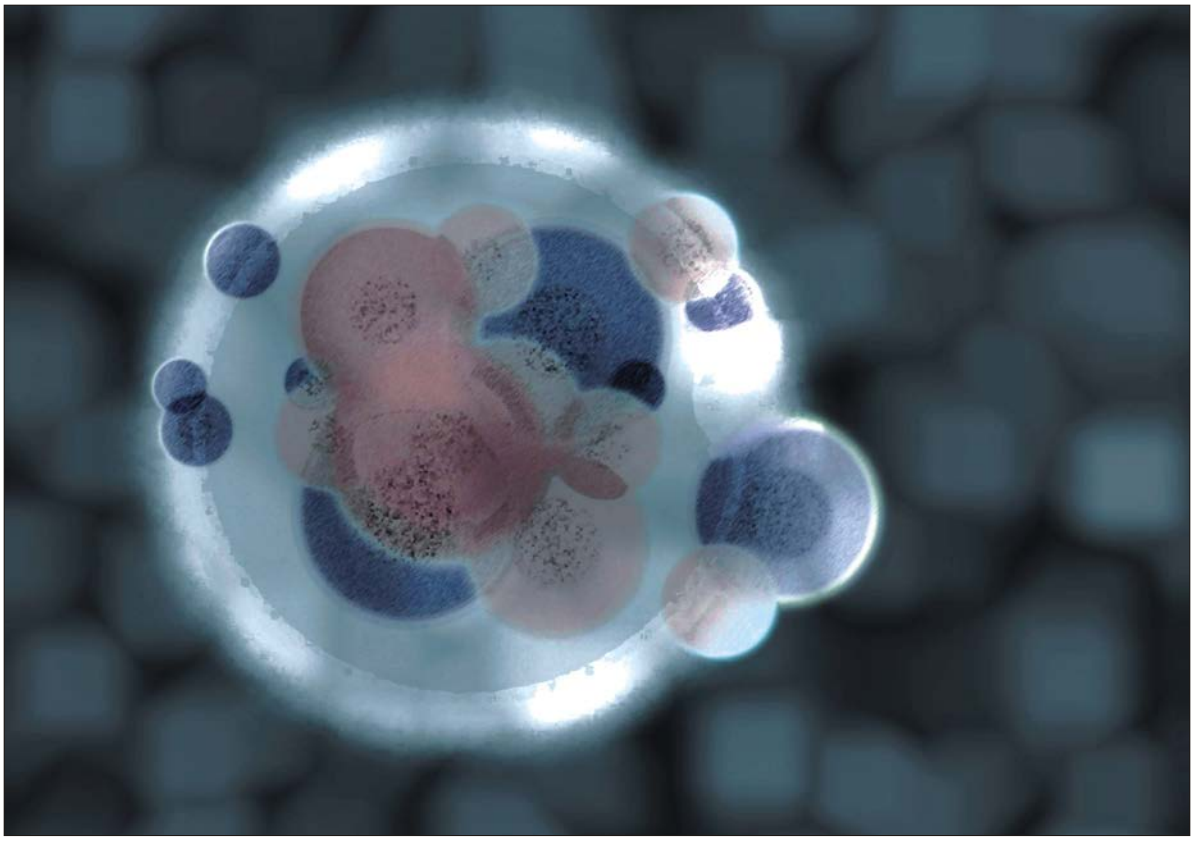

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# Female Gametophyte in Tristichoideae (Podostemaceae): Re-Investigation

[1]S. Sikolia and [2]P. Ochora
[1]Department of Botany and Horticulture, Maseno University, P.O. Box 333, Maseno, Kenya
[2]Department of Botany, Jomo Kenyatta University, P.O. Box 62000(00200), Nairobi, Kenya

