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CONTRIBUTION TO THE CYTOLOGY OF HYMENOMYCETES : X. KARYOLOGICAL STUDIES IN *HEXAGONA APIARIA* (PERS.) LLOYD

By

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(With 3 Text-figures)

The karyological study of *Hexagona apiaria* has been divided into its somatic and reproductive phases.

The somatic phase starts with the germination of uninucleate basidiospores. Prior to germination of each basidiospore considerable enlargement of tue spore takes place in every case. Nuclear division starts within the basidiospore during which it becomes fairly dilated. Various stages of nuclear division has also been noted. The germ tube is formed after one, two or more mitotic divisions of the nucleus within the basidiospore. All of the nuclei excepting one, which remain in the sporecase, migrate into the germ-tube. Wall-formation starts from the terminal part of the germ-tube which is either simultaneous or successive. Ultimately the spore-case is delimited from the germ-tube by a septum. In several cases, formations of germ-tubes have been found to occur at opposite ends of a basidiospore. Subsequent nuclear divisions in the germ-tube are followed by wall formation and on branching it produces a septate monokaryophasic mycelium without clamp-connexion.

Compatible primary mycelia produce typical dikaryophasic mycelium when crosse in pairs. The binucleate hyphal cells in the dikaryophasic mycelium are provided with clamp-connexions almost at every septum.

In the reproductive phase, formation of basidia and basidiospores takes place as usual in the hymenium of the fructifications. The terminal cell of a hypha forms the young basidium which is characteristically binucleate. The two nuclei therein fuse to form the synkaryon. This nucleus enlarges considerably with the enlargement of the basidium and generally migrates to the upper part of the basidium. It then undergoes three successive divisions of which the first one is reductional. During early prophase I chromatin reticulum appears with a fairly large nucleolus. The reticulum disappears finally with the formation of typical bivalents in metaphase I and the diploid (2n) chromosome number

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for the fungus is 14. The orientation of the spindle is transverse or obliquely longitudinal and that of successive divisions are extremely variable. The reduction division is followed by two equational ones. The eight nuclei thus formed within the mature basidium at first remain scattered and four of them eventually migrate into the four developing basidiospores through the sterigmata. The remaining four nuclei gradually become indistinct in the collapsing basidium.

Introduction

The available literature reveals that considerable amount of work has already been done in this line during the past decades of the present century. Olive (1953) in his excellent review has recognized Kharbush (1929), Sass (1929), Wakayama (1930, 1932), Pinto-Lopes (1949) and a few others as some of the pioneer workers in this field of study. Our knowledge has further been supplemented by the recent studies of the workers like Boidin (1954), Bakerspigel (1959). Ward and Cuirysek (1961), Motta (1967) and Wilson and Aist (1967) with various species of Hymenomycetes. In India, Bose (1937) is the first to undertake such problems and has reported partly the nuclear behaviour in eleven species of Polyporaceæ. His lead have been successfully followed by Banerjee and his co-workers (1955, 1956, 1957, 1960, 1961, 1962, 1966a, b, 1967) who have contributed valuable informations on the karyological behaviour in the life-cycles of some species of Polyporaceæ, Thelephoraceæ and Agaricaceæ.

Material and Methods

Fresh basidiocarps of *H. apiaria* were collected from the garden during the months of March to November, 1964, growing on the decayed branches of *Euphoria longan*. Small, rectangular pieces (5×5 mm. approximately) were cut carefully from the peripheral regions of the fresh basidiocarps, slightly soaked with water for keeping them in the sporing condition, and were killed, fixed in different fixatives, such as 'Bouin-Allen', 'Sass', 'Carnoy', 'Navaschin' (A and B) and Formalin-Acetic-Alcohol This was done at different hours in a day (24 hours) at intervals of 3 hours. Rapid penetration of the fixatives in the materials was done by keeping them in reduced atmospheric pressure for a few minutes (Sass, 1929). The materials were then transferred to fresh fixing fluids and allowed to remain for the scheduled time recommended for each case. Of all the fixing fluids 'Bouin-Allen' was found to be the most suitable one for this study, although good results were also obtained with 'Sass'. The materials were then washed thoroughly, dehydrated with ethyl alcohol (70 percent) and passed through n-butyl in different concentrations (25, 35, 55, 75 and 100 percent) for further dehydration to avoid difficulties in sectioning (Ehrlich and McDonough, 1949). The dehydrated pieces of the basidiocarps were finally infiltrated with and embedded in paraffin.

