A CYTOLOGICAL STUDY OF ASPERGILLUS VARIECOLOR
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ASPERGILLUS VARIECOLOR (BERK. & BR.) THOM & RAPER

by

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INTRODUCTION AND LITERATURE REVIEW

With the improvement of cytological techniques more detailed and accurate studies of nuclear behaviour in fungi have been made possible. Using modern staining methods and squash preparations many detailed investigations have been made in recent years of the meiotic prophase nucleus, which is the largest in the life cycle of many fungi. Studies of this nucleus and the subsequent processes of reduction division have provided most of the information for the basis of fungal cytology. They have also helped to elucidate some of the genetical and taxonomical problems within this group of organisms. In fact, there has been an increased integration of the fields of taxonomy, genetics and cytology in the fungi over the past years, as there has been in the higher plants. Knowledge for one organism drawn from all three fields helps to clarify basic aspects of its life history, its reproductive processes, the mechanisms of inheritance, and its phylogenetic relationships with other species.

One of the most detailed and integrated studies of this nature is one which followed the establishment of meiosis in the resistant sporangia of Euallomyces (Emerson and Wilson, 1949; Wilson, 1952). In 1954, Emerson and Wilson described the interspecific hybrids, the cytogenetics, and the cytotaxonomy of Euallomyces. Allomyces produces gametangia, male and female together on hyphal tips. In Allomyces arbuscula the male cell is subterminal, and in
A. javanicus var. macrogynous it is terminal. Emerson (1941) crossed these two species, and an F₁ generation was produced which showed the parental types and a whole series of intermediate strains. However, the illustrated data of Emerson and Wilson (1954) showing the chromosome numbers and configurations in meiosis I of the F₁, F₂ and F₃ generations explained these peculiarities. They discovered that a series of natural polyploid strains exists for each of the two species they had used, and that they had in fact crossed two polyploid gametophytic strains. In meiosis I in the F₁ generation they observed that not all the chromosomes formed bivalents and that the univalents became randomly distributed at the poles. These facts explained the series of intermediate strains and the low viability they found in F₁ meiospores. From the cytological, morphological and genetical evidence they also concluded that Allomyces javanicus var. macrogynus is a species, A. macrogynus.

El-Ani also used cytological information to clarify aspects of taxonomy in the Hypocreales. In 1956a he established that the four different sexual strains of Hypomyces solani f. cucurbitae did not result from a difference in chromosome number as Hirsch thought in 1949. He found a constant chromosome number of four in the ascus nuclei of all four strains. He also found a chromosome number of four in Gibberella cyanogena in 1956b, but in
Nectria peziza in 1959 he found a chromosome number of five, although the meiotic divisions and ascus development were similar to that in Hypomyces.

Cytology can also help to clarify aspects of sexuality such as heterothallism and homothallism. Olive in 1956, and Carr and Olive in 1958 and 1959 published a series of cytogenetic studies for Sordaria fimicola. In 1958 they gave an excellent account of the meiotic divisions in ascospore formation, describing precocious synapsis, the morphology of the seven elongated pachytene chromosomes and the centriole-fibril mechanism in ascospore delimitation. They also found that in the absence of antheridia and conidia in S. fimicola, heterokaryosis originated by hyphal anastomoses. On the basis of their observations they attempted to explain the possible origin of heterothallism from homothallism, perhaps by way of unbalanced heterothallism.

These few examples of detailed cytological studies which used modern techniques illustrate the impetus given to fungal cytology by McClintock in 1945 when she applied the orcein squash technique to the asci of Neurospora crassa in order to study the meiotic prophase nuclei. With this technique she made the first cytological study of an Ascomycete along the lines of higher plant cytology. McClintock gave a detailed, morphological description of the seven pachytene chromosomes, and worked out the approximate position of the centromere on each one. These direct observations of pachytene
chromosomes, and more recently those of Barry (1965), have confirmed the theoretical interpretation that abnormal linkage relationships can be caused by segmental interchange between non-homologous chromosomes. McClintock found that synapsis occurred at an early stage between contracted chromosomes which then elongated during a prolonged pachytene. She described the delimitation of the eight ascospores by fibres radiating from the centrioles. Her work complemented a genetical study by Beadle and Tatum (1945) also on *N. crassa*.

Singleton (1953) continued the work of McClintock on *N. crassa*, giving an excellent account of nuclear division from crozier formation to mitosis in the ascospores. He confirmed her observations of precocious synapsis and produced excellent photographs to validate his chromosome maps showing the positions of the chromomeres and probable positions of the centromeres. This map drawn from microscopic observations correlates well in number and detail to the linkage maps compiled for this species from genetical evidence by Barratt and Strickland in 1961. Singleton's photographs of the large centrioles at Telophase III and their radiating fibrils add further evidence to the centrosome-astral ray mechanism in ascospore delimitation described by Harper (1905) for the powdery mildews, and by Dodge (1927) for *Neurospora tetrasperma*. Singleton determined spindle orientation of the different nuclei, emphasizing
the importance of their relationships on the relative positions of the ascospores within the ascus. He found very little overlap of the spindles following Telophase II and III, the four meiotic products forming a row and the eight resulting nuclei remaining in four adjacent pairs, each pair being the product of one mitotic division.

With the lead of McClintock and Singleton many accounts of meiotic nuclear division have been published. These have resulted in the emergence of a general pattern of meiosis in the fungi. In Prophase I precocious synapsis occurs. This was suggested by Wheeler et al (1948) for Glomerella, and since the descriptions by McClintock (1945) and Singleton (1953), it has been demonstrated repeatedly, for example, in Cochliobolus sativus (Shoemaker, 1955; Hrushovetz, 1956); in Venturia inaequalis (Day, Boone and Keitt, 1956; Julien, 1958); and in Rosellinia limoniiispora (Hayman, 1963). Contraction of the chromosomes follows and at diplotene chiasmata appear (Singleton, 1953; Wells, 1956). Although the exact time of chromosome replication is not known, the four-strand nature of the bivalents has been indicated by I.M. Wilson (1937) for Aleuria rutilans, and more recently from cytogenetic studies on Sordaria fimicola (Olive, 1959; Kitani et al., 1962). The short bivalents of Metaphase I are described for some fungi as scattered irregularly over the central part of the spindle, and the following Anaphase
disjunction is often asynchronous (Wilson 1952; El-Ani 1959). The second and third divisions are very similar to the first except that the chromosomes are visibly smaller. Two mechanisms have been described for ascospore delimitation, one involving the centrioles and astral rays, as demonstrated by Singleton (1953), by Wells (1956) and by Carr and Olive (1958); and the other method entailing simple cleavage of the cytoplasm around the spores (Heim, 1952; Elliott, 1960), sometimes accompanied by the presence of small oil vacuoles along the delimiting lines (Jenkins, 1934; Hayman, 1963). There seems to be no general pattern of behaviour for the nucleolus, however. Different workers have reported various occurrences according to the species they have investigated. In some species the two nucleoli fuse shortly after the two chromatin masses have fused (Wheeler et al., 1948; Hrushovetz, 1956; Carr and Olive, 1958). The nucleolus may then disappear in late pachytene or early diplotene and not reappear in later divisions (Wheeler et al., 1948; El-Ani, 1956a), or it may persist in the cytoplasm through Metaphase I (McClintock, 1945; Singleton, 1953; Olive, 1950; Day, Boone and Keitt, 1956). Wells (1956) reported that the two nucleoli in the young ascus disappeared and a new single nucleolus formed later.

