number of perithecia with n asci containing only black spores; Yn = number of perithecia with n asci containing only yellow spores; Sn = number of perithecia with n segregating asci; BSn, YSn, BY = number of mixed perithecia containing more than one ascus type (black/segregating, yellow/segregating and black/yellow repectively). These values correspond to the totals for each type of perithecium in Table 4.2, with the exception of several perithecia containing broken asci, which are omitted here because they cannot be classified accurately.

Table 4.6

Het.	Perithecial contents																															
	Black						Yellow									Segregating							Mixed										
	B1	B2	B3	B4	B5	B6	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	S1	S2	S3	S4	S5	S6	S7	BS2	BS3	BS4	BS6	YS2	YS3	YS4	YS6	YS8	BY	T
1	4	0	0	0	0	0	10	3	0	0	0	0	0	0	0	9	2	1	1	0	0	0	0	0	0	0	1	0	1	1	0	0	31
2	10	7	0	0	0	0	14	3	1	0	0	0	0	0	0	19	7	3	2	0	0	0	2	1	0	0	2	0	0	0	0	0	71
3	22	4	4	0	1	0	23	8	2	2	1	0	0	0	0	40	15	8	0	0	0	0	2	0	1	0	1	3	0	1	0	0	138
4	14	6	1	1	0	0	19	11	4	4	0	0	0	0	0	22	10	5	0	2	0	0	0	0	1	0	2	1	0	0	1	0	104
5	3	2	0	0	0	0	66	38	17	2	4	1	1	1	1	16	6	3	1	0	1	0	0	0	0	0	3	4	2	0	0	0	172
6	61	14	7	3	1	1	23	9	4	1	2	0	1	0	0	32	20	7	3	3	1	1	1	1	0	1	1	0	0	0	0	0	198

Figure 4.24

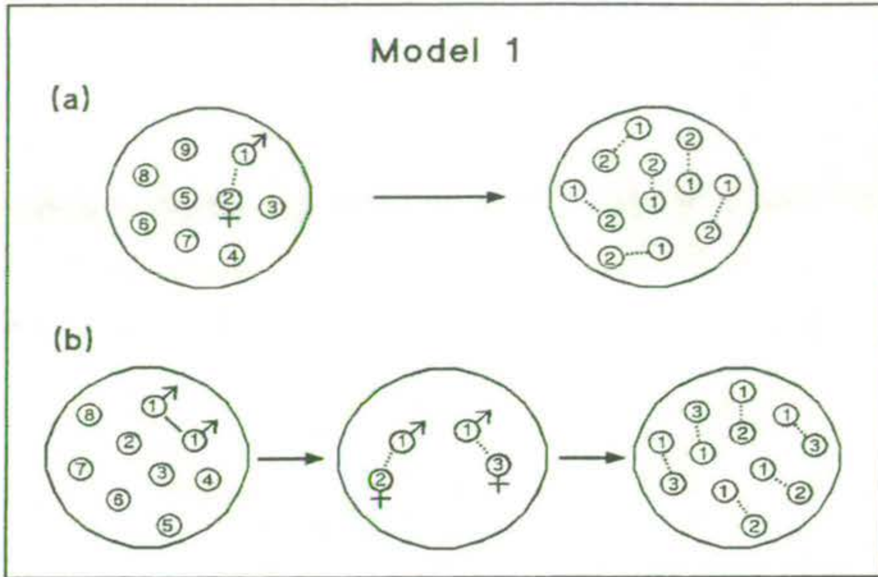
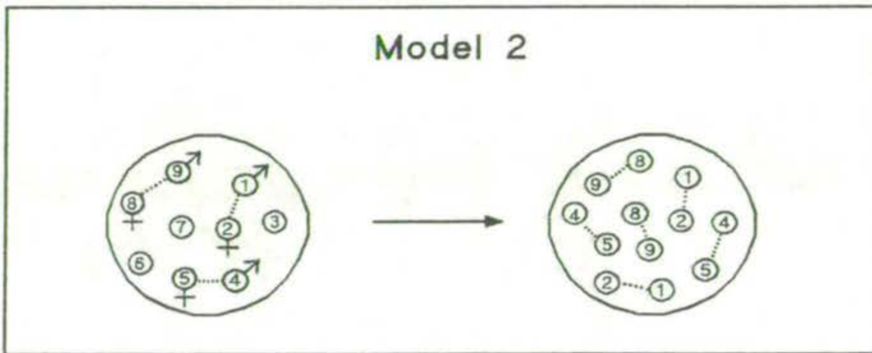


Figure 4.25



Figures 4.24 and 4.25: Models for nuclear behaviour during homothallic development in *Sordaria brevicollis*. A different number is used to denote each nucleus within the ascogonium prior to pairing, although this does not imply that all nuclei are genetically distinct. Paired nuclei are linked by a dotted line. **Figure 4.24:** Model 1 (a): One nucleus assumes a "male" function, and pairs with a single "female" nucleus. This pair of nuclei passes through several conjugate nuclear divisions and gives rise to all asci within the perithecium. Model 1 (b): One nucleus assumes a male function, and undergoes a single mitotic division. The two products of this division then pair with two different female nuclei, so that one nucleus is the "father" of all asci within the perithecium, although two female nuclei are involved. **Figure 4.25:** Model 2: Several nuclei assume male functions, and pair with a number of female nuclei. Various pairs of male and female nuclei may then give rise to asci.

achieved or maintained, particularly during homothallic spore development. Whilst it is convenient to call one of the ascogonial nuclei "male" this can lead to confusion when attempting to compare homothallic and heterothallic development and is probably more accurate simply to refer to ascogonial nuclei as being "receptive". Nevertheless, model 1 (b) requires that a single nucleus is somehow distinguished from the other nuclei within the ascogonium, dividing once and then pairing with two different partners. The mechanism by which this might be achieved is unknown.

Amongst perithecia from unfertilised heterokaryons, the frequency of fruitbodies containing both black and yellow spores never substantially exceeded 50%, suggesting that model 2 did not operate, and further evidence to this effect was obtained from inspection of the data concerning mixed perithecia. In model 1, all the asci within a perithecium have one nucleus in common, and perithecia containing asci with 8 black spores and asci with 8 yellow spores are not expected. Model 2 however, predicts the occurrence of such perithecia. Table 4.6 shows that no such perithecia were ever observed, although as already noted, the number of homothallically-derived perithecia with two or more asci was relatively low, and black/yellow perithecia might have been missed. Using the nuclear ratios for each heterokaryon (Table 4.2), the relative probability for the three types of mixed perithecia (black and segregating, yellow and segregating, and yellow and black) under models 1 and 2 were calculated, and the relative likelihood of the two models was calculated for the data in Table 4.6. This revealed that model 1 was 70 times more likely than model 2. Application of standard maximum likelihood techniques (Weir, 1990) to the same data showed that the best fit was obtained with 60% of perithecia having two founder nuclei (one female and one male) (model 1 (a)), and 40% having three founder nuclei (two female and one male)(model 1 (b)). The standard error of this estimate was 6.5%.

A third line of evidence in support of model 1 was obtained from the distribution of ascus types in mixed perithecia from crossed heterokaryons. Figure 4.19 illustrates such a perithecium, and shows that the different ascospore phenotypes were typically clumped into two distinct regions within the centrum. If such perithecia were produced as suggested in model 2, from several pairs of nuclei, then the two ascus types might be expected to be more evenly distributed. A similar conclusion was reached by Johnson (1977) in "mosaic perithecia" from *N. crassa.*

The statistical analysis described above makes a number of assumptions concerning nuclear behaviour during ascogonial development. First, it is assumed that there is no correlation between the number of founder nuclei and the number of asci which are formed in each perithecium. Secondly, it is supposed that the nuclear ratio within each ascogonium is in proportion to the nuclear ratio for the heterokaryon as a whole. Justification for the latter assumption is obtained from the proportions of mixed perithecia seen in uncrossed heterokaryons. After initiation as a side branch from a vegetative hypha, the ascogonial coil becomes multinucleate (e.g. Uecker, 1976), and although the mechanism by which this is achieved is unknown, there would seem to be two possibilities:-

(a) Many nuclei migrate from the mycelium into the ascogonium. Taking heterokaryon 1 as an example, this would predict the development of a relatively uniform population of protoperithecia, each with ascogonial nuclei in proportion to the nuclear ratio (37:63).

(b) Only a small number of nuclei enter the ascogonium. This number must be greater than one, however, or all the asci within each homothallic perithecium would be uniform. If two nuclei from the vegetative mycelium enter the ascogonial coil and undergo several successive nuclear divisions, ascogonia containing all yellow, all black, and both yellow and black nuclei will be formed in proportions determined by the nuclear ratio, and predicted by the Hardy-Weinberg formula (i.e. yellow 40%, black 14%, yellow and black, 46% for heterokaryon 1). Ascogonia of the last type will have black and yellow nuclei in equal proportion.

The probability of selecting a black "female" nucleus will be the same in either case: 1×0.37 for (a), and $0.14 + 0.46 \times 0.50 = 0.37$ for (b). Within mixed perithecia from uncrossed heterokaryons however, (a) predicts that the nuclear ratio for the heterokaryon will be reflected in the number of black/segregating and yellow/segregating perithecia produced, while (b) suggests that the numbers of black/segregating and yellow/segregating perithecia should be equal. Table 4.6 illustrates that (a) is probably correct. In heterokaryon 5 for example, where the nuclear ratio is 16:84 in favour of yellow nuclei, all 9 of the mixed perithecia detected, contained yellow and segregating asci.

The nature of the relationship between nuclear behaviour in crossed and uncrossed perithecia is not entirely clear. The data concerning crossed perithecia suggested that in 20-60% of fruitbodies, two or more ascogonial nuclei contributed to the centrum, but that only rarely (in less than 1% of perithecia) did fertilisation by more than one conidial nucleus take place. The latter observation apparently held true for uncrossed perithecia, in so far as all of the asci within individual perithecia shared at least one parental nucleus. Statistical analysis suggested that in uncrossed heterokaryons around 60% of perithecia might be founded by two ascogonial nuclei, and 40% by three nuclei, although it is not clear how many of these might function as "females" if a protoperithecium was fertilised. The development of both types of perithecia can also be considered in terms of the behaviour of the "male" nucleus. It may be argued that all of the nuclei within an unfertilised ascogonium are competent to act as female parents, but that their ability to do so is limited by the number of male nuclei available with which to pair. If, in an uncrossed heterokaryon, "maleness" is essentially a facility to form pairs, conferred perhaps by an epigenetic event, then the number of pairs which can form will be determined by the number of divisions of the male nucleus prior to pairing. A similar argument may be used to explain nuclear behaviour in crossed perithecia, although here the male nucleus is genetically distinct. If the male nucleus pairs with an ascogonial nucleus, and the two undergo a series of conjugate divisions, then only one female nucleus will contribute to the asci. If, however, the male nucleus undergoes at least one division prior to pairing, then two or more female nuclei will contribute.

4.3.3 Failure to enhance homothallic fruiting by the addition of extracts from mated cultures

The failure of an extract from mated cultures of *S. brevicollis* to stimulate increased homothallic fruiting in unmated cultures was perhaps not surprising. Although Siddiq (1989) reported that an extract from *P. brassicae* caused increased development of sterile perithecia in unmated cultures of *S. brevicollis,* she found no such effect produced by an extract from *S. brevicollis* itself. Amongst several possible explanations suggested, was that the time of application was critical to the stimulation of fruiting and that the optimal time had not been identified. During the present study, extract was added to mycelia of a range of ages but no stimulation was detected at any stage. This may have reflected inadequacies in the extraction process employed. However, as many *mtA* strains of *S. brevicollis* have been demonstrated to possess a constitutive

ability to produce homothallic perithecia, it is possible that some components of extracts from mated cultures, which stimulate sexual development in some fungal species, are already present in unmated *mtA* cultures of *S. brevicollis*. It might have been expected however that homothallic fruiting in *mta* strains could be stimulated by application of a biologically active extract, and a further detailed examination of this mating type might prove productive. Although both lipid and protein extracts have been reported to stimulate sexual reproduction in fungi (see Dyer *et al.*, 1992 and references therein), the development induced is often incomplete, and it is possible that full development requires a combination of numerous different factors.

4.3.4 Evolution of homothallism in *Sordaria* and *Neurospora*

The possible evolution of homothallism and heterothallism in fungi has been considered by many authors (e.g. Olive, 1958; Lundqvist, 1972), and has recently become a subject of research using molecular techniques (e.g. Taylor and Natvig, 1989). Whilst it is generally assumed that homothallism preceded heterothallism in the early development of fungal breeding systems, there is also evidence to suggest that some modern homothallic species have arisen from heterothallic ancestors, and it seems that evolution may proceed in either direction according to selective pressures (Olive, 1958). The Sordariales includes several genera which contain homothallic, pseudohomothallic and heterothallic species, and this group would thus appear to provide excellent material for the study of the evolution of breeding systems.

Within the genus *Sordaria*, relatively few heterothallic species have been identified, and the most commonly isolated species are homothallic. Amongst the heterothallic species *S. sclerogenia* (Fields and Grear, 1966), *S. tormento-alba* (Cailleux, 1971), and an as yet unnamed species isolated in New Zealand by D. P. Mahoney (pers. comm.), show no signs of homothallic fruiting (D. J. Bond, pers. comm.). In *S. heterothallis* however, many of the protoperithecia in uncrossed cultures were reported to enlarge to the size of mature perithecia, although asci were never observed (Fields and Maniotis, 1963). When *S. heterothallis* was crossed with the closely related *S. thermophila*, the perithecia which formed were found to contain two types of viable spores (Fields, 1963; Lewis, 1969a, 1969b). Non-pigmented spores (produced at a frequency of 20-30 asci per perithecium) were shown to contain elements from the genomes of both parents, a mixture which caused impaired mycelial growth and inhibited spore pigmentation amongst the progeny (Lewis, 1969b). Within each ascus of this type only a single

spore was seen to germinate, the remainder showing varying degrees of abortion. That the viable progeny were interspecific hybrids was confirmed by the recovery of both parents following a series of back-crosses. Interspecific crosses also yielded occasional asci with 8 pigmented spores (Fields, 1963; Lewis 1969a). Up to three such asci were detected in approximately 3% of perithecia (a frequency equivalent to 0.01% of all asci examined from interspecific crosses). Ascus dissection showed that all of the spores within a single ascus were of the same mating type and a single genotype, with *S. heterothallis* being recovered most frequently.

Although the formation of the second type of ascus suggests certain parallels with the situation observed in *S. brevicollis* there are several significant differences. Both mating types of *S. heterothallis* were recovered at approximately equal frequencies, and in two of the crosses analysed, the microconidial nucleus was inherited (Lewis, 1969a). The latter suggested that the results could not be explained solely on the basis of events occurring in the somatic mycelium. It seems likely, however, that the mechanisms facilitating homothallic spore production in *S. brevicollis* and the development of homozygous asci from interspecific crosses between *S. heterothallis* and *S. thermophila,* are fundamentally similar. It is possible that detailed examination of isolated homokaryons of either of the latter species might have revealed the type of spore production observed in *S. brevicollis,* although the results indicated that some kind of interaction between the two genomes was required. Unfortunately, fertile cultures of both *S. heterothallis* and *S. thermophila* appear to have been lost, so these possibilities cannot be distinguished by further experiments.

Although the molecular basis for heterothallism appears to be similar in *Sordaria* and *Neurospora*, analysis of homothallic species from both genera has revealed an interesting divergence: those homothallic strains of *Sordaria* which have been examined, contain both mating type idiomorphs (closely linked in the case of *S. macrospora*), whilst all but one of the homothallic *Neurospora* genomes contains only the *mtA* sequence (Glass *et al.*, 1988, 1990b). The exception is *N. terricola*, in which both sequences were present on the same restriction fragment and are therefore closely linked (Glass *et al.*, 1990b). It would therefore appear that when producing spores in uncrossed *mt*A cultures, *S. brevicollis* more closely resembles homothallic *Neurospora* species such as *N. africana* or *N. lineolata* than the homothallic *Sordarias.*

Since very similar mating type sequences are present in *Neurospora* and *Sordaria*, these sequences must have been in existence in an ancestral species before the genera diverged. Two alternative hypotheses which might explain the subsequent evolution of mating systems are illustrated in Figs. 4.26 and 4.27. The ancestral species may have been homothallic, containing both mating type sequences in the same haploid nucleus (Fig. 4.26). If these idiomorphs were closely linked as in *N. terricola* (Glass *et al.*, 1990b) or some of the homothallic *Sordarias* (D. J. Bond and A. J. Leigh-Brown, pers. comm.) then heterothallism has arisen independently in these genera following recombination events which separated the two sequences. Additional modes of homothallism might then have evolved from the heterothallic species, with a by-pass for the *mta* requirement giving rise to species such as *N. africana*, while division II spindle overlap resulted in the development of pseudohomthallic species such as *N. tetrasperma*.

Alternatively the ancestral species might have been heterothallic, with separate mating type sequences as in the modern heterothallic species (Fig. 4.27). In this case all of the homothallic species would be secondarily derived, either by by-passing the requirement for *mta*, packaging the two sequences within the same spore, or by putting the sequences onto a common restriction fragment following a recombination event. A similar scheme is envisaged in *Neurospora* by Metzenberg and Glass (1990), although these authors suggest that evolution of homothallism took place via pseudohomothallic species, rather than directly from heterothallic ancestors. Pseudohomothallic species have yet to be identified in *Sordaria,* however, and homothallism of the kind seen in *S. brevicollis* appears to represent an intermediate stage between true heterothallism and homothallism of the type seen in *N. africana.*

Figures 4.26 and 4.27: Two alternative scenarios for the evolution of breeding systems in *Sordaria* and *Neurospora*. The square boxes, bordered by solid lines represent the genotype at the mating type locus of a homothallic species. The rectangular boxes, divided by a dotted line, represent the possible genotypes at the mating type locus in a heterothallic species. The unusual status of *S. brevicollis* (and possibly *S. heterothallis*) is denoted by a combination - a square box representing *mtA* functioning as a homothallic species, and a rectangular box, with *mta* contributing to heterothallic crosses.

Figure 4.26

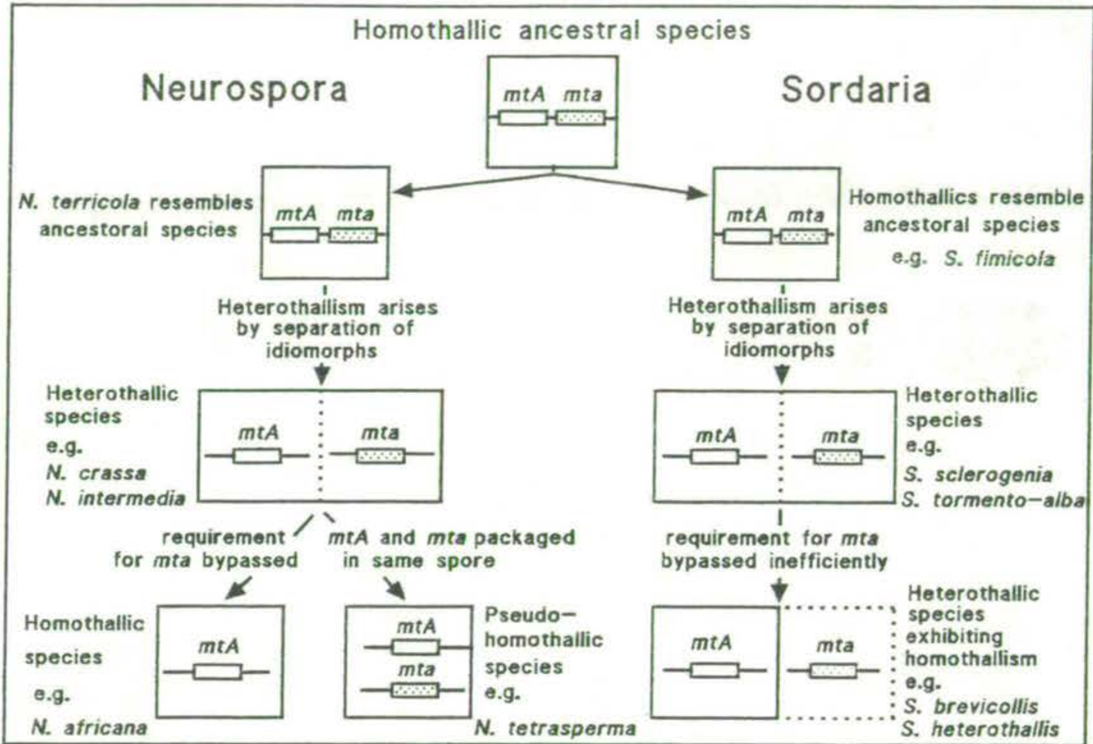
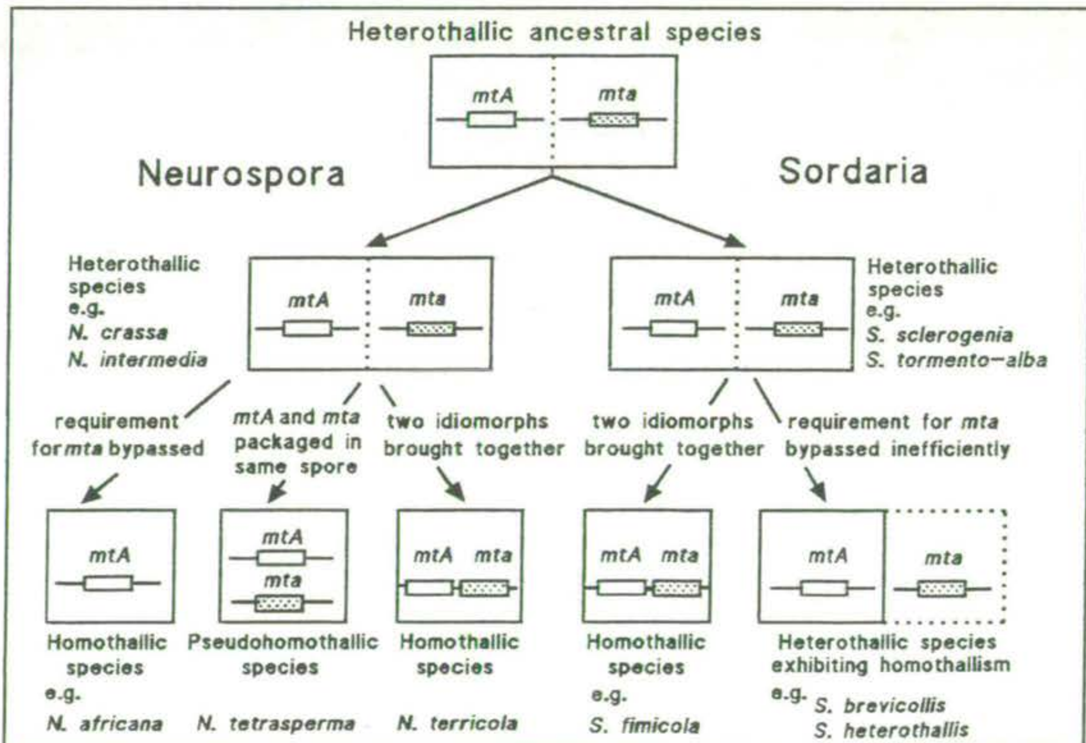


Figure 4.27



CHAPTER FIVE

ENVIRONMENTAL REGULATION OF MULTICELLULAR DEVELOPMENT IN SORDARIA BREVICOLLIS

5.1 Introduction

Many environmental variables have been shown to influence the regulation of multicellular development in fungi (Moore-Landecker, 1992) and the effects of several of these factors on the development of *S. brevicollis* have been examined during the present study. In Chapter Three it was shown that growth in continuous light, stimulates development of vegetative hyphal aggregates (VHAs), but inhibits protoperithecial production, so that perithecial formation is typically optimal in complete darkness. Photoinhibition of sexual reproduction is an unusual phenomenon in fungi, and illumination has more frequently been found to exert either stimulatory or neutral effects on fruitbody development (section 1.7.1). In consequence, the effect of light on the balance between formation of protoperithecia, perithecia and VHAs was examined in order to investigate both the regulatory mechanisms underlying sexual morphogenesis, and the relationship between the various pathways of multicellular development.

Although the effects of light are probably of greatest importance, several other environmental factors which influence development, including nutritional status and temperature, are examined later. The first set of experiments described is concerned with microconidial concentration, a factor which has a profound effect on the formation of perithecia under laboratory conditions. It is not known whether spermatisation plays a significant rôle in fertilisation of protoperithecia in the wild, however, and it may be that under natural conditions, perithecia are usually formed when mycelia of opposite mating type meet. Nevertheless, since the microconidia produced by *S. brevicollis* are apparently incapable of acting as asexual propagules (Olive and Fantini, 1961; Sanni, 1984), some other selective advantage, such as a spermatial activity, may be attached to their continued production. If microconidia do function as spermatia in the wild, it seems unlikely that they ever reach the saturating conditions produced in the laboratory, and it is therefore of interest to examine the effects of lower microconidial concentration on perithecial production, and indirectly, on other

developmental pathways. In the following series of experiments, the conditions required for optimal microconidial production were established, and the effects of microconidial concentration on the formation of crossed and uncrossed perithecia were examined.

5.2 Results

5.2.1 Effect of microconidial concentration on sexual development

5.2.1.1 Optimisation of microconidial production

Preliminary experiments suggested that although microconidial production was usually reduced by light, this effect was highly variable (data not shown). In comparison, the number of microconidia which developed on vigorous dark grown cultures of either mating type proved relatively consistent, in the range $2.0\text{-}2.5 \times 10^5$ per 8.5 cm Petri plate. Microconidia were also examined using the vital stain fluorescein diacetate (Butt *et al.*, 1989), which showed that over 95% of microconidia were viable, whether produced in the light or in the dark. In order to standardise subsequent experiments as far as possible, dark grown microconidia were used. Microconidial number, like ascospore germination, was enhanced by growing cultures on CMA containing 0.7% (w/v) sodium acetate.

5.2.1.2 Effect of microconidial concentration on perithecial production

5.2.1.2.1 *Crossed perithecia*

To determine the concentration at which microconidia became limiting to protoperithecial fertilisation, a concentrated suspension of microconidia from *mta* plates was diluted to produce a range of concentrations from 250 to 5×10^4 microconidia ml^{-1}. These suspensions were used to cross 4 day old, dark-grown cultures of *mtA* (10 ml suspension per plate). After a further 6 days incubation in the dark, the number of mature perithecia on each crossed plate was determined. Fig. 5.1 shows that microconidia became limiting when applied at less than 5.0×10^4 per plate, and were severely limiting below 2.5×10^4 per plate. This result was unexpected as viable microconidia appeared to be present in considerable excess of the number of perithecia produced.

In a second experiment using the same isolates, 10 ml of a suspension of 2.5×10^4 microconidia/ml was used to fertilise a series of 20 *mtA* plates. The suspension was poured on to the first plate, allowed to stand for 5 minutes, poured onto the next plate in the series, and so on until all plates had been crossed. After a further 6 days incubation in the dark, the first and twentieth

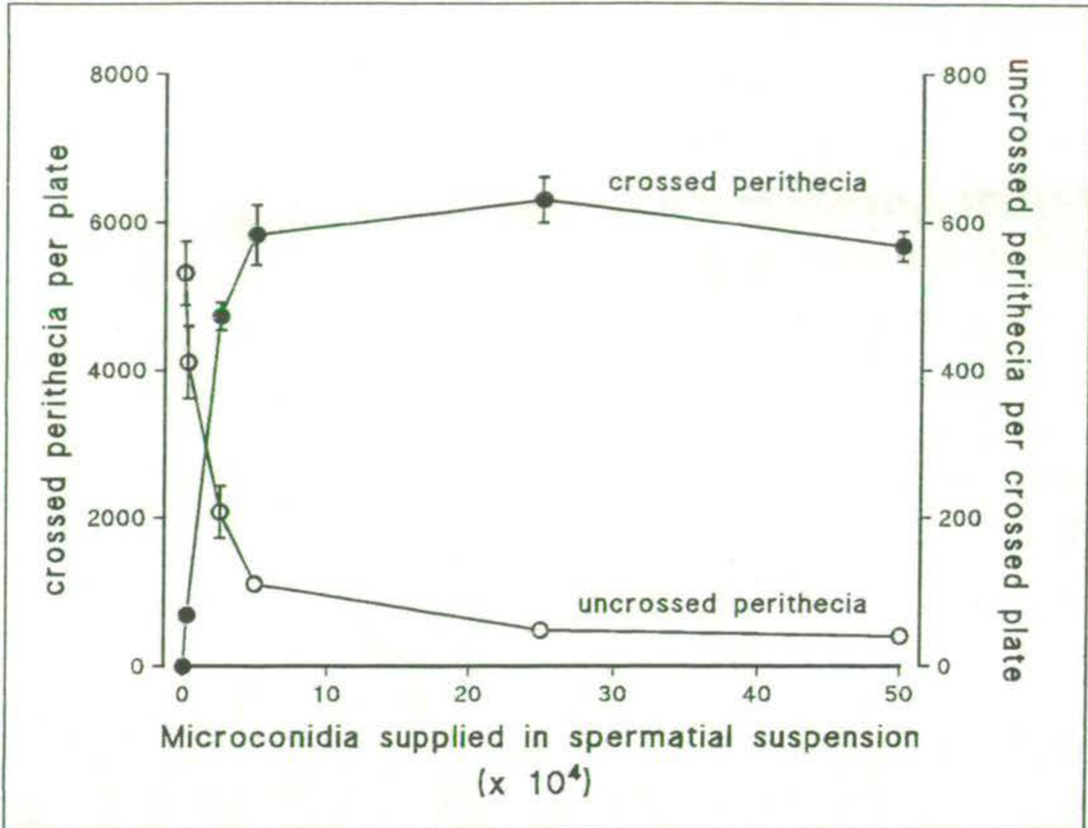


Figure 5.1: Effect of microconidial concentration on the frequency of crossed and uncrossed perithecia in a wild type strain of *mtA*. Dark grown cultures were crossed by spermatisation when 4 days old, and perithecia counted in 10 day old cultures. Each point represents the mean for 10 replicate plates ± standard error. Error bars are omitted when smaller than data point symbols. Note difference in scales on Y axes.

plates were examined, and found to produce 6224 and 6008 perithecia, respectively. Since all of the intermediate plates in the series developed similar numbers of perithecia, it was calculated that approximately 1.2 x 10^5 protoperithecia had been fertilised, utilising about half of the microconidia in the original suspension. These results demonstrated that a large proportion of the microconidia in a spermatial suspension were poured away when excess liquid was drained from the protoperithecial culture.

Although the second technique was clearly less wasteful in terms of the microconidial suspension required, the first method was used routinely because cross-contamination of female plates was avoided. In all subsequent experiments, 10 ml of a suspension of approximately 6 x 10^4 microconidia /ml was used to fertilise protoperithecial cultures.

5.2.1.2.2 *Uncrossed perithecia*

During the course of the first experiment described above, it was noted that at low microconidial concentrations, not all perithecia on 10 day old cultures were fully developed in terms of pigmentation, neck elongation or spore production. Upon closer examination of these perithecia (which were excluded from the count of mature fruitbodies) it became evident that they resembled the uncrossed perithecia found on certain unfertilised mycelia. The cultures were therefore recounted for uncrossed perithecia. Fig. 5.1 illustrates the relationship between the frequencies of crossed and uncrossed perithecia, and shows that significant numbers of uncrossed perithecia formed only when microconidial concentration was limiting to the formation of crossed perithecia.

5.2.2 Effect of light on multicellular development

5.2.2.1 VHA formation

Preliminary experiments designed to quantify VHA production generated little useful information because of the difficulty in distinguishing individual structures. Although light exposure clearly stimulated VHA formation, and it was possible to assign test cultures a score on a +/- basis (Table 5.1), this system relied on a visual assessment, and hence was subjective. Two further approaches were therefore adopted in an attempt to quantify VHA production. The first was based on the observation that the volume of VHA tissue present on light-grown plates appeared far greater than that of protoperithecia on dark-grown cultures, and that this might be accompanied by increased fresh or dry weight.

Illumination conditions		
Days 0 - 4 (female culture)	Days 4 - 10 (crossed culture)	VHA frequency
Dark	Dark	-
Dark	Light	+
Light	Dark	++++
Light	Light	++++

Table 5.1: Effect of illumination conditions on the production of vegetative hyphal aggregates (VHAs). Cultures incubated under the light regimes shown were crossed by spermatisation at 4 days old, then incubated for a further 6 days to allow perithecial maturation. The intensity of VHA formation was assessed on the following scale:

-	=	0
+	=	1- 1,000
++++	=	> 10,000

The fresh and dry weights of mycelia produced by an isolate of *mtA* are shown in Fig. 5.2. The results illustrate that contrary to expectation, light-grown cultures produced lower fresh weights than dark-grown plates. In the case of dry weight, the distinction between light and dark grown cultures was less pronounced, indicating that dark-grown cultures had a higher moisture content than those grown in the light. A very similar pattern was followed by *mta* cultures (results not shown).

A second approach to the quantification of VHA production utilised the fact that light-grown cultures of *S. brevicollis* accumulated a pinkish-orange pigment (Figs. 3.15 - 3.18), which was absent from cultures incubated in the dark. Attempts were therefore made to correlate VHA production and the development of this pigment. After inoculation, cultures were incubated in the dark for 48 hours, and then exposed to light for periods between 30 minutes and 6 hours (Fig. 5.3 (a)). At the end of each light treatment, the presence or absence of VHAs was monitored, and pigment was extracted from the mycelium according to Davies and Grierson (1989). In a parallel experiment, plates were returned to the dark after light exposure. Approximately 54 hours after inoculation, these cultures were examined for the presence of VHAs, and pigment extractions were carried out. The pigment extracted in both cases showed a main peak at 474 nm, with shoulders at 448 and 504 nm (Fig. 5.3 (b)), similar to that obtained for a pigment extracted from illuminated cultures of *N. crassa* (Fig. 5.3 (b)). The orange pigments produced by *N. crassa* have been identified as carotenoids (Zalokar, 1957) and it would appear that the pigment produced in light-grown mycelia of *S. brevicollis* is also a carotenoid.

