Macrosporogenesis and development of the female gametophyte in *Gentiana punctata* (*Gentianaceae*)

Radka Bicheva, Penka Robeva-Davidova & Petka Yurukova-Grancharova

Institute of Botany, Bulgarian Academy of Sciences, Acad. G. Bonchev St., bl. 23, 1113 Sofia, Bulgaria, e-mail: radkagb@yahoo.com

Received: May 28, 2003 ▹ Accepted: June 25, 2003

Abstract. A comparative embryological study of natural Bulgarian populations of *Gentiana punctata* was carried out. Macrosporogenesis and development of the female gametophyte have been studied. The ovule is anatropous, tenuinucellate and unitegmic with 1-3-cellular archesporium. Usually, archesporogenesis runs without formation of parietal cells, but sporadically 1-2 parietal cells were found. The single archesporial cell differentiates into a macrospore mother cell and after meiosis a linear tetrad forms in the ovule.The embryo sac develops after *Polygonum* (monosporic)- type and in single ovules according to *Allium* (bisporic)-type. It was established that *Gentiana punctata* is a strongly amphimictic species.

Key words: embryo, embryology, embryo sac, endosperm, *Gentiana punctata*

Introduction

Genus *Gentiana* L. is represented by 11 species in the Bulgarian flora (Tutin 1972; Kozhuharov & Petrova 1982). *G. punctata* L. belongs to section *Gentiana.* It is a protected species and entered in the *Red Data Book of PR Bulgaria* (Velchev 1984).

The species was presented by small populations distributed mainly in the wet, rocky, grassy, shrub-covered, and woody places in the Balkan Range (*Central* & *Western*), Mt Vitosha, Pirin and Rila Mts at altitudes above 1500 m. The national reserves of this species are rather limited in these territories but in the Rila Mts are stronger in numbers (Bondev 1995).

Embryologically, the species is poorly studied. The available data are very sparse and relate only to certain stages of the microsporogenesis, development of the male gametophyte (Shamrov 1986; Rao & Chinnappa 1995) and the specificities of the female generative sphere (Shamrov 1988). Out of the 316 *Gentiana* species, only 17 were object of embryological research. In this respect, Bulgarian populations of species from genus *Gentiana* are studied for the first time. So far only *G. asclepiadea* and *G. pyrenaica* have been studied karyologically (Nikolov 1991).

The present work marks the beginning of a complete embryological study of the Bulgarian representatives of genus *Gentiana*, many of which are medicinal, endemic, rare or threatened species.

Material and methods

The material of study (flower buds and flowers in different age) has been collected periodically during two consecutive years (2001-2002) from six natural populations: three from Mt Vitosha (No 3E – at peak Maluk Rezen, No 2E – under Cherni Vruh, No 8E – at the Stenata locality); two in the Rila Mts (No 6E – Popova Kapa lake and No 5E – Smradlivoto lake) and one in the Norhtern Pirin Mts (No 9E – Mouratovo lake).

The material was fixed with FAA (formalin: glacial acetic acid: 70 % alcohol = 5 : 5 : 90 parts) and treated according to the classical paraffin methods (Nikolov &

Daskalov 1966). The serial paraffin sections 10-18 μm thick were stained with Heidenhain's haematoxylin. The observations were carried out with Amplival light microscope.

The herbarium specimens were deposited in SOM.

Results and discussion

In the studied species the pistil was formed of two carpels, cenocarpous, with an upper unilocular ovary, a nectar disc at the base and two stigmas set on a short style. The ovules in the ovary were set in two rows along the walls, varying greatly and occasionally numbering as 50–60**.** The setting of a great number of ovules in one ovary is considered as a primitive embryological feature by Poddubnaya-Arnoldi (1964).