Even though only a few examples of cytological studies have been cited here, it is still apparent that most studies use Ascomycetes, particularly pyrenomycetes and discomycetes which have
linear asci. There has been little investigation of Plectomycetes with non-linear asci, as for example, the \textit{Aspergillaceae}. Members of this family, particularly \textit{Aspergillus nidulans}, are frequently used in genetical experiments (Pontecorvo \textit{et al}, 1953; Käfer, 1957 and 1958), but Elliott's work in 1960 on \textit{A. nidulans} is the first detailed cytological study of meiosis in this group of fungi. He gave an account of meiosis in haploid and diploid strains, and presented photographs which clearly indicated a haploid chromosome number of eight, not four as suggested earlier by Pontecorvo (1953) and Elliott (1956). This number confirmed the eight linkage groups established by Käfer (1958) from genetical analysis.

\textit{Aspergillus variecolor} (Berk. and Br.) Thom and Raper, to be investigated in this study, is a member of the \textit{A. nidulans} group which is characterised by short, columnar conidial heads with primary and secondary sterigmata; a cleistothecium containing purple-red ascospores with equatorial crests; and large, thick-walled, globose Hülle cells. The distinctive feature of \textit{A. variecolor} is the ascospore with its wide equatorial crests (3.0-4.0 \textmu m wide) which are deeply dissected giving the spore a star-like appearance.

The history of \textit{A. variecolor} is complicated and illustrated a little of the chaos of nomenclature in the \textit{Aspergillus} and \textit{Penicillium} groups. Berkeley and Broome in 1857 first described
A. variecolor as "doubtfully a Gasteromycete or possibly a lichen" (Thom & Raper, 1945) and called it Emericella variecolor. In 1883 Eidam described the same fungus under the name Sterigmato-cystis nidulans, and Borzi in 1885 called it Inzengaea erythrospora. Winter (1887) recognising from Eidam's illustrations its relationship to the Aspergilli, transferred it to this genus and most mycologists accept this placing. However, Langeron (1922) renamed it as the type species of his new genus Diplostephanus, and Vuillemin in 1927 proposed that Eidam's species be moved to Emericella. Recently Benjamin (1955) also tried to reinstate Emericella for all ascosporic forms of A. nidulans. Of these proposals, none received much acceptance. In 1934 Curzi, apparently unaware of the earlier nomenclature, described the same fungus and called it Aspergillus stellatus. This is, however, a synonym to the earlier designation of A. variecolor.

Previous studies of the Aspergillaceae are mainly concerned with ascogonial and cleistothecial development. The first species in which sexual stages were described was Eurotium Aspergillus glaucus de Bary (A. herbariorum Wiggers) by de Bary in his researches of 1870. Fraser and Chambers in 1907 also used this fungus and described a tightly coiled archicarp divided into multinucleate stalk, ascogonium and trichogyne. They described an antheridium but did not think that it actually fused with the trichogyne.
Therefore they concluded that a "reduced fertilization" took place. Asci were thought to arise frequently, though not always, from the penultimate cell of a hook-like process, but no fusion between the tip cell and the stipe was mentioned. Fusion of two nuclei in the ascus was observed, but no details for the "three karyokinetic divisions" were given.

In 1907 Dangeard described the ascogonial coils and subsequent cleistothecial development of several Aspergillus species and Dale (1909) described the ascogonium of A. repens de Bary, but could not observe details of the three nuclear divisions in the ascospore formation. She did describe, however, two fusions between nuclei, first, a fusion of two nuclei in the ascogonium, which she called a "reduced form of sexuality" occurring in the absence of an antheridium, and second, a fusion in the young binucleate ascus. Dale also stated that the young asci developed from a penultimate cell, but no mention was made of true crozier formation.

In 1944 Olive gave an account of cleistothecial and crozier formation in A. Fischeri using the aceto-orcein squash technique. He described the ascogonium as divided into multinucleate cells which eventually became binucleate. He, too, observed several nuclei in the ascogonium which appeared to be fusing, but also stated the possibility of the small nuclei appearing superimposed. Typical crozier formation was prominent. Nuclear fusion occurred
in the young asci, but no detail was given for the meiotic nuclear divisions.

Moreau and Moreau (1953) described cleistothecial development in *A. ruber* and the formation of asci either from croziers or directly from binucleate cells without crozier formation.

Pontecorvo in 1953 attempted to clarify the "three successive nuclear divisions" in spore formation. He showed the meiotic Metaphase I and Anaphase I nuclei, but he could only count four chromosome pairs.

In 1960 Elliott published the first detailed description of the meiotic nuclear divisions in haploid and diploid strains of *A. nidulans*. Elliott found the pachytene chromosomes to be fully paired throughout their length, but during diakinesis the bivalents became separated except for a single point of contact at one end. During diakinesis the chromosomes paired again forming two parallel rods, and Elliott was able to observe some of their morphology at this stage.

In Metaphase I the eight contracted bivalents were arranged regularly around the edge of a narrow spindle, forming a ring when seen in polar view. Elliott thought that ascospore delimitation was by cleavage of the cytoplasm around each nucleus. In the diploid strains, in which haploid nuclei fuse in the vegetative mycelium, Elliott found that a single diploid nucleus divides meiotically in the ascus to form haploid ascospores. Chromosome
configurations during this reduction division were the same as in the wild type, haploid x haploid cross. No evidence of tetraploid meiosis was seen. The chief differences between the two strains were first, the presence of coiled hyphae with uninucleate cells in the diploids rather than true croziers as in the wild type; and second, cleistothecia of the diploid strains had many more sterile hyphae, some of which appeared like multinucleate, inflated sacs. Many asci degenerated at Metaphase I in the diploid strains, though development appeared to proceed normally up to that stage.