Although not detected in dark-grown cultures of *S. brevicollis*, pigment was present within 30 minutes of first exposure to light (Fig. 5.3 (c)). Visual inspection of cultures indicated that pinkish colouration was actually present after as little as 10 minutes illumination, and it is possible that the precursors of the pigment were already present in dark-grown mycelia. After a period of 4 hours illumination, pigment levels were similar to those from light-grown control cultures (extracted at 48 and 54 hours after inoculation). After being exposed to light for 6 hours, test cultures contained significantly higher levels of pigment than the controls (Fig. 5.3 (c)). As these results are expressed as pigment/g fresh weight, conversion to dry weight (see above) increases the apparent excess of pigment produced by dark-grown cultures after exposure to light.

No VHAs were visible after illumination of up to and including 4 hours, although they had begun to form around the edge of the Petri plate after 6 hours.

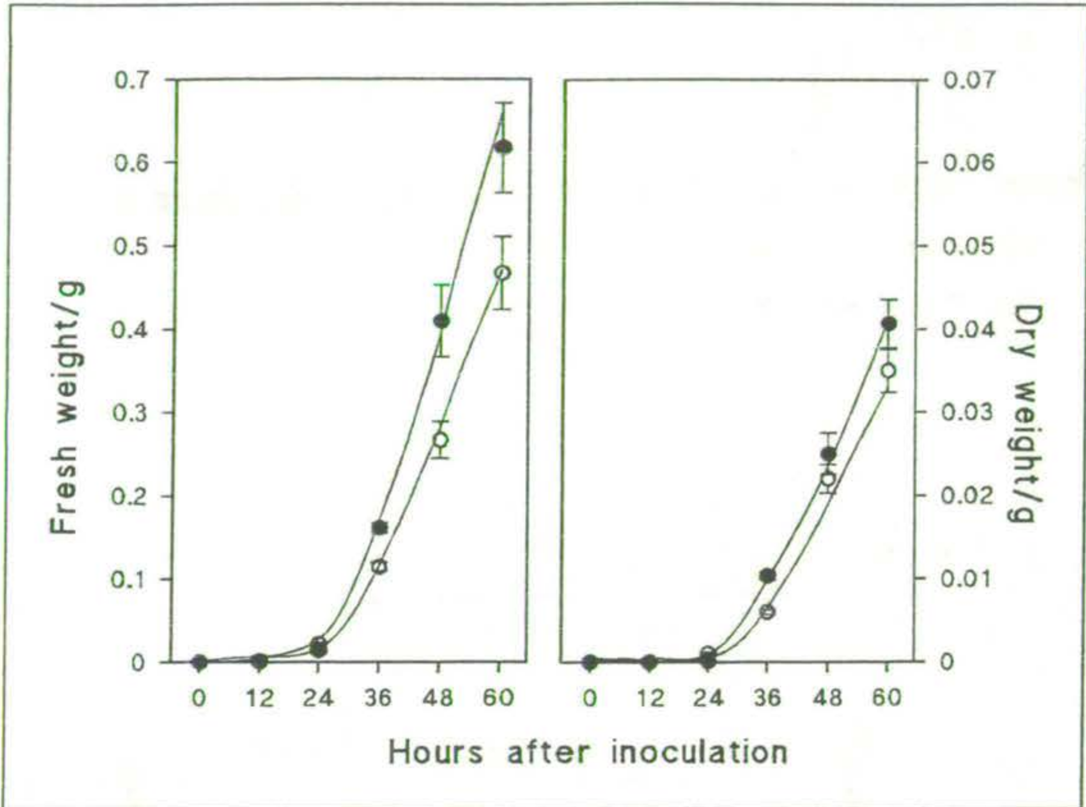


Figure 5.2: Effect of illumination on fresh and dry weight (per 8.5 cm Petri plate). Plates containing CMA overlaid with cellophane membranes were inoculated with a strain of *mtA*, and incubated either in the dark or in continuous light. Each point is the mean for 10 replicate plates ± standard error. Solid circles = dark grown, open circles = light grown.

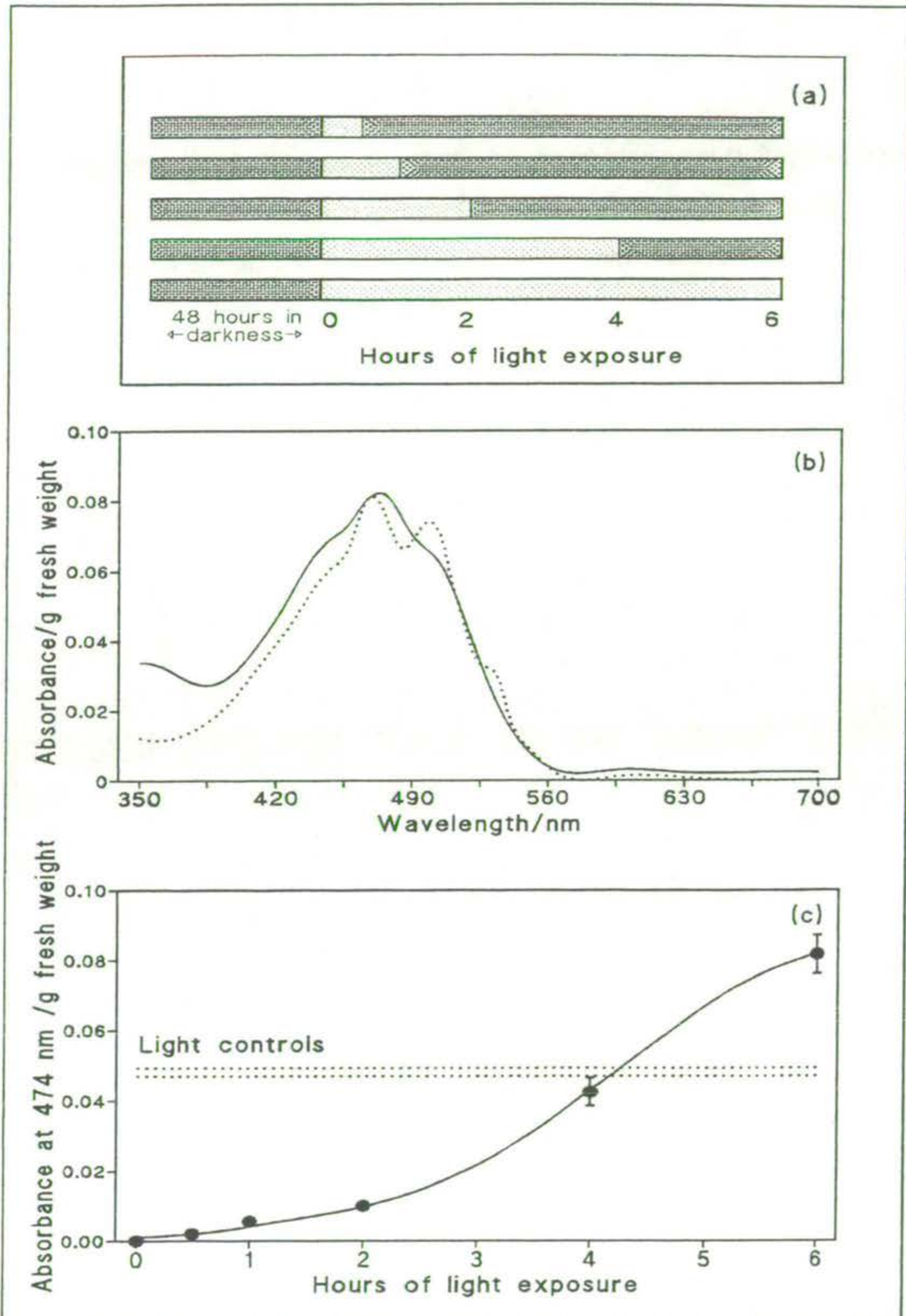
Figure 5.3: Extraction and characterisation of pigment from mycelia of *S. brevicollis* after exposure to white light.

(a) Diagrammatic illustration of experimental design. All cultures were incubated in darkness for 48 hours after inoculation, and then exposed to white light for periods between 30 minutes and 6 hours. At the end of each light treatment, half the plates were extracted, and half returned to the dark until the end of the 6 hour period, so that the period of dark incubation after light exposure varied from 0 - 5.5 hours. Control plates maintained in complete darkness or continuous light were extracted at 48 or 54 hours after inoculation.

(b) Absorption spectra in hexane for the carotenoid-like pigment extracted from illuminated cultures of *S. brevicollis* (solid line), and for a similar pigment extracted from light-grown cultures of *N. crassa*. The spectrum for *S. brevicollis* has a main peak at 474 nm, and that for *N. crassa* at 472 nμm. The scale on the Y axis relates to *S. brevicollis* only: the level of extractable pigment from *N. crassa* was approximately 5 times that from *S. brevicollis* (on a fresh weight basis) and is plotted at 1/5 concentration to facilitate comparison.

(c) Absorbance at 474 μm, at the end of the light treatments illustrated in Fig. 5.3(a). Each point represents the mean for three replicate experiments $\pm$ standard error. Dark controls showed zero absorbance. Light controls (dotted lines) extracted at 48 and 54 hours, exhibited peak absorbances of 0.048 ± 0.004 and 0.051 ± 0.005 respectively.

Figure 5.3



All those plates which had been exposed to light and then returned to the dark had developed a few VHAs by the end of the 6 hour period. It was therefore concluded that although both pigment formation and VHA production were light-stimulated, pigmentation preceded the development of VHAs, and could not be used as a convenient method to quantify their production. That the carotenoid-like pigment produced by *S. brevicollis* is not concerned with perithecial pigmentation was demonstrated by its presence in the hyaline mutant, at levels equivalent to those found in the wild type strains described above (data not shown). The same pigment was also produced in senescing cultures which had lost both female fertility and the ability to produce VHAs (data not shown).

5.2.2.2 Sexual development

5.2.2.2.1 *Effect of white light on perithecial formation*

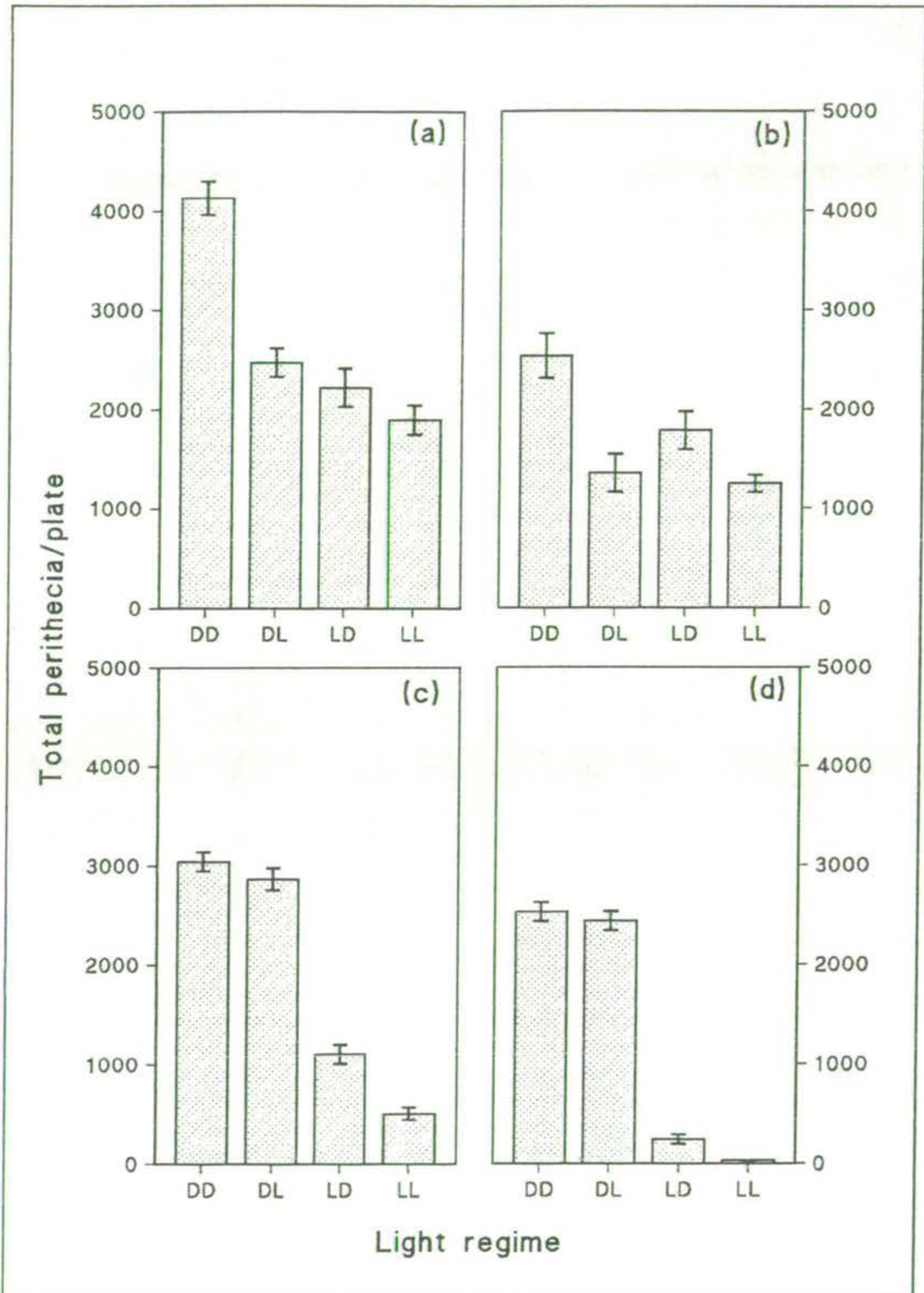
During morphological studies it became apparent that perithecial formation in *S. brevicollis* was inhibited by light. Although many aspects of fungal reproduction have been shown to be light-regulated, illumination has typically been found to exert either stimulatory or neutral effects on fruitbody formation (section 1.7.1), so the inhibition observed in *S. brevicollis* was unusual.

In preliminary experiments, four crosses were subjected to the light treatments detailed in Table 5.1. Four day old protoperithecial cultures were crossed by spermatisation, and mature perithecia scored after a further 6 days. Fig. 5.4 shows the frequency of perithecia for each cross and light treatment, and illustrates two important points. First, it should be noted that although perithecial frequencies were relatively consistent between the replicates for each cross (standard errors are comparatively small), there was marked variation between crosses, both in the maximum number of perithecia produced in complete darkness, and in the inhibitory effect of light. Certain strains consistently produced more perithecia than others, although all were isolated from the same stock crosses. Despite this variation, which was not associated with mating type, certain trends could be identified:- (i) For each cross, maximum perithecial production occurred in complete darkness (DD); (ii) Exposure to light from 0-4 days after inoculation (i.e. before crossing), reduced perithecial numbers (compare DD and LD); (iii) Exposure to light from 4-10 days after inoculation (i.e. after crossing), also reduced perithecial frequency, (compare DD and DL). While crosses (a) and (b) showed a substantial reduction in perithecial number when cultures were illuminated after crossing, similar illumination of crosses (c) and (d) produced relatively little effect.

Figure 5.4 (a-d): Effect of light on perithecial frequency in 4 wild type strains of *Sordaria brevicollis*. (a) and (c) are *mtA* female, (b) and (d) *mta* female. Light regimes: DD = 10 days dark; DL = 4 days dark, 6 days light; LD = 4 days light, 6 days dark; LL = 10 days light. Four day old plates were crossed by spermatisation, and perithecial frequencies were determined after 10 days. Each bar represents the mean for 10 replicate plates ± standard error.

Figure 5.5 (a-d): Effect of light on perithecial distribution in 4 wild type strains of *Sordaria brevicollis*. Strains and light treatments are as Fig. 5.4. Each histogram in Fig. 5.5 illustrates the distribution of perithecia whose total frequencies were shown in Fig. 5.4. (Thus Fig. 5.5 (a) DD represents the distribution of the perithecia whose total frequency is shown in Fig. 5.4 (a) DD.) Each bar represents the mean for 10 replicate plates ± standard error.

Figure 5.4



Figs. 5.5 (a-d) illustrate the distribution of perithecia for the same crosses, and show that when these strains were incubated in the dark throughout protoperithecial formation and perithecial maturation (DD), perithecia were typically produced at highest concentration towards the edge of the Petri plate (Figs. 5.5 (a-c))). The exception was cross (d), where perithecial distribution was relatively even (Fig. 5.5 (d)). When previously dark-grown cultures were illuminated after crossing (DL), the distribution of perithecia was very similar to that for DD plates, although the total number of perithecia was reduced (e.g. Fig. 5.5 (a) DD-DL).

Exposure to light during protoperithecial formation (from 2-4 days after inoculation), not only reduced the total number of fruitbodies which matured (when compared with completely dark grown plates), but also caused a marked change in perithecial distribution. A reduction in perithecial density at the edge of the Petri plate, was typically accompanied by an increase in perithecial formation in the centre of the culture, so that the overall distribution was more uniform (compare Fig. 5.5 (a) DD and LD). Cultures incubated in constant light (LL) also showed relatively even perithecial distributions. Close examination of LD and LL cultures revealed that over 50% of perithecia developed below the surface of the growth medium. Although the necks of such perithecia sometimes developed to several millimetres in length, they often failed to reach the surface, and mature ascospores were frequently ejected into the media. Similar perithecia were also produced on DD and DL plates, but represented only a small proportion of total perithecial production. Thus exposure to light resulted in a reduction in total perithecial production, and a three dimensional alteration in the distribution of those perithecia which formed.

The results indicated that although light affected both protoperithecial formation (which occurred predominantly between 2-4 days after inoculation), and perithecial maturation (which took place from 4-10 days after inoculation), the early phase of development exhibited greater sensitivity, and this period was therefore examined in more detail. To check that little protoperithecial formation took place more than 96 hours after inoculation, uncrossed cultures (incubated either in the dark or in continuous white light) were scored for protoperithecial frequency at 12 hour intervals between 96 and 144 hours after inoculation. The results (data not shown) indicated that protoperithecial frequency remained constant over the period examined, irrespective of light regime. Protoperithecial frequency was also monitored in crossed cultures during the 24 hour period following spermatisation, during which no increase in

Figure 5.5(a)

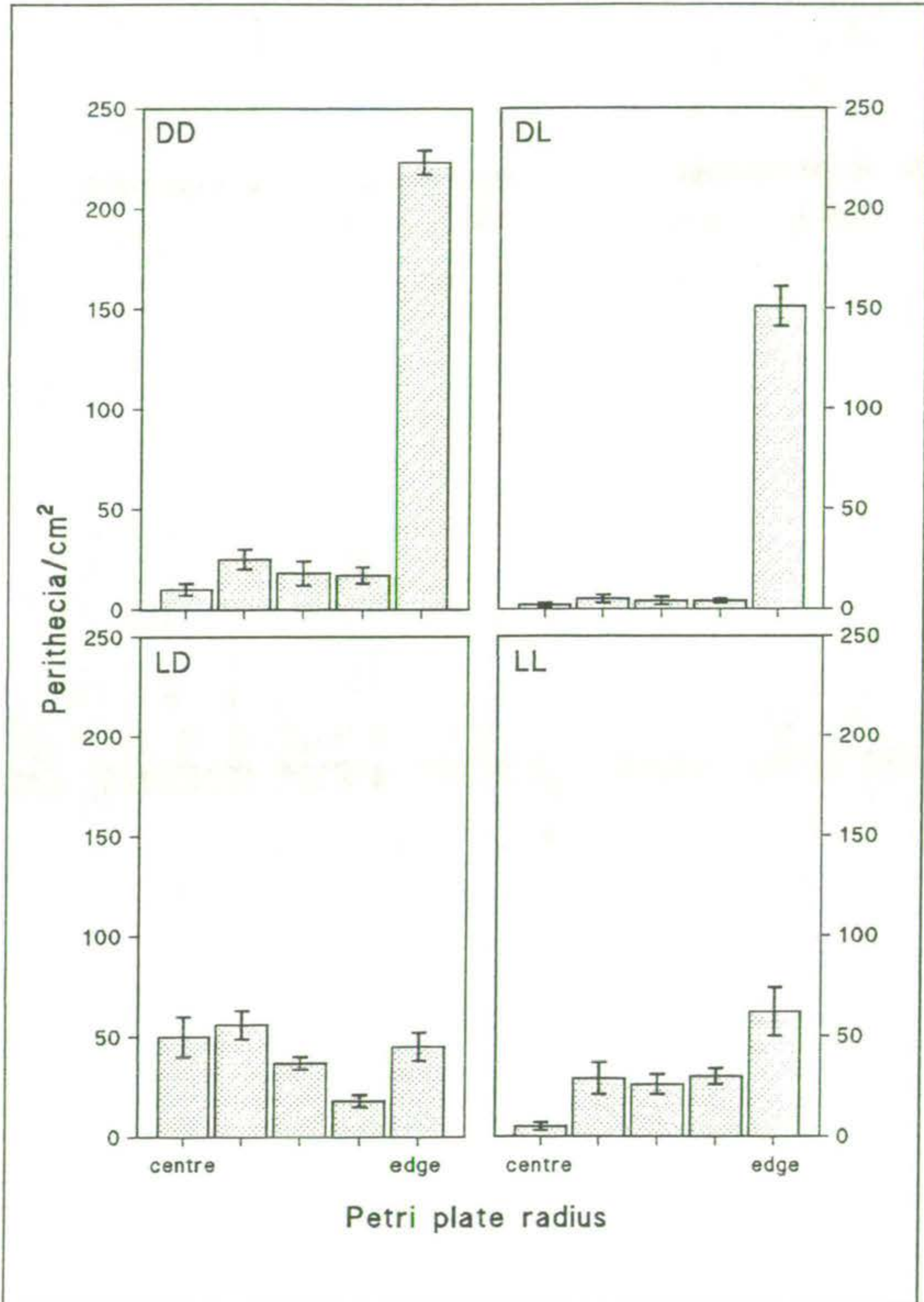


Figure 5.5(b)

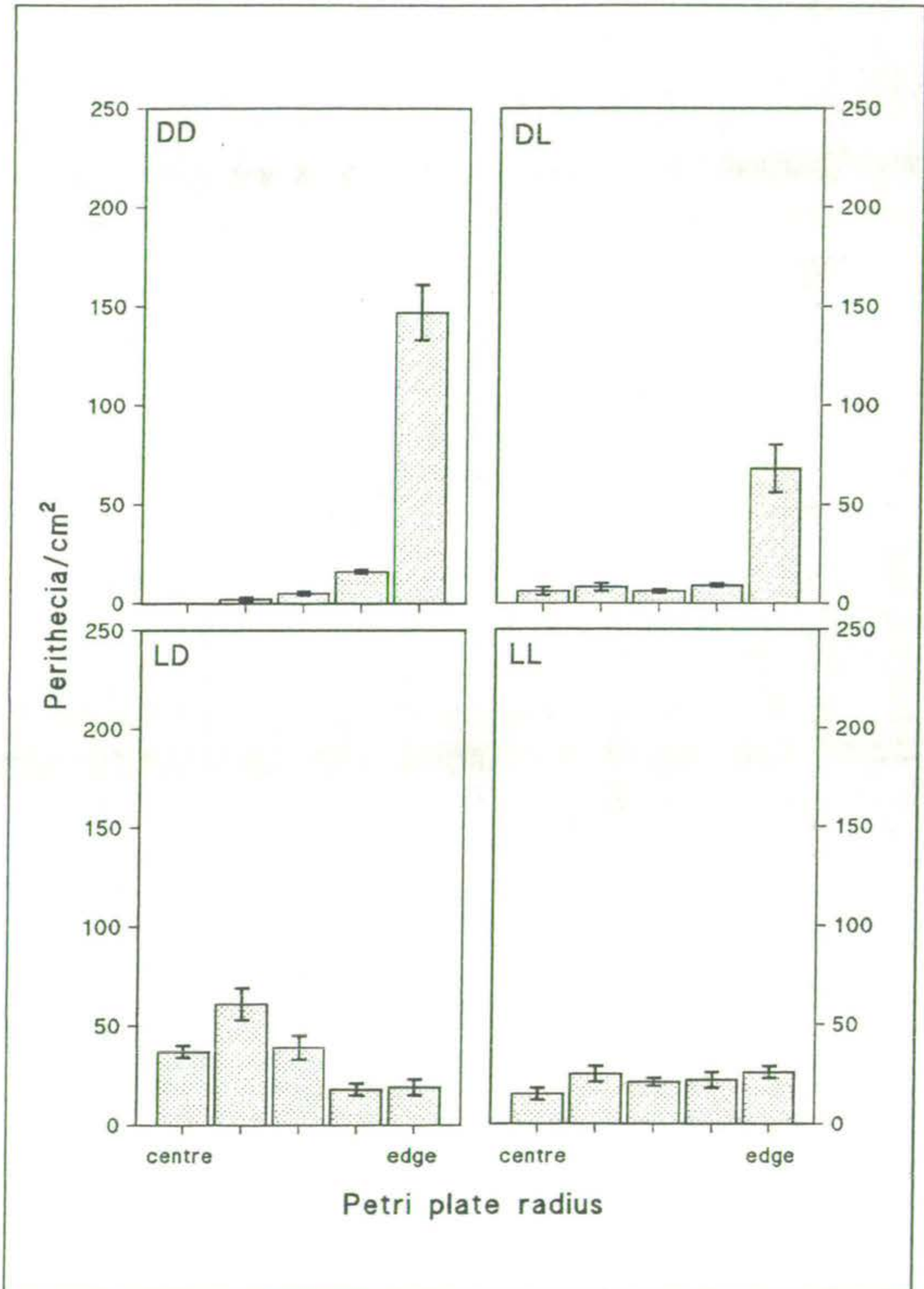


Figure 5.5(c)

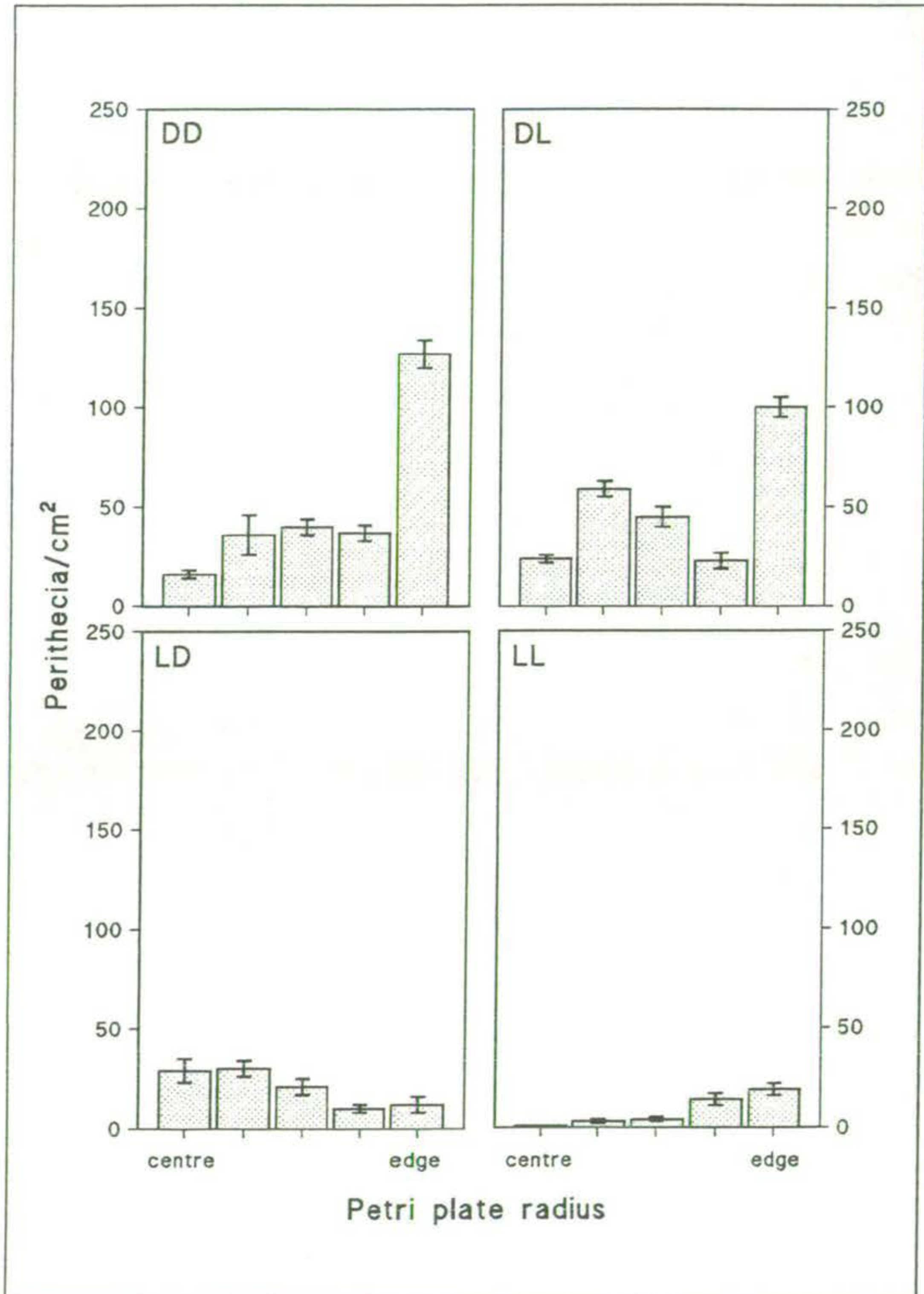
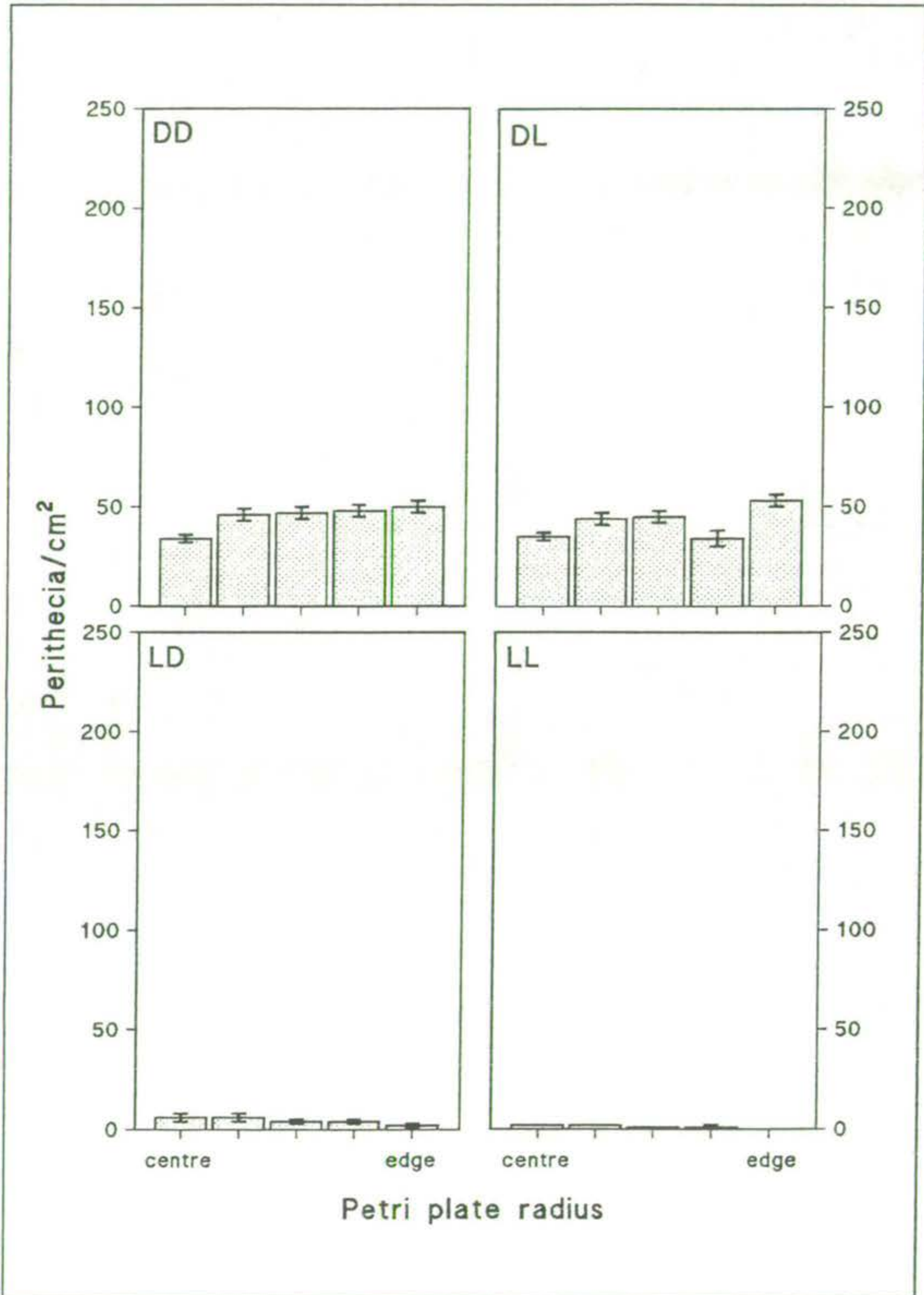


Figure 5.5(d)



frequency was detected. Although the subsequent expansion of fertilised protoperithecia prevented observation of the underlying mycelia, it seems unlikely that significant numbers of new protoperithecia were formed after cultures have been crossed.

5.2.2.2.2 *Effect of white light during protoperithecial formation*

An *mtA* strain which exhibited relatively even perithecial production was selected as the protoperithecial parent, for a series of experiments designed to establish the effect of light at different stages during the first 4 days of development. After inoculation onto plates containing CMA either with or without cellophane membranes, cultures were incubated in the dark for 4 days, with 8 hour periods of exposure to white light as illustrated in Fig. 5.6. Four days after inoculation, half of the cultures grown on membranes were scored for protoperithecial production, after staining with Nile red; all the remaining cultures were crossed with spermatia of the opposite mating type, and incubated in the dark for 6 days. Ten days after inoculation, the frequency of perithecia in crossed cultures was determined.

Figs. 5.7 (a-l) illustrate variation in protoperithecial frequency and distribution caused by exposure to 8 hour periods of white light. Before examining the results in detail, it is should be noted that the mycelium reached the edge of the Petri plate approximately 46 hours after inoculation, and prior to this, was not competent to differentiate protoperithecia (section 3.1.1). Thus any experimental variation in protoperithecial frequency which resulted from light exposure earlier than 46 hours after inoculation (i.e. treatments a-e) required some form of signal storage in the mycelium until protoperithecial formation could take place. It should also be remembered that the total area of mycelium illuminated in early light treatments was only small, so that any effect of illumination might be "diluted" by subsequent hyphal growth.

