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Development of Female Gametophyte in *Gladiolus italicus* Miller (Iridaceae)

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Abstract. In this study gynoecium, megasporogenesis, megagametogenesis and female gametophyte of *Gladiolus italicus* Miller were examined cytologically and histologically by using light microscopy techniques. Ovules of *G. italicus* are of anatropous, bitegmic and crassinucellate type. Embryo sac development is of monosporic Polygonum type. Polar nuclei fuse before fertilization to form a secondary nucleus near the antipodals. The female gametophyte development of *G. italicus* was investigated for the first time with this study.

Keywords: *Gladiolus italicus*, Iridaceae, embryo sac, Polygonum type.

INTRODUCTION

Iridaceae family is represented by approximately 66 genera and 2245 species in the world (Christenhusz and Byng 2016; Burgt et al. 2019). This family includes ornamentals like *Gladiolus*, *Belamcanda*, *Iris*, *Crocus*, *Eleutherine*, etc. and also plants of commercial value like *Crocus sativa* and *Iris spp.* (Venkateswarlu et al.1980). Genus *Gladiolus* L. has more than 260 species (Goldblatt 1996). 11 *Gladiolus* species are found in various regions of Turkey and 6 of them; *G. anatolicus* (Boiss.) Stapf, *G. attilae* Kit Tan, B. Mathew & A. Baytop, *G. halophilus* Boiss. & Heldr., *G. humilis* Stapf, *G. micranthus* Stapf, *G. osmaniyensis* Sağıroğlu are endemic (Tan et al. 2006; Güner et al. 2012; Sağıroğlu and Akgül 2014). *Gladiolus italicus* is in IUCN Red List category of Turkey. *G. italicus* is distributed in Macaronesia, Mediterranean basin to central Asia. It is also introduced and naturalized in California. It naturally grows in many parts of Turkey (Demir and Çelikel 2019). It is a monocotyledon with spectacular flowers (Tan and Edmondson 1984).

Gladiolus corms are used in the treatment of dysentery and gonorrhoea in some countries of Africa (Nguedia et al. 2004). In Turkey, it is known as an aphrodisiac and it is known to have emetic property (Baytop 1999). *G. italicus* and *G. atroviolaceus* corms are used in ice cream and also in other

dairy foods (Öztürk and Özçelik 1991). The chemical composition of *Gladiolus* plants is studied. Demeshko et al. (2020) studied carboxylic acid content of *G. hybridus* leaves. The chemical composition of the essential oil and the antibacterial, antifungal and antioxidant properties of the essential oil extract of *G. italicus* are investigated by Üçüncü et al. (2016). The seed testa structure of *G. italicus* is studied with scanning electron microscope by Erol et al. (2006). Üzen (1999) studied *G. italicus* morphologically and anatomically. It is also studied karyologically and cytologically. The chromosome numbers of the species are resulted $2n=30$, 60 (Iran) and $2n=120$ (Aegean Islands and Spain) in several populations (Perez and Pastor 1994; Kamari et al. 2001; Fakhraei et al. 2011). In addition, chromosome numbers such as $2n = 60, 90, 110-120, 120, \sim 170, 176$ are reported by other researchers (Ohri and Khoshoo 1985; Van Raamsdonk and De Vries 1989). Mensinkai (1939) reported that cytomixis was observed in meiosis in a study of four *Gladiolus* species (*G. tristis*, *G. byzantinus*, *G. primulinus*, and *G. dracocoe*) mitosis and meiosis divisions. The pollen morphology of *G. italicus* is examined using light and scanning electron microscopy by Dönmez and Işık (2008). They described the pollen grains of *G. italicus* as monosulcate, heteropolar, elliptic, spinulose-perforate and tectate-collumellate (Dönmez and Işık 2008).

Embryological studies in family Iridaceae are rather limited. Studies about the development of the embryo sac are reported by Davis (1966) in *Iris japonica* and *I. tenax*. Then other studies are done in *Sisyrinchium striatum* and *S. californicum* by Lakshmanan and Philip (1971) and *Crocus sativus* and *C. thomasii* by Chichiricco (1987, 1989).

The aim of this study is to determine the development of female gametophyte in *G. italicus*. Cytological and embryological features of *G. italicus* have not been studied yet. This study is also an attempt toward a better understanding of taxonomic relationships between closely related taxa within the Iridaceae and are indirectly useful to the efforts to protect this species *in vitro*.