The well developed ovule is anatropous, tenuinucellate and unitegmic. The nucellar epidermis is not ephemeral and remains intact until the uninucleate stage of the embryo sac (ES) development. Its degeneration runs from the micropyle to the chalaze. In the chalazal part of some ovules still vital cells of the nucellar epidermis have been observed even after uninucleate stage of the ES. At the beginning of the meiosis in the macrospore mother cell (MMC) the integument of the ovule was built of 4–6 cell layers and after the binucleate stage of ES it becomes 7–8- layered.

The cells of the outermost layer of the integument differ distinctly from the rest ones. They are rectangular, strongly lengthened radially, uninucleate, thin-walled, and their outer walls are rounded to undulate. The nucleus in them was usually set closer to the inner cell wall and has a strong basophilous nucleolus.

Mention deserves the fact that the innermost layer of the only integument in *G. punctata* does not differentiate into integumentary tapetum. This specificity of the ovule structure, typical of the whole family *Gentianaceae,* was reported by Shamrov (1987) and Poddubnaya-Arnoldi (1982). However, the histochemical studies carried out later by Shamrov (1990) made possible the finding of integumentary tapetum in *G. cruciata*.

The anatropous ovule of *G. punctata* has a deep and narrow micropyle and a well developed funiculus. Neither epistasy and hypostasy, nor an obturator have been observed in any of the studied populations of this species.

Other species of the genus *Gentiana* and *Gentianaceae* have similar ovule feature like *G. cruciata* (Bouman & Schier 1979), *Centaurium ramosissimum* Druce (Vijayaraghavan & Padmanaban 1969), *Swertia minor* (Rao & Nagaraj 1982), etc. There are, however, some exceptions. Shamrov (1988), for instance, had observed an obturator in *G. lutea*.

During the ontogenesis of the ovule, when it is still orthotropous, usually an unicellular archesporium has been set hypodermally in it (Plate I, Fig. 1). In the population No 3E we have observed also the setting of a multicellular archesporium (1–3 cells) (Plate I, Fig. 2). Other authors have also reported the formation of a multicellular archesporium in other species: two-cellular in *G. lutea* (Kordjum 1978; Shamrov 1987, 1988); and two- and three-cellular in *C. ramosissimum* (Vijayaraghavan & Padmanaban 1969). These facts testify that the general inference made by Poddubnaya-Arnoldi (1982) about the setting only of unicellular female archesporium in all *Gentianaceae* is untenable.

The setting of a multicellular archesporium is regarded as a more primitive embryological feature and does not correspond to the high specialization of *Gentianaceae.*

Archesporogenesis in the family runs, as a rule, without formation of parietal cells (Shamrov 1987; Poddubnaya-Arnoldi 1982). As an exception, in single ovules of the population No 3E archesporogenesis ran with formation of 1–2 parietal cells. These cases showed and intermediate type of archesporogenesis in *G. punctata*, which probably was a manifestation of heterobatmy judging by that embryological feature.

Irrespective of the setting of a multicellular archesporium in some ovules, it is only one of the cells that undergoes further development and after a period of differentiation directly functions as a macrospore mother cell (MMC). Evidence of this was the presence of only one embryo sac within the ovule in *G. punctata*. Meiosis in MMC ends with the formation of a linear macrospore tetrad (Plate I, Fig. 3), but in the populations No 3E and No 8E we have observed the formation of diads (Plate I, Fig. 4) in some ovules. The same fact has been mentioned by other authors for other species of *Gentianaceae*. Shamrov (1988) described both the formation of linear macrospore tetrads and diads in *G. lutea* and *G. cruciata*, Rao & Nagaraj (1982) had found the same in *S. minor*, and Vijayaghavan & Padmanaban (1969) in *C. ramosissimum*. Formation of tetrads and diads as a result of the macrosporogenesis has shown that besides according to the basic *Polygonum* (monosporic)-type, the ES can also develop according to the deviating *Allium* (bisporic)-type (Plate I, Fig. 4). For *G. punctata* this specificity is reported for the first time and does not agree with the inference made by Poddubnaya-Arnoldi (1982) about the existence only of *Polygonum*-type of development of the female gametophyte in all representatives of *Gentianaceae*.