Illumination of cultures soon after inoculation (Figs. 5.7 a-b) resulted in protoperithecial distributions and frequencies which differed only slightly from the dark control. Light exposure between 16-24 hours after inoculation, however, caused a more substantial deviation from the dark control, with increased protoperithecial density of protoperithecia in the area immediately around the inoculum, accompanied by a slight increase in protoperithecial frequency in the outer part of the plate (Fig. 5.7 (c)). After illumination between 24-40 hours (Figs. 5.7 (d-e)), protoperithecial density in the centre of the plate dropped below the level of the dark control. When light exposure was given 40-48 hours after

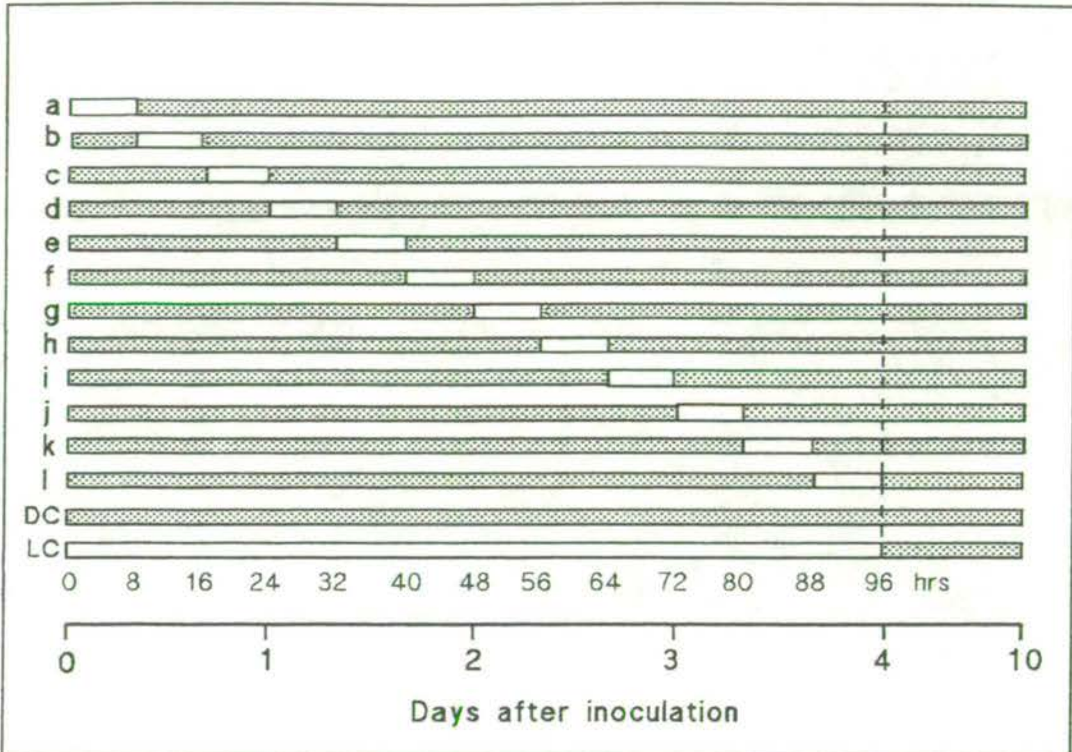


Figure 5.6: Effect of white light on protoperithecial development in *Sordaria brevicollis*: Experimental design. Petri plates containing CMA overlaid with cellophane membranes were inoculated with a wild type strain of *mtA*, and incubated in the dark for 96 hours, with 8 hour periods of illumination as shown. Shaded boxes represent dark, unshaded boxes represent light. After 96 hours (dotted line), half of the cultures for each light treatment were scored for protoperithecial frequency, and the remainder were crossed by spermatisation. Ten days after inoculation, perithecial frequencies were determined. A further set of Petri plates containing CMA without cellophane membranes, was exposed to the same light regimes. All these plates were crossed by spermatisation, and perithecia were scored 10 days after inoculation. No attempt was made to determine the frequency of protoperithecia on plates without membranes. Each light treatment is denoted by a letter (a - l), which also applies to the data shown in Figs. 5.7, 5.8 and 5.9. DC = dark control; LC = light control.

Figure 5.7 (a-l): Effect of light exposure on protoperithecial distribution. Strips of cellophane membrane bearing 4 day old mycelium were stained with Nile red, and protoperithecia were scored along a narrow radius. Each histogram shows the effect of a period of 8 hours light exposure, together with points representing the dark control. Each bar represents the mean for 16 radii ± standard error (4 radii from each of 4 replicate plates). Graphs (a-f) also show the position of the mycelial front, at the beginning and end of light exposure (double-headed arrow). Error bars for the dark control are shown in the first graph on each page, but omitted thereafter. Light controls (data not shown) exhibited uniformly low protoperithecial density.

Figure 5.7(a-d)

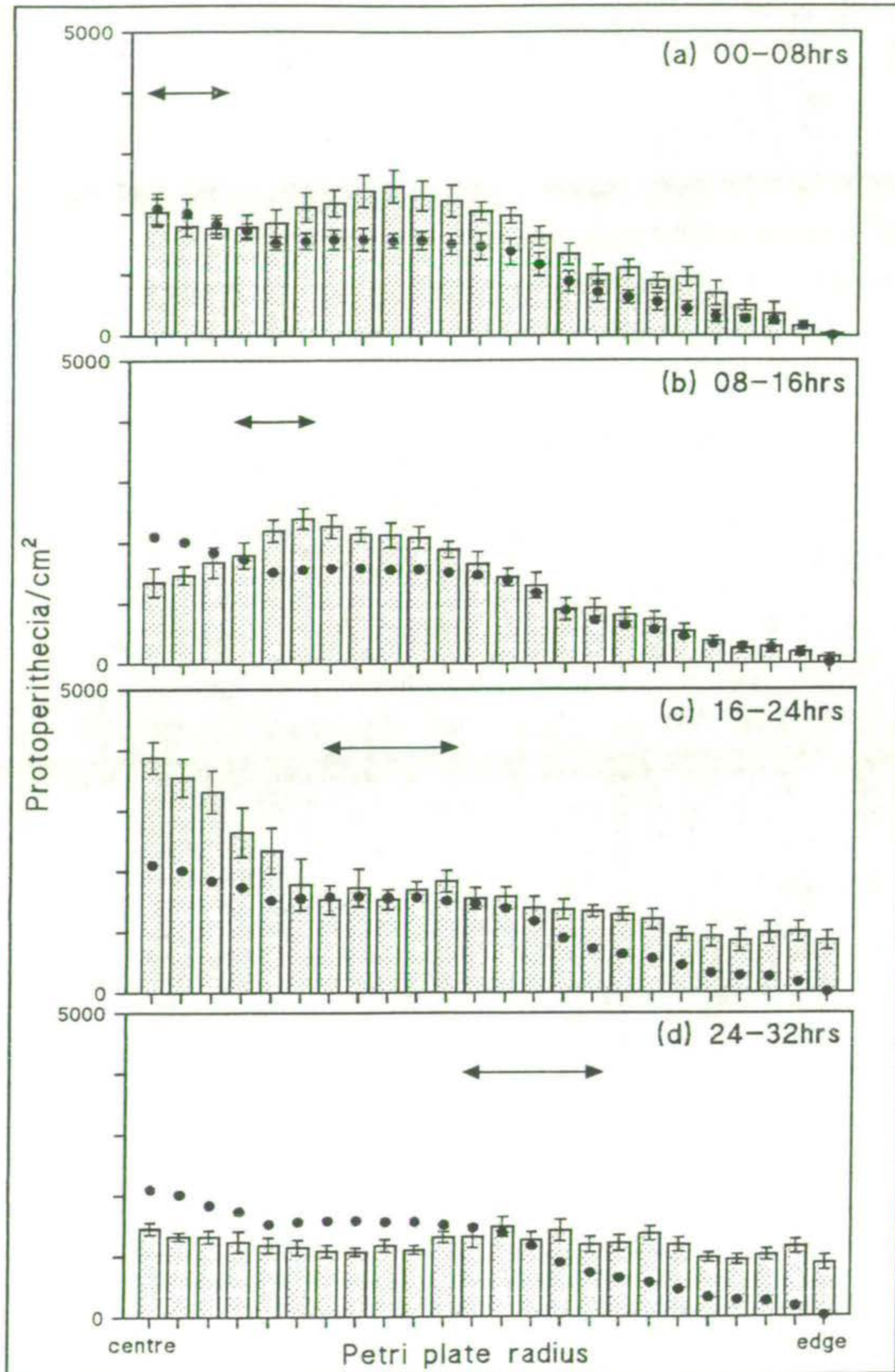


Figure 5.7(e-h)

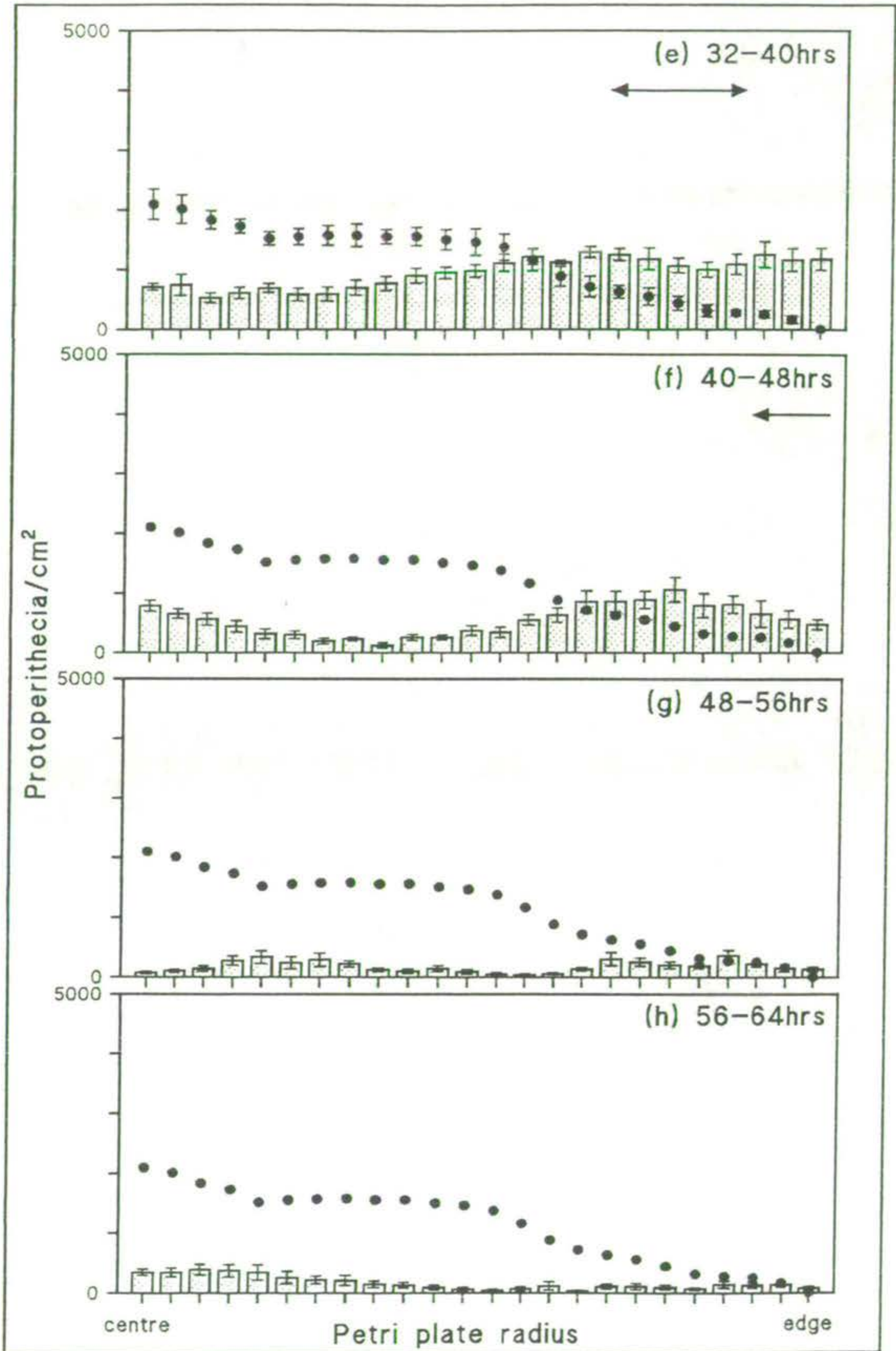
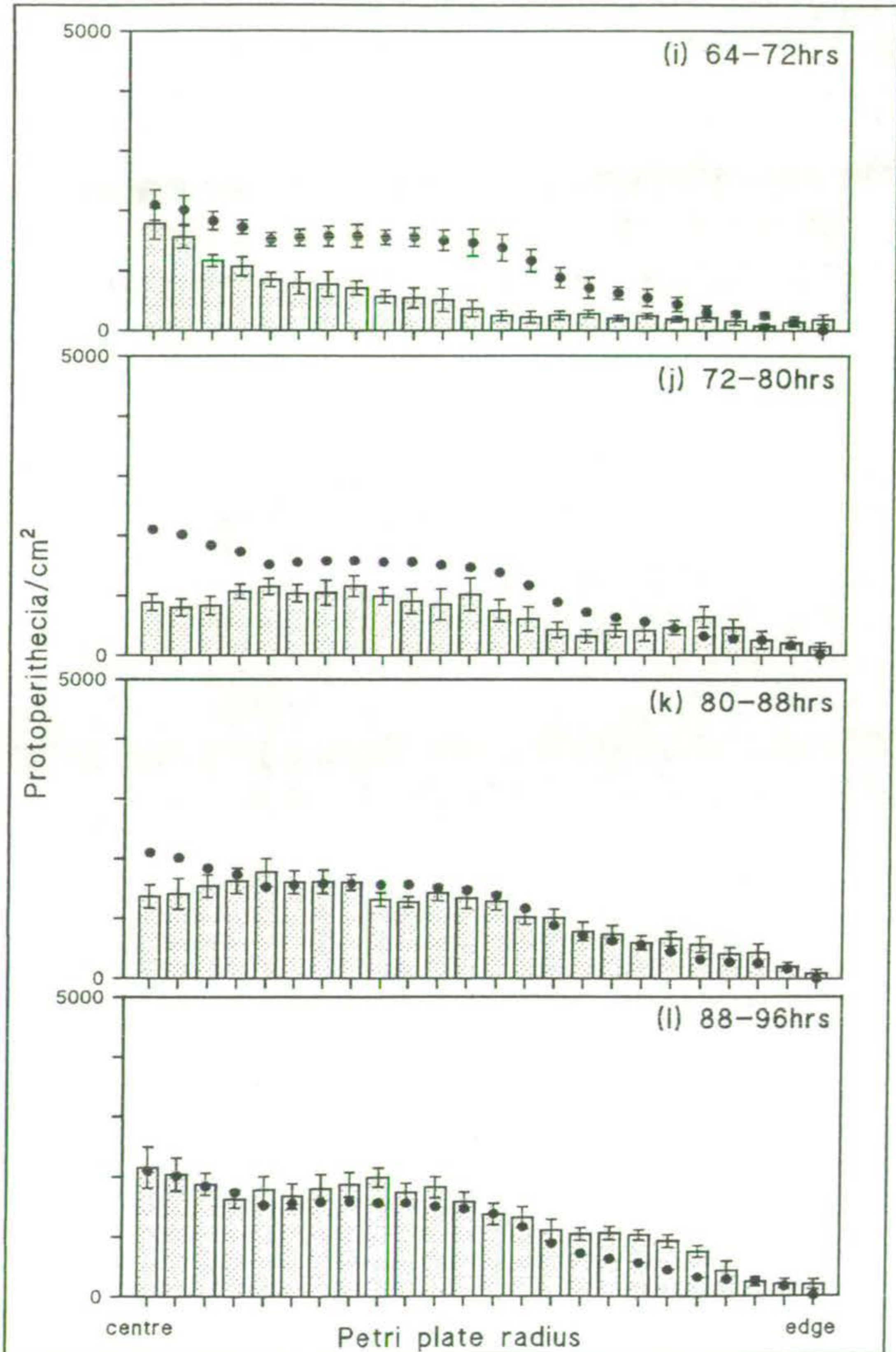


Figure 5.7(i-l)



inoculation (Fig. 5.7 (f)), protoperithecial formation in the outer part of the plate was also reduced, a trend which continued after illumination between 48-64 hours, resulting in protoperithecial formation at a uniformly low level (Figs. 5.7 (g-h). Thereafter, exposure to light appeared to have progressively less effect on protoperithecial production, with cultures illuminated between 80-96 hours showing protoperithecial formation very similar to the dark control (Figs. 5.7 (k-l).

Total protoperithecial frequencies for each light treatment (Fig. 5.10 (a) show that although protoperithecial formation was lowest when plates were exposed to light 48-64 hours after inoculation (immediately after the mycelial front reached the edge of the Petri plate), protoperithecial formation had been falling at a relatively constant rate after light exposure during the previous 24 hours. Exposure to light after 64 hours probably produced a lesser inhibitory effect because many protoperithecia had already been formed prior to illumination. These results confirmed the previous finding that protoperithecial formation was largely complete by 96 hours after inoculation, and indicated that the majority of protoperithecial development had taken place after 80 hours.

Perithecial distribution in 10 day old cultures could be seen to follow a very similar pattern to that for protoperithecia, although at a lower frequency (Figs. 5.8 (a-l)). Maximum perithecial formation occurred following illumination between 16-24 hours after inoculation, then declined to a minimum level after illumination between 48-64 hours (Fig. 5.10 (b)). Thereafter, perithecial frequency increased to a level slightly above that of the dark control (Fig. 5.10 (b)). In Figs. 5.9 (a-l), similar results for perithecial distribution plates without cellophane membranes are illustrated. Although growth on CMA overlaid with cellophane membranes resulted in greater perithecial frequency, perithecial distribution was not altered significantly. The peak in perithecial frequency occurred following illumination between 32-40 hours, fewest perithecia developed after illumination between 48-64 hours, and illumination after 72 hours produced little effect on subsequent perithecial development (Fig 5.10 (c)).

Fig. 5.10 summarises the effects of the various light treatments on total protoperithecial and perithecial frequencies. Although variation in total frequencies exhibit very similar patterns, it is interesting to note that the proportion of protoperithecia which developed into perithecia varied inversely with protoperithecial frequency (Fig. 5.10 (b)). Clearly at high protoperithecial densities, the number of fruitbodies which subsequently matured might be limited by nutritional as well as spatial considerations. It is not obvious, however,

Figure 5.8 (a-l): Effect of light exposure during protoperithecial formation on subsequent perithecial formation, on CMA overlaid with cellophane membranes. Perithecial frequency was determined by dividing each sample (a segment equivalent to 1/16 of the Petri plate) into 8 sectors. (Each bar in Fig. 5.8 therefore corresponds to a length of radius equivalent to 3 bars in Fig. 5.7.) Each bar represents the mean for 4 plates (16 samples in all) ± standard error. The results for the dark control are included in each graph, although the error bars for the control data, shown in the first graph on each page, are omitted thereafter.

Figure 5.9 (a-l): Effect of light exposure during protoperithecial formation on subsequent perithecial formation, on CMA without cellophane membranes. Details as for Figure 5.8.

Figure 5.8(a-d)

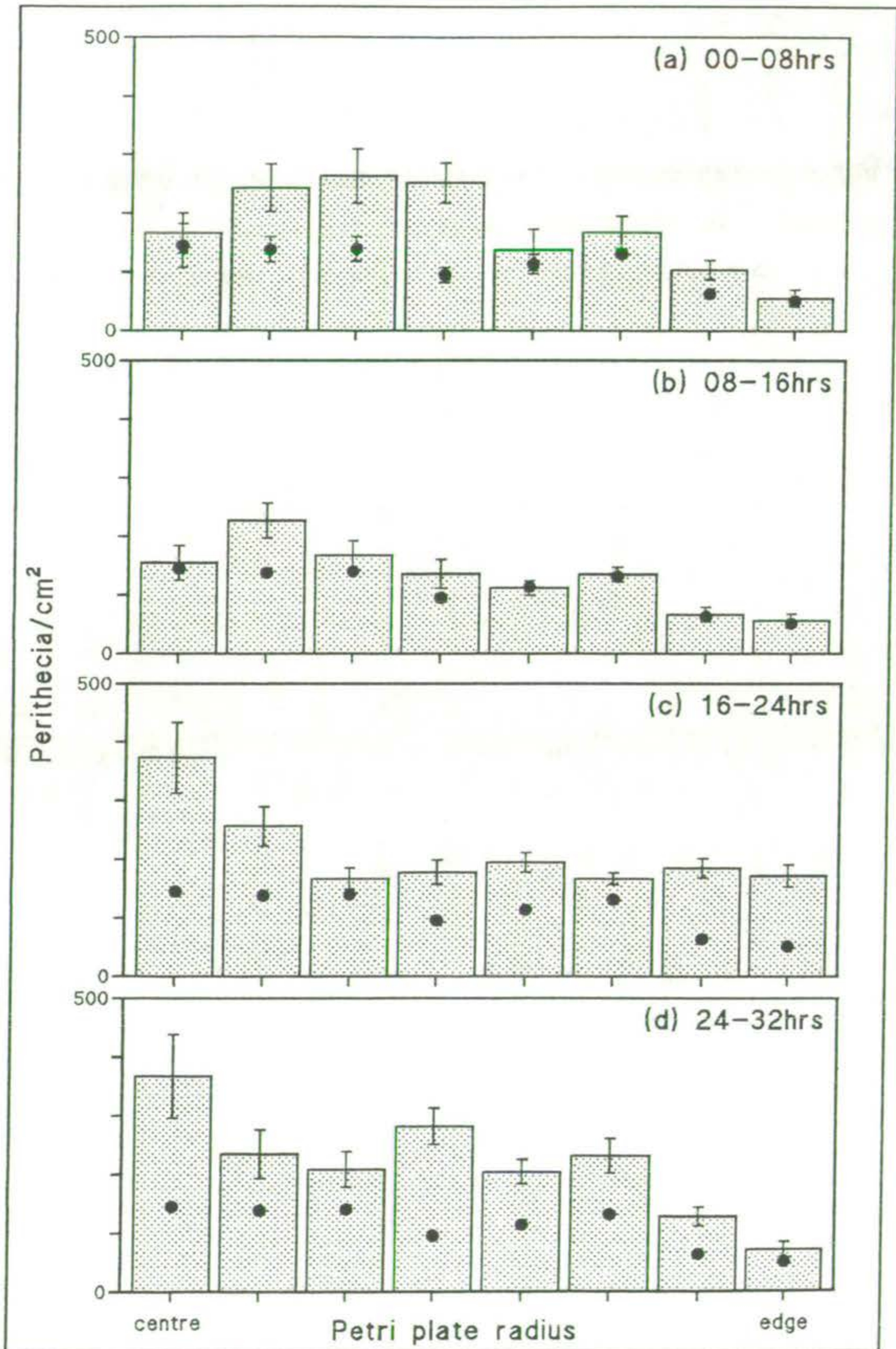


Figure 5.8(e-h)

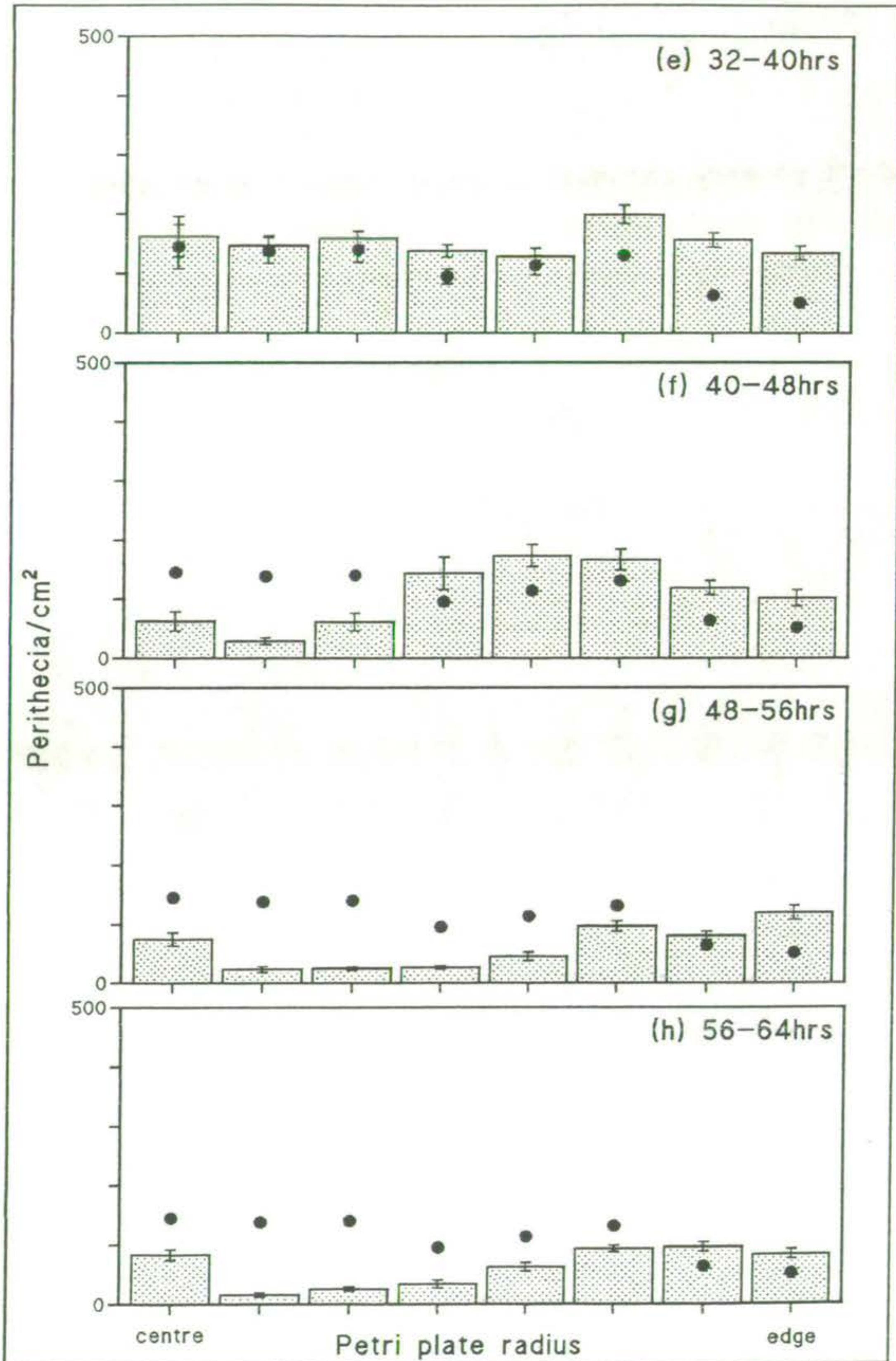


Figure 5.8(i-l)

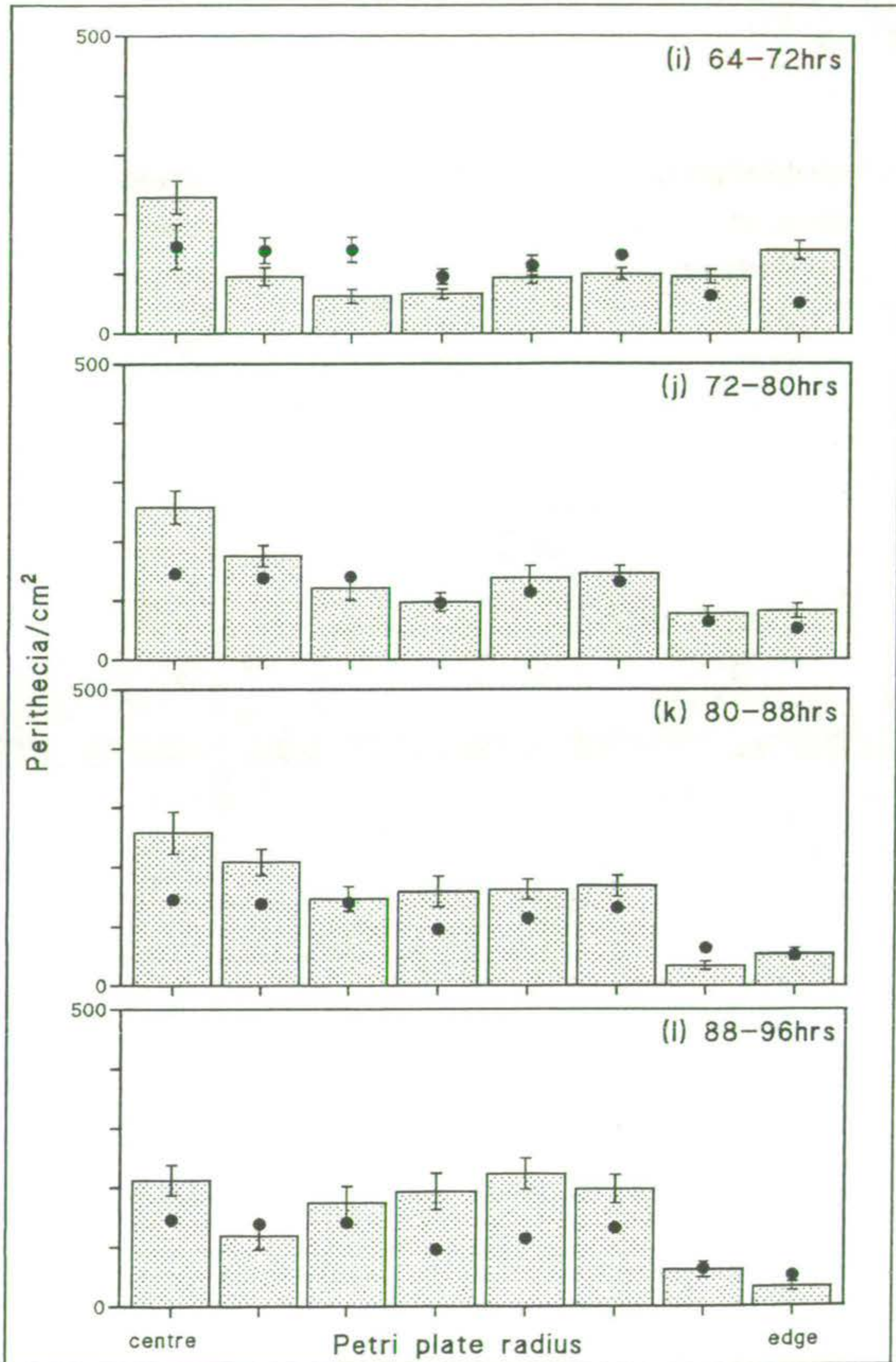


Figure 5.9(a-d)

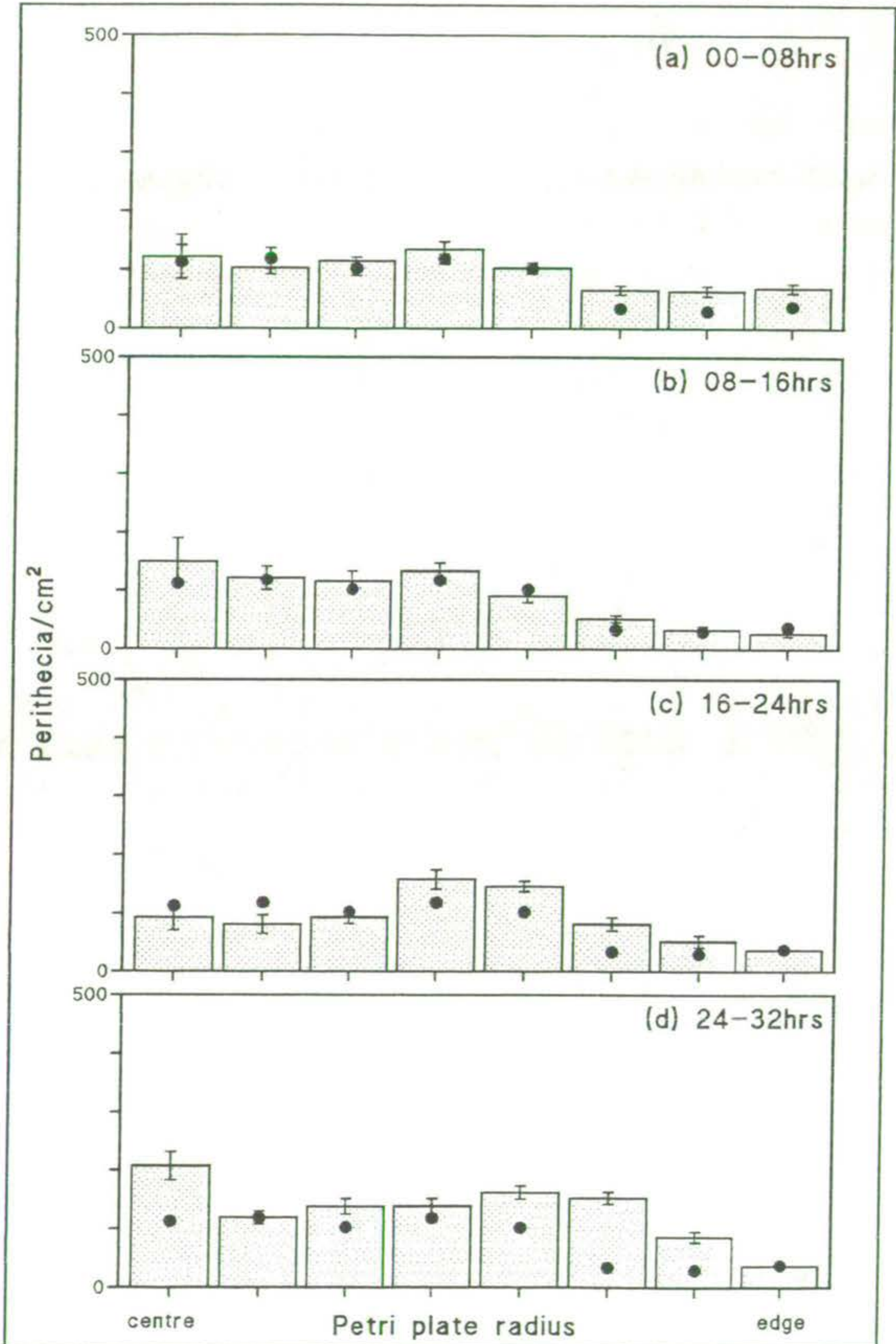


Figure 5.9(e-h)