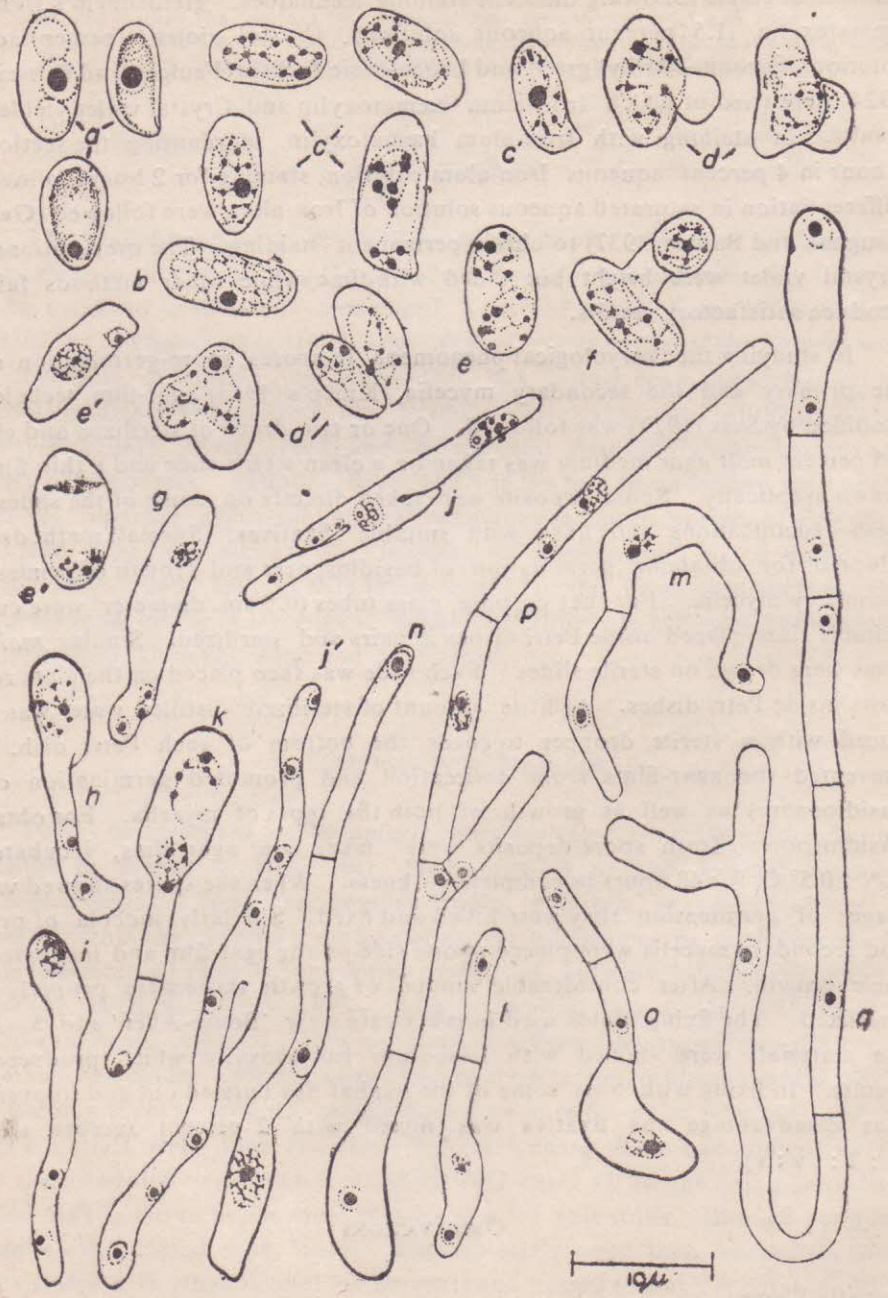
Microtome sections, 6 – 8μ thick, were cut and those were stained with a number of stains following different staining techniques. Heidenhein's Iron-alum hæmatoxylin (1.5 percent aqueous solution), Crystal violet (1 percent aqueous solution), Pyronin-Methyl green and Leuco-basic fuchsin (Feulgen and Rossenbeck, 1924) were tried of which Iron-alum hæmatoxylin and Crystal violet yielded best results. In staining with Iron-alum hæmatoxylin, mordanting the sections for 1 hour in 4 percent aqueous Iron-alum solution, staining for 2 hours followed by differentiation in saturated aqueous solution of Iron-alum were followed (Gwynne-Vaughan and Barnes, 1937) to obtain permanent staining. The preparations with Crystal violet were bright but faded with time, while other methods failed to produce satisfactory results.

