Bothalia 9, 1:195–202.

The Cytology of Mycosphaerella pinodes*

by

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ABSTRACT

The behaviour of nuclei during ascus and spore formation and maturation in *Mycosphaerella pinodes* (Berk. & Blox.) Stone was studied by means of the HCl-Giemsa technique. The mechanisms of dikaryotization could not be determined but typical croziers were formed. Nuclear fusion occurred in the young ascus and was followed by four conventional successive divisions. At pachytene the chromosomes could be counted and measured. The haploid chromosome number was found to be 15. Ascospore delimitation could be seen at metaphase IV so that each spore is homokaryotic. Two nuclear divisions occurred in the ascospores so that each cell became quadrinucleate.

INTRODUCTION

In an earlier paper (van Warmelo, 1966) the development of the ascocarp of *Mycosphaerella pinodes* (Berk. & Blox.) Stone was described. As it is parasitic on pea plants it was thought that knowledge of the possible variation potential could be of importance in the study and control of this fungus. A review of the literature showed that there was considerable variation in the nuclear behaviour of different species. The cytology was, therefore, investigated to obtain an indication of the variation potential and to obtain data which may be of value in taxonomic studies of this large group.

The development of the ascocarp and nuclear behaviour preceding ascus formation in many species of *Mycosphaerella* is well known from the work of Barr (1958), Higgins (1920, 1929, 1936), Jenkins (1930, 1938), Latham (1934) and Wolf (1940a, b).

The formation of archicarps and spermatia was reported in several species of *Mycosphaerella*, for example *M. tulipiferae* (Higgins, 1936) and *M. arachidicola* and *M. berkeleyii* (Jenkins, 1938). Each archicarp consisted of a basal carpogonium and trichogyne, embedded in the ascostroma. The haploid spermatial nucleus migrated down the trichogyne to initiate the dikaryophase by association with the single carpogonial nucleus. After association of the nuclei the carpogonium became the ascogonium. Conjugate division of the ascogonial nuclei led to the formation of ascogenous hyphae. In *M. tassiana* (Barr, 1958) the ascogenous hyphae developed from ascogenous cells. No archicarps, spermatia or ascogonia were found and the origin of the ascogenous cells was not determined.

The presence of croziers has been reported in several species of *Mycosphaerella* (Higgins, 1929, 1936; Jenkins, 1938) whereas no croziers were found in other species (Higgins, 1920; Jenkins, 1930; Latham, 1934). Barr (1958) reported that croziers were not always formed in *M. tassiana*.

* Based on a thesis submitted to the University of Natal, Pietermaritzburg, in partial fulfilment of the requirements for the degree of M.Sc. Agric.

The number of asci which may develop from one ascogenous hypha was found to vary in different species of *Mycosphaerella*. In *M. bolleana* only one ascus arose from each ascogenous hypha due to the lack of branching ascogenous hyphae and the absence of croziers (Higgings, 1920). After conventional ascus formation in *M. tulipiferae* (Higgins, 1936) and *M. brassicicola* (Dring, 1961) the terminal and basal cells of the crozier fused. The nuclei passed into a branch developing from the basal cell, thus forming a second ascus. In *M. personata* (Higgings, 1929) multiple croziers were formed by repetition of the process outlined above, resulting in the development of several asci from one ascogenous hypha.

Barr (1958) investigated nuclear behaviour after ascus formation in *Mycosphaerella tassiana* and *M. typhae*. In *M. tassiana* karyogamy took place after ascus formation. Growth of the ascus and nuclear divisions following karyogamy were conventional. Spore formation occurred coincidentally with the production of eight nuclei in the ascus. The spore initials became binucleate and, after septum formation, two further divisions followed resulting in ascospores with quadrinucleate cells. The mycelial cells in this fungus were uninucleate except for the multinucleate tips. Barr (l.c.) reported the haploid chromosome number in *M. tassiana* to be four.

Barr (1958) did not observe meiotic and mitotic stages in *Mycosphaerella typhae*. The nuclear sequence, however, appeared to be similar to that in *M. tassiana*. A noticeable difference between these two species was the uninucleate ascospore cells in *M. typhae*. The chromosome number was not determined.

Considerable differences in nuclear behaviour and ascus development are thus evident in different species of *Mycosphaerella*.

Materials and Methods

Culture.—The fungus studied was isolated from ascocarps on naturally infected pea straw. Cultures were maintained as described earlier (van Warmelo, 1966).

