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Embryological features, pollen and seed viability of *Arnica montana* (Asteraceae) – a threatened endemic species in Europe

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Abstract – The embryological features, mode of reproduction and reproductive capacity (pollen and seed viability) on two naturalized populations of *Arnica montana* in Bulgaria were studied. The embryological study shows that *A. montana* is a facultative apomictic species in which sexual reproduction predominates. In this species, it was established that there is a comparatively high viability of the mature pollen and embryos, which enables the successful realization of its reproductive capacity. The results of the study on *A. montana* reveal that both sexual and asexual vegetative reproduction with rhizomes undoubtedly play more important roles than the apomixis (namely diplospory) for support and preservation of the populations.

Keywords: apomixis, *Arnica montana*, embryology, male and female gametophyte, pollen and seed viability, sexual reproduction

Introduction

Arnica montana L. is a rosette-forming perennial plant of the family *Asteraceae* Dum., subfamily *Asteroideae*, tribe *Madieae* (Noyes 2007). It is a diploid species with 2n = 38 (Ekenäs 2008), highly self-incompatible with entomophily (Luijten et al. 1996, 2000). *Arnica montana* has been used for medical purposes since the 1500s because of the contents of various active compounds with antiseptic, antifungal, antimicrobial and antibiotic activities. It occurs from the lowlands to the alpine belt. In the mountains, *Arnica montana* is a characteristic species of *Nardus stricta* grasslands and hay meadows (Oberdorfer 1994). The excessive exploitation of this medicinal plant has affected the state of its natural reserves and reproductive capacity. At present *A. montana* is considered a rare and endemic species in Europe (Maguire 1943, Ferguson 1976, Ekenäs 2008), and is included in many European countries in the category "endangered" and listed in Annex V of the EU-FFH-directive (Directive 92/43/EEC 1992). The species is regarded as: "critically endangered" in Belgium, Bosnia, Croatia and Luxembourg; "endangered" in Belarus and the Netherlands; "vulnerable" in Estonia, Germany, Latvia, Lithuania, Portugal and Romania; and "near threatened" in Denmark and Norway.

In Bulgaria, *A. montana* was found more than 100 years ago in the Rila Mts (Herbarium specimen SO 86331 deposited, exists in the Herbarium of Biological Faculty of the Sofia University "St. Kliment Ohridski") but up to now its occurrence in the country has not been confirmed. Hitherto, this species has been primarily an object of chorological, karyological and phytochemical studies but scanty and fragmentary data exist on its embryology in the accessible literature. The aim of the present study is to reveal some of the main characteristics of the reproductive biology of *Arnica montana*: peculiarities of the male and female gametophyte; embryo- and endospermogenesis; pollen and seed viability, in connection with the realization of its reproductive capacity that influence the shape and size of the populations.

Material and methods

The material of two naturalized populations (two-years old) of *A. montana* were studied, as follows: Beli Rid experimental station – on Mt Vitosha (a population from the Carpathian Mountains, Ukraine) and the experimental station in the village of Beglica – Rhodopi Mts (Western) – a population from the Botanical garden, Chemnitz, Germany. The Mt Vitosha population consists of 167 individuals and

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the Rhodopi Mts population contains 146 individuals. Voucher specimens were deposited in the Herbarium of Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (SOM 168516, 168517 – from Mt Vitosha and SOM 169535 – from the Rhodopi Mts).

Embryological study

For embryological study, 50 flower buds and capitula at different stages of development were collected from 20 individuals of each of the two naturalized populations (above mentioned) and fixed in a mixture of FAA (formalin: glacial acetic acid: 70% ethanol in correlation 5:5:90 parts). Consecutively, the plant material was treated according to the classical paraffin methods (Sundara 2000), embedded in paraffin and cut into 10–25 µm sections with a rotary microtome. The sections were stained with Heidenhain's haematoxylin and embedded in Enthelan in order to get permanent slides.

Pollen and seed viability study

Mature pollen grains were isolated from the two studied populations and their viability was estimated using the acetocarmine test (Singh 2003). For this purpose, anthers from 50 open flowers from 20 plants of each population studied were collected, placed in 1% acetocarmine solution, dispersed on the slides and the stained (viable) and unstained (unviable) pollen grains were counted in 30 anthers from each population using a light microscope (visual field, enlargement 100×).

