

FLOWER ONTOGENY AND REPRODUCTIVE BIOLOGY OF *SALVIA VIRIDIS* L.

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Abstract

The flower ontogeny and the developmental features of male and female gametophyte were investigated in *Salvia viridis* L. It has purple and hermaphrodite flowers. The stamen primordia differentiate shortly before the initiation of carpel primordia from floral meristem. The anther is bisporangiate. Although the anther wall consists of an epidermis, endothecium, middle layer and secretory tapetum in young stage, only epidermal and endothelial cells preserve their vitality until the period of anther dehiscence. The microspore tetrad is tetrahedral and the mature pollen grains are pericarpate and tectate. The pistil has a superior ovary with four loculi and a thick, closed style with forked, unapiculate stigma. The anatropous, tenuicellate ovule differentiates by axilar placentation. The development of the monosporic embryo sac follows by the Polygonum type.

Key words: Developmental biology, Ontogeny, Reproductive biology, *Salvia viridis*, Sexual reproduction

Introduction

Salvia viridis L. is a member of the genus *Salvia*, which is the largest genus of the family Lamiaceae, with more than 900 species (Rungsimakan & Rowan, 2014). The genus *Salvia* is represented by 86 species in Turkey including *Salvia viridis* L. (Davis, 1982). *S. viridis* is the only annual species of the genus in Turkey (Özdemir *et al.*, 2009).

The hermaphrodite flowers are produced in racemes, or panicles in *S. viridis* L. The flowers are zygomorphic and they are used for the preparation of drugs and treatment of diseases such as sore throat, tonsillitis and gingival disease. Moreover, they are used for perfumery and food industry (Neugebauerova *et al.*, 2015).

The developmental and embryological features in angiosperms are significant subjects because they are constant characteristics functioning as a meaningful indicators of systematic familiarity (Von Teichman & Van Wyk, 1991; Vardar, 2013). They provide useful data for the discipline of cell biology, taxonomy, seed production and outbreeding mechanisms, as well.

The studies on the reproductive biology of *S. viridis* are limited. This may be due to the medical and pharmacological features are taken more attention. The purpose of this work is to find out the details on the flower ontogeny and development of female and male reproductive organs of the species.

Materials and Methods

The flower buds of *S. viridis* were picked from Zeytinburnu Medicinal Plant Garden (Istanbul/Turkey). Firstly, the diameter of the flowers were measured by stereomicroscope (Olympus 970931). The flowers were carefully excised and fixed in acetic-alcohol (1:3, v/v) and they were embedded in paraffin. Ultimately, they were sliced at 5-10 µm in thickness with Leica RM2235 rotation microtome.

For histological studies, the sections were stained with Delafield's hematoxylin. For cytochemical analysis, periodic acid-Schiff (PAS) (Feder & O'Brien, 1968) for insoluble polysaccharides, Coomassie Brilliant Blue (Fisher *et al.*, 1968) for proteins, Sudan Black B for lipids

(Pearse, 1961), Auramine O (Heslop-Harrison & Shivanna, 1977) for sporopollenin and exine were applied to the sections. Moreover, the sections were stained with DAPI to elucidate nucleus morphology (Schweizer, 1976).

The preparations were photographed with the KAMERAM software, assisted by a KAMERAM digital camera and an Olympus BX-51 microscope. DAPI and Auramine O stained preparations were investigated with Olympus BX-51 microscope fluorescence microscope at 440 nm and 365 nm wavelength respectively and photographed with KAMERAM software, assisted by a KAMERAM digital fluorescence camera.

For SEM analysis, the plant material was fixed according to the literature of Platt *et al.* (1983), dried with ethanol-HMDS (Topçuoğlu *et al.*, 2009), coated with 11 nm of gold and examined with a SEM (JEOL JMS-59 10LV).

Results

S. viridis has the raceme type of inflorescence (Fig. 1A), bearing purple, zygomorphic, hermaphrodite flowers consisting of two lever-like stamens and a pistil. The filament bearing the fertile anther has a protrusion near the base, which remains as a project throughout the development.

