STRUCTURE AND DEVELOPMENT OF THE OVULE AND EMBRYO-SAC OF *PIPER LONGUM* L.

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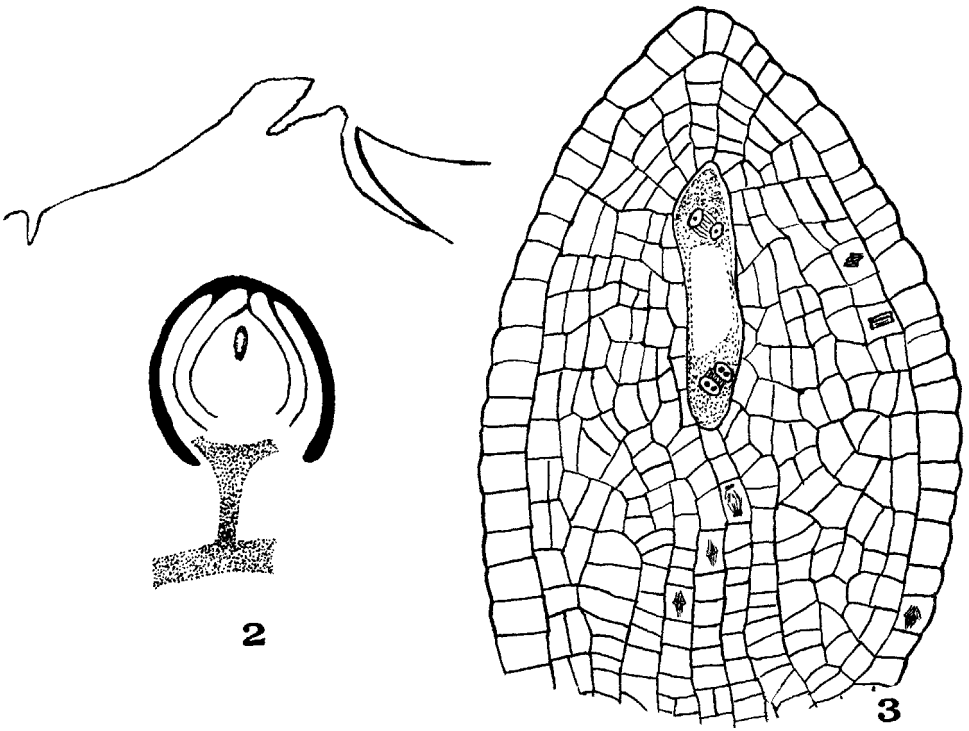
The Piperaceae has been a family of unusual interest to students of angiospermous embryology ever since the end of the last century, when Campbell (1899, 1901) and Johnson (1900) almost simultaneously discovered the 16-nucleate type of embryo-sac in *Peperomia*. This discovery soon drew the attention of botanists to other genera of the family. Johnson (1902) himself immediately took up the study of *Piper* and *Heckeria*, investigating two species of each genus, namely *Piper adunca*, *P. medium*, *Heckeria umbellata* and *H. peltata*. *Piper medium* and *Heckeria umbellata* were investigated in detail. He reported in all species the presence of a single primary archesporial cell, the formation of a primary wall cell and the *Lilium*-type of embryo-sac. The megaspore-mother cell was found to form no tetrad of megaspores, but as a result of three free nuclear divisions followed by cell formation gave rise directly to a 7-celled 8-nucleate embryo-sac. Such a type of embryo-sac development according to the present terminology is described as the *Adoxa*-type. Johnson (1910) studied later the embryo-sac of *Piper Betel* var. *monoicum* and found the same type of development in this species also. Fischer (1914) and Palm (1915) investigated the embryo-sac development in *Piper tuberculatum* and *P. subpeltatum* respectively. Their observations agreed with those of Johnson. The *Fritillaria*-type of embryo-sac was discovered by Bambacioni (1928) 15 years ago. Since its discovery first in *Fritillaria persica* and later in other members of the Liliaceae and other families (cf. Schnarf 1936 and Maheshwari 1937), the occurrence of the *Adoxa*-type of embryo-sac in *Piper* and *Heckeria* has been much doubted. Schnarf (1931 and 1936), Capoor (1937) and Maheshwari (1937) have all expressed the opinion that the development of the embryo-sac in these genera from the published figures of the different authors appears to follow the *Fritillaria*-type. Maheshwari recently in collaboration with Gangulee (Maheshwari and Gangulee, 1942) has actually verified this supposition for *Heckeria*. The genus *Piper*, however, remains to be reinvestigated.

