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# Embryological study of Bulgarian populations of *Gentiana lutea* (*Gentianaceae*)

## Abstract

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Embryological study on native Bulgarian populations of *Gentiana lutea* L. has been carried out. The peculiarities of the anthers, ovules, micro- and macrosporogenesis, the development of male and female gametophyte as well as the embryo- and endospermogenesis are established. The results of the study reveal a specialization of this species, in particular regarding the female gametophyte development not only according to the basic *Polygonum* (monosporic)-type but to the *Allium* (bisporic)-type too and a tendency to gametophytic apomixis (apospory). Some of relatively primitive features are established in the male generative sphere but they are not so much and important to change the status of *G. lutea* as an evolutionary advanced species and they are only considered as a manifestation of the heterobathmy.

*Key words*: male and female gametophyte, embryogenesis, endospermogenesis.

## Introduction

*Gentiana lutea* L. is a medicinal plant, included in the Red Data Book of PR Bulgaria within the category "endangered species" (Velchev 1984). The species is represented with loose populations or single individuals at herbaceous places and stony slopes rarely in the Central Balkan Range, Vitosha, Rila, Pirin and Central Rhodopi Mts at altitude from 1200 to 2600 m and its national reserves are near to the critical minimum (Bondev 1995).

From ancient times, the roots drug of *G. lutea* is known as a remedy for augmentation of the gastric secretion and digestion. In the folk medicine it is used also for decrease of the temperature, externally – for compresses and baths and in the cases of the infected wounds to facilitate the epithelization (Petkov 1982).

The peculiarities of male and female generative spheres of this species are studied only by Shamrov (1986, 1988) and the data for its embryogenesis are fragmentary (Shamrov 1987).

Up to now, Bulgarian populations of *G. lutea* are studied karyologically (Andreev 1982), pharmacologically (Boichinov & Boeva 1953; Assenov & Nikolov 1989), ecologically and phytochemically (Stoyanov & Kitanov 1960; Kozhuharova 1994; Georgieva 2007), but embryological investigation is carried out for the first time.

In the present work, as a part of complete embryological study undertaken on the Bulgarian representatives of the genus *Gentiana* L., the results of embryological investigation on *G. lutea* are given.

## Material and methods

Material of the study (flower buds and flowers at different age) has been collected from five native populations of *G. lutea* (Vitosha Mt, at the peak Golyam Rezen; Rhodopi Mt (Western), the village Kesten; Rila Mt, above the Suhoto lake; Pirin Mt, Banderitsa mountain hostel; Balkan Range (Western), Kozya stena summit) and fixed in FAA mixture (formalin: glacial acetic acid: 70 % ethanol in correlation 5:5:90 parts). The serial paraffin cuts with thickness 12-25 mm, maded with rotary microtome are stained with Heidenhain's haematoxylin (Rajan S. Sundara 2000). The glass made slides are embedded in Canada balsam. The observations have been carried out with "Amplival" light microscope and the microphotographs are made with digital camera "Canon".

## Results

The embryological features are basically the same in all studied populations of *G. lutea*, unless in the following descriptions particular comments are given.

*Male generative sphere*

The anthers are tetrasporangiate. The anther's wall formation conforms to the Dicotyledonous-type (Davis 1966) and consists of epidermis, endothecium, two middle layers and glandular tapetum (Fig. 1) that are clearly distinguished during the heterotypic division of the meiosis in microspore mother cells (MMCs).

The epidermal cells clearly enlarge tangentially during the anther ontogenesis. The cells of the initially one-layered endothecium divide in radial direction, forming two additional rows that develop typical fibrous thickenings after the formation of microspore tetrads. In comparison with the other layers of the anther wall, the epidermis and endothecium preserve long time, remaining entire and vital up to the mature pollen stage (Fig. 5). The cells of the two middle layers become flattened, pressed from the growing cells of the endothecium and tapetum and degenerate to the end of heterotypic division of the meiosis in MMCs.