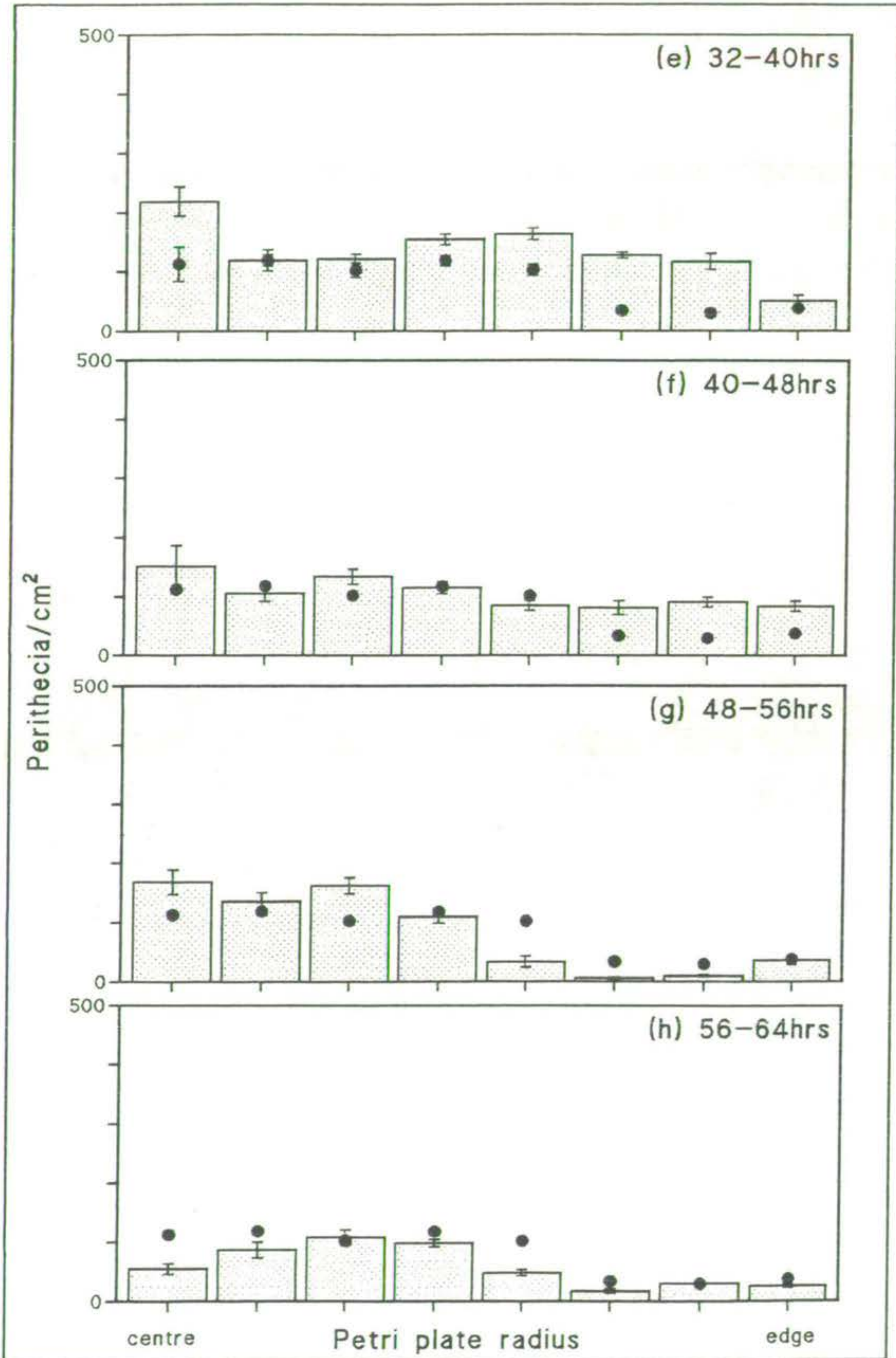


Figure 5.9(i-l)

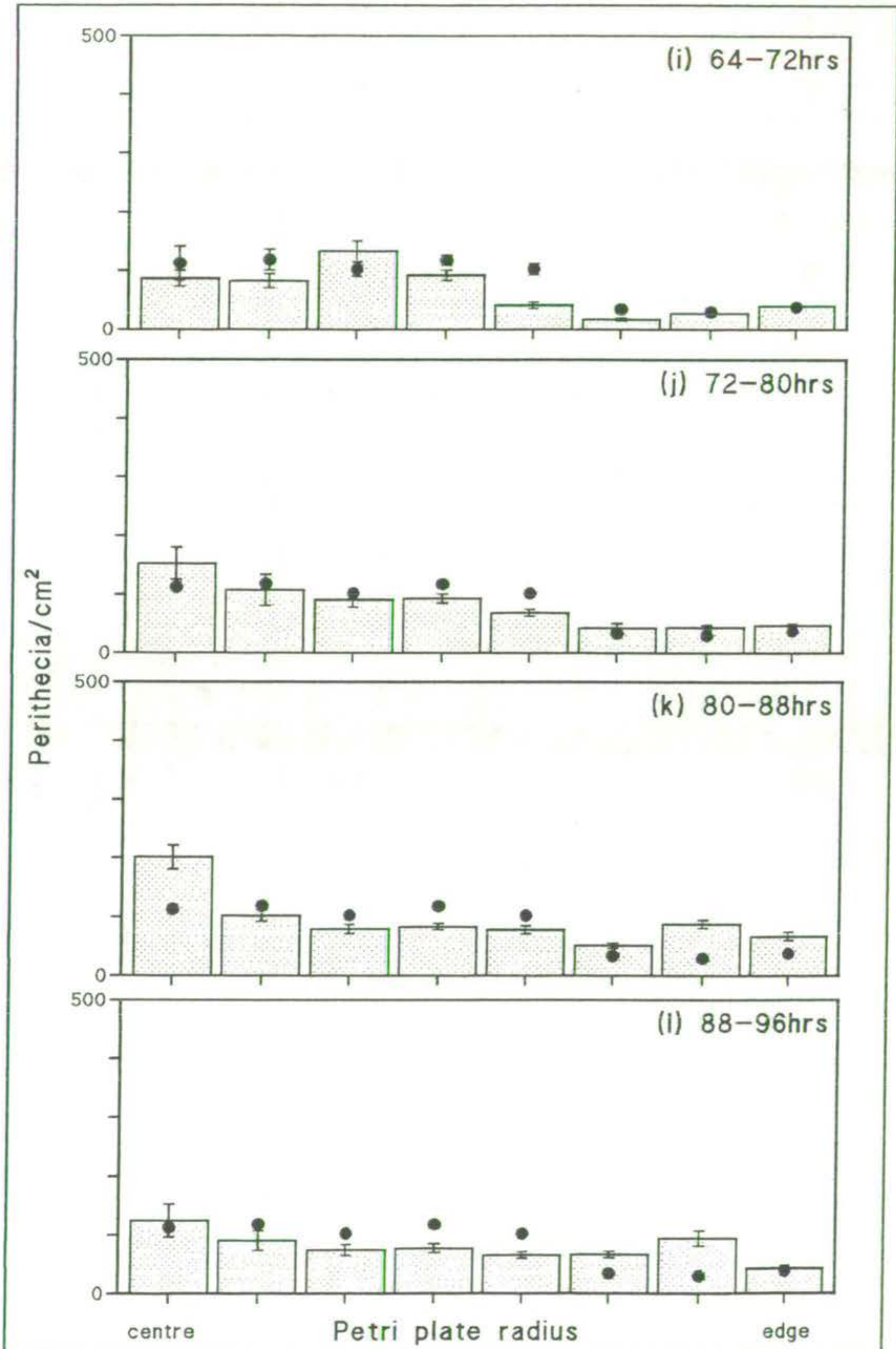


Figure 5.10 (a-c): Effect of light exposure during protoperithecial formation on protoperithecial and perithecial frequencies: Summary. Figs. 5.7 - 5.9 illustrate the distribution of protoperithecia or perithecia produced in response to various light treatments. Fig 5.10 shows the total frequency of protoperithecia or perithecia for the same treatments.

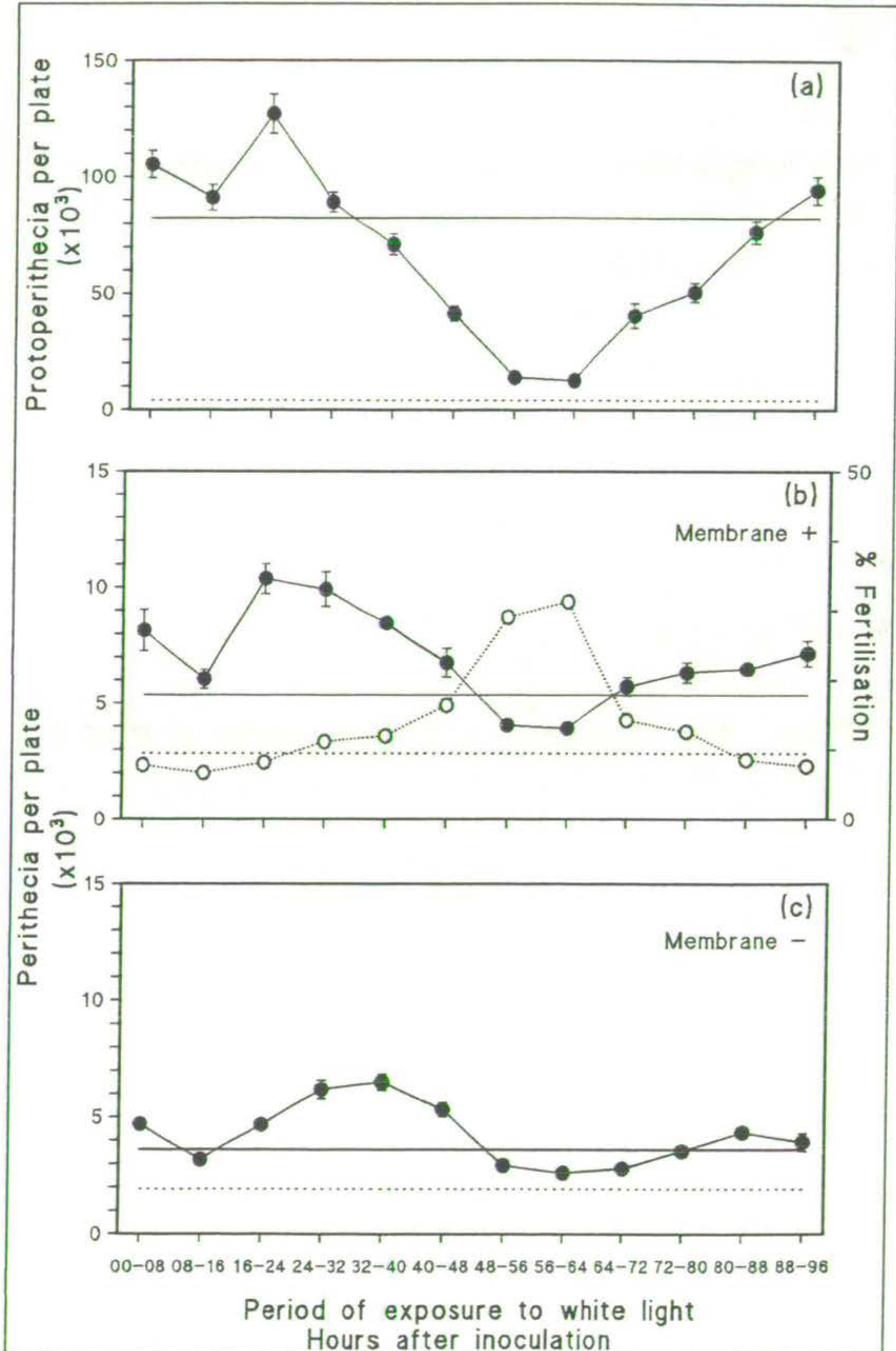
(a) Protoperithecia: dark control (solid horizontal line) = 80,122 ± 4,012; light control (dotted horizontal line) = 4,159 ± 1,878.

(b) Perithecia (membrane +): dark control = 5,261 ± 324; light control = 2,807 ± 368. Closed circles are perithecial frequencies. Open circles represent the % of protoperithecia which developed into perithecia, and illustrate that when protoperithecial frequency was lowest, the proportion of protoperithecia which developed into mature perithecia was greatest. (The use of "% fertilisation" as a label for the Y2 axis may not be strictly accurate, since it is possible that all protoperithecia are fertilised, but that subsequent development is blocked.)

(c) Perithecia (membrane -): dark control = 3,591 ± 127; light control = 1,966 ± 218.

All data points represent the mean for 16 replicates ± standard error.

Figure 5.10



why only 30% of those protoperithecia which formed at low density should mature. There would appear to be two possible explanations: (a) light exposure during protoperithecial differentiation, may have resulted not only in reduced protoperithecial frequency, but also in reduced viability of the protoperithecia which formed; or (b) only around 30% of all protoperithecia detected were functional, irrespective of the light treatment. Staining with Nile red revealed the presence of numerous compact, spherical structures with high lipid content, which were scored as protoperithecia, but it is possible that not all of these structures were fully functional. In either event, perithecial density was limited not only by the number of protoperithecia, but also by factors such as nutrient availability, and the net effect was that variation in perithecial production represented a "damped" version of protoperithecial frequency.

5.2.2.3 Effect of light wavelength on multicellular development

Experiments were carried out to determine which light wavelength(s) caused the predominantly inhibitory effects described in the previous sections. Petri plates inoculated with a strain of *mtA* were incubated under coloured or neutral density filters for 4 days, with control plates incubated under white light or in the dark. After 4 days, VHA formation was assessed, and protoperithecial frequency was determined. Other cultures were crossed by spermatisation and then incubated in the dark for 6 days, before the frequency of mature fruitbodies was determined. In a parallel experiment, uncrossed cultures were incubated under the various light treatments for 10 days, and the frequency of uncrossed perithecia was determined.

After 4 days incubation under white light, VHAs had formed over almost the entire surface of the plate, apart from the area immediately around the inoculum, while dark-grown cultures produced no VHAs, and those incubated under neutral density filters showed reduced VHA formation. No VHAs were formed on cultures grown under red light, while under blue light, VHA frequency and distribution were equivalent to those under white light (data not shown). The results indicated that VHA production was stimulated by blue light.

Fig. 5.11 shows that protoperithecia were most numerous in the dark, and were produced at lowest frequency under blue or white light. Incubation under neutral density filters resulted in the production of intermediate numbers of protoperithecia, suggesting an inhibitory effect which was dependent not only upon wavelength but also upon light intensity. The apparent inhibitory effect on protoperithecial formation exerted by red light was unexpected, but was

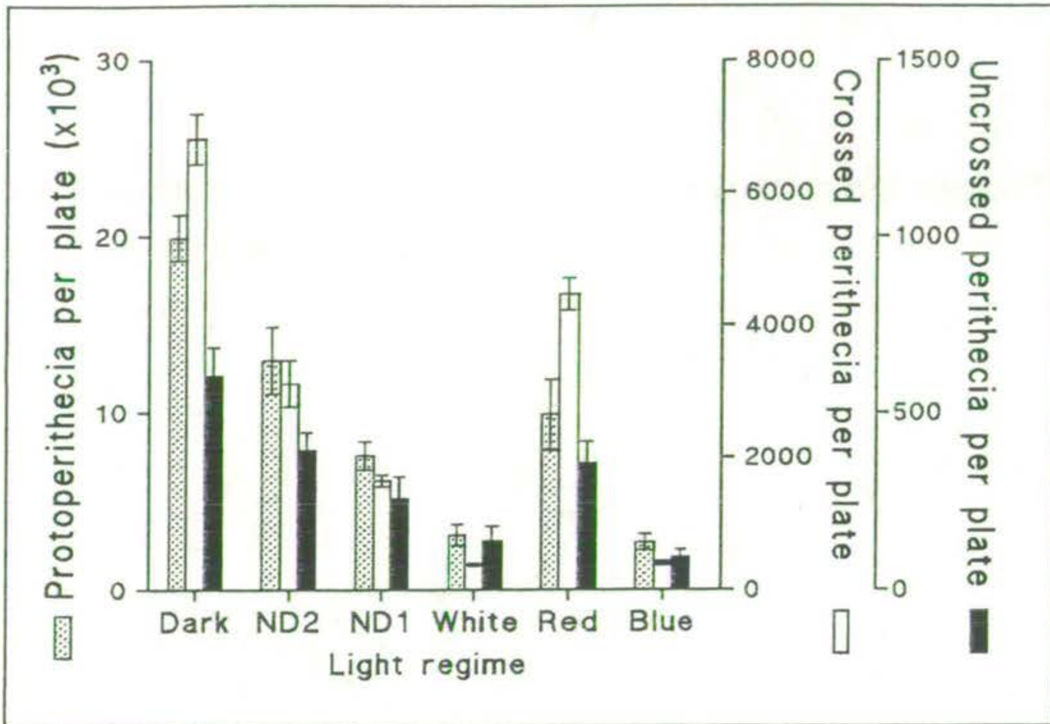


Figure 5.11: Effect of light wavelength and intensity on the formation of protoperithecia, crossed and uncrossed perithecia. Newly-inoculated *mtA* cultures were incubated either in the dark, under white light, or inside packets constructed from the following polycarbonate lighting filters: (Figure in brackets is the % transmission for each filter.) Blue (18.70); red (18.90); neutral density 2 (13.73); neutral density 1 (23.54). After 96 hours under each light treatment, protoperithecial frequency was determined. Crossed and uncrossed cultures were incubated in the dark for a further 6 days, and then perithecial frequency determined. Each bar represents the mean for 8 replicate plates $\pm$ standard error.

reproduced in two replicate experiments. Although blue light responses are well documented in fungi (Senger, 1984), red light effects are more rarely reported.

The effect of light wavelength on perithecial production was broadly similar to that on protoperithecial formation, with the greatest frequencies of both crossed and uncrossed perithecia produced in the dark, and lowest numbers under blue or white light (Fig. 5.11). Incubation under blue or white light resulted in the formation of very few uncrossed perithecia, largely because protoperithecial formation was reduced in illuminated cultures. Of the 20×10^3 protoperithecia produced on a typical dark-grown culture, around 3 % developed into uncrossed perithecia: under blue or white light, protoperithecial frequency fell to less than 5×10^3, and 4.5 % of protoperithecia developed into uncrossed perithecia.

The effect of short periods of illumination with blue or red light was also examined. Cultures were incubated in the dark for 96 hours, apart from 12 hour periods of illumination with blue or red light. Protoperithecial frequency was determined in 4 day old cultures. This procedure resembled that used for investigation of protoperithecial formation under white light (Fig. 5.6), although the period of exposure to filtered light was extended to compensate for reduced light intensity. In consequence, the number of time points was reduced, and some variation may have been lost. Nevertheless, Fig. 5.12 shows that exposure to blue light produced a similar effect to illumination with white light (Fig. 5.10 (a)). Exposure to blue light after the mycelium reached the edge of the Petri plate (approximately 48 hours after inoculation) resulted in a pronounced reduction in protoperithecial frequency. Later light treatments had progressively less effect on protoperithecial formation, and after exposure to blue light from 84-96 hours after inoculation, protoperithecial frequency was not significantly different to the dark control. Similar periods of exposure to red light indicated that the observed reduction in total protoperithecial frequency (Fig. 5.11) was largely the result of an effect exerted on young mycelia, several hours before protoperithecial formation commenced (Fig. 5.12). This inhibitory effect was greatest in plates exposed to red light between 12-24 hours after inoculation, and illumination in the period from 36-96 hours after inoculation produced no significant deviation from the dark control.

5.2.2.4 Effect of photoperiod on multicellular development

When considering the effects of photoperiod, it should be remembered that although an alternating light:dark cycle approximates to natural conditions

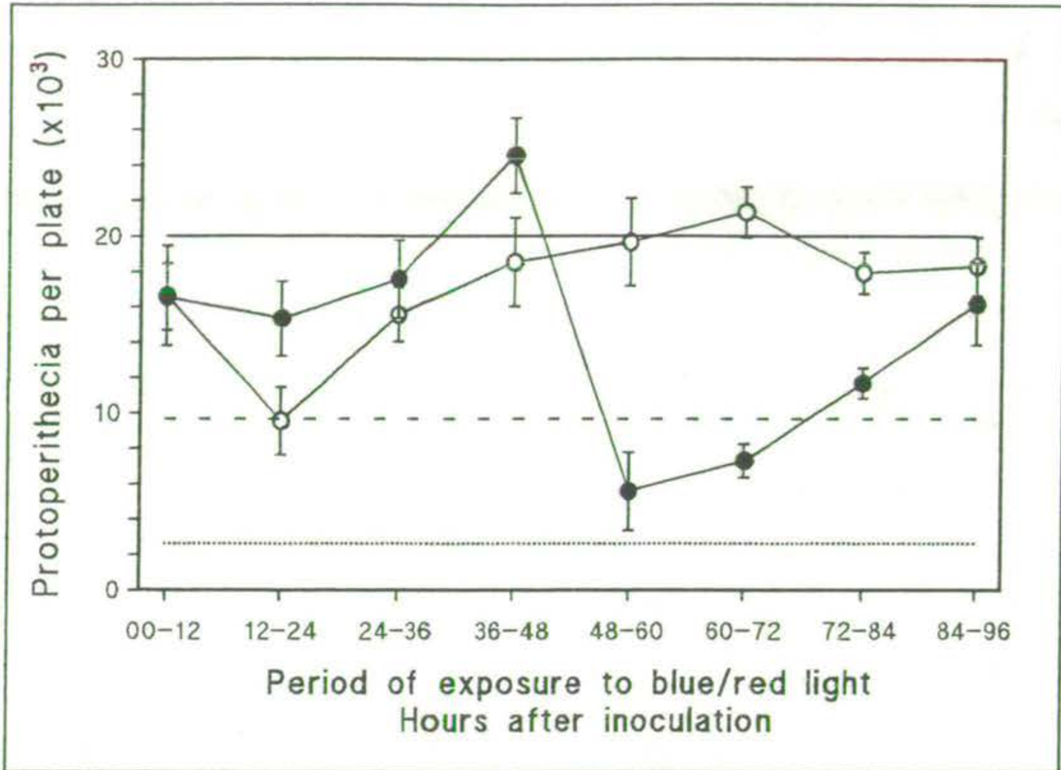


Figure 5.12: Effect of exposure to 12 hour periods of blue or red light on subsequent protoperithecial formation. In a modified version of the experiment outlined in Fig. 5.6, dark-grown cultures were subjected to 12 hours illumination with blue or red light. Protoperithecial frequency was determined 96 hours after inoculation of cultures. Open circles represent values for red light, closed circles for blue light. Each point represents the mean for eight replicate plates ± standard error. Horizontal lines represent the controls for dark incubation (solid line), 96 hours red light (dashed line), and 96 hours blue light (dotted line). For the standard errors attached to these values see Fig 5.11.

much more closely than does continuous illumination, it does not reproduce the gradual transition from light to dark, nor the changes in light quality, to which a fungus is exposed during a typical diurnal cycle. Photoperiod experiments carried out under laboratory conditions do not provide accurate simulations of the natural environment, and should not be interpreted as such.

Cultures were incubated for 4 days on a 12:12 light:dark cycle (with white, blue or red light), beginning with the light phase. Control plates were incubated in continuous darkness. The results showed that the formation of protoperithecia and perithecia under alternating light:dark conditions (Fig. 5.13) was very similar to that under observed under constant light of similar wavelength (Fig. 5.11). VHA formation and pigment production in cultures grown under blue:dark or white:dark cycles were also similar to cultures incubated under constant blue or white light. In view of these results, no further investigations of the effect of photoperiod were carried out.

5.2.3 Effect of carbon source on multicellular development

5.2.3.1 VHA formation

In Chapter Three it was suggested that the standard growth conditions provided in the laboratory did not supply the necessary combination of factors to allow full development of the VHA pathway. CMA is routinely used as a culture medium which promotes sexual reproduction, and may not contain substances which stimulate full development of other pathways. Such a hypothesis was supported by the observation of enhanced VHA development on media overlaid with cellophane membranes, or containing intact rabbit pellets (section 3.2.1.4.2). A series of experiments was therefore carried out in which the carbon sources and concentrations in the growth media were varied, and the carbon/nitrogen ratio thereby adjusted. Rigorous nutritional investigations would clearly require the substitution of CMA with chemically defined media whose components could be varied systematically. Corn meal agar, although providing a combination of nutrients which supports mycelial growth and perithecial production, is of variable composition. The manufacturers (Oxoid) are unable to provide even approximate figures for carbon and nitrogen contents, and the variety of grain used to provide the corn meal is changed according to cost and availability. Nevertheless, CMA has long been used as a growth medium for *S. brevicollis* (e.g. Olive and Fantini, 1961; Shaw, 1962: MacDonald and Bond, 1976), and was used throughout the morphological and genetic studies described in this thesis. Hence it was not felt that a change in growth medium was advisable, particularly

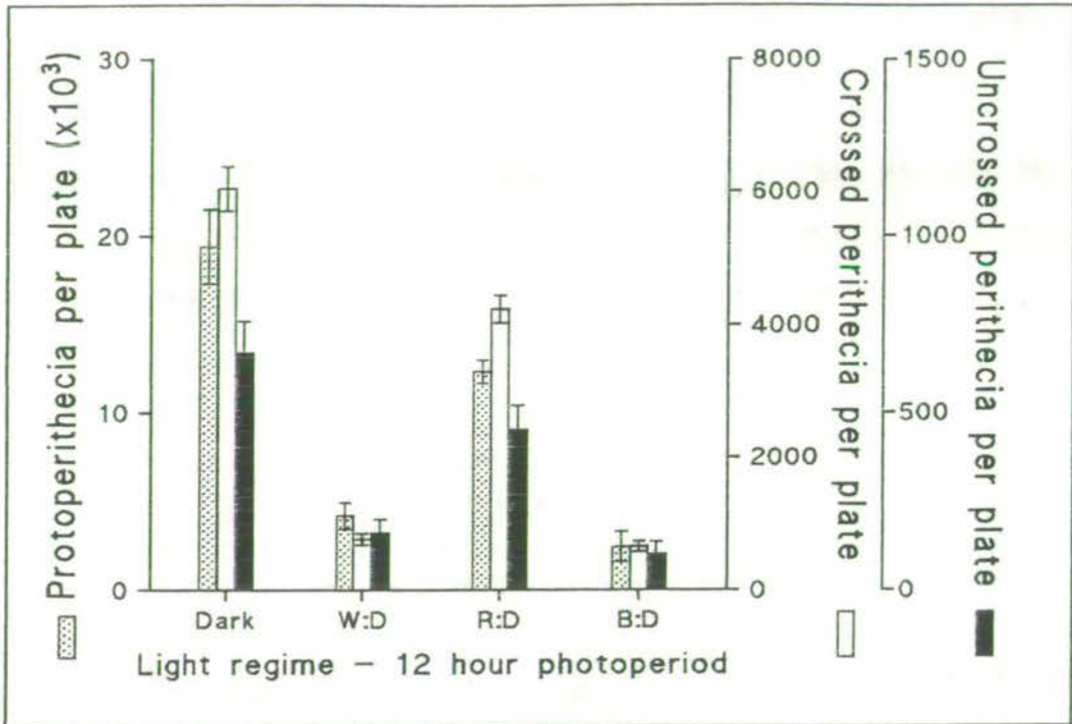


Figure 5.13: Effect of 12:12 dark:light photoperiod on formation of protoperithecia and perithecia. Plates inoculated with a strain of *mtA* were incubated in alternating light:dark, beginning in the light phase. After 96 hours, protoperithecial frequency was determined. Crossed and uncrossed cultures were incubated in the dark for a further 6 days, and perithecial frequency determined. Each bar represents the mean for 8 replicate plates ± standard error. X axis legend: W:D = white light:dark; R:D = red light:dark; B:D = blue light:dark.

since the primary objective of these nutritional studies was to differentiate between the structures formed on CMA. The observations recorded in the following sections therefore refer only to the effects of variation in the *additional* carbon source (0.3 % sucrose and 0.2 % glucose in CMA). The corn meal extract added to technical agar to make corn meal agar is predominantly starch, a polysaccharide which is clearly utilised by *S. brevicollis* to produce modest growth and perithecial formation on corn meal agar with no added carbon source (see later).

The sucrose and glucose in CMA were substituted with alternative carbon sources at equivalent carbon concentrations (170 mM carbon), with control plates containing corn meal agar (17 g per litre distilled water) with no added carbon source. The growth rates of light and dark incubated mycelia on different media are illustrated in Fig. 5.14 (a, b). Irrespective of light regime, the most rapid mycelial growth was supported by media containing sodium acetate, followed by glucose, sucrose or xylose (which were virtually indistinguishable), while the medium containing no added carbon source produced better hyphal growth than that containing lactose.

No VHAs were produced on dark grown plates, irrespective of the carbon source provided in the growth medium. All carbon sources except xylose supported similar degrees of VHA development when cultures were incubated in the light, although few VHAs were produced on media with no additional carbon source (data not shown). Cultures growing on medium supplemented with xylose developed a greenish-brown pigmentation when grown in the light, and did not produce VHAs. It is not known whether the production of the more typical carotenoid-like pigment was absent from such cultures, or simply masked by the darker pigment.

The effect of concentration of two sugars (glucose and sucrose) on VHA production was also examined. Cultures were inoculated on media supplemented with either sucrose or glucose (in the range 1-400 mM), and incubated in continuous light for 4 days. VHAs were produced in approximately equal numbers on media containing either sugar at concentrations up to and including 200 mM (data not shown). At 400 mM, hyphal growth was relatively poor and few VHAs were produced. At no concentration did VHAs develop to any greater extent than that seen on CMA.

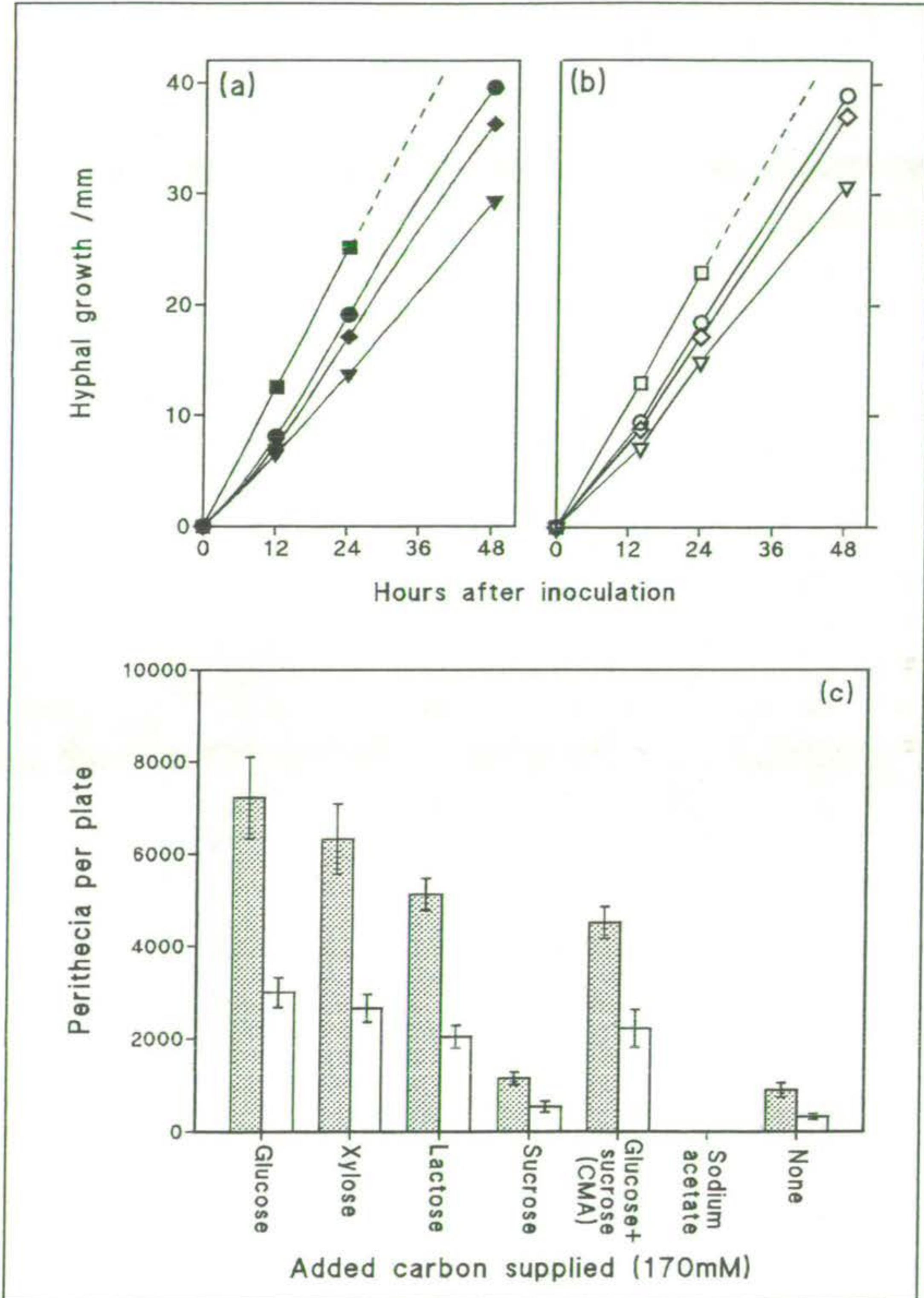
Figure 5.14: Effect of various carbon sources and illumination conditions on mycelial growth and perithecial formation.

(a) Mycelial growth in darkness on media containing alternative added carbon sources. In each instance, the concentration of added carbon was equivalent to that in CMA (170 mM). Each data point represents the mean for four radii measured on 8 replicate plates (32 measurements in all) $\pm$ standard error. Standard errors which plot within the datapoints and are omitted. Symbols for carbon sources are as follows: square = sodium acetate; circle = glucose, sucrose and xylose; diamond = CMA, and "none" - media containing cornmeal agar supplemented with yeast extract but no added carbon source; triangle = lactose.