**Abstract:** A trilocular ovary possesses several anatropous, tenuinucellate and bitegmic ovules on the axile placenta. Selective callose deposition in cell walls and effects of integumentary directional tension (or stress) forces during first meiotic division and natural competition between dyads exists. Micropylar dyad cell degenerates; shift in nutrient supply at megagametogenesis relates to the degeneration of the primary chalazal nucleus. Primary micropylar nucleus undergoes two free nuclear (mitotic) divisions producing four nuclei that alone organize a four-celled female gametophyte consisting of two synergids, a large central egg cell and a polar cell. Filiform apparatus are present in the synergids. Female gametophyte development is of the monosporic category, Apinagia type, form B; syngamy occurs. The cell walls of nucellar cells fails to resist acropetal net tension force of the inner integument, disorganize, break and release naked protoplast in the cavity formed resulting in a structure referred as nucellar plasmodium; role of lytic enzymes is pointed out. Nucellar plasmodium organizes during post-fertilization period in *Trisicha trifaria* of the Tristichoideae (Podostemaceae).

**Key words:** Tristichoideae, female gametophyte, apinagia type-form B, filiform apparatus, callose

## INTRODUCTION

Podostemaceae is a family of embryological, morphological and ecological unusual aquatic angiosperms. They occur on rocks in swift flowing streams, rapids and waterfalls in the tropics and subtropics. When the water-level falls during the dry season, the plants emerge above the water to flower and fruit. Thus, Podostemaceae and Hydrostachyaceae only survive this extreme variable seasonal aquatic condition amongst the angiosperms (Van Steenis, 1981).

The morphology of Podostemaceae is characterized by lack of the radicle at the base of the hypocotyls but has adventitious root on the lateral side (Suzuki *et al*., 2002). The roots are prostrate on and adhere to rocks, bearing sterile and fertile shoots on the lateral and apical axes. The root apical meristem and cap organization is least specialized in the Tristichoideae and Weddellinoideae (Koi *et al*., 2006). Podostemoideae has bifacial ventral layer that give rise to the root cap acroscopically. The shoot is no exception enigmatic organs, usually deviating from the norm course of shoot development pattern in the angiosperms (Koi and Kato, 2007). For example, there is no obvious shoot apical meristem in some species of Podostemoideae (Rutishauser, 1995, 2000; Rutishauser and Grubert, 1999), but occur in the Tristichoideae and Weddellinoideae subfamilies of Podostemaceae (Koi *et al*., 2006). The female gametophyte development is no different to this unique characteristic course of differentiation (Sikolia and Onyango, 2008).

The literature on the female gametophyte was reviewed by Battaglia (1971) and pointed out the need for re-investigation in several taxa of Podostemaceae. Arekal and Nagendran (1975a, b, 1976, 1977a, b), Nagendran and Arekal (1976), Nagendran *et al*. (1976), Nagendran *et al*. (1977) and Nagendran *et al*. (1980) studied the female gametophyte in many Indian taxa. Battaglia (1987) critically analyzed the observations and interpretations on the female gametophyte ontogeny. Battaglia (1987) categorically stated that all embryological data not assigned to the Apinagia type are doubtful cases and therefore require reinvestigation. Some embryological observations made for *Tristicha trifaria* by Jager-Zurn (1967) needs clarification. Out of 73 species in 16 genera recorded for the Africa continent (approx. 26% of the total taxa known), only seven species representing three genera, *Inversodicraea*, *Polypleurum* and *Tristicha* have been studied so far. On the account of the endemism, accessibility for collection and availability of flowers and fruits only during a limited span of time, several taxa have not been investigated. Cusset and Cusset (1988) based on the morphological, anatomical, embryological and physiological data proposed a new class Podostenopsida

**Corresponding Author:** S. Sikolia, Department of Botany and Horticulture, Maseno University, P.O. Box 333, Maseno, Kenya

beside the magnoliopsida and the Liliopsida. In view of the above, the female gametophyte ontogeny in *Tristicha trifaria* of the Podostemaceae was investigated.