MATERIALS AND METHODS

In this study, *G. italicus* plants were collected from Höyükütatar village of Edirne A1 (E) in European Turkey. They were brought to the Botanical Garden of Trakya University. Voucher specimens were placed in the Herbarium of Trakya University (EDTU). Ovaries were examined under an Olympus SZ61 stereomicroscope. For cyto-histological studies, flowers and buds were fixed in Carnoy's fluid (3:1, ethyl alcohol: acetic

acid). Dehydration process was done with increasing alcohol series for 24 hours (70%, 80%, 90%, 96%, absolute alcohol). Then, the material was kept in a mixture of 1 absolute alcohol: 1 basic resin + activator for 24 hours. After being kept in pure resin for 24 hours, the next day, they were embedded in historesin (Leica, Historesin-embedding kit), which was prepared by adding hardener to the basic resin and activator mixture in an appropriate ratio according to manufacturer's protocols (Leica Microsystems, Nussloch). Semi-thin (2 μ m) sections taken from the materials embedded in historesin with Leica RM2255 rotary microtome and stained with 1% Toluidine blue (O'Brien et al. 1964). Slides were examined with an Olympus CX31 microscope and photographed by Progress C12 camera.

RESULTS

Gynoecium

Gynoecium of *Gladiolus italicus*, contains a pistil with inferior ovary, a long style and a three-lobed, spatulate stigma (Figure 1). *G. italicus* has a trilocular, syncarpous ovary. In the ovary 22-24 ovules are marginal-central placented (Figure 2).

Megasporangium

Ovules of *G. italicus* are anatropous, crassinucellate and bitegmic. The outer integument consisted of 5-6 cell layers and the inner integument consisted of 2 cell lines. The micropyle is formed by the inner integument. The inner and outer integuments are five- to seven-layered around the micropyle (Figure 3a).

Megasporogenesis

One of the sub-epidermal cells at the tip of the ovule of *G. italicus* differentiates to form a megaspore mother cell (MMC). The MMC cell forms deep within the nucellus. It has a large volume and larger nucleus and is easily distinguishable from other cells (Figure 3b).

As the outer integument become apparent, the first meiotic division begins in the MMC (Figure 3c). The volume of the MMC increases during meiosis (Figure 3d). A dividing wall is formed between the two nuclei after meiosis I. After a short period, also meiosis II is completed. A linear megaspore tetrad forms as a result of meiosis of the MMC (Figure 3e).

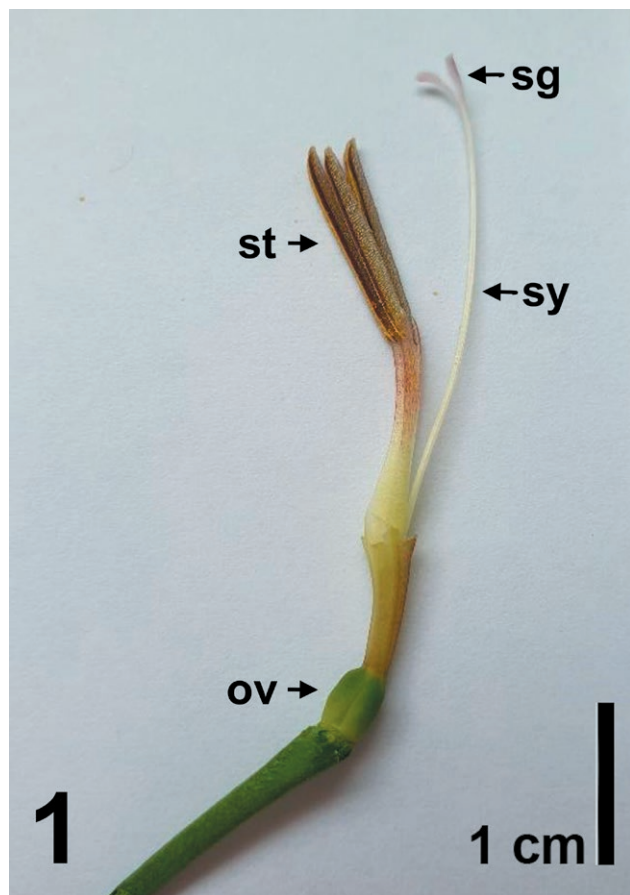


Figure 1. Pistil and stamens of *Gladiolus italicus*. (ov, ovary; sg, stigma; st, stamens; sy, style).

Megagametogenesis

After megalporogenesis, atrophy of the three megaspores on the micropylar side occurred. Then, the active megaspore on the chalazal side began mitosis. When the active megaspore divided into two nuclei at the end of the first mitosis, they moved towards the opposite poles and a large central vacuole was formed between them (Figure 3f).