(b) Replicate experiment with cultures incubated in constant white light. In both light and dark, mycelia grown on media containing sodium acetate reached the edge of an 8.5 cm Petri plate in less than 48 hours.

(c) Effect of carbon source and concentration on perithecial formation. Carbon sources and concentrations as described above. (Determination of protoperithecial frequency was not attempted since this would have required inoculation of cultures on cellophane membranes, which were themselves metabolised.) Each bar represents the mean for 8 replicate plates $\pm$ standard error. Shaded bars = dark-grown cultures, open bars = light-grown cultures

Figure 5.14



5.2.3.2 Fruitbody formation

The effect of differing carbon sources on perithecial formation was also examined (Fig. 5.14 (c)). Where fruitbodies were produced, perithecial frequency was higher in the dark than in the light, with greatest perithecial formation (in excess of 6 x 10^3 fruitbodies per plate) occurring on media containing glucose (C6) or xylose (C5). Perithecial production on media containing sucrose was not significantly different to that on media with no added carbon source (around 1 x 10^3 fruitbodies per plate). No perithecia were produced on media containing sodium acetate, although numerous microconidia developed on conidiophores which were readily visible to the naked eye.

Varying the concentration of glucose and sucrose in the growth medium had a more pronounced effect on perithecial production than on VHA formation. Perithecial frequency was optimal on media containing added glucose at a concentration of 30 mM (equivalent to 180 mM carbon) (Fig. 5.15 (a)). At glucose concentrations above 100 mM, hyphal growth became irregular with increased apical branching. The mycelium, typically hyaline in dark-grown cultures grown on CMA, accumulated a greenish-brown pigmentation on media containing glucose at concentrations above 150 mM. Centrum development was also atypical at high glucose concentrations, with relatively few asci produced within each perithecium, and asynchronous spore development within individual asci (Fig 5.15 (b)). The gelatinous sheath which surrounded each ascospore was unusually thick, and the ascospores themselves were seen to be narrower than normal. No spore discharge was observed at glucose concentrations above 150 mM.

Few perithecia developed on media containing sucrose at concentrations in the range 1-150 mM, and the development of similar numbers of perithecia on media with no added sugar suggested that sucrose was not utilised for fruitbody production (Figs. 5.14 (c) and 5.15 (a)). Only at concentrations of 200-400 mM sucrose were additional perithecia produced, and these matured rather slowly (discharging spores 14 days after inoculation). Each perithecium contained numerous asci, and within each ascus, spore maturation was synchronous.

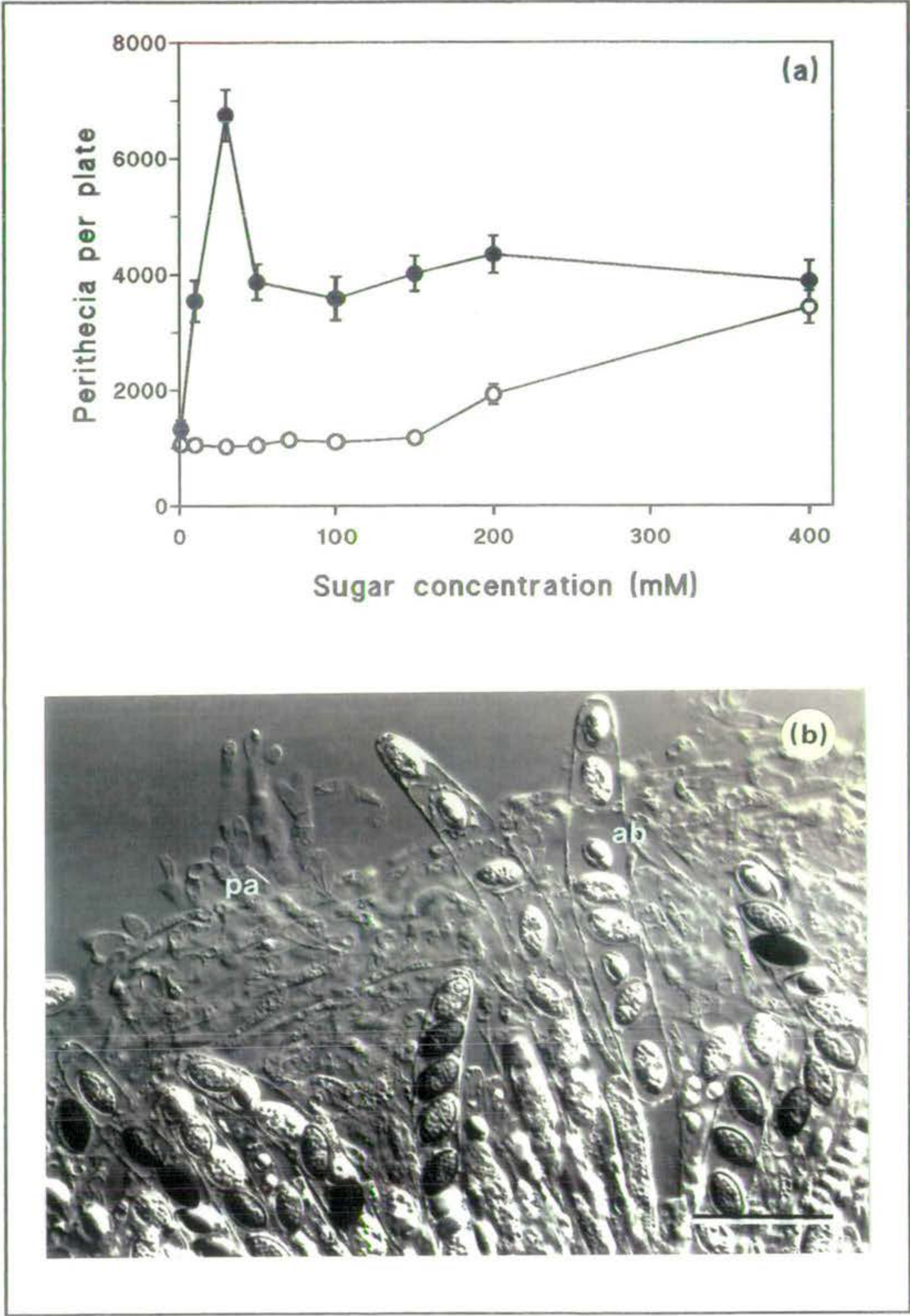
5.2.4 Effect of nitrogen source on VHA development

The experiments described above varied the carbon source, carbon concentration, and carbon:nitrogen ratio present in the growth media, without generating any development of the VHA pathway beyond that seen on CMA. Although the composition of animal dung is highly variable (Tisdale and Nelson,

Figure 5.15: Effect of glucose and sucrose concentration on perithecial formation. (a) Perithecial frequency. Perithecial formation was monitored on media containing added sucrose or glucose over a range of concentrations from 1-400 mM. Closed circle = glucose; open circle = sucrose. Each point represents the mean for 8 replicate plates ± standard error. Error bars which plot within the data points are omitted.

(b) Part of the centrum from a 12 day old, crossed perithecium grown on media containing 400 mM added glucose. Relatively few asci are produced within each perithecium, and paraphyses (pa) persist. Within each ascus, ascospore maturation is asynchronous, and several spores appear to have aborted (ab). Bar = 50 μm.

Figure 5.15



1956; Smith, 1958) a figure of 0.50% nitrogen would appear to be a reasonable approximation for whole manure, a figure well in excess of the 0.01% nitrogen provided in CMA by the addition of yeast extract (0.1% (w/v) yeast extract, which contains 9.8 % nitrogen). Nitrogen will also be present in corn meal agar (since the manufacturers produce corn meal extract using hot water, which may dissolve some proteins) but it is unlikely that the total nitrogen present in CMA approaches that in manure.

In initial experiments, the yeast extract in CMA was substituted with the nitrogen sources provided in other growth media (ammonium nitrate (Vogel, 1956) or potassium nitrate (Westergaard and Mitchell, 1947)), or with urea, the chief form of nitrogen in manure (Tisdale and Nelson, 1956). In each case, the added nitrogen concentration provided was equivalent to 0.01% (w/v). Cultures were incubated either in the dark or in constant white light for 4 days, after which VHA formation was assessed visually. No VHA formation took place on dark-grown cultures. In the light, VHA formation was limited on media containing urea, but occurred normally on media containing NH_4NO_3 or KNO_3. In subsequent experiments, the nitrogen concentration in the media was varied between 0.01 and 1.00%. At high nitrogen concentrations (in excess of 0.05%) mycelial growth was disrupted and few or no VHAs were produced, while at lower nitrogen levels VHA production was similar to that on CMA (data not shown).

None of the nitrogen sources or nitrogen concentrations examined were observed to promote intense VHA development on artificial media of the kind observed on rabbit pellets. Nor was any evidence obtained that alteration of the carbon or nitrogen components of the media could stimulate the development of VHAs into more "sclerotial" structures. The most intense VHA production observed in culture throughout the present study remained that seen on plates overlaid with cellophane membranes (Figs. 3.17 and 3.18).

5.2.5 Effect of temperature on multicellular development

The effect of one further environmental variable, temperature, was also examined. It is a well established phenomenon in fungi that the temperature range over which mycelial growth can take place is wider than that facilitating reproduction (reviewed by Moore-Landecker, 1992), and this was found to be the case in *S. brevicollis.* Mycelial growth in race tubes was examined between 0-50°C. All tubes were incubated in the dark for a period of 160 hours, after which the position of the mycelial front was recorded. The results in Fig. 5.16

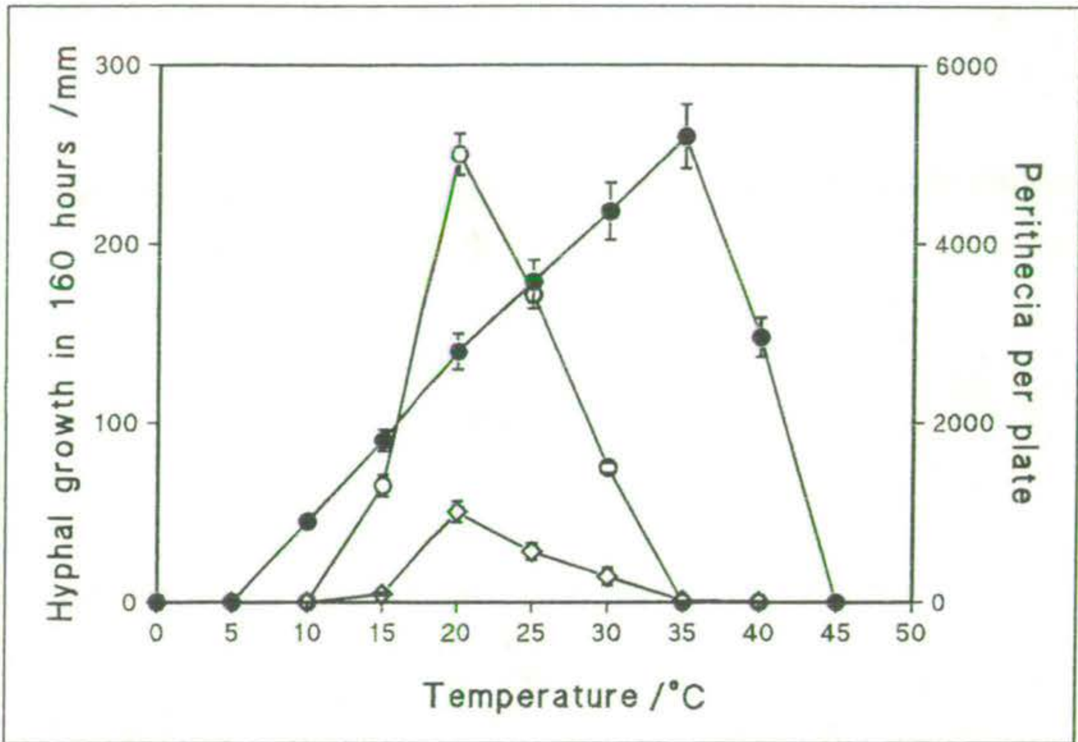


Figure 5.16: Effect of temperature on mycelial growth and perithecial formation. Closed circle = hyphal growth; open circle = crossed perithecia; open diamond = uncrossed perithecia. Hyphal growth was monitored over a range from 0-50°C. Mycelial extension was measured after 160 hours incubation in 30 mm Race tubes (each poured with 20.0 ml CMA). Each point represents the mean for 8 race tubes ± standard error. Error bars which plot within the data symbol are omitted. Development of crossed and uncrossed perithecia was monitored within the range 10-35°C, over growth periods adjusted to give the same number of temperature-hours as 10 days growth at 25°C (see Table 5.2). Each point represents the mean for 8 replicate plates ± standard error.

Temperature °C	Days before crossing (a)	Days after crossing (b)	Total incubation period (c)
10	10d	15d	25d
15	6d 16hrs	10d	16d 16hrs
20	5d	7d 12hrs	12d 12hrs
25	4d	6d	10d
30	3d 8hrs	5d	8d 8hrs
35	2d 21hrs	4d 7hrs	7d 4hrs
40	2d 12hrs	3d 18hrs	6d 6hrs

Table 5.2: Variation of incubation time according to temperature (length of growth period corresponding to 10 days at 25°C). Cultures were crossed by spermatisation after the times shown in column (a), then incubated for the times shown in column (b) to allow perithecial maturation. Uncrossed cultures were incubated for the same total time period (column (c)).

show that at temperatures between 10-35°C, mycelial growth rate increased by 0.05 mm hr^{-1} $°C^{-1}$. At 40°C mycelial growth became very sparse, and no hyphal growth occurred at 45°C. When the experiment was repeated in the light, all growth rates were reduced slightly, although maximum hyphal extension was still observed at 35°C (data not shown). Production of VHAs took place over the temperature range which permitted mycelial growth, although relatively few structures formed below 20°C or above 35°C. The extent of development of individual VHAs was not influenced by temperature.

In order to examine the effect of temperature on sexual development, it was necessary to adjust the length of the incubation period to allow equivalent developmental stages to be compared. Perithecial formation was monitored within the interval from 10-40°C, over the time periods set out in Table 5.2. The formation of both crossed and uncrossed perithecia was optimal at 20°C and took place over a narrower temperature range than that which permitted hyphal growth, and at 35°C (when hyphal growth was greatest), no fruitbodies were produced. The frequency of spore production in uncrossed perithecia was examined within the range 15-30°C. No spores were detected within uncrossed perithecia produced at 15°C, but since the total number of such perithecia was low, it was not inconceivable that spore production could take place at this temperature. Between 20-30°C, the percentage of spore-bearing fruitbodies was approximately 10% of all uncrossed perithecia, and homokaryotic spore production showed no clear variation with temperature.

5.3 Discussion

5.3.1 Effect of light on pigmentation and multicellular development

The effects of light on the development of *S. brevicollis* were found to be as follows:-

(i) Blue light stimulated the formation of a carotenoid-like pigment (with a main absorption peak at 474 nm) which was not detectable in mycelia grown under red light, or in the dark. Exposure of dark-grown cultures to blue light resulted in rapid pigment synthesis.

(ii) Exposure to blue light also caused the formation of vegetative hyphal aggregates (VHAs), whose structure is described in Chapter Three. VHA formation lagged behind carotenoid production and was detected only after several hours of illumination.

(iii) Short periods (8 or 12 hours) of exposure to blue or white light were found to have a stimulatory, inhibitory or neutral effect on protoperithecial

production, according to the age of the mycelium when illuminated. Continuous light resulted in reduced protoperithecial formation. Largely as a consequence of the inhibitory effect on protoperithecial production, exposure to blue or white light resulted in a reduction in the number of perithecia which matured after crossing.

(iv) Those crossed perithecia which matured in light-grown cultures were morphologically indistinguishable from perithecia which developed at equivalent density in the dark. Upon illumination, both light- and dark-grown perithecia exhibited phototropic neck curvature and light-stimulated spore discharge.

(v) Exposure to blue or white light also resulted in a reduction in the number of uncrossed perithecia which developed, again reflecting reduced protoperithecial frequency. The number of uncrossed perithecia which contained ascospores was lower in light-grown cultures than those incubated in the dark (Fig 4.23).

(vi) Numbers of protoperithecia, crossed and uncrossed perithecia were also reduced following exposure to red light, although the inhibitory effect was less pronounced than that produced by blue or white light.

(vii) The maximum inhibitory effect on protoperithecial production caused by a single period of illumination was similar to the effect of 96 hours continuous light exposure. This suggested that the receptor(s) could be saturated by a relatively short period of illumination at an appropriate stage in the developmental sequence.

The observation that production of carotenoid-like pigments in *S. brevicollis* is stimulated by blue light is not surprising, since many fungi produce similar pigments in response to short wavelength radiation (Senger, 1984; Moore-Landecker, 1992). The absorption spectrum for the pigment produced in light-grown cultures of *S. brevicollis*, showed marked similarities to that for neurosporoxanthin (4'-apo-ß-caroten-4'-oic acid), a pigment extracted from light-grown cultures of *N. crassa* (Zalokar, 1957). Carotenoid formation in the latter species has been studied extensively (e.g. Ruddat and Garber, 1983), and phytoene (a colourless precursor of several carotenoid pigments) has been shown to accumulate in dark-grown mycelia. A similar accumulation of pigment precursors in *S. brevicollis* would explain the rapid development of pigmentation in dark-grown cultures following brief exposure to light. Carotenoid production has also been shown to be stimulated by short wavelength radiation in *S. fimicola* (Ingold and Hadland, 1959b), although these authors reported the formation of

some pigment in the dark, and no such pigmentation could be detected in *S. brevicollis*.

The inhibition of perithecial formation observed in *S. brevicollis* can be considered as a combination of two effects: inhibition of protoperithecial formation, and inhibition of perithecial maturation. It would appear that the former is a direct response to blue light exposure during the period of protoperithecial development, and is apparently not reversed by return to the dark: exposure to 8 hours of white light in the period from 48-56 hours after inoculation produced an inhibitory effect equivalent to that which resulted from 96 hours illumination (Fig. 5.10 (a)). Short periods of illumination did not stimulate significant VHA production however, although numerous VHAs were produced under continuous light. It therefore seems reasonable to conclude that, while protoperithecial inhibition and VHA production are both blue light effects, the two responses are not directly linked. It is possible, however, that VHA development may reduce the number of protoperithecia which subsequently mature into perithecia, as a consequence of limiting the nutrient supply. Experiments using a variety of carbon sources indicated that provision of increased amounts of readily utilised monosaccharides such as glucose, resulted in the formation of greater numbers of perithecia than were produced on CMA (Fig. 5.14 (c)). Failure to detect storage products within VHAs does not necessarily contradict a nutrient limitation hypothesis: VHAs are composed of numerous fine hyphae whose formation requires the synthesis of relatively large amounts of cell wall material (particularly chitin, see Gooday (1978) for a review). The production of VHAs may therefore divert the components of such material away from developing perithecia.

Evidence in favour of possible nutrient limitation of perithecial formation in illuminated cultures was obtained from observation of varying patterns of perithecial distribution in dark- and light-grown plates. Although the total number of perithecia formed in the light was lower than in the dark, the density of fruitbodies in the central part of light-grown cultures was often greater (e.g. Fig. 5.5 DD and LD). The central area of the plate was also the region where VHA density was at a minimum. A relatively large proportion of the perithecia which matured on light-grown plates developed under the surface of the growth media, while VHAs were always restricted to the surface. These results were compatible with a hypothesis that VHAs inhibited perithecial development, either as a consequence of nutrient limitation, or possibly by occupying the surface of the media and restricting the space available for perithecial expansion.

An alternative explanation for perithecial formation below the surface of the growth medium may be found in the report of Bennett and Howe (1980) on sexual development in *Neurospora tetrasperma*. These authors discovered that although the first protoperithecia which developed on a young mycelium were almost all produced at the surface, the majority of later protoperithecia were differentiated beneath the surface. It might therefore be argued that the sub-surface perithecia seen in *S. brevicollis* arise from protoperithecia which are produced at a later stage in development, possibly when the degree of light sensitivity has declined. (In order to account for the fertilisation of protoperithecia developed below the surface of the medium it must be supposed that either these structures have trichogynes long enough to reach the surface, or that microconidia can germinate and produce at least limited mycelial growth prior to plasmogamy.)

Although a reasonable case can be made in favour of a nutrient diversion hypothesis, other evidence suggests that light may also have a direct effect in reducing perithecial numbers. When cultures incubated in darkness for 96 hours were illuminated after crossing, fewer perithecia developed than in cultures maintained in continuous darkness (Fig. 5.4). DL plates developed a few VHAs, predominantly around the edge of the Petri plate, but these were far less numerous than in cultures incubated in continuous light. In the case of strains such as those illustrated in Fig. 5.4 (a) and (b), it was hard to envisage how such limited VHA formation could lead to the observed reduction in perithecial number when cultures were illuminated after crossing. It would thus appear that perithecial maturation can also be directly inhibited by light exposure.

The development of VHAs on the surface of young mycelia may also have had an inhibitory effect on gaseous exchange in the underlying hyphae. Experiments on *N. tetrasperma* indicated that while fermentative glycolysis supported the production of ascogonial coils, their subsequent development into protoperithecia and perithecia was dependent on oxidative metabolism (Viswanath-Reddy and Turian, 1975). Thus the development of anaerobic conditions as a result of reduced gaseous exchange might lead to the inhibition of protoperithecia underneath a layer of VHA tissue.

An obvious method which might have been used to clarify the possible relationship between VHA formation and perithecial inhibition was mutant analysis, - examination of the effect of light on fruiting in strains which produce protoperithecia and perithecia, but not VHAs. Unfortunately such strains were not available, and time did not allow for an extensive programme of mutagenic

treatment and screening. None of the individuals isolated from stock crosses, nor those generated in various crossing experiments, exhibited female fertility without VHA formation, however, and this failure to isolate spontaneous mutants may indicate that the two pathways will prove difficult to separate genetically. Brief examination of a female-sterile double mutant (*per 1- per 2-*) supplied by D. J. Bond, revealed that in this case, loss of protoperithecial formation was accompanied by a failure to produce VHAs. After reaching the edge of the Petri plate, *per 1- per 2-* cultures exhibited premature mycelial senescence and marked loss of viability when subcultured, a phenomenon shown by MacDonald and Bond (1976) to be correlated with a sharp fall in the level of glucose 6-phosphate (G6P) in the growth medium. (In contrast, concentration of G6P remained more-or-less constant when wild type strains reached the edge of the Petri plate.) The same authors also reported that female sterile strains often reverted to fertility after being subcultured several times (Bond and MacDonald, 1976). Genetic analysis of these strains indicated that the mycelium had become heterokaryotic with revertant nuclei, (*per 1+* or *per 2+*) which were believed to have a selective advantage when a senescing mycelium was subcultured. In the context of the present study, it would be interesting to determine whether female-sterile mycelia which reverted for protoperithecial production also reverted for VHA formation. Observations to this effect would lend weight to a hypothesis that since *per 1* and *per 2* (or linked genes) influenced both VHA and protoperithecial formation, then the two developmental pathways may be more closely associated than would be deduced from morphological and environmental studies.

It is not clear to what extent, if any, the method of inoculation may affect subsequent development of protoperithecia and perithecia. Exposure of cultures to white, blue or red light in the period soon after inoculation caused fluctuations in the level of protoperithecial production (Figs. 5.10 (a) and 5.12). Such behaviour may have been influenced not only by light, but also by substances diffusing from the inoculum. These could have been nutritional factors, or compounds produced as a response to the severing of hyphae when inocula were cut from parent cultures. Since it has been observed that wounding can induce fruiting in both ascomycete and basidiomycete species (e.g. Pollock, 1975; Leonard and Dick, 1973; Leslie and Leonard, 1979b), it is possible that a diffusible substance produced by wounded hyphae in the inoculum block might influence protoperithecial formation in the daughter culture, particularly in the central part of the colony. Examination of protoperithecial distribution in

cultures irradiated with white light from 16-24 hours after inoculation - a light treatment which caused a modest stimulation of protoperithecial number relative to the dark control (Fig. 5.10 (a)) - showed that the highest density of protoperithecia was in the area immediately around the inoculum (Fig. 5.7 (c)). The same interpretation might also explain the apparent stimulation of protoperithecial formation by blue light (Fig. 5.12). It could be argued that irradiation prior to the mycelium reaching the edge of the Petri plate was of neutral effect, and that any increase in protoperithecial frequency was caused by substances in the media.

The inhibitory effect of red light on protoperithecial formation was caused by illumination during the first 36 hours after inoculation, and irradiation of older mycelia produced no significant reduction in numbers: there was no inhibitory effect immediately after the mycelium reached the edge of the Petri plate analogous to that observed with blue or white light. The observation that red light did not induce VHA formation, even though protoperithecial formation was reduced, lends further support to the idea that the two pathways are subject to separate control mechanisms.

The pathways leading to formation of crossed and uncrossed perithecia seem to be under very similar control, however, as might be expected. Almost all the factors which affected the formation of protoperithecia and crossed perithecia has similar effects on the number of uncrossed perithecia. The exception, noted in the previous chapter, was that the proportion of uncrossed perithecia which contained spores (i.e. the number of homothallic perithecia) was reduced in light-grown cultures, and those cultures on media overlaid with cellophane membranes. This apparent light inhibition of spore formation was not seen in crossed perithecia, and so may have been caused by nuclear events which were specific to uncrossed protoperithecia or perithecia. At no time was evidence for significant differential fertilisation of protoperithecia obtained, confirming the finding of MacDonald and Bond (1976) that perithecial distribution reflected that of protoperithecia. This was generally also the case for the development of protoperithecia into uncrossed perithecia, although a slight tendency was noted for uncrossed perithecia to form towards the edge of the Petri plate.

Although the examples of morphogenesis reviewed in Chapter One all are taken from work on ascomycete species, many of the most comprehensive studies to date have been carried out on basidiomycete genera such as *Coprinus* (Manachère (1988) and references therein.) In species such as *C. congregatus* where fruitbody production is induced by light, it has been shown that the

mycelium is not uniformly competent to differentiate primordia (Ross, 1982). Only a narrow zone representing the youngest hyphae receives the light stimulus, resulting in the formation of a ring of primordia. In *S. brevicollis*, where protoperithecial differentiation occurs in complete darkness, and the effects of light are largely inhibitory, no such localised response to light has been observed. There is an added degree of complexity in the study of basidiomycete fructification, since the form of individual sporocarps is also subject to light control. It has been shown for example, that continuous illumination of *C. congregatus* results in the production of substances in the cap which inhibit growth of the stipe (Robert and Bret, 1987). No comparable variation in morphology was ever observed between light- and dark-grown perithecia in *S. brevicollis*, even in sectioned material. A combination of light and age has been reported to cause morphological variation in *Gelasinospora reticulispora*, however, with either perithecia or cleistothecia being produced according to environment and mycelial age (Fukuda *et al.*, 1971).

Irrespective of the precise nature of its mechanism, the photoinhibition of perithecial formation observed in *S. brevicollis* is not a typical phenomenon in fungi, where the majority of light-sensitive species exhibit light-stimulated reproduction (section 1.7.1.1.2). Although a slight enhancement of perithecial frequency could be obtained by short exposure to blue light before the mycelium had become competent to differentiate protoperithecia, illumination during protoperithecial formation caused a reduction in protoperithecial frequency, and a similar level of inhibition was seen in response to constant light, or to a 12:12 light:dark photoperiod. The potential ecological consequences of these observations are considered in Chapter Six.

5.3.2 Effect of nutrient status on multicellular development

5.3.2.1 Protoperithecia, crossed perithecia and VHAs

It has long been recognised that the carbon sources which support optimal vegetative growth in fungi may be different to those which promote sexual reproduction (e.g. Hawker, 1957). The conclusions which can be drawn from observations made on carbon nutrition in *S. brevicollis* during the present study are necessarily limited, since defined media was not used. Nevertheless, the differential responses to added carbon sources are of interest.

S. brevicollis produced more perithecia on media supplemented with glucose than with sucrose (up to a concentration of 400 mM). This observation

was contrary to that of Bretzloff (1954) who found that on media containing 2 % sugar (approximately 60 mM sucrose, or 110 mM glucose), *S. fimicola* produced more numerous perithecia with sucrose. When the residual sugar present in the growth medium was determined, the same author found that very little sucrose had been utilised, and concluded that either *S. fimicola* did not produce sucrase (invertase), or that this enzyme was confined to the interior of the cells. Superior perithecial production on sucrose media was attributed to nutrient starvation, while glucose media supported excellent vegetative growth. Sugar uptake and metabolism by *S. fimicola* was also investigated by ap Rees *et al.* (1984). who showed that the fungus could neither take up sucrose, nor hydrolyse it extracellularly. The results obtained during the present study suggest a similar situation in *S. brevicollis.* An inability to utilise sucrose would not be particularly surprising, given that herbivore dung is likely to contain significant amounts of cellulose (which is broken down into glucose, via cellobiose), but very little free glucose or sucrose. Hence the invertase which would be necessary to hydrolyse sucrose (to glucose and fructose) may not be produced in this coprophilous species. It seems likely that at high concentrations, sucrose in the growth medium was partially hydrolysed during autoclaving, resulting in the presence of glucose, which then supported fruitbody formation. The failure of *S. fimicola* and *S. macrospora* to produce significant numbers of perithecia on media containing sucrose was reported by Hodgkiss (1969), who found lactose to be the best carbon source for fruitbody production, followed by cellulose, glucose or fructose, and starch. Growth on media containing lactose resulted in the formation of 5 times as many perithecia as growth on glucose. Although media containing lactose supported moderate perithecial formation in *S. brevicollis*, media containing glucose produced greater numbers of perithecia.

Perithecial production was optimal on corn meal agar supplemented with 30 mM glucose (equivalent to approximately 0.5 % w/v). At concentrations in excess of 100 mM glucose, perithecial number was approximately constant at 4 x 10^3 fruitbodies per plate, but few ascospores matured and none were discharged. This may have been an osmotic effect. Although the centra from perithecia which developed on corn meal agar supplemented with 400 mM sucrose matured much more evenly, and exhibited normal spore discharge, it is probable that sucrose was not transported (Jennings, 1974). Poor ascospore development may also have been caused by an effect analogous to catabolite repression in yeast, whereby the synthesis of certain enzymes is suppressed by high glucose concentrations (Entian and Barnett, 1992). The enzymes typically suppressed by

glucose are those of primary metabolism, however, and there is no obvious reason why the centrum should be affected in the manner observed, although the metabolic rate in developing asci may be far higher than that in the rest of the perithecium. A further possibility is that high glucose levels in the growth medium affected transport processes within the hyphae (Jennings (1974, 1976) and references therein).

Sodium acetate, commonly added to media at a concentration of 0.7% (w/v) in order to stimulate spore germination, was found to have several effects on morphological development in *S. brevicollis*. Although hyphal extension, microconidial formation, and VHA development took place on media containing acetate, all cultures were female sterile. Acetate has also been shown to induce female sterility in *N. crassa*, in this case by inhibiting the later stages of protoperithecial development (Turian *et al.*, 1962; Turian and Viswanath-Reddy, 1971). Acetate has been shown to inhibit glucose uptake in *Saccharomyces cerevisiae* (Okada and Halvorson, 1964) and *Aspergillus nidulans* (Romano and Kornberg, 1969), and similar inhibition in *S. brevicollis* might adversely affect protoperithecial formation. In contrast, xylose facilitated mycelial growth and the formation of numerous perithecia, but few or no VHAs. Although it seems likely that the lack of development of the VHA pathway was due to signalling rather than nutritional effects, carbon metabolism in fungi is complex (e.g. Burnett, 1976; Cochrane, 1976) and analysis of these differential effects is beyond the scope of this thesis. Nevertheless, the fact that a particular carbon source facilitated the production of a specific multicellular structure is of interest when considering the regulatory processes involved in differentiation.

The effects of various nitrogen sources on multicellular development in *S. brevicollis* were less striking than those exerted by carbon, and again interpretation is complicated by the use of corn meal agar rather than defined media. Although urea was not utilised to any great extent, ammonium nitrate and potassium nitrate both supported similar levels of VHA production to those observed on media supplemented with yeast extract. Different patterns of VHA production on Vogel's medium (containing ammonium nitrate) and Westergaard and Mitchell's medium (containing potassium nitrate)(Vogel, 1956; Westergaard and Mitchell, 1947), have been observed by D. J. Bond and S. J. Broxholme (unpublished results). It was found that on medium containing a Vogel's salt solution, multicellular structures were produced at the centre of the colony, before the mycelium reached the edge of the Petri plate, while on medium with Westergaard and Mitchell's solution, such structures were formed only after the

edge of the plate was reached. The implication is that the aggregates formed on Vogel's media were VHAs, and those formed on Westergaard and Mitchell's media were protoperithecia. Inhibition of protoperithecial formation has also been correlated with the presence of ammonium in the growth medium in *N. crassa* (Sommer *et al.*, 1987), and the failure of the present study to reveal any variation in multicellular development on media containing different nitrogen sources may reflect the presence of other components of the corn meal agar. The unpublished results of Bond and Broxholme lend further weight to the hypothesis that the developmental pathways leading to the formation of VHAs and protoperithecia are quite distinct.

Several previous studies have explored the effect of carbon and nitrogen in regulating fruitbody formation in *Sordaria* species (Lilly and Barnett, 1947; Molowitz *et al.*, 1976; Roure and Bouillant, 1986). The latter study showed that perithecial development in *S. macrospora* was prevented with ammonium nitrate as the nitrogen source, but only when the pH of the culture medium was below a critical level of 6.9. In the present study, the pH of the growth medium was adjusted to 6.8 $\pm$ 0.1, and thus any pH-dependent effects of nitrogen source may have been overlooked. In addition, the growth medium was not buffered, so pH may have altered during growth.

Amongst the conclusions reached by Molowitz *et al.*, (1976) from their investigation of the effects of biotin and L-arginine on fruitbody formation in *S. macrospora*, was that protoperithecial formation only took place once hyphal density reached a critical level. Although there have been alternative interpretations of similar effects seen in other fungal species (section 3.1.1), this hypothesis is of interest in the context of VHA formation in *S. brevicollis*, which can take place before the edge of the Petri plate is reached. It seems likely therefore that VHA formation occurs at lower hyphal density than that required for protoperithecial formation.