The chalazal macrospore of the tetrad, after intensive growth and distinct vacuolization, usually functions as an embryo sac mother cell (EMC), while the remaining three macrospores degenerate and are observed as a dark coloured spots above it (Plate I, Fig. 3).

The one-nucleate ES is well vacuolized and its nucleus is situated in various parts of the embryo sac cavity. At this stage, the nucellar epidermis represented by one layer of cells starts getting disorganized, with the process of degradation more clearly expressed in the micropylar part of ES.

During the binucleate and tetranucleate stage of the development of ES, the nuclei undergo a normal polarization for the *Polygonum*-type (Plate I, Fig. 5, 6). During the binucleate stage of ES (Plate I, Fig. 5), usually complete degeneration of the nucellar epidermis sets in, but even after its destruction the integumentary tapetum does not differentiate.

In single cases, during the stage of eight-nucleate ES some non-polarized nuclei have been observed, which subsequently did not differentiate into elements of the ES.

The mature ES is spindle-shaped elongated, widened at the micropylar end and slightly narrow in the chalazal end, with an organization of the elements after the *Polygonum*-type of development: 3-celled egg apparatus, 2 polar nuclei and 3-celled antipodal complex.

At the beginning of its differentiation, the egg cell and the synergids do not differ in shape and size (Plate II, Fig. 1). Shamrov (1988) made a similar observation in *G. lutea*. Subsequently, the egg cell becomes distinctly pyriform, with vacuolization and situation of the nucleus in it (Plate II, Figs 2, 3) typical for the representatives of *Gentianaceae* (Poddubnaya-Arnoldi 1982; Shamrov 1987). The synergids are strongly elongated, cylindrical to ribbonlike (Plate II, Figs 2, 3), without hook outgrowths and filiform apparatus observed in *G. lutea* by Shamrov (1988). Their life is relatively short, because of that they cannot be observed after fertilization, contrary to other species of *Gentianaceae*, such as *Gentianella azurea* (Quan & He 1996), in which they remain more longer.

The fusion of two polar nuclei and formation of a central cell (secondary nucleus) of ES takes place prior to fertilization. The central cell is huge in size and its place is variable: one can observe it not only close to the egg cell which is its normal position before fertilization (Plate II, Figs 2, 3), at the centre of the embryo sac cavity (Plate II, Fig. 4) as well as close to the chalaze.

The antipodal complex differentiates later than the egg apparatus and is represented by 3 cells with linear (Plate II, Fig. 5) or T-shape arrangement (Plate II, Fig. 6). Polyploidization, as well as multiplication of the nuclei in some antipodal cells (up to 6–7 nuclei per cell) have been observed (Plate III, Fig. 1). A similar antipodal apparatus was observed in *G. lutea*, *G. cruciata* (Shamrov 1987, 1988) and in *C. ramosissimum* (Vijayaghavan & Padmanaban 1969), while in *S. minor* multiplication of the antipodals up to 6–8 cells was noticed (Rao & Nagaraj 1982). The antipodals in ES of *G. punctata* are not ephemeral. They continue to exist until to the beginning of embryogenesis, after which they degenerate.

In the investigated species, the formation of the embryo and endosperm is a result from the porogamous, double fertilization. Mention deserves the established polyspermy: penetration of more than two sperms into ES (up to 10–12), close to the egg cell and central cell (Plate III, Fig. 2). As polyspermy occurs seldom in the Angiosperms (Poddubnaya-Arnoldi 1976), its identification for the first time in *G. punctata* is a contribution not only to the embryology of genus *Gentiana*, but to family *Gentianaceae* too.

The first division of the fertilized central cell of ES runs much earlier that the first division of the zygote. This is proved by the presence of great number of free endospermal nuclei in the stage of two-celled embryo (Plate III, Fig. 3).