The nuclei in the poles enters in the second mitosis and an embryo sac with 4 nuclei is formed (Figure 3g). After the third mitosis, there are eight nuclei in the embryo sac.

Female gametophyte

The embryo sac of *G. italicus* is of the *Polygonum* type. It has eight nuclei and seven cells. It shows a clear polarization. The mature embryo sac contains an egg apparatus, three antipodal cells and a central cell with

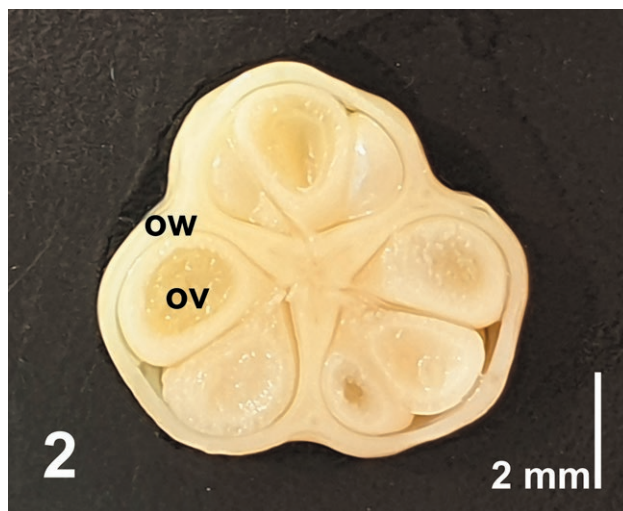


Figure 2. Cross section of *Gladiolus italicus*' ovary (ov, ovule; ow, ovary wall).

two polar nuclei. The polar nuclei fuse before fertilization and form a secondary nucleus near the antipodal cells.

Antipodal cells

Antipodal cells are haploid. They have densely stained cytoplasm and evident, large nucleolus (Figure 3h). They are surrounded by whole cell wall. Their chalazal sides are embedded within the hypostasis. (Figure 3i).

Central cell

The central cell is located in the middle of the embryo sac, initially contains a polar nucleus on the micropylar side and a polar nucleus near the antipodal cells on the chalazal side. The nucleus on the micropylar side moves to the side of the polar nucleus, which is located close to the chalazal side (Figure 3j). These two polar nuclei fuse before fertilization and they form the secondary nucleus near the antipodal cells. (Figure 3k).

Egg apparatus

The egg apparatus is located on the micropylar side of the embryo sac. It consists of two synergid cells and an egg cell. The synergid cells form the filiform apparatus with their walls towards the micropylar side. The filiform apparatus enlarges the wall surface of the synergid cells. This facilitates nutrient and water intake. One of the synergid cells begins to degenerate before fertiliza-