During the anther ontogenesis, the glandular tapetum undergoes some changes. As a result of a mitotic division its initially one-nucleate cells become two-nucleate. The tapetum cells enlarge and intrude into the anther locules to form "placentoids" ("trabecules") that separate the sporogenous tissue and later on the microspore tetrads (Fig. 2). After that, the tapetum cells, disintegrate, undergo a lysis and penetrate between the microspore tetrads, forming together unusual mass in which the individual protoplasts are clearly distinguished (Fig. 4). The tapetum begins to degenerate and at the stage of mature pollen grains even its traces are not observed. The wall of the mature anthers in *G. lutea* consists of the persistent epidermis and fibrous endothecium.

Sporogenous tissue is 3-, 4-layered and its consisting cells initially are tightly packed to each other. Later on, they grow, round up, separate from each other and differentiate into

MMCs (Fig. 1). Meiosis runs normally and only insignificant deviations are registered, as: single lagging chromosomes; chromosomes scattered along the division spindle; asymmetrical disposition of the spindles during homeotypic division of the meiosis in MMCs. As a result of a simultaneous microsporogenesis predominantly tetrahedral, isobilateral, T-shaped, rarely decussate and single linear tetrads are formed (Fig. 3).

Soon after their formation, the tetrads disintegrate and each of the microspores differentiates into young one-celled pollen grain. The mature pollen is 3-celled at the time of anther dehiscence (Fig. 6). During the development of male gametophyte some degenerating processes are observed predominantly at 2- or 3-celled pollen stage in different amount in the studied populations but more clearly expressed in the population from Pirin Mt.

### *Female generative sphere*

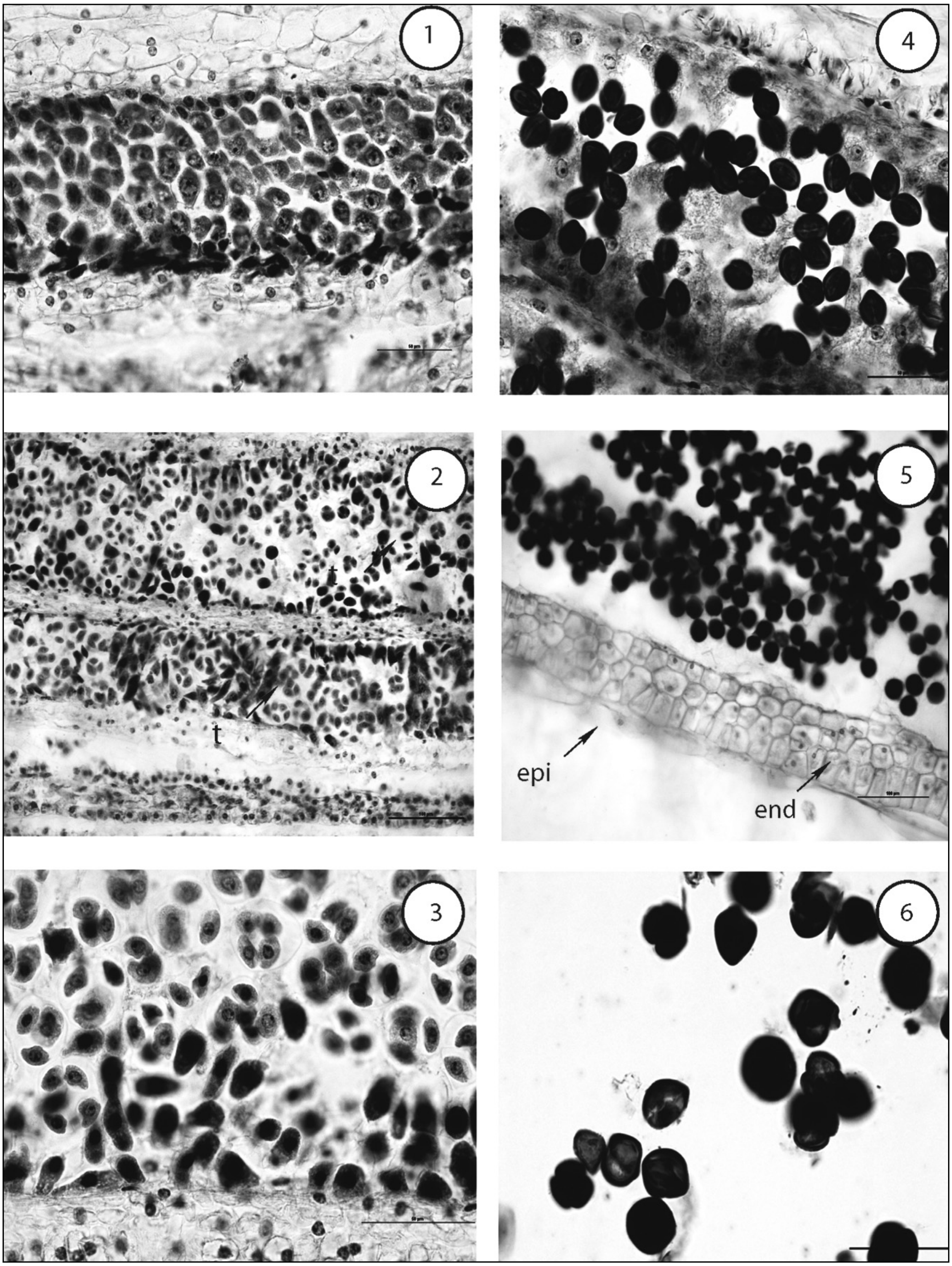
Within the one-loculate superior ovary of *G. lutea,* the ovules arranged in two rows form (occasionally numbering as 50-60).

