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*Regular Article*

# Male gametophyte development of *Silene sangaria* Coode & Cullen (Caryophyllaceae), an endemic species from Turkey

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**Summary** In this study, microspore formation (microsporogenesis), gamete formation (microgametogenesis), and pollen features of *Silene sangaria*, a species endemic to Turkey, was examined cytoembryologically and histochemically. The species is distributed along the Black Sea coast of Turkey. The materials were collected from the coast of Igneada village (Kirklareli province). The anthers, separated by size, were passed through ethyl alcohol concentration series, and embedded in historesin. Sections were sliced using a rotary microtome and stained with toluidine blue O for general histological observations, Coomassie brilliant blue for proteins, and periodic acid-Schiff for insoluble polysaccharides. The aceto-orcein squash technique was used for cytological observations, and lactophenol-aniline blue solution was used to assess pollen viability. The anthers of *S. sangaria* are tetrasporangiate, and its anther wall development is of basic type. The tapetum is secretory type, and cytokinesis is simultaneous type. As a result of meiotic division of microspore mother cells, 43.5% decussate, 28.2% rhomboidal, 21.1% tetrahedral and 7.2% isobilateral tetrads occur. The released microspores first pass through the first pollen mitosis to form vegetative and generative cells, then the generative cell passes through the second pollen mitosis to form two sperm cells. Pollen grains are three-celled when released from the anther. Pollen viability rate is high (91.82%). Mature pollen grain contains a high concentration of insoluble polysaccharide and protein.

**Keywords** *Silene sangaria*, Caryophyllaceae, Anther wall, Microsporogenesis, Microgametogenesis, Histochemistry.

The family Caryophyllaceae, represented by about 100 genera and 3,000 species, is mainly distributed in the temperate and warm regions of the northern hemisphere. The center of diversity of the family is the Eastern Mediterranean and Irano-Turanian floristic regions (Hernandez-Ledesma *et al.* 2015). In Turkey, the family is represented by 35 genera and more than 540 species, ranking third in the Flora of Turkey in terms of the number of taxa (Güner *et al.* 2012). *Silene* L., the largest genus of the Caryophyllaceae family with approximately 700 species, has a wide distribution in the northern hemisphere (Martin-Gomez *et al.* 2022). Turkey and the Southern Balkan Peninsula are the main centers of diversity for the genus (Greuter 1995). The genus is distributed in Turkey with 166 species, 72 of which are endemic, and the endemism ratio is 43.3% (Coode and Cullen 1967, Davis *et al.* 1988, Güner *et al.* 2012).

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*Silene sangaria* Coode & Cullen is a tetraploid ($2n=4x=48$) endemic species among the flora of Turkey and grows only in a limited area on the Black Sea coast (Sakarya, Istanbul and Kirklareli provinces). Its habitat comprises shores, sandy areas, and dunes (Meric and Guler 2013). According to the Red Book of Plants of Turkey, the conservation status of *S. sangaria* is vulnerable (VU) (Ekim *et al.* 2000). In addition, the species is listed in Appendix 1 (species of flora strictly protected), which covers the Convention on the Protection of European Wildlife and Natural Habitats (Council of Europe 1979). Although the genus *Silene* includes about 700 species worldwide, studies on its reproductive biology are very few. In these papers, embryo and endosperm development of *S. conoidea*, the ultrastructure of endosperm and perisperm of *S. alba*, the pollen and tapetum ontogeny of *S. dioica*, and pollen wall ontogeny of *S. pendula* have been investigated (Cook 1909, Heslop-Harrison 1963, Audran and Batcho 1981, Mohana Rao *et al.* 1988). Recently, male and female gametophyte development of an arctic species, *S. involucrata*, and female gametophyte development of a gynodioecious species, *S. muradica*, have been studied in detail (Kellmann-Sopyla *et al.* 2017, Kartal and Tekin 2021).