The present work is based on a study of *Piper longum* Linn., the long pepper. This species is found both wild and cultivated in the tropical parts of India, Ceylon and Malaya. Its spikes contain a volatile oil, an acrid resin and piperine, and are commonly employed in native and household medicine as a stimulant and carminative. The material used in the investigation was collected by the writer in the month of December 1936 from a plant growing in the Royal Botanic Gardens, Peradeniya, Ceylon. It was fixed in formalin acetic alcohol, and later studied according to the customary methods. Heidenhain's and Delafield's haematoxylin were employed as stains. As the flowers are arranged almost perpendicularly on the axis of the inflorescence and the ovules are orthotropous, serial transverse sections of the spikes are found to yield perfect longitudinal sections of the gynoecium and the ovule. This is clear from the photograph reproduced here as Fig. 1. Every flower of the spike is thus sectioned in the most favourable manner for following the development of the ovule and embryo-sac.

STRUCTURE OF THE GYNOECIUM.

Piper longum is a dioecious species. The female flowers are borne in dense spikes about $\frac{1}{2}$–1 inch long. The ovaries and the bracts of the naked flowers are pressed very closely and they are all fused with one another (Plate III, Fig. 1). Transverse sections of a flower reveal the presence of four vascular bundles in the wall of the unilocular ovary.

Two of these are the dorsal bundles and two represent the fused ventrals. Each ovary is topped by two sessile stigmas. The gynoecium is thus clearly bicarpellary.



Figs. 2-3.—*Piper longum*. Fig. 2, Longitudinal section of a gynoecium showing the form and structure of the ovule. The vascular tissue is dotted. Fig. 3, Longitudinal section of a nucellus with the embryo-sac at the second 4-nucleate stage. Fig. 2, × 50; Fig. 3, × 500.

Both the wall of the ovary and the bracts are composed of an almost uniform parenchymatous tissue except for the outer and inner epidermal layers and the vascular strands. There is no differentiation of the palisade or spongy mesophyll in the bracts and the ovary wall is not differentiated into three distinct regions as described by Johnson (1902) in *Piper medium*. Special cells secreting volatile oil are present scattered throughout the wall of the ovary and the mesophyll of the bracts. They are larger than the surrounding cells and their protoplasts and nuclei remain active until the ripening of the fruits as described and illustrated by Johnson (1902) in *Heckeria umbellata*.

Structure of the Ovule.

The ovary contains a single basal orthotropous ovule characteristic of the family, with two integuments, of which the inner alone forms the micropyle (Fig. 2)[1]. The outer integument is 3-4 and the inner 2–4 cells thick at the 8-nucleate embryo-sac stage. The nucellus is well developed (Fig. 3) and a characteristic feature is the presence of a meristematic zone at its chalazal end which goes on adding new cells until a very late stage of development. The meristematic activity from the chalazal end extends gradually also to the sides of the nucellus, which consequently continuously increases in

[1] Johnson (1902) does not say anything in the text about the formation of the micropyle in species of *Piper* investigated by him, but in his Figs. 5 and 6 he shows the micropyle formed from both the integuments. Such variation in the formation of the micropyle is not observed among closely related plants, and it appears probable that his illustrations in this respect are not quite accurate.

size. At the mature embryo-sac stage there are approximately 8 layers of cells above, 8–15 on the sides and about 60 below the embryo-sac. The development of a meristematic zone at the chalazal end of the nucellus has been observed by the author also in several families of the allied order Centrospermales. In *Piper longum*, however, the cells of the nucellus epidermis never divide periclinally and there is no epidermal cap formation as is characteristic of the Centrospermales (Joshi, 1939). The ovule receives a single vascular trace, which spreads out peltately in the chalaza (Fig. 2). The micropylar apex of the nucellus is generally pointed.

The Development of the Embryo-sac.