The development of a flower starts with the distinction of apical meristem, appearing as a big, roundish bulge and composed of consecutive cell layers (Fig. 1B). Afterwards, the peak of apical meristem flattens, widens and transforms into the floral meristem which contain sequential cell layers as in apical meristem. The cells of floral meristem are pronounced with their bigger volume and denser cytoplasm (Fig. 1C).

Two stamen primordia differentiate as a protrusion on the both sides of the floral meristem (Fig. 1D). Concomitant with the differentiation of stamen primordia, the straight of floral meristem becomes invisible. Afterwards, the stamen primordia starts to grow up and develop. The filament differentiation is followed by the anther formation (Fig. 1E, 1G). Thereafter, two carpel primordia emerge on the floral meristem (Fig. 1F). Freshly formed carpel primordia are not as roundish as

stamens. In the following stages, two carpel primordia grow longer and form a solid style. Additionally, four ovaries arise below the carpel primordia (Fig. 1H).

In order to follow the duration of development easily, the width and height of flower buds in essential stages were measured (Table 1).

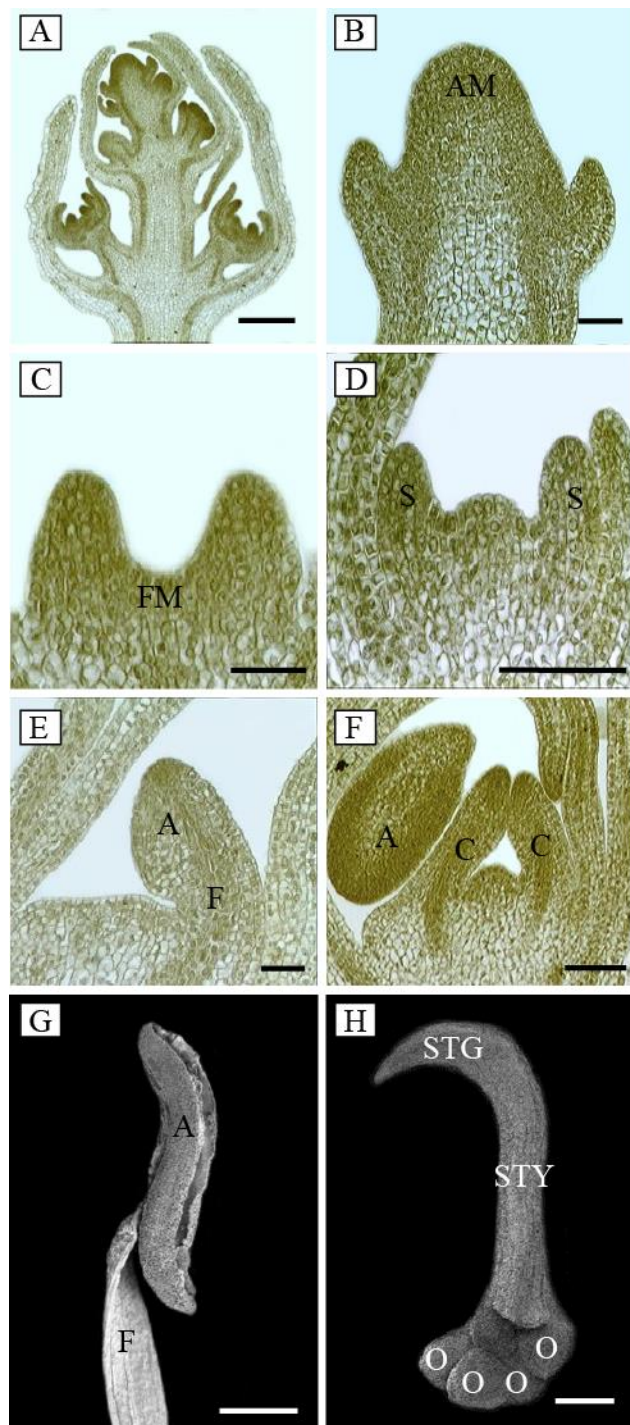


Fig. 1. Flower ontogeny of *Salvia viridis*. A. Inflorescence (Raceme). B. Apical meristem. C. Floral meristem. D. Stamen primordia. E. Anther-filament differentiation. F. Carpel primordia. G. SEM micrograph of a mature stamen. H. SEM micrograph of a mature pistil. AM: Apical meristem, FM: Floral meristem, S: Stamen primordia, A: Anther, F: Filament, STG: Stigma, STY: Style, O: Ovary. Bar: 300 μ m (A), 50 μ m (B, C, D, E), 100 μ m (F), 200 μ m (H), 500 μ m (G).

Table 1. Flower bud size of *Salvia viridis* in essential developmental stages.