As can be seen, studies on the reproductive biology of the genus *Silene*, which includes diploid, tetraploid, hermaphrodite, dioecious, and gynodioecious species, are quite limited. The aim of this study is to reveal the male gametophyte development of *S. sangaria* in detail. Abnormalities in meiosis can be seen in tetraploid species, and these abnormalities cause pollen sterility. In this study, the effect of ploidy level on pollen viability of the species was also investigated.

## Materials and methods

Samples were collected from the coast of Igneada village (41°52′58″N, 27°59′32″E, Demirköy district, Kirklareli province, Turkey) in July–August and fixed using ethyl alcohol and acetic acid solution (3 : 1, v/v) in the field. After fixation for 24 h, they were washed in 96% ethyl alcohol and stored in 70% ethyl alcohol at 4°C. The anthers were separated according to their size. To examine meiotic division, slides were prepared by aceto-orcein (1%) squash method (La Cour 1941). For pollen viability, mature pollen grains from the fully opened flowers were harvested into petri dishes. Lactophenol-aniline blue staining solution was used to examine pollen viability (Kearns and Inouye 1993). After 20 min, fully stained pollen grains were considered fertile, and unstained pollen grains were considered sterile (nonliving). A total of 5,000 pollen grains were counted, and the viability percentage was calculated.

For histological observations, anthers of various sizes were dehydrated through a series of increasing ethyl alcohol concentration, then embedded in historesin according to the manufacturer's instructions (Leica Historesin Embedding Kit, Leica Biosystems Heidelberg, Germany) (Kartal and Tekin 2021). Sections of 4 µm thickness were taken from the embedded blocks using a rotary microtome with a tungsten carbide blade (RM 2255, Leica Biosystems, Germany). Sections taken were stained in 0.5% toluidine blue O solution for 2 min at 60°C on a hot plate, then washed in distilled water for 30 s and dried in air (O'Brien *et al.* 1964, modified). Slides were sealed with Entellan. Microscopy images were taken using an Olympus CX21 microscope (Tokyo, Japan) with a Kameram software program (Argenit, Türkiye).

For insoluble polysaccharides, the periodic acid-Schiff reaction (PAS) was applied. Sections of 4 µm thickness were kept in 1% periodic acid solution in 90% ethyl alcohol for 30 min, then rinsed with distilled water. After staining with Schiff's reagent for 30 min, the slides were immersed in 0.5% sodium metabisulfite solution three times for 5 min. They were then washed for 5 min in running water and rinsed in distilled water. Next, the slides were dried in air and mounted with Entellan (Jensen 1962). For proteins, the sections were stained with 0.025% Coomassie brilliant blue (w/v) in distilled water/acetic acid/methanol (v/v/v, 87 : 10 : 3) solution for 10 min in an oven (60°C). Then, they were washed for 2 min in distilled water. Finally, the slides were dried in air and sealed with Entellan (Heslop-Harrison *et al.* 1973).

## Results

### *Anther wall development*

In *S. sangaria*, the anthers are tetrasporangiate. The anther wall consists of an epidermis, an endothecium,

