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Cytoembryological study of *Crepis bithynica* (Asteraceae) from Bulgaria

Abstract

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A cytoembryological study of *Crepis bithynica* - a Balkan subendemic species from Pirin Mt., Bulgaria has been carried out. The major characteristics of the male and female gametophytes and the trends of evolution of the embryological structures and processes of *C. bithynica* were revealed. The anther wall develops according to the Dicotyledonous-type and it is four-layered with epidermis, endothecium, one ephemeral middle layer and ameboid tapetum. A reduction of the anther locules from four to two was observed. After simultaneous microsporogenesis predominantly tetrahedral tetrads are formed. The mature pollen is in a high degree fertile. The archesporium in the ovule is unicellulate. The embryo sac develops after basic Polygonum (monosporic)-type. Somatic apospory and integumentary embryony were observed. Some of the primitive embryological features correspond with the relatively primitive morphology of *C. bithynica*. The study reveals a relatively high specialization and plasticity especially of the female gametophyte. This provides better trophical function, larger adaptability and successful reproduction, thus supporting the potential possibilities for survival and expansion of the populations of *C. bithynica*.

Introduction

Crepis bithynica Boiss. belongs to sect. Macropodes Babc. of the genus *Crepis* L. (Babcock 1947b). The species is a Balkan subendemic distributed in the SW part of Asia Minor (Bithynian Olympus) and in the mountains of the S Balkan Peninsula. It grows in calcareous stony habitats, mostly rock fissures and screes. In Bulgaria the species is found only in several localities in Pirin Mt. (Dimitrova 2002).

So far, only four species of *Crepis* from Bulgarian localities have been studied embryologically: *C. sancta* (L.) Babc. and *C. zacintha* (L.) Babc. (Yurukova-Grancharova 1983a), *C. foetida* L. (Yurukova-Grancharova 1987) and *C. setosa* Haller f. (Yurukova-Grancharova 1994, 2000). All are annuals, with short reproductive cycle and morphological adaptations for wide distribution and survival in arid habitats.

Crepis bithynica is a taxon of restricted distribution and international conservation interest. It is included under the category "rare" in the Red Data Book of Bulgaria (Velchev

1984) and the IUCN Red List (Walter & Gillet 1998). The chromosome number, karyotype structure and nuclear DNA amount of this species have already been studied from Bulgarian accessions (Kuzmanov & Kozuharov 1970; Kuzmanov 1975; Dimitrova & Greilhuber 2000, 2001).

We consider that a detailed study of the embryology of *C. bithynica* is important to reveal the reproductive potential of the species. This information can be relevant to the choice of the most appropriate measures for protection of *C. bithynica* in the Bulgarian flora.

