RESEARCH ARTICLE

Ivanka Semerdzhieva [1]
Elina Yankova-Tsvetkova [2]

Embryological characteristics on three species of the genus *Gentianella (Gentianaceae)*

Authors' addresses:
[1] Department of Botany and Agrometeorology, Agriculture University, Plovdiv 4000, Bulgaria.
[2] Department of Plant and Fungal Diversity, Division of Flora and Vegetation, Institute of Biodiversity and Ecosystem Research, BAS, 1113 Sofia, Bulgaria.

Correspondence:
Ivanka Semerdzhieva
Department of Botany and Agrometeorology, Agriculture University, 12 Mendeleev Blvd., Plovdiv 4000, Bulgaria.
e-mail: v_semerdjieva@abv.bg

ABSTRACT

Embryological study on native populations of three *Gentianella species* has been carried out. The anther is tetrasporangiate. Its wall develops according to Dicotyledonous-type and consists of epidermis, fibrous endothecium, two middle layers and glandular tapetum. The simultaneous microsporogenesis leads to formation of predominantly tetrahedral microspore tetrads. The mature pollen grains are 3-celled. The ovule is hemianatropous, tenuinucellate and unitegmic with unicellular archesporium. The development of the embryo sac follows the *Polygonum*-type. The legitimate embryo and endosperm develop after double fertilization. The embryogenesis runs according to Solanad-type. Initially, the endosperm is nuclear

Key words: *Gentianella*, embryology, male and female gametophyte, embryo, endosperm

Introduction

In the last years, there is a growing worldwide concern about the status of biodiversity and in particular the growing awareness of the importance of plant diversity. A variety of approaches and techniques both in situ and ex situ have been proposed and implemented for conservation of plant resources. The in situ strategy emphasizes on the protection of natural ecosystems for the conservation of overall diversity of genus, species and ecological processes. Biosphere Reserves, National Parks and Wildlife Sanctuaries have been set up. The ex situ strategies rely on botanical gardens, conservation stands, seed and pollen banks and germplasm banks to help conserve species outside the natural habitats. The ex situ approach finds more application for economic plants, whereas the in situ ones – for the wild plants (Koul Moza & Bhatnagar, 2007). Any conservation approach has to be based on an in depth study of plant reproductive biology. The processes of gamete development, pollination, endosperm and embryo development and other reproductive features can provide important clues regarding the reproductive constrains of plants that need conservation. The studies can also help in developing certain protocols to combat the problems that impede regeneration. In this sense, the embryological investigations play a key role, as they allow for the detection of weak points in the chain of reproduction processes and further elaborate methods of overcoming them. The results of investigations of this kind can serve as a basis for the further ex situ conservation of rare species.

Gentianella ciliata, *Gentianella germanica* and *Gentianella bulgarica* are such species that belong to genus *Gentianella* of the *Gentianaceae* family. *G. ciliata* (known also as *Gentiana ciliata* and *Gentianopsis ciliata*) and *G. germanica* are included in Duthch Red List of vascular plants (Van der Meijden et al., 2000), *G. ciliata* – in the category of "Critically Endangered" species, and *G. germanica* – in the category of "Vulnerable" species. *G. bulgarica* is a bolcan endemic (Petrova & Kožuharov, 1982). In Bulgaria this three species are not under protection, but its distribution in the country is restricted (Asyov & Petrova, 2006).

The embryological data on genus *Gentianella* are scanty. Up to now, a plane embryological characteristic is made only of *G. azurela* (Juan-Quan L, Ting-Nong, 1996b) and partially (the megagametogenesis, embryo and endospermogenesis) are studied *G. germanica* and *G. caucasea* (Akhalkatsi & Wagner, 1997).

RESEARCH ARTICLE

The aim of the present study is to reveal the main aspects of reproductive biology of *Gentianella ciliata*, *Gentianella germanica* and *Gentianella bulagarica*, namely the peculiarities of the male and female gametophyte, embryo- and endospermogenesis in connection with realization of its reproductive capacity that influences the character and size of the populations

Materials and Methods

At one native population of the three investigated *Gentianella* species were studied, namely:

1. *G. ciliata* – Vitosha Mauntain
2. *G. bulgarica.* – locality Tiha Rila, Rila Mauntain
3. *G. germanica.*- the top Malyovitsa, Rila Mauntain

The material of the study (flower buds and flowers at different stage of development) was collected from the native populations of the studied species and fixed in FAA mixture (formalin: glacial acetic acide:70 % ethanol in correlation 5:5:90 parts) and embedded in paraffin according to classical paraffin method (Sundara 2000). The serial paraffin cuts with thickness 9-15 μm, made with rotary microtome, were stained with Heidenhain's haematoxylin. The permanent slides were embedded in Enthelan. Observations and photographs were carried out using an Olympus CX 21 microscope and digital "Infinity lite" Camera, 1,4Mpx.