5.3.2.2 Protoperithecia, crossed perithecia and uncrossed perithecia

It would appear that the protoperithecia of *S. brevicollis* remain receptive to fertilisation longer than those of *N. tetrasperma*, since Bennett and Howe (1980) reported that protoperithecia in the latter species became unreceptive when 36-42 hours old. Although routine spermatial crosses in *S. brevicollis* were carried out 96 hours after inoculation (when protoperithecia were estimated to be up to 48 hours old), much older mycelia developed crossed perithecia when fertilised. When 10 day old heterokaryons (which had previously been examined

for the presence of homothallic perithecia) were crossed by spermatisation, perithecia matured within 4-5 days (too rapidly for new protoperithecial formation to have taken place prior to perithecial expansion). This observation also revealed a significant difference between the formation of crossed and uncrossed perithecia in *S. brevicollis*: although crossing inhibited the formation of uncrossed perithecia (Fig. 5.1), the production of spore-bearing uncrossed perithecia did not inhibit the subsequent development of crossed perithecia on the same mycelium. It is possible that inhibition of homothallic fruiting in crossed cultures was a nutrient-dependent effect. Fertilisation of 4 day old protoperithecia triggered rapid expansion of the peridium during the following 48 hour period, possibly diverting nutrients away from uncrossed perithecia (which expanded slowly, even in unfertilised cultures)). Nutrient limitation was not likely to occur when cultures which had already produced uncrossed perithecia were crossed, since relatively few uncrossed perithecia formed.

Nutrient-dependent inhibition of perithecial formation has previously been reported in sequential crosses in *N. tetrasperma* (Howe and Prakash, 1969; Calhoun and Howe, 1972). It was shown that if a portion of a mycelium was crossed by spermatisation, subsequent crosses on different parts of the same mycelium did not produce perithecia. Evidence that the effect was nutrient-dependent was obtained from experiments which demonstrated that addition of nutrients, particularly sucrose and glucose, could reverse the inhibition. (In contrast with *S. brevicollis* and *S. fimicola* which apparently lack invertase activity, sucrose was rapidly hydrolysed by *N. tetrasperma* (Calhoun and Howe, 1972).) A further study of the same species by Bennett and Howe (1980), showed that immediately after fertilisation, the production of new protoperithecia was strongly inhibited, although this inhibition declined slightly as the mycelium aged. This result was the reverse of that expected if the nutrients which remained in the mycelium became increasingly depleted with increased age. Bennett and Howe suggested the presence of a chemical inhibitor which was released after fertilisation, and gave priority to perithecial development at the expense of the formation of additional protoperithecia. The production of a similar diffusible inhibitor has also been proposed in *S. fimicola* (Pollock and Johnson, 1972; Pollock, 1973). Although an inhibitor released by crossed perithecia in *S. brevicollis* would explain the inhibition of the homothallic pathway after crossing, the reverse situation (inhibition of heterothallic fruiting after the production of homothallic perithecia) does not appear to take place. Whilst it may be argued that homothallic perithecia are relatively few in number, and that

the concentration of any inhibitory substance might therefore be low, it is also possible that such an inhibitor was simply not produced by uncrossed perithecia.

5.3.3 Effect of temperature on multicellular development

The optimal temperatures for mycelial growth and perithecial formation were different in *S. brevicollis*. Mycelial elongation increased at almost constant rate between 10-35°C, followed by a sharp drop to zero growth at 45°C. Fruiting occurred over a narrower temperature range (15-30°C), with maximum perithecial frequency observed at 20°C. Broadly similar results have been reported for *N. tetrasperma*, where maximum growth rate was achieved at 35°C, while optimal protoperithecial frequency was obtained at 20°C (Viswanath-Reddy and Turian, 1975). Perithecial frequency in *N. tetrasperma* was optimal at 30°C, however, demonstrating a degree of differential development of protoperithecia which was not observed in any of the experiments carried out on *S. brevicollis*.

Observation of light-grown cultures incubated over a range of temperatures, provided further evidence to suggest that VHA formation and protoperithecial production are part of distinct developmental pathways: VHA formation occurred over a broader temperature range than that which permitted protoperithecial development (although not the entire range which facilitated hyphal growth). The primary light requirement for VHA formation remained however, and at no temperature were VHAs produced in the dark.

The effect of temperature on sexual development has also been examined in *S. fimicola* (Pollock, 1973), *S. macrospora* (Esser, 1980), various species of *Nectria* (Hanlin, 1961; Dietert *et al.*, 1983) and *N. crassa* (McNelly-Ingle and Frost, 1965). In the latter study, the effect of temperature on protoperithecial formation was found to be variable and strain-specific in the range 15-30°C. Within perithecia produced at extremes of temperature, ascus development was often found to be abnormal, with aborted or misshapen spores, and at high temperature (above 27.5°C) occasional asci were observed to contain more than 8 spores. No such phenomenon was observed in the wild type strains of *S. brevicollis* examined during the present study. Viswanath-Reddy and Turian (1975) reported the development of "lassos" in cultures of *N. tetrasperma*, and attributed their formation to changes in temperature. These looped hyphae, were described by Turian (1975) as abortive ascogonia, although this interpretation has been questioned (N. D. Read, in preparation). Whilst similar structures have

occasionally been observed in *S. brevicollis* during the present study, their formation was not apparently linked to incubation temperature.

Although both temperature and nutritional factors have been observed to modify development in *S. brevicollis*, it is clear that illumination is of primary importance, causing inhibition of protoperithecial and perithecial formation, and the stimulation of VHA production. Since it has not proved possible to achieve significant additional development of the VHA pathway by modifying the light regime, nutritional status or incubation temperature, it must be concluded for the present that there is no "sclerotial" stage in *S. brevicollis*, and that structures seen in culture represent the end point of this pathway. It should also be noted however that only a small number of carbon and nitrogen sources have been examined, and that numerous substances including RNA base analogues, sterols and various amino compounds, have been shown to influence aspects of multicellular development in related species (e.g. Lindenmayer and Schoen, 1967; Elliot, 1969; Roure and Bouillant, 1986). In addition, substances produced at very low concentrations by certain fungi have been shown to influence morphological development in other species (Siddiq, 1989). When isolated in the laboratory, *S. brevicollis* is deprived of interaction with other species which might stimulate further development of the VHA pathway.

5.3.4 Summary

5.3.4.1 Are VHAs aberrant protoperithecia?

Although sexual reproduction in fungi is often stimulated by light, illumination typically *inhibited* protoperithecial formation in *S. brevicollis*. In contrast, blue or white light stimulated the production of VHAs, so it might be argued that VHAs represent defective sexual primordia of some kind. There is some evidence that the two pathways may not be entirely distinct: although no attempt has been made to produce mutants, no fertile strains which produce VHAs have been found to arise spontaneously during the course of numerous crosses. In addition, a female sterile mutant *(per 1- per* 2-) did not develop VHAs. However, a far greater body of evidence points to VHA production and protoperithecium formation being separate pathways, with distinct regulatory mechanisms:-

(i) Light effects: Exposure of dark-grown cultures to short periods of blue or white light during protoperithecial formation, resulted in reduced protoperithecial frequency, without stimulating significant VHA production. Red

light also inhibited protoperithecial development, but did not cause any VHA formation. When dark-grown cultures were illuminated after crossing, perithecial numbers were often reduced, although few VHAs formed.

(ii) Nutritional effects: Media containing sodium acetate supported the development of VHAs but not protoperithecia, while the reverse was true of media supplemented with xylose. The data of Bond and Broxholme suggested differential production of VHAs and protoperithecia according to the nitrogen source provided.

(iii) Temperature effects: The range of temperatures at which protoperithecial production took place was narrower than that over which VHA formation occurred.

(iv) Morphological observations: As illustrated in Chapter Three, there is little structural similarity between VHAs and protoperithecia. In addition, VHAs may be formed before the mycelium reaches the rim of the Petri plate, so VHA production is not subject to the edge effect.

5.3.4.2 Does VHA formation inhibit protoperithecial development?

There is evidence to suggest that VHAs may act as nutrient sinks and thus inhibit protoperithecial formation. Although VHAs did not appear to store nutrients, the formation of numerous fine hyphae inevitably required large quantities of cell wall material. Addition of extra glucose to the growth medium stimulated the development of greater numbers of fruitbodies, suggesting that perithecial frequency on CMA was limited by nutrient supply. Furthermore, the perithecia which matured on light-grown cultures were predominantly in areas where VHA density was relatively low. (An alternative explanation for the latter observation was that VHAs inhibited protoperithecial formation either by limiting the space available on the surface of the growth media, or by restricting gaseous exchange.) Other results, however, indicated that protoperithecial inhibition was a direct effect of exposure to light. As noted above, light could inhibit sexual differentiation without stimulating significant production of VHAs, and on media supplemented with xylose, illumination reduced perithecial frequency even though no VHAs were produced.

Although these results do not provide unambiguous answers, it seems likely that VHA production was stimulated by blue light, and that blue light also inhibited protoperithecial formation, but that the two processes were not related. VHA formation may, however, have had an indirect effect on perithecial maturation.

CHAPTER SIX

GENERAL DISCUSSION

The primary objective of this General Discussion is to relate observations made in the laboratory to the probable life cycle of *Sordaria brevicollis* in the wild. Whilst it must be recognised at the outset that the morphological and genetical pathways seen in culture may not be expressed under natural conditions, this fungus is unusual in both its sexual system and its response to light, and it may prove useful to examine these apparent peculiarities in an ecological context.

6.1 Ecology of coprophilous fungi

The life cycle of a typical coprophilous ascomycete is illustrated in Fig. 6.1. After ascospore germination on dung, mycelial proliferation is followed by ascocarp development. When mature, ascospores are discharged onto the surrounding vegetation, and when leaves are consumed by a herbivore, the spores are also ingested. After passing undigested through the animal's gut, spores are deposited in fresh dung where the cycle is repeated. Ascospores show a number of adaptations to this mode of existence: various appendages or a mucilaginous/ gelatinous sheath help spores to adhere to vegetation; dark pigments in the spore wall protect the protoplasm from the effects of ionising radiation; and spore walls are resistant to chemical and enzymatic degradation during passage through the digestive tract (Wicklow, 1981; Read and Lord, 1991)). In addition, the liberation of spores is often explosive, shooting propagules several centimetres into the airstream, and thereby aiding wider dispersal (Burnett, 1976; Ingold and Hadland, 1959a).

Faecal substrates have been likened by Wicklow (1981) to "sinking islands on which fungal colonists are doomed as the dung decomposes and becomes incorporated into the soil", and most coprophilous species are dependent upon spores as a means of escaping from spent substrates. Spore production, conveniently assessed in terms of sporocarp density, is therefore a direct measure of ecological fitness in coprophilous habitats. The formation of sporocarps is influenced not only by environmental variables such as light and temperature, but also by competitive interactions: the greater the number of species within a single pellet, the greater the likelihood that competition for factors such as space

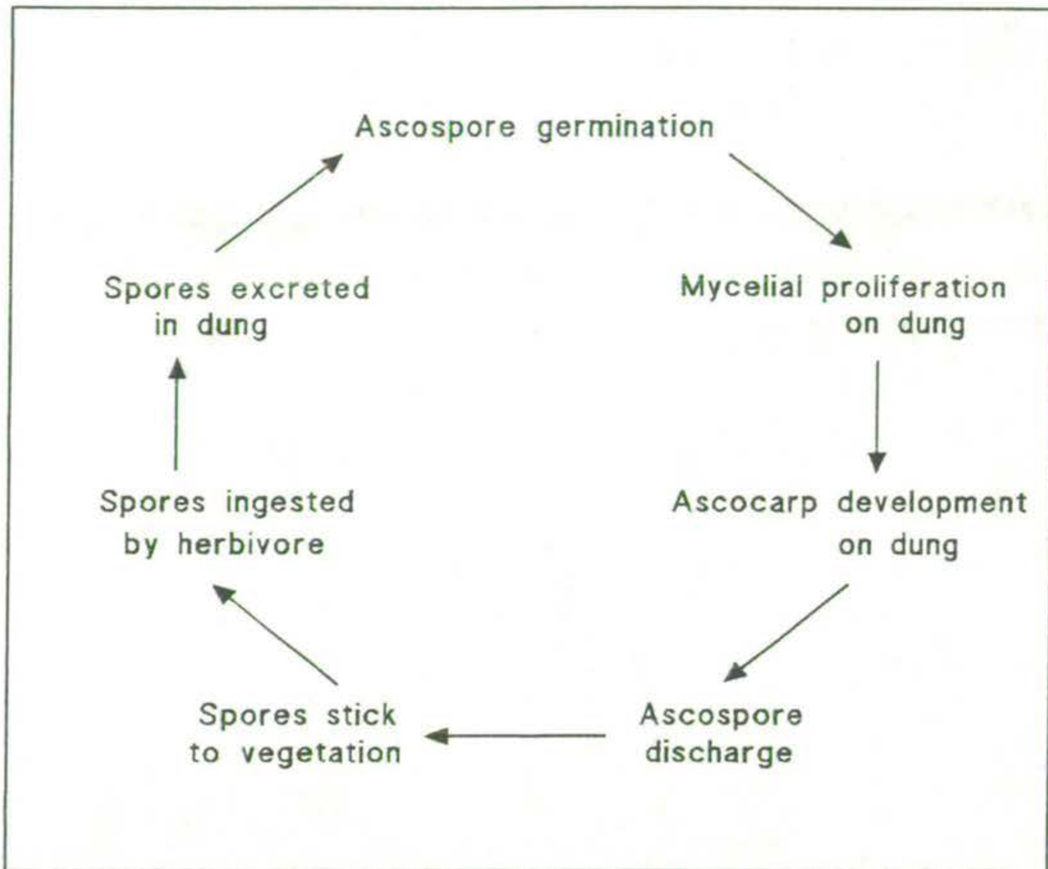


Figure 6.1: Generalised life cycle of a coprophilous ascomycete. After ascospore germination (usually on herbivore dung) a phase of mycelial proliferation takes place, prior to the formation of ascocarps. When liberated (often by explosive discharge mechanisms), mature ascospores stick to surrounding vegetation until they are ingested by a grazing herbivore. The spores of many coprophilous species have either mucliagious or gelatinous sheaths, or a range of other appendages which insure that they remain attached to the vegetation until it is consumed. During this period, dormant ascospores are probably protected from harmful radiation by the dark pigments present in the spore wall. Passage through the digestive tract triggers the germination of spores, so that fresh faecal material is rapidly colonised.

and nutrients will occur. Reproductive capacity may also be limited by genetic factors: many coprophilous species are homothallic, a mating strategy with obvious advantages when individual spores germinate in reproductive isolation. In contrast, a single spore isolate of a typical heterothallic species cannot produce sexual spores, and in the absence of asexual propagules or resting structures such as sclerotia, is destined to expire when the substrate is exhausted.

6.2 *Sordaria brevicollis* as a coprophilous species

Sordaria brevicollis is a heterothallic species which has been reported relatively infrequently in the wild, although it is apparently of world-wide distribution: records exist for zebra dung in New York zoo (Olive and Fantini, 1961), buffalo dung in Africa (Khan and Krug, 1989) and opossum dung in New Zealand (D. P. Mahoney pers. comm.). Although several other heterothallic species have been isolated (e.g. *S. heterothallis* (Fields and Maniotis, 1963) and *S. tormento-alba* (Cailleux, 1971)), all of the most commonly recorded species of *Sordaria* are homothallic (Lundqvist, 1972). Although heterothallic species are more frequently encountered in the related genus *Neurospora* (Perkins *et al.*, 1976), these species often produce copious quantities of asexual conidia, which are lacking in *Sordaria*.

6.2.1 Breeding systems

After many years of maintenance in the laboratory, several *mtA* strains of *S. brevicollis* have been observed to produce homothallic ascospores. Although it is by no means certain that this phenomenon occurs under natural conditions, the ability of an isolated homokaryon to produce sexual spores could clearly be of great value as a survival mechanism. The spores produced would be better equipped to withstand deteriorating conditions than the vegetative mycelium, although on germination they would produce mycelia genetically identical to the parent. However, heterokaryon formation between two different *mtA* homokaryons could facilitate the production of spores which were genetically distinct from both parents. The ease with which heterokaryons formed in the laboratory (without forcing) does not necessarily indicate that heterokaryosis is a widespread phenomenon in the wild, however, since the stock cultures used during the present study are descendants of a small number of ascospores isolated by Olive and Fantini (1961), and are relatively homogeneous with respect to incompatibility loci (D. J. Bond, pers. comm.). The occurrence of heterokaryosis in the wild was reviewed by Caten and Jinks (1966) who

concluded that although heterokaryosis took place in homothallic species, its frequency was rather lower than previously suggested (e.g. Snyder, 1961). It is perhaps safer to assume therefore, that if *S. brevicollis* exhibits any homothallic spore production in the wild, then this is confined to isolated homokaryons.

The observation that development of crossed perithecia largely inhibited the formation of uncrossed fruitbodies, but not *vice versa*, is consistent with a hypothesis that facultative homothallism is a mechanism which allows an individual to escape from a declining substrate, but that heterothallic spore production predominates when both mating types are present. It is not inconceivable, however, that several generations of homothallic spore production would result in the development of a population of individuals which were all of *mtA*, and that the *mta* sequence could effectively be lost. It is also possible that strains derived from "homothallic" parents may show an increased tendency to produce spores in uncrossed culture. Although no rigorous investigations have as yet been conducted, it was noted during genetic analyses of homothallic fruiting (section 4.2.2) that heterokaryon 6 (one of whose component homokaryons was germinated from a homothallically-derived ascus) showed the highest percentage of uncrossed perithecia containing spores (Table 4.1).

Whilst it is possible that homothallic spore production by this heterothallic species has developed only after 30 years in the laboratory, there is evidence to suggest that a similar phenomenon may take place in other heterothallic *Sordaria* species, particularly *S. heterothallis* (section 4.3.4). The studies conducted by Lewis (1969a,b) on *S. heterothallis* (Fields and Maniotis, 1963) and *S. thermophila* (Fields, 1968) were conducted only a few years after the isolation of these species, and give some indication that the genes responsible for atypical sexual behaviour may have been present in the original isolates.

6.2.2 Ageing and loss of fertility

Fungi inhabiting coprophilous substrates may be subject not only to deteriorating environmental conditions, but also to a progressive loss of fertility as the mycelium ages. It is not known whether the mycelial senescence observed in laboratory cultures occurs under natural conditions, but such ageing could foreseeably reduce any advantage gained from homothallic spore production.

The effects of ageing on the fertility of a strain of *S. brevicollis* in culture may be illustrated by reference to the experiments described in Chapter Five. All of the detailed investigations carried out on light responses in *S. brevicollis*

employed a single *mtA* strain as the protoperithecial parent. Nevertheless, the numbers of protoperithecia produced (even in dark controls) in the blue/red light experiments (Fig. 5.12) were only about a quarter of those produced in earlier experiments using white light (Fig. 5.10 (a)). This variation in the magnitude of protoperithecial production was attributed to the increasing age of the strain concerned. Although the number of perithecia produced in dark controls remained relatively constant between experiments (Fig. 5.10 (b), Fig. 5.11) the observed drop in protoperithecial frequency was probably the first sign of mycelial ageing. Since protoperithecia were produced in considerable excess in younger strains, the proportion of protoperithecia which developed into mature perithecia increased as the strain aged, masking the fall in protoperithecial formation. Approximately six weeks after these experiments were conducted, perithecial numbers began to decline, and mycelial growth rate was reduced.

Reduced mycelial growth and progressive loss of female fertility in culture has previously been reported in other species of *Sordaria* and *Melanospora* (Lindenmayer and Schoen, 1967; Hawker, 1951), and mycelial senescence is a well documented phenomenon in the coprophilous species, *Podospora anserina*, where hyphal growth is restricted to a strain-specific length (e.g. Smith and Rubenstein, 1973). Thereafter growth rate declines, and eventually the hyphae die. It has been shown that the effects of ageing in *P. anserina* can be reduced by the application of inhibitors of mitochondrial DNA replication and protein synthesis (Tudzynski and Esser, 1979), and it is believed that senescence is caused by the excision and autonomous replication of short segments from the mitochondrial genome (Osiewacz and Esser, 1984). Mycelial senescence has also been examined in strains of *Neurospora intermedia* from the Hawaiian archipelago (Rieck *et al.*, 1982; Griffiths and Bertrand, 1984). Although phenotypically similar to the phenomenon observed in *P. anserina*, senescence in *N. intermedia* is thought to result from the accumulation of copies of a "foreign" sequence within the mitochondrial DNA (Bertrand *et al.*, 1985). Continuous vegetative propagation results in loss of mitochondrial functions and eventual cell death. As in *P. anserina*, senescence in *N. intermedia* is maternally transmitted, and senescence is "escaped" via spermatia, which allow the nucleus from an ageing mycelium to be passed on without any maternal cytoplasm. If the ageing observed in *S. brevicollis* is also maternally transmitted, then any homothallically-derived spores may be expected to produce mycelia containing the same defective mitochondria.

6.2.3 Photoresponses

It was concluded elsewhere that VHA formation and protoperithecial inhibition in *S. brevicollis* are both blue light effects, but that the development of VHAs probably does not cause the observed reduction in protoperithecial frequency (section 5.3.1). Thus it would appear that light has a direct inhibitory effect on the development of sexual primordia in *S. brevicollis*, an unexpected result in a species whose mature perithecia show phototropic neck curvature and light-stimulated spore discharge. While it may be argued that spores excreted in animal dung may germinate in darkness, the need for effective spore dispersal would seem to dictate that fruitbodies are formed at or near the surface of the substrate, where mycelia will, in all probability, be exposed to light.

The development of VHAs, whilst not apparently inhibiting protoperithecial production, may lead to a reduction in the number of perithecia which subsequently mature, and thereby reduce the number of ascospores which are produced. It is therefore difficult to explain why VHAs should be produced, unless the fungus derives some benefit from their formation which is not apparent in the laboratory. It might be speculated for example that by rapidly covering the surface of the substrate with a layer of VHA tissue, the fungus could limit colonisation by potential competitors. A layer of VHA tissue might conceivably reduce the rate of water-loss, and thus maintain higher humidity (favourable for perithecial development) within the substrate. Formation of a layer of orange VHA tissue over the surface of the substrate might also filter out the blue wavelength radiation which is the major cause of protoperithecial inhibition, and could thereby effectively increase perithecial formation.

However, the failure of previous workers on *S. brevicollis* to report the development of VHA-type structures in their isolates (e.g. Olive and Fantini, 1961; Berg, 1962; Shaw, 1962) fuels speculation that VHAs have arisen following a mutation in culture. Mutants (sometimes called saltants) have arisen in laboratory cultures of many fungal species, and frequently exhibit superior growth to the wild type (Caten, 1987). Such a phenomenon is not without parallel in *S. brevicollis* where Shaw (1962) reported the presence of an orange mutant (*or*) which differed from her "dark, grey-brown" wild type strains, by producing a pale mycelium which developed an orange pigment. This mutant, which was present in the original stock cultures of Olive and Fantini (1961), outgrew the wild type under laboratory conditions, and all subcultures of the *mtA* stock eventually became orange (Shaw, 1962). The orange mutation, which was linked

to the *mtA* locus, also resulted in female sterility, and so it is not possible that the strains of *S. brevicollis* now considered to be wild type are really of this mutant type. Nevertheless, all the "wild type" strains used during this study produced hyaline mycelia in the dark on CMA, developed carotenoid-like pigments when exposed to light, and are apparently distinct from the wild type strains described by Shaw. VHA formation may well have arisen as a spontaneous mutation in culture, in which case, VHAs may be entirely absent in the wild.

6.3 *Sordaria brevicollis* as a model system: future work

Although it will inevitably remain difficult to prove conclusively whether homothallic spore production and VHA formation take place in the wild or are simply features of prolonged growth in the laboratory, *S. brevicollis* provides an interesting, easily manipulated system for the study of multicellular development in fungi. Further investigations could take a number of different directions:-

(i) The incidence of homothallism in a heterothallic species is of particular interest in view of the recent cloning and sequencing of the mating type alleles in *N. crassa* (Vollmer and Yanofsky, 1986; Glass *et al.*, 1988; Glass *et al.*, 1990a; Staben and Yanofsky, 1990)) and the continuing investigation of the rôle of the mating type sequence in regulating morphological development (Glass and Lee, 1992). Although there have been reports that the sexual cycle in homokaryons of *N. crassa* can be induced by the addition of substances from mated cultures (Islam and Weijer, 1972; Vigfusson and Cano, 1974) these results do not appear to have been replicated, and none of the mating type mutants generated by repeat-induced point (RIP) mutation have shown an ability to by-pass the requirement for the opposite mating type for completion of the sexual cycle (Glass and Lee, 1992). A detailed comparison of the *mtA* sequences in *S. brevicollis* and *N. crassa* might shed light on the nature of the mechanism which permits homothallic spore production in the former species, as well as providing interesting information on the evolution of the mating type sequences in these related genera.

(ii) Examination of the manner in which homothallism is inherited may also prove productive. Although homothallic spore production has never been observed in *mta* strains, suggesting that homothallism is controlled by the *mtA* sequence, by no means all *mtA* mycelia produce spores in uncrossed cultures. It has been noted in passing, although not confirmed by systematic experimentation, that individuals produced by several successive generations of inbreeding do not seem to produce homothallic spores, although they retain the

ability to produce uncrossed perithecia. It may be the case that while homothallic spore production is a function of the *mtA* locus, an unlinked gene or genes may regulate its expression.

(iii) Further investigation of the cytology of ascus development in uncrossed perithecia would also be of interest, particularly in homokaryons or heterokaryons which exhibit a relatively high frequency of spore production, where the potential for finding asci at the required stages of development would be increased. It may also prove possible to study the behaviour of nuclei in ascogenous hyphae, croziers and young asci using confocal microscopy to examine intact perithecia.

(iv) Clarification of the effect of VHA formation on sexual development could be achieved by investigation of the behaviour of mutants which produce protoperithecia without VHAs. Since no such mutants have arisen spontaneously, it would be necessary to carry out a programme of mutagenesis followed by screening of progeny.

(v) It may also be of interest to compare the pattern of developmentally regulated proteins in light-grown cultures of *S. brevicollis* with the results already obtained for dark-grown material (Broxholme *et al.*, 1991). Preliminary studies using polyacrylamide gel-electrophoresis indicated the presence of a protein with a molecular weight of approximately 28 kDa which was present in 3 day old, light-grown cultures, but absent from comparable dark grown plates (data not shown). (These cultures were inoculated on 14 cm Petri plates, so that after 72 hours growth the mycelium had yet to reach the edge of the plate, and no protoperithecial differentiation had taken place.)

(vi) In order to determine the precise light wavelengths affecting multicellular development it will be necessary to determine action spectra for protoperithecial inhibition and VHA formation. Although the former should be a relatively simple procedure, no satisfactory method has yet been developed for the quantification of VHA formation.

(vii) The effects of nutrient sources on multicellular development are worthy of further examination using defined media. Of particular interest are the possible rôles of acetate, xylose and various nitrogen sources.

(viii) More detailed examination of development in hyaline perithecia may also prove of interest, since the observations made here indicate that the pseudoparenchyma which forms the wall of wild type perithecia is absent in the hyaline mutant (*hya*). In the walls of 11 day old, hyaline perithecia, individual hyphae can still be distinguished (Figs. 3.10 and 3.11)). Since the mutant

perithecia show strong laccase activity (Fig. 3.29), it may be argued that the proposed rôle for laccase in the cross-linking of adjacent hyphal walls (Bu'lock, 1967) is not correct in this case. Alternative laccase assay techniques may reveal the presence of different laccases within the developing perithecium, and it is possible that the function of the laccase localised during the present study (using the technique of Hermann *et al.*, (1983)) has another function, unrelated to hyphal adhesion. It would also be of interest to embed and section hyaline protoperithecia and young perithecia in order to carry out a comparative study of fruitbody development in mutant and wild type strains. This study might also be extended to other spore colour mutants (such as the buff mutant) where spore colour abnormalities are accompanied by altered pigmentation of the perithecial wall and by atypical perithecial morphology (notably the shape of the neck). Both buff and hyaline mutants were observed to produce uncrossed perithecia, but these were never found to contain spores, and it is possible that various aspects of the structural development of the perithecium may have a bearing on the development of the fertile tissues.

The work described in this thesis illustrates that there is considerable scope for further investigation of multicellular development in *Sordaria brevicollis*, a species which has already yielded much useful information on genetic and developmental processes in fungi, and which may also hold important clues to the evolution of fungal breeding systems.

APPENDIX 1

ASCOMYCETE SYSTEMATICS

The taxonomy of the Ascomycotina has been the subject of much study and numerous revisions (eg. Lutrell, 1951; Gäumann, 1964; Ainsworth and Bisby, 1971; Ainsworth, 1973; Barr, 1976, 1983; Eriksson, 1982; Hawksworth *et al.*, 1983; Hawksworth, 1985). The scheme of Ainsworth (1973), still cited in some mycological textbooks (eg. Moore-Landecker, 1982), divides the Ascomycotina into six classes; Hemiascomycetes, Plectomycetes, Discomycetes, Pyrenomycetes, Laboulbeniomycetes and Loculoascomycetes. This classification relies heavily upon ascus type and fruitbody morphology. Thus for example, cleistothecia are the typical ascocarps of Plectomycetes, apothecia are characteristic of Discomycetes, and perithecia are produced by many Pyrenomycetes. More recent developmental studies have revealed however that the arrangement is flawed; convergent evolution has lead to the development of similar structures in unrelated taxa, and classes such as the Plectomycetes have been shown to be polyphyletic (Malloch, 1981). In later schemes a greater range of taxonomic criteria have been employed (for examples see Reynolds (1981)), and emphasis has been shifted to classification at the ordinal level (Eriksson, 1982; Hawksworth *et al.*, 1983), splitting the Ascomycotina into smaller groups of related families. It is widely recognised however that contemporary classification schemes are still far from perfect and that further data on a wide variety of morphological, biochemical and cytological features are required before the problems can be resolved (eg. Hawksworth, 1985). Advances in understanding fungal evolution and improving classification may also be made using a range of molecular techniques (Dutta and Ojha, 1972; Taylor, 1986; Al-Sagur, 1989; Duran and Gray, 1989; Taylor and Natvig, 1989; Berbee and Taylor, 1992).

The Sordariales, amongst the orders studied most extensively to date, includes five families whose taxa were previously assigned to the Pyrenomycete order Sphaeriales (Müller and von Arx, 1973). Within the Sordariales, Hawksworth *et al.* (1983) recognise the Chaetomiaceae, Lasiosphaeriaceae, Melanosporaceae, Nitschkiaceae and Sordariaceae. These families are often still referred to as pyrenomycete (with a small "p").

It is from the Sordariaceae that many of the examples used to illustrate the processes of morphological differentiation and sexual development described in Chapter 1 are drawn. The family includes eight genera, notably *Neurospora*, *Gelasinospora* and the type-genus, *Sordaria*. The primary diagnostic feature for the separation of these genera is the nature of the ascospore wall. The ascospores of *Neurospora* species have ribbed walls, *Gelasinospora* spores have pitted walls, and the walls of *Sordaria* are smooth (see photographs in Dutta *et al.*, 1981). The genera also differ in their preferred substrates, *Neurospora* often being found on burned vegetation whilst *Sordaria* and *Gelasinospora* species are largely coprophilous. The chromosome complements are very similar however, all having seven haploid chromosomes (Perkins, 1985).

APPENDIX 2

STATISTICAL ANALYSIS OF SEGREGATING ASCI: RATIONALE FOR THE REALLOCATION OF 3RD DIVISION OVERLAP ASCI

A X^2 test was carried out to test the significance of observed variations in the sizes of the 3rd division overlap class of segregating asci between uncrossed perithecia from heterokaryons (*S187*/wild type) and crossed perithecia (*S187* x wild type). For convenience, the data upon which this analysis was based is reproduced below (this data forms part of Table 4.3).

	Total asci	Frequency of each ascus type			
Source	examined	MI	asym MII	sym MII	3rd div n overlap
Combined heterokaryons	456	212	134	39	71
Control cross	570	243	218	87	22

Prior to the calculation of X^2, a 2 x 2 contingency table was constructed according to Hale (1965):-

71 (a)	385 (c)	456 (e)
22 (b)	548 (d)	570 (f)
93 (g)	933 (h)	1026 (N)

X^2 was then calculated from the formula: $X^2 = \frac{N(bc-ad)^2}{efgh}$

In this case $X^2 = \frac{9.51 \times 10^{11}}{2.26 \times 10^{10}} = 42.15$

From probability tables, a value of $X^2 \geq 3.84$ indicates significance ($p \leq 0.05$, one degree of freedom). The difference in the frequency of 3rd division overlap is therefore highly significant (even at $p \leq 0.001$), and this variation presented a problem when comparing other ascus types. It was therefore necessary to reallocate 3rd division overlap asci as accurately as possible before a true comparison can be made between the sizes of the other classes.

The following table shows the precise sequences of spores in 3rd division overlap asci, the frequency of each category, and the basic ascus type (MI, asymmetrical or symmetrical MII) from which they were derived. It was assumed that the observed sequence was produced in the simplest possible manner.

	Spore sequence	Frequency	Derived from
Heterokaryons	3113	15	MI
	221111	16	asym MII
	211112	13	asym MII
	11111111	5	asym MII
	211211	3	asym MII
	23111	1	asym MII
	2123	7	MI or asym MII
	1232	6	asym or sym MII
	111221	4	asym or sym MII
	22121	1	asym or sym MII
Cross	3113	9	MI
	211112	11	asym MII
	2123	2	MI or asym MII

A problem arose with asci such as those with a 1232 spore sequence, which could be derived from either of two of the basic types (in this case symmetrical or asymmetrical MII). Clearly to allocate half of such asci to each class would have been erroneous, as the symmetrical MII type was present at a much lower frequency, because of 2nd division overlap. The asci should properly have been divided according to the relative sizes of the symmetrical and asymmetrical MII classes, but these could not be calculated accurately until the 3rd division overlap asci were included. As a good approximation the following system was adopted: asci of doubtful origin were allocated according to the proportions of the three basic ascus types after those with clear derivation had been included. Thus, those 3rd division asci derived from either MI or asymmetrical MII types were allocated 4:3 in favour of MI, and those from either asymmetrical or symmetrical MII types were allocated 11:2 in favour of asymmetrical MII. A similar principle was applied to crossed perithecia. The corrected data sets were then as follows:

Source	Total asci examined	Frequency of each ascus type MI	asym MII	sym MII
Combined heterokaryons	456	231	184	41
%		51	40	9
Control cross	570	253	230	87
%		44	40	15

Values of X^2 were then calculated as explained previously:-

MI $X^2 = \frac{2.73 \times 10^{11}}{6.82 \times 10^{10}} = 4.00$

asymmetrical MII $X^2 = \frac{0.00}{6.82 \times 10^{10}} = 0.00$

symmetrical MII $X^2 = \frac{2.73 \times 10^{11}}{2.98 \times 10^{10}} = 9.13$

The proportion of excess asymmetrical MII asci was calculated from the formula of Chen and Olive (1965) i.e.

$$\frac{\text{Apparent asymmetrical MII asci - Symmetrical MII}}{\text{Total number of asci}} \times 100$$

For the combined asci from heterokaryons this was 31%, and for crossed perithecia, 25%, suggesting that 2nd division overlap occurred at broadly similar frequencies in both cases.