## MATERIALS AND METHODS

The study was carried out between 2000-2004 duration. *Tristicha trifaria* (Bory ex wild) Sprengel was collected from the following habitats:

- Webuye falls - 7 km from Webuye Town, Bungoma district, Western Province, Kenya
- Fourteen Falls - 14 km from Thika Town, Murang'a district, Central Province Kenya

The plant material collected was fixed in Formalin-Acetic-Alcohol (FAA) and preserved in 70% ethanol. Fixed plant specimens were identified under a binocular stereomicroscope. Individual flower buds of all stages were dehydrated in the ethanol-xylol series and imbedded in paraffin wax 52°C melting point. Serial microtome sections were cut at 7-12 μm thicknesses. Sections were stained in Heidenhain's Iron alum-hematoxylin and counterstained in erythrosine or fast green in clove oil. DPX mountant was used to prepare permanent slides. Camera Lucida drawings of the female gametophyte stages were drawn at table level using a Leitz monocular microscope at different magnifications.

## RESULTS

The gynoecium is superior, syncarpous and tricarpellary. Ovary is trilocular. In a transverse section of a young ovary, several ovular primordial in each locule rise from a massive axile placenta as protuberances (Fig. 1a). In the developing ovular primordial, a central row of nucellar cells is observed (Fig. 1b). A hypodermal cell in the nucleus differentiates early and is visible as a densely cytoplasmic, large nucleated cell (Fig. 1c). This archesporial cell enlarges without further divisions and directly transforms as the megaspore mother cell (Fig. 1d). The megaspore mother cell undergoes the first meiotic division resulting in two nuclei followed by wall formation (Fig. 1e). This results in the formation of two dyad cells (Fig. 1f). The micropylar dyad cell degenerates regularly and is only recognized as a crescent-shaped cap of dark mass in later stages of the developing embryo sac (Fig. 1g-i, Fig. 2a-e). Meanwhile, the chalazal dyad cell enlarges and completes meiosis- II. The resulting two nuclei are separated by a distinct vacuole (Fig. 1g) of the two nuclei; the chalazal one degenerates without further

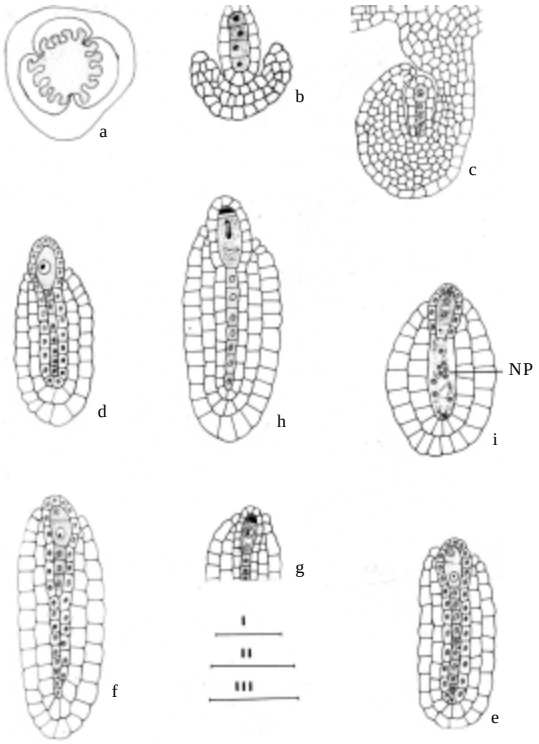


Fig. 1: Early female gametophyte of *Tristicha trifaria*, Bar I for (a) = 350 μm; Bar II for (b, c, e, f, g and i) = 120 μm and Bar III for (d and h) = 70 μm)

division (Fig. 1h) and in subsequent stages assumes a picnotic appearance. The other nucleus located at the micropylar end divides mitotically in a plane parallel to the long axis of the embryo sac (Fig. 1e). The resultant two nuclei move a part (Fig. 1i). Both these nuclei again divide mitotically and often synchronously (Fig. 2a) to produce four nuclei in the embryo sac (Fig. 2b) distributed cross-wise. At this stage, the remains of the degenerated chalazal megaspore nucleus are reduced to crescent-like structure. Now the embryo sac undergoes cellular organization (Fig. 2c) and results in a four-celled embryo sac (Fig. 2d). Thus, the micropylar quartet of nuclei alone contributes to the organization of the mature embryo sac that consists of two juxtaposed synergids, an egg cell and micropylar polar cell. In the present study, filiform apparatus (SY) have been observed in both the synergids (Fig. 2e). The development of the female gametophyte in this taxon conforms to the Apinagia type, form B of Battaglia (1971). Antipodal cells have not been observed.