The primary archesporial stages and the cutting off of the primary wall cell were not observed, but later stages (Figs. 4 and 5) indicate a single archesporial cell and the formation of a primary wall cell, as in other investigated species of *Piper*. The primary wall cell undergoes repeated divisions soon after its differentiation, so that even before the megaspore-mother cell undergoes the first division it has given rise to four layers of parietal tissue. By the time the ovule reaches the mature embryo-sac stage it has formed about eight layers of parietal cells.

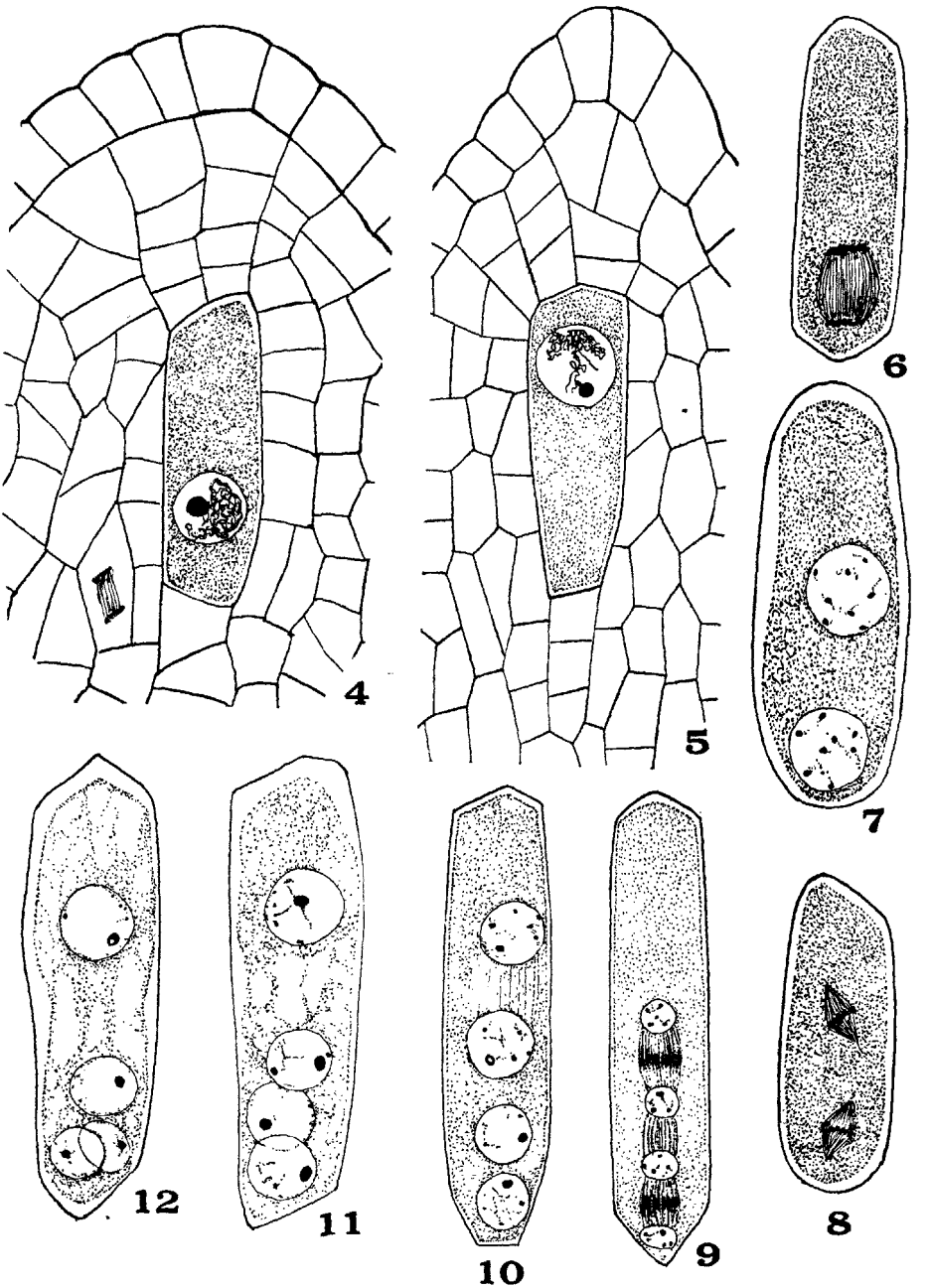
The megaspore-mother cell is approximately three times as long as broad (Figs. 4 and 5). Its most notable feature is the position of the nucleus, which is situated not in the centre or close to the micropylar end, as is the case among the flowering plants in general, but near the chalazal end (Fig. 4). This position is almost constant and sometimes the nucleus even touches the chalazal end wall. Only in one case the nucleus of the megaspore-mother cell was found near the micropylar end (Fig. 5). Bambacioni-Mezzetti (1931) also has figured the nucleus in the megaspore-mother cells of *Tulipa praecox* and *T. sylvestris* near the chalazal end, but this position does not appear to be so common. Nor we find this position of the nucleus in the megasporocytes of *Fritillaria persica* (Bambacioni, 1928), *Lilium Henryi* (Cooper, 1935), *Tamarix dioica* (Joshi and Kajale, 1936) and *Gagea fascicularis* (Joshi, 1940), other plants with the same type of embryo-sac development.