Material and methods

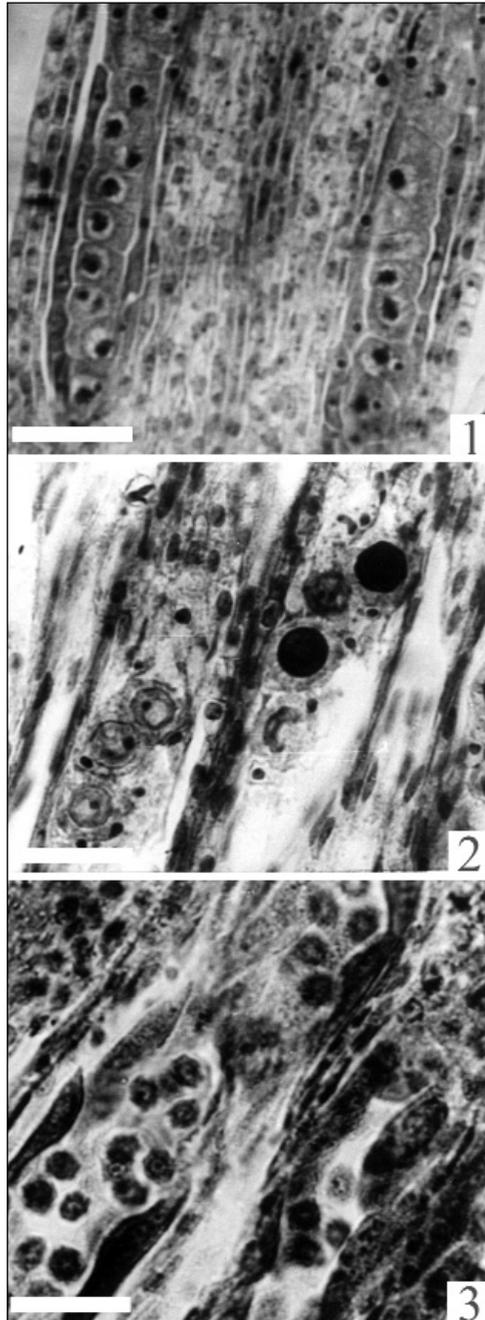
Flower buds, capitula and achenes at various stages of development were collected from a natural population of *C. bithynica* (SOM157221: Pirin mountain - calcareous screes along the path from Banderitza chalet to Kazanite locality, ca. 1900 m alt.; Dimitrova, 10 Aug. 1999). The plant material was fixed in FAA (5 parts formalin : 5 parts glacial acetic acid : 90 parts 70 % ethanol). Classical paraffin method was applied (Romeis 1948). Serial microtom cuts 8-14 μm thick were made. The paraffin sections were stained with Heidenhain's haematoxylin (Heidenhain 1886) and mounted in Canada balsam. The permanent slides were examined with light ZEISS-Amplival microscope. The microphotos were made with "Nf-matic" camera.

The voucher specimens are deposited in the Herbarium of the Institute of Botany of the Bulgarian Academy of Sciences (SOM).

Results

Anther and development of male gametophyte - The anthers of *C. bithynica* are tetrasporangiate. In individual flowers, only in several capitula, a reduction of the anther locules from four to two as a result of disintegration of the connective tissue between them is observed. The anther wall develops centrifugally following the Dicotyledonous type (Davis 1966). It consists of four layers: epidermis, endothecium, middle layer and tapetum (Fig. 1).

The epidermis consists of a single row of almost rectangular uninucleate cells with clearly expressed vacuolization. They are larger than the cells of the endothecium and the middle layer. The endothecium cells are uninucleate and almost rectangular in shape as well, but narrower and shorter compared to the epidermal ones. Fibrous thickenings of the endothecium cells are observed after the one-celled stage of the pollen grains. The narrow, polygonal uninucleate cells of the middle layer of the anther wall are strongly elongated tangentially and tightly pressed on both sides by the large endotheical and tapetal cells. In *C. bithynica* the middle layer is ephemeral and usually degenerates towards the end of prophase I of the meiosis in PMCs, but sporadically during metaphase I - anaphase I darkly stained, degenerating parts of this structure can be observed. Initially, the tapetum of the anther wall is glandular. It consists of wide, square to rectangular uninucleate cells arranged in a single layer. During ontogenesis, as a result of comparatively fast subsequent mitotic divisions, the nuclei in the tapetum cells multiply - from one to four - six, rarely -



Figs 1-3. Anther and male gametophyte. Fig. 1. Four-layered anther wall with glandular tapetum and one-layered sporogenous tissue. Fig. 2. One-celled pollen and amoeboid tapetum. Fig. 3. Tetrahedral microspore tetrads and glandular tapetum in an anther. Scale bar = 50 μ m.

eight. Mitotic disturbances, i.e. fusion of metaphase or anaphase plates, cause polyploidization in some tapetal cells. After the one-celled pollen stage in the anthers, the glandular tapetum transforms into amoeboid false periplasmodium (Fig. 2) that exists very shortly. Usually, by the time of formation of the mature pollen grains only the epidermis is completely preserved in the anther wall. More rarely the endothecium is observed too but in rather destroyed state.

The sporogenous tissue in the anthers is composed by one, rarely two - three rows of comparatively small uninucleate polygonal cells tightly packed to one another with one - two darkly stained nucleoli (Fig. 1). Very quickly the sporogenous cells grow, round up, separate from each other and differentiate into PMCs.

