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Cytological and histological studies on female gametophyte of *Leucojum aestivum* (Amaryllidaceae)

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Abstract: In this study, gynoeceum, development of megasporangium, megasporogenesis, megagametogenesis and female gametophyte of *Leucojum aestivum* were examined cytologically and histologically. Ovules of *L. aestivum* are of anatropous, bitegmic and crassinucellate type. Inner integument forms the micropyle. Archesporial cell develops directly into a megasporocyte. Embryo sac development is of bisporic *Allium* type. Filiform apparatus is observed in synergids. Polar nuclei fuse before fertilization to form secondary nucleus near the antipodals.

Key words: *Leucojum aestivum*; Amaryllidaceae; megasporogenesis; megagametogenesis

Introduction

Family Amaryllidaceae has 60 genera and 800 species according to recent records (Watson & Dallwitz 2005). Genus *Leucojum* has only two species: *L. vernum* and *L. aestivum* but *L. aestivum* has two sub-species: subsp. *aestivum* and *pulchellum* (Crellin 2005). *L. aestivum* is spread naturally in the North Africa, Europe, Southeast Asia and in the Mediterranean. (Darlington & Ammal 1945; Crellin 2005). *Leucojum* is represented by "*L. aestivum* subsp. *pulchellum*" in Turkey. This species is distributed in very specific 8 habitats in Turkey (Davis 1984).

In recent years molecular studies done by Meerow et al. (1999) and Ito et al. (2005) provide good support for the monophyly of the Amaryllidaceae and indicate Agapanthaceae as its likely sister family. Aliaceae are in turn sister to the Amaryllidaceae/Agapanthus clade. *Galanthus* and *Leucojum* are supported as sister genera by the Bootstrap (Meerow et al. 1999). It is strongly suggested that the Amaryllidaceae originated in Africa (Ito et al. 2005).

Embryological studies done with Amaryllidaceae are generally missing in almost every genus because male and female gametophytes of these plants develop underground. Embryological characteristics of this family have been gained from the studies done with species in limited numbers and most of the characteristics of this family are doubtful. Both *Allium* and *Polygonum* types of embryo sacs have been observed in the genera of this family (Davis 1966). Embryo sac of *Endymion* type has also been determined in recent records (Dane 1999). The ovules are anatropous to hemianatropous, uni- or bitegmic, rarely without integuments. In some species secondary multiplication of antipodals has been reported. Antipodal cells can be ephemeral or persistent (Watson & Dallwitz 2005). Hypostase is present or absent. Endosperm formation is nuclear (Davis 1966) or hellobial (Dane 1999).

The early developmental stages of buds occur underground. This may be the reason of seldom embryological studies done in Amaryllidaceae. Only endosperm formation of *L. aestivum* has been determined and it is nuclear (Davis 1966). Franke et al. (1977) had made some observations with endosperm cells.

The aim of this study is to determine development of the megasporangium, megasporogenesis, megagametogenesis and organization of the mature embryo sac of *L. aestivum*. It is also an attempt toward a better understanding of taxonomic relationships with closely related taxa within the Amaryllidaceae.

Material and methods

In this study, *L. aestivum* plants (Fig. 1a) were collected from the natural population at Tavuk Forest of Edirne A1 (E) in European Turkey, between April and May of 2004 and 2005. 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 binocular microscope. For cyto-histological studies, flowers and buds (Fig. 1b) were fixed in Carnoy's fluid (3 : 1, ethyl alcohol : acetic acid). Customary methods of dehydration, infiltration, paraffin embedding, microtoming and staining were followed (Johansen 1940). Serial sections of ovaries were cut at the thickness of 6–15 microns and stained with Delafield's hematoxylin. Some of the ovules were fixed in 3% glutaraldehyde (GA) in 0.1M Milloning's phosphate buffer pH 6.8, for 2 hours (Milloning 1962). The ovules were then washed several times in buffer, fixed overnight with 1% buffered OsO_4 and dehydration was made with gradually increasing aceton-propylenoxide series.