Results

During the study, we observed no essential embryological differences between the three studied *Gentianella* species. The embryological features reported in the following descriptions, therefore, are common to all populations examined, unless particular comments are given.

Anther and development of the male gametophyte.

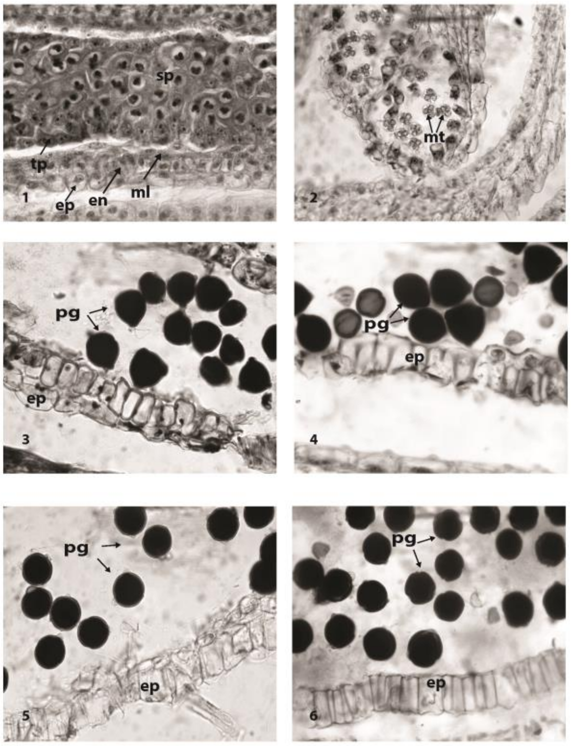
The anthers are tetrasporangiate. The anther wall develops according to the Dicottyledonous-type (Davis 1966) and prior to maturation usually comprises five layers: an epidermis, an endothecium, two middle layers and tapetum that are clearly distinguishable still at the time of prophase I in the microspore mother cells (MMCs) (Figure 1).

The epidermal cells are large in size, almost rectangular and one-nucleate. During the anther ontogenesis they tangentially lengthened and rounded up. The cells of endothecium are smaller in size but wider. They develop the typical for the family *Gentianaceae* fibrous thickenings after the microspore tetrads formation in the anthers (Poddubnaya-Arnoldi, 1982, Shamrov, 1987).

The two middle layers are ephemeral that begin to degenerate to the end of the meiosis in MMCs. Then, their cells become flattened pressed from the enlarging cells of the endothecium and the tapetum and only a few darkly stained parts of this layers present at the uninucleate pollen stage.

The tapetum is one-rowed, glandular, consisting of large, one-nucleate rectangular or almost quadratic cells. During the anther ontogenesis, in result of mitotic divisions (still at the stage of prophase I in MMCs), they become two-, four-nucleate. At about the time of one-nucleate pollen grains formation, the tapetum degenerates forming together unusual mass in which the individual protoplasts are clearly distinguished. The tapetal cells degenerate completely when mature pollen grains form. At the stage of maturity, the anther wall comprises epidermis and fibrous endothecium (Figures 3-5). During the maturation of pollen grains the epidermal cells of anther wall in *G. ciliata* y *G. bulgarica* elongated considerably in radial direction and at the time of shedding only this cells preserve conserving the entirety of epidermal layer of the anther wall (Figure 6).The same manner of development of this two layers of anther wall was observed in other *Gentianella* species (*G. azurea* - Juan-Quan &, Ting-Nong, 1996b) and other representatives of the family *Gentianaceae* (*G. cruciata* - Yankova & Yurukova, 2010, *Swertia punctata* – Yankova-Tsvetkova et al., 2011; *Comastoma pulmonarium* – Juan-Quan & Ting-Nong, 1996a, etc). In *G. germanica*, in contrary, the endothecium cells are bigger than the epidermal ones (Figure 3).