This study presents, as far as was seen, the cytological details of the nuclear divisions accompanying the development of the ascus and the production of ascospores in _A. variecolor_. It is hoped that this study will add to the small amount of cytological data already published for the _Aspergillus_ group, and that it may, possibly, be of future use in explanation of phylogenetic relations and in genetical experiments within this group of fungi.
Materials and Methods

The isolate of *Aspergillus variecolor* (Berk. and Br.) Thom and Raper, was obtained from Dr. Ralph Emerson. Emerson received the culture from Dr. K. Raper at the Northern Regional Research Laboratory, Peoria, Ill., where it was designated as NRRL 212. The isolate has been kept in this laboratory for several years on potato dextrose agar.

Cytological observations were made on preparations obtained by one of two procedures:

i) cleistothecial squash,

ii) growth on cellophane.

i) **Cleistothecial Squash**

a) **Cultural methods**

Squash techniques of cleistothecial contents are excellent for observing nuclear behaviour in developing asci. This method requires immature cleistothecia as free from the covering of aerial mycelium and Hülle cells as possible.

Single or multiple spore cultures grown on corn meal agar and Czapek's solution agar were incubated at 27°C for three days, or at room temperature for six days. They produced sparse mycelial and conidial growth and a small number of scattered, large cleistothecia, with only a thin covering of Hülle cells. On potato dextrose agar and yeast dox agar, growth
was faster and more luxurious but the cleistothecia were much smaller and immersed in a thick covering of Hülle cells and aerial hyphae which became hard and sclerotal-like, making it difficult to dissect out the cleistothecia without rupturing them.

b) Staining

Many combinations of different fixatives, hydrolysing agents and stains were tried, but the best preparations were obtained with a 2% solution of proprionic-orcein, using a 1:3 proprionic-alcohol fixative during the dissection of the cleistothecium. A trace of lactic acid was added to the stain on the slide to reduce staining of the cytoplasm.

The stain was prepared by adding Gurr's powdered orcein dye to 100 mls. boiling, 45% proprionic acid, cooling the solution rapidly and filtering.

Aceto-carmine was also used in some preparations. It was prepared by refluxing 2 gms. carmine (Matheson Comp., Inc.) in 100 mls. 75% acetic acid for 1 hour, then cooling and filtering the solution. A few drops of saturated ferric acetate solution were added to the stain until it became plum-coloured.

Special treatment was needed to show the elongated chromosomes of early Prophase I which are difficult to stain. Hydrolysis of intact cleistothecia for 5 mins. at 60°C in 1N HCl caused these chromosomes to show up after several days stored
at 4°C. The cytoplasm stained much less with this procedure, but many refractive particles interfered with the observation of other meiotic stages.

c) Preparation of slides

Several cleistothecia were placed on a slide in a drop of fixative to prevent them from drying out. Under a dissecting scope the surrounding Hülle cells and hyphae were scraped off the cleistothecial wall. The wall was slit carefully and the inner contents forced out as an intact, globular mass, which could be transferred to a drop of stain on a clean slide. With this method, the inner contents only, of the cleistothecium were squashed, without the thick wall or large Hülle cells covering the tissue or preventing adequate squashing. Very young cleistothecia whose walls were still pale yellow, not brown, were freed of Hülle cells and transferred directly to the stain. In this case, the cover slip was pushed laterally over the cleistothecium causing the wall to peel from the ascogenous hyphae. Once the cover slip was added the slide was left for 5-10 mins. for the stain to penetrate the tissues. Heavy pressure on the cover slip was then applied to flatten the asci. Preparations were sealed with a gum-mastic paraffin preparation.

Temporary slides prepared in this way showed the contracted chromosomes in a few hours, but were much better if left over-
night. Slides at 4°C could be used for two weeks but after this time the cytoplasm became too densely stained for accurate observation.

ii) Growth on Cellophane

A method for observing the initial stages of ascogonial development and cleistothecial initiation was required.

The very young stages are best studied in intact material. Lifting them from a culture and smearing them on a slide causes too much disturbance to the structures. In order to observe these structures in situ the hanging drop method was used, and slide cultures (i.e. a thin film of inoculated agar spread on a slide in a sterile moist petri dish) were tried, but though low carbohydrate content agar was used, too many conidia developed. The conditions were poor and cleistothecia took a long time to form.

The cellophane culture technique modified by Kondo et al (1959) was found to be extremely useful. Discs of cellulose dialysis membrane (approximately 7 cms.) were alternated between moist filter papers in a petri dish and autoclaved. The discs were placed on an agar surface moistened with sterile distilled water. By keeping the agar surface moist, wrinkling of the cellophane and air bubbles beneath the cellophane surface were avoided.
Water agar and corn meal agar were used. The cellophane surface was inoculated with ascospores and conditions were usually moist enough for germination. If the cultures appeared very dry after one or two days, sterile distilled water was pipetted onto the surface.

Fruiting colonies developed in about 2 weeks. The mycelium was sparse and few conidia were produced. Very early stages of ascogonial formation, when the hypha becomes coiled and twisted could be recognised easily through a low power dissecting scope.

A portion of cellophane carrying initials was cut out and peeled from the agar. The cellophane was put onto a slide so that no air bubbles were caught beneath it, and stain was added. Aceto-carmine and propionic-orcein were used. A cover slip was added and the slide squashed lightly between layers of absorbent paper. The cover slip was then sealed.

The cellophane did take up some stain but not enough to cause difficulties. In fact, the mycelium took up less stain, but this was an advantage.

These preparations were excellent for revealing complete hyphal systems in their normal relation to each other. The slides remained in good condition for many weeks, especially when kept refrigerated.

Microscopy

A Zeiss Opton microscope was used fitted with apochromatic objectives and 10X oculars. Contrast was increased by
using a Wratten Filter No. 58.

Photographs were taken with a Model L Bausch and Lomb Photographic equipment. The film used was Kodak Contrast Process Panchromatic (4" x 5" plates) developed in Kodak Dll developer and printed on Kodabromide paper.

