

# Male and female gametophyte development in *Achillea tenuifolia* (Asteraceae)

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## ABSTRACT

The anther, pollen and ovule development in *Achillea tenuifolia* were studied with a bright field microscopy. Results showed that the anther is of tetrasporangiate type and the anther wall is composed by four layers: an epidermis, an endothecium, one middle layer and a tapetum layer. Tapetum is of secretory type and its cells showed polyploidy. Pollen tetrads were tetrahedral, microspores were very irregular and contained large amounts of starch at the time of dispersion. Pollen grains were generally tricolporate, and in some cases were tetracolporate or even pentacolporate with spines on surface. The size of the pollen grains varied and ranging from 18-42.5  $\mu\text{m}$  at the polar axis and from 16.5-35.5  $\mu\text{m}$  at the equatorial axis. Ovule is anatropous, unitegmic and tenuicellate. The archesporium may consist of one or more archeosporial cells, but only one of them undergoes meiosis, forming a linear or T-shaped tetrad. A 7-celled embryo sac is formed corresponding to the *Polygonum* type. Embryo sac is very tiny at the beginning of its development, its size was increased considerably at late growth stages. The relationship between Asteraceae, Calyceraceae and Goodeniaceae are discussed but based on embryological evidence, Goodeniaceae appear to be the putative sister group of Asteraceae. To understand more exact relationships within the order Asterales, embryological studies are recommended.

**Keywords:** Pollen grain; Ovule development; Developmental biology; Ontogeny; Asterales.

## Introduction

The study of the reproductive biology of flowering plants is considered to have had a shorter history dating back to the early nineteenth century, beginning with the discovery of the pollen tube (1). However, sustained investigations on this topic began with the discovery of the actual fusion of the male and female gametes during fertilization in *Monotropa hypopitys* L. (2, 3).

The Asteraceae (Compositae) is the species rich vascular plant family in the world, with 24000-30000 species and 1600-1700 genera (4). They are easily distinguished by the florets grouped in capitula, and the fruit forming a cypsela with pappus. Asteraceae taxa are variable in life-form including herbs, succulents, lianas, epiphytes, trees, or shrubs, and they reach every environment and continent, except Antarctica (4). Relationships among the Asteraceae and other families remained still obscure (5).

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Morphological and molecular data proposed the closed relationship between Goodeniaceae, Calyceraceae and Asteraceae (6). In addition, there are significant similarities in floral and inflorescence morphology between the Asteraceae and Calyceraceae (6, 7). Important similarities to the highly specialized secondary pollen-presentation mechanism of the Asteraceae have been documented in Goodeniaceae (8). DeVore and Stuessy (9) argued in favor of a sister group relationship between Asteraceae and Calyceraceae, mainly drawing evidences from morphology, however, the Asteraceae-Calyceraceae sister group relationship was soon challenged by Goodeniaceae. Since then there have been three competing hypotheses: a clade of Asteraceae + Calyceraceae with Goodeniaceae as its sister group (10-20), or a clade of Goodeniaceae + Calyceraceae with Asteraceae as its sister group (21-24), or a clade of Asteraceae + Goodeniaceae with Calyceraceae as its sister group (13, 25). It is possible to find at least some characters in favor of any of these relationships, but as shown by various authors (9, 11, 26) the morphology is mainly in favor of the Calyceraceae-Asteraceae sister group relationship, while some molecular markers suggested the other two alternatives. Recently, there have been many improvements in the resolution of the taxonomic relationships and the classification of the Asteraceae at the subfamilial and tribal level (27). It seems that embryological studies may have complementary role in the solving of problems in this family (Asteraceae) and others close families (27).