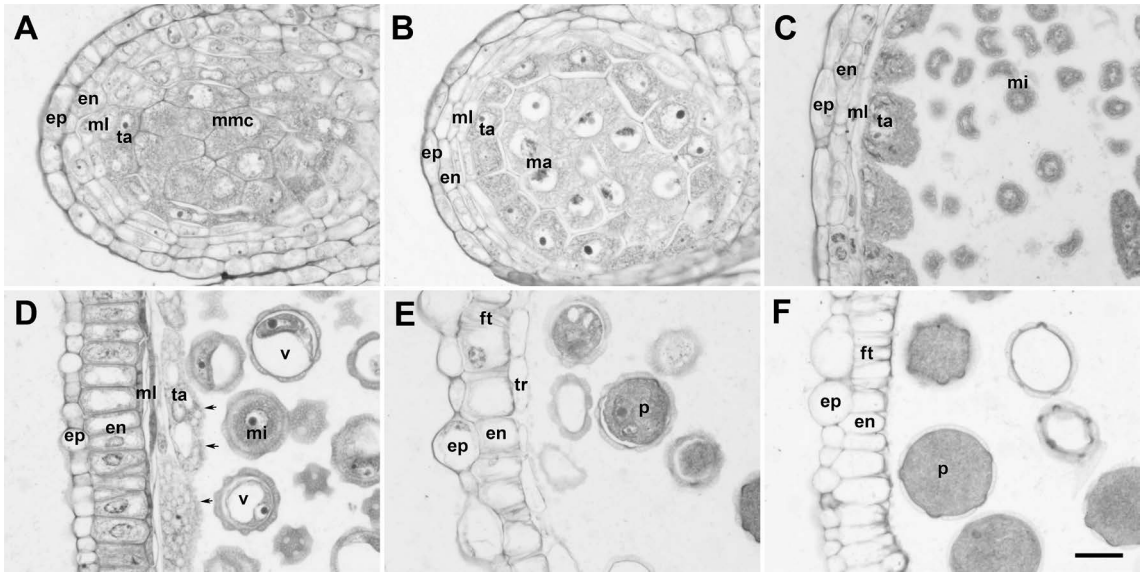



**Fig. 1. Anther wall development stages of *S. sangaria*.** (**A**) microspore mother cell stage; (**B**) microsporocyte stage; (**C**) early microspore stage; (**D**) vacuolated microspore stage (the arrows show secretion of tapetal cells); (**E**) two-celled pollen stage; (**F**) mature pollen grain stage. en: endothecium; ep: epidermis; ft: fibrous thickenings; ma: microsporocytes; mi: microspore; ml: middle layer; mmc: microspore mother cell; p: pollen grain; ta: tapetum; tr: tapetal remnants; v: vacuole. Scale bar=20 µm.

two middle layers, and a tapetum. Anther wall formation is of the basic type, and tapetum is of the secretory type (Fig. 1D). Tapetum cells begin to divide before microspore mother cells (Fig. 1A). When the microspore mother cells begin to divide by meiosis, the tapetum cells increase significantly in size (Fig. 1B). When meiotic division is complete and microspores are released in the anther locule, the middle layers flatten, and the tapetum cells further increase in size (Fig. 1C). At the vacuolated microspore stage, endothecium cells elongate perpendicular to the axis of the anther wall, and the middle layers become flatter. Tapetum cells begin to deteriorate by vacuolating, and the secretory substances they give to the anther locule become evident (Fig. 1D). At the two-celled pollen stage, fibrous thickenings are seen in the endothecium cells, which increase in size. The middle layers disappear completely, and only the walls of the tapetum cells remain (Fig. 1E). At the mature pollen stage, the anther wall consists of an epidermis and a fibrous thickened endothecium (Fig. 1F).

*Microsporogenesis*

Microspore mother cells of *S. sangaria* were observed with large nuclei and prominent nucleoli (Fig. 2A). The chromosomes begin to appear at the leptotene stage. They become more prominent due to chromosome pairing during the zygotene stage, and the callus wall begins to accumulate at this stage (Fig. 2B). The chromosomes continue to shorten and thicken in the pachytene and diplotene stages (Fig. 2C, D); after the diakinesis stage, the nuclear membrane and nucleoli disappear (Fig. 2E). During metaphase I, the chromosomes are aligned on the equatorial plate and during anaphase I, homologous chromosomes are pulled to the poles (Fig. 2F, G). At the telophase I stage, the nuclear membrane and nucleoli reappear (Fig. 2H). The cytokinesis of *S. sangaria* is of simultaneous type. After a short period of interkinesis and prophase II stages (Fig. 2I), the chromosomes shorten and thicken, and they enter metaphase II stage. During the metaphase II stage, the chromosomes are aligned on the equatorial plate, and in the anaphase II stage, the chromatids of the chromosomes are regularly pulled to the poles (Fig. 2J, K). In telophase II stage, chromatids reaching opposite poles begin to unravel, and then the nuclear membrane and nucleolus reappear (Fig. 2L). At the end of the meiotic division in *S. sangaria*, four types of tetrads are formed; decussate, rhomboidal, tetrahedral, and isobilateral. The shape of the tetrad is determined by the arrangement of the spindle apparatus during metaphase II stage. The type of tetrad is clearly observed during telophase II stage (Fig. 3A–D). The ratios of the tetrad types seen are 43.5% decussate (Fig. 3E), 28.2% rhomboidal (Fig. 3F), 21.1% tetrahedral (Fig. 3G), and 7.2% isobilateral (Fig. 3H). The meiosis of *S. sangaria*, a tetraploid plant, is quite regular. Abnormalities such as chromosome bridges and lagging chromosomes are rarely seen during meiosis (2%).