In studying the karyological phenomena in spores, spore-germination and in the primary and the secondary mycelia, Kniep's (1913) agar-film technique as modified by Sass (1929) was followed. One or two drops of sterilized and cleared 0.5 percent *malt-agar* medium was taken on a clean sterile slide and a thin film was drawn aseptically. Spore-deposite were taken directly on some of the slides from fresh fructifications and fixed with suitable fixatives. Special methods were adopted for obtaining germination of basidiospores and growth of primary and secondary mycelia. For that purpose, glass tubes (0.5 cm. diameter) were cut into suitable sizes, placed inside Petri dishes in pairs and sterilized. Similar *malt-agar* films were drawn on sterile slides. Each slide was then placed on the glass rods in pairs inside Petri dishes. A little amount of sterilized distilled water was introduced with a sterile dropper to cover the bottom of each Petri dish. This prevented the agar-films from desiccation and promoted germination of the basidiospores as well as growth of both the types of mycelia. For obtaining basidiospores fresh spore-deposits were taken on agar-films, incubated at 30°(±0.5°)C. for 48 hours in complete darkness. When the spores showed various stages of germination they were killed and fixed. Similarly, inocula of primary and secondary mycelia were placed at one side on the agar-film and incubated for their growth. After considerable amount of growth those were properly killed and fixed. The fixing fluids used in every case were 'Bouin-Allen' and 'Sass' and the materials were stained with Iron-alum hæmatoxylin which produced best results. In fixing with 'Sass' some of the hyphal tips bursted out and to overcome that disadvantage the fixative was mixed with 2 percent sucrose solution (1 : 1 : : v : v).