In the investigated species the endosperm was initially nuclear. The free endospermal nuclei are situated along the walls within the ES cavity (Plate III, Fig. 3), enclosing besides the young embryo from all sides (Plate III, Fig. 4). At the globular stage of the embryo and in the presence of several dozens free endospermal nuclei as a result of fast cytokinesis, initially nuclear endosperm transforms into cellular. Shamrov (1988) noted that in *G. lutea* this process had taken place in the presence of 128 free endospermal nuclei, while in the investigated species-in the presence of several dozens of nuclei, i.e. much earlier.

For order *Gentianales* only the *Solanad*-, *Caryophyllad*- and *Asterad*-type of embryogenesis have been mentioned so far (Johansen 1950).

The instability in the formation of the cell wall after the first division of the zygote reported by Shamrov (1987) in *G. lutea* has been observed by us in *G. punctata* (from oblique to clearly longitudinal). Longitudinal formation of the first cell wall in the beginning of embryogenesis (Plate III, Fig. 3) has been found only for the *Piperad*-type (Johansen 1950). This shows that *G. punctata* relates particularly to that type of embryogenesis. In Angiosperms, the *Piperad*-type embryogenesis is extremely rare and was reported by Johansen (1950) only for seven families.

The multicellular globular embryo in *G. punctata* has a short, two-rowed suspensor built of 3–4 cells in each row (Plate III, Fig. 5).

The mature embryo is erect, with two equal cotyledons and no heterocotyly has been observed. The endosperm was not completely consumed in the mature seeds.