Developmental stage	Flower bud width	Flower bud height
Apical meristem	200 μ m	180 μ m
Floral meristem	240 μ m	200 μ m
Initiation of stamen primordia	440 μ m	400 μ m
Initiation of carpel primordia	550 μ m	520 μ m
Sporogenous tissue	900 μ m	700 μ m
Ovule initiation	1000 μ m	900 μ m
Pollen mother cell	1020 μ m	950 μ m
Microspore tetrad	1140 μ m	1000 μ m
Mature pollen	2500 μ m	1050 μ m
Megaspor mother cell	2700 μ m	1200 μ m
Megaspore tetrad	3000 μ m	1500 μ m
Mature embryo sac	4500 μ m	3200 μ m

The undifferentiated anther of *S. viridis* is ovoid shaped and consists of meristematic cells surrounding by a single layer of epidermis. One or two archesporial cells differentiate in the sporangium and these distinguishable cells possess prominent nuclei and denser cytoplasm (Fig. 2A). Afterwards, the archesporial cells undergo mitotic divisions to form the parietal and the sporogenous layer (Fig. 2B). Whereupon, the cells of parietal layer result in the formation of anther wall layers by successive periclinal and anticlinal divisions and the sporogenous cells undergo mitotic divisions to give pollen mother cells (PMCs) (Fig. 2C). Concurrent with development and divisions, the anthers become bisporangiate (Fig. 2C, D). Mature pollen grains are spread out to the nature by deployment of the anther at the end of the development (Fig. 2E).

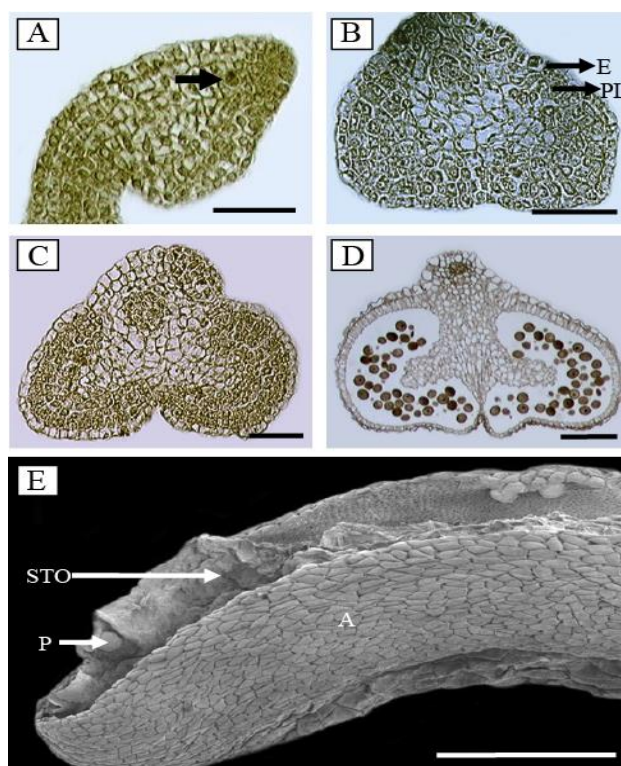


Fig. 2. Anther ontogeny of *Salvia viridis*. A. Undifferentiated anther and archesporial cell (arrow). B. Parietal layer and epidermis. C. Bisporangiate anther. D. Mature anther with pollen grains. E. SEM micrograph of anther dehiscence. E: Epidermis, PL: Parietal layer, STO: Stomium, P: Pollen, A: Anther. 50 μ m (A, B, C), 200 μ m (D, E).