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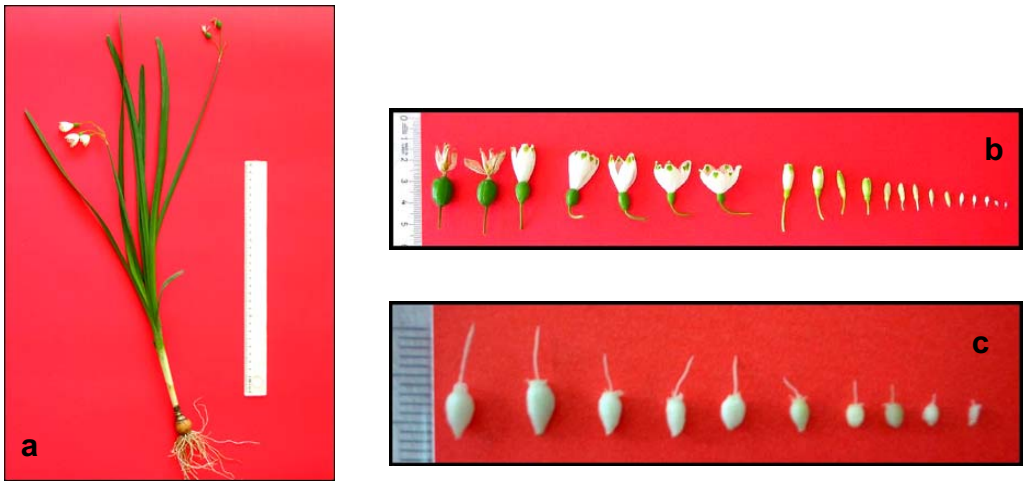

Fig. 1. a – *Leucojum aestivum* L., b – Flowers and buds of *Leucojum aestivum* in different size, c – Gynoeciums of *Leucojum aestivum* in different size.

Fig. 2. a – Cross section of *Leucojum aestivum*'s mature ovary, b – Ovules of *Leucojum aestivum*.

The material was embedded in Epon (Freeman & Spurlock 1962). Semithin sections (1 μm thick) of ovules were stained with toluidin blue (Feder & O'Brien 1968). The slides were examined and photographed by an Olympus photomicroscope.

Results

Gynoeceum

Gynoeceums of *L. aestivum* (Fig. 1c), which were between 3–14 mm in length, were dissected from buds and flowers (Fig. 1b). *L. aestivum* has the trilocular inferior ovary (Fig. 2a). It is observed that 30–40 ovules (Fig. 2b) are marginal-central placented (Fig. 2a).

Development of megasporangium

The ovules are bitegmic (Fig. 3c). Integument protuberances were seen in the ovules at preceding phases when archesporial cell differentiated (Fig. 3a). During the megasporogenesis integuments develop and the ovule becomes hemi-anatropous (Fig. 3b). During megagametogenesis integument protuberances continue to grow and to acquire a shape and the ovule becomes anatropous (Fig. 3c). Micropyle is formed by inner integument. The inner integument is 2– to 3– layered and the outer one 5– to 6– layered around the micropyle (Fig. 3c).

Megasporogenesis

Ovules of *L. aestivum* are crassinucellate (Fig. 4a). The archesporial cell enlarges and functions directly as the megaspore mother cell under 2–3 cell layered epidermis (Fig. 4b, c). Regular meiosis is seen in MMC. Zygonema (Fig. 4d), diakinesis (Fig. 4e, f), metaphase I (Fig. 4g), anaphase I (Fig. 4h) are regular. During diakinesis 11 bivalents were observed (Fig. 4e, f). Caryokinesis is followed by cytokinesis in telophase I (Fig. 4i). It divides meiotically forming 1 dyad (Fig. 4j). Later the micropy-

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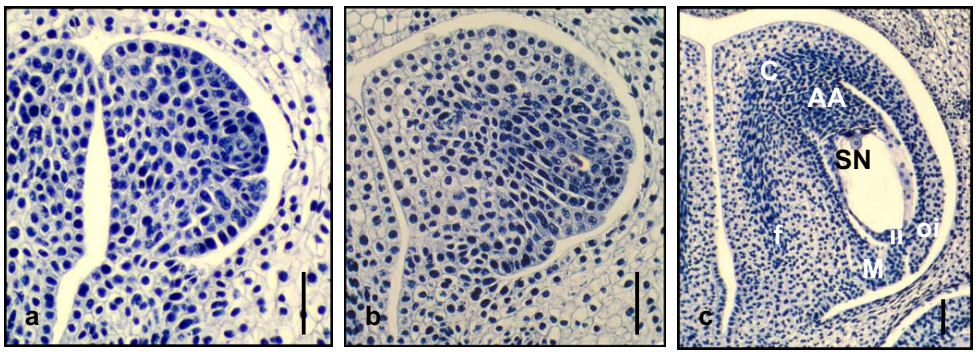