In most cases the meiosis in PMCs runs normally which is proved by the formation of five bivalents ($n=5$) observed during diakinesis and metaphase I. However, in a small number of PMCs in some capitula, certain deviations during meiosis are observed: 1. single lagging chromosomes; 2. individual chromosomes scattered along the whole length of the division spindle; 3. sporadically one - two univalents together with bivalents in PMCs; 4. atypical orientation of the spindle during the homeotypic division. The simultaneous microsporogenesis results predominantly in tetrahedral (Fig. 3), rarely isobilateral tetrads.

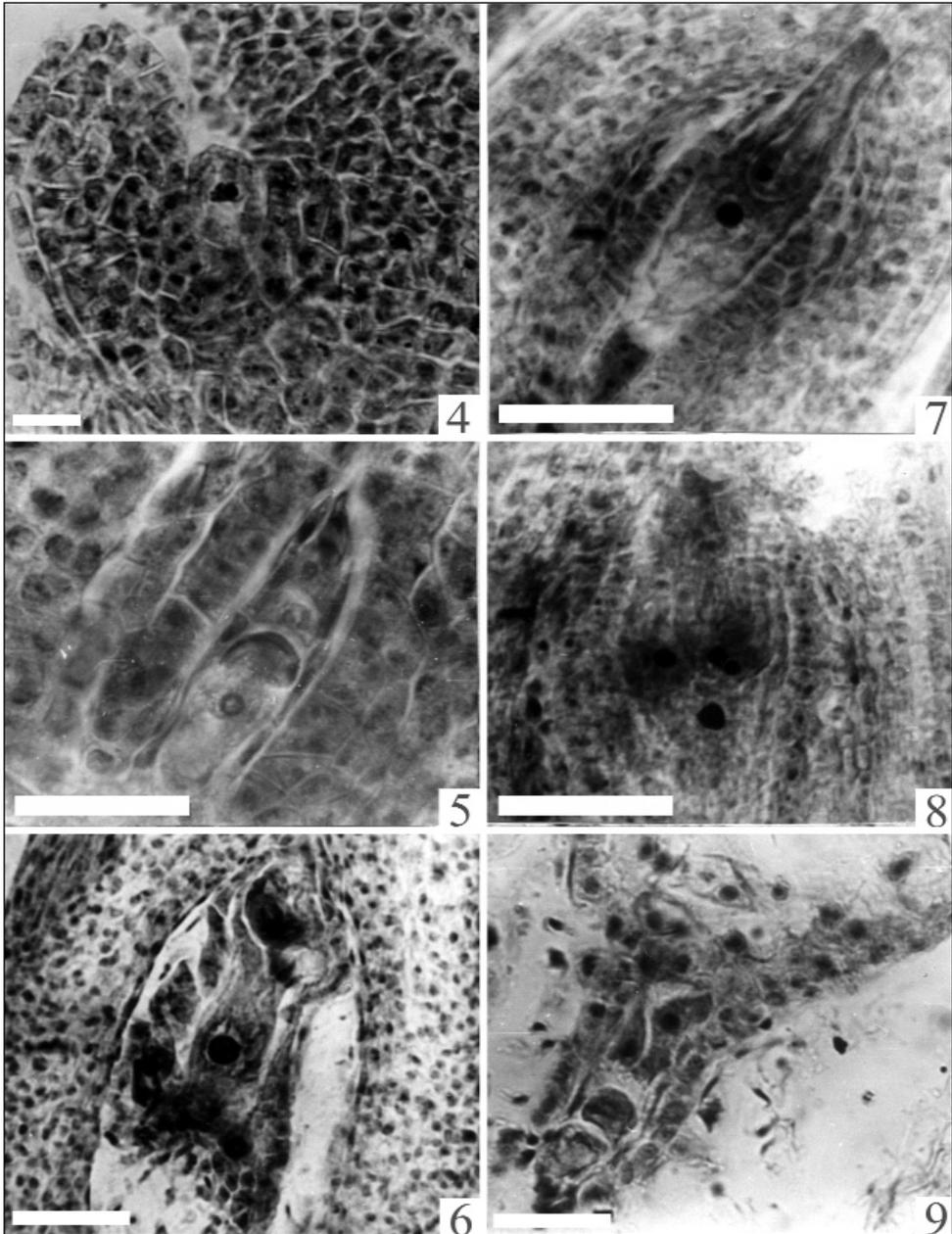
The mature pollen is morphologically uniform, three-celled, three-colporate with echinate exine. Empty, almost transparent sterile pollen grains, normal in size with a smooth surface and disturbed colporate complex are often found. In some anthers degenerating pollen tetrads as well as darkly stained pollen grains without a visible nucleus are observed. Usually, in these cases the tapetum of the anthers is completely destroyed.