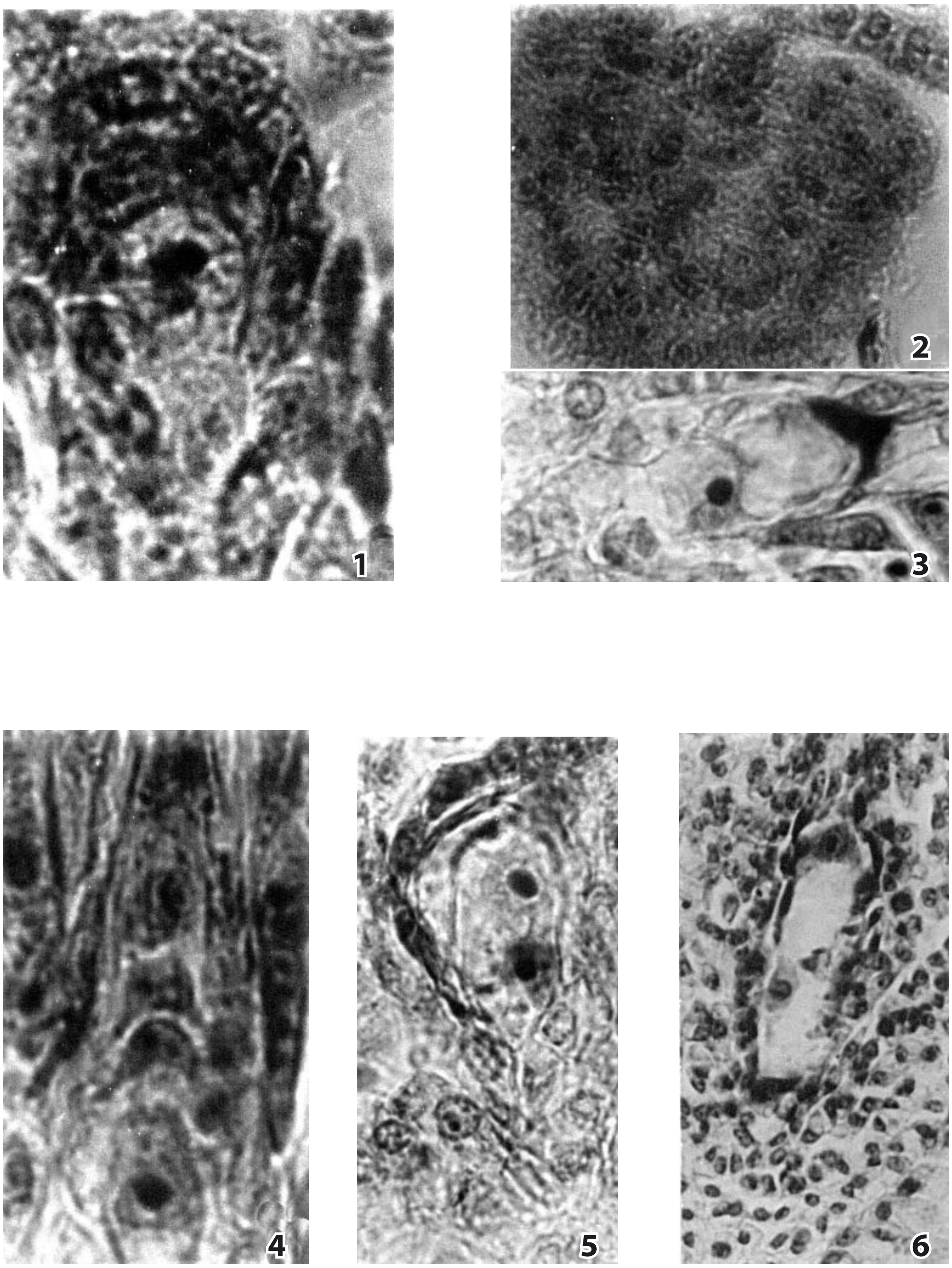
Conclusion

As a result of the study of *G. punctata,* marking the start of an entire embryological investigation of the Bulgarian representatives of *Gentiana*, the peculiarities of the structures and processes in the female generative sphere of this species were revealed. One of the data confirmed so far known peculiarities typical for genus *Gentiana* and *Gentianaceae.* A number of new, until now unreported features have been found out, which contribute to the enrichment of the embryological characteristics not only of the investigated species, but of the genus and the family in general. Some of them are:

- More than one archesporial cell in the ovule forms, which does not confirm the general conclusion made by Poddubnaya-Arnoldi (1982) that in all representatives of *Gentianaceae* only an unicellular female archesporium exists.
- Archesporogenesis of the intermediate type with formation of one or two parietal cells, probably is a manifestation of heterobatmy by that embryological feature. This runs contrary to the stand-point of Poddubnaya-Arnoldi (1982) only of a direct functioning of the primary female archesporium in all *Gentianaceae,* without formation of any parietal cells.
- Besides of tetrads, formation of diad of macrospores after the meiosis in the MMC of the ovule. This testifies that the development of female gametophyte can follow not only the basic *Polygonum* (monosporic)-type, but also the deviating *Allium* (bisporic)-type.
- Untypical polarization of the elements in the 8-nucleate ES, which does not correspond to either of the described types of the female gametophyte development (Poddubnaya-Arnoldi 1976).