The mature ovule is anatropous, tenuinucellate, unitegmic with a short funiculus and deep and narrow micropyle. During the early stages of its development, a funicular obturator forms (Fig. 7). It is clearly distinguished from the other somatic cells of the ovule, consisting of radial enlarged cells that grow toward to the tip of the funiculus. Except the obturator, other specialized structures like epistase and hypostase are not found but we observed another specific feature of the ovule, so called “wings” that form at its raphal, chalazal and anti-raphal sides (Fig. 7).

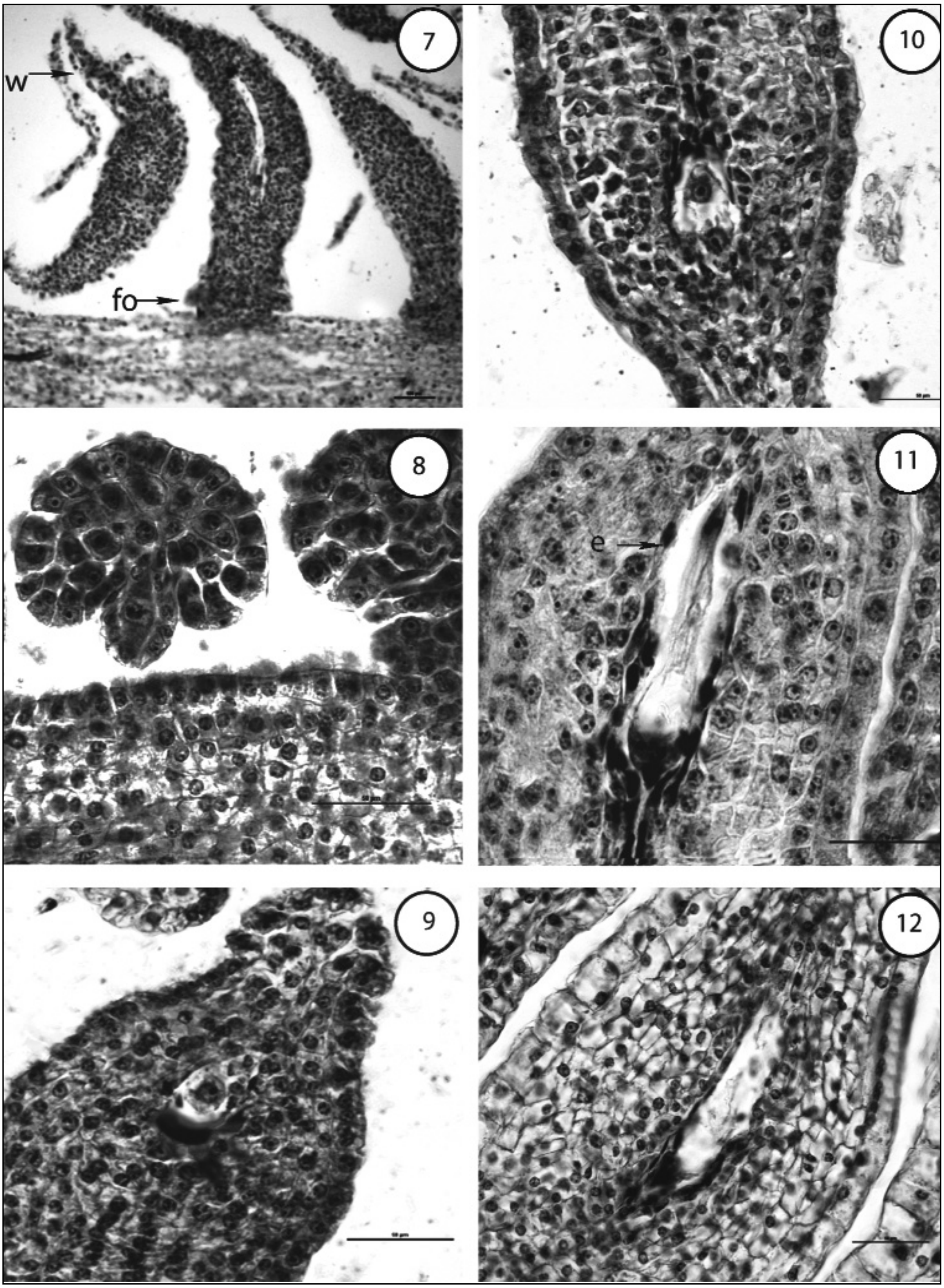
The tenuinucellate ovule possesses the nucellar epidermis with a single row of cells and one-celled archesporium. Its degeneration begins during the early stages of the embryo sac (ES) development. At the four-nucleate ES stage no traces of the nucellus are observed. In the mature ovule, the single integument is vigorous, comprising up to 15 rows of cells. The cells of its outermost layer are one-nucleate, bigger than the other somatic cells and during ovule’s ontogenesis strongly lengthen radially. At the stage of four-nucleate ES, from the innermost layer of a single integument, an endothelium differentiates and its cells are more clearly lengthened in tangential than radial direction (Fig. 11).