REFERENCES

Ainsworth, G. C. (1973) Introduction and keys to higher taxa. In *The Fungi: An Advanced Treatise, Volume IVA*. (ed. G. C. Ainsworth, F. K. Sparrow and A. S. Sussman) pp. 1-7. Academic Press, New York.

Ainsworth, G. C. and Bisby, G. R. (1971) *Dictionary of the Fungi,* 6th edn. Commonwealth Mycological Institute, Kew, England.

Al-Sagur, A. M. (1989) Genetical and biochemical studies of five *Neurospora* populations from Iraq. *Journal of Biological Sciences Research* **20**: 397-406.

Anagnostakis, S. L. (1982) The origin of ascogenous nuclei in *Endothia parasitica. Genetics* **100**: 413-416.

Ander, P. and Erikkson, K. E. (1976) The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentum. Archives of Microbiology* **109**: 1-8.

Anderson, J. G. (1978) Temperature-Induced Fungal Development. In *The Filamentous Fungi, Volume III, Developmental Mycology*. (ed. J. E. Smith and D. R. Berry) pp. 358-375. Edward Arnold (Publishers) Ltd., London.

ap Rees, T., Wilson, P. M. and Wright, B. W. (1984) The ability of *Sordaria fimicola* to take up and metabolise glucose and sucrose. *Journal of General Microbiology* **130**: 3235-3238.

Arpin, N. and Bouillant, M. L. (1981) Light and mycosporines. In *The Fungal Spore: Morphogenetic Controls*. (ed. G. Turian and H. R. Hohl) pp. 435-454. Academic Press, London.

Babai-Ahary, A., Daboussi-Bareyre, M. J. and Parisot, D. (1982) Isolation and genetic analysis of self-sterility and perithecial pigmentation mutants in a homothallic isolate of *Nectria haematococca. Canadian Journal of Botany* **60**: 79-84.

Backhouse, D. and Stewart, A. (1987) Anatomy and histochemistry of resting and germinating sclerotia of *Sclerotium cepivorum. Transactions of the British Mycological Society* **89**: 561-567.

Backus, M. P. (1939) The mechanics of conidial fertilisation in *Neurospora sitophila. Bulletin of the Torrey Botanical Club* **66**: 63-76.

Bahn, M. and Hock, B. (1973) Morphogenese von *Sordaria macrospora*: Die Induktion der Perithezienbildung. *Berichte der Deutschen botanischen Gesellschaft* **86**: 309-311.

Barbesgaard, P. O. and Wagner, S. (1959) Further studies on the biochemical basis of protoperithecia formation in *Neurospora crassa. Hereditas* **45**: 564-572.

Bar-Nun, N., Tal-lev, A., Harel, E. and Mayer, A. M. (1988) Repression of laccase formation in *Botrytis cinerea* and its possible relation to phytopathogenicity. *Phytochemistry* **27**: 2505-2509.

Barja, F., Jaramillo-Maranon, M., Rossier, C. and Turian, G. (1992) Differential effects of griseofulvin and benomyl on morphology and nuclear distribution during the germination of *Neurospora crassa* macroconidia. *Mycological Research* **96**: 147-150.

Barr, M. E. (1976) Perspectives in the Ascomycotina. *Memoirs of Brooklyn Botanic Garden (New York)* **28**: 1-8

Barr, M. E. (1983) The ascomycete connection. *Mycologia* **75**: 1-13.

Barron, G. L. (1968) *The Genera of Hyphomycetes from Soil.* pp. 246-247. The Wilkins and Wilkins Company, Baltimore.

Barry, E. G. (1969) The diffuse diplotene stage of meiotic prophase in *Neurospora. Chromosoma* **26**: 11-129.

Beckett, A. (1981a) The ascus with an apical pore: development, composition and function. In *Ascomycete Systematics, The Luttrellian Concept.* (ed. D. R. Reynolds) pp. 7-26. Springer-Verlag, New York.

Beckett, A. (1981b) The ultrastructure and behaviour of nuclei and associated structures within the meiotic cells of Euascomycetes. In *The Fungal Nucleus.* (ed. K. Gull and S. G. Oliver) pp. 37-61. Cambridge University Press.

Beckett, A. (1981c) The ultrastructure of septal pores and associated structures in the ascogenous hyphae and asci of *Sordaria humana. Protoplasma* **107**: 127-147.

Bell, W. R. and Therrien, C. D. (1977) A cytophotometric investigation of the relationship of DNA and RNA synthesis to ascus development in *Sordaria fimicola. Canadian Journal of Genetics and Cytology* **19**: 359-370.

Bennett, S. N. and Howe, H. B. (1980) A quantitative study of protoperithecial and perithecial development in *Neurospora tetrasperma. Transactions of the British Mycological Society* **74**: 51-59.

Benny, G. L., Samuelson, D. A. and Kimborough, J. W. (1980) Ascocarp development in *Chaetomidium arxii* (Chaetomiaceae) with special emphasis on the ontogeny of its cephalothecoid peridium. *Botanical Gazette* **141**: 347-352.

Berbee, M. L. and Taylor, J. W. (1992) Two ascomycete classes based on fruiting body characters and ribosomal DNA sequence. *Molecular Biology and Evolution* **9**: 278-284.

Berg, C. M. (1966) Biased distribution and polarized segregation in asci of *Sordaria brevicollis. Genetics* **53**: 117-129.

Bertrand, H., Chan, B. S. S. and Griffiths, A. J. F. (1985) Insertion of a foreign nucleotide sequence into mitochondrial DNA causes senescence in *Neurospora intermedia. Cell* **41**: 877-884.

Bistis, G. N. (1956) Studies on the genetics of *Ascobolus stercorarius* (Bull.) Schröt. *Bulletin of the Torrey Botanical Club* **83**: 35-61.

Bistis, G. N. (1981) Chemotropic interactions between trichogynes and conidia of opposite mating-type in *Neurospora crassa. Mycologia* **73**: 959-975.

Bistis, G. N. (1983) Evidence for diffusible, mating-type-specific trichogyne attractants in *Neurospora crassa. Experimental Mycology* **7**: 292-295.

Bjorling, K. (1944) *Ascobolus equinus*, ein apomiktischer Ascomycet nebst einigen Bemerkungen über die Sexualität der Euascomyceten. *Kungliga Fysiografiska Sallskapets i Lund Förhandlingar* **14**: 1-21.

Blaich, R. and Esser, K. (1971) The incompatibility relationships between geographical races of *Podospora anserina. Molecular and General Genetics* **111**: 265-272.

Bond, D. J. (1969) Genetic recombination in *Sordaria brevicollis.* Ph.D. thesis, University of Cambridge.

Bond, D. J. and MacDonald, D. J. (1976) The genetic control of protoperithecial production in *Sordaria brevicollis. Genetical Research* **27**: 59-67.

Boone, D. M. and Keitt, G. W. (1956) *Venturia inaequalis* (Cke.) Wint. VIII. Inheritance of colour mutant characters. *American Journal of Botany* **43**: 226-233.

Boucherie, H., Bégueret, J. and Bernet, J. (1976) The molecular mechanism of protoplasmic incompatibility and its relationship to the formation of protoperithecia in *Podospora anserina. Journal of General Microbiology* **92**: 59-66.

Boucherie, H. and Bernet, J. (1978) Protoplasmic incompatibility and self-lysis in *Podospora anserina*; enzymes associated with cell destruction. *Canadian Journal of Botany* **56**: 2171-2176.

Boucherie, H., Dupont, C. H. and Bernet, J. (1981) Polypeptide synthesis during protoplasmic incompatibility in the fungus *Podospora anserina. Biochimica et Biophysica Acta* **653**: 18-26.

Bretzloff, C. W. (1954) The growth and fruiting of *Sordaria fimicola. American Journal of Botany* **41**: 58-67.

Broxholme, S. J. (1988) The molecular analysis of sexual morphogenesis in *Sordaria brevicollis.* Ph.D. thesis, University of Edinburgh.

Broxholme, S. J., Read, N. D. and Bond, D. J. (1991) Developmental regulation of proteins during fruit-body morphogenesis in *Sordaria brevicollis. Mycological Research* **95**: 958-969.

Bu'lock, J. D. (1967) Fungal metabolites with structural function. In *Essays in Biosynthesis and Microbial Development.* E. R. Squibb Lectures on Chemistry of Microbial Products. pp. 1-18. Wiley, New York.

Bullock, S., Willetts, H. J. and Ashford, A. E. (1980) The structure and histochemistry of sclerotia of *Sclerotinia minor* Jagger. I. Light and electron microscope studies on sclerotial development. *Protoplasma* **104**: 315-331.

Burnett, J. H. (1975) *Mycogenetics.* John Wiley and Sons, London.

Burnett, J. H. (1976) *Fundamentals of Mycology.* 2nd edition. Edward Arnold (Publishers) Ltd., London.

Buston, H. W. and Rickard, B. (1956) The effect of a physical barrier on sporulation of *Chaetomium globosum. Journal of General Microbiology* **15**: 194-197.

Butler, G. M. (1966) Vegetative structures. In *The Fungi: An Advanced Treatise, Volume II.* (ed. G. C. Ainsworth and A. S. Sussman) pp. 83-112. Academic Press, New York.

Butt, T. M., Hoch, H. C., Staples, R. C. and St. Leger, R. J. (1989) Use of fluorochromes in the study of fungal cytology and differentiation. *Experimental Mycology* **13**: 303-320.

Byrne, B. J. (1975) Freeze-etch and thin section studies of *Neurospora crassa* ascospores. *Protoplasma* **84**: 357-377.

Cailleux, R. (1971) Recherches sur la Mycoflore coprophile Centafricaine. Les genres *Sordaria, Gelasinospora, Bombardia*; écologie. *Bulletin trimestriel de la Société Mycologique de France* **8**: 461-626.

Calhoun, F. and Howe, H. B. (1972) Nutrient-dependent inhibition of perithecial development due to sequential crosses on the same mycelium of *Neurospora tetrasperma. Planta* **108**: 289-302.

Carr, A. J. H. and Olive, L. S. (1958) Genetics of *Sordaria fimicola.* II. Cytology. *American Journal of Botany* **45**: 142-150.

Carroll, G. C. (1969) A study of the fine structure of ascosporogenesis in *Saccobolus kerverni. Archives of Microbiology* **66**: 321-339.

Casselton, L. A. (1978) Dikaryon Formation in Higher Basidiomycetes. In *The Filamentous Fungi, Volume III, Developmental Mycology.* (ed. J. E. Smith and D. R. Berry) pp. 273-297. Edward Arnold (Publishers) Ltd., London.

Catchside, D. G. (1944) Polarised segregation in an Ascomycete. *Annals of Botany* **8**: 119-130.

Caten, C. E. and Jinks, J. L. (1966) Heterokaryosis: Its significance in wild homothallic ascomycetes and fungi imperfecti. *Transaction of the British Mycological Society* **49**: 81-93.

Caten, C. E. (1987) The genetic integration of fungal life styles. In *Evolutionary Biology of the Fungi.* (ed. A. D. M. Rayner, C. M. Brasier and D. Moore) pp. 215-229. Cambridge University Press.

Champe, S. P. and El-Zyat, A. A. E. (1989) Isolation of a sexual sporulation hormone from *Aspergillus nidulans. Journal of Bacteriology* **171**: 3982-3988.

Champe, S. P., Rao, P. and Chang, A. (1987) An endogenous inducer of sexual development in *Aspergillus nidulans. Journal of General Microbiology* **133:** 1383-1387.

Chen, K. C. (1965) The genetics of *Sordaria brevicollis.* I. Determination of seven linkage groups. *Genetics* **51:** 509-517.

Chen, K. C. and Olive, L. S. (1965) The genetics of *Sordaria brevicollis.* II. Biased segregation due to spindle overlap. *Genetics* **51:** 761-766.

Chet, I. and Henis, Y. (1968) The control mechanism of sclerotial formation in *Sclerotium rolfsii* Sacc. *Journal of General Microbiology* **54:** 231-236.

Chet, I and Hüttermann, A. (1982) *De novo* synthesis of polyphenol oxidase (Laccase) during formation of sclerotia in *Sclerotium rolfsii. FEMS Microbiology Letters* **14:** 211-215.

Clutterbuck, A. J. (1972) Absence of laccase from yellow-spored mutants of *Aspergillus nidulans. Journal of General Microbiology* **70:** 423-435.

Cochrane, V. W. (1976) Glycolysis. In *The Filamentous Fungi, Volume II, Biosynthesis and Metabolism.* (ed. J. E. Smith and D. R. Berry) pp. 65-91. Edward Arnold (Publishers) Ltd., London.

Coley-Smith, J. R. (1979) Survival of plant pathogenic fungi in soil in the absence of host plants. In *Soil Borne Plant Pathogens.* (ed. B. Schippers and W. Gams) pp. 39-57. Academic Press, London.

Collinge, A. J. and Trinci A. P. J. (1974) Hyphal tips of wild type and spreading colonial mutants of *Neurospora crassa. Archiv für Mikrobiologie* **99:** 353-368.

Colson, B. (1934) The cytology and morphology of *Neurospora tetrasperma* (Shear and Dodge). *Annals of Botany* **48:** 211-224.

Cooke, R. C. (1971) Physiology of sclerotia of *Sclerotinia sclerotiorum* during growth and maturation. *Transactions of the British Mycological Society* **56:** 51-59.

Curtis, C. R. (1972) Action spectrum of the photoinduced sexual stage in the fungus *Nectria haematococca* Berk. and Br. var. *cucurbitae* (Snyder and Hansen) Dingley. *Plant Physiol*ogy **49:** 235-239.

Davies, K. M. and Grierson, D. (1989) Identification of cDNA clones from tomato (*Lycopersicon esculentum* Mill) mRNAs that accumulate during fruit ripening and leaf senescence in response to ethylene. *Planta* **179:** 73-80.

Degli-Innocenti, F, Pohl, U. and Russo, V. E. A. (1983) Photoinduction of protoperithecia in *Neurospora crassa* by blue light. *Photochem*istry *and Photobiology* **37:** 49-51.

Degli-Innocenti, F. and Russo, V. E. A. (1984a) Genetic analysis of blue light-induced responses in *Neurospora crassa.* In *Blue Light Effects in Biological Systems.* (ed. H. Senger) pp. 213-219. Springer-Verlag, Berlin.

Degli-Innocenti, F. and Russo, V. E. A. (1984b) Isolation of new white collar mutants of *Neurospora crassa* and studies on their behaviour in the blue light-induced formation of protoperithecia. *Journal of Bacteriology* **159**: 757-761.

Dehorter, B. and Bernillon, J. (1983) Photoinduction des périthèces de *Nectria galligena*: production et activité photomorphogène des mycosporines. *Canadian Journal of Botany* **61**: 1435-1442.

Dehorter, B. and Lacoste, L. (1979) Photoinduction des périthèces du *Nectria galligena*. I. Influence de la lumière blanche. *Canadian Journal of Botany* **58**: 2206-2211.

DeLange, A. M. and Griffiths, A. J. F. (1980a) Meiosis in *Neurospora crassa*. I. The isolation of recessive mutants defective in the production of viable ascospores. *Genetics* **96**: 367-378.

DeLange, A. M. and Griffiths, A. J. F. (1980b) Meiosis in *Neurospora crassa*. II. Genetic and cytological characterisation of three meiotic mutants. *Genetics* **96**: 379-398.

Dengler, I. (1937) Entwicklungsgeschichtliche Untersuchungen an *Sordaria macrospora* Auersw., *S. uvicolla* Viale et Mars. und *S. brefeldii* Zopf. *Jahrbucher fur wissenschaftliche Botanik* **84**: 427-448.

Dodge, B. O. (1927) Nuclear phenomena associated with heterothallism and homothallism in the ascomycete *Neurospora*. *Journal of Agricultural Research* **35**: 289-305.

Dodge, B. O. (1932) The non-sexual and sexual functions of microconidia of *Neurospora*. *Bulletin of the Torrey Botanical Club* **59**: 347-360.

Dodge, B. O. (1935) The mechanics of sexual reproduction in *Neurospora*. *Mycologia* **27**: 418-437.

Duran, R. and Gray, P. M. (1989) Nuclear DNA, an adjunct to morphology in fungal taxnomy. *Mycotaxon* **36**: 205-220.

Dutta, S. K., Frederick, L. and Austin, W. L. (1981) Some recent observations on the relationships between homothallic and heterothallic species of *Neurospora*. *Journal of the Elisha Mitchell Scientific Society* **97**: 126-135.

Dutta, S. K. and Ojha, M. (1972) Relatedness between major taxonomic groups of fungi based on the measurement of DNA nucleotide sequence homology. *Molecular and General Genetics* **114**: 232-240.

Dyer, P., Ingram, D. S. and Johnstone, K. (1992) The control of sexual morphogenesis in the Ascomycotina. *Biological Reviews* **67**: 421-458.

Dyer, P., Ingram, D. S. and Johnstone, K. (1993) Evidence for the involvement of linoleic acid and other endogenous lipid factors in perithecial development of *Nectria haematococca* mating population VI. *Mycological Research* **97**: 485-496.

El-Ani, A. S. and Olive, L. S. (1962) The induction of a balanced heterothallism in *Sordaria fimicola*. *Proceedings of the National Academy of Sciences U.S.A.* **48**: 17-19.

Elliott, C. G. (1960) The cytology of *Aspergillus nidulans. Genetical Research* **1**: 462-476.

Elliot, C. G. (1969) Effects of inhibitors of sterol synthesis on growth of *Sordaria* and *Phytophthora. Journal of General Microbiology* **56**: 331-343.

Ellis, J. J. (1960) Plasmogamy and ascocarp development in *Gelasinospora calospora. Mycologia* **52**: 557-573.

Entian, K. D. and Barnett, J. A. (1992) Regulation of sugar utilisation by *Saccharomyces cerevisiae. Trends in Biochemical Science* **17**: 506-510.

Eriksson, O. (1982) Outline of the ascomycetes-1982. *Mycotaxon* **15**: 203-248.

Esser, K. (1980) Effect of temperature and light on perithecial development in *Sordaria macrospora. Mycologia* **72**: 619-622.

Esser, K. and Meinhardt, F. (1977) A common genetic control of dikaryotic and monokaryotic fruiting in the basidiomycete *Agrocybe aegerita. Molecular and General Genetics* **155**: 113-115.

Esser, K. and Minuth, W. (1970) The phenol oxidases of the ascomycete *Podospora anserina.* V. Genetic regulation of the formation of laccase. *Genetics* **64**: 411-458.

Esser, K. and Straub, J. (1958) Genetische Untersuchungen an *Sordaria macrospora* Auersw., Kompensation und induktion bei genbedingten Entwicklungs-defekten. *Zeitschrift für Vererbungslehre* **89**: 729-746.

Esser, K., Saleh, F. and Meinhardt, F. (1979) Genetics of fruitbody production in higher basidiomycetes. 2. Monokaryotic and dikaryotic fruiting in *Schizophyllum commune. Current Genetics* **1**: 85-88.

Fennell, D. I. (1973) Plectomycetes; Eurotiales. In *The Fungi: An Advanced Treatise, Volume IVA.* (ed. G. C. Ainsworth, F. K. Sparrow and A. S. Sussman) pp. 45-68. Academic Press, New York.

Fields, W. G. (1963) Genetic investigation in the *Sordaria heterothallis* complex. Ph.D. thesis. Wayne State University, Detroit, Michigan, U.S.A.

Fields, W. G. (1968) A new species of *Sordaria. Mycologia* **60**: 1117-1118.

Fields, W. G. and Grear, J. W. (1966) A new heterothallic species of *Sordaria* from Ceylon. *Mycologia* **58**: 524-528.

Fields, W. G. and Maniotis, J. (1963) Some cultural and genetic aspects of a new heterothallic *Sordaria. American Journal of Botany* **50**: 80-85.

Fincham, J. R. S., Day, P. R. and Radford, A. (1979) *Fungal Genetics.* 4th edn. Blackwell Scientific Publications, Oxford.

Fitzgerald, P. H. (1963) Genetic and epigenetic factors controlling female sterility in *Neurospora crassa. Heredity* **18**: 47-62.

Fox, F. M. (1986) Ultrastructure and infectivity of sclerotium-like bodies of the ectomycorrhizal fungus *Hebeloma sacchariolens*, on birch (*Betula* spp.). *Transactions of the British Mycological Society* **87**: 359-369.

Froeyen, O. J. (1980) Perithecium growth and expansion in *Chaetomium globosum*. *Canadian Journal of Botany* **58**: 375-383.

Fukuda, Y., Inoue, Y. and Furuya, M. (1971) Developmental anatomy of experimentally induced ascocarps in *Gelasinospora reticulispora*. *Botanical Magazine* **84**: 330-348.

Furtado, J. S. (1970) Ascal cytology of *Sordaria brevicollis*. *Mycologia* **62**: 453-461.

Garnjobst, L. (1955) Further analysis of genetic control of heterokaryosis in *Neurospora crassa*. *American Journal of Botany* **42**: 444-448.

Garnjobst, L. and Tatum, E. L. (1967) A survey of new morphological mutants in *Neurospora crassa*. *Genetics* **57**: 579-604.

Garnjobst, L. and Wilson, J. F. (1956) Heterokaryosis and protoplasmic incompatibility in *Neurospora crassa*. *Proceedings of the National Academy of Sciences U.S.A.* **42**: 613-618.

Garrett, S. D. (1970) *Pathogenic Root-infecting Fungi*. pp. 92-106. Cambridge University Press.

Gaudet, D. A. and Kokko, E. G. (1986) Development of microsclerotia by *Coprinus psychromorbidus*. *Canadian Journal of Botany* **65**: 26-33.

Gäumann, E. (1964) *Die Pilze*. Berkhäuser Verlag, Basel.

Gillies, C. B. (1972) Reconstruction of the *Neurospora crassa* pachytene karyotype from serial sections of synaptonemal complexes. *Chromosoma*, Berlin, **36**: 119-130.

Glass, N. L., Grötelueschen, J. and Metzenberg, R. L. (1990a) *Neurospora crassa A* mating-type region. *Proceedings of the National Academy of Sciences U.S.A.* **87**: 4912-4916.

Glass, N. L. and Lee, L. (1992) Isolation of *Neurospora crassa A* mating type mutants by repeat induced point (RIP) mutation. *Genetics* **132**: 125-133.

Glass, N. L., Metzenberg, R. L. and Raju, N. B. (1990b) Homothallic Sordariaceae from nature: The absence of strains containing only the *a* mating type sequence. *Experimental Mycology* **14**: 274-289.

Glass, N. L., Vollmer, S. J., Staben, C., Grötelueschen, J., Metzenberg, R. L. and Yanofsky, C. (1988) DNAs of the two mating-type alleles of *Neurospora* crassa are highly dissimilar. *Science* **241**: 570-573.

Gooday, G. W. (1978) The enzymology of hyphal growth. In *The Filamentous Fungi, Volume III, Developmental Mycology*. (ed. J. E. Smith and D. R. Berry) pp. 51-77. Edward Arnold (Publishers) Ltd., London.

Greenspan, P., Mayer, E. P. and Fowler, S. D. (1985) Nile red: A selective fluorescent stain for intracellular lipid droplets. *Journal of Cell Biology* **100**: 965-973.

Gregory, P. H. (1984) The fungal mycelium - an historical perspective. In *The Ecology and Physiology of the Fungal Mycelium*. (ed. D. H. Jennings and A. D. M. Rayner) pp. 1-22. Cambridge University Press.

Griffiths, A. J. F. and Bertrand, H. (1984) Unstable cytoplasms in Hawaiian strains of *Neurospora intermedia*. *Current Genetics* **8**: 387-398.

Griffiths, A. J. F. and DeLange, A. M. (1978) Mutations of the *a* mating-type gene in *Neurospora crassa*. *Genetics* **88**: 239-254.

Griffiths, A. J. F. and Rieck, A. (1981) Perithecial distribution patterns in standard and variant strains of *Neurospora crassa*. *Canadian Journal of Botany* **59**: 2610-2617.

Gull, K. (1978) Form and function of septa in filamentous fungi. In *The Filamentous Fungi, Volume III, Developmental Mycology*. (ed. J. E. Smith and D. R. Berry) pp. 78-93. Edward Arnold (Publishers) Ltd., London.

Hackett, C. J. and Chen, K. C. (1976) Ultrastructure of developing ascospores in *Sordaria brevicollis*. *Journal of Bacteriology* **126**: 883-894.

Hale, L. J. (1965) *Biological Laboratory Data*. Methuen & Co. Ltd., London.

Hall, R. (1971) Effect of carbon-nitrogen ratios on production of perithecia by *Sordaria fimicola*. *Canadian Journal of Microbiology* **17**: 132-134.

Hanlin, R. T. (1961) Studies in the genus *Nectria* - I. Factors influencing perithecial formation in culture. *Bulletin of the Torrey Botanical Club* **88**: 95-103.

Harding, R. W. and Melles, S. (1983) Genetic analysis of phototropism of *Neurospora crassa* perithecial beaks using white collar and albino mutants. *Plant Physiology* **72**: 996-1000.

Harding, R. W. and Shropshire, W. (1980) Photocontrol of carotenoid biosynthesis. *Annual Review of Plant Physiology* **31**: 217-238.

Harper, J. E. and Webster, J. (1964) An experimental analysis of the coprophilous fungus succession. *Transactions of the British Mycological Society* **47**: 511-530.

Harris, J. L., Howe, H. B. and Roth, I. L. (1975) Scanning electron microscopy of surface and internal features of developing perithecia of *Neurospora crassa*. *Journal of Bacteriology* **122**: 1239-1246.

Hawker, L. E. (1951) Morphological and physiological studies on *Sordaria destruens* (Shear) comb. nov. (syn. *Melanospora destruens*), *Sordaria fimicola* and *Melanospora zamiae*. *Transactions of the British Mycological Society* **34**: 174-186.

Hawker, L. E. (1957) *The Physiology of Reproduction in Fungi*. Cambridge University Press.

Hawker, L. E. (1966) Environmental influences on reproduction. In *The Fungi: An Advanced Treatise, Volume II.* (ed. G. C. Ainsworth and A. S. Sussman) pp. 435-511. Academic Press, New York.

Hawksworth, D. L. (1985) Problems and prospects in the systematics of the Ascomycotina. *Proceeding of the Indian Academy of Science* (*Plant Science*) **94**: 319-339.

Hawksworth, D. L. (1987) The evolution and adaptation of sexual reproductive structures in the Ascomycotina. In *Evolutionary Biology of the Fungi.* (eds A. D. M. Rayner, C. M. Brasier and D. Moore) pp. 179-189. Cambridge University Press.

Hawksworth, D. L., Sutton, B. C. and Ainsworth, G. C. (1983) *Ainsworth and Bisby's Dictionary of the Fungi*, 7th edn. Commonwealth Mycological Institute, Kew, England.

Heath, M. C. and Heath, I. B. (1975) Ultrastructural changes associated with the haustorial mother cell septum during haustorium formation in *Uromyces phaseoli* var. *vignae. Protoplasma* **84**: 297-314.

Henderson, L. E., Elliott, M. and Ross, I. K. (1983) Sclerotium formation in *Coprinus congregatus. Mycologia* **75**: 738-741.

Hereward, F. V. and Moore, D. (1979) Polymorphic variation in the structure of aerial sclerotia of *Coprinus cinereus. Journal of General Microbiology* **113**: 13-18.

Hermann, T. E., Kurtz, M. B. and Champe, S. P. (1983) Laccase located in hulle cells and cleistothecial primordia of *Aspergillus nidulans. Journal of Bacteriology* **154**: 955-964.

Herskowitz, I. (1988) Life cycle of the budding yeast *Saccharomyces cerevisiae. Microbiological Revue* **52**: 536-553.

Heslot, H. (1958) Contribution à l' étude cytogénétique des Sordariacées. *Revue de Cytologie et de Biologie Végétales* **19**: 1-209.

Hirsch, H. M. (1954) Environmental factors influencing the differentiation of protoperithecia and their relation to tyrosinase and melanin formation in *Neurospora crassa. Physiologia Plantarum* **7**: 72-97.

Ho, C. C. (1972) Mutations blocking development of the protoperithecium in *Neurospora. Neurospora Newsletter* **19**: 15-16.

Hock, B., Bahn, M., Walk, R-A. and Nitschke, U. (1978) The control of fruiting body formation in the ascomycete *Sordaria macrospora* Auersw. by regulation of hyphal development. An analysis based on scanning electron and light microscopic observations. *Planta* **141**: 93-103.

Hodgkiss, I. J. (1969) The effect of various carbon and nitrogen sources on fruiting and sporulation of certain pyrenomycetes. *Nova Hedwegia* 18: 209-214.

Hohl, H. R. and Streit, W. (1975) Ultrastructure of ascus, ascospore and ascocarp in *Neurospora lineolata. Mycologia* **67**: 367-381.

Horowitz, N. H., Feldman, H. M. and Pall, M. L. (1970) Derepression of tyrosinase synthesis in *Neurospora* by cycloheximide, actinomycin D and puromycin. *Journal of Biological Chemistry* **245**: 2784-2788.

Horowitz, N. H., Fling, M., Macleod, H. and Watanabe, Y. (1961) Structural and regulative genes controlling tyrosinase synthesis in *Neurospora. Cold Spring Harbour Symposium on Quantitative Biology* **26**: 233-238.

Hotson, H. H. (1942) Some species of *Papulaspora* associated with rots of *Gladiolus* bulbs. *Mycologia* **34**: 391-399.

Howe, H. B. (1956) Crossing over and nuclear passing in *Neurospora crassa. Genetics* **41**: 610-622.

Howe, H. B. and Prakash, V. (1969) A regulatory system controlling inhibition of the sexual cycle in *Neurospora. Canadian Journal of Genetics and Cytology* **11**: 689-705.

Huang, L. C. (1976) Developmental morphology of *Triangularia backusii* (Sordariaceae). *Canadian Journal of Bot*any **54**: 250-267.

Huang, L. C. (1983) Histology, amino acid leakage, and chemical composition of normal and abnormal sclerotia of *Sclerotinia sclerotiorum. Canadian Journal of Botany* **61**: 1443-1447.

Huebschman, C. (1952) A method for varying the average number of nuclei in the conidia of *Neurospora crassa. Mycologia* **44**: 599-604.

Ilott, T. W., Ingram, D. S. and Rawlinson, C. J. (1986) Evidence of a chemical factor involved in the control of sexual development in the light leaf spot fungus, *Pyrenopeziza brassicae* (Ascomycotina). *Transactions of the British Mycological Society* **87**: 303-308.

Ingold, C. T. (1953) *Dispersal in fungi.* Clarendon Press, Oxford.

Ingold, C. T. and Dring, V. J. (1957) An analysis of spore discharge in *Sordaria. Annals of Botany* **21**: 465-477.

Ingold, C. T. and Hadland, S. A. (1959a) The ballistics of *Sordaria. New Phytologist* **58**: 46-57.

Ingold, C. T. and Hadland, S. A. (1959b) Phototropism and pigment production in *Sordaria* in relation to quality of light. *Annals of Botany* 23: 425-429.

Inoue, Y. (1984) Re-examination of action spectroscopy in blue/near-UV light effects. In *Blue Light Effects in Biological Systems* (ed. H. Senger) Springer-Verlag, Berlin.

Inoue, Y. and Furuya, M. (1970) Perithecial formation in *Gelasinospora reticulispora.* I. Effects of light at two different growth states. *Development, Growth and Differentiation* **12**: 141-150.

Inoue, Y. and Furuya, M. (1974a) Perithecial formation in *Gelasinospora reticulispora.* II. Promotive effects of near-ultraviolet and blue light after dark incubation. *Plant & Cell Physiology* **15**: 195-204.

Inoue, Y. and Furuya, M. (1974b) Perithecial formation in *Gelasinospora reticulispora.* III. Inhibitory effects of near-UV and blue light during the inductive dark period. *Plant & Cell Physiology* **15**: 469-475.

Inoue, Y. and Furuya, M. (1975a) Perithecial formation in *Gelasinospora reticulispora.* IV. Action spectrum for the photoinduction. *Plant Physiology* **55**: 1098-1101.

Inoue, Y. and Furuya, M. (1975b) Perithecial formation in *Gelasinospora reticulispora.* V. Microscopically versus photobiologically recognisable differentiation of hyphae. *Botanical Magazine* **88**: 31-40.

Inoue, Y. and Watanabe, M. (1984) Perithecial formation in *Gelasinospora reticulispora.* VII. Action spectra in UV region for the photoinduction and the photoinhibition of photoinductive effect brought by blue light. *Plant & Cell Physiology* **25**: 107-113.

Islam, M. S. (1981) Sex pheromones in *Neurospora crassa.* In *Sexual Interactions in Eukaryotic Microbes.* (ed. D. H. O'Day and P. A. Horgen) pp. 131-154. Academic Press, New York.

Islam, M. S. and Weijer, J. (1969) Sex hormones in *Neurospora crassa. Neurospora Newsletter* **15**: 24-25.

Islam, M. S. and Weijer, J. (1972) Development of fertile fruit bodies in a single-strain culture of *Neurospora crassa. Folia Microbiologia* **17**: 316-319.

Itoh, T. and Morishita, K. (1971) Effects of cytoplasm on protoperithecium formation in *Neurospora crassa. Japanese Journal of Genetics* **46**: 7-15.

Iyengar, G. A. S., Deka, P. C., Kundu, S. C. and Sen, S. K. (1977) DNA synthesis in course of meiotic development in *Neurospora crassa. Genetical Research* **29**: 1-8.