*Pollen development*

Within the anther locule of *S. sangaria*, sporogenous tissue is formed directly into microspore mother cells (Fig. 4A). Microspore mother cells undergo meiosis

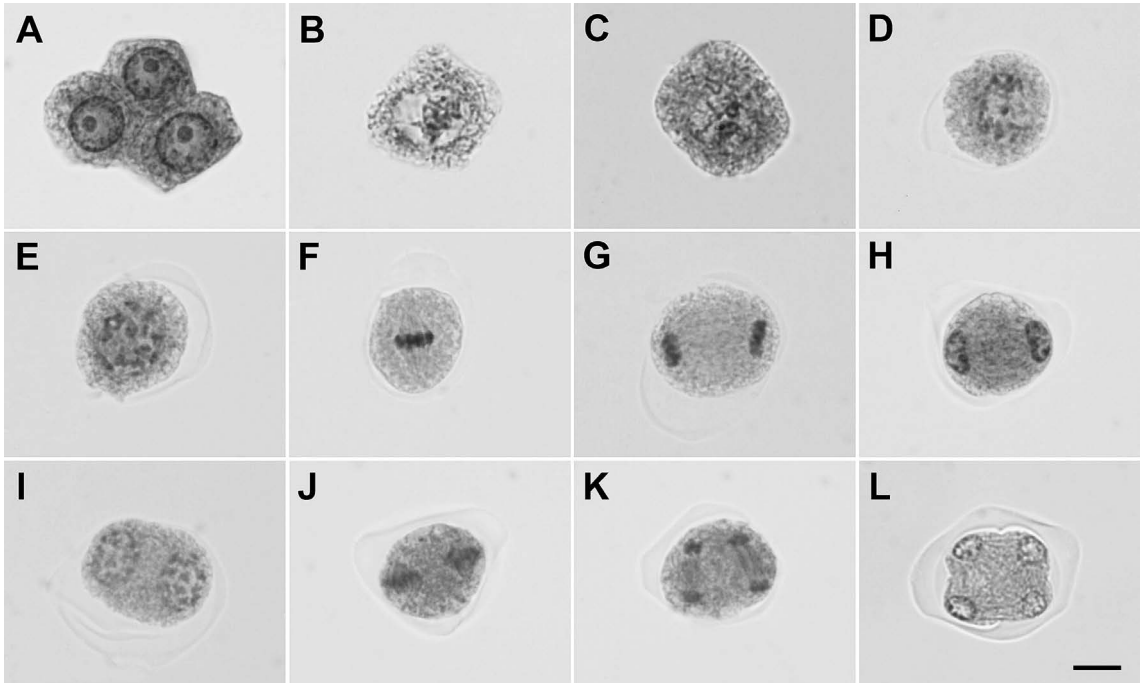



**Fig. 2. Stages of microsporogenesis in *S. sangaria*.** (**A**) microspore mother cells; (**B**) zygotene; (**C**) pachytene; (**D**) diplotene; (**E**) diakinesis; (**F**) metaphase I; (**G**) anaphase I; (**H**) telophase I; (**I**) prophase II; (**J**) metaphase II; (**K**) anaphase II; (**L**) telophase II. Scale bar=10 μm.