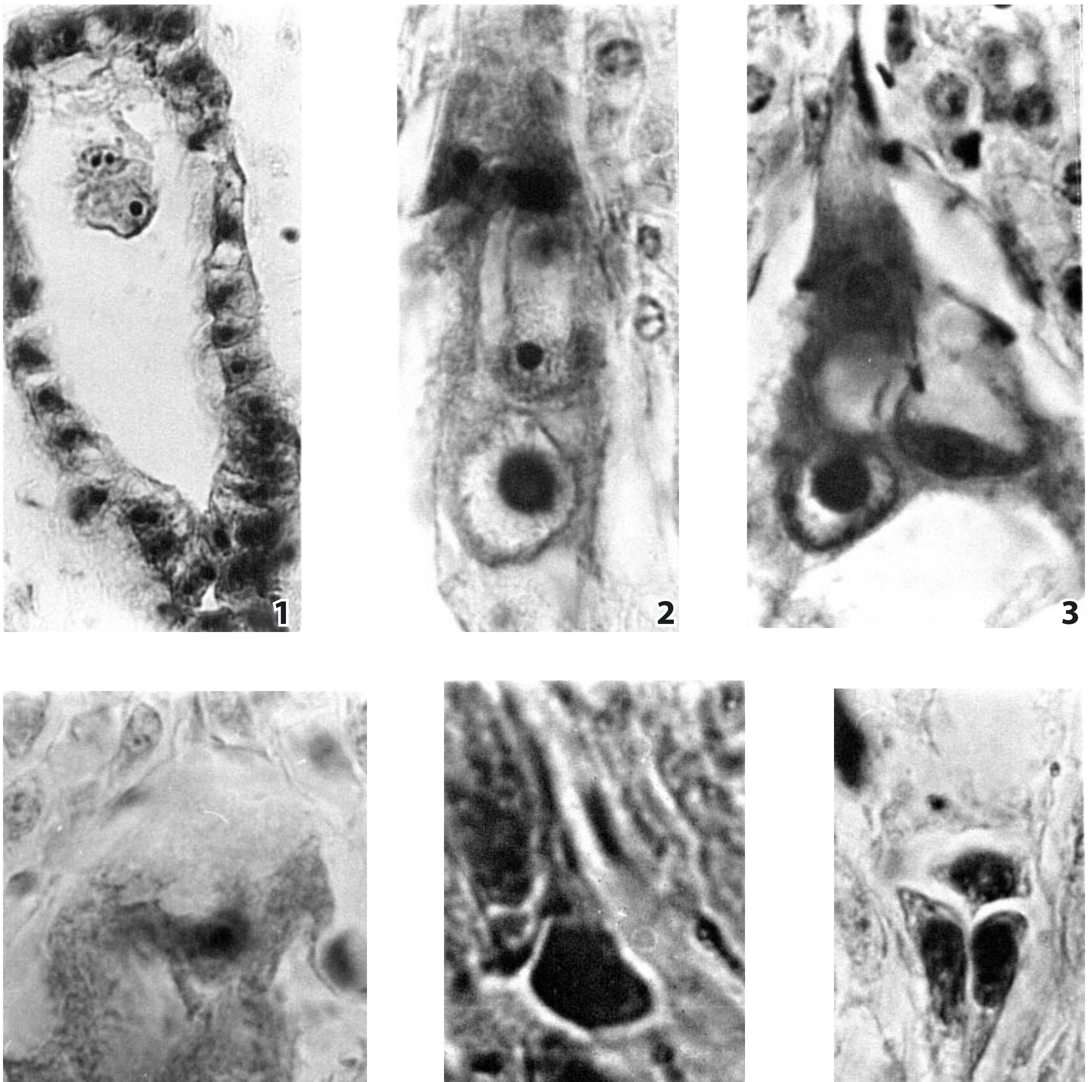
The embryological study carried out has shown that *G. punctata* is an amphimictic species, combining both relatively primitive and evolutionary more progressive embryological features.