Fertilization is porogamous and the Pollen Tube (PT) enters the embryo sac overlapping one of the synergids (Fig. 2e). In the protoplasmic mass of the pollen tube and degenerating synergid, two degenerating

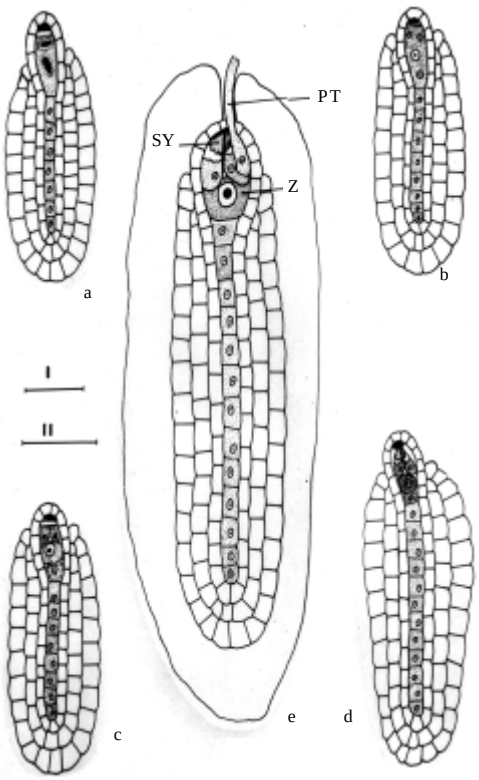


Fig. 2: Late female gametophyte of *T. trifaria*. SY: Filiform apparatus, PT: Pollen tube, Z: Zygote), Bar I for (a, b, c and d) = 25 μm; Bar II for (e) = 50 μm

nuclei were observed. One of them could be the undischarged second male gamete and the other one, the synergid nucleus. Fusion of the egg cell and a male gamete results in the formation of zygote (Z) (Fig. 2e). The zygote is enlarged and extended towards the chalazal part and sideways in the central region of the embryo sac. No fusion of the polar cell and second male gamete was observed. There is single fertilization.

During the ontogeny of the ovule, the nucellar cells at the base of the megaspore mother cell become prominent and elongate in the longitudinal direction (Fig. 1d-e). These cells are bound only by the inner integuments which continuously enclose two-third of the female gametophyte portion in a chalazal-micropylar axis. The cell walls of nucellar cells remain intact until the period fertilization occurs (Fig. 2e) when the zygote begins to divide, these nucellar cells have been observed to lose their walls and organize a structure commonly referred as pseudo-embryo sac.

## DISCUSSION

The embryological studies of the female gametophyte depict a classical botanical problem of differentiation during the ontogeny in the family. A trilocular ovary possesses numerous tenuinucellate, anatropous and bitegmic ovules on a swollen axile placenta. Similar observations are reported (Arekal and Nagendran, 1977b; Sikolia and Onyango, 2008) and fail to confirm previous contention of the ovary being unilocular with free central placentation (Rendle, 1925; Ramamurthy and Joseph, 1964) in the family.