For the study on seed viability, 400 mature seeds were collected from the two populations. To estimate the seed (embryo) viability, a quick tetrazolium test was applied (Peters 2000). The embryos were isolated with a dissection needle, incubated in water at 30–35 °C and subsequently in a diluted 1% solution of 2,3,5-triphenyltetrazolium chloride for 24 hours. Initially, the tetrazolium solution is colourless, but it changes to red when it comes to contact with hydrogen (a reduction process), deriving from enzymes of the respiration process of the seeds.

The observations during the present study were carried out using a stereomicroscope "Leica EZ4", LM "Olympus" CX2. The microphotographs were made with Digital Camera 1.4 Mpx.

Results

Embryological features

Anther and development of the male gametophyte

The anthers are tetrasporangiate. The anther wall formation follows the dicotyledonous type and consists of four layers: an epidermis, an endothecium, one middle layer and a tapetum (Fig. 1A). The epidermis comprises one row of almost rectangular uninucleate cells that vastly enlarge during the anther ontogenesis. The middle layer is ephemeral and degenerates up to end of the meiosis in microspore mother cells (MMCs). The endothecium develops not clearly expressed fibrous thickenings of its consisting cells. This layer becomes completely disorganized at the stage of the mature three-celled pollen. Initially, the tapetum is glandular, consisting of one row of uninucleate cells (Fig. 1A). During the meiosis in MMCs, a rapid lengthening and multiplication of the nuclei of tapetum cells (as result of consecutive mitoses) is observed (Fig. 1C) and they become four- to eight-nucleate at the stage of microspore tetrads. After the formation of uninucleate pollen grains, the tapetum transforms from glandular to ameboid (Fig. 1E). At the time of anther dehiscence, the anther wall comprises only epidermis

The sporogenous tissue is one-, two-rowed (Fig. 1A). The meiosis in MMCs passes with some deviations, such as: individual lagging chromosomes (Fig. 1C) and chromosome out of the division spindle, especially during the first (heterotypic) division of the meiosis. After simultaneous cytokinesis, the resultant microspore tetrads (Fig. 1B) were classified on the basis of 50 enumerated tetrads, according to Schmid (1982), as: "usually" tetrahedral (71%), "occasionally" isobilateral (22%), "quite occasionally" T-shaped (5%) and "rarely" linear (2%). Sporadically monads and dyads were also observed. In a number of florets of some capitula degenerating microspore tetrads presented (Fig. 1D).At the time of shedding, the pollen grains are usually morphologically uniform, three-celled, tricolporate with

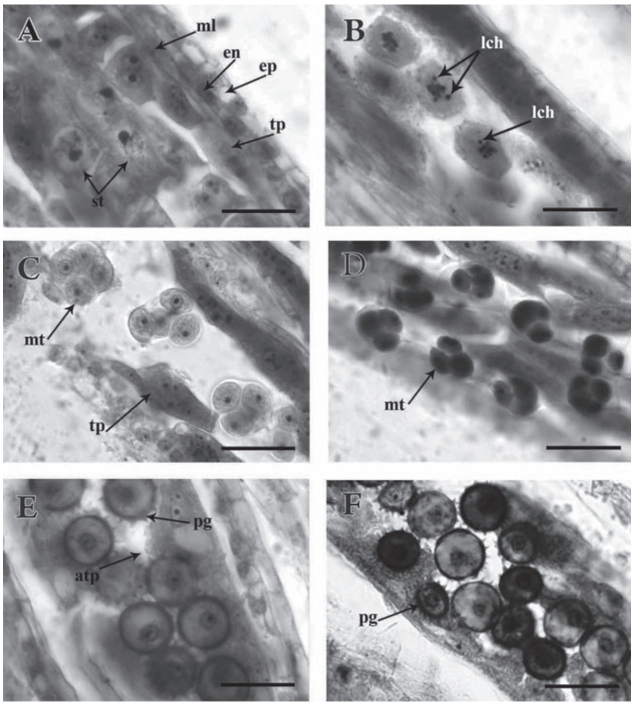

Fig. 1. Anther and development of the male gametophyte: A) anther wall and two-rowed sporogenous tissue, B) lagging chromosomes in the metaphase I of the meiosis in microspore mother cells, C) different type of microspore tetrads and one-rowed glandular tapetum with multinucleate cells, D) degenerating microspore tetrads in the anther locules, E) one-nucleate pollen and ameboid tapetum, F) viable and sterile pollen grains in an anther locule. Ep -epidermis, en – endothecium, ml – middle layer, tp – tapetum, st – sporogenous tissue, mt – microspore tetrad, pg – pollen grain, atp – amoeboid tapetum, lch – lagging chromosome. Scale bars = 20 µm.