In the young ovule, one-celled archesporium is formed hypodermally (Fig. 8). Archesporogenesis runs without formation of parietal cells. The single archesporial cell enlarges and functions directly as megaspore mother cell (MMC). After meiosis in it, a linear megaspore tetrad forms in the ovule. The chalazal megaspore becomes functional as embryo sac mother cell and successively develops after three mitotic divisions into two-, four- (Figs 10 and 11) and finally eight-nucleate ES. In this way, the ES formation follows the *Polygonum* (monosporic)-type with three-celled egg apparatus, two polar nuclei and three-celled antipodal complex. In the population from Rila Mt we observed in some ovules the formation of a megaspore dyad instead of tetrad (Fig. 9). In these cases the ES development follows the *Allium* (bisporic)-type from the chalazal megaspore of the dyad.

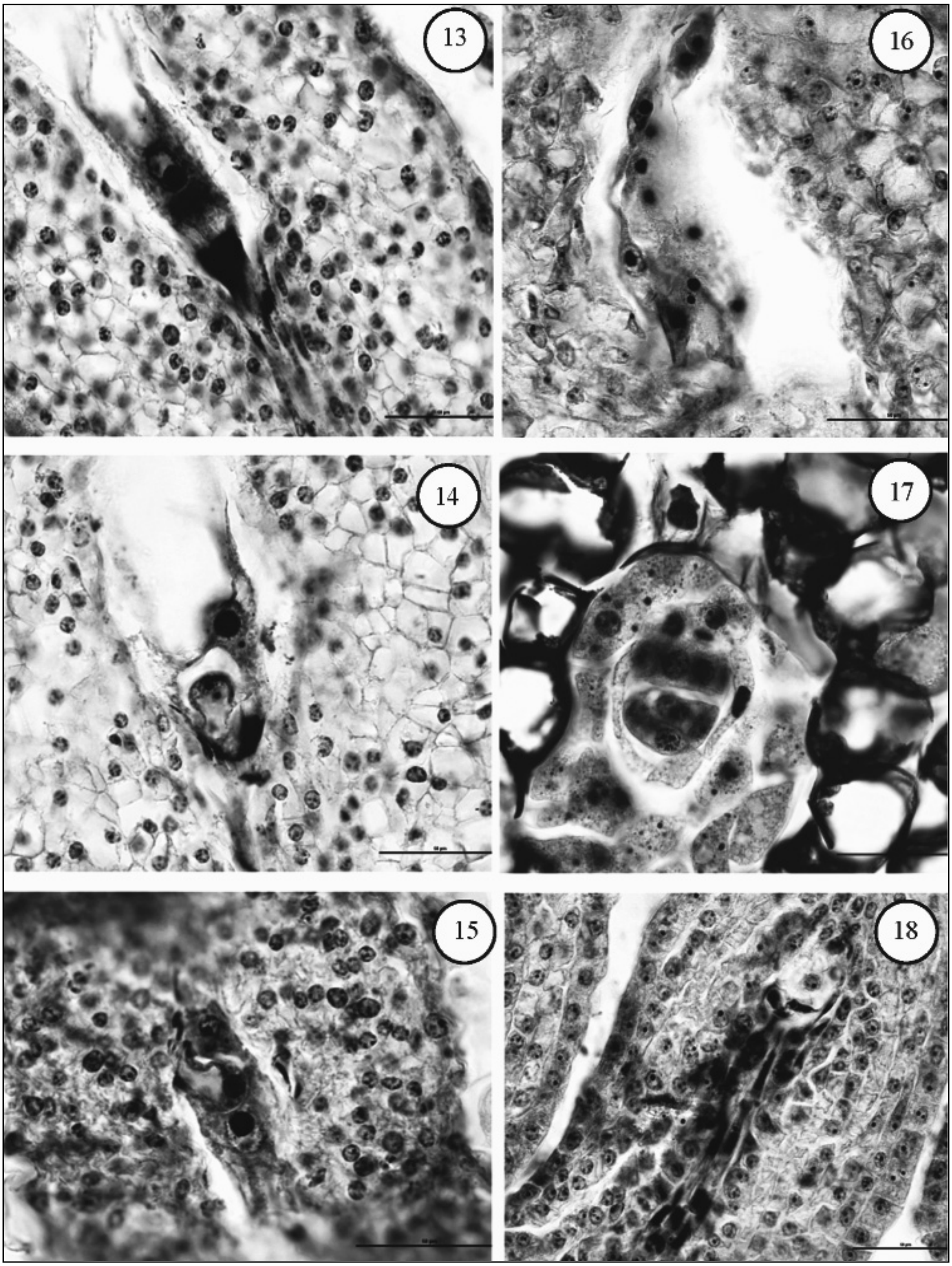
At the beginning of the mature ES differentiation, the cells of egg apparatus appear morphologically similar. Subsequently the egg cell and the synergids become pyriform in shape with typical vacuolization and position of their nucleus. Two synergids are hooked, with filiform apparatus (Fig. 13) and degenerate after the fertilization.