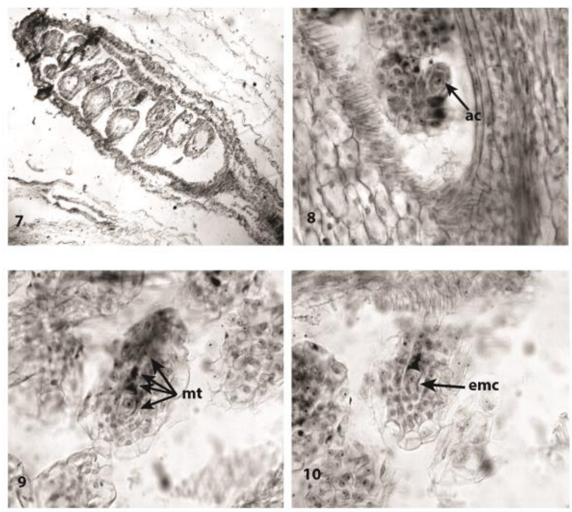
The sporogenous tissue is 3-4 layered (Figure 1) and consists of small one-nucleate polygonal, with dense cytoplasm cells that initially tightly packed one another with one. Later on, the sporogenous cells grow, round up, separate from each other and differentiate into MMCs. Meiosis runs normally with some insignificant deviations, most frequently expressed in the presence of lagging chromosomes behind the division spindle; chromosome bridges (predominantly during the heterotypic division); asymmetrical disposition of the spindles during the homeotypic division of the meiosis. After simultaneous microsporogenesis, predominantly tetrahedral tetrads form (Figure 2). The mature pollen grains are 3-celled as the observed ones in *Gentianella azurea* (Juan-Quan L, Ting-Nong, 1996b).



Figures 1-6. *Anther and development of the male gametophyte: 1, Sporogenous tissue and anther wall in G. ciliata (x400); 2, Microspore tetrads in the anther in G. ciliate (x400); 3, Mature pollen and anther wall consisting of epidermis and endothecium in G. germanica (x400); 4, Mature pollen and anther wall consisting of epidermis and endothecium in G. ciliata (x400); 5, Mature pollen and anther wall consisting of epidermis and endothecium in G. bulgarica (x400); 6 Mature pollen and anther wall consisting of epidermis at the time of shedding in G. ciliata (x400); ep – epidermis, en - endothecium, ml - middle layer, tp – tapetum, sp - porogenous tissue, mt – microspore tetrad, pg - pollen grain.*

Ovule and development of the female gametophyte.

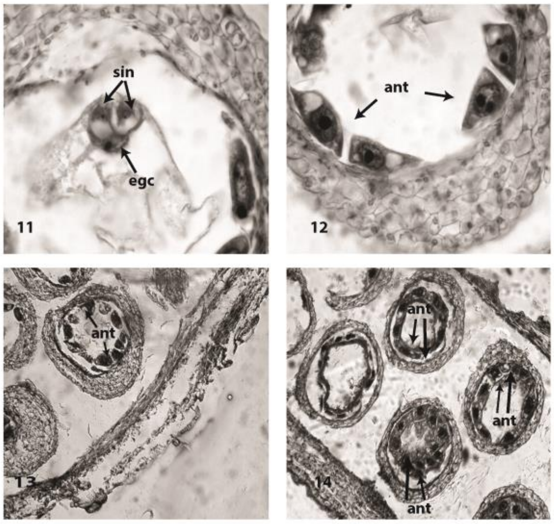
As in all *Gentianaceae* species (Shamrov, 1987, 1988), the pistil in the studied *Gentianella* species is paracarpous and consists of two carpels. The ovary is upper, unilocular, with a great number of ovules in it on parietal placentation (Figure 7). The well-developed ovule is hemianatropous as the announced one in *G. azurea* (Juan-Quan L & Ting-Nong, 1996b), *G.caucasea* and *G. germanica* (Akhalkatsi & Wagner, 1997), tenuinucellate, typical variation, recorded from Shamrov (2000) for the representatives of *Gentianacea* family, unitegmic with poor nucellus that consists of a single archesporial cell surrounded by one-layered nucellar epidermis. The nucellar epidermis begins to degenerate when the one-nucleate embryo sac (ES) forms and at the stage of four-nucleate ES its completely disappears. The single integument consists of four, five layers of cells and forms a short micropyle. The announced by us formation of integumentary tapetum (endothelium) from the cells of two innermost integumental layers in *G. lutea* (Yankova & Yurukova, 2009) and *G. cruciata* (Yankova & Yurukova, 2010) is observed in the studied species too (Figures 11 and 18).