echinate exine. Often, besides the normal viable pollen grains, small-sizedand dark-stained sterile grains were observed in the anthers of some florets in one and the same capitulum (Fig. 1F).

Ovule and development of the female gametophyte

The gynoecium is syncarpous, inferior with a unilocular ovary in which only one ovule forms. The well-developed ovule is anatropous, tenuinucellate and unitegmic. In it unicellular archesporium forms hypodermally. The archesporium cell functions directly as a megaspore mother cell (Fig. 2A), which later undergoes meiosis to produce a linear megaspore tetrad (Fig. 2B). The embryo sac (ES) development runs according to the *Polygonum* (monosporic)-type from the chalazal megaspore of tetrad that functions as an embryo sac mother cell. After three mitoses, successively two-, four- and eight-nucleate ES forms. The mature ES consists of a three-celled egg apparatus (a usually pear-shaped egg cell and two synergids), two polar nuclei (after their fusion a secondary nucleus forms) and a three-celled antipodal apparatus in the chalazal part of ES (Fig. 2C). The synergids degenerate after fertilization. The antipodals are ephemeral and begin to degenerate after the formation of a secondary nucleus. The endothelium differentiates from the innermost layer of the single integument after one-nucleate ES and the cells of which it consists progressively lengthen in a radial direction during the ES development.

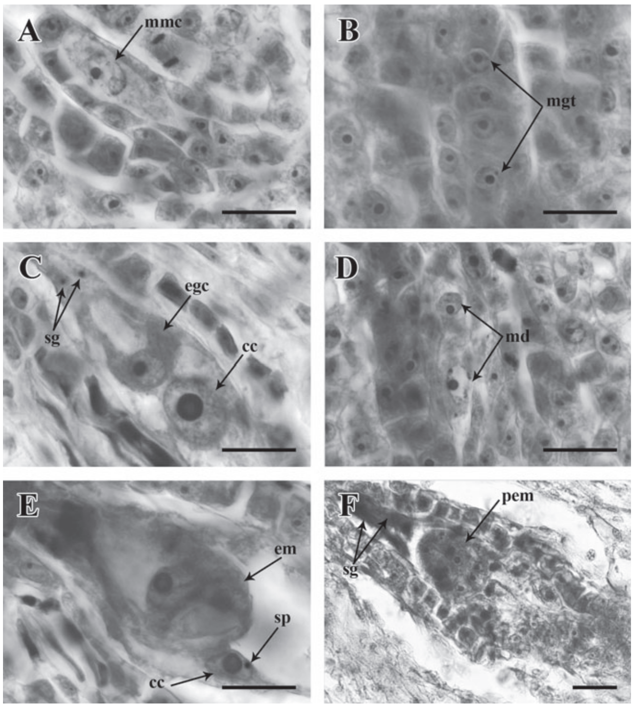

Fig. 2. Ovule and development of the female gametophyte: A) megaspore mother cell in the ovule, B) lineare megaspore tetrad in the ovule, C) three-celled egg apparatus (egg cell and two synergides) and secondary nucleus in the embryo sac, D) megaspore dyad (initiation of the diplosporous *Taraxacum*-type development of embryo sac from the chalazal megaspore), E) two-celled pro-embryo and developing endosperm, F) parthenogenetic globular embryo together with preserved two synergids and nuclear endosperm. Mmc – megaspore mother cell, mgt – megaspore tetrad, sg – synergid, egc – egg cell, cc – central cell, md – megaspore dyad, em – embryo, sp – sperm, pem – parthenogenetic embryo. Scale bars = 20 µm (A–E) and 50 µm (F).