In the legends for the figures:

(c) = aceto-carmine stain,

(d) = propionic-orcein stain.
OBSERVATIONS

ORIGIN OF THE ASCOGONIUM

The vegetative hyphae are septate, with multinucleate cells, and with occasional anastomoses between adjacent hyphae. The main hyphae, or leading hyphae, are thick and contain granular cytoplasm. They anastomose more frequently than the branch hyphae and sometimes their cells become distinctly binucleate after such a fusion (Fig. 1). The ascogonial initials are short, coiled branches of the thicker hyphae. They soon become septate and Figs. 2 and 3 show ascogonial coils with at least four septa. The exact number of septa, and the nuclear condition of the cells is difficult to observe without sectioned material, since surrounding vegetative hyphae branch copiously (Fig. 4), and soon cover the initial. Darkly staining branches arise from the base of the ascogonium and surround it tightly (Fig. 5). These form the cleistothecial wall, which in amature cleistothecium consists of a single layer of thickened, pigmented hyphae which branch and anastomose as shown in Fig. 6.

Meanwhile, the vegetative hyphae immediately surrounding the ascogonium proliferate rapidly. Sometimes, as shown in Fig. 2 the hyphae only start to branch once the ascogonium is coiled; but in many cases, short branches appear before the ascogonial initial itself can be recognized. Some of the cells
Fig. 1 (c) Anastomosing hyphae with binucleate cells. (X1900)

Fig. 2 (c) Ascogonial coil with at least four septa. Surrounding mycelium beginning to proliferate. (X1900)

Fig. 3 (c) Ascogonial coil with at least three septa. (X960)

Fig. 4 (c) Proliferous growth in region of ascogonial initiation. Some intercalary and terminal swellings are developing. (X960)
Fig. 5. (c). Young cleistothecium, with darkly-staining hyphae enclosing the ascogonium. Mature Hüllle cells already surround cleistothecium. (X960)

Fig. 6. (c).

Branched pigmented and thickened hyphae of cleistothecial wall. In right hand corner, immature Hüllle cells from surrounding hyphae. (X960)

Fig. 7. (c). Chains of intercalary swollen cells in proliferating hyphae. (X960)
of the surrounding hyphae and the proliferous growth, also become swollen during these developments. The swollen cells are either intercalary, forming chains, as shown in Fig. 7, or are produced at the tips of the proliferating hyphae (Fig. 8). Usually, though not always, Hülle cells develop from the swollen cells. The Hülle cells are characteristic of cleistothecial formation in the Aspergillus nidulans group and do not appear to form on other parts of the mycelium. During initial stages of their development, the cells become slightly enlarged and the protoplasmic contents become dense and granular (Figs. 8 and 9). In Fig. 6 a nucleus is still apparent in the cytoplasm. As the cells enlarge further, the nucleus disappears and the cytoplasm shrinks from the outer walls (Fig. 9). In many developing Hülle cells there appear in the cytoplasm about eight contorted, globular bodies which stain densely with aceto-carmine or -orcein (Fig. 9). These may represent the disintegrating nucleus. The cell continues to enlarge and the cytoplasm becomes a small central mass which extends into the base of the terminal cells (Fig. 9), or extends towards each adjacent cell in the intercalary cells (Fig. 10). There is a septum at the base of each terminal cell and on either side of the intercalary cell. Hülle cells appear to be very thick walled (Fig. 10) and are usually described as such in the literature. However, the outer, thin primary wall, which can be seen in Fig. 9, stains blue with lactophenol cotton
Fig. 8. (c). Young terminal swollen cells containing dense cytoplasm and globular bodies. (X1000)

Fig. 9. (c).

Central - Immature Hülle cell with dense cytoplasm.
Left - Mature Hülle cell with "thickened" wall, and stained globular bodies in the cytoplasm.
Right - Thin primary wall of mature cell.
(X1000)

Fig. 10. (c). Hülle cells with dense central cytoplasm. Lower two intercalary cells show cytoplasmic extensions towards adjacent cells, and septa between cells. (X960)
blue, but the inner 'thickening' does not. There is a possibility that the 'thickening' may be a liquid-filled space left by shrinkage of the cytoplasm as suggested by Bent and Morton (1963) for their "large" cells in *Penicillium* species, or it may be a deposit of a different chemical nature from the primary wall. As many Hüle cells develop and as they enlarge they become detached from the hyphae and massed together forming continuous layers around the cleistothecium.

**DEVELOPMENT OF THE ASCOGENOUS HYPHAE**

Ascogenous hyphae are only found in young cleistothecia in which the wall has not yet turned brown. Squashes of immature cleistothecia show a mass of thick contorted, ascogenous hyphae with irregular-shaped inflations along their length (Fig. 11), and young rounded asci appear to develop by simple proliferation of binucleate cells which subsequently enlarge. However, the asci actually develop from croziers.

During crozier initiation short lateral branches are produced at the tip and along the length of the ascogenous hyphae. The tip of the hypha rarely bends round to form the crozier as in typical crozier formation. Instead, the tip cell of the crozier is formed by a "lateral branch" which remains perpendicular, or nearly so, to the main hypha as in Fig. 12a. The penultimate cell of the crozier is the tip of the ascogenous hypha which has
continued to grow forwards, as shown at 'b' in Fig. 12. One nucleus is in the lateral protrusion and the other is below in the ascogenous hypha (Fig. 12c). Mitotically dividing nuclei were rarely seen, but binucleate penultimate cells with uninucleate tip and basal cells were observed (Fig. 13). In Fig. 13 the fusion nucleus in the penultimate cell appears to be in early Prophase I already. There is a nucleus in the basal cell and a septum between the basal and penultimate cells. Only the clear karyolymph of the nucleus in the tip cell is seen at this focus, and no septum between this cell and the penultimate cell is visible. The two nuclei of the penultimate cell usually fuse immediately. The tip cell now bends round to fuse with the basal cell, and Fig. 14 shows the cells just after fusion. There are two nuclei; the tip cell nucleus is only just in focus. Once the tip cell and basal cell have fused reforming the dikaryon, secondary croziers develop immediately as in Fig. 15. These are much less elongated than the original ones and are more typical in their development. So many secondary croziers develop that asci are formed in large groups, for example, Fig. 31. Growth is so rapid throughout this period that different nuclear stages are seen in varying sizes of asci.

DEVELOPMENT OF THE ASCUS

The young ascus grows rapidly into an enlarged rounded cell during fusion of the two nuclei, and the cytoplasm remains very dense. Fig. 15 shows a cell just at the stage of nuclear fusion.
Fig. 11 (c). Contorted ascogenous hyphae, with groups of young asci in lower part of picture. (X1900)

Fig. 12 (c). Developing crozier with uninucleate 'lateral branch' (a); anucleate penultimate cell (b); and uninucleate basal cell (c). (X1900)

Fig. 13 (c). Elongated crozier prior to fusion of tip and basal cells. Penultimate cell contains early Prophase I nucleus. (X2800)

Fig. 14 (c) Binucleate penultimate cell, and fused basal and tip cells with two nuclei (X2500)
The chromatin material is slightly separated into two closely adjacent masses at the edge of a clear karyolymph. In Fig. 16 fusion is complete and there is a large dense fusion nucleus in a rounded ascus. Prophase I follows immediately.