Examined taxon in this study is the *Achillea tenuifolia* Lam. (Compositae) with small yellow flowers and several times pinnately leaves which have worm shaped resemblance. This plant is known for many years in the folk medicine. It has been used to reduce sweating and to stop bleeding. It helps regulation of the menstrual cycle and reduces heavy bleeding and pain (28). The aim of the present work was to study different developmental stages of anther, pollen grain, ovary and ovule in *A. tenuifolia* and to compare them with the data available on other members of Asteraceae as well as Goodeniaceae, and Calyceraceae. Although there are some reports about other members of Asteraceae (29-31), this is the first embryological investigation on *A. tenuifolia*.

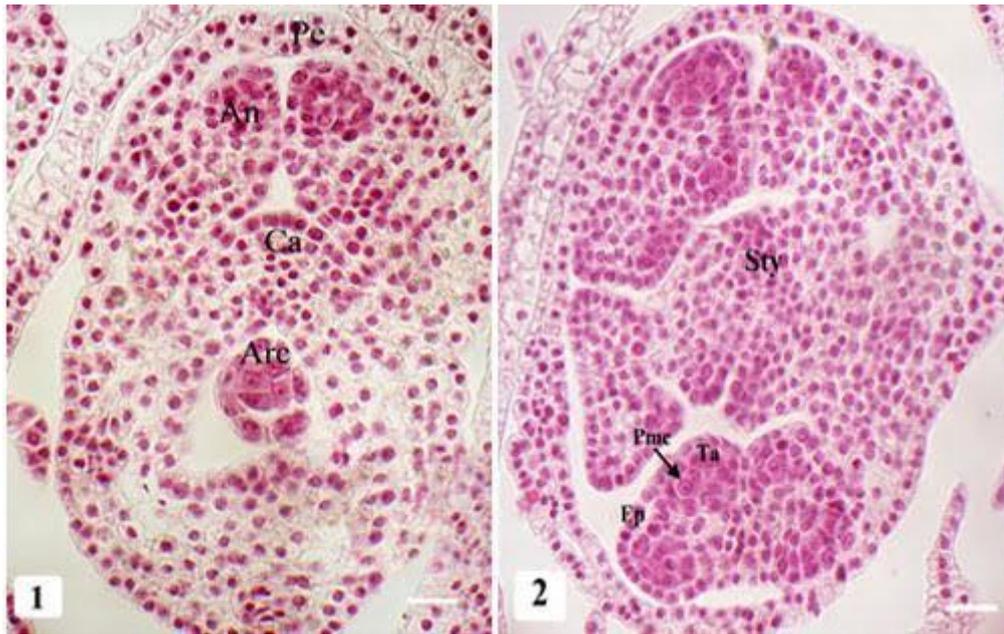
## Material and Methods

Samples of the *Achillea tenuifolia* plants, in the different stages of development were collected from its naturally growing habitat in Hamadan, 40 km from Asad Abad at an altitude 2100-2180 m. Specimens were deposited at the herbarium of the Bu-Ali Sina University (HBAS). Flowers and tiny buds in different sizes were removed, fixed in FAA<sub>70%</sub>, stored in 70% ethanol and were passed from alcohol gradient with increasing density, then were passed from toluene and paraffin bath and embedded in paraffin and sliced at 7-10  $\mu\text{m}$  with a Micro DC 4055 microtome (Dideh Sabz, Urmia, Iran). Staining was carried out with PAS (Periodic Acid Schiff) according to a common protocol (32) and contrasted with Meyer's Hematoxylin (33). For each developmental stage, several sections observed under a Zeiss Axiostar Plus bright field microscope (Germany). Several samples were studied for each developmental stage and photomicrographs were made from the best ones. The pollen grains were prepared (34) and examined by bright field microscope.