Ovule and development of female gametophyte - In the inferior, unilocular ovary of *C. bithynica* basally a single ovule develops in which hypodermally unicellular archesporium forms. The fully mature ovule is anatropous, tenuinucellate, unitegmic. In the later stages of the ovule development a hypostase appears in its chalazal part.

The single archesporial cell is usually clearly distinguished from the somatic cells of the ovule by its larger size, dense cytoplasm and more prominent darkly stained nucleus. It differentiates into a megaspore mother cell (MMC) after a short period of intensive growth and vacuolization (Fig. 4). Archesporogenesis runs without formation of parietal cells. After meiosis in MMCs a linear megaspore tetrad is formed in the ovule. Usually the chalazal megaspore soon differentiates into an embryo sac mother cell (EMC) (Fig. 5). The three sister megaspores towards the micropyle degenerate progressively and only their darkly stained remnants can be observed even after the differentiation of the one-nucleate embryo sac (ES). Sometimes two functional megaspores in a tetrad were found that after three mitotic divisions develop into two mature ESs in a row within an ovule (Fig. 6).

The endothelium differentiates at the one-nucleate stage of the ES from the innermost layer of the single integument (Fig. 5). The endothelium consists of one layer of morphologically uniform uninucleate cells that lengthen radially during the ES development. In some ovules only towards the chalazal part of the ES two-layered endothelium is observed.

The ES development follows the basic, monosporic Polygonum-type. After three subsequent mitotic divisions in the EMC, two-, four-, and eight-nucleate ES is formed. The polarization of the nuclei, vacuolization in each of the above mentioned ES stages, and the structure of the mature ES are typical for the Polygonum-type female gametophyte (Fig. 7).



Figs 4-9. Ovule and female gametophyte. Fig. 4. Ovule with hypodermally located megaspore mother cell (MMC). Fig. 5. Linear megaspore tetrad with chalazal megaspore functioning as embryo sac mother cell (EMC). Fig. 6. Elements of two mature ESs in an ovule. Fig. 7. Mature ES with egg apparatus, central cell and three antipodals. Fig. 8. Mature ES with two egg cells in an egg apparatus, a synergid and the central cell. Fig. 9. Four antipodals in the chalazal narrow side of the ES. Scale bar = 50 μ m.

The differentiation of the elements in the mature ES starts from its micropylar towards chalazal pole with the three-celled egg apparatus. The egg cell is large, pyriform with a clearly expressed vacuolization and a big nucleus in its wider basal end (Fig. 7). Sporadically an egg apparatus with two morphologically equal egg cells and only one synergid can be found (Fig. 8). The two synergids are smaller than the egg cell, almost pyriform, rarely cylindrical in shape with filiform apparatus. Two polar nuclei move almost to the central part of the ES where they fuse into a central cell (Fig. 7).