Observations

Somatic Phase

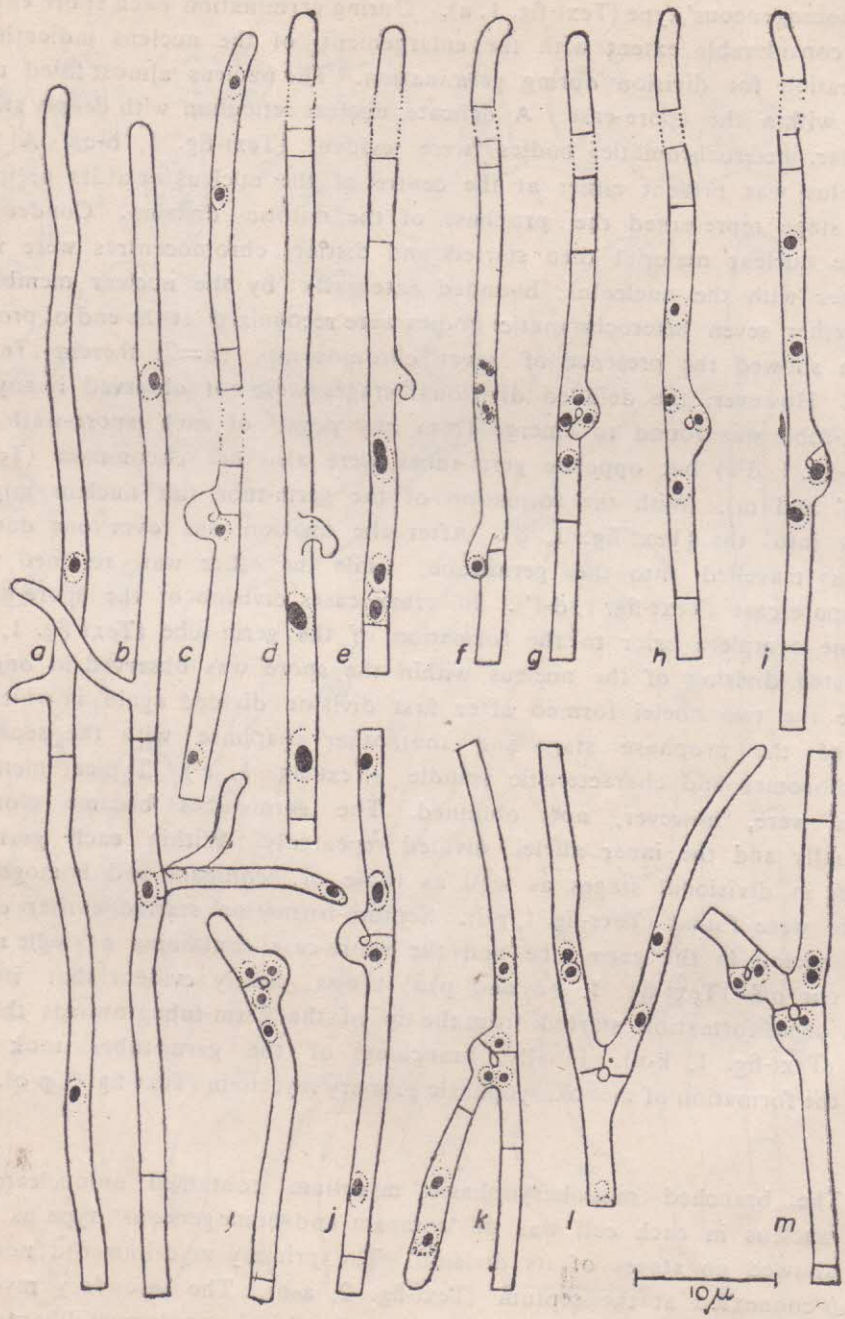
The basidiospores were slightly thick-walled, boat-shaped, oblong in outline with a distinct apiculus. Each basidiospore was uninucleate after discharge from the sterigma with the nucleus (about 2μ across) of 'compact



Text-fig. 1. Nuclear phenomena in basidiospores and germinating basidiospores of *Hexagona apiaria* (Pers) Lloyd

and homogeneous' type (Text-fig. 1, a). During germination each spore enlarged to a considerable extent with the enlargement of the nucleus indicating its preparation for division during germination. The nucleus almost filled up the space within the spore-case. A delicate nuclear reticulum with deeply stained, granular, heterochromatic bodies were evident (Text-fig. 1, b-c). A single nucleolus was present either at the centre of the nucleus or at its periphery. That stage represented the prophase of the mitotic division. Condensation of the nuclear material then started and distinct chromocentres were visible together with the nucleolus, bounded externally by the nuclear membrance. Altogether seven heterochromatic bodies were recognized at the end of prophase which showed the presence of seven chromosomes (n=7) therein (Text-fig. 1, c'). However, the detailed divisional stages were not observed in any case. Germ-tube was found to emerge from any point of each spore-wall singly (Text-fig. 1, d-f) but opposite germ-tubes were also not uncommon (Text-fig. 1, j-j' and m). With the formation of the germ-tube the nucleus migrated partly into the (Text-fig. 1, d). After the division was over one daughter nucleus travelled into the germ-tube, while the other was retained within the spore-case (Text-fig. 1, d-f'). In other cases division of the spore-nucleus became complete prior to the formation of the germ-tube (Text-fig. 1, e-e'). Repeated division of the nucleus within the spore was observed in one case where the two nuclei formed after first division divided again in which one showed the prophase stage and the other anaphase with the separating chromosomes and characteristic spindle (Text fig. 1, e'). Typical metaphase plates were, however, not obtained. The germ-tubes became elongated gradually and the inner nuclei divided repeatedly. Within each germ-tube nuclei in divisional stages as well as those of 'compact and homogeneous' nature were found (Text-fig. 1, g-i). Septum-formation started earlier or at a later period in the germ-tube and the spore-case containing a single nucleus was cut off (Text-fig. 1, l-o and p). It was clearly evident that in most cases septa-formation started from the tip of the germ-tube towards the basal part (Text-fig. 1, k-n). Finally, branching of the germ-tubes took place with the formation of monokaryophasic primary mycelium (Text-fig. 1, p-q).