Fixation.—Ascocarp material was fixed overnight in a 3: 1 mixture of absolute ethyl alcohol and glacial acetic acid. The fixed material was then stored in 70 per cent ethyl alcohol at room temperature after washing for ten minutes in 90 per cent alcohol followed by ten minutes in 80 per cent alcohol.

Staining.—Mounts for examination were prepared by dissecting out centra from fixed ascocarps in distilled water. The centra were then transferred to 1 N HCl at room temperature for ten minutes and hydrolyzed for 6½ minutes in 1 N HCl at 60°C. After hydrolysis the centra were washed in distilled water and taken through a phosphate buffer (pH 6·9) to the staining solution.

The Giemsa staining technique as used in several other investigations (Hrushovetz, 1956; Knox-Davies and Dickson, 1960; Ward and Ciurysek, 1962) was employed. Two drops of stain per ml of buffer solution were used. Staining was done overnight in tightly sealed glass vials. After staining the centra were mounted in a drop of buffer on a slide and squashed. The preparations were not permanent so that nuclear stages were photographed immediately after preparation.

Results

No ascogonia, trichogynes or spermatia were observed so that the mechanism by which dikaryotization occurs is still unknown.

Centrum.—Within individual centra a wide variety of stages could be found. Most of the early stages were found in 10-day old cultures whereas centra consisting mainly of mature asci occurred in 14-day old cultures.

Crozier formation.—Croziers, which developed in the conventional manner, were common in centra from 10-day old cultures (Figs. 1, 2, 3, 4). Nuclear fusion did not occur in the subterminal cell and the two nuclei passed into the young ascus formed by the enlargement of the subterminal cell (Fig. 5). The origin of the ascogenous hyphae could, however, not be traced.

Croziers with anucleate terminal cells were sometimes observed and it is assumed that the nucleus in the terminal cell degenerated after septum formation. At no time was fusion of the terminal cell with the basal cell observed.

Ascus formation and development.—The young binucleate asci could be readily distinguished from the invariably uninucleate cells of the basal tissue (Fig. 29). Karyogamy took place in the young ascus (Fig. 6) resulting in the diploid nucleus (Fig. 7), after which the ascus enlarged considerably. Occasionally fusion failed to occur as two pachytene stages were sometimes seen in one ascus.

Nuclear divisions in the ascus.

Division I.—The young asci grew rapidly during the period from nuclear fusion to pachytene, after which stage the rate of growth diminished. The diplophase was of short duration as the reductional division was initiated soon after fusion.

Meiotic prophase was typical consisting of five stages. At leptotene (Fig. 8) the nuclear membrane was still present. The chromosomes were long and somewhat indistinct and a large nucleolus could usually be distinguished.

At zygotene (Fig. 9) pairing of chromosomes could be observed. The chromosomes were long and thin and a nucleolus was usually visible.

At pachytene the paired chromosomes had become much more definite in appearance. Centromeres could, however, not be distinguished. It was possible to count and measure individual chromosomes at this stage (Fig. 10) and they were numbered (Fig. 11) following the system employed by Singleton (1953). The approximate lengths of the chromosomes were: $1 = 9{\cdot}5\mu$; $2 = 8{\cdot}5\mu$; $3 = 8{\cdot}5\mu$; $4 = 7{\cdot}0\mu$; $5 = 6{\cdot}5\mu$; $6 = 6{\cdot}0\mu$; $7 = 5{\cdot}5\mu$; $8 = 5{\cdot}5\mu$; $9 = 5{\cdot}5\mu$; $10 = 5{\cdot}0\mu$; $11 = 4{\cdot}0\mu$; $12 = 4{\cdot}0\mu$; $13 = 3{\cdot}5\mu$; $14 = 3{\cdot}5\mu$; $15 = 3.0\mu$. Chromosome 15 was associated with the nucleolus.

At diplotene the chromosomes were much contracted and fuzzy in appearance (Fig. 12), as has been reported by Carr and Olive (1958) and Knox-Davies and Dickson (1960).

Diakinesis was not observed.

At metaphase I (Fig. 13) individual chromosomes could not be distinguished except where movement separated one or more chromosomes from the rest. Rod-like centrioles appeared and the spindles were always parallel to the long axis of the ascus.

At anaphase I the chromosomes were spread irregularly in a band between the two centrioles (Fig. 14). The distance between the centrioles at mid-anaphase was larger than at metaphase and it appeared that movement of the centrioles occurred during anaphase separation. As this stage was seldom seen it was inferred to be of short duration.