The anther wall consists of four layers from outer to inner: epidermis, endothecium, middle layer and secretory tapetum (Fig. 3A). The epidermis is a single row with rectangle, flattened and ordered cells. Even though epidermal cells lose their rectangular appearance and flatten throughout the development, the cells preserve their vitality until the period of anther dehiscence (Fig. 3A, B, C, D). The monolayer endothecium comprises of flattened and radially elongated cells and develops up the period of anther dehiscence (Fig. 3A, B, C, D). In addition, fibrous wall thickening in the endothecial cells starts to be discernible when the microspore tetrads are visible in the pollen sacs (Fig. 3C). The endothecial layer maintains its vitality until the period of anther dehiscence such as epidermis (Fig. 3D). The middle layer with flattened cells is ephemeral (Fig. 3A, B, C, D). Concurrent with the progression of meiosis in the PMCs, middle layer enters a slow atrophy process (Fig. 3C) and completely invisible when the mature pollen grains are formed (Fig. 3D).

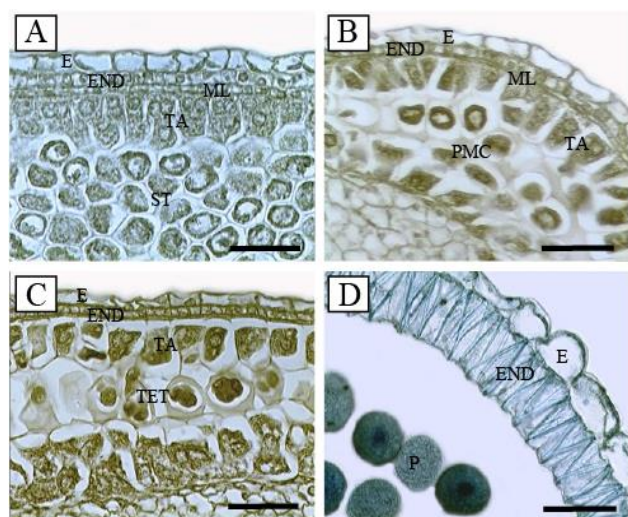


Fig. 3. Anther wall layers in *Salvia viridis*. A. At the sporogenous tissue stage. B. At the PMCs stage. C. At the microspore tetrad stage. D. At the mature pollen stage. E: Epidermis, END: Endothecium, ML: Middle layer, TA: Tapetum, ST: Sporogen tissue, PMC: Pollen mother cell, TET: Microspore tetrad, P: Pollen. Bar: 50 μ m (A, B, C, D).

S. viridis has monolayered secretory tapetum. The tapetal cells are distinguishable from others by their larger volume, denser cytoplasm and larger spherical nuclei. In the early developmental stage, a tapetal cell has usually single nucleus (Fig. 3A, Fig. 4A). Simultaneously with meiosis in PMCs, tapetal cells extend radially (Fig. 3B, C). Additionally, while meiotic division continues, one or two mitotic divisions take place in tapetal cells and nuclear divisions are not followed by cytokinesis. In this manner, tapetal cells with 2-4 nuclei can be observed (Fig. 4B). After the tetrad formation, tapetal cells become degenerate and completely disappear when mature pollen grains are formed (Fig. 3D).

The PMCs of *S. viridis* are isodiametric cells with rich cytoplasm (Fig. 3B). They undergo regular meiotic division which is followed by simultaneous cytokinesis. As a result, tetrahedral tetrads are formed, surrounding by

callose walls (Fig. 3C). The callose wall is dissolved at the end of meiosis and young microspores disperse into anther locus. In the course of the development, free microspores turn into the mature pollen grains (Fig. 3D).