The branched monokaryophasic mycelium contained uninucleate cells. The nucleus in each cell was of 'compact and homogeneous' type as before and showed no stages of its division. The primary mycelium did not show clamp-connexion at the septum (Text-fig. 2, a-b). The secondary mycelium, on the other hand, contained binucleate cells showing its typical dikaryophasic nature (Text-fig. 2, c). The hyphæ contained simple clamp-connexion almost at every septum. The nuclei were also of 'compact and homogeneous' nature

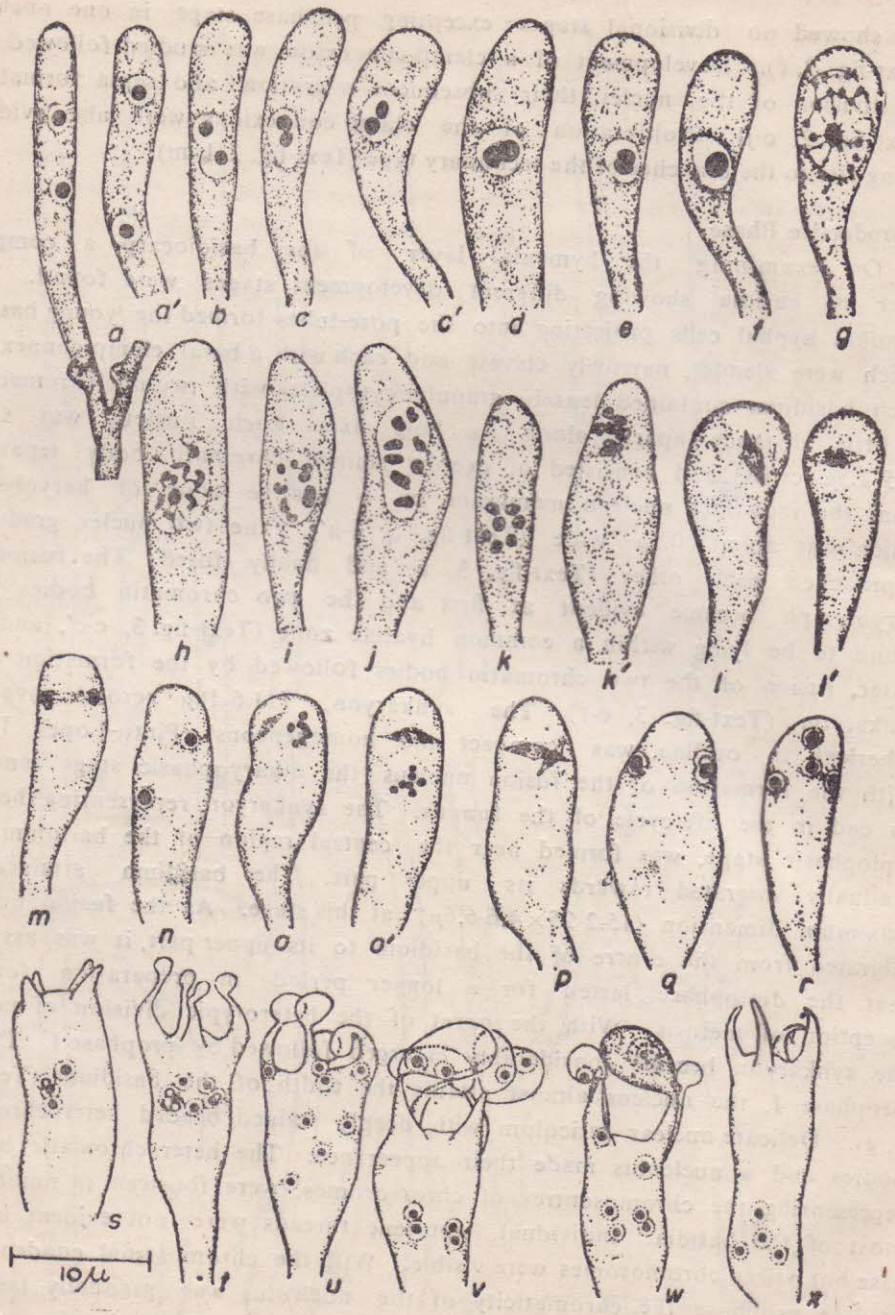


Text-fig. 2. Nuclear phenomena in primary and secondary mycelia of *Hexagona apiaria* ((Pers.) Lloyd