In Fig. 17 two small nucleoli are visible, one to either side of the early Prophase I nucleus. The nucleoli probably fuse, since in most asci at a slightly later stage there is one larger nucleolus, as in Fig. 18. Sometimes asci are seen where nucleolar fusion is delayed until just before pachytene. The nucleolar chromosome is shown in Fig. 19. It is longer than the other chromosomes and is frequently bent or curved near the centre. This is also shown in Fig. 20; the chromosome extending from the chromatic mass is probably the nucleolar chromosome (the nucleolus is not stained here).

Early stages of Prophase I show a compacted mass of contracted chromosomes, lying to one side of a clear karyolymph. Often fine strands extend from the heterochromatic mass, as shown in Fig. 20. These appear to be single chromosome arms. The nucleus gradually becomes less compacted and appears very heterochromatic (Fig. 21a). Often at this stage, as the chromosomes are beginning to elongate they are seen to be paired. In Fig. 21a, just below the arrow, there is a pair of chromosomes, with a slight gap between the two members. These two chromosomes appear to have a sub-terminal constriction. In the larger ascus, Fig. 21b, the chromosomes have
Fig. 15. (c). Fusion of two nuclei in penultimate cell. Below it is a secondary crozier. (X2800)

Fig. 16. (c). Young rounded ascus with large, dense fusion nucleus. (X2800)

Fig. 17. (c). Two small nucleoli on either side of early Prophase I nucleus. Chromosome threads just beginning to appear. (X1900)

Fig. 18. (c). Ascus with large, single nucleolus. (X1900)
Fig. 19. (c). Rounded ascus with nucleolus and curved nucleolar chromosome attached to it. (x1900)

Fig. 20. (o). Rounded ascus with early Prophase I nucleus. Fine strands extend from the mass. The long, curved one is probably the nucleolar chromosome (nucleolus is unstained) (x2800)

Fig. 21. (o).

Three asci with nuclei at progressive stages of early Prophase I. The chromosomes are beginning to appear as threads. Those below arrow at (a) are paired and have a subterminal constriction. (x2800)
taken up more stain and appear thicker and more distinct.

In Fig. 22 the chromosomes are elongating and are visible as thick threads which are clearly paired at 'a'. These chromosomes also show the sub-terminal constriction seen in Fig. 21a. At 'b' in Fig. 22, the tips of the chromosome are very fine and seem to be separated. Precocious synapsis, then, appears to occur. As pachytene is reached the chromosomes become scattered throughout the karyolymph and are so elongated, that they stain poorly and are only visible as diffuse, paired strands, (Fig. 23). In this photograph the members of a pair are separated, but later they become twisted around each other, as shown in Fig. 24.

The chromosomes contract from diplotene throughout diakinesis until maximum contraction at Metaphase I. They appear as darkly-staining rods scattered irregularly in the nucleus (Fig. 25). They are distinctly paired at 'a' in Fig. 25, and the curved nucleolus chromosome is visible at 'b'. Fig. 26 shows the highly contracted pre-Metaphase chromosomes, which in Fig. 27 are becoming aligned at the Metaphase plate. In figs. 28 and 29, the chromosomes are almost globular and are positioned in a compact group just prior to Metaphase proper. In polar view the grouping at Metaphase I appears as a ring corresponding to the outside of the spindle. In Figs. 30a and b seven bivalents form the ring and one bivalent is centrally located. There is then, a haploid chromosome number of eight for *Aspergillus variecolor*. Fig. 31 shows
Fig. 22. (c). Chromosomes are thick, double threads. At (a) paired chromosomes with sub-terminal constriction. Above (b) two fine unpaired strands at tip of bivalent. (X2800)

Fig. 23. (c).

Pachytene. Chromosomes are elongated and paired, with narrow gap between the two members. (X2800)

Fig. 24. (c) Diplotene. Ascus with chromosomes pairs twisted around each other. (X2800)
Fig. 25 (c). Early diakinesis. Contracting, paired chromosomes. At (a) they are "V" shaped. The curved nucleolar chromosome shows at (b). (X2800)

Fig. 26 (c) (X1900)

Mid-diakinesis.

Bivalents are short dark rods.

Fig. 27 (c) (X2800)

Fig. 28 (c) Late diakinesis. Contracted bivalents grouped close together. (X2800)
eight homologous chromosomes just beginning to separate, aligned along the length of the spindle. The smaller chromosomes appear more widely separated than the larger ones, indicating an asynchronous Anaphase. The ascus in this figure is larger than in Fig. 30. This is because Fig. 30 shows a squash of an immature cleistothecium, and in these, nuclear divisions occur more rapidly. It is possible to see all Prophase I stages up to Metaphase I in a small rounded ascus. In older cleistothecia the divisions are slower and the ascus enlarges more during Prophase I. Few nuclei were seen at Telophase and Interphase I suggesting these stages are of short duration.

The Prophase II nuclei are large and diffuse, (Fig. 32). The two nuclei often appear superimposed in squashed preparations, and this may be a result of chromosome disjunction in Anaphase I; the two sets of chromosomes do not move very far apart. The asci enlarge during Prophase II and usually reach full size. Their cytoplasm becomes much less dense and some asci show the typical lobed appearance seen in Figs. 29 and 30. Figs. 33a and b are photographs taken at two different focal planes. The two nuclei are at Metaphase II or early Anaphase II, and are clearly aligned perpendicular to each other. The metaphase plate in this division is much smaller than that in Metaphase I. In Fig. 33a, the smallest chromosome has begun to disjoin, and Fig. 34 shows both nuclei at Anaphase II. Again the nuclei are clearly at right angles to
Fig. 29. (o) Pre-Metaphase I. Eight contracted bivalents becoming grouped at spindle equator. (X2800)

Fig. 30. (o) Metaphase I

Polar view of eight bivalents, seven on the outside, and one in the centre. (X2800)

Fig. 31. (o) Early Anaphase I. Eight disjoining bivalents spread out along the length of the spindle. Ascus becoming lobed. (X2800)
**Fig. 32** (o). Prophase II. 
Two large nuclei in an unusually small ascus. 
(X2800)

**Fig. 33** (c).

Metaphase II. 