An ovular primodium consists of four cells formed in axial row. Of these, a hypodermal cell differentiates early in the ovule, which bends in the form of a hook causing its distal components to move close to the placenta. At this time, the integuments are initiated at the base of the ovule and their growth accompanies the maturation of the sporogenous cell into the megasporocyte. The differentiation of the integuments is simultaneous, but the rate of growth of the outer one surpasses the inner, which lags behind. Meanwhile, a densely cytoplasmic, large nucleated hypodermal archesporial cell enlarges and directly transforms into a megaspore mother cell. It is at this stage that the outer integument alone organizes the micropyle. The micropylar end-section of the regular ovule is enclosed upto its two-third portion in a chalazal-micropylar axis. This confirms the reports of Mukkada (1969) in the investigated, *Dalzellia zeylanica*. Thus, the origin and maintenance of the nucellus involves a specific act of single cell differentiation. This single archesporial cell is placed in the genetically homogenous protoplasmic mass of the growing nucellus. The ultimate results of this event are immediately seen in the fully developed female gametophyte, as it will be revealed soon.

The megaspore mother cell undergoes the first meiotic division to form two unequal dyad cells, within the nucellar tissue. This is associated with proliferation of the nucellar cells that forms an enlarged densely protoplasmic layer. Then, the tightly enclosed meristematic region is likely subjected to physical (or mechanical) stress arising from the nucellar layer during the differentiation process. One of the two non-isodiametric cells, the smaller micropylar one degenerates regularly without further division and is seen as an inverted cap of dark mass in the upper region of the forming megagametophyte. Similar observations have been recorded in diverse taxa studied in the family (Arekal *et al*., 1975a, b, 1977b; Nagendran *et al*., 1976, 1977, 1980). But the present observation fails to confirm the reported occasional atypical division (meiosis- II) of the micropylar dyad cell or its persistence without further division suggested

by Battaglia (1987). Further, there is no functional significance of these meiotic products of the upper dyad cell in terms of its participation in the cellularization process of the female gametophyte. It is therefore proposed that they represent degenerating meiotic products that constitute deviation from the norm pathway of the ontogeny in the family as shown in the *Zeylanidium olivaceum* investigated (Arekal and Nagendran, 1977a). Furthermore, it depicts non-conformity course of the established ontogeny of monosporic category where the upper dyad cell divides (meiosis-II) to produce two mononucleate cells instead of a binucleate state in the bisporic type; a case unlikely in *Z. olivaceum* (Arekal and Nagendran, 1977a) and a fact emphasized by Battaglia (1971). Probably, there are variations of the degeneration phase of the upper dyad cell and be confirmed in *T. trifaria* but seen later as a dark mass in the family. Furthermore, it is without functional rationale during ontogeny in the present study. The degeneration of this cell may be due to callose deposition in the walls of the upper cell as seen in *Oenothera* sp. (Haig, 1986) and therefore nutritionally isolated from the maternal food supply, or natural competition ensues amongst the dyad cells, until one degenerates within the ovule (Noher de Halac, 1980).

From a mechanical effect point of view, Lintilhac (1974) and Lintilhac and Jensen (1974) explain that the initial differentiation of the megaspore mother cell within the free-standing nucellar primodium appears to be dependent on its maintenance of a stressed equilibrium structure and the production of a predictably located region of null stress. This condition is considerably modified by the mechanical enclosure of the inner integuments over the micropylar tip of the nucellus. The compressive stress produced by the axial meristem and the growth of the nucellus is controlled and directed within the nucellus by the pressure of integuments. Thus, the localized region of pure tension which can be used for morphogenetic advantage is maintained without these or any change in their organization and the distribution of stress within the nucellus is altered. This results into the destruction of regular concentricity of comprehension lines through the cell division area and the stability of the chalazal female gametophyte is destroyed. The micropylar female gametophyte is spared the physical stress and strain damage and enters second meiosis division. Lintilhac and Jensen (1974) points out the orientation of the cell walls is directly dependent at some stage to new cell wall precursor (pre-prophase' microtubules cell plate) which is sensitive to mechanical stress or its effects. For instance, they may be shear-sensitive elements capable of processing into constant and predictable orientations with respect to externally applied stress. After meiosis I, the differentiation of integuments is complete and only the outer one form the micropyle. Then, the micropylar region of the forming female gametophyte is impaired in terms of corrective and regular organization of the stresses. Thus, the normal cell-plate formation followed by cell division of the upper dyad cell cannot take place properly. May be this explain the unexpected binucleate states which is not complete in terms of cell wall formation. However, the chalazal dyad cell within the whole field-effects of the integuments in the provision of normal stress patterns undergoes the second meiotic division without impairment. In this respect, the theory of megaspore conflict of Haig (1986) suggests that, the somatic spores of their derivatives having a role in successful gametophyte functions should have a tendency to become non-functional.