and showed no divisional stages excepting prophase stage in one nucleus (Text-fig. 2, f). Development of a clamp-connexion was studied followed by the division of the nuclei, their subsequent migration and septa formation (Text-fig. 2, c-j). Proliferation of the clamp-connexions were also evident giving rise to the branches of the secondary type (Text-fig. 2, k-m).

Reproductive Phase

On examining the hymenial layer of the basidiocarp a compact layer of basidia showing different development stages were found. The terminal hyphal cells projecting into the pore-tubes formed the young basidia which were slender, narrowly clavate and each with a basal clamp-connexion. Each basidium contained densely granular cytoplasm with two nuclei remaining a little distance apart along its long axis. Each nucleus was small (1.5-2.5μ across) and consisted of deeply stained chromatin body separated from the indistinct nuclear membrane by a hyaline zone of karyolymph which was about 0.5μ wide (Text-fig. 3, a-a'). The two nuclei, gradually approached each other (Text-fig. 3, b) and finally fused. The fusion of karyolymph became evident at first and the two chromatin bodies were found to be lying within a common hyaline zone (Text-fig. 3, c-c', and d). Later, fusion of the two chromatin bodies followed by the formation of a synkaryon (Text-fig. 3, e-f). The synkaryon, (11.6-16μ across), oval to spherical in outline, was 'compact and homogeneous' (Pinto-Lopes, 1949). With the formation of the fusion nucleus the dikaryophasic stage came to an end in the life-cycle of the fungus. The synkaryon, representing the only diplophasic stage, was formed near the central region of the basidium and gradually migrated towards its upper part. The basidium attained its maximum dimension (15.2-25$\times$4.6-6.6μ) at this stage. As the fusion nucleus migrated from the centre of the basidium to its upper part, it was assumed that the diplophase lasted for a longer period in preparation for the inception of meiosis. With the onset of the heterotypic division of meiosis, the synkaryon became considerably enlarged followed by prophase I. During prophase I, the nucleus almost attains the width of the basidium (Text-fig. 3, g). Delicate nuclear reticulum with deeply stained, beaded heterochromatic bodies and a nucleolus made their appearance. The heterochromatic bodies, representing the chromosentres of chromosomes, were fourteen in number in most of the basidia. Individual leptotene threads were not evident in any case but paired chromosomes were visible. With the chromosomal condensation at late prophase the chromaticity of the nucleolus was gradually lost and it disappeared finally (Text-fig. 3, h-j). The chromosomes became clearly visible within a distinct nuclear membrane and with the disappearance of the nucleolus the nuclear membrane also disappeared with the formation of



Text-fig. 3. Nuclear phenomena in the basidia of *Hexagona apiaria* (Pers.) Lloyd