- **a** = narrow metaphase plate of first nucleus. Smallest bivalents starting to disjoin.
- **b** = different focal plane showing second metaphase nucleus perpendicular to first. 
(X2800)

**Fig. 34** (c) Early Anaphase II. Two perpendicular nuclei showing asynchronous disjunction and narrow metaphase plate. 
(X2800)
each other. During Division II and in the following mitotic division the chromosomes are much smaller than in the first meiotic division. They are rarely distinct enough to count after Anaphase I. The width of the spindle, too, is shorter; compare (Fig. 31 and 33).

In Prophase III the nuclei are large and even more diffuse (Fig. 35). The chromosomes contract up to Metaphase III and form the same ring pattern as in Metaphase I. Figs. 37 & 38 show asci with nuclei at Anaphase III. They also show the polar view of one set of chromosomes which are still in their Metaphase III grouping. Figs. 37 and 38 also show the characteristic position of the division II nuclei towards one end of the ascus. The divisions of all the nuclei are synchronized here as in the other divisions in this fungus. During the following resting stage, the eight resulting nuclei become diffuse bodies, and spread throughout the ascus, almost filling it (Fig. 39).

During the following ascospore delimitation the nuclei appear to remain in Prophase of the fourth division (Figs. 40, 41 and 42). The cytoplasm appears to divide by cleavage around the nuclei. No oil vacuoles were seen accompanying the delimitation and no centrioles have been seen to suggest a different method of delimitation. The wall becomes thick, (Fig. 43) so it is difficult to follow the nuclear events after spore formation, but Figs. 42 &
Fig. 36. (c). Late Prophase III nuclei. Bottom left nucleus shows metaphase ring formation. (X2800)

Fig. 37. (o). Early Anaphase III showing nuclei in characteristic position resulting from perpendicular orientation in division II. (X2800)

Fig. 38. (c). Anaphase III. Polar view of centre nucleus shows ring formation of Metaphase stage. Chromosome are much smaller. (X2800)
Fig. 39. (o). Late Anaphase III or Telophase III showing eight diffuse nuclei in lobed ascus. (X2800)

Early stages in ascospore delimitation. Nuclei remain in Prophase IV. Cytoplasm appears to divide by cleavage. (X2800)

Fig. 43. (o). Lobed ascus containing delimited spores. Not all cytoplasm is included within spore wall. Thick-walled ascospore shown to the right. (X2800)
43 show delimited spores with nuclei at late Prophase IV.

Not all the cytoplasm of the ascus is included within the spore when it is delimited (Fig. 43) and that which remains is aggregated around the outside of each spore. This cytoplasm becomes much less dense, and the points of the equatorial crests are sometimes seen as faint striations in this cytoplasm (Fig. 44).

When finally formed, the points of the spore crests project into the lobes of the asci, Fig. 46.

During the long period of delimitation and maturation the spores remain uninucleate, but later, often just before germination, a further mitotic division occurs, making the spores binucleate (Fig. 46a). Immediately prior to germination the spores swell, pushing apart the two "halves" of the spore wall between, and parallel to, the two equatorial crests (Fig. 47a). The contents are sometimes so swollen that they seem to emerge as a whole from the spore walls (Fig. 47). Two germ tubes grow from the contents and one nucleus migrates into each one.
Fig. 44. (o). Initial stages of crest formation. Arrow points to faint striations in cytoplasm surrounding delimited spores. (X2800)

Fig. 45. (o). Underexposed photograph showing uni-nucleate, fully developed spores. (X960)

Fig. 46. (o). Eight developed spores with crests projecting into lobes of ascus. Spore at (a) is binucleate. (X960)

Fig. 47. Slide culture of germinating asco-spores. The two 'valves' have opened between the equatorial crests. The spore contents have emerged at (a) and produced two germ tubes. (X1900)
DISCUSSION

With the use of the squash technique and aceto-orcein stain, this study of *Aspergillus variecolor* has shown some of the processes involved in cleistothecial production and in the divisions of the fusion nucleus during ascospore formation.

The ascogonium is a coiled lateral branch very similar to those shown for *A. Fischeri* by Olive (1944). The present study showed the ascogonium to be a much tighter coil than Benjamin (1955) diagrammed for *Emericella (Aspergillus) variecolor*. Looser coils similar in appearance to the drawings by Benjamin were found on hyphae at the extremities of the colony, but in such cases the hyphae were very different from the shorter, lateral branch described as the ascogonium in this study. These hyphae were extremely long and thin and often the hyphal tip was found to grow out beyond the coil, and even to coil again. In these regions of the mycelium, hyphae were often seen to twist loosely around each other, either in pairs or in groups. This phenomenon is a recognized occurrence, particularly of aerial hyphae which are not in direct contact with the nutritive medium, or of hyphae in slightly starved conditions. From observations in the present study it is considered that these are not the true ascogonial coils.

The ascogonium developed as a branch of a thicker, vegetative, anastomosing hypha, in which the nuclei are often found in pairs. The same hypha was often seen to produce several ascogonia. Ernest
(1949) also observed that perithecia in *Glomerella* arise from certain thicker hyphae. Anastomoses between hyphae of the same or different strains of fungi are a means for promoting heterokaryosis and a parasexual cycle. This is known to occur in *A. nidulans*, through the work of Pontecorvo and Roper (1952), and Pontecorvo (1953 and 1954), and in other Aspergillaceae for example, *A. oryzae* and *A. sojae* (Ishitani et al., 1956). In other fungi such as *Sordaria fimicola* (Carr and Olive, 1958) hyphal anastomoses can be used as a means to produce heterokaryosis when antheridia are absent. In *Aspergillus variecolor*, a homothallic species in which no evidence of an antheridium was found, it is reasonable to assume that parasexuality can occur, and that a greater possibility of heterokaryosis, especially in the ascogonium, is provided by hyphal anastomosis.

In this preliminary study of the coiled ascogonium using only squashed material, septation was difficult to see although there appeared to be at least four septa. Fraser and Chambers (1907) using sections of *A. herbariorum* cited a unicellular trichogyne and ascogonium, and a septate stalk, Dale (1909) using *A. repens* and Olive (1944) using *A. Fischeri* observed an indefinite number of septa, and Moreau and Moreau (1953) described the ascogonial tip ("trichogyne des auteurs") of *A. ruber* as degenerating. This division of the coil into definite parts may be too rigid a nomenclature.
Proliferation of the surrounding hyphae occurs very rapidly, often before the ascogonium has begun to coil. Such immediate response might suggest the possibility of a stimulus of some kind from the developing ascogonium or the hypha bearing it. Also, if degree of staining is any indication of the "condition" of the mycelium, then it is interesting to note that the portion of hypha bearing the ascogonium, plus a small length on either side of it; the proliferating branches; and any part of a vegetative hypha which is in close proximity to the ascogonium, stains much more densely than other portions of the vegetative mycelium.