Figs 1-6. Male generative sphere. Fig. 1. Five-layered anther wall and MMCs in prophase I of the meiosis. Fig. 2. Anther with microspore tetrads and “trabecules” (t). Fig. 3. Microspore tetrads and glandular tapetum. Fig. 4. One-celled pollen and degenerating tapetum. Fig. 5. Degenerating mature pollen grains and anther wall with epidermis (epi) and three endotecium layers (end). Fig. 6. Mature pollen grains. Scale bar = 50 µm.



Figs. 7-12. Female generative sphere. Fig. 7. Mature ovule with funicular obturator (fo) and wings (w). Scale bar = 100 µm. Fig.8. Megaspore mother cell in the ovule. Fig. 9. Megaspore dyad with functional chalazal cell. Fig. 10. Two-nucleate ES. Fig. 11. Four-nucleate ES with endothelium (e). Fig. 12. Degenerating antipodal complex in the ES. Scale bar = 50 µm (Figs 8-12).



Figs 13-18. Female gametophyte, embryo- and endospermogenesis. Fig. 13. Egg apparatus and central cell in the ES. Fig. 14. Double fertilization in the ES. Fig. 15. Zygote and primary endosperm nucleus. Fig. 16. Two-celled embryo and free nuclei of the cellular endosperm. Fig. 17. Globular embryo and cellular endosperm. Fig. 18. One-nucleate aposporous ES and degenerating legitimate ES in an ovule. Scale bar = 50 µm.