(Fig. 4B–D), and free microspores are formed (Fig. 4E). The microspores, initially small in size, swell by absorbing fluid from the anther locule. A large vacuole forms inside the pollen, pushing the nucleus aside, and the first pollen mitosis occurs (Fig. 4F). Thus, two cells, vegetative and generative, are formed in the pollen (Fig. 4G). Spermatogenesis (formation of sperms by second pollen mitosis) takes place while the pollen is inside the anther locule. The prophase (Fig. 4H), metaphase (Fig. 4I), anaphase (Fig. 4J) and telophase (Fig. 4K) stages of

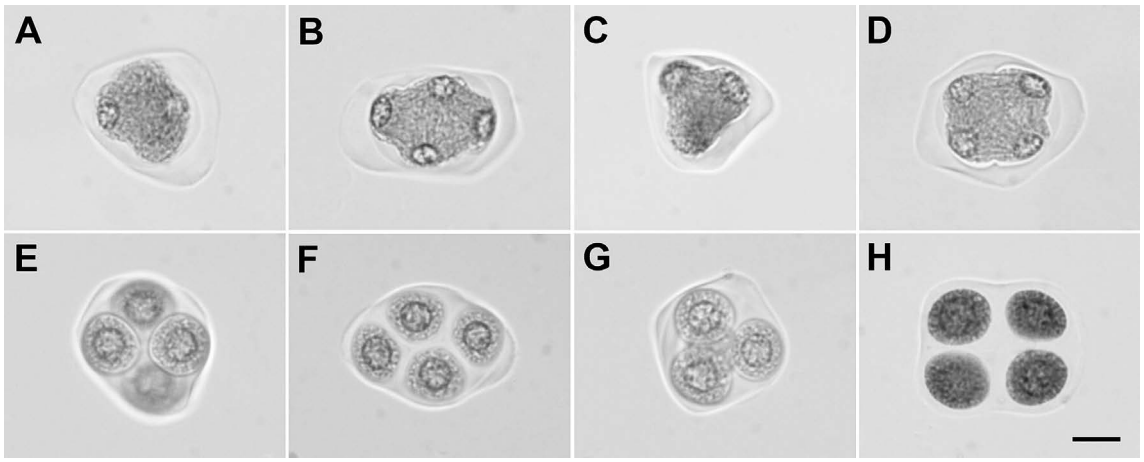



**Fig. 3. Telophase II and tetrad types in *S. sangaria*.** (**A**) decussate-type telophase II; (**B**) rhomboidal-type telophase II; (**C**) tetrahedral-type telophase II; (**D**) isobilateral-type telophase II; (**E**) decussate-type tetrad; (**F**) rhomboidal-type tetrad; (**G**) tetrahedral-type tetrad; (**H**) isobilateral-type tetrad. Scale bar=10 μm.