Fig. 3. Developmental stages of *L. aestivum*'s anatropous ovule. a – Phase of the archesporial cell differentiation, b – Megasporogenesis phase, scale bars = 50 μm, c – Megagametogenesis phase, scale bar = 120 μm (a–c, longitudinal sections stained with Delafield's hematoxylin) (A – antipodal cell; C – chalaza; f – funiculus; ii – inner integument; M – micropyle; oi – outer integument; SN – secondary nucleus).

lar megaspore degenerates and the chalazal megaspore functions (Fig. 4k), forming the active megaspore. Polarity is observed in the active megaspore. A vacuole in micropylar side and nucleus in chalazal side were observed (Fig. 4l).

Megagametogenesis and female gametophyte

Embryo sac of *L. aestivum* is of the *Allium* type. It has 8 nuclei. Increase in volume of active megaspore on the chalazal side was observed before mitosis (Fig. 4l). Three mitotic divisions of the chalazal megaspore lead to the formation of 2, (Fig. 4m) 4 (Fig. 4n) and 8 nucleate embryo sacs (Fig. 4o). A large central vacuole between the two groups of 2, 4, nuclei was observed (Figs 4m, n). After the 3rd division there are eight nuclei in the embryo sac; three at the chalazal end (antipodals); three at the micropylar end (egg apparatus) and two central polar nuclei. Then polar nuclei migrate near antipodals (Fig. 5a, b) and fuse before fertilization and form secondary nucleus near antipodals (Fig. 9, 10).

Mature embryo sac

Mature embryo sac has two synergid cells. They have filiform apparatus and a dense cytoplasm. Numerous small vacuoles were observed on the chalazal side and nuclei were seen on the micropylar poles of the synergids (Fig. 6). Nucleus of the egg cell is on the chalazal pole and a large vacuole was observed on the micropylar pole (Fig. 7). Three antipodal cells are bigger than the other cells of embryo sac and remain preserved in the mature female gametophyte (Fig. 8). They do not degenerate (Fig. 10). Two polar nuclei fuse before fertilization and form secondary nucleus near antipodal cells (Fig. 9, 10). The inner integument cells of the mature embryo sac at micropylar side are formed by 3–5 cell layers. Inner epidermis cells of the inner integument that surround embryo sac are elongated and differentiated like endothelium (Fig. 10, 11).

Discussion

In this study, developmental stages of female gametophyte in *L. aestivum* which grows naturally in Edirne-Tavuk Forest is examined. *L. aestivum* species is represented by *L. aestivum* subsp. *pulchellum* in Turkey (Davis 1984).

Ovules of *L. aestivum* are anatropous, bitegmic and crassinucellar like in most species of the family Amaryllidaceae (Davis 1966). Ovules of *Allium textile* (Alliaceae) (Khaleel & Mitchell 1982) and *Galanthus nivalis* (Amaryllidaceae) (Chudzik & Sniezko 2003) are anatropous, bitegmic and teninucellar but ovules of *Sternbergia lutea* (Amaryllidaceae) (Dane 1999) are hemianatropous, bitegmic and pseudocrassinucellar. Themidaceae (Berg 2003) has also anatropous and crassinucellar ovules. In *L. aestivum* the micropyle is formed by the inner integument like *Zephyranthes* Herbert (Amaryllidaceae) (Davis 1966), *A. textile* (Khaleel & Mitchell 1982), *S. lutea* (Dane 1999) and Themidaceae (Berg 2003).

Integuments of *Hymenocallis occidentalis* (Amaryllidaceae) contain a dense distribution of chlorophyll and stomata. They have the ability of photosynthesis. The presence of chlorophyll was also confirmed in the integuments of *Amaryllis belladona* L. (Amaryllidaceae), *Gladiolus* L. (Iridaceae) and *Lilium martagon* L. (Liliaceae) (Bouman 1974; Unal 2004) but these characteristics were not seen in *L. aestivum*.