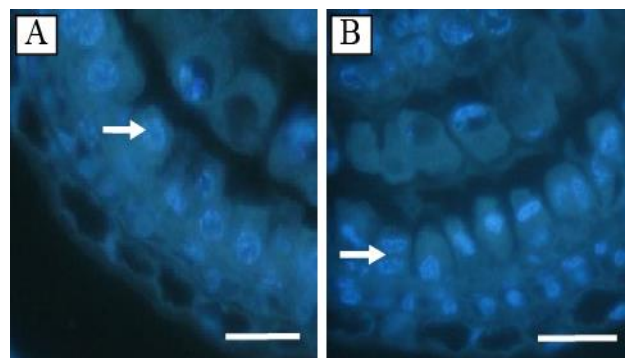


Fig. 4. Anther wall layers stained with DAPI in *Salvia viridis*. A. Tapetal cells with a large, spherical nuclei (arrow), B. Tapetal cells with two or three nuclei (arrow). Bar: 10 μ m (A, B).

The pollen grains of *S. viridis* are pericarpate and tectatae (Fig. 5A, B, C, D, E). The pollen wall is composed of innermost layer, intin and outermost layer, exine (Fig. 5A). Exine is made up of lipoidal substances but intine is consist of insoluble polysaccharides, as revealed by Auromine O and Sudan Black B and PAS, respectively (Fig. 5B, C, D). Exine pattern is reticulate type (Fig. 5E). Cytochemical tests remarked that cytoplasm of a mature pollen grain is filled with insoluble polysaccharides, proteins and lipids (Fig. 5A, B, C).

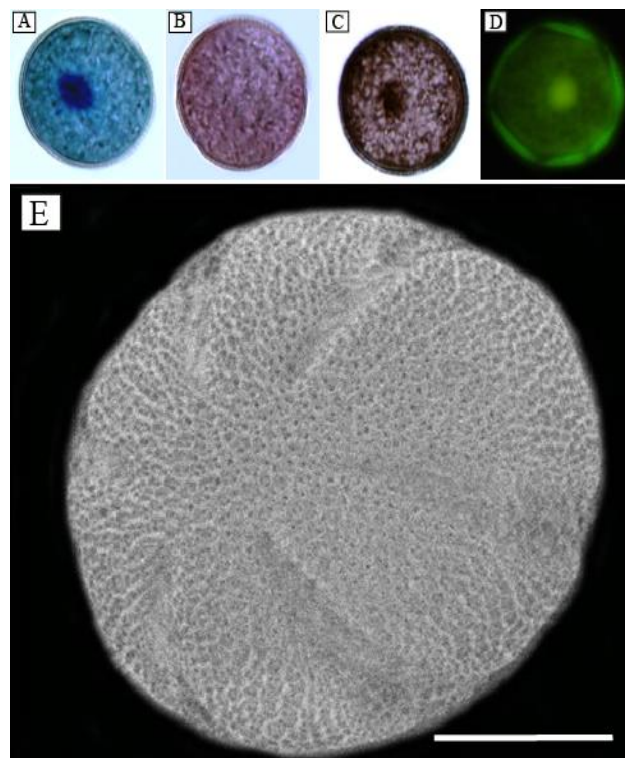


Fig. 5. Morphology and cytochemistry of pollen grains in *Salvia viridis*. A. A Pollen stained with Coomassie brilliant blue, B. Stained with PAS, C. Stained with Sudan Black B, D. Stained with Auromine O, E. SEM micrograph of a mature pollen. Bar: 10 μ m (A, B, C, D, E).