At telophase I the chromosomes were closely grouped at the spindle poles (Fig. 15). Lagging chromosomes, which were frequently found in *Trichometasphaeria turcica* (Knox-Davies and Dickson, 1960), were not observed. One case was seen where the two telophase I nuclei were connected by strands of aggregated spindle fibres (Fig. 16).

Division II.—This, and all succeeding divisions were mitotic. During prophase of the second division recondensation of chromosomes took place. At metaphase II the spindles were again parallel to the long axis of the ascus (Fig. 17). Anaphase II figures were not observed. The structure of the telophase II (Fig. 18) and interphase II (Fig. 19) nuclei was identical with that of telophase I and interphase I nuclei respectively.

Division III.—Prophase III nuclei (Fig. 20) were similar to those found at prophase II. At prometaphase III (Fig. 21) the arrangement of the chromosomes was still random. No metaphase or anaphase figures were seen, but the arrangement of nuclei suggested that the spindles were either parallel with or oblique to the long axis of the ascus. Telophase III nuclei were closely aggregated, as before (Fig. 22).

Division IV.—Prophase IV (Fig. 23) was similar to previous prophase stages. At metaphase IV the spindles were not visible but individual chromosomes could be clearly seen (Figs. 24, 25). At this stage chromosomes could again be counted. The average number of fifteen found here agreed with the number determined at pachytene. Occasionally cleavage planes could be detected in the cytoplasm at metaphase IV, but at telophase IV (Fig. 26) eight one-celled binucleate ascospores were usually clearly delimited. Transverse septa started to form in the young ascospores soon after telophase IV.

Nuclear division in the ascospore. In the immature ascospores two further divisions of the nuclei took place during development. In the mature state the spores contained four nuclei per cell (Figs. 27, 28). Ascospores were released from the asci only after this stage had been reached.

Somatic mycelium.—The cells of young hyphae which developed from germinating spores were uninucleate although multinucleate terminal cells were occasionally seen. Cells in the older mycelium were, however, usually multinucleate.

Discussion

In *Mycosphaerella pinodes* no trichogynes, spermatia or ascogonia were observed, the earliest stage seen being the early binucleate crozier. It thus appears that ascus development in this fungus is similar to that of *M. tassiana* as described by Barr (1958).

The chromosomes of *M. pinodes* appear to be longer than those of *M. tassiana* (Barr, 1958) but are shorter than the chromosomes of *Neurospora crassa* (Singleton, 1953), *Sordaria fimicola* (Carr and Olive, 1958) and *Trichometasphaeria turcica* (Knox-Davies and Dickson, 1960).

Mycosphaerella tassiana, with chromosome number of 4 (Barr, 1958), is the only other species of *Mycosphaerella* of which this character is known. This number is appreciably lower than the high number of 15 found in *M. pinodes*. The regularity and stability of the divisions in the latter species and the lack of lagging and aberrant chromosomes indicate that the chromosome complement is not an incomplete tetraploid.

The haploid chromosome number of *Mycosphaerella pinodes* is exceeded only by the sixteen chromosomes observed in *Aleuria* (*Peziza*) *rutilans* by Wilson (1937). Hirsch reported twelve chromosomes in *Pyronema confluens* but the highest number counted previously among the perithecial and ascolocular forms was ten in *Phyllactinia corylea* (Colson, 1938). *M. pinodes* thus has the highest number of chromosomes known among the ascolocular and perithecial forms.

The large difference in haploid number between *M. tassiana* and *M. pinodes* suggests that there may be different groups within this genus. The available evidence indicates a possible relationship between a moniliaceous imperfect stage (*Cladosporium herbarum*) and a low chromosome number (in *M. tassiana*) and a sphaeropsidaceous

imperfect stage (*Ascochyta pinodes*) and a high chromosome number in (*M. pinodes*). A large number of form genera of Fungi Imperfecti are associated with species of *Mycosphaerella*, for example *Ramularia* (Plakidas, 1941), *Cercosporella* (Doidge, 1950), *Scolecotrichum* (Wehmeyer, 1946), *Cercospora* (Jones, 1944), *Stigmina* (Smith and Smith, 1941), *Phyllosticta* (Wolf, 1940b), *Phoma* (Brooks, 1953), *Piggotia* (Wolf and Davidson, 1941), *Ascochyta* (Wiant, 1945), *Phleospora* (Brooks, 1953), *Septoria* (von Arx, 1949), *Marssonia* (Wolf and Davidson, 1941), *Septogloeum* (Doidge, 1950) and *Cylindrosporium* (Doidge, 1950). It may thus be possible to correlate chromosome number with the imperfect stages of various species of *Mycosphaerella*. Such correlation may allow division of the genus into a number of smaller taxa. These relationships, however, require verification by similar studies of other species in this genus.