The nucleus of the megaspore-mother cell undergoes the I meiotic division near the chalazal end (Fig. 6), just where it is generally situated in the resting condition. No wall is formed after this division. Thus the result of the first division in the megaspore-mother cell is not the formation of a dyad, but of a 2-nucleate embryo-sac (Fig. 7). This differs from a normal 2-nucleate embryo-sac in the absence of any signs of vacuolation and in the position of the nuclei. One of the nuclei is situated at the chalazal pole, but the other is found near the centre of the embryo-sac instead of being situated at the micropylar end.

During the second division of the megaspore-mother cell, one of the spindles is found near the chalazal end, the second near the middle of the embryo-sac (Fig. 8). No walls are laid even at the end of this division. It results in the formation of a 4-nucleate embryo-sac (Figs. 9 and 10), which again differs from a normal 4-nucleate embryo-sac in the absence of a large central vacuole. The nuclei are situated more or less in a row. If the successive nuclei from the chalazal to the micropylar end of the embryo-sac are numbered as 1, 2, 3 and 4, we find that the first one is situated close to the chalazal end, the fourth near the middle of the embryo-sac (never quite close to the micropylar end), and the rest two in between the first and the fourth. All are at first nearly equally spaced and of the same size.

The two spindles at the end of the II nuclear division in the embryo-sac do not disappear immediately after telophase, but they are on the contrary reinforced by secondary spindle fibres. A secondary spindle also develops between the second and third nuclei, so that all the four nuclei become connected with one another by spindles (Fig. 9), as already reported by the author in *Gagea fascicularis* (Joshi, 1940). The spindles between the sister nuclei become somewhat thickened about the middle, but even evanascent cell-plates are never organised. The dissolution of the spindles starts from the chalazal end, the spindle towards the micropylar pole being visible even after the other two have

disappeared (Fig. 10). As the spindles disappear, the nuclei undergo considerable increase in size. The nuclei near the centre enlarge much more than those towards the chalazal end of the embryo-sac. This unequal enlargement appears to be entirely due to the greater space available to the nuclei near the centre.



Figs. 4–12.—*Piper longum*. Various stages in the development of the embryo-sac from the megaspore-mother cell stage up to the first 4-nucleate stage showing the 1+3 arrangement of the nuclei. For further explanation see text. Magnification of all figures approximately 1000.

Another change observed in the embryo-sac after the second nuclear division and before the onset of the next division is in the position of the nuclei. After the spindle fibres have disappeared, the two central nuclei begin to move towards the chalazal pole. This results in the formation of an embryo-sac with one nucleus near the micropylar end and three nuclei lying close together at the opposite end (Figs. 11 and 12). The mechanics of this 1 plus 3 arrangement of the nuclei or the physical forces which bring about such an arrangement are little understood. In *Piper longum*, the spindles do not appear to play any rôle in this connection. The two chalazal spindles disappear before the third towards the micropylar side. Hence they cannot be considered to hold the three chalazal nuclei together, while the micropylar one moves apart. The spindle between the two micropylar nuclei also does not lengthen appreciably after the formation of the nuclei so as to push one of them towards the chalazal end. Both of these points are shown quite clearly by a comparison of Figs. 9 and 10. On the other hand, we observe about this time the development of small vacuoles between the two micropylar nuclei (Figs. 11 and 12). These vacuoles appear to gradually enlarge and ultimately give rise to the large central vacuole characteristic of the later stages of the embryo-sac. The development of these vacuoles about this time and in this particular position between the third and the fourth nuclei appears to be the chief force concerned in bringing about the 1 plus 3 arrangement of the nuclei. This pushes the two central nuclei towards the chalazal pole, and later the development of the large central vacuole pushes the solitary micropylar nucleus from its earlier position near the centre to the micropylar end of the embryo-sac.

