SHORT COMMUNICATION

Modified Ovule Clearing Technique to Examine Monosporic Apinagia-type Embryo sac Development in *Indotristicha ramosissima* (Podostemaceae- Tristichoideae)

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Abstract

In this current study, a re-examination of various stages of monosporic embryo sac development in *Indotristicha ramosissima* (Podostemaceae-Tristichoideae) has been attempted utilizing a modified ovule-clearing technique specifically tailored for this unique angiosperm family. It is worth noting that ovules within the family Podostemaceae typically contain silica, which poses challenges for tissue clearing. Consequently, there has been limited success in ovule clearing within this family. My investigation confirmed the presence of a 4-nucleate and monosporic Apinagia type of embryo sac in this species, in contrast to prior beliefs of embryo sac following a bisporic *Allium* type pattern. Additionally, my findings revealed that the embryo sac develops only after the degeneration of one of the dyads during megasporogenesis.

Keywords: Haptophytes, Podostemaceae, Tristichoideae, Female Gametophyte, Monosporic Embryo sac

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Introduction

The family Podostemaceae comprises a significant group of aquatic dicotyledons, encompassing 54 genera and around 300 species (Koi *et al*., 2012; Cheek *et al*., 2017; Krishnan *et al*., 2019b). These plants are predominantly found in tropical and subtropical regions around the world. They thrive in fast-flowing waters of rivers and streams, firmly anchoring themselves to rocks and stones through rhizoids and haptera. Of the 28 species of Podostemaceae present in India, 23 are endemic (Khanduri *et al*., 2015).

Despite their remarkable diversity, our understanding of the reproductive characteristics of this family is limited, with only 29 species having been investigated to date (Philbrick and Novelo, 1997; Khosla *et al*., 2000a, 2001b; Hiroshi and Masahiro, 2002; Gupta and Sehgal, 2009; Sehgal *et al*., 2009; Sobral-Leite *et al*., 2011; Khanduri *et al*., 2014; Krishnan *et al*., 2019a; Silva-Batista *et al*., 2020). Furthermore, detailed embryological information is scarce for this unique botanical family. Some of the distinctive embryological features observed in most members of this family include the formation of a nucellar plasmodium (pseudo-embryo sac), the absence of antipodal cells, triple fusion, and the subsequent development of endosperm. (Arekal and Nagendran, 1975; Ram and Sehgal, 1992; Jäger-Zürn, 1997).

The present study reports a re-investigation of the development of embryo sac (ES) in a podostemad, *I. ramosissima* (sub-family: Tristichoideae) using an ovule clearing technique (Siddiqi *et al.,* 2000) which was modified to suit the family. The ovules in Podostemaceae are bitegmic in nature and exhibit silica deposition in their cells. The presence of silica in the cells is a major hindrance in the tissue-clearing process leading to various artifacts under a differential Interference Contrast (DIC) microscope. Thus, most of the embryological studies in the family are based on sections obtained using microtomy which is a cumbersome and time-consuming process. In the present study, the details of the development of an ES are elucidated based on the ovule-clearing technique described in detail by Siddiqi and others (2000) and its further modification to study the different developmental stages of female gametophyte in the family Podostemaceae.

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Material And Methods

I.ramosissima plants typically initiate flowering during the month of September while being submerged, coinciding with the period of swollen rivers. For this particular study, plant specimens were gathered from the Netravati River, located in Belthangadi, Karnataka, India, in December 2002.

To study the details of the ES development in *I. ramosissima* flower buds (n = 50) of different developmental stages were fixed in FAA (3.7% formalin, 5% acetic acid, 50% ethanol) overnight at 4°C, rinsed and stored in 70% ethanol. Ovules were dissected and dehydrated in graded acetone series (10–100%) and were subsequently kept in Methyl salicylate for two hours followed by overnight (10 hours) storage in Spurr's resin (7:1). The cleared ovules were carefully mounted on a glass slide and observed under Zeiss Axioscope microscope equipped with differential interference contrast optics. In the original protocol for clearing (Siddiqi *et al.,* 2000), the pistils stored in FAA were

washed in 50% acetone followed by dehydration in acetone series, and subsequently cleared in methyl benzoate. In the present investigation, the protocol was modified to suit the podostemads, wherein the pistils were rinsed in 70% ethanol and further cleared and embedded using methyl salicylate instead of methyl benzoate which was used for embedding in the original protocol. The current protocol envisages augmenting the studies of various reproductive structures in a quick and efficient manner.