In *L. aestivum* the archesporial cell functions directly as the megaspore mother cell like in *S. lutea* (Dane 1999). Cytokinesis in the megaspore mother cell of *L. aestivum* follows only the first meiotic division and chalazal diad of cells develops into an *Allium* type embryo sac like in *A. textile* (Khaleel & Mitchell 1982). The *Allium* type embryo sac is developed in most members of Amaryllidaceae, although *Polygonum* type occurs in *Allium mutabile* (Alliaceae), *Crinum* spp., *Nothoscordum fragrans, N. Striatum*, *Pancratium maritimum* (Davis 1966), *G. nivalis* (Chudzik & Sniezko 2003) and *Endymion* type occurs in *S. lutea* (Dane 1999).

Synergids of *L. aestivum* exhibit the filiform apparatus like in *S. lutea* (Dane 1999) and *G. nivalis* (Chudzik & Sniezko 2003). Nucleus of the egg cell in *L. aestivum* is on the chalazal side of the cell and there

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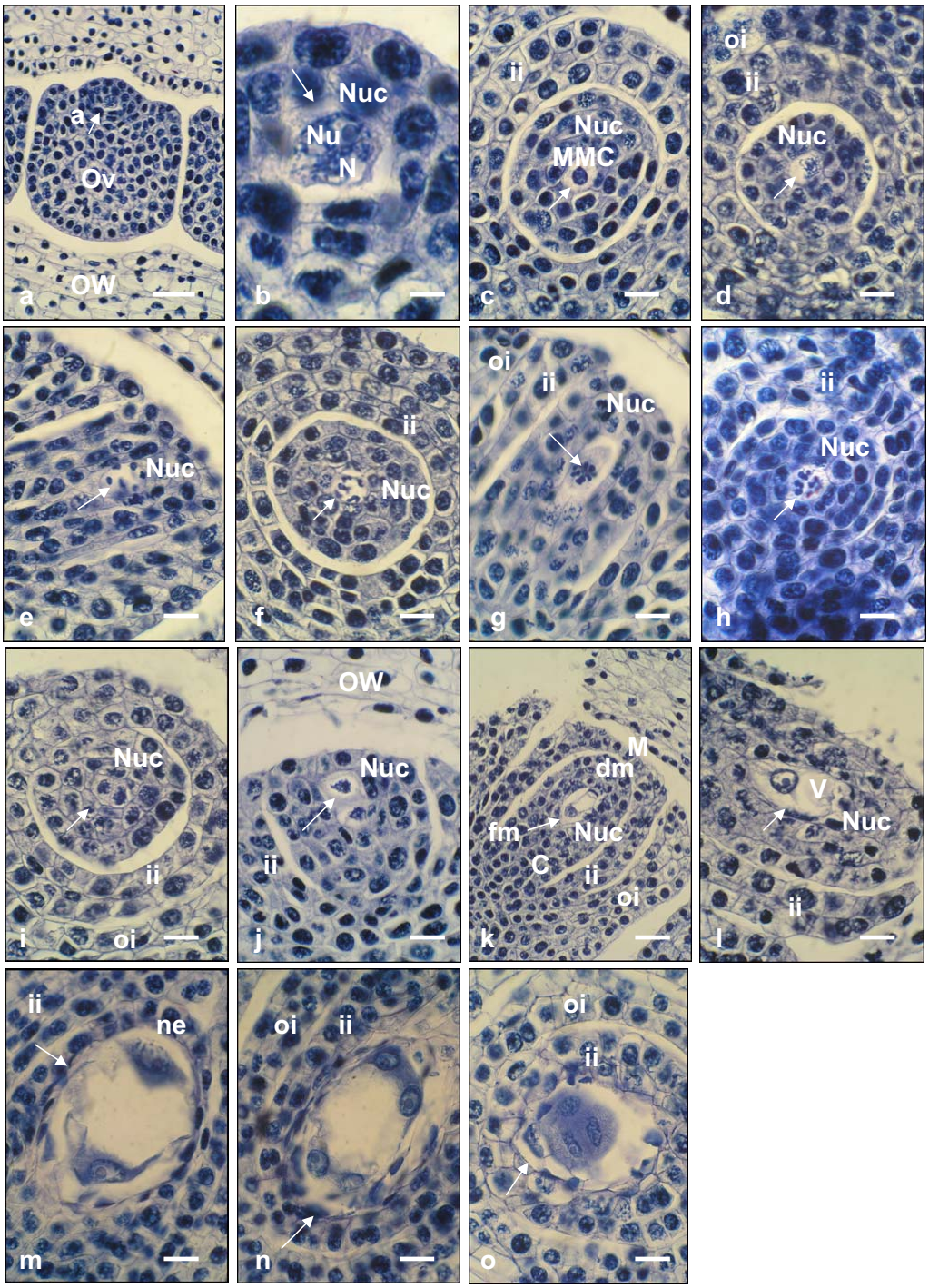