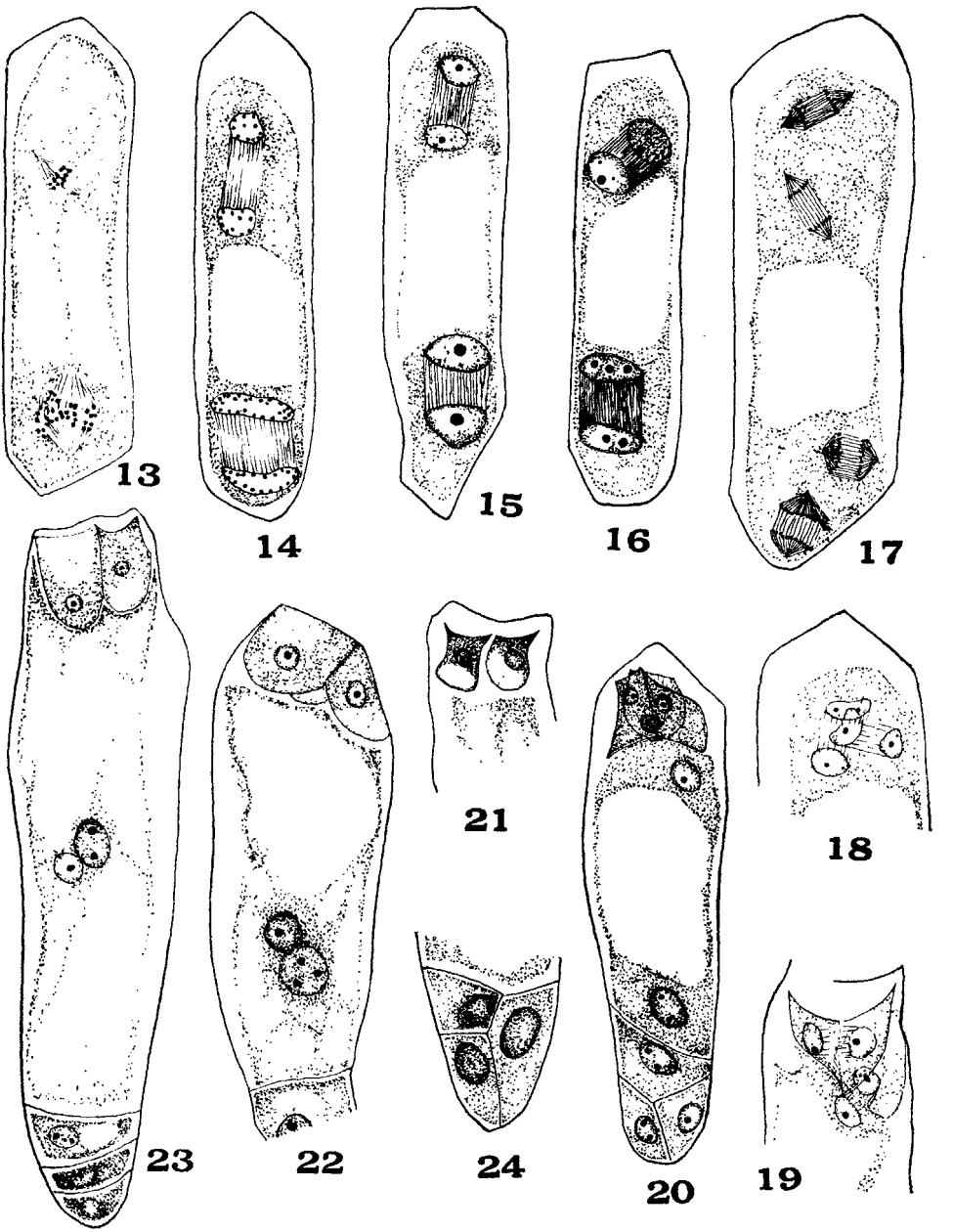
During the next division in the embryo-sac, the three chalazal nuclei fuse, and there is organised a large spindle at the chalazal end and a small spindle near the micropylar end (Fig. 13). The diploid number of chromosomes has been determined from dividing nucellus cells to be somewhere between 20 and 24. The exact number could not be determined due to the absence of suitably fixed material for this purpose and the extremely small size of the chromosomes. An attempt was also made to count the number of chromosomes on the large and the small spindles at this stage of the embryo-sac. This also was only partly successful, but it was ascertained that the larger chalazal spindle carries approximately three times as many chromosomes as the smaller micropylar spindle. This supports the above statement that the three chalazal nuclei fuse during this division. The result of the third nuclear division in the development of the embryo-sac is the establishment of a second 4-nucleate embryo-sac (Figs. 14–16). This differs from the earlier 4-nucleate stage in that the nuclei are found in pairs. The chalazal nuclei are larger and stain more deeply. They possess generally two nucleoli each, though sometimes there is only one (Fig. 15). Three nucleoli in a chalazal nucleus after this stage of the embryo-sac are comparatively rare (Fig. 16). This is different from what is seen in other plants possessing the same type of embryo-sac. The central vacuole of the embryo-sac becomes quite conspicuous during the third nuclear division (Figs. 13–16).