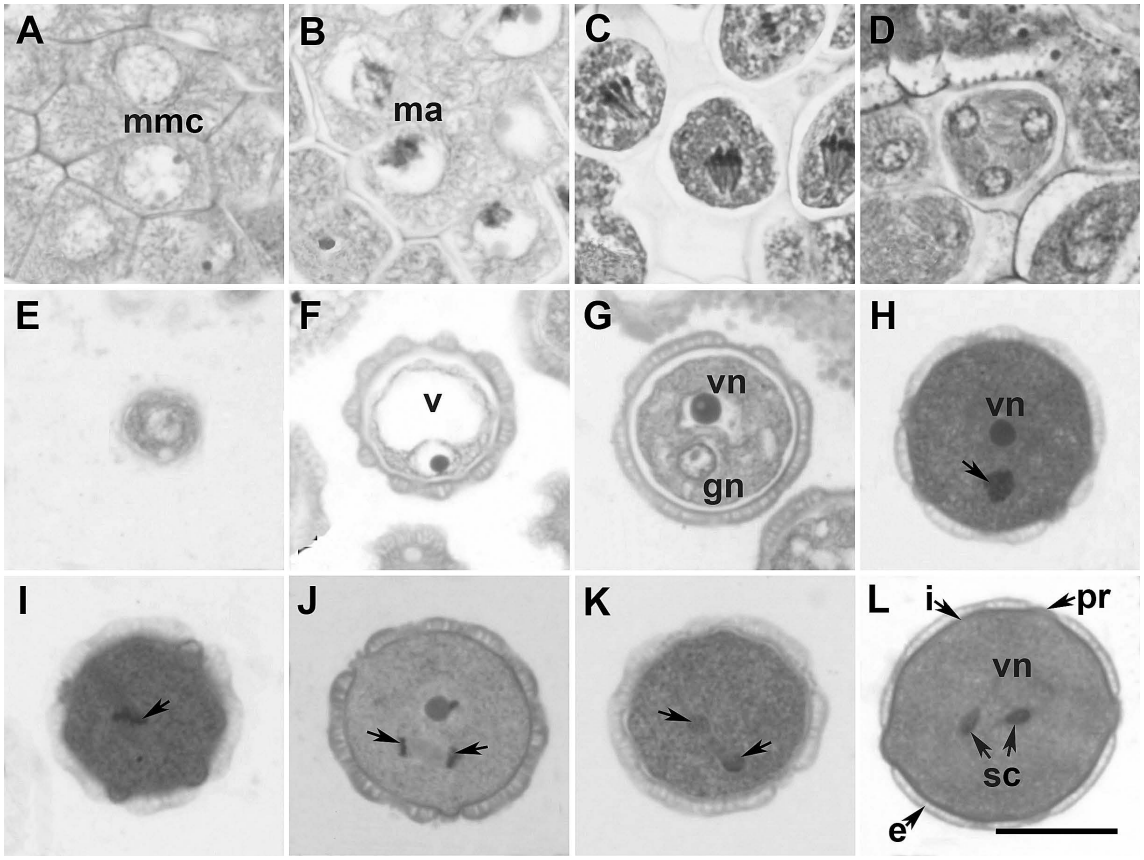



**Fig. 4. Pollen development in *S. sangaria*.** (**A**) microspore mother cells; (**B**) microsporocytes; (**C**) metaphase I stage of meiosis; (**D**) telophase II stage of meiosis; (**E**) microspore; (**F**) vacuolated microspore; (**G**) two-celled pollen; (**H**) prophase stage of second mitosis (arrow); (**I**) metaphase stage of second mitosis (arrow indicates chromosomes arranged on the metaphase plate); (**J**) anaphase stage of second mitosis (arrows indicate chromosomes at poles); (**K**) telophase of second mitosis (arrows indicate chromosomes at poles); (**L**) three-celled mature pollen. e: exine; gn: generative nucleus; i: intine; ma: microsporocytes; mmc: microspore mother cells; pr: germination pore; sc: sperm cells; vn: vegetative nucleus. Scale bar=20 μm.

mitosis occur regularly. At the end of the second pollen mitosis, two sperm cells are formed with very little cytoplasm, surrounded by a plasma membrane (Fig. 4L). Sperm cells are initially round, then take the form of spindles. When pollen is expelled from the anther, it is three-celled. Pollen viability is high (91.82%) as a result of regular meiosis and mitosis divisions. Fertile pollen grains are stained, large, and uniformly shaped, while sterile pollen grains are unstained and small.

*Histochemistry of the anther wall and pollen*

At the beginning of development, all wall layers and microspore mother cells give positive reaction to staining with Coomassie brilliant blue in the anthers (not shown). Also in the meiosis stages, the epidermis, endothecium, middle layer, tapetum cells, and meiocytes show positive reaction (Fig. 5A). The middle layer and tapetum, which give a strong positive reaction in the young pollen stage, break down in the mature pollen stage and transfer the proteinaceous substances to the maturing pollen grains (Fig. 5B). At this stage, the epidermis and endothecium cells are poor in cytoplasmic protein content, while the cytoplasm of the mature pollen grain is full of protein (Fig. 5C).