Fig. 4. a,b – Archesporial cell (arrow) and young nucellus, c – MMC at interphase, d – Zygonema- Prophase I, (arrow), e,f – Diakinesis – Prophase I, (arrows), g – Metaphase I (arrow), h – Anaphase I (arrow), i – Telophase I (arrow), j – Dyad cells (arrow), k – Functional and degenerated megaspores at diad phase (arrow), l – Functional megaspore showing polarity due to vacuole (arrow), m – 2–nucleated embryo sac (arrow), n – 4-nucleated embryo sac (arrow), o – 8-nucleated embryo sac (arrow). (a–d, f, h, i,m–o, cross sections; e, g, j–l, longitudinal sections, all sections stained with Delafield's hematoxylin) a, k, scale bar = 30μm; b, scale bar = 10μm; c–j, l–o, scale bars = 20μm. (a, archespore; C, chalaza; dm, degenerated megaspore; fm, functional megaspore; ii, inner integument; M, micropyle; MMC, megaspore mother cell; N, nucleus; ne, nucellar epidermis; Nu, nucleolus; Nuc, nucellus; oi, outer integument; Ov, ovule; OW, ovary wall; V, vacuole).

is a large vacuole on the micropylar side.

The polar nuclei fuse before fertilization and secondary nucleus moves close to the antipodals as the other Amaryllidaceae members (Davis 1966) and in *Bellevalia edirnensis* (Hyacinthaceae) (Dane 2006). The antipodals of *L. aestivum* persist as in *S. lutea* (Dane 1999) and in *B. edirnensis* (Dane 2006). Antipodal cells are stained densely like those of *Fumaria* (Saksena 1954; Sehgal & Tiwari 1977). A limited degree of secondary multiplication of antipodals has been reported in *Notoscordum* (Amaryllidaceae) (Davis 1966).

In conclusion, cytological and embryological char-

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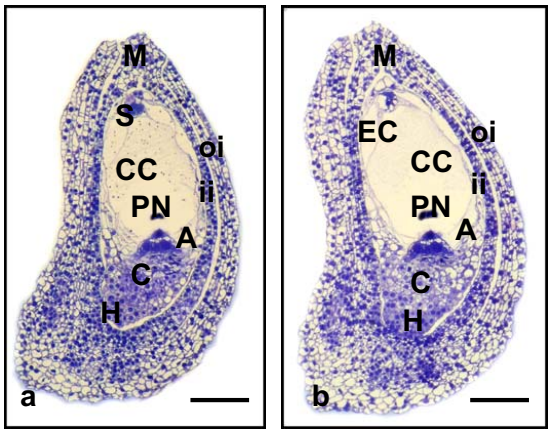

Fig. 5. a – Section of mature embryo sac of *L. aestivum* with synergids. b – Section of the same mature embryo sac of *L. aestivum* with egg cell. (a, b longitudinal semi-thin sections stained with toluidin blue) Scale bars = 120 μm (A, antipodal cell; oi, outer integument; F, filiform apparatus; H, hypostase; ii, inner integument; C, chalaza; M, micropyle; CC, central cell; PN, polar nucleus; S, synergid cell; EC, egg cell)

acteristics of *L. aestivum* were studied for the first time. Development of female gametophyte is normal. Nucellus and embryo sac types show some differences in the sister genera *Leucojum* and *Galanthus*. Polarity was determined in synergids, egg cell and functional megaspore of *L. aestivum* as described earlier (Ekici & Dane 2004). Data we gained from this study will also contribute to the embryological characteristics used in taxonomy of Amaryllidaceae, which were revised (Meerow et al. 1999) in recent years. Ultrastructure of embryo sac in *L. aestivum* will be the object of further research.