The nuclei of the second 4-nucleate embryo-sac undergo one more mitotic division (Fig. 17). The two spindles at each pole during this division are orientated mostly at right angles to each other. Further, the chalazal spindles are larger and carry more chromosomes than the micropylar. Cell walls are laid after this division and an 8-nucleate 7-celled embryo-sac is formed (Fig. 20), which differs from a normal 8-nucleate embryo-sac in that the four chalazal nuclei are somewhat larger and stain more deeply than the nuclei at the opposite pole. The chalazal nuclei also possess generally two nucleoli, though sometimes there are three or only one nucleolus. The study of the organisation of the egg apparatus clearly illustrated by Figs. 18 and 19 shows that the nuclei of the two synergids are derived from a common mother nucleus and same is the case with the egg and the upper polar nuclei. This contradicts the observations of Schürhoff (1919 and 1928), who regards one of the synergids as sister to the egg and the other as sister to the upper polar nucleus.

The Mature Embryo-sac.

The mature embryo-sac is a cylindrical or broadly spindle-shaped structure (Figs. 20, 22 and 23). The egg apparatus and the antipodals occupy a comparatively small part

of the embryo-sac. The central cell on the other hand is very large. The cells of the egg apparatus are rather poorly differentiated. The egg may or may not show a well marked vacuole at the micropylar end. Similarly the synergids may be non-vacuolate



Figs. 13–24.—*Piper longum*. Various stages in the development of the embryo-sac from the third nuclear division up to the mature embryo-sac. Figs. 18 and 19 show the differentiation of the egg apparatus. Fig. 21 shows only the synergids and 24 only the antipodals. In Fig. 22, the antipodals are excluded and Fig. 23 does not show one of the synergids. All other figures represent complete embryo-sacs. For further explanation see text. Magnification of all figures approximately 1000.

or show only a small vacuole in the chalazal part. The micropylar ends of the synergids facing the periphery of the embryo-sac are generally pointed (Fig. 21). The antipodal cells, which do not show any notable peculiarity, may be full of protoplasm or may show small vacuoles. They are generally arranged in a triangular fashion (Figs. 20 and 24), but sometimes are found in one row also (Fig. 23). They always remain uni-nucleate. The two polar nuclei differ in size and staining capacity. They meet either near the centre of the embryo-sac (Fig. 23) or close to the antipodals (Fig. 22), but do not fuse immediately. Perhaps they remain free till the time of fertilisation.

Discussion.