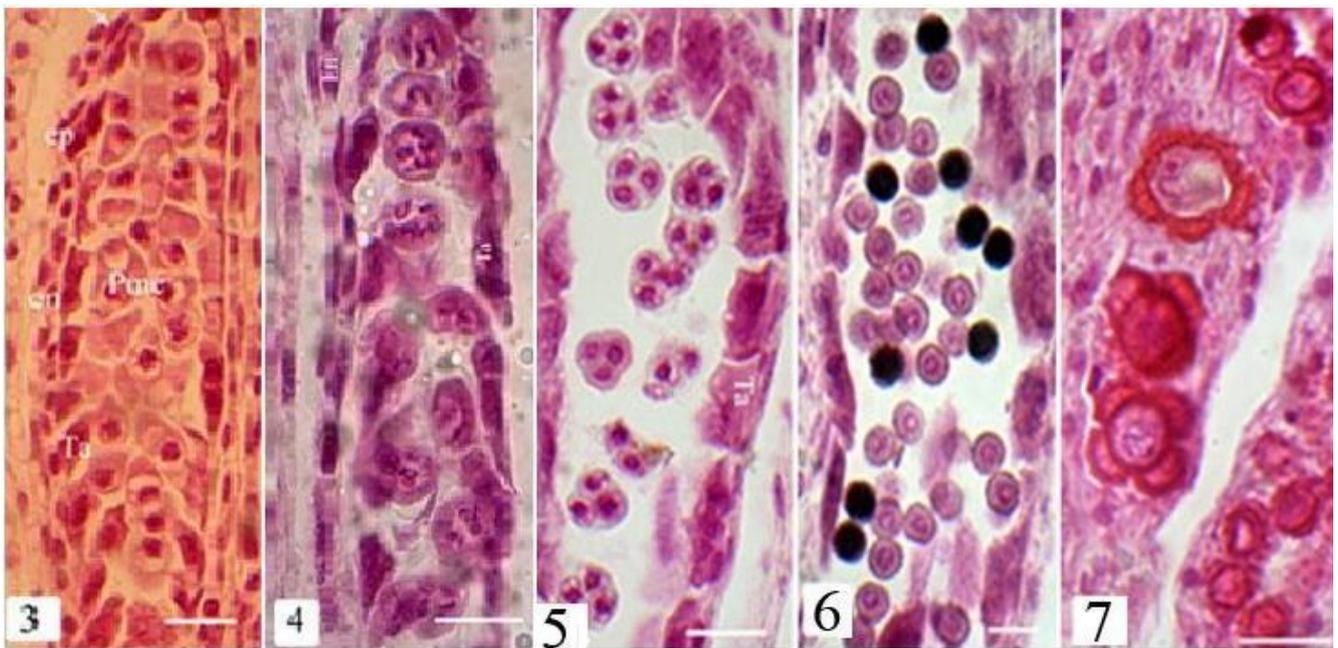
## Results

### *Development of stamen, anther and pollen grains*

Development of stamens begins with emergence oval-shaped cell masses on the receptacle. These masses are composed of similar cells (Fig. 1). Results showed that the anther of *A. tenuifolia* is tetrasporangiate (Fig. 2). In early stages of development, several rows of archesporial cells differentiate beneath the epidermis of the anthers. They have dense cytoplasm and prominent nuclei. They divide periclinally, form inner sporogenous cells and outer parietal cells. Parietal cells are divided and cause to form anther wall that consists of four layers (Fig. 3): one epidermis layer, an endothecium layer, one middle layer and one tapetum layer in the proximity the microsporocyte. Sporogenous cells are divided and formed microsporocytes with large nuclei that showed high-density color (Fig. 3). Epidemism in this species is lacking any trichome and extensions. Middle layer is thin and have spindle-shaped and long cells. The tapetum cells are oval-round shape, uni-nucleate or bi-nucleated at the stage of microsporocyte and growing toward anther cavity (Figs. 4, 5).



Figures 1-2. Development of reproductive organs in *Achillea tenuifolia*: 1, longitudinal section of young bud that showed early stages of development of stamens, carpel, and ovule; 2, cross section of a young anther with four pollen sac that contains pollen mother cells (PMC) and the formation of anther wall. Abbreviations: Pe (Petal), Sty (Style), St (Stamen), Are (Arceospore), Ep (Epidermis), Ta (Tapetum), Ca (carpel), Pmc (Pollen mother cells). Scale bars = 50  $\mu$ m.