Since cytoplasmic cleavage planes occur in the ascus before the 16-nucleate stage, each ascospore contains two identical nuclei at telophase IV. The mature ascospore thus contains eight identical nuclei. This confirms the results of Hare and Walker (1944) who demonstrated by cultural studies that the two cells of the ascospore of *M. pinodes* are genetically identical. This mode of ascospore formation is important in fungi in which the ascospores are not obligately uniseriate. If metaphase IV spindles are irregularly arranged and spore delimitation occurs at telephase IV, a two-celled ascospore may incorporate two nuclei of different compatibility reaction. In heterothallic fungi this could give rise to pseudohomothallism (Raper, 1960). But, in *M. pinodes*, which is homothallic (Hare and Walker, 1944), it would have no effect.

The significance of the quadrinucleate condition of the mature ascospore cells of *M. pinodes* is not clear at present but the similarity with *M. tassiana* is noteworthy. The multinucleate condition may be a mechanism of survival as has been suggested for other fungi with multinucleate spores (Knox-Davies. Pers. comm.).

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EXPLANATION OF FIGURES

All figures magnified 2000x.

FIG. 1.—Early binucleate crozier.

FIG. 2.—Binucleate crozier.

FIG. 3.—Conjugate division in a crozier.

FIG. 4.—Quadrinucleate crozier.

FIG. 5.—Binucleate ascus.

FIG. 6.—Ascus with fusing nuclei.

FIG. 7.—Diploid ascus.

FIG. 8.—Leptotene.

FIG. 9.—Zygotene.

FIG. 10 & 11.—Pachytene.

FIG. 12.—Diplotene.

FIG. 13.—Metaphase I.

FIG. 14.—Anaphase I.

FIG. 15.—Telophase I.

FIG. 16.—Telophase I with strands of aggregated spindle fibres.

FIG. 17.—Metaphase II.

FIG. 18.—Telophase II.

FIG. 19.—Interphase II.

FIG. 20.—Prophase III.

FIG. 21.—Prometaphase III.

FIG. 22.—Telophase III.

FIG. 23.—Prophase IV.

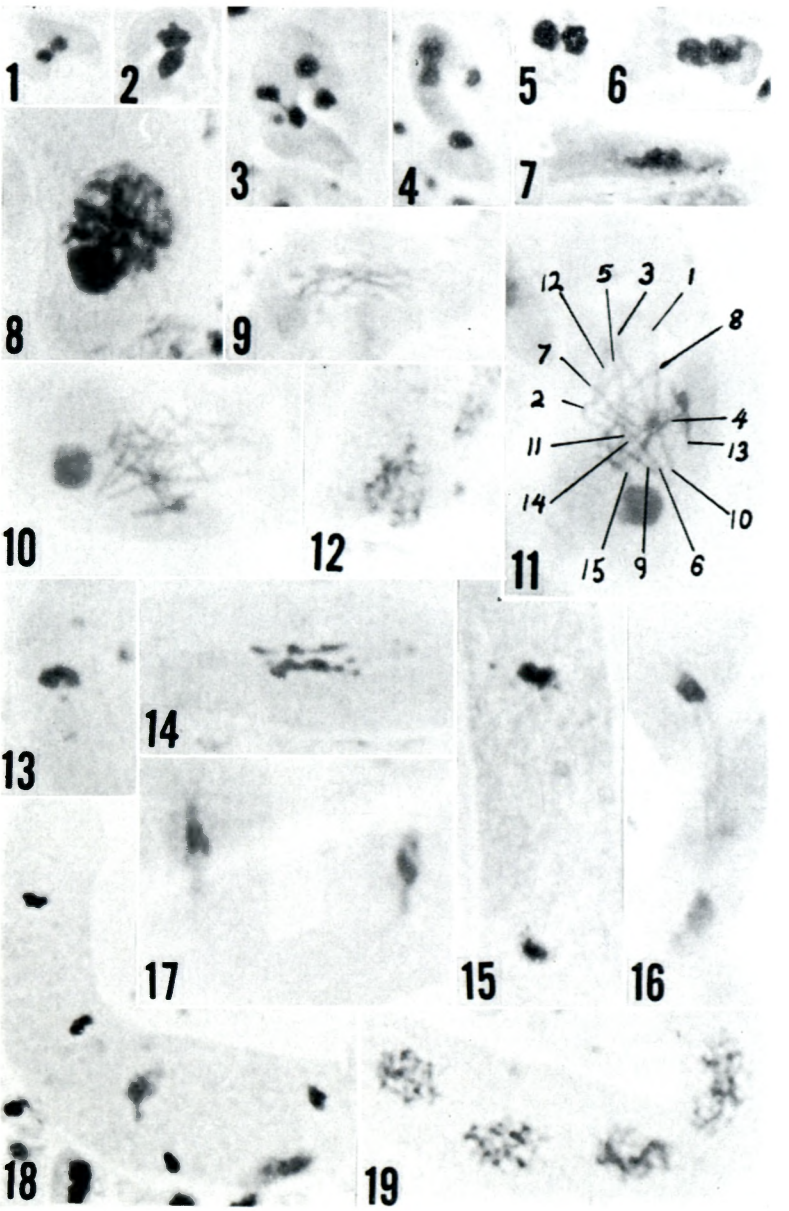
FIG. 24 & 25.—Metaphase IV.