The nucleus of the chalazal dyad cell undergoes the second meiotic division and two megaspore nuclei are produced without cell wall formation between them. This confirms the observation made in the taxa of *Polypleurum* (Mukkada, 1964; Nagendran *et al*., 1977) contrary to that in *P. elongatum* (Magnus, 1913) and *Griffithella hookeriana* (Razi, 1949). Of the two nuclei separated by a distinct vacuole at the primary two-nucleate stage, only the micropylar one undergoes the first and second nuclear mitotic divisions in a successive manner, producing four nuclei. Consequently, the micropylar quartet of nuclei does only organize into two synergids, an egg cell and a polar cell. The latter cell degenerates. Unlike in the present study, if the opposite mode of ontogeny happened, then the chalazal quartet of nuclei derived from the primary chalazal nucleus could organize three antipodal cells and the lower polar cell. On the contrary there is gradual degeneration of the primary chalazal nucleus, which completely disappears, in the female gametophyte. Battaglia (1971, 1987) points out, this may be because the chalazal region of the developing embryo sac is physiologically depressive or hypofunctional. For instance, the nucleus decrease in size as a reflection of reduction in physiological activities and its division must be a quite an uncommon event (Battaglia, 1971, 1987). The nuclear reduction in size is only an indication of degeneration. This can happen because they can be a shift in nutrient supply to the developing megagametophyte as seen in *Spinacia* sp. (Wilms, 1980). The chalazal component would be disadvantaged in placement towards the direction of material nutrient supply compared to the micropylar one. Due to a precocious degeneration and disappearance of the chalazal megaspore nucleus, the female gametophyte never attains a five-nucleate stage. This occurs before

cellularization of the female gametophyte is completed in *T. trifaria* contrary to the reports of its persistence (Jäger-Zürn, 1967). Therefore the reinterpretation of the development and organization of the female gametophytes in *T. trifaria* by Battaglia (1971) as the Apinagia type, form A does not arise. Too, the antipodal compliment is eliminated by the failure of the chalazal megaspore nucleus to undergo all the post-meiotic mitoses. This 'strike' phenomenon has been reported in all the investigated taxa of Podostemaceae (Nagendran *et al*., 1977) and *Epipogium roseum* (Arekal and Karanth, 1981). Similar process takes place partially or wholly in the monosporic, bisporic as well as tetrasporic female gametophyte, through the family, Orchidaceae (Abe, 1972). Then, how can the antipodal cell(s) form, if its predecessor, the primary chalazal nucleus completely disappears without further division during the female gametophyte ontogeny?