At the beginning of development in the microspore mother cell stage, the epidermis, endothecium, and middle layers contain small amounts of insoluble polysaccharides. No polysaccharides are observed in the tapetum and microspore mother cells (not shown). During meiotic division, intense polysaccharide deposition is observed in the epidermis and endothecium cells, while the middle layers, tapetum, and microspore mother cells show weak PAS positive reaction (Fig. 5D). At the young pollen stage, the epidermal, endothecial, and middle layers show positive PAS reaction, while the tapetum and microspores show negative PAS reaction (Fig. 5E). In the mature pollen stage, no polysaccharides are observed in the anther wall layers, while the cytoplasm of the pollen is filled with insoluble polysaccharides. The intine gives positive reaction for insoluble polysaccharides, while the exine gives negative PAS reaction (Fig. 5F).

## Discussion

In this study, male gametophyte development of endemic *S. sangaria*, which was introduced to the scientific world in 1967 based on specimens collected from Karasu town of Sakarya province, was investigated cytoembryologically and histochemically. The distribution area of the species in Turkey includes Sakarya (Karasu), İstanbul (Kumkoy), and Kirklareli (Igneada) (Coode and Cullen 1967, Meric and Guler 2013). The chromosome number of the species is $2n=4x=48$ (Meric and Guler 2013). There are no studies on the embryology of the species.

*S. sangaria* shows the characteristics of the Caryophyllaceae family in terms of anther structure and development (Davis 1966). Anthers are tetrasporangiate, and the anther wall consists of an epidermal layer, an endothecial layer, two middle layers, and a tapetum layer. The anther wall development of *S. sangaria* is of the basic type. In previous studies, basic, monocotyledonous, and dicotyledonous anther wall development types have been reported in the family Caryophyllaceae (Davis 1966, Qu *et al.* 2010, Wang *et al.* 2017). The species has a secretory-type tapetum, which is the general type of the family Caryophyllaceae. Similar findings have been reported in *S. dioica* by Audran and Batcho (1981) and