Figures 7-10. *Development of female gametophyte: 7, Ovary with ovules at axial placentation in G. bulgarica (x100); 8, Unicellular archesporium in G. ciliata (x400); 9, Megaspore tetrad in G. ciliata (x400); 10, Embryo sac mother cell in G. ciliata (x400); ac – archesporial cell, mt – microspore tetrad, emc - embryo sac mother cell.*

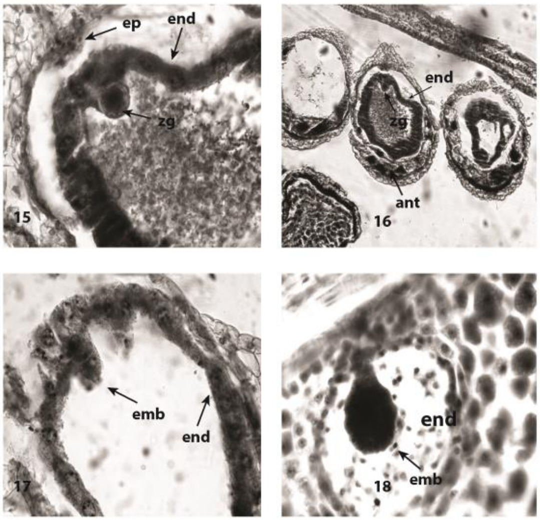
This structure is not typical for the representatives of the *Gentianaceae* family and is reported only for *G. cruciata* (Shamrov, 1990, 2000) and *Exacum pumilum* (Davis, 1966; Poddubnaja-Arnoldi, 1982). Like the established in *G. cruciata*, the cells of endothelium in the three studied species are elongated tangentially (not radially) that is not typical for this structure (Poddubnaja-Arnoldi, 1976). In the mycropile a specific structure known as epistase was observed (Figure 15). The formation of such structure is reported also for *G. azurea* (Juan-Quan L & Ting-Nong, 1996b). In the ovule an unicellular archesporium forms hypodermally, that differentiates directly into megaspore mother cell (megasporocyte) without formation of parietal cells (Figure 8).

The megasporocyte undergoes meiosis and a linear tetrad of megaspores forms (Figure 9). Usually the chalazal megaspore becomes functional (Figure 10) from which the embryo sac develops after *Polygonum* type after consecutive mitotic divisions. The mature ES is globose-shaped with the typical differentiation of its elements – egg apparatus consisting of two sinergids and one egg cell with the typical for this cells vacualization and location of the nuclei (Figure 11); a central cell formed after the fusion of the two polar nuclei and antipodal apparatus which cells undergo continued divisions and their number becomes from three to 9-12 in *G. ciliata* y *G. bulgarica* and 9-16 in *G germanica*. The prolifetation of the antipodal cells is observed in other studied *Gentianella* species too (*G. azurea* – Juan-Quan L, Ting-Nong, 1996b, *G. germanica* and *G. caucasea* – Akhalkatsi & Wagner, 1997) and may be considered as typical characteristic for the genus *Gentianella*. The antipodal cells increase considerably in size containing several nuclei or one hypertrophic nucleus with numerous nucleoides (Figure 12) and situate in a ring into the ES cavity in *G. ciliata* and *G. bulgarica* (Figure 13) or in a two row forming an antipodal tissue in *G. germanica* (Figure 14).



Figures 11-14. *Development of the female gametophyte: 11, Egg apparatus in G. ciliata (x400); 12, Antipodals in G. ciliate (x400); 13, Antipodal complex in G. ciliata (x100), 14, Antipodal complex in G. germanica (x100); ec – egg cell, sin – sinegid, antipodal cell.*

The sinergids degenerate after the fertilization whereas the antipodals are longlived – they persist until the endosperm becomes cellular (Figure 16). The long-lived antipodals are typical for other representatives of family *Gentianaceae* too - the species of genus *Swertia*: in *S. franchetiana* and *S. tetraptera* they persist until the stage of four-celled embryo and in *S. erythrosticta* Maxim., up to the stage of two-celled embryo (Xue et al., 2007).



Figures 15-18. *Embryo- and endospermogenesis:15, Zygote, nuclear endosperm and epistase in G. germanica (x400); 16, Zygote, nuclear endosperm and antipodal cells in G. germanica (x100); 17, Four-cellular young Solanad-type embryo and nuclear endosperm in G. germanica (x400); 18, Globular embryo and cellular endosperm in G. germanica (x400); zg – zygote, ant – antipodal cell, ep – epistase, end – endosperm, emb – embryo.*

The legitimate embryo and endosperm form after double porogamous fertilization. The first division of the primary endosperm nucleus precedes that of the zygote. The proof of this, are the free endosperm nuclei observed in the ES cavity at the stage of zygote – 28 in *G. germanica*, 12 – in *G. bulgarica* and 9-10 in *G. ciliata*. The direction of the cell wall setting, during the first divisions of the zygote, show that the embryogenesis follows the Solanad-type (Figure 17). This type embryogenesis is marked as a characteristic one for the family *Gentianaceae* (Davis, 1966; Shamrov, 1987). The endospermogenesis passes a free nuclear stage and differentiates into completely cellular at the globular embryo stage (Figure 18).