FIG. 26.—Telophase IV.

FIG. 27.—Ascospores with binucleate cells.

FIG. 28.—Ascospores with quadrinucleate cells.

FIG. 29.—Uninucleate pseudoparenchyma.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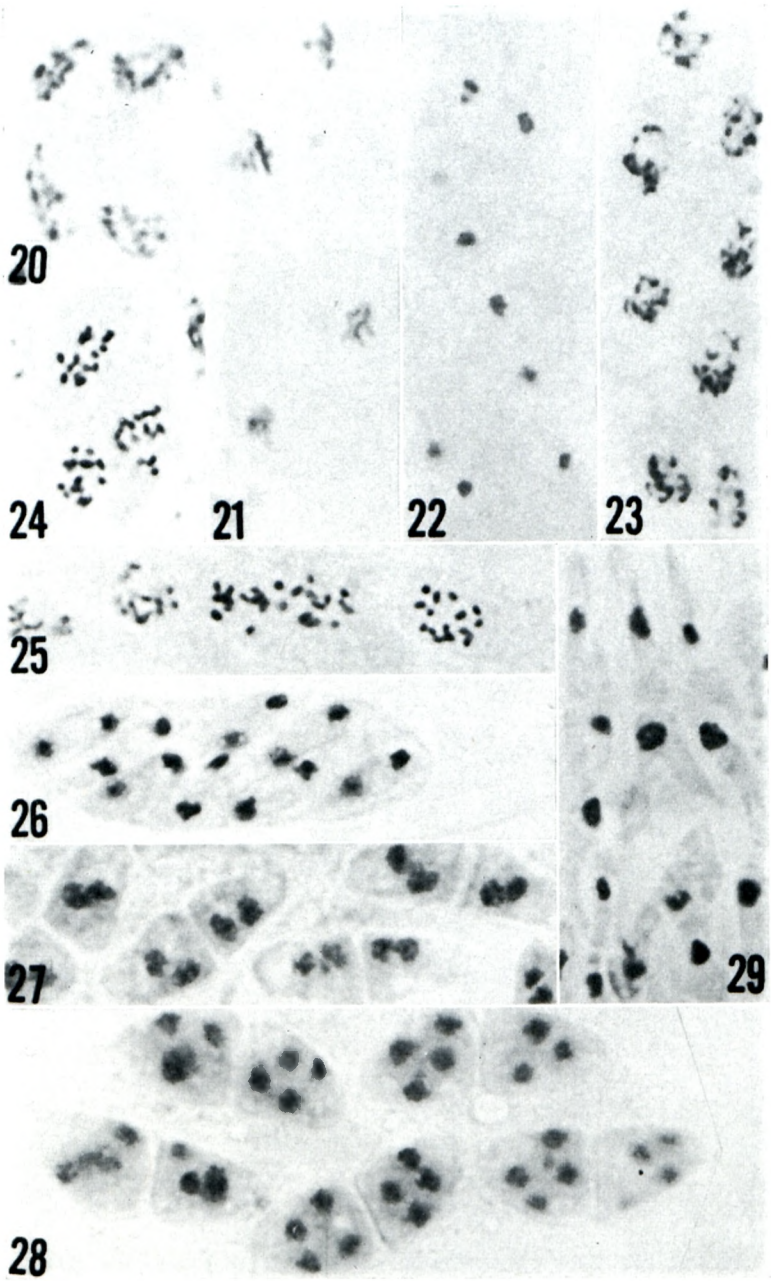
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