Figures 3-7. Microsporogenesis and male gametogenesis in *Achillea tenuifolia*. 3, longitudinal section of anther showing pollen mother cells and developing anther wall; 4, Pollen Mother cell at the Prophase I; 5, Tetrahedral microspore tetrads; 6, Young microspores just released from tetrads; 7, Mature tricolpate pollen grains. Abbreviations: En (Endothecium), Pmc (Pollen mother cells), Ep (Epidermis), tr (transitional layer), Ta (Tapetum). Scale bars = 30  $\mu$ m.

Tapetum cells keep their individuality therefore this layer is of secretory type (Figs. 4-7). Tapetum layer is degenerated at the stages of unicellular or bi-cellular pollen grains (Fig. 7).

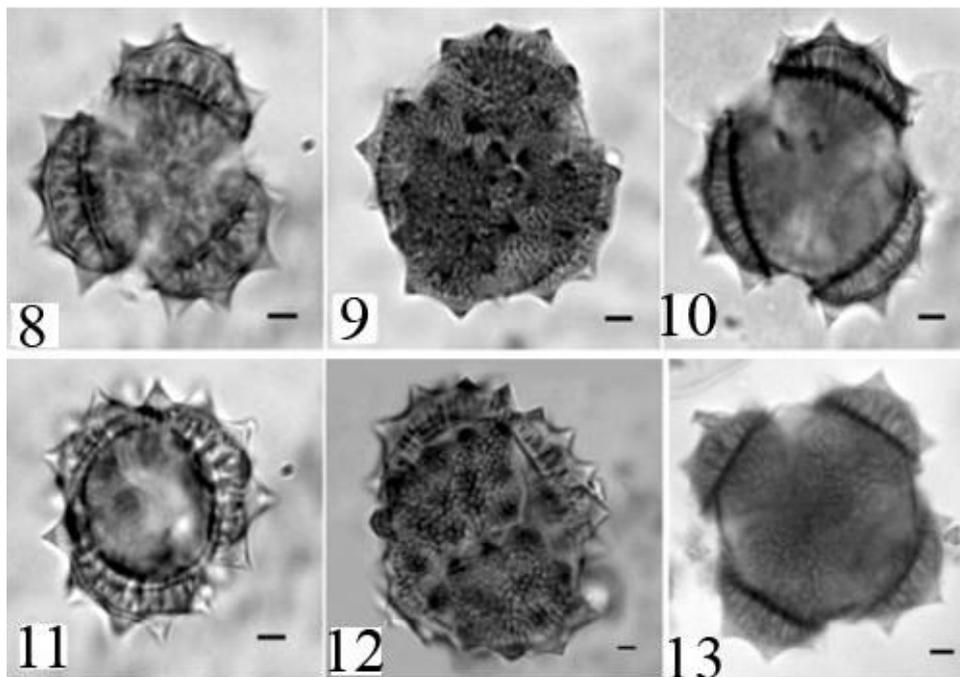
Primary sporogenous cells (Fig. 3) were developed directly to microsporocytes. Microsporocytes have the large nucleus and are located in the center of cells that is representing preparation of these cells to division

(Fig. 4). During the microsporangium process, each microsporocyte undergoes meiosis and results in a microspore tetrad. The tetrad type is tetrahedral (Fig. 5). In the two neighboring pollen sacs, development of microspores is not synchronized. Callosic wall is formed around the tetrad and between each monad widely (Fig. 5). Then callosic wall degenerated and the microspores released from tetrads. Microspores are non-vacuolated, irregular shaped, contained dense cytoplasm and visible nucleus in the cell center. Microspores showed high levels of starch that is darkened in this staining (Fig. 6). Its nucleus takes up a peripheral position together the central vacuole develops, i.e., forming a large vacuole squashes the