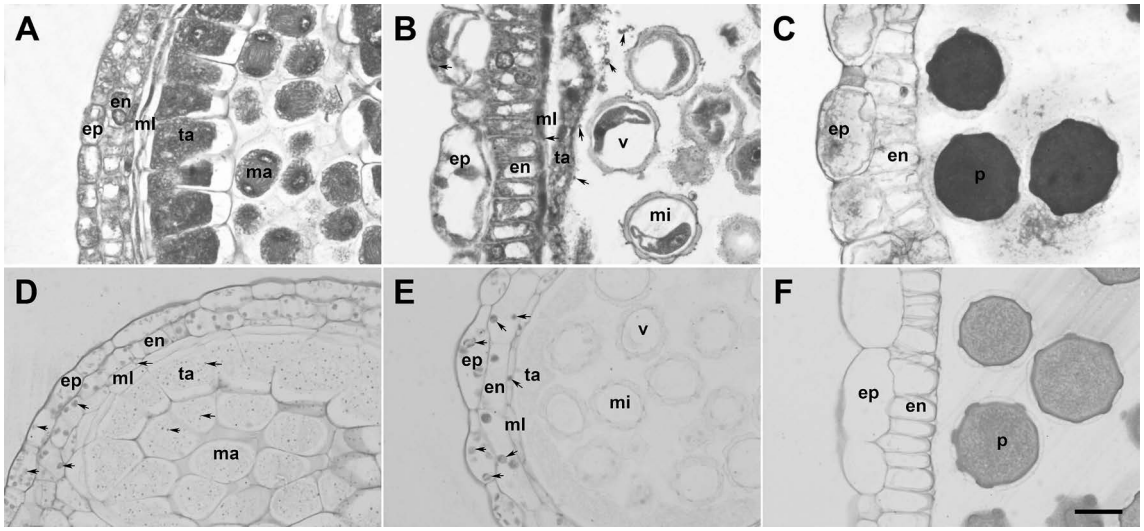



**Fig. 5. Protein and insoluble polysaccharide content in anther wall and pollen of *S. sangaria*.** (**A**) protein content at the microsporocyte stage; (**B**) protein content at the vacuolated microspore stage (arrows indicate protein secretion of tapetum cells); (**C**) protein content at the mature pollen stage; (**D**) insoluble polysaccharide content at the microsporocyte stage (arrows); (**E**) insoluble polysaccharide content at the vacuolated microspore stage (arrows); (**F**) insoluble polysaccharide content at the mature pollen stage. en: endothecium; ep: epidermis; ma: microsporocytes; mi: microspore; ml: middle layer; p: pollen grain; ta: tapetum; v: vacuole. Scale bar=20 μm.

in *S. involucrata* by Kellmann-Sopyla *et al.* (2017).

In microspore mother cells of *S. sangaria*, meiosis is usually normal (98%), and simultaneous cytokinesis is observed. At the end of meiosis, 43.5% decussate, 28.2% rhomboidal, 21.1% tetrahedral, and 7.2% isobilateral tetrads are formed. Matamoro-Vidal *et al.* (2016) report that tetrads formed in *S. latifolia* are 81.5% tetrahedral, 17.9% rhomboidal, and 0.5% isobilateral. Also, Prieu *et al.* (2019) report that tetrads were 81% tetrahedral and 19% rhomboidal in *S. pendula*. In this study, the decussate tetrad type is reported for the first time in the genus *Silene.* Although *S. sangaria* is a tetraploid species (Meric and Guler 2013), meiosis and pollen mitosis are quite regular (98%). Chromosome stickiness, chromosome bridges, micronuclei, triads, pentads, and unreduced pollen grains (2*n*) in the meiotic division of three *Silene* species (*S. gynodiocia, S. crispans*, and *S. indeprensa*) have been reported by Sheidai *et al.* (2011). The mature pollen of *S. sangaria* is expelled from the anther as three-celled (vegetative cell and two sperm cells). This is a general feature for the Caryophyllaceae family (Davis 1966). Pollen viability of *S. sangaria* is high (91.82%). Mayer and Gottsberger (2000) report pollen viabilities of 89% for *S. alba*, 84% for *S. pendula*, 82% for *S. vulgaris*, and 93% for *S. dichotoma*. These data show that the pollen viability of *Silene* genus is high.

In *S. sangaria*, all anther wall layers and microspore mother cells contain equal levels of protein at the microspore mother cell stage. During the later stages, the protein in the epidermal and endothelial layers migrates to the middle layers and tapetum. It is then secreted by the tapetum and transferred to pollen grains. The mature pollen grain contains dense protein. There are no studies on the protein contents of the anther wall and pollen in the genus *Silene*, and our study presents the first data on this subject. In the microspore mother cell stage, the epidermal, endothecial, and middle layers contain small amounts of insoluble polysaccharides. Tapetum cells and microspore mother cells are PAS negative. During the meiosis stages, all layers show PAS positive reaction (dense in epidermis and endothecium cells; weak in middle layers, tapetum, and microspore mother cells). The mature anther wall does not contain insoluble polysaccharides, while the mature pollen grains are filled with starch. Baker and Baker (1979) report that the Caryophyllaceae family is among the non-starchy-pollen-containing families. On the other hand, Davis (1966) reports that mature pollen of the genus *Stellaria* contains starch.

This study is the first report on anther wall and pollen development in endemic *S. sangaria*. It has tetrasporangiate anthers and displays a basic type of anther wall development, secretory tapetum, and simultaneous cytokinesis. The resulting tetrad types are decussate (43.5%), rhomboidal (28.2%), tetrahedral (21.1%), and isobilateral (7.2%). Although it is a tetraploid species, meiosis is generally normal (98%), and pollen viability is high (91.82%). The mature pollen grains are three-celled and contain abundant protein and starch. *S. sangaria* has the general characteristics of the Caryophyllaceae family in terms of microsporangium and male gametophyte development.

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