It is clear from the above description that the embryo-sac in *Piper longum* develops directly from the megaspore-mother cell and is of the tetrasporic type. Further, four nuclear divisions intervene between the megaspore-mother cell and the formation of the 8-nucleate embryo-sac. After the second division the nuclei of the embryo-sac show a 1 plus 3 arrangement. During the next division the three chalazal nuclei fuse, forming a large spindle at the chalazal end. The result of this division is the establishment of a second 4-nucleate stage characterised by larger triploid chalazal nuclei and smaller haploid micropylar nuclei. These characteristics demonstrate clearly that the development of the embryo-sac in *Piper longum* follows the *Fritillaria*-type.

The family Piperaceae includes 9 genera. We know nothing at present about the development of the embryo-sac or other embryological characters of *Zippelia*, *Macropiper*, *Chavia*, *Nematanthera*, *Verhuellia* and *Symbryon*. This is so on account of their restricted distribution and the difficulty of obtaining their material suitable for such investigations. Embryological studies in the family are confined to the remaining three genera,—*Peperomia*, *Piper* and *Heckeria*—, which are more widely distributed. All species of *Peperomia* have been found to possess a tetrasporic 16-nucleate type of embryo-sac, which is called after this genus as the *Peperomia*-type. Believing that the embryo-sacs of *Piper* and *Heckeria* developed according to what is now described as the *Adoxa*-type (then called the *Lilium*-type), Johnson (1905) emphasised a great difference between the embryo-sacs of the different genera of the Piperaceae. The demonstration of *Fritillaria*-type of development in *Piper* and *Heckeria*, however, shows that the embryo-sacs of these genera are not very dissimilar from those of *Peperomia* species, for both the *Fritillaria* and the *Peperomia* types of embryo-sacs are tetrasporic and in both cases four nuclear divisions intervene between the megaspore-mother cell and the mature embryo-sac. Consequently, the statement of Johnson (1905) based on his studies of the Piperales that "the structure and mode of development of the megaspores and the gametophyte of angiosperms is not a satisfactory index of genetic relationship" appears not quite correct.

Summary.

The study of the development and structure of the ovule and embryo-sac of *Piper longum* L. shows that the micropyle of the single basal orthotropous ovule is formed only by the inner integument. The nucellus is large and increases continuously in size by the meristematic activity of the cells at its base and to some extent also at the sides.

The primary archesporial cell cuts off a wall cell, which forms about 8 layers of parietal tissue. The nucleus of the megaspore-mother cell is situated generally close to the chalazal end. Megaspores are not formed. The embryo-sac develops directly from the megaspore-mother cell and is tetrasporic. Four nuclear divisions intervene between the megaspore-mother cell stage and the mature embryo-sac. After the second division, the four nuclei, which are arranged almost in a row, become connected with one another by secondary spindle fibres. As these spindles disappear, the two middle nuclei move towards the chalazal end, resulting in a 1+3 arrangement of the nuclei. Such an arrangement appears to be brought about chiefly by the development of small vacuoles, precursors of the large central vacuole of the later embryo-sac stages, in between the two nuclei nearest to the micropylar pole of the embryo-sac. During the third division, the three chalazal nuclei fuse. This

results in a second 4-nucleate embryo-sac, with large triploid chalazal and small haploid micropylar nuclei. The fourth division is normal. The development of the embryo-sac in *Piper longum* agrees with the *Fritillaria*-type. The two synergid nuclei during the differentiation of the egg apparatus are observed to descend from a common mother nucleus, and the same is the case with the egg and the upper polar nuclei.

The mature embryo-sac is nearly cylindrical. It shows no unusual features except for the differences in the size, staining capacity and internal constitution of the chalazal and the micropylar nuclei. The two polar nuclei meet near the centre of the embryo-sac or close to the antipodals.

The demonstration of *Fritillaria*-type of development in *Heckeria* and *Piper* shows that the embryo-sacs of the different genera of the Piperaceae are not very dissimilar from one another. The study of the Piperaceae does not support the statement of Johnson (1905) that the characters of the female gametophyte are not a satisfactory index of phylogenetic relationship.

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Fig. 1.—*Piper longum*. Transverse section of a female spike, showing flowers and ovules cut longitudinally.