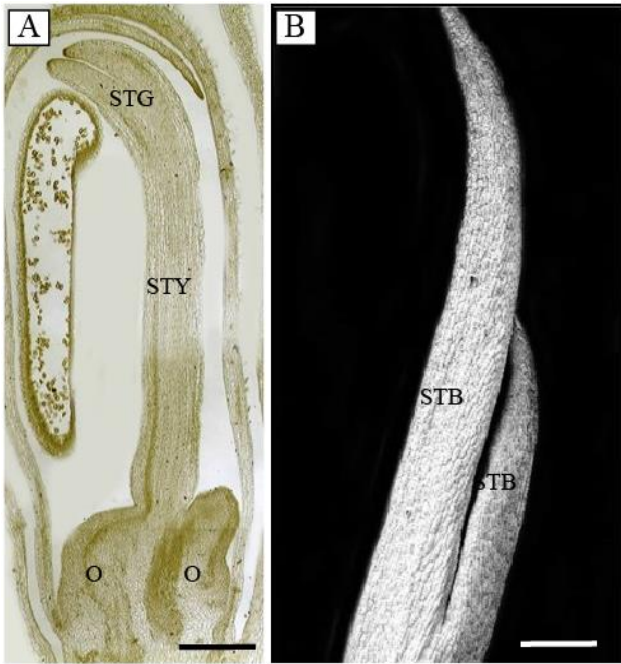


Fig. 6. Pistil of *Salvia viridis*. A. Longitudinal section of the mature pistil. B. SEM micrograph of the stigma. STG: Stigma, STY: Style, O: Ovary, STB: Stigmatic branches. Bar: 200 µm (A, B).

The pistil of *S. viridis* has a superior ovary and a thick style with deep cleft stigma (Fig. 6A, B). The ovary has four loculi and each loculus contains an anatropous ovule with axile placentation (Fig. 7A). The stigma is dry and the receptive surface is not papillose (Fig. 6B). The style is closed type.

The ovules differentiate as a small bulge on the placenta in ovary, consisting of cells with dense cytoplasm and small nuclei (Fig. 7A). Later, the funiculus grows and begins to curl. The obturator differentiates at the base of funiculus (Fig. 7B). The ovules are unitegmic. With the differentiation of integument independently (Fig. 7B), the curling of ovule increases (Fig. 7C). The ovule becomes anatropous at four megaspore tetrad stage and the obturator grows towards to the micropyle (Fig. 7C, D).

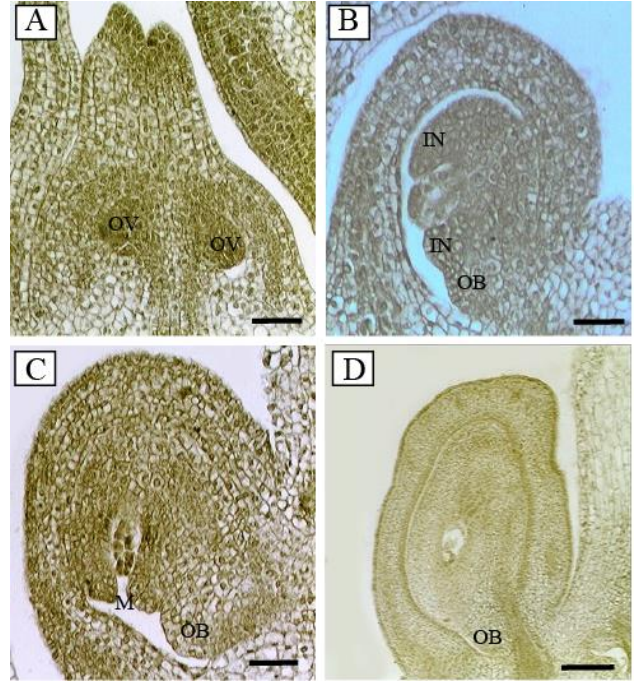


Fig. 7. Ovule development in *Salvia viridis*. A. Ovule differentiation. B. Integument and obturator differentiation. C. Ovule curling. D. A mature anatropous ovule. OV: Ovule, IN: Integument, OB: Obturator, M: Micropyle. Bar: 50 µm (A, B, C), 200 µm (D).