cytoplasm and the nucleus toward the microspore margin. Nucleus of microspores then undergoes mitosis and resulted to form two unequal nuclei, a large vegetative and small generative one thus to form bi-nucleate pollens (Fig. 7), further two cell ones. Although most mature pollen grains are tricolporate, pollen grains with 4 and 5 colpi were also observed (Figs. 8-13). The polar diameter length and equatorial diameter varies from 18-42.5 and 16.5-35.5  $\mu\text{m}$ , respectively. The mean of the spine length and exine thickness of the pollen grains in this species is 2.4 and 2.7  $\mu\text{m}$  respectively (Table 1). As shown in Table 1 and Fig. 8-13 the tetracolporate pollen grains is larger than the other ones.

**Table 1. Pollen grain size in six individuals of *Achillea tenuifolia***

Characters	Spine length	Exine thickness	Equatorial diameter	Polar diameter
Sample	( $\mu\text{m}$ )	( $\mu\text{m}$ )	( $\mu\text{m}$ )	( $\mu\text{m}$ )
1	3	3.5	22.5	25.5
2	1.2	2	24.5	26
3	2.9	3	23.1	23.5
4	2.2	2.5	16.5	18
5	2.6	2.5	35.5	42.5
6	2.5	2.8	30.5	31.4
Average	2.4	2.7	25.4	27.8



**Figures 8-13. Micrograph of pollen grains in different individuals of *Achillea tenuifolia*. 8, Polar view of a tricolporate pollen grain. 9, Equatorial view of a tricolporate pollen grain. 10, 11, Polar and equatorial view of a another tricolporate pollen grain. 12, 13, Equatorial and polar view of a tetracolporate pollen grain. Scale bars = 5  $\mu\text{m}$ .**

**Development of ovary, ovule and megasporogenesis**

Simultaneous with the formation of the stamens, carpel and ovular primordium is formed (Fig. 1). In this species, young ovaries have only one ovule and when the ovular primordium appears, the carpel already was closed (Fig. 1). The ovule is anatropous bitegmic, tenuicellular and the nucellus epidermis consist of one layer of spherical cell. The ovule primordium starts bending at an early stage. The initiation of the