The concept that, since the days of Hofmeister and Strasburger--- the antipodal cell (Strasburger's Antipoden der Gegenfusslerinen) is a cell situated at the chalazal end of the mature ES, usually I- nucleate, regularly degenerating during fertilization or, rarely, showing mitotic activity (phenomenon of Polyantipody), (Battaglia, 1987); could be admissible only if it is a derivative of the primary chalazal nucleus during the female gametophyte ontogeny. It follows that, the status of any component must reveal its origin at megagametogenesis and functional consequences attained before its morphological location can be taken into consideration. The present study refutes the view of antipodal cell re-evaluation in the family (Kapil and Bhatnagar, 1978) and consequently accepts the contention of its absence (Nagendran *et al*., 1980). Therefore, the view by different embryologists who have investigated the female gametophyte of Podostemaceae that the cell in the chalazal region of the embryo sac is a pro endospermic cell with one polar cell contrary to Battaglia's (1971, 1987) as the antipodal cell is acceptable in the present study. Therefore the organized female gametophyte consists of two synergids at the micropylar region, a large central egg cell and the upper polar cell. The polar cell degenerates. The synergids are justified because the present investigation confirmed it possess the filiform apparatus and the middle cell being the egg cell, in the embryo sac. The ensuing mode of ontogeny in *T. trifaria* conforms to the monosporic category described by Maheshwari (1937) and Nagendran (1974). This is because in the present investigation, only one megaspore nucleus at the prime stage of the megagametophyte undergoes the first and second nuclear mitotic divisions resulting all the four nuclei that alone take part in the cellularization and are present in an organized female gametophyte. The development and organization of the female gametophyte, which is a four-nucleate and four-celled unit conforms to the Apinagia type, form B of Battaglia (1971). Furthermore, Battaglia (1987) withdrew forms C and D of the Apinagia type was based on the observations recorded by Arekal and Nagendran (1977b). More studies are deemed necessary, for the remaining taxa of Podostemaceae, to establish the existence and validity of the Apinagia type, form A that is considered doubtful, in the present investigation.

Fertilization is porogamous. The pollen tube enters the female gametophyte destroying one of the synergids on its pathway, contrary to Razi's (1955) view that it takes place between the two synergids. The remaining synergid degenerates during embryogenic stages. The mode of pollen tube pathway observed in *Dalzellia zeylanica* (Chopra and Mukkada, 1966), *Indotristicha ramosissima* (Mukkada, 1969), *Mourera fluviatilis* (Went, 1908) is confirmed in the present study. The nature of the pollen tube contents including the undischarged second male gamete in the female gametophyte till fertilization stage as recorded earlier (Went, 1908; Chopra and Mukkada, 1966; Mukkada, 1969) does occur. The degeneration of the polar cell commences before the pollen tube penetrates the female gametophyte. Similar observations have been recorded in other taxa of Podostemaceae (Arekal and Nagendran, 1975b, 1977a, b; Nagendran *et al*., 1980). The chances of the polar cell being fertilized by second female gamete does not arise, because none of the present preparations depicted the fusion between them contrary to the reports by Razi (1955). This is reflected by the failure of the primary endosperm nucleus formation in the ovule, which is also reported in the investigated taxa of the family (Mukkada, 1969; Arekal and Nagendran, 1975b; Nagendran *et al*., 1976). The function of the endosperm is taken over by the nucellar plasmodium in Podostemaceae. The zygote formed increase in size, both towards the chalazal region of the female gametophyte as well as sideways. Various investigators (Went, 1908; Walia, 1965; Chopra and Mukkada, 1966; Mukkada, 1969; Arekal and Nagendran, 1975b, 1977a, b; Nagendran *et al*., 1976) have recorded similar observations. Therefore, only single fertilization takes place in the family of Podostemaceae.

The pseudo-embryo sac ontogenetically corresponds to the nucellar cells situated at the base of the gametophyte. Initially, the smaller nucellar cells elongate and depict a densely protoplasmic mass; enclosed by the integument layer. The latter is associated with rapid cell division in contrast to the cells of the nucellar tissue, acropetally. This results in the differential growth rates between the layers. Although the outer integumentary

layer shows faster growth rate than the former two layers, its effects are minimal and form an enclosure system. The differential growth rates between the nucellar and inner integumentary layers progressively intensify while the cell walls of the former become thin, weak and take very slight stain. Thus opposing tensions are created between the layers. The net tension of the thicker inner integumentary walls leads to the thin walls of the nucellar layer to disorganize by stretching and shearing stresses followed by disruption. The ensuing hyaline cell walls may be summarily broken down due to the action of lytic enzymes.