The ovules are tenuinucellate and the megaspore mother cell (MMC) differentiates just below the nucellar epidermis (Fig. 8A). Subsequently, the MMC undergoes meiosis to form a dyad (Fig. 8B) and then a linear megaspore tetrad (Fig. 8C). The chalazal megaspore is functional and bigger than the others. It undergoes 3 successive mitotic divisions and forms 8-nucleated embryo sac. In the micropylar pole, three nuclei develop into an egg apparatus consisting of an egg and two synergids and three nuclei developing into antipodal cells in the chalazal region (Fig. 8F). Two polar nuclei are located in the centre of the embryo sac (Fig. 8E). The development of the embryo sac follows by the monosporic Polygonum type.

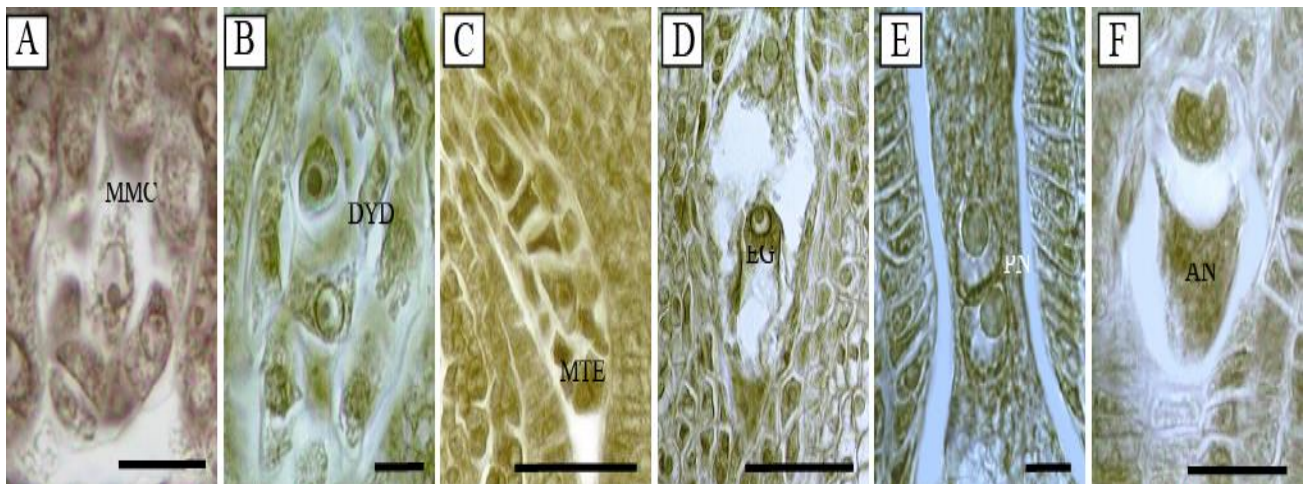


Fig. 8. Development of embryo sac in *Salvia viridis*. A. Megaspor mother cell. B. Dyad cells. C. Megaspor tetrad. D. Egg cell. E. Polar nuclei. F. Antipodal cells. MMC: Megaspor mother cell, DYD: Dyad, MTE: Megaspor tetrad, EG: Egg cell, PN: Polar nucleus, AN: Antipod. Bar: 10 µm (A, B, E), 50 µm (C, D, F).

Discussion

In Lamiaceae, the flower ontogeny has been studied in only a few genera for instance *Marrubium*, *Phlomis*, *Stachys* and *Mentha* (Naghiloo *et al.*, 2013). This is the first detailed report on the flower ontogeny and development of female and male reproductive organs in *S. viridis*.

Although *Salvia sclarea* has paniculate type of inflorescence (Özdemir & Şenel, 1999), *Scutellaria pinnatifida* (Naghiloo *et al.*, 2014) and *S. viridis* have raceme type of inflorescence. But the flowers are zygomorphic in all the species.