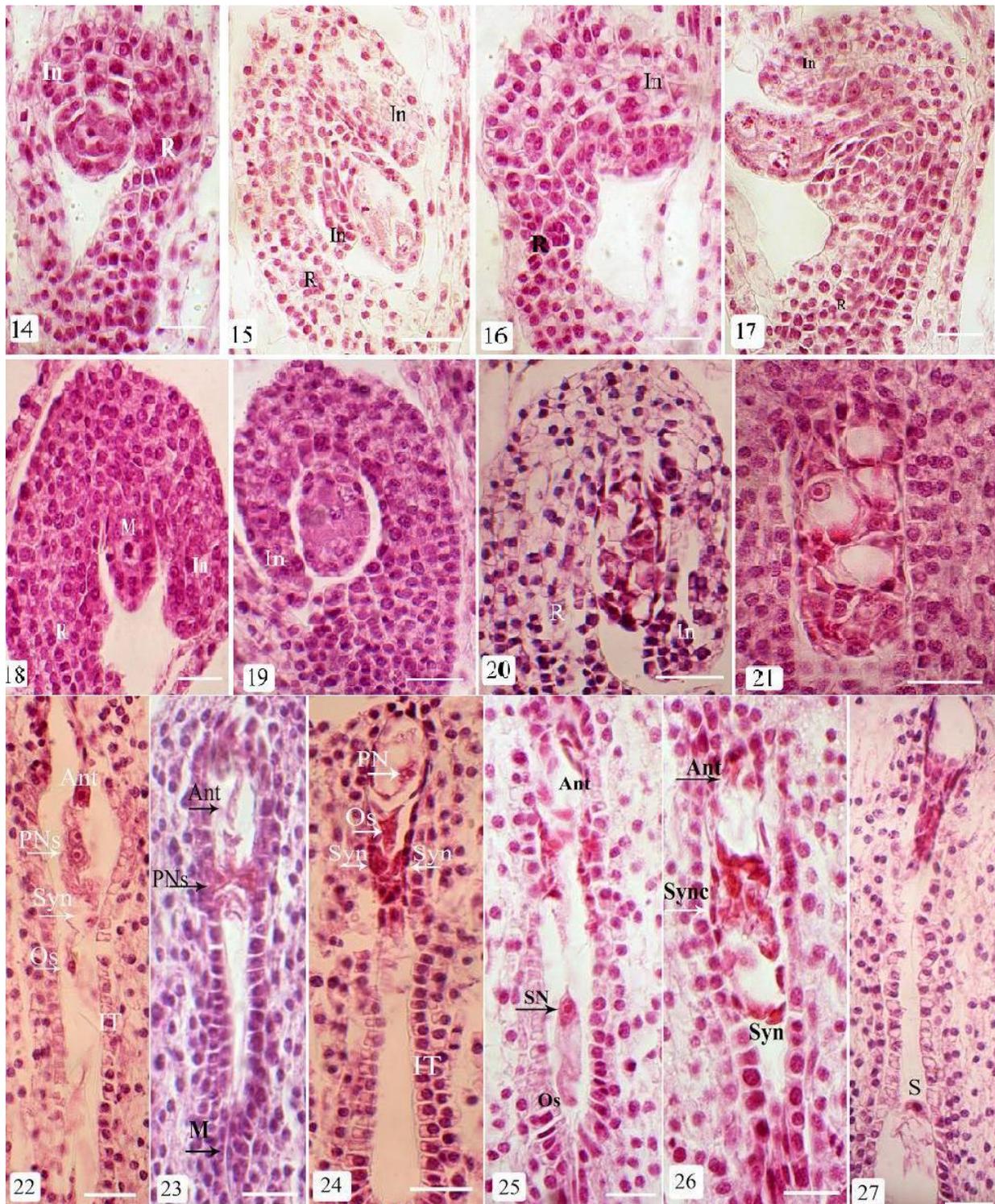
integuments takes place when the ovule shows a nearly 90° curvature. The integument is initiated from periclinal and oblique divisions of dermal cells. The original integument on the opposite side of funicular, grows asymmetrically faster than the lateral integument but the lateral integument degenerated at the early stages (Figs. 14, 15). During growth, the free end of the original integument in the ovule funicular pole constitutes the micropyle with right curve.

**Table 2. Embryological comparison of *Achillea tenuifolia* with other Asteraceae, Calyceraceae and Goodeniaceae**

Character	<i>Achillea tenuifolia</i>	Asteraceae	Calyceraceae	Goodeniaceae
<b>Anthers and microspores</b>				
Number of microsporangia	4	4	?	4
Tapetum type	Amoeboid, Secretory	Amoeboid	Secretory	Secretory
Mature pollen grains	2-Celled	3-Celled	2-Celled	2-Celled
<b>Ovary and ovule</b>				
Ovary	Inferior, 1 locular	Inferior, 1 locular	Inferior, 1 locular	Inferior, (1 or)2 locular
Hypostase	Not formed	Not formed or formed	?	Formed
Antipodal cells	Persistent, multinucleate	Persistent, multinucleate	Ephemeral	Ephemeral
Thickness	?	12-25 cells	?	18-20 cells
<b>Fertilization, endosperm, and embryo</b>				
Path of pollen tube	Poregamous	Poregamous	?	Poregamous
Mode of endosperm formation	Nuclear	Nuclear	Cellular	Cellular or Nuclear
Endosperm in mature seed	Scanty	Scanty or absent	Copious	Scanty
Embryogenesis	Asterad type	Asterad type	?	Solanad type
<b>References</b>	Present paper	(9), (19), (47), (36)	(34), (48)	(27), (6)