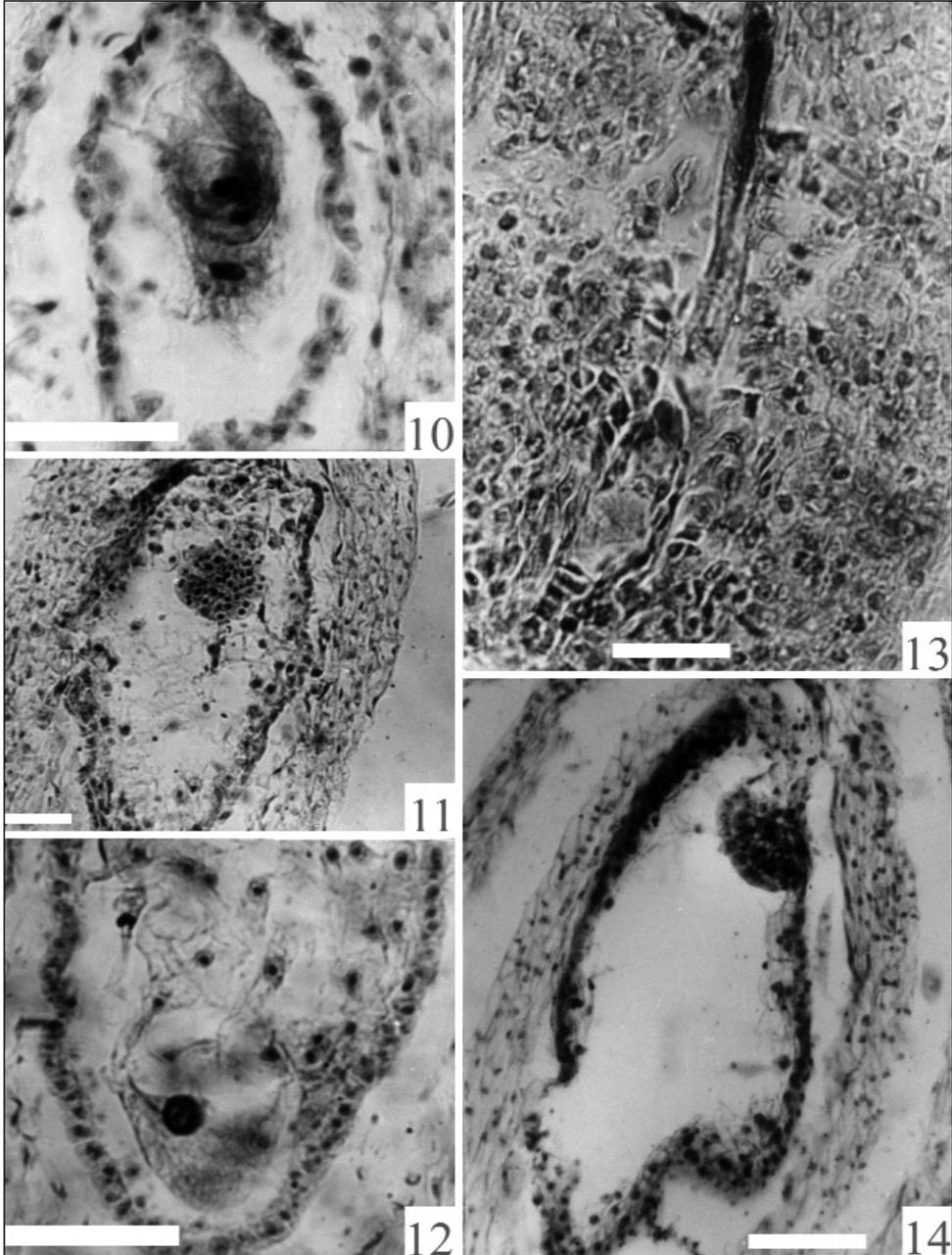
The antipodal complex is differentiated in the narrow chalazal part of the ES. Generally, it consists of three uninucleate cells (Fig. 7) with T-shaped or linear arrangement. Sometimes, the antipodals are cuneiform and their narrow ends enter deeply into the chalazae. They often show a tendency to haustorization. The antipodals of *C. bithynica* are not ephemeral and remain vital till the latest stages of embryo- and endospermogenesis. After fertilization, in most ES they multiply from three to four - six as a result of mitotic divisions and subsequent cytokinesis (Fig. 9). Sporadically, three-celled antipodal complex occurs that resembles morphologically an egg apparatus inverted towards the micropyle. After the formation of the central cell of the ES the pollen tube penetrates through the micropyle usually destroying one of the synergids which later rapidly degenerates.