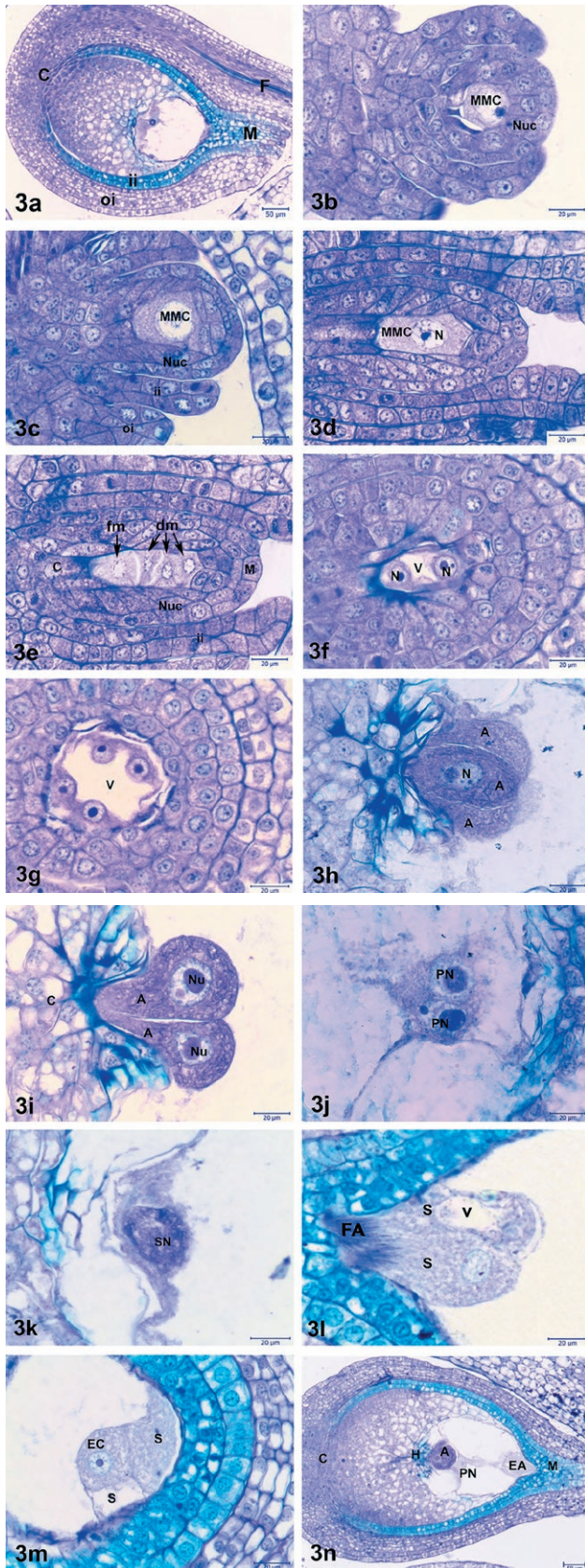


Figure 3. Female gametophyte in *Gladiolus italicus* and its developmental stages; **3a**, ovule of *G. italicus*; **3b**, Crassinucellate ovule of *G. italicus*; **3c**, Bitegmic ovule of *G. italicus*; **3d**, Prophase I phase of meiosis in megaspore mother cell in *G. italicus*; **3e**, *G. italicus*' linear type of megaspore tetrad; **3f**, Two-nucleate embryo sac in *G. italicus*; **3g**, Four-nucleate embryo sac in *G. italicus*; **3h**, Antipodal cells of *G. italicus*; **3i**, Densely stained cytoplasm and nucleoli in the antipodal cells of *G. italicus*; **3j**, Polar nuclei of *G. italicus*; **3k**, Secondary nucleus of *G. italicus*; **3l**, Synergid cells of *G. italicus*; **3m**, Egg apparatus of *G. italicus*; **3n**, Hypostase in *G. italicus*. (A, antipodal cell; C, chalaza; dm, degenerated megaspores (arrows); EA, egg apparatus; EC, egg cell; F, funiculus; FA, filiform apparatus; fm, functional megaspore (arrow); H, hypostase; ii, inner integument; MMC, megaspor mother cell; M, micropyle; N, nucleus; Nu, nucleolus; Nuc, Nucellus; oi, outer integument; PN, polar nucleus; S, synergid; SN, secondary nucleus; V, vacuole).

tion and a vacuole is formed (Figure 3l). The egg cell is located between the two synergid cells in the embryo sac (Figure 3m).

Hypostasis

In the advanced stages of embryo sac development, tissue differentiation is observed on the chalazal side, in *G. italicus*. This tissue is named as hypostasis and it differentiates from the tissue in the nucellus between integuments and the chalaza. The hypostasis in the embryo sac of *G. italicus* consists of cells with thickened walls and it is cup-shaped (Figure 3n).

DISCUSSION

In this study, the developmental stages of the embryo sac in *G. italicus* is presented for the first time by using light microscopy techniques.

G. italicus shows the characteristics of the Iridaceae family in terms of ovules and embryo sac development (Davis 1966; Lakshmanan and Philip 1971; Chichiricco 1987; 1989; Zhang et al. 2011). 22-24 ovules are located in the inferior, trilobular ovary in *G. italicus*. They are marginal-central placented. Zhang et al. (2011) reported that *Iris mandshurica* had also inferior, trilobular ovary, but its ovules were axial placented. Ovules of *G. italicus* are anatropous, bitegminous and crassinucellate like *Iris mandshurica*. In *G. italicus*, 5-6 cell lines forms the outer integument and 2 cell lines forms the inner integument. The micropyle is formed by the inner integument in both *G. italicus* and *Iris mandshurica* (Zhang et al. 2011). Similar features are observed in *Sisyrinchium striatum* and *S. californicum* (Lakshmanan and Philip 1971), *Crocus sativus* (Chichiricco 1987), *C. thomasi*

(Chichiricco 1989). In previous studies, it was reported that both outer and inner integuments were formed by 2 cell lines in *Sisyrinchium striatum* and *S. californicum* (Lakshmanan and Philip 1971). The inner integument is formed by 2 cell lines in *Crocus thomasi* (Chichiricco G. 1989). The outer integument is formed by 6-8 layers and the inner integument was formed by 4-6 cell lines in *Iris mandshurica* (Zhang et al. 2011). These findings showed that *G. italicus* had characteristics of the Iridaceae family in terms of ovule type and development. It is also closer to *Crocus* and *Sisyrinchium* genera in terms of number of cell lines of the inner integument.