Went (1908) pointed out that the development of the pseudo-embryo sac by the stretching and dissolution of the cell walls of the nucellar layer, indicates in many cases that the developing female gametophyte exercise a solvent action on the surrounding tissue of the nucellus, which can take place on the nucellar cells towards the chalazal region. Davis (1961) suggested that there is digestion of the middle lamella (or hyaline layer) of the adjoining nucellar epidermis by lytic enzymes liberated from the tip of the functional megaspore in *Podolepis jaceoides*. Further, similar contentions are confirmed through histochemical studies carried out in the diverse taxa of the angiosperms (Poddubnaya-Arnold and Zinger, 1961). The disappearances of hyaline layer after wall disruption in the nucellar cells provide ample evidence to this effect of the lytic activity in *T. trifaria*. Thus, the physical factor as a result of tension set up by different growth rates of the nucellar layer and inner integument, also suggested by Hammond (1937) and the chemical factor (=lysis of hyaline walls of the nucellar cells by enzymatic action) could be part of the whole process responsible for the disintegration of walls of the nucellar cells. This process commences at the chalazal part of the ovule and proceeds basipetal.

The wall disintegrating of the nucellar cells results in vacuolated cytoplasmic mass containing large and health nuclei contrary to Went (1908) and Engler (1930) views of the nuclei in degenerated or fragmented phase, respectively. The present observation confirm the report of Jäger-Zürn (1967), Arekal and Nagendran (1975a, b) and Nagendran *et al.* (1976, 1980), regarding the nature of the nuclei in the forming cavity. In the process of zygote formation, the naked protoplasts commences to fuse one by one, often clumping at a point and in longisection of the ovule, their number often less than eight. Later, the long cavity consisting of multinucleate protoplasts is formed. The resultant structure organized never appears like an embryo sac, either in its ontogeny or organization and appearance. By the time it is fully organized, the multinucleate protoplasts do not depict their walls. Precision lacks in referring in the cavity as pseudo-embryo sac. This is because it is organized by the fusion of individual nucleate protoplast as a consequence of the disintegration of cell walls separating them in the nucellus. Thus, the term Nucellar Plasmodium of Arekal and Nagendran (1975b) is more acceptable. Subsequently the embryo grows into it and completes its development. It is now an established fact that the organization of the nucellar plasmodium in the investigated genera of Podostemoideae (Arekal and Nagendran, 1976, 1977b) takes place before fertilization while it is a post-fertilization phenomenon in Tristichoideae (Arekal and Nagendran, 1977b) as recorded in the present investigation.

Embryologists who have studied the family of Podostemaceae have unreservedly pointed out the significance of the nucellar plasmodium as a source of nourishment for the growth of the gametophyte. This is valid because the inner integumentary cells in closer proximity to the developing proembryo stain negative for starch in contrast to the cells of the outer integumentary. This has been reported in Podostemaceae (Razi, 1955; Mukkada, 1969), diverse taxa of *Rhododendron* (Palser *et al.*, 1992) and other angiospermous taxa (Palser *et al.*, 1992; Jensen, 1965). There is provision to receive the growing embryo, which subsequently occupies the entire space of the nucellar plasmodium. Because of the limited size of the female gametophyte to provide enough space to the developing embryo, its area with that of nucellar plasmodium suffices.

During the embryogenic stages, the liquid medium of the nucellar plasmodium becomes useful, because the plants are suddenly exposed as water level in the streams subsides. An adaptation which has enabled a successful mode of life in all the members of Podostemaceae in aquatic ecosystem. In this connection, Arber (1920) calls the nucellar plasmodium (pseudo-embryo sac) as an ideal water reservoir. It follows that in the absence of the endosperm, an alternative, the nucellar plasmodium serve to conserve food materials, drawn by the developing embryo, suspended in its fluid of the cytoplasmic mass. This unit, also maintain the internal maternal ovular environment from collapsing through the tension effect of its fluid mass to counteract the inward pressure from the surrounding sporophytic tissues and other external sources where the plant grows. Probably, it furnishes certain morphogenetic substances necessary for differentiation of the developing embryo (Mukkada, 1969). These may include enzymes, growth hormones and osmoregulatory fluids. The supply of these components and their subsequent roles as they affect the developing embryo needs detailed investigations.

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