The legitimate embryo and endosperm of *C. bithynica* form after the double porogamous fertilization. The zygote undergoes its first transversal division before the first mitosis of the fertilized central cell of the ES (Fig. 10). Both the first division of the zygote and the development of the young embryo show that embryogenesis in *C. bithynica* follow the Asterad-type, typical for Asteraceae (Johansen 1950; Davis 1966). The globular embryo has a comparatively long four - five, rarely six-, seven-celled vigorous suspensor (Fig. 11). Very often haustorization and clear vacuolization at least of the last cell of the suspensor (towards the micropyle) is observed.

Initially the endosperm is of nuclear type. It differentiates into cellular one after the globular embryo stage. The cytokinesis between the free endosperm nuclei runs from the periphery towards the central part and from the micropylar towards the chalazal pole of the ES cavity - a direction typical for most Angiosperms (Hudijak 1963). In the course of this cytokinesis the chalazal endosperm haustorium begins to differentiate in the lower part of the endosperm (Fig. 12).

The mature embryo is straight with two morphologically equal cotyledons. At this stage the endosperm is usually completely consumed and only one layer of its cells remains close to the still vital endothelium of ES.

In single ovules an aposporous ES develops from a somatic cell deeply disposed into the chalazae (Fig. 13). This aposporous ES is usually found after the degeneration of the legitimate one and is separated from it by one - two layers of somatic cells (Fig. 13). The development of such somatic ES usually reaches only the two-nucleate stage but not maturity and embryo formation. Sporadically integumental embryony has been also observed (Fig. 14), when an additional somatic embryo develops from a cell/cells of the inner layers of the single integument of the ovule.



Figs 10-14. Female gametophyte, embryogenesis and endospermogenesis. Fig. 10. First division of the zygote and primary endosperm nucleus. Fig. 11. Globular embryo with suspensor and nuclear endosperm. Fig. 12. One-nucleate endosperm haustorium in the chalazal part of the ES. Fig. 13. One-nucleate aposporous ES and degenerated legitimate ES above. Fig. 14. An integumentary multicellular embryo. Scale bar = 50 μ m.

Discussion

From the about 200 existing *Crepis* species (Bremer 1994) less than twenty are examined embryologically (Solntseva 1987). Most data concern the polyploid American apomictic species where apospory, parthenogenesis, and apogamy have been observed (Babcock 1938; Stebbins & Babcock 1939; Stebbins & Jenkins 1939). In Bulgarian flora 17 species of *Crepis* are distributed (Dimitrova 2002) of which so far only four have been studied embryologically.

The following major embryological characteristics typical for tribe *Lactuceae* have been registered in *C. bithynica*: Dicotyledonous-type of development of four-layered anther wall; ephemeral middle layer; initially glandular anther tapetum that transforms into ameboid one; three-celled mature pollen; unicellulate female archesporium; archesporogenesis without formation of parietal cells; embryo sac of Polygonum-type; embryogenesis of Asterad-type; mature straight embryo with two equal cotyledons.

In the same time *C. bithynica* has several distinctive embryological characteristics that are absent or only sporadically found both in *Lactuceae* as a whole and in genus *Crepis* in particular. These characteristics concern both the male and female gametophytes.

Male gametophyte

1. In some anthers the typically four anther locules are reduced to two that is considered as a more advanced feature. Such phenomenon has been found also in *C. sancta* from Bulgarian localities (Yurukova-Grancharova 1983a).

2. Either none or only in some endothecium cells fibrous thickenings of their walls are observed. Similar was the case in the previously studied *C. foetida* (Yurukova-Grancharova 1987) and *C. setosa* (Yurukova-Grancharova 1994). Supposedly, this is a general trend in *Lactuceae* in contrast to *Asteroideae* where such thickenings are clearly expressed as observed for instance in the Bulgarian representatives of sect. *Filipendulinae* (DC.) Afan. of the genus *Achillea* L. (Yurukova-Grancharova & al. 2002).