To elucidate the ontogeny of female gametophyte by microtomy, flowers (n = 50) of different developmental stages were also fixed in Karnowsky's fixative which were further dehydrated in graded series of ethanol and were later infiltrated and embedded with Glycol methacrylate monomer mixture (Feder and O'Brien, 1968). In 1 to 2 µm thick sections were obtained using rotary microtomy and sections were stained with Toluidine Blue 'O' and observed under an optical photomicroscope.

Results

The gynoecium in *I. ramosissima* is tricarpellary and syncarpous, comprising of numerous tenuinucellate ovules. The development of the ES begins with the differentiation of the uppermost cell of the nucellus just below the nucellar epidermis as an archesporial cell in the ovule (Fig. 1A). This cell undergoes enlargement and accumulates a high cytoplasmic density, featuring a prominent nucleus. Subsequently, it assumes the role of the megaspore mother cell (MMC) directly (Fig. 1B). At this stage, the ovules lack integuments, and the nucellus along with MMC remains naked and projected. After the differentiation of MMC; the cells below the nucellus give rise to the integuments of the ovule. The development of the outer integument starts from the base of the archesporial cell.

At the onset of megasporogeneis, the micropylar end of the MMC gets occupied by its large nucleus, while the chalazal end is characterized by the presence of a large vacuole (Fig. 1B). Intriguingly, the nuclear division of meiosis I in the MMC occurs at the 90° right angles to the longitudinal axis of the ovule (Fig. 1C). Immediately after Meiosis I, a transverse wall is laid between newly formed nuclei leading to the formation of a dyad which is unequal in size with the smaller upper cell (towards micropyle) and larger lower cell (at chalazal end side). Subsequently, the smaller upper cell of the dyad undergoes degeneration, forming a dark crescent-shaped cap over the chalazal cell of the dyad in subsequent stages (as illustrated in Fig. 1D). At this juncture, the nucellus cells undergo disorganization, giving rise to a nucellar *Plasmodium*, a distinctive trait within the Podostemaceae family. Simultaneously, the outer integument experiences rapid growth towards the upper part of the nucellus, leaving the inner integument behind, which exhibits slower growth. The outer integument is notably robust, consisting of three layers, and it exclusively forms the micropyle. In contrast, the inner integument typically shows limited development, characterized by the presence of only two to three cell layers (Fig. 1D).

Further development of the ES is taken up by the chalazal cell of the dyad which undergoes the second meiotic division (Meiosis II) leading to the formation of two megaspore nuclei. The two megaspore nuclei occupy the two ends of the sac being

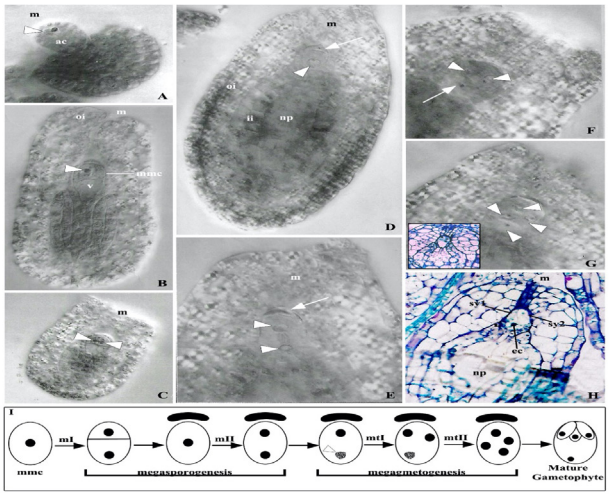

Fig. 1. Different stages of Monosporic Apinagia type of ES development in I*ndotristicha ramosissima*. A: An archesporial cell (ac) with a prominent nucleus (arrowhead). B. With the development of the outer integument (oi), a prominent micropyle (m) can be seen. An enlarged MMC exhibiting a prominent nucleus (arrowhead), dense cytoplasm, and a chalazal vacuole (v). C. Two nuclei after meiosis I (arrowheads). D. Dyad formed after meiosis I with the upper dyad cell being smaller than the lower cell. The arrow points to the crescent-shaped degenerating upper cell of the dyad. E. Lower cell of dyad undergoing meiosis. Two well-formed nuclei at telophase II can be seen arranged vertically (arrowheads). The arrow points to the degenerated upper cell of the dyad. F. The nucleus towards the micropyle undergoes mitosis I to form two nuclei (arrowheads). The degenerating nucleus of the chalazal end can be seen as a pycnotic body (arrow). G. Four nuclei (arrowheads) resulting after the second round of mitotic division (mitosis II); Inset: the egg apparatus as seen in the section of the ovule. H. A mature and organized ES with two synergids (sy1 and sy2), an egg cell (ec), and a nucellar plasmodium (np) as seen in thin sections. Micropyle (m) is also prominent. I. Schematic presentation of mega sporogenesis and mega gametogenesis leading to the formation of 4-nucleate Monosporic Apinagia type of ES (mI-meiosis I, mII- Meiosis II, mit I-mitosis I and mit II- mitosis II).