Two polar nuclei fuse before the fertilization and form a big central cell that is located closely to the egg cell (Fig. 13).

Antipodal complex consists of three one-nucleate cells with T-shape or linear arrangement. In the antipodals, a multiplication of the nuclei runs as well as polyploidization, in particular in the population from the Balkan Range. Usually, the antipodals degenerate after the fertilization (Fig. 12).

The legitimate embryo and endosperm form after double porogamous fertilization (Fig. 14). The first mitotic division of the zygote (Fig. 15) is transversal (Fig. 16) and the embryogenesis follows the Solanad-type.

Initially, the endosperm is nuclear (Fig. 16). Later on, the endosperm differentiates into cellular one at the globular embryo stage (Fig. 17). Endospermogenesis begins before the embryogenesis and even at the stage of zygote in the ES cavity 15-20 free endosperm nuclei present. It is of interest to mention, that sporadically, in ovules of the population from Rila Mt, an additional aposporous ES forms above the one-nucleate degenerating legitimate one (Fig. 18).

## Discussion

During the study on *G. lutea*, typical of the family *Gentianaceae* L. and genus *Gentiana* embryological characteristics are established, as follows: tetrasporangiate anthers, Dicotyledonous-type of the anther's wall development with more than one middle layer; fibrous endothecium; 3-celled mature pollen grains; anatropous, tenuinucellate, unitegmic ovule; one-celled female archesporium; archesporogenesis without formation of parietal cells; *Polygonum*-type female gametophyte; *Solanad*-type embryogenesis and cellular-type of the endosperm (Davis 1966; Poddubnaya-Arnoldi 1982; Shamrov 1987). Besides, a number of new embryological peculiarities are found, announced up to now only for some other species *Gentiana* as well as the peculiarities that are not identically interpreted from different authors.

### *Male generative sphere*

Shamrov (1986), concerning the anther's wall in *G. lutea* announces for the formation of two additional layers in it, referring them to the middle layers that consisting cells enlarge and develop fibrous thickenings like the endothecium. Our observations during the study, give us the reason to interpret this fact by a different manner. Because of the additional layers are morphologically identical with the endothecium (including the fibrous thickening of their cells and an early degeneration of the middle layers), their origin probably have to be connected with the endothecium than the middle layers. We consider that histological investigations are needed also to be carried out in order to receive more definite data for clarification of the origin of these additional layers in the anther's wall of *G. lutea*.

The early degeneration of the middle layers to the end of the heterotypic division of meiosis in MMCs defines them as ephemeral. Contrary, Shamrov (1986), shows that these layers preserve even at the stage of mature pollen grains in the anthers. A longer preservation of the middle layers is noticed also for other species of the family

*Gentianaceae,* as *Megacodon stylophorus* (C.B.Clark) H. Smith and *Veratrilla baillonii* Franch (Xue & Li 2005).

Poddubnaya-Arnoldi (1982) and Shamrov (1987) describe the anther's tapetum of the family *Gentianaceae* as glandular without transformation in ameboid or periplasmodial one, in particular in *G. lutea* too (Shamrov 1986). Davis (1966), gives a contrary opinion, considering that the tapetum in this family is glandular with transformation into ameboid one. The same conclusion is made for *G. carpatica* Kit. from Schnarf (1923) as well as for *Canscora diffusa* (Vahl) R. Brown ex Roem. & Schult. and *C. decussata* (Roxb.) Roem. & Schult. (Maheshwari 1962). Periplasmodial tapetum is announced for *Gentiana asclepiadea* L. (Wóycicki 1935), *G. pedicellata* (D. Don) Griseb., *G. quadrifaria* Blume and *Canscora decurrens* Dalz. (Sankara Rao & Chinnappa 1983).

Our observations reveal that the anther's tapetum in *G. lutea* is glandular without transformation into ameboid one that support the opinion of Shamrov (1986) concerning to this species. The unusual state of the tapetum established in *G. lutea*, when its cells penetrate between the microspore tetrads might to be considered as a beginning of degeneration than a transformation of glandular tapetum into ameboid or periplasmodial one.