The hyphae ensheathing the ascogonium arise from two sources. The cleistothecial wall consists of flattened hyphae arising from the basal portion of the ascogonium, and the proliferating vegetative hyphae form the loose outer sheath of Hüllle cells and hyphae. This is very similar to what Corlett found for the related genera *Microascus longirostris* and *M. trigonosporus* (1963). Olive (1944), although he mentioned the two origins of the hyphae did not specify any difference in their final differentiated forms. Moreau and Moreau (1953) also described the development of the cleistothecial wall from hyphae arising from the ascogonial stalk.

An interesting aspect of cleistothecial production in *Aspergillus variecolor* is the inevitable Hüllle cell formation. These arise from terminal, and sometimes intercalary, swellings of the hyphae surrounding the cleistothecial wall. The initial swellings
are of the same type as those described by several workers such as De Lamater (1937); Lilly and Barnett (1951); Frey (1927) and McGahen and Wheeler (1951), for other fungus species. Studies have shown that they arise more abundantly in vegetative hyphae when the medium is of higher acidity (Frey 1927). As the swellings develop into Hüle cells the walls do not appear to thicken, but the cell becomes vacuolated and the protoplasm shrinks away from the wall forming a small granular mass which often extends into the "stalk" or base of the Hüle cell. Intercalary Hüle cells sometimes have thin strands of protoplasm extending to both cross walls, although it appears that the protoplasmic strands of adjacent cells are not continuous. When Hüle cells are massed together forming a continuous layer they look very similar to the sclerotal cells of Penicillium shown by Porter and Coats 1957, in which they found protoplasmic connections between the cells.

Young Hüle cells arising from terminal swellings also look very similar to the photographs presented by Smith (1943) of abnormal conidial heads in Aspergillus proliferans. Smith thought these structures were homologous to sterigmata. "Swollen" or "giant" cells have been described as homologous to sporophores by several workers. Bent and Morton (1963) described some environmental factors leading to formation of "large" cells in Penicillium griseofulvum and related fungi, including Aspergillus niger. Their photographs showed very similar structures to those
found in Hülle cell development. "Large cells" were produced in very acid media but only in conditions conducive for sporulation at a more favourable level of pH. They described the walls as staining blue with iodine and appearing "no thicker than other hyphal walls", and they said that in some cells the cytoplasm "was contracted from the wall leaving a liquid filled space in which occasional small particles showed Brownian movement". They concluded that the "large cells" were homologous to "sporophores which failed to complete normal development at low pH."

Although the Hülle cells seen in this study appear very similar to photographs of "giant", "large" or "swollen" cells, and to some sclerotial cells, it is difficult to tell whether they are morphologically similar structures. Certainly particles were never seen between the outer wall and the protoplasm, and the outer wall did not stain blue with iodine. It is difficult to say from observations alone, whether the inside of the wall is thickened or whether it is a liquid filled space. It failed to stain at all with lactophenol, methylene-blue, iodine or propriono-orcein. Hülle cells have been described as "thick-walled" for a long time but no morphological studies have been made of them.

In the present literature several isolated descriptions of "abnormal" cells occur. The descriptions all show some resemblances and some differences to each other, and all show intermediate forms
according to the extremity of the conditions and the stage of development. Perhaps these cells described are homologous but are showing variations of form due to their local environment. The conditions around a cleistothecium must be somewhat different also, since Hülle cells occur only around the cleistothecia themselves. Perhaps, too, the sclerotia of certain Aspergillaceae, for example, *Aspergillus niger*, show an even more extreme condition since in many of them no fruiting bodies occur at all. In these cases conditions must be unfavourable, since it has been found that some sclerotia, for example those of *A. alliaceus*, occasionally produce cleistothecia (Fenelli and Warcup 1959). Much more work is required to show the inter-relationships between environmental factors, initiation of asexual and sexual reproduction and "large cell" formation.

The asci develop from large croziers arising along the length of ascogenous hyphae. As Pontecorvo found for *Aspergillus nidulans* (1953), "The immature perithecium is filled with thick contorted hyphae of such irregular shapes that it is difficult to grasp their details. On these hyphae ('ascogenous hyphae') the ascus primordia bud out like grapes in a bunch." The croziers are difficult to recognize since they are very elongated, particularly the tip cell, which usually bends to fuse with the basal cell only after the penultimate cell has been cut off. Unlike primary croziers in many other fungi, the tip of the hypha does not bend
over to form the tip cell. Instead the hyphal tip continues to grow forwards whilst the tip cell of the crozier is produced as a lateral protrusion which then bends backwards. This sort of development may be a result of the very rapid growth at this stage and is more reminiscent of clamp formation in the Basidiomycetes, except that the ascogenous hyphae are initially coenocytic. Smaller, more typical secondary croziers proliferate rapidly from the original one. Croziers have also been described in studies of other *Aspergillus* species.

Growth of asci with early Prophase I nuclei is very rapid, so the nucleoli, which disappear as pachytene is reached, are relatively short lived. Two nucleoli were seen in young asci after the chromatin material had fused. At a slightly later stage a larger, single nucleolus was seen. Disintegrating nucleoli were not observed. It is probable, then, that the nucleoli fuse in this species. The nucleolus became gradually smaller and by the time pachytene was reached it had disappeared altogether. It was not seen to reappear in subsequent divisions. The nucleolar chromosome is one of the longest of the eight chromosomes and stains darker than the others in early Prophase I stages.

The Prophase I chromosomes were difficult to see and little of their morphology and synaptic behaviour could be made out. The only definite fact is that they appear to be paired, though with a slight gap between members of a pair, by the time they are fully
extended. (Fig. 23). Elliott (1960) also had difficulty with these chromosomes, but he found the pachytene chromosomes of the diploid strains easier to analyse than those of the haploid strains. However, precocious synapsis is almost an expected fact in meiosis in fungi since it has been described so often, particularly for the Ascomycetes. The only exception so far seems to be *Sporormia obliquepelta* (Wells, 1956), but synaptic behaviour was not emphasized in the study, and onset of synapsis was not mentioned. From the photographs presented, some chromosomes appeared to be paired before the stage of maximum extension, although there were several unpaired chromosome arms apparent. Singleton (1953) also noted some cases where chromosomes were unpaired at late stages of elongation.