The initial archesporial cell is distinguished from the other nucellar cells (Fig. 1), because it presents a larger volume, dense cytoplasm, and distinct nucleolus. Some of the cells of the nucellar tissue develop directly into the archesporial cells, one of which divides into a primary parietal cell and a megaspore mother cell (MMC) (Fig. 14) and other cells were degenerated. The megaspore mother cell (MMC) divides meiotically and undergoes 2 successive divisions resulting the equal sized dyad cells at the meiosis I (Fig. 15) and the linear-shaped (Fig. 16) or T-shaped (Fig. 17) tetrad cells. At this

stage of development the integument of ovule surrounds ovule bulk more than three-quarters. The 3 micropylar megaspores degenerate, and the chalazal one develops into megagametophyte (Fig. 18). Three successive mitotic karyokineses give rise to an 8-nucleate embryo-sac (Figs. 19-21). One central vacuole is formed and 4 nuclei are positioned in the micropylar end of the cytoplasm, and the other 4 nuclei in the chalazal end (Fig. 22). Subsequent nuclear migration and cytokinesis occurred during megagametogenesis, eventually resulting in a mature embryo sac with the typical eight-nucleate one.



Figures 14-27. Megasporogenesis and megagametophyte development in *A. tenuifolia*. 14, Megaspore mother cell. 15, First meiosis resulted in formation of equally-sized dyad cells that shows developing integuments. 16, Linear-shaped tetrads. 17, Ovule with tetrads that are arranged as T-shaped forms. 18, Formation the functional micropylar megaspore. 19, Bi-nucleate embryo sac. 20, Four-nucleated embryo sac. 21, Embryo sac of eight-nucleated. 22, 23, Maturing embryo sac showing two polar nuclei, oosper and antipodal cells. 24, Maturing embryo sac showing egg apparatus that contains oosper cell and synergid cells. 25, Mature embryo sac that secondary nucleus resulted by the fusion of polar nuclei in the micropylar end and increases of antipodal cells number. 26, Longitudinal section of mature embryo sac showing the formation of endosperm and decrease of antipodal cells. 27, Migration of sperm cell toward the egg apparatus and formation of the egg cell. Abbreviations: In (integument), R (raphe), PNs (polar nuclei), PN (polar nucleus), SN (secondary nucleus), Os (oosper cell), Syn (synergid cell), Og (egg apparatus), Ant (antipodal cell), M (micropyle), S (sperm cell), Sync (endosperm), IT (endothelium), Em (embryo). Scale bars = 60  $\mu$ m.

After the 8-nucleate stage, the coenocytic megagametophyte becomes partly cellular (Figs. 23, 24). This process is simultaneous at the micropylar and chalazal ends. Mature embryo consists of 7 cells: 2 synergid cells and one egg cell comprised egg apparatus, 3 antipodal cells and one central cell that contained 2 nuclei (Figs. 24-27). Egg cells are larger and distinguishable from synergids by its position (Fig. 24). The polar nuclei are visible in the center of embryo sac that are fusing just before fertilization and produced a secondary nucleus (Fig. 25). Antipodal cells were divided and increased their number (Figs. 25, 26). At the late stages of development embryo sac, after fertilization, the number of antipodal cells was decreased but they were not degenerated completely (Fig. 27). In this species, division of the endosperm nucleus was prior to the formation of zygote and its successive divisions cause to form albumin storage tissue that it is of coenocyte (nuclear) type (Fig. 26). Thus embryo sac is ready for fertilization and forms the egg cell (Fig. 27). The cells in endothelium layer, in this species, have the high stability and remains in the all stages of embryo sac development (Figs. 22-27).