During this study, a specific feature in *G. lutea* is observed, as formation of rows of cells, crossing the anther locules in radial direction that Shamrov (1986) found in the same species and named them ”trabecules”. This peculiarity is established too in other representatives of the family *Gentianaceae* like *Megacodon stylophorus*, *Veratrilla baillonii* (Xue & Li 2005) and species *Swertia* L. (Xue & al. 2007) that Steffen & Landmann (1958) named “placentoids”. Because of the similarity between the tapetal cells and “trabecules”, Shamrov (1986) considers that “…it is the tapetum itself, which cells to the side of connecting tissue and outer anther's wall during their development intrude between the sporogenous cells, tie together and form these peculiar barriers”.

### *Female generative sphere*

The ovule of *G. lutea* is anatropous indicated as a typical for the family *Gentianaceae* by Poddubnaya-Arnoldi (1982). Shamrov (1987) defines the ovule in the representatives of this family as a preliminary anatropous, rarely – orthotropous in *Halenia elliptica* D. Don, *Cothylanthera tenuis*, species *Voyria* Aubl. or hemitropous in *Gentiana tenella* Rottb. and *Gentianella lingulata* G. Agardh. It is indicated that in *Gentianella azurea* (Bunge) Holub (Liu & He 1996), *G. caucasea* (Loddiges ex Sims) Holub, *G. germanica* (Willd.) Boerner (Akhalkatsi & Wagner 1997) and *G. ciliata* (L.) Borkh. (our unpublished data) a hemitropous ovule is presented and orthotropous one is shown in *Swertia tetraptera* Maxim (Xue & al. 1999).

Shamrov (1999), gives a new classification of the ovule's types on the basis of the nucellar structure and development and shows that in the family *Gentianaceae* the ovule is tenuinucellate – “standard (simpetal) variation” in which the nucellus is presented by the nucellar epidermis that envelopes the megasporocyte during its development and destroys before the fertilization. Our observations on the ovule ontogenesis of *G. lutea* show that it might be refer to the same ovule type, namely “standard (simpetal) variation”.

We observe a funicular obturator, described by Shamrov (1988) in this species too as well as the so colled “wings” that Bouman & Schrier (1979) announce for *G. asclepiadea*.

In *G. lutea* the endothelium is formed, that is announced up to now for *Gentianaceae* only in *G. cruciata* L. (Shamrov 1990) and *Exacum pumilum* Griseb. (Poddubnaya-Arnoldi 1982).

In the ovule of *G. lutea* the one-celled archesporium forms, that is typical of the family *Gentianaceae* and genus *Gentiana* (Poddubnaya-Arnoldi 1982, Shamrov 1987), but in this species Kordjum (1978) and Shamrov (1987, 1988) show also two-celled archesporium.

In the ovules of *G. lutea* predominantly megaspore tetrad forms, but in some ovules of the population of Rila Mt a dyad of megaspores forms instead of a tetrad and ES not develops according to *Polygonum (*monosporic)-type but follows *Allium* (bisporic)-type of development. The *Allium*-type female gametophyte is announced also in *G. cruciata* (Schamrov 1988), *G. punctata* L. (Bicheva & al. 2004) and other species of *Gentianaceae* as *Centaurium ramosissimum* (Vijayaraghavan & Padmanabhan 1969) and *Swertia minor* Griesb. (Sankara Rao & Nagaraj 1982). In the Rila Mt population of *G. lutea*, the formation of an aposporous ES is observed in the chalazal part of some ovules, but the embryo formation is not found. Rudenko (1961) shows nucellar adventive embryony not only in this *species* but also in *G. punctata*, *G. livonica* Eschf. and *G. carpatica* Wettst.

The peculiarities of the male and female generative sphere established during the present embryological study on Bulgarian populations of *G. lutea* reveal a specialization of this species, in particular regarding the female gametophyte pronounced in the following features: anatropous, tenuinucellate, unitegmic ovule; funicular obturator; one-celled female archesporium; archesporogenesis without formation of parietal cells; *Allium* (bisporic)–type female gametophyte together with the basic *Polygonum* (monosporic)-type; endothelium; tendency to gametophytic apomixis (apospory). Some of relatively primitive features observed in the male generative sphere (multiplication of number of the anther wall layers and the multilayered sporogenous tissue) are not so significant in evolutionary aspect and essential to change the status of *G. lutea* as an evolutionary advanced species and they are only considered as a manifestation of the heterobathmy.

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