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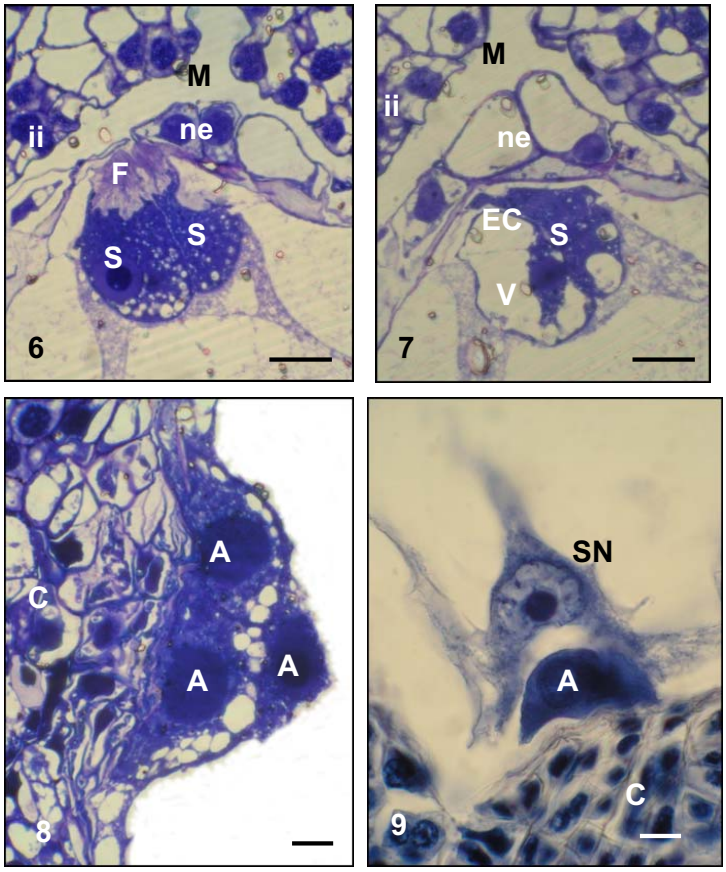

Fig. 6. Synergid cells in mature embryo sac of *L. aestivum.* (Longitudinal semi-thin section stained with toluidin blue). Scale bar = 30 μm (F, filiform apparatus; ii, inner integument; M, micropyle; ne, nucellar epidermis; S, synergid cell).
Fig. 7. Egg cell and synergid cell in mature embryo sac of *L. aestivum.* (Longitudinal semi-thin section stained with toluidin blue). Scale bar = 30 μm (EC, egg cell; ii, inner integument; M, micropyle; ne, nucellar epidermis; oi, outer integument S, synergid; V, vacuole)
Fig. 8. Antipodal cells on the chalazal side of mature embryo sac of *L. aestivum.* (Longitudinal semi-thin section stained with toluidin blue). Scale bar = 30 μm (A, antipodal cell; C, chalaza).
Fig. 9. Secondary nucleus and antipodal cell in the mature embryo sac of *L. aestivum.* (Longitudinal section stained with Delafield's hematoxylin). Scale bar = 30 μm (A, antipod cell; C, chalaza; SN, secondary nucleus).

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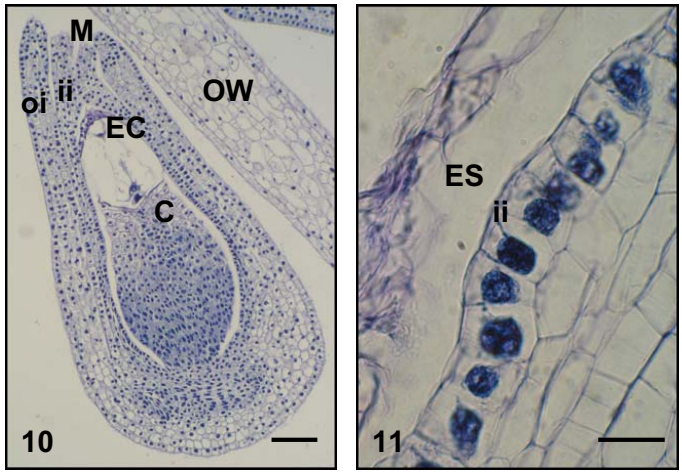

Fig. 10. Mature embryo sac of *L. aestivum*, general view. (Longitudinal section stained with Delafield's hematoxylin). Scale bar = 150 μm; (C, chalaza; EC, egg cell; ii, inner integument; M, micropyle; oi, outer integument; OW, ovary wall).
Fig. 11. Differentiation of inner integument cells in the mature embryo sac. (Longitudinal section stained with Delafield's hematoxylin). Scale bar = 10 μm (ES, embryo sac; ii, inner integument).

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