placed at each pole of the spindle (Fig. 1E). The megaspore nucleus which is present at the chalazal end soon decreases in size and eventually degenerates which can be later seen as a pycnotic body (Fig. 1F). The upper megaspore nucleus undergoes two successive mitotic divisions and gives rise to four nuclei (Fig. 1G). This quartet of nuclei gets organized into a 4-nucleate mature ES comprising of an egg cell, flanked by two synergids, and a polar nucleus in the central cell (Fig. 1H). The nucleus of the central cell degenerates soon. Here, it could be ascertained that only the upper megaspore nucleus (i.e., towards micropyle) is instrumental in the formation of the embryo sac in the species, thus the ES in the species corresponds to the monosporic Apinagia type of ES. The disintegration of the nucellus into a plasmodium, a key feature of the family is also evident prior to fertilization.

Discussion

Though all the ovules in the family are anatropous, tenuinucellate, and bitegmic, there is a lot of variation observed in the development and organization of ES in different species of

the family. While Battaglia (1971) has elucidated Apinagia, Dicraea and Podostemum types of embryo sacs in the family, other scientists (Arekal and Nagendran, 1977; Nagendran *et al.,* 1977) have reported Apinagia, Podostemum, Polypleurum and Willisia type of embryo sac development has been recorded in the family. Although the development of ES in *I. ramosissima* has been worked out by several researchers, there still remains confusion about it being a bisporic Apinagia type. Like in several members of the sub-family Podostemoideae, the gynoecium in *I. ramosissima* is also tricarpellary, syncarpous, and trilocular, possessing a superior ovary with axile placentation as compared to bicarpellate, syncarpous, and bilocular ovary observed in other podostemads.

The archespore in *Weddellina squamulose* shares a hypodermal position, much akin to the arrangement seen in *Indotristicha*. In both cases, the uppermost cell of the inner nucellar row assumes the role of the megaspore mother cell, as documented by Jäger-Zürn in 1997. While Arekal and Nagendran (1977) reported simultaneous development of both integuments in *I. ramosissima*, the present study observed a sequential development, with the outer integument leading the way, followed by the inner integument. As is characteristic of other Podostemaceae members, the inner integument extends only to the base of the chalazal side of the embryo sac (ES), leaving the ES partially exposed. In contrast, the outer integument entirely encloses the ES, forming the micropyle. The current study reports the disintegration of the chalazal nucleus after Meiosis II (arrowhead), leaving the micropylar nucleus alone to take over the formation of the embryo sac. In *Zeylanidium lichenoides*, too, the development is similar but the degeneration of the central cell and eventually the polar nucleus within leads to the formation of 3-celled and 3-nucleate structure (Chaudhary *et al.,* 2014). Though a similar type of embryogeny is also observed in *Marathrum schiedeanum*, the central cell undergoes a programmed death before maturity leading to the formation of an ES which is reduced to a 3-nucleated, 3-celled state, consisting of two synergids and an egg cell (Jiménez-Durán *et al.,* 2021).

Nagendran (1974) has reported a bisporic *Allium* type of ES in *I. ramosissima*. Later, an Apinagia type of ES development (form B of Battaglia, 1971) was reported by Arekal and Nagendran (1977). A four-celled monosporic embryo sac of the Apinagia type similar to *Indotristicha* has also been reported in *Vanroyenella* (Murguía-Sánchez *et al.,* 2002). The various stages of ES development as observed by the author following the clearing of ovules in methyl salicylate, outlined in the current paper reaffirms a monosporic Apinagia type of ES development in *I. ramosissima*.

Conclusion

Re-examination of different stages of embryo sac development in *Indotristicha ramosissima* (Podostemaceae-Tristichoideae) utilizing a modified ovule-clearing technique specifically designed for this unique angiosperm family has led the investigator to conclude that the embryo sac here is a 4-nucleate and monosporic Apinagia type of embryo sac, and is not a bisporic *Allium* type.

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Author Contribution

Charu Khosla Gupta conceived and wrote the perspective article.

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Data Availability Statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts Of Interest

The author declares no conflict of interest.

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