RESEARCH ARTICLE

Conclusion

The results of the embryological study on Bulgarian native population of three species from genus *Gentianella* confirm data announced for other species of the genus and family *Gentianaceae*: tetrasporangiate anthers; Dicotyledonous-type of anther wall formation; glandular tapetum; simultaneous microsporogenesis; tetrahedral microspore tetrads formation; superior unilocular ovary; tenuinucellate and unitegmic ovule; *Polygonum*-type of ES formation; multicellular antipodal complex, porogamous fertilization, Solanad-type embryogenesis; nuclear endosperm.

The observed embryological features and absence of apomixis characterize the studied species as sexually reproducing taxons. The regularity of processes in the generative sphere provides a high reproductive capacity of the studied species that grantee the conservation of size of its populations.

References

Akhalkatsi M., Wagner J. 1997. Comparative embryology of three *Gentianaceae* species from the Central Caucasus and the European Alps. Pl. Syst. Evol. 204: 39-48.

Asyov B, Petrova A. 2006.Conspectus of the vascular flora of Bulgaria. Distribution Maps and Floristic Elements. Ed. 3. BBF, Sofia, p. 190-191.

Davis G. 1966. Systematic Embryology of the Angiosperms, New York, London, Sydney, John Wiley & Sons, Ltd.

Juan-Quan L, Ting-Nong H. 1996a. The embryological studies of *Comastoma pulmonarium* (*Gentianaceae*). Acta Phytotax. Sinica 34 (6): 577-585.

Juan-Quan L, Ting-Nong H. 1996b. Embryological studies of Gentianella azurea. Acta Bot. Yunn. 18 (2): 151-158.

Koul Moza M, Bhatnagar AK. 2007. Plant reproductive biology studies crucial for conservation. Curr. Sci. 92 (9): 1207.

Petrova A, Kožuharov St. 1982. Gentianella Moench. In: Velĉev V (ed.). Flora of Bulgaria, vol VIII, Aedibus Acad. Sci. Bulgaricae, Serdicae, Sofia, p. 408-414.

Poddubnaja-Arnoldi VA. 1976. Cytoembryology of Angiosperms. Moskva, Nauka, 1976.

Poddubnaja-Arnoldi VA. 1982. Characteristics of the Angiosperms families on cytoembryological features. Moskva, Nauka, p.171-172.

Shamrov I. 1987. Family *Gentianaceae*. In: Comparative embryology of flowering plants (eds T.B. Batygina, M.S. Jakovlev). Leningrad, Nauka, 4:137-145.

Shamrov I. 1988. Ovule and structural characteristics of embryo sac in some members of the Gentianaceae family. Bot. Zhurn. 73 (2): 213-222.

Shamrov I. 1990. The ovule of *Gentiana cruciata* (*Gentianaceae*): structural-functional aspects of development. Bot. Zhurn. 75 (10): 1363-1379.

Shamrov I. 2000. Translocation pathways for metabolites in developing ovules of *Gentiana cruciata* L., *Gymnadenia conopsea* (L.) R.Br., *Gagea stipitata* Merklin and *Luzula pedemontana* Boiss.Et Rent. Acta Biol. Cracov. 42 (1): 61-77.

Sundara R. S. 2000. Practical manual of plant anatomy and Embryology, New Delhi. Anmol Publ. PVT LTD.

Van der Meijden R, Oder B, Groen CLG, Witte JPM, Bal D. 2000. Bedreigde en kwetsbare vaatplanten in Nederland. Basisrapport met voorstel voor de Rode Lijst. Gorteria 26, 85–208.

Xue CY, Ho TN, Li DZ. 2007. Embryology of *Swertia* (*Gentianaceae*) relative to taxonomy. Bot. J. Lin. Soc. 155 (3): 383-400.

Yankova-Tsvetkova E., Yurukova-Grancharova P. 2009. Embryological study of Bulgarian populations of Gentiana lutea (Gentianaceae). Fl. Medditerranea 19:189-197.

Yankova E, Yurukova P. 2010. Embryological study of Gentiana cruciata L. (Gentianaceae) from Bulgaria. Journ. Appl. Biol. Sciences 4 (2): 19-23.

Yankova-Tsvetkova E., Baldjiev G., Yurukova-Grancharova P. 2011. Reproductive biology of *Swertia punctata* (*Gentianaceae*) – a glacial relict in the Bulgarian flora. Comp. Rend. Acad. Bulg. Sci. 64 (7): 959-966.