3. The sporogenous tissue in *C. bithynica* usually is one-layered, but in some anthers two or three layers are observed. Such structure of the sporogenous tissue was found in other *Lactuceae* from Bulgaria - *Leontodon crispus* Vill. (Yurukova-Grancharova 1978) and *Chondrilla juncea* L. (Yurukova-Grancharova 1979) but not in *C. sancta* and *C. zacyantha* (Yurukova-Grancharova 1983a), *C. foetida* (Yurukova-Grancharova 1987) and *C. setosa* (Yurukova-Grancharova 1994). The multilayered sporogenous tissue is considered to be evolutionary more primitive embryological feature than the single-layered one. This corresponds with the relatively primitive morphology of *C. bithynica*: tap-rooted perennial, with medium-sized capitula and comparatively large, unbeaked achenes.

4. Although *C. bithynica* is a diploid with presumably balanced meiosis certain deviations in chromosome behaviour during PMC meiosis have been registered. It causes disturbances in the production of pollen grains that reduces their vitality and fertilizing ability. This fact together with the harsh environmental conditions of the habitat can threaten the successful reproduction of the populations of *C. bithynica*.

Female gametophyte

1. In some ESs of *C. bithynica*, two instead of one egg cells in an egg apparatus were observed. Such phenomenon was also found in *C. sancta* (Yurukova-Grancharova 1983a) and *Urospermum picroides* (L.) Scop. ex F. W. Schmidt (Yurukova-Grancharova 1983b) from Bulgaria.

2. In *C. bithynica* the synergids have clearly expressed fibrillar apparatus, that is not very typical for the other embryologically studied Bulgarian *Crepis* and *Lactuceae* representatives (Yurukova-Grancharova 1979). Moreover, the synergids of *C. bithynica* show a tendency to haustorization, but typical synergid haustoria like in *Cichorium intybus* L. (Yurukova-Grancharova 1979) are not found.

3. The antipodal complex in *C. bithynica* is usually three-celled, but very often after fertilization the antipodals multiply up to six deeply situated in the chalazal part of ES. More than three antipodals were found in *C. sancta* (Yurukova-Grancharova 1983a), *C. foetida* (Yurukova-Grancharova 1987), *Mycelis muralis* (L.) Dumort and *Chondrilla juncea* (Yurukova-Grancharova 1979), and *Lapsana communis* L. (Yurukova-Grancharova 1995). In *Crepis blattaroides* (L.) Vill., *C. dioscoridis* L., and *C. virens* L. (Dahlgren 1920; Schnarf 1931) multiplication of the antipodals has also been observed. Sometimes, the antypodal complex of ES in *C. bithynica* resembles an egg apparatus inverted to the micropyle. This was also found in some ES of *C. zacynta* (Yurukova-Grancharova 1983a).

4. In *C. bithynica* as well as in *C. foetida* (Yurukova-Grancharova 1987), *Leontodon hispidus* L. (Yurukova-Grancharova 1978) and *Sonchus asper* (L.) Hill (Yurukova-Grancharova 1995) elements of two ESs within an ovule were observed. This proves a tendency towards polyembryony that increases the reproductive potential and adaptability of *C. bithynica*.

5. In the advanced stages of the endospermogenesis an endosperm haustorium differentiates in the chalazal part of the ES cavity of *C. bithynica* similar to *Leontodon hispidus* (Yurukova-Grancharova 1978).

6. In contrast to the statement that apomixis is typical only for the American polyploid *Crepis* (Babcock 1947a) in *C. bithynica* we observe a tendency towards somatic apospory and integumental embryony is observed. This is the most important embryological feature of *C. bithynica* as a diploid taxon that distinguishes it from the other Bulgarian *Crepis* and *Lactuceae* taxa studied so far. Such tendency towards somatic apospory was also found in *Picris hieracioides* and *Leontodon hispidus* (Bergman 1932).

In conclusion it can be noticed that *C. bithynica* demonstrates a relatively high plasticity and specialization, especially of the female gametophyte, expressed in the presence of all above mentioned features. They provide in its turn better trophic function of the embryo of this species, larger adaptability, successful reproduction, thus supporting the potential possibilities for survival and expansion of the populations of *C. bithynica*.

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