The development of a flower starts with the transformation of apical meristem into the floral meristem in *S. viridis*. The morphological changes, observed during the transformation of apical meristem to floral meristem, the apical meristem grows in size, apical apex widens and flattens (Grant, 1994; Fuentes-Granados & Widrechner, 1996; Ikeda *et al.*, 2007; Çetinbaş & Ünal, 2012). According to the study of Teeri *et al.* (2006), the flattening and expansion of floral meristem occurs as a result of the increase in division rates of the cells in the centre of floral meristem. As in other plants (Uhl, 2011), all floral organ primordia in *S. viridis* develop as a result of periclinal division of floral meristem cells. Similar with *Mentha piperita* (Naghiloo *et al.*, 2014), firstly stamens and then carpels differentiate from floral meristem in *S. viridis*. Female organ development starts with the arise of carpel primordium in the center of floral meristem, as it is a usual manner in flowering plants (Gasser & Beers, 1993). Two carpels appear in the center of the flower as in *Mentha piperita* (Naghiloo *et al.*, 2014).

Even though, the androecium consists of four stamens in *Scutellaria pinnatifida* (Naghiloo *et al.*, 2014), *S. viridis* has two stamens. Genus *Salvia* is featured by lever-like stamens. The stamen structure of *S. viridis* is quite similar with that of *S. argentea*. According to the study of Bockhoff *et al.* (2004), the filament of *S. argentea* which has a protrusion close to the base, ends with an inefficient anther. In contrast, the protrusion near the base of the filament does not bear an anther and show no further differentiation in *S. viridis*.

In Lamiaceae, the anthers are bisporangiate as in *Rosmarinus officinalis* (Jiménez *et al.*, 1995) and in *S. viridis* or tetrasporangiate as in *Calamintha umbrosa* (Dwivedi, 1985). In *S. viridis*, the anther wall typically consists of epidermis, endothecium, middle layer and the tapetum resemble with *Rosmarinus officinalis* (Jiménez *et al.*, 1995). The tapetum of *S. viridis* is of the secretory (parietal) type, that is a prevalent situation in Lamiaceae (Johri *et al.*, 1992). But in some Lamiaceae species such as *S. mellifera* (Carlson & Stuart, 1936), and *Calamintha umbrosa* (Dwivedi, 1985) tapetum is dimorphic. The tapetal cells of *Rosmarinus officinalis* (Jiménez *et al.*, 1995), are initially uninucleate, become binucleate while development goes on. The endothecium develops fibrous thickenings as in *Calamintha umbrosa* (Dwivedi, 1985).

The meiotic division of pollen mother cell is of simultaneous type in *Salvia viridis*. Although microspore tetrads are tetrahedral types in *S. viridis*, *Calamintha umbrosa* have decusate type of microspore tetrads (Dwivedi, 1985).

Pollen grains of *S. viridis* are pericarpate and tectatae. In *R. officinalis* (Jiménez *et al.*, 1995), mature pollen are hexacolpate and they are hexaporate in *Calamintha umbrosa* (Dwivedi, 1985). Although, the intine is clearly bilayered in *R. officinalis* (Jiménez *et al.*, 1995), is monolayered in *S. viridis*.

S. viridis and *Mentha suaveolens* have bifid stigma (Naghiloo *et al.*, 2014). Similarly to all examples of family Lamiaceae (Junell, 1934; Kamelina & Dzevaltovsky, 1987; Ryding, 1995), the ovary in *S. viridis* is in upper position and has 4-loculi. In each loculus, an anatropous, tenuinucellate unitegmic ovule forms and develops as in *S. nemorosa* (Daskalova, 2004) and *Calamintha umbrosa* (Dwivedi, 1985). Noral macrosporogenesis in the megaspore mother cell takes place, resulting in a linear macrospore tetrad. Development of the female gametophyte follows the Polygonum type, the only type specified so far within the entire family Lamiaceae (Davis, 1966; Kamelina & Dzevaltovsky, 1987) and in most of the examined species of genus *Salvia* (Carlson & Stuart, 1936).

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