Plate I



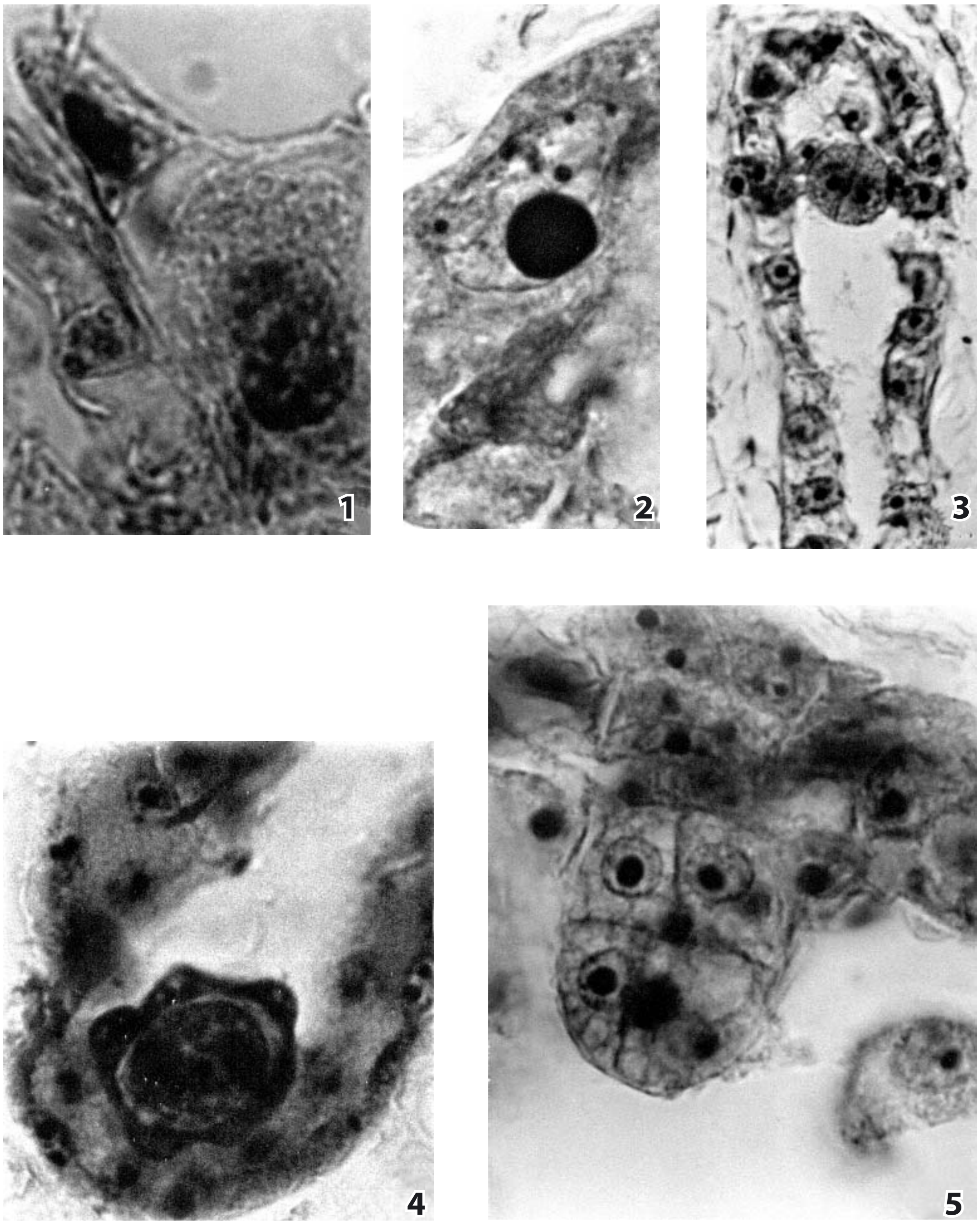
Figs 1-6. Ovule, macrosporogenesis and development of the female gametophyte in *G. punctata*:
1, unicellular archesporium in the ovule (× 400); **2,** two archesporial cells in the ovule (× 160); **3**, linear marcrospore tetrad and chalazal cell functions as EMC (× 400); **4,** macrospore diad and the beginning of development of ES after the *Allium*-type (× 160); **5,** binucleate ES (× 160); **6,** tetranucleate ES (× 160).

Plate II



Figs 1-6. Female gametophyte in *G. punctata*:
1, three-celled egg apparatus (× 160); **2,** egg apparatus and central cell in the mature ES (× 400); **3,** cental cell in ES close to the egg cell prior to fertilization (× 400); **4,** central cell at the centre of the ES cavity (× 400); **5**, three-celled antipodal complex with linear arrangement of its elements (× 400); **6**, three-celled antipodal complex with T-shaped arrangement of its elements (× 160).