During diakinesis and Metaphase I in *Aspergillus variecolor*, the highly contracted chromosomes and their bivalent nature can be seen more clearly, and chromosome counts can be made. The haploid chromosome number observed for *A. variecolor* is eight. Elliott (1960) also found a chromosome number of eight for *A. nidulans*. As was also found by Elliott (1960) in *A. nidulans*, during early diakinesis in *A. variecolor*, the bivalents separate except for a single point of contact. This has not been described often for fungi. Towards the end of diakinesis the chromosomes pair again and their bivalent nature is clearly seen as they arrange themselves on the metaphase plate. Unlike in many other
fungi, and reminiscent of higher plants, this arrangement is regular, with seven bivalents around the edge of the plate and the eighth in the centre. Elliott (1960) also had a distinct metaphase plate but all eight bivalents were arranged around the edge of the plate. None were centrally located. Very few Metaphase I plates were seen, indicating that this may be a very rapid stage. The metaphase plates of divisions II and III also appeared in the form of a ring, but the whole plate and the chromosomes were even smaller at these stages making chromosome counts difficult.

Disjunction of chromosomes is asynchronous and typical of meiosis in fungi. In *A. variecolor* the chromosomes do not move very far apart during Anaphase I or II, even though the ascus has enlarged considerably by this time. The resulting nuclei remain together in a small region of the ascus. This indicates that the spindle is very short, although actual spindles were not seen even after keeping the material in fixatives for long or short periods of time, or after hydrolysing in NHCl. Spindle orientation in *A. variecolor* in the first division was longitudinal, as in most Ascomycetes, and in the second division the chromosome disjunctions of the two nuclei were clearly orientated at right angles to each other. (Fig. 33).

No evidence, using these staining methods, indicated centrioles or astral rays, although during initial delimitation of the asco-
spores a darkened semi-circle was seen, sometimes to one side of the nucleus. This is reminiscent of spore delimitation in *Neurospora* depicted by Harper (1900) showing the "eyebrow" arc around each nucleus, and it is possible that this represented some astral-ray mechanism. However, it is easy to misinterpret darker-coloured "lines" around delimiting spores, particularly since they are being observed through the ascus wall. In *Aspergillus nidulans* Elliott (1960) decided that cleavage of the cytoplasm around each nucleus seemed to occur, and several other workers have stated this to be the case.

This strain of *Aspergillus variecolor* is distinctive in that during division II the ascus takes on a distinctly lobed appearance. This is a very consistent feature. At first it was thought that the lobes may result from squashing the ascus, but the lobes appear even in unsquashed preparations. The only other explanation was that they helped to accommodate the points of the very wide, dissected crests of the mature ascospore, and indeed, in a mature ascus the equatorial crests of the spores seem to penetrate all free space and every available projection. However, the fact that the lobes appear at such an early stage is off-putting, and also in 1955 Fennell and Raper described a new variety of *Aspergillus variecolor*, *A. variecolor* var. *astellata*, possessing all the *A. variecolor* characters and having lobed asci, but the ascospores have slightly smaller, frilled equatorial crests which are
undissected, so the lobes are unaccounted for in this case.

Fennell and Raper (1955) described this variety as "apparently unique" in having lobed asci. Benjamin (1955) also described *Emericella (Aspergillus) variecolor var. astellata* as unique in having lobed asci. In 1964 Rai et al. isolated a strain of *A. variecolor* (Wis. 4696) with lobed asci. In their paper they state that Raper "(personal communication)" has also observed this character in some isolates of *A. variecolor*. Lobed asci may be a very variable character since it has been observed in different isolates and also in this study in which the *A. variecolor* used is a subculture of the type species (NRRL 212). Lobed asci have not been described for the type species, but it definitely occurred in the isolate used in this study.

The process of reduction division in *A. variecolor* is, as a whole, extremely rapid. Squashed cleistothecia from a culture four days old show a mass of ascogenous hyphae and many young asci at the binucleate and at the fusion nucleus stage. Some asci have mid-prophase nuclei. Cleistothecia from a five day old culture have many asci with various stages of Prophase I nuclei, and several asci at early division II stages. By the sixth day delimiting ascospores can already be seen. Prophase I takes a relatively long time, but the other divisions are rapid. In contrast, actual maturation of the delimited spores takes another one to two weeks.

This study of the nuclear cycle in the ascus of *A. variecolor*
shows great similarity to meiosis in *A. nidulans*. Figures shown by Elliott (1960) are almost identical for most stages, to the ones shown in this study. The chief exception is Metaphase I. This similarity of the sexual stage contributes to evidence that classification of the *Aspergillus* species according to asexual characters and the grouping of similar species is justified.

This study has also contributed to evidence showing the basic similarity of meiosis in fungi to that in higher plants. From the results of studies of many fungi, the main differences in the meiotic processes are precocious synapsis of homologous chromosomes; an irregular metaphase plate; and asynchronous chromosome disjunction. *A. variecolor* shows the latter difference and possibly the first. Otherwise, meiosis is identical to that in higher plants.
SUMMARY

1. *Aspergillus variecolor* (Berk. and Br.) Thom and Raper, a member of the *Aspergillus nidulans* group, is a plectomycete with nonlinear asci produced in a cleistothecium.

2. Coiled ascogonia arise as short branches of thicker, anastomosing hyphae.

3. The cleistothecial wall develops from hyphae arising from the ascogonial stalk, and rapid proliferation of surrounding vegetative hyphae form a loose envelope of hyphae and Hülle cells around the cleistothecium.

4. Hülle cells develop as swollen tips of lateral branches of the sterile hyphae surrounding the cleistothecium. Their protoplasm is shrunken from the outer wall and contains darkly staining bodies which may represent the disintegrating nucleus. The thin outer wall of the cell stains with lactophenol cotton blue, but the wide region between the outer layer and the protoplasm does not stain. This region may be either filled with liquid, or a deposit of a different chemical composition from that of the wall.

5. Asci arise from proliferating croziers.

6. The nuclear cycle in the developing ascus follows the same general pattern as in other higher Ascomycetes, and is almost identical to that in *Aspergillus nidulans*. 

7. There is fusion of the nuclei in the young ascus followed by fusion of the nucleoli; probably early synapsis of the chromosomes; rapid elongation of the chromosomes and gradual disappearance of the nucleoli into late pachytene of Prophase I; contraction of the chromosomes; and formation of eight nuclei by two meiotic and one mitotic divisions.

8. The metaphase plate is regular and anaphase disjunction asynchronous.

9. Spindles, centrioles and astral rays are not revealed with the staining methods used in this study.

10. Asci are characterized by distinct lobes which appear during division II.

11. Ascospores are binucleate when fully mature.

12. The haploid chromosome number for *Aspergillus variecolor* is eight.
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