## Discussion

Results of this research showed that development of the four-layered anther wall occurred as dicotyledonous-type reported previously for Asteraceae (35, 36). Archisporial cells are recognized by their prominent nuclei and compact cytoplasm. They are divided periclinally resulted to form outer primary parietal and inner sporogenous cells that is accordance with previous findings (29). Presence of middle layer reported earlier in other species of Asteraceae (30) is also visible in *A. tenuifolia*. Two basic types of tapetum are recognized in Angiosperms: secretory and amoeboid type (37). In *A. tenuifolia* the tapetum is of secretory type with multiplication of the nuclei (1-3 nuclei per cell) that is consistent with the results of investigation in *Cichorium intybus* L. (29). Each microsporocyte undergoes the common mode of meiosis to produce tetrahedral tetrads known in other Asteraceae (36). The nucleus of microspores divided by the mitosis into two nuclei, so called bi-nucleated pollen grain that is different from earlier results in the other

Asteraceae (35) where are 3-celled are reported to shed. Pollen grains in this species are mostly tricolporate but tetracolporate or even pentacolporate ones were observed and which show different in sizes. Pollen grains with variable number of aperture have been reported in the Asteraceae and other species of the genus *Achillea* (38). Akyalcin et al. (39) compared cellular organization in the ovule primordium with the shoot apical meristem. Our results showed that in the species, ovule initiation is basipetal and originates as a small protuberance. The ovule is anatropous according to the ontogenetic classification performed earlier (40). In *A. tenuifolia*, the chalazal megaspore of the linear-shaped tetrad gives rise to form *Polygonum* type of embryo sac as described for more than 70% of angiosperm (1, 41, 42). The Linear tetrads (29, 30) and T-shaped tetrads (35) were reported in the other species of Asteraceae. A remaining megaspore produced 8-nucleated and then cellularized embryo sac. In mature embryo sac three cells were differentiated at the micropylar end that consists of an oosphere and two synergids. In *Polygonum* type development of embryo sac, antipodal cells are placed on the chalazal end of embryo sac. The antipodal cells are usually three and variable in size and number (7, 39), as reported in Asteraceae (30). Our results indicated that the most antipodal cells are degenerated in the late stages of embryo sac development but they were not degenerated completely. In this species, it seems that antipodal cells have not any specific role, but their function is importing of nutrients into the embryo sac at the early developmental stages (43).

Embryological features of Asteraceae, Calyceraceae and Goodeniaceae are summarized in Table 2. Embryology of Asteraceae has been extensively studied (28, 44) and results indicated that Asteraceae species are varied in many of embryological characters. This family always has anatropous, unitegmic, tenuinucellate ovules and also bears an endothelium though with varying layers (36, 45-47). The embryological studies on Calyceraceae are rare so that most of information was obtained from *Acicarpha tribuloides* Juss. (48). Calyceraceae have tenuinucellate, unitegmic and anatropous ovules which develop the *Polygonum* type embryo sac with ephemeral antipodal cells. They form the endothelium but lack any endosperm haustorium (49). There is not

any information available for the thickness of the integument that is an important character in critical evaluating similarities or dissimilarities within Asteraceae or Goodeniaceae. Goodeniaceae lack also the endosperm haustorium and filamentous suspensor. The integument in this family is formed of 18-20 cell layers (50). Goodeniaceae resemble Asteraceae, embryologically, in having thick integument, seeds with scanty endosperm and the nuclear type of endosperm.

Within Asterales the monophyly of the clade Calyceraceae-Goodeniaceae-Asteraceae is supported by lacking of endosperm haustorium (51). There are clear differences in embryology between Calyceraceae and Asteraceae: Calyceraceae lack nuclear endosperm, bear scanty endosperm and thick

integument. Likewise, close relationships between Goodeniaceae and Asteraceae are supported by the seed with scanty endosperm and the thick integument (46). Asteraceae are characterized by two autapomorphies, i.e., amoeboid tapetum and nuclear type endosperm indicating its advanced phylogenetic position (43). Our embryological evidence supports the hypothesis that Goodeniaceae as the closest sister group of Asteraceae.

Our results show the potential application of embryological studies in assessing the phylogenetic relationships among asterids. As such data are poorly available in this group, including more taxa as representatives of various genera in asterids might provide valuable information in clarifying the borders between taxa mostly at family or generic rank.

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