One of the subepidermal cells in the ovule of *G. italicus* differentiates from others to form the megaspore mother cell (MMC). The MMC appears below the nucellus. When the outer integument initiated, the first meiosis was taking place. A cell wall is formed between the two nuclei after meiosis I. After a short rest period, meiosis II is completed. Linear megaspore tetrad occurs as a result of meiosis. Megasporogenesis is of the Polygonum type. In this kind of embryo sac, in the beginning of megagametogenesis, 3 micropylar megaspores are degenerated. The degeneration of the three supernumerary meiotic products is hence a case of developmental programmed cell death (PCD) and it is of interest to investigate its features with the aim of checking resemblance to apoptotic morphological syndrome as described in general and as described for plants (Papini et al. 2011). TEM observations on the degenerating nonfunctional megaspores in *Larix leptolepis* (Sieb. et. Zucc.) Gordon (Pinaceae) showed morphological features that are typical of PCD (Cecchi Fiordi et al. 2002). Then, megasporogenesis and PCD in *Tillandsia* (Bromeliaceae) were studied in a comprehensive study by Papini et al. (2011). The general shrinkage of the cell protoplast and the condensation of the cytoplasm and particularly of the nucleus observed in the degenerating supernumerary megaspores are signs of PCD (Pennell and Lamb 1997). The PCD of the supernumerary megaspores in angiosperms is a deletional PCD, since the developmental program leading to the female gametophyte formation and maturation implies their disappearance (Papini et al., 2011). The chalazal megaspore becomes functional and mature embryo sac is formed after three sequential mitosis. Similar findings have been observed in the previously studied species; *Sisyrinchium striatum*, *S. californicum* (Lakshmanan and Philip 1971) *Crocus sativus* (Chichiricco 1987), *C. thomasi* (Chichiricco 1989), and *Iris mandshurica* (Zhang et al. 2011).

A large number of tissue remodeling occurs during the seed development, with some of the cells being eliminated as a result of PCD. Synergids die during double fertilization (Doronina et al. 2020) Vacuolization is

one of the morphological patterns accompanying PCD in synergids. In *Nicotiana tabacum* (Tian and Russell, 1997), cytoplasmic vacuolization, in *Proboscidea louisianica* (Mogensen, 1978), *Pennisetum glaucum* (Chaubal and Reger, 1993), *Nicotiana tabacum* (Huang and Russell, 1994), *Helleborus bocconeii* (Bartoli et al., 2017) vacuole rupture were seen in synergid cells. In *G. italicus*, vacuolization is also occurred in one of the synergids due to early stage of fertilization.

In *G. italicus*, bowl-like hypostasis with thickened walls is seen and it is densely stained. In some plants, although the walls of the hypostasis are thickened due to substances such as cutin, suberin and lignin, in some plants they remain thin walled. They have a secretory cell structure (Johri et al. 1992). It is reported that thickened walled hypostasis was seen in *Crocus sativus* (Chichiricco 1987) and *C. thomasi* (Chichiricco 1989). In *Leucojum aestivum* (Amaryllidaceae), hypostasis cells are thin-walled and have abundant cytoplasm (Ekici and Dane 2008). There are no reports on hypostasis in *Sisyrinchium striatum*, *S. californicum* (Lakshmanan and Philip 1971) and *Iris mandshurica* (Zhang et al. 2011). Ünal (2011) reported that hypostasis developing from nucellar cells beneath the embryo sac plays a role in preventing embryo growth. They also deliver nutrients from the vascular bundles to the embryo sac. In some taxa they play a role in maintaining the water balance. In the light of these findings, it is seen that *G. italicus* is close to genus *Crocus* in terms of hypostasis.

CONCLUSION

In conclusion, the ovule and the development of female gametophyte of *G. italicus* were studied for the first time and it was seen that the findings obtained from this study were compatible with the previously examined species belonging to the Iridaceae family. PCD occurred when functional megaspore formed at the end of megasporogenesis. It has also occurred in the degeneration of synergid cells. *G. italicus* showed characters of the Iridaceae family in terms of female gametophyte development. Data gained from this study will also contribute to the general knowledge about the embryological characters used in the taxonomy of Iridaceae family.

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