Plate III



Figs 1-5. Female gametophyte, embryo- and endospermogenesis in *G. punctata*:
1, multiplication of the cells in an antipodal complex (×400); **2**, polyspermy in the central cell (×400); **3**, first division of the zygote and nuclear endosperm (×63); **4**, young embryo enclosed by free endospermal nuclei (×160); **5,** globular embryo with a short suspensor (×400).

References

Bondev, I. (ed.). 1995. Chorological atlas of Bulgarian medicinal plants. Acad. Publishing House "Prof. Marin Drinov", Sofia (in Bulgarian).

Bouman, F. S. & Schier, S. 1979. Ovule ontogeny in seed coat development in *Gentiana,* with a discussion on the evolutionary origin of the single integument. – Acta Bot. Neerl., **28**(6): 468-478.

Johansen, D. 1950. Plant Embryology. Waltham, Mass.

Kordjum, E. 1978. Evolutionary cytoembryology of Angiosperms. Naukova Dumka, Kiev (in Russian).

Kozhuharov, S. & Petrova, A. 1982. *Gentianaceae*. – In: **Jordanov, D.** (ed.), Fl. Reipubl. Popularis Bulgaricae**,** Vol. **8,** pp. 384- 418. In Aedibus Acad. Sci. Bulgaricae, Serdicae (in Bulgarian).

Nikolov, N. A. 1991. Chromosome numbers of the Bulgarian angiosperms from the Northern Pirin Mts: Bayuvi Doupki, Dzindzhiritsa Reserve. – Fitologiya**, 41**: 70-75.

Nikolov, H. & Daskalov, S. 1966. Cytological technics. Agric. Acad. Sci., Sofia (in Bulgarian**).**

Poddubnaya-Arnoldi, V. A. 1964. General embryology of Angiosperms. Nauka, Moscow (in Russian).

Poddubnaya-Arnoldi, V. A. 1976. Cytoembryology of Angiosperms. Basis and Perspectives. Nauka, Moscow (in Russian).

Poddubnaya-Arnoldi, V. A. 1982. Characteristics of the Angiosperms families by cytoembryological features. Nauka, Moscow (in Russian).

Quan, L. J. & He, T. N. 1996. Embryology of *Gentianella azurea*. – Acta Bot. Yunnan, **18**(2): 151-158.

Rao, K. S. & Nagaraj, M. 1982. Studies into *Gentianaceae.* Embryology of *Swertia minor* (*Gentianaceae*). – Canad. J. Bot., **60**(2): 141-151.

Rao, K. S. & Chinnappa, C. C. 1983. Pericolporate pollen in *Gentianaceae.* – Canad. J. Bot., **61**(1): 174-178.

Shamrov, I. I. 1986. Anther development of *Gentiana lutea* (*Gentianaceae*). – Bot. Zhurn., **71**(6): 733-739.

Shamrov, I. I. 1987. *Gentianaceae.* – In: **Batygina, T. B. & Jakovlev, M. S.** (eds), Comparative Embryology of the Flowering Plants, Vol. **4**, pp. 137-145. Nauka, Leningrad (in Russian).

Shamrov, I. I. 1988. Ovule development and structural characteritics of the embryo sac in some members of *Gentianaceae*. – Bot. Zhurn., **73**(2): 213-222 (in Russian).

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

Tutin, T. G. 1972. *Gentiana* L. – In: **Tutin, T. G. & al.** (eds), Flora Europaea. Vol. **3,** pp. 59- 63. Cambridge Univ. Press, Cambridge.

Velchev, V. (ed.).1984. Red Data Book of the People's Republic of Bulgaria. Vol. **1** Plants. Publishing House Bulg. Acad. Sci., Sofia (in Bulgarian).

Vijayaraghavan, M. R. & Padmanaban, U. 1969. Morphology and embryology of *Centaurium ramosissimum* Druce and affinities of the family *Gentianaceae*. – Beitr. Biol. Pflanzen, **46**: 15-37.