transverse or obliquely longitudinal spindle in metaphase I. During metaphase I seven highly chromatic bivalents (2n=14) were distinctly visible in the polar view while the bivalents remained oriented at the equator of the spindle (Text-fig. 3, k-k′) Metaphase I was very soon followed by anaphase I when each of the seven pairs of chromosomes separated from each other and the two sets of seven chromosomes (genome) moved towards the two poles of the achromatic spindle. The chromosomes remained closely associated at this stage and their individual identity were not ascertained either in polar or in transverse view (Text-fig. 3, l-l′). During late anaphase I, the chromosomes were found to be associated at the two poles but their number could not be counted. The chromosomal fibres remained extended from pole to pole (Text-fig. 3, m) which later disappeared at telophase I. Eventually, the chromosomes at each pole fused to form a daughter nucleus. The two daughter nuclei thus formed, were of 'compact and homogeneous' type and remained side by side either in a transverse plane or in an obliquely longitudinal plane with respect to the long axis of the basidium (Text-fig. 3, n). The heterotypic division ended with the formation of the two daughter nuclei which was followed by homotypic one. The two nuclei then divided in succession (Text-fig. 3, o-o′) but sometimes the divisions were also simultaneous (Text-fig. 3, p). The detailed chromosome behaviour was, however, not observed and stages with one nucleus in the metaphase II and the other in the anaphase II were evident (Text-fig. 3, o-o′). The metaphase spindles were variously oriented during the second divison. In some both the spindles were transverse but at right angles to each other (Text-fig. 3, o′) while in others one remained transverse and the other obliquely longitudinal to the long axis of the basidium (Text-fig. 3, o-p). Those observations were in support of the view of Rogers (1934) who criticised the taxonomic value of spindle orientation in the classification of basidiomycetes. The four nuclei formed after second division of meiosis were alike, 'compact and homogeneous'. The orientation of the four nuclei were extremely variable and were either closely associated with one another or more or less scattered at the centre (Text-fig. 3, s), or in the upper part of the basidium (Text-fig. 3, q-r). With the end of second division rudiments of sterigmata, four in number, were found to develop from the upper end of the basidium (Text-fig. 3, s). Within the basidium a further homotypic division of the tetracyte nuclei took place and the eight nuclei, thus formed, either remained closely associated (Text-fig. 3, t) or were scattered (Text-fig. 3, u). Throughout the basidium divisional stages and spindle-formation of the third division were, however, not found. During the third division distinct swelling of the tips of the sterigmata was observed and those gradually became large in

size (Text-fig. 3, t-u). Four nuclei from the basidium migrated (Text-fig. 3, u) through the sterigmata, one in each, into the developing basidiospores, formed at their apices (Text fig. 3, v) The basidiospores were uninucleate and were ultimately abjointed from the sterigmata. The migration of the nuclei into the basidiospores and their development were in succession rather than simultaneous (Text-fig. 3, w). The nucleus, in each spore, was medianly placed or in contact with the spore-wall (Text-fig. 3, v-w). After the formation of the basidiospores, the remaining four nuclei within the basidium degenerated gradually (Text-fig. 3, x). The basidium after spore-discharge, became shorter with drooping sterigmata and finally collapsed.

Discussion

The investigation on the karyological phenomena in the life-cycle of *Hexagonia apiaria* point to the fact that the fungus is a heterothallic one. The uninucleate basidiospores are phenotypically similar and produce primary mycelia having uninucleate hyphal cells. The single nucleus in each basidiospore and hyphal cell represents the monokaryophase of the life-cycle. On the other hand, the secondary mycelia, obtained by mating suitable compatible monokaryophasic types, possess two nuclei in each hyphal cell with clamp-connexions almost at every septum. The secondary mycelium thus represent the dikaryophase of the life-cycle. This dikaryophase continues for an indefinite period which is terminated in the young basidia where the two nuclei in each basidium fuse to from the synkaryon. As usual the basidium is always the ultimate cell of a dikaryophasic hypha. The synkaryon within the basidium is the only diplophase in the life-cycle of the fungus. This diplophasic condition is soon followed by meiotic division which is regarded as the antithesis of syngamy. At the advent of nuclear division 'interphasic' enlargment of the synkaryon is evident. With the initiation of meiosis I, which is heterotypic in nature, a number of small, deeply stained chromatin bodies and a nucleolus become visible within the nucleus. Wakayama (1930, 1932), Banerjee *et al.* (1956, 1960, 1961, 1962, 1966a, 1966b, 1967) and others also have reported the presence of such chromatin bodies. Kharbush (1929) has interpreted these as 'protochromosomes' while Olive (1953) considers the chromatin bodies as ture chromosomes or heterochromatic bodies on the chromosomes. However, there still lies a gap in our knowledge in understanding the significance of these chromatin bodies in the meiotic prophase. The heterotypic division is followed by two successive homotypic ones resulting in the production of eight nuclei in each basidium. Of the daughter nuclei, four migrate to the four developing basidiospores, one in each, through the

sterigmata and the other four nuclei remaining within the basidium finally degenerate. The significance of third homotypic division and also of the degeneration of four daughter nuclei in the collapsing basidium remain still an open challenge to the workers in this line of study.

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