Jarvis, W. R. and Traquair, J. A. (1985) Sclerotia of *Aspergillus aculeatus. Canadian Journal of Botany* **63**: 1567-1572.

Jennings, D. H. (1974) Sugar transport into fungi: An essay. *Transactions of the British Mycological Society* **62**: 1-24.

Jennings, D. H. (1976) Transport and translocation in filamentous fungi. In *The Filamentous Fungi, Volume II, Biosynthesis and Metabolism.* (ed. J. E. Smith and D. R. Berry) pp. 32-64. Edward Arnold (Publishers) Ltd., London.

Jensen, J. D. (1981) The developmental morphology of *Hypoxylon serpens* in culture. *Canadian Journal of Botany* **59**: 40-49.

Jensen, J. D. (1982) The development of *Gelasinospora reticulispora. Mycologia* **74**: 724-737.

Jensen, J. D. (1983) The development of *Diaporthe phaseolorum* variety *sojae* in culture. *Mycologia* **75**: 1074-1091.

Jha, K. K. and Olive, L. S. (1975) Genetics of Sordaria fimicola. VIII. Nitrosoguanidine mutagenesis and isolation of protoperithecial mutants. *Mycologia* **67**: 45-55.

Johnson, T. E. (1976) Analysis of pattern formation in *Neurospora* perithecial development using genetic mosaics. *Developmental Biology* **54**: 23-36.

Johnson, T. E. (1977) Mosaic analysis of autonomy of spore development in *Neurospora. Experimental Mycology* **1**: 253-258.

Johnson, T. E. (1978) Isolation and characterisation of perithecial development mutants in *Neurospora. Genetics* **88**: 27-47.

Johnson, T. E. (1979) A *Neurospora* mutation that arrests perithecial development as either male or female parent. *Genetics* **92**: 1107-1120.

Jong, S. C. and Davis, E. E. (1973) Stromatic *Neurosporas. Mycologia* **65**: 458-464.

Khan, R. S. and Krug, J. C. (1989) New records of the Sordariaceae from East Africa. *Mycologia* **81**: 862-869.

Kohn, L. M. and Grenville, D. J. (1989) Anatomy and histochemistry of stromatal anamorphs in the Sclerotiniaceae. *Canadian Journal of Botany* **67**: 371-393.

Krug, J. C. and Cain, R. F. (1974) New species of *Hypocopra* (Xylariaceae). *Canadian Journal of Botany* **52**: 809-843.

Labarère, J and Bernet, J. (1978) A pleiotropic mutation affecting protoperithecium formation and ascospore outgrowth in *Podospora anserina. Journal of General Microbiology* **113**: 19-27.

Labarère, J. and Noël, T. (1992) Mating type switching in the tetrapolar basidiomycete *Agrocybe aegerita. Genetics* **131**: 307-319.

Lakin-Thomas, P. L., Coté, G. G. and Brody, S. (1990) Circadian rhythms in *Neurospora crassa*: Biochemistry and genetics. *Critical Reviews in Microbiology* **17**: 365-416.

Lamb, B. C. (1966) Polarised segregation in Ascomycetes and the differential bursting of asci. *Genetical Research* **7**: 325-734.

Lauter, F. R. and Russo, V. E. A. (1990) Light-induced dephosphorylation of a 33-kDa protein in the wild-type strain of *Neurospora crassa*: The regulatory mutants *wc*-1 and *wc*-2 are abnormal. *Journal of Photochemistry and Photobiology B, Biology* **5**: 95-104.

Leatham, G. F. and Stahmann, M. A. (1981) Studies on the laccase of *Lentinus edodes*: Specificity, localisation and association with the development of fruiting bodies. *Journal of General Microbiology* **125**: 147-157.

Leonard, T. J. and Dick, S. (1973) Induction of haploid fruiting by mechanical injury in *Schizophyllum commune. Mycologia* **65**: 809-822.

Leonard, T. J. and Phillips, L. E. (1973) Study of phenoloxidase activity during the reproductive cycle in *Schizophyllum commune*. *Journal of Bacteriology* **114**: 7-10.

Leslie, J. F. and Leonard, T. J. (1979a) Monokaryotic fruiting in *Schizophyllum commune*: Phenoloxidases. *Mycologia* **71**: 1082-1085.

Leslie, J. F. and Leonard, T. J. (1979b) Three independent systems that control initiation of a fungal fruiting body. *Molecular and General Genetics* **171**: 257-260.

Lewis, L. A. (1969a) Genetics of an interspecific cross in the genus *Sordaria*. I. Analysis of 8-spored asci homozygous for the mating type factor. *Genetics* **62**: 79-101.

Lewis, L. A. (1969b) Genetic evidence for hybridisation in an interspecific cross in the genus *Sordaria*. *Journal of General Microbiology* **59**: 359-367.

Lilly, V. G. and Barnett, H. L. (1947) The influence of pH and certain growth factors on mycelial growth and perithecial formation by *Sordaria fimicola*. *American Journal of Botany* **34**: 131-138.

Lindenmayer, A. and Schoen, H. F. (1967) Selective effects of purine and pyrimidine analogues and of respiratory inhibitors on perithecial development and branching in *Sordaria*. Plant Physiology 42: 1059-1070.

Lu, B. C. and Galeazzi, D. R. (1978) Light and electron microscopic observations of a meiotic mutant of *Neurospora crassa*. *Canadian Journal of Botany* **56**: 2694-2706.

Lundqvist, N. (1972) Nordic Sordariaceae s. lat.. *Symbolae Botanicae Upsalienses* **20**: 1-374.

Luttrell, E. S. (1951) Taxonomy of the pyrenomycetes. *University of Missouri Studies* **24**: 1-120.

Lysek, G. (1976) Formation of perithecia in colonies of *Podospora anserina*. *Planta* **133**: 81-83.

MacDonald, D. J. and Bond, D. J. (1974) Preferential ascus discharge during cross maturation in *Sordaria brevicollis*. *Genetics* **76**: 245-253.

MacDonald, D. J. and Bond, D. J. (1976) Genetic and environmental factors influencing the production and distribution of protoperithecia in *Sordaria brevicollis*. *Journal of General Microbiology* **95**: 375-380.

Mai, S. H. (1976) Morphological studies in *Podospora anserina*. *American Journal of Botany* **63**: 821-825.

Mai, S. H. (1977) Morphological studies in *Sordaria fimicola* and *Gelasinospora longispora*. *American Journal of Botany* **64**: 489-495.

Mainwaring, H. R. and Wilson, I. M. (1968) The life cycle and cytology of an apomictic *Podospora*. *Transactions of the British Mycological Society* **51**: 663-677.

Malloch, D. (1981) The Plectomycete Centrum. In *Ascomycete Systematics. The Luttrellian Concept.* (ed. D. R. Reynolds) pp. 73-91. Springer-Verlag, New York.

Manachère, G. (1988) Regulation of sporophore differentiation in some macromycetes, particularly in Coprini: An overview of some experimental studies, from fruiting initiation to sporogenesis. *Cryptogamie: mycologie* **9**: 291-323.

Martin, P. G. (1969) Apparent self-fertility in *Neurospora crassa. Journal of General Microbiology* **20**: 213-222.

Marx, D. H. (1980) Ectomycorrhizal fungusinoculations: a tool for improving forestation practices. In *Tropical Mycorrhiza Research 2.* (ed. P. Mikola) pp. 13-71. Oxford University Press.

Mathieson, M. J. (1956) Ascospore dimorphism and mating type in *Chromocrea spinulosa* (Fuckel) Petch n. comb. *Annals of Botany* **64**: 449-466.

Mayer, A. M. (1987) Polyphenol oxidases in plants - recent progress. *Phytochemistry* **26**: 11-20.

Mayer, A. M. and Harel, E. (1979) Polyphenol oxidases in plants. *Phytochemistry* **18**: 193-215.

McCoy, R. E., Horst, R. K. and Dimock, A. W. (1975) Environmental factors regulating sexual and asexual reproduction by *Mycosphaerella ligulicola. Phytopathology* **62**: 1188-1195.

McLeish, J. and Sunderland, N. (1961) Measurements of deoxyribosenucleic acid (DNA) in higher plants by Feulgen photometry and chemical methods. *Experimental Cell Research* **24**: 527-540.

McNelly-Ingle, C. A. and Frost, L. C. (1965) The effect of temperature on the production of perithecia by *Neurospora crassa. Journal of General Microbiology* **39**: 33-42.

Metzenberg, R. L. (1972) Genetic regulatory systems in *Neurospora. Annual Review of Genetics* **6**: 111-132.

Metzenberg, R. L. and Glass, N. L. (1990) Mating type and mating strategies in *Neurospora. BioEssays* **12**: 53-59.

Meyer, J. (1957) Évolution nucléaire et organogenèse chez *Gelasinospora calospora* (Mout.) Moreau. *Cellule* **58**: 345-362.

Mirza, J. H. and Khatoon, A. (1973) Studies on *Sordaria humana* (Fuckel) Winter: The cytology of ascus development and developmental morphology of perithecium. *Pakistan Journal of Botany* **5**: 19-28.

Mitchell, M. B. (1959) Detailed analysis of a *Neurospora* cross. *Genetics* **44**: 847-856.

Mitchell, M. B. (1960) Evidence of non-random distribution of ascus classes in fruiting bodies of *Neurospora crassa. Genetics* **45**: 1245-1251.

Mooney, J. L. and Yager, L. N. (1990) Light is required for conidiation in *Aspergillus nidulans. Genes and Development* **4**: 1473-1482.

Molowitz, R., Bahn, M. and Hock, B. (1976) The control of fruiting body formation in the ascomycete *Sordaria macrospora* Auersw. by arginine and biotin: a two factor analysis. *Planta* **128**: 143-148.

Moore-Landecker, E. (1975) Effect of cultural conditions on apothecial morphogenesis in *Pyronema domesticum. Canadian Journal of Botany* **53**: 2759-2769.

Moore-Landecker, E. (1979a) Effect of cultural age and a single photoperiod on morphogenesis of the discomycete *Pyronema domesticum. Canadian Journal of Botany* **57**: 1541-1549.

Moore-Landecker, E. (1979b) Effect of light regimens and intensities on morphogenesis of the discomycete *Pyronema domesticum. Mycologia* **71**: 699-712.

Moore-Landecker, E. (1982) *Fundamentals of the Fungi.* 2nd edn. Prentice-Hall Inc. New Jersey.

Moore-Landecker, E. (1983) Chemical induction of *Pyronema domesticum* apothecia in the dark. *Mycologia* **75**: 832-838.

Moore-Landecker, E. (1992) Physiology and biochemistry of ascocarp induction and development. *Mycological Research* **96**: 705-716.

Moore-Landecker, E. and Shropshire, W. (1982) Effects of aeration and light on apothecia, sclerotia, and mycelial growth in the discomycete *Pyronema domesticum. Mycologia* **76**: 820-829.

Moreau, C. (1953) Les genres *Sordaria* et *Pleurage*: Leurs affinités systématiques. *Encyclopédia Mycologique* **24**: 1-330.

Moreau, M. and Moreau, F. (1950) Étude du développment du *Triangularia bambusae* (van Beyma) Boedijn. *Revue de Mycologie* **15**: 146-158.

Morinaga, T., Ito, S. and Minoura, K. (1980) Fungal succession on deer dung-The effects of light on the development of certain fungi. *Transactions of the Mycological Society of Japan* **21**: 351-357.

Motta, J. J. (1971) Cytology and morphogenesis in the rhizomorph of *Armillaria mellea. American Journal of Botany* **56**: 610-619.

Mu'Azu, S. (1973) Cytogenetic studies on *Sordaria brevicollis.* Ph. D. thesis, University of Cambridge.

Müller, E. and von Arx, J. A. (1973) Pyrenomycetes: Melioales, Coronophorales, Sphaeriales. In *The Fungi: An Advanced Treatise, Volume IVA*. (ed. G. C. Ainsworth, F. K. Sparrow and A. S. Sussman) pp. 87-132. Academic Press, New York.

Muñoz, V. and Butler, W. L. (1975) Photoreceptor pigment for blue light in *Neurospora crassa. Plant Physiology* **55**: 421-426.

Mylyk, O. M. and Threlkeld, S. F. H. (1974) A genetic study of female sterility in *Neurospora crassa. Genetical Research* **24**: 91-102.

Nakamura, K., and Egashira, T. (1961) Genetically mixed perithecia in *Neurospora. Nature* **190**: 1129-1130.

Nawrath, C. and Russo, V. E. A. (1990) Fast induction of translatable messenger RNA by blue light in *Neurospora cra*ssa wt: The *wc*-1 and *wc*-2 mutants are blind. *Journal of Photochemistry and Photobiology, B Biology* **4**: 261-272.

Nelson, A. C., and Backus, M. P. (1968) Ascocarp development in two homothallic *Neurosporas. Mycologia* **60**: 16-28.

Nelson, R. R. (1971) Hormonal involvement with sexual reproduction in the fungi with special reference to F-2, a fungal oestrogen. In *Morphological and Biochemical Events in Plant-Parasite Interaction.* (ed. S. Akai and S. Ouchi) pp. 181-205. The Phytopathological Society of Japan, Tokyo.

O'Brien, T. P. and McCully, M. E. (1981) *The Study of Plant Structure; Principles and Selected Methods.* Termarcarphi Pty. Ltd. Melbourne, Australia.

Okada, H. and Halvorson, K. O. (1964) Uptake of α-thioethyl-D-glucopyranocide by *Saccharomyces cerevisiae.* II. General characteristics of an active transport system. *Biochimica et Biophysica Acta* **82**: 547-555.

Olive, L. S. (1954) Cross-karyogamy and segregation in a homothallic fungus. *Bulletin of the Torrey Botanical Club* **81**: 95-97.

Olive, L. S. (1958) On the evolution of heterothallism in fungi. *American Naturalist* **92**: 233-251.

Olive, L. S. and Fantini, A. A. (1961) A new, heterothallic species of *Sordaria. American Journal of Botany* **48**: 124-128.

Osiewacz, H. D. and Esser, K. (1984) The mitochondrial plasmid of *Podospora anserina*: a mobile intron of a mitochondrial gene. *Current Genetics* **8**: 299-305.

Ott, M. and Ardizzi, J. (1989) The effects of griseofulvin on the vegetative growth and sexual phase development of the fungus *Neurospora tetrasperma* Dodge. *Journal of the Pennsylvania Academy of Science* **63**: 181-185.

Page, R. M. (1965) The physical environment for fungal growth. III. Light. In *The Fungi: An Advanced Treatise, Volume I.* (ed. G. S. Ainsworth and A. S. Sussman) pp. 559-574. Academic Press, New York.

Perkins, D. D. (1985) Aspects of the *Neurospora* genome. In *Molecular Genetics of Filamentous Fungi.* (ed. W. E. Timberlake) Alan R. Liss, Inc., New York.

Perkins, D. D. (1987) Mating-type switching in filamentous ascomycetes. *Genetics* **115**: 215-216.

Perkins, D. D., Radford, A., Newmeyer, D. and Björkman, M. (1982) Chromosomal loci of *Neurospora crassa. Microbiological Reviews* **46**: 426-570.

Perkins, D. D. and Raju, N. B. (1986) *Neurospora discreta*, a new heterothallic species defined by its crossing behaviour. *Experimental Mycology* **10**: 323-338.

Perkins, D. D. and Turner, B. C. (1988) *Neurospora* from natural populations: Toward the population biology of a haploid eukaryote. *Experimental Mycology* **12**: 1-44.

Perkins, D. D., Turner, B. C. and Barry, E. G. (1976) Strains of *Neurospora* collected from nature. *Evolution* **30**: 281-313.

Perry, J. W., and Evert, R. F. (1984) Structure of microsclerotia of *Verticillium dahliae* in roots of "Russet Burbank" potatoes. *Canadian Journal of Botany* **62**: 396-401.

Picard, M., Debuchy, R. and Coppin, E. (1991) Cloning the mating types of the heterothallic fungus *Podospora anserina*: Developmental features of haploid transformants carrying both mating types. *Genetics* **128**: 539-547.

Pollock, R. T. (1973) Environmental factors affecting the pattern of perithecium development in *Sordaria fimicola. Bulletin of the Torrey Botanical Club* 100: 78-83.

Pollock, R. T. (1975) Control of the pattern of perithecium development in *Sordaria fimicola* on agar medium. *Growth* **39**: 223-232.

Pollock, R. T. and Johnson, G. A. (1972) The blackberry mutant of *Sordaria fimicola. Canadian Journal of Genetics and Cytology* **14**: 943-948.

Pontecorvo, G. (1956) The parasexual cycle in fungi. *Annual Review of Microbiology* **10**: 393-400.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D. and Bufton, A. W. J. (1953) The genetics of *Aspergillus nidulans. Advances in Genetics* **5**: 141-238.

Prillinger, H. and Esser, K. (1977) The phenoloxidases of the ascomycete *Podospora anserina.* XIII. Action and interaction of genes controlling the formation of laccase. *Molecular and General Genetics* **156**: 333-345.

Radford, A. and Threlkeld, S. F. H. (1970) Genetically mixed perithecia and somatic recombination in a pseudo-wild type strain of *Neurospora crassa. Canadian Journal of Genetics and Cytology* **12**: 547-552.

Raju, N. B. (1978) Meiotic nuclear behaviour and ascospore formation in five homothallic species of *Neurospora. Canadian Journal of Botany* **56**: 754-763.

Raju, N. B. (1980) Meiosis and ascospore genesis in *Neurospora. European Journal of Cell Biology* **23**: 208-223.

Raju, N. B. (1986) A simple fluorescent staining method for meiotic chromosomes of *Neurospora. Mycologia* **78**: 901-906.

Raju, N. B. (1992a) Functional heterothallism resulting from homokaryotic conidia and ascospores in *Neurospora tetrasperma. Mycological Research* **96**: 103-116.

Raju, N. B. (1992b) Genetic control of the sexual cycle in *Neurospora. Mycological Research* **96**: 214-262.

Raju, N. B. and Perkins, D. D. (1978) Barren perithecia in *Neurospora crassa. Canadian Journal of Genetics and Cytology* **20**: 41-59.

Raper, J. R. (1960) The control of sex in fungi. *American Journal of Botany* **47**: 794-808.

Rau, W. (1980) Blue light-induced carotenoid biosynthesis in microorganisms. In *The Blue Light Syndrome.* (ed. H. Senger) pp. 283-298. Springer-Verlag, New York.

Rayner, A. D. M., Powell, K. A., Thompson, W. and Jennings, D. H. (1985) Morphogenesis of vegetative organs. In *Developmental Biology of the Higher Fungi.* (ed. D. Moore, L. A. Casselton, D. A. Wood and J. C. Frankland) pp. 249-279. Cambridge University Press.

Read, N. D. (1983) A scanning electron microscopic study of the external features of perithecium development in *Sordaria humana. Canadian Journal of Botany* **61**: 3217-3229.

Read, N. D. (in press) Cellular nature and multicellular morphogenesis of higher fungi. In *Shape and Form in Plants and Fungi.* (ed. D. S. Ingram) Cambridge University Press.

Read, N. D. and Beckett, A. (1985) The anatomy of the mature perithecium in *Sordaria humana* and its significance for fungal multicellular development. *Canadian Journal of Botany* **63**: 281-296.

Read, N. D. and Lord, K. M. (1991) Examination of living fungal spores by scanning electron microscopy. *Experimental Mycology* **15**: 132-139.

Read, N. D., Porter, R. and Beckett, A. (1983) A comparison of preparative techniques for the examination of the external morphology of fungal material with the scanning electron microscope. *Canadian Journal of Botany* **61**: 2059-2078.

Reynolds, D. R. ed. (1981) *Ascomycete Systematics. The Luttrellian Concept.* Springer-Verlag, New York.

Rieck, A., Griffiths, A. J. F. and Bertrand, H. (1982) Mitochondrial variants of *Neurospora intermedia* from nature. *Canadian Journal of Genetics and Cytology* **24**: 741-759.

Rigling, D., Heiniger, U. and Hohl, H. R. (1989) Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica. Phytopathology* **79**: 219-223.

Robert, J. C. and Bret, J. P. (1987) Release of an inhibitor of stipe elongation from illuminated caps of *Coprinus congregatus* mushrooms. *Canadian Journal of Botany* **65**: 505-508.

Robinson, W. (1926) The conditions of growth and development of *Pyronema confluens* Tul. (*P. omphalodes* (Bull) Fuckel). *Annals of Botany* **60**: 245-272.

Rogers, J. B. (1975) *Xylaria polymorpha.* II. Cytology of a form with typical robust stromata. *Canadian Journal of Botany* **53**: 1736-1743.

Romano, A. H. and Kornberg, H. L. (1969) Regulation of sugar uptake by *Aspergillus nidulans. Proceedings of the Royal Society, Series B* **173**: 475-490.

Ross, I. K. (1979) *Biology of the Fungi. Their Development, Regulation and Associations.* McGraw-Hill Book Company, New York.

Ross, I. K. (1982) Localisation of carpophore initiation in *Coprinus congregatus. Journal of General Microbiology* **128**: 2755-2762.

Roure, M and Bouillant, M. L. (1986) Development and application of a bioassay to study the effects of nutrients, pH, and active substances on *Sordaria macrospora* fruiting. *Canadian Journal of Microbiology* **32**: 930-936.

Ruddat, M. and Garber, E. D. (1983) Biochemistry, physiology and genetics of carotenogenesis in fungi. In *Secondary Metabolism and Differentiation in Fungi.* (ed. J. W. Bennett and A. Ciegler) pp. 95-151. Marcel Dekker, Inc., New York and Basel.

Russo, V. E. A. (1986) Are carotenoids the blue-light photoreceptor in the photoinduction of protoperithecia in *Neurospora crassa*? *Planta* **168**: 56-60.

Russo, V. E. A. (1988) Blue light induces circadian rhythms in the *bd* mutant of *Neurospora*: Double mutants *bd, wc*-1 and *bd, wc*-2 are blind. *Photochemistry and Photobiology* (*B: Biology*) **2**: 59-65.

Sang, H. (1978) Studies on genetic recombination in *Sordaria brevicollis.* Ph. D. thesis, University of Cambridge.

Sanni, M. O. (1982) Perithecial development of *Gelasinospora longispora. Botanical Gazette* **143**: 542-545.

Sanni, M. O. (1984) Perithecium development in *Sordaria brevicollis. Nordic Journal of Bot*any **4**: 65-69.

Sansome, E. R. (1947) The use of heterokaryons to determine the origin of the ascogenous nuclei in *Neurospora crassa. Genetica* **24**: 59-64.

Sengbusch, P. V., Hechler, J. and Müller, U. (1983) Molecular architecture of fungal cell walls. An approach by use of fluorescent markers. *European Journal of Cell Biology* **30**: 305-312.

Senger, H. ed. (1984) *Blue Light Effects in Biological Systems.* Springer-Verlag, Berlin.

Shaw, J. (1962) Asymmetrical segregation of mating type and two morphological mutant loci in *Sordaria brevicollis. Bulletin of the Torrey Botanical Club* **89**: 83-91.

Siddiq, A. A. (1989) The control of sexual morphogenesis in *Pyrenopeziza brassicae*, cause of light leaf spot of brassicas. Ph. D. thesis, University of Cambridge.

Siddiq, A. A., Ingram, D. S., Johnstone, K., Friend, J. and Ashby, A. M. (1989) The control of asexual and sexual development by morphogens in fungal pathogens. *Aspects of Applied Biology* **23**: 417-426.

Siddiq, A. A., Johnstone, K. and Ingram, D. S. (1990) Evidence for the production during mating of factors involved in suppression of asexual sporulation and the induction of ascocarp formation in *Pyrenopeziza brassicae. Mycological Research* **96**: 757-765.

Simonet, J. M. and Zickler, D. L. (1972) Mutations affecting meiosis in *Podospora anserina.* I. Cytological studies. *Chromosoma* **37**: 327-351.

Singleton, J. R. (1953) Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa. American Journal of Botany* **40**: 124-144.

Smith, A. M. (1958) *Manures and fertilisers.* Thomas Nelson & Son Ltd. Edinburgh.

Smith, D. A. (1975) A mutant affecting meiosis in *Neurospora. Genetics* **80**: 125-133.

Smith, J. R. and Rubenstein, I. (1973) The development of "senescence" in *Podospora anserina. Journal of General Microbiology* **76**: 283-296.

Snyder, W. C. (1961) Heterokaryosis as a natural phenomenon. *Recent Advances in Botany, Volume I.* 371-374. University of Toronto Press.

Sommer, T., Chambers J. A. A., Eberle, J., Lauter, F. R. and Russo, V. E. A. (1989) Fast light-regulated genes of *Neurospora crassa. Nucleic Acid Research* **17**: 5713-5723.

Sommer, T., Degli-Innocenti, F. and Russo, V. E. A. (1987) Rôle of nitrogen in the photoinduction of protoperithecia and carotenoids in *Neurospora crassa. Planta* **170**: 205-208.

Song, P. S. (1984) Photophysical aspects of blue light receptors: The old question (flavins versus carotenoids) re-examined. In *Blue Light Effects in Biological Systems.* (ed. H. Senger) pp. 75-80. Springer-Verlag, Berlin.

Springer, M. L. and Yanofsky, C. (1989) A morphological and genetic analysis of conidiophore development in *Neurospora crassa. Genes and Development* **3**: 559-571.

Srb, A. M. and Basl, M. (1969) The isolation of mutants affecting ascus development in *Neurospora crassa* and their analysis by a zygote complementation test. *Genetical Research* **13**: 303-311.

Staben, C. and Yanofsky, C. (1990) *Neurospora crassa a* mating type region. *Proceedings of the National Academy of Sciences, U.S.A.* **87**: 4917-4921.

Stahl, U. and Esser, K. (1976) Genetics of fruitbody production in higher basidiomycetes. I. Monokaryotic fruiting and its correlation with dikaryotic fruiting in *Polyporus ciliatus. Molecular and General Genetics* **148**: 183-197.

Stamberg, J. and Koltin, Y. (1981) The genetics of mating systems: fungal strategies for survival. In *The Fungal Community. Its Organisation and Rôle in the Ecosystem.* (ed. D. T. Wicklow and G. C. Carroll) pp. 157-170. Marcel Dekker, Inc., New York and Basel.

Syrop, M. J. and Beckett, A. (1972) The origin of ascospore-delimiting membranes in *Taphrina deformans. Archiv für Mikrobiologie* **86**: 185-191.

Tan, K. K. (1978) Light-induced fungal development. In *The Filamentous Fungi, Volume III, Developmental Mycology.* (ed. J. E. Smith and D. R. Berry) pp. 334-357. Edward Arnold, London.

Tan, S. T. and Ho, C. C. (1970) A gene controlling the early development of protoperithecia in *Neurospora crassa. Molecular and General Genetics* **107**: 158-161.

Taylor, J. W. (1986) Fungal evolutionary biology and mitochondrial DNA. *Experimental Mycology* **10**: 259-269.

Taylor, J. W. and Natvig, D. O. (1989) Mitochondrial DNA and evolution of heterothallic and pseudohomothallic *Neurospora* species. *Mycological Research* **93**: 257-272.

Thompson-Coffe, C. and Zickler, D. (1992) Three microtubule organising centres are required for ascus growth and sporulation in the fungus *Sordaria macrospora. Cell Motility and the Cytoskeleton* **22**: 257-273.

Tisdale, S. L. and Nelson, W. L. (1956) *Soil fertility and fertilisers.* MacMillan, New York.

Townsend, B. B. and Willetts, H. J. (1954) The development of sclerotia of certain fungi. *Transactions of the British Mycological Society* **37**: 213-221.

Trinci, A. P. J. (1974) A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. *Journal of General Microbiology* **81**: 225-236.

Tudzynski, P. and Esser, K. (1979) Chromosomal and extrachromosomal control of senescence in the ascomycete *Podospora anserina. Molecular and General Genetics* **173**: 71-84.

Turian, G. (1975) Differentiation in *Allomyces* and *Neurospora. Transactions of the British Mycological Society* **64**: 367-380.

Turian, G. (1978) *Sexual morphogenesis in the Ascomycetes.* In *The Filamentous Fungi, Volume III, Developmental Mycology.* (ed. J. E. Smith and D. R. Berry) pp. 315-333. Edward Arnold, London.

Turian, G., Seydoux, J. and Volkmann, D. (1962) Activités isocitratasiques et type de sporulation chez *Neurospora tetrasperma* et chez *N. sitophila*, souche normale et mutant microconidien. *Pathological Microbiology* **25**: 737-751.

Turian, G. and Viswanath-Reddy, M. (1971) Metabolic and ultrastructural aspects of sexual differentiation in *Allomyces* and *Neurospora. Journal of the Indian Botanical Society, Golden Jubilee Volume* **50A**: 88-89.

Uecker, F. A. (1976) Development and cytology of *Sordaria humana*. *Mycologia* **68**: 30-46.

Vigfusson, N. V. and Cano, R. J. (1974) Artificial induction of the sexual cycle of *Neurospora crassa*. *Nature* **249**: 383-385.

Vigfusson, N. V. and Weijer, J. (1972) Sexuality in *Neurospora crassa*. II. Genes affecting the sexual development cycle. *Genetical Research* **19**: 205-211.

Viswanath-Reddy, M. and Turian, G. (1972) Temperature-induced synchronous differentiation of ascogonia in *Neurospora*. *Experientia* **28**: 99-100.

Viswanath-Reddy, M. and Turian, G. (1975) Physiological changes during protoperithecial differentiation in *Neurospora tetrasperma*. *Physiologia Plantarum* **35**: 166-174.

Vogel, H. J. (1956) A convenient growth medium for *Neurospora* (medium N). *Microbial Genetics Bulletin* **13**: 42-43.

Vollmer, S. J. and Yanofsky, C. (1986) Efficient cloning of genes of *Neurospora crassa*. *Proceedings of the National Academy of Sciences U.S.A.* **83**: 4869-4873.

von Arx, A. J. (1973) Ostiolate and nonostiolate Pyrenomycetes. *Proceedings (of the) Koninklijke Nederlandse Akademie van Wetenschappen, Series C.* **76**: 289-296.

Walkey, D. G. A. and Harvey, R. (1967) Spore discharge rhythms in pyrenomycetes. II. Endogenous and exogenous rhythms of spore discharge. *Transactions of the British Mycological Society* **50**: 229-240.

Waters, H., Butler, R. D. and Moore, D. (1975) Structure of aerial and submerged sclerotia of *Coprinus lagopus*. *New Phytologist* **74**: 199-205.

Watkinson, S. C. (1978) Growth of rhizomorphs, mycelial strands, coremia and sclerotia. In *Fungal Walls and Hyphal Growth*. (ed. J. H. Burnett and A. P. J. Trinci) pp. 93-113. Cambridge University Press.

Wehmeyer, L. S. (1926) A biologic and phylogenetic study of stromatic spheriales. *American Journal of Botany* **13**: 514-645.

Weijer, J. and Vigfusson, N. V. (1972) Sexuality in *Neurospora crassa* I. Mutations to male sterility. *Genetical Research* **19**: 191-204.

Weijer, J. and Dowding, E. S. (1960) Nuclear exchange in a heterokaryon of *Neurospora crassa*. *Canadian Journal of Genetics and Cytology* **2**: 336-343.

Weir, B. S. (1990) *Genetic Data Analysis*. Sinauer Assoc. Inc., Sunderland, Mass.

Weresub, L. K. and Le Clair, P. M. (1971) On *Papulaspora* and bulbilliferous basidiomycetes *Burgoa* and *Minimedusa*. *Canadian Journal of Botany* **49**: 2203-2213.

Wessels, J. G. H. (1986) Cell wall synthesis in apical hyphal growth. *International Review of Cytology* **104**: 37-79.

Weste, G. (1970) Factors affecting vegetative growth and the production of perithecia in culture by *Ophiobolus graminis*. II. Variations in light and temperature. *Australian Journal of Botany* **18**: 11-28.

Westergaard, M. and Hirsch, H. (1954) Environmental and genetic control of differentiation in *Neurospora. Proccedings of the Symposium of the Colston Research Society* **7**: 171-183.

Westergaard, M. and Mitchell, H. K. (1947) *Neurospora*. V. A synthetic medium favouring sexual reproduction. *American Journal of Botany* **34**: 573-577.

Whetzel, H. H. (1945) A synopsis of the genera and species of the Sclerotiniaceae, a family of stromatic inoperculate discomycetes. *Mycologia* **37**: 468-714.

Whitehouse, H. L. K. and Haldane, J. B. S. (1946) Symmetrical and asymmetrical reduction in Ascomycetes. *Journal of Genetics* **47**: 208-212.

Wicklow, D. T. (1981) The coprophilous fungal community: a mycological system for examining ecological ideas. In *The Fungal Community. Its Organisation and Rôle in the Ecosystem*. (ed. D. T. Wicklow and G. C. Carroll) pp. 47-76. Marcel Dekker, Inc., New York and Basel.

Willetts, H. J. (1971) The survival of fungal sclerotia under adverse environmental conditions. *Biological Reviews* **46**: 387-407.

Willetts, H. J. (1972) The morphogenesis and possible evolutionary origins of fungal sclerotia. *Biological Re*views **47**: 515-536.

Willetts, H. J. (1978) Sclerotium formation. In *The Filamentous Fungi, Vol. III, Developmental Mycology*. (ed. J. E. Smith and D. R. Berry) pp. 197-213. Edward Arnold (Publishers) Ltd., London.

Willetts, H. J. and Bullock, S. (1982) Studies on the ontogeny and ultrastructure of the sclerotium of *Botrytis cinerea* Pers. ex Nocca & Balbis. *Canadian Journal of Microbiology* **28**: 1347-1354.

Willetts, H. and Bullock, S. (1992) Developmental biology of sclerotia. *Mycological Research* **96**: 801-816.

Worrall, J. J., Chet, I. and Hüttermann, A. (1986) Association of rhizomorph formation with laccase activity in *Armillaria* spp. *Journal of General Microbiology* **132**: 2527-2533.

Yang, X. B., Snow, J. P., and Berggren, G. T. (1989) Morphogenesis of microsclerotia and sasakii-type sclerotia in *Rhizoctonia solani*, anastomosis group 1, intraspecific groups IA and IB. *Mycological Research* **93**: 429-434.

Zalokar, M. (1957) Isolation of an acidic pigment in *Neurospora. Archives of Biochemistry and Biophysics* **70**: 568-571.