It is important to notice that in the megaspore mother cells of some ovules in the florets of the capitula a restitution nucleus forms after inhibited meiosis that leads to a dyad of megaspores, instead of a tetrad (Fig. 2D). Later on, in these cases, from the chalazal megaspore a meiotic diplosporous ES of *Taraxacum*-type develops.

Usually, the embryo and endosperm form after double fertilization accompanied with a destruction of one synergid from the pollen tube penetrating it through the micropyle of ovule. The first division of the zygote is transverse and usually runs before the endospermogenesis (Fig. 2E). The embryogenesis follows the Asterad type. In the mature seed, the embryo is nearly straight with two equal cotyledons.

In five ovules (18% from 28 observed in total with globular embryo), the presence of a globular embryo together with two undamaged, long living synergids (in contrast to the stage of legitimate globular embryo in which the synergids are missing) provides reason to suppose that in these cases the embryo forms parthenogenetically from the unfertilized egg cell in ES that develops after a meiotic diplosporous *Taraxacum*-type, mentioned above (Fig. 2F).

At the beginning, the endosperm passes from a free nucleate to a cellular stage, when a globular embryo forms. The mature seed completely lacks endosperm (Fig. 4D).

Pollen and seed viability

After acetocarmine staining, the cytoplasm and nuclei of viable pollen grains were stained in red while unviable, empty and shrunken pollen grains remain unstained (Figs. 3A and D). The results of the study show the high viability of the mature pollen (over 80%) in the two studied naturalized populations of *A. montana* (Tab. 1). According to the intensity of staining with tetrazolium solution, the viable embryos are stained in red, while embryos partially stained

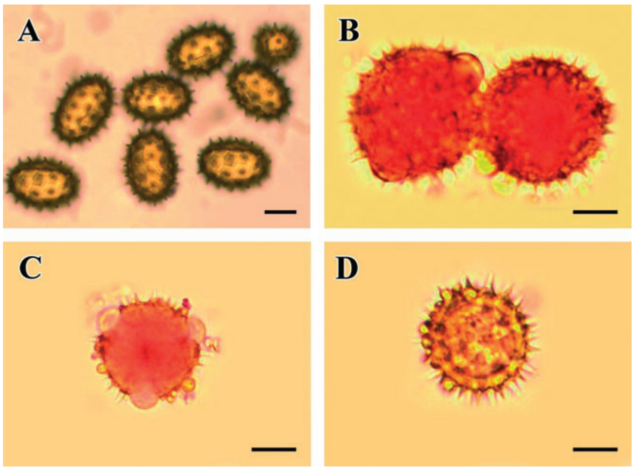

Fig. 3. Pollen of *Arnica montana* analyzed by acetocarmine test: A) mature pollen grains without acetocarmine staining, B) viable pollen grains, stained in red, C) tricolporate viable pollen grain, stained in red, D) unviable, unstained pollen grain. Scale bars = 100 µm (A), and 20 µm (B–D).

Tab. 1. Pollen viability in studied populations, analyzed by acetocarmine staining. No. – number.

Populations	No. of pollen grains	Viability (% ± SD)
Germany (Rhodopi Mts)	2897	79.99 ± 4.22
Ukraine (Vitosha Mt)	2810	83.77 ± 7.12

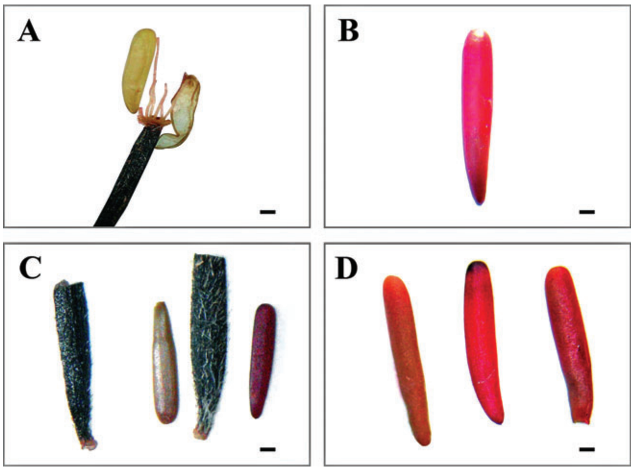

Fig. 4. Seed (embryo) viability examined by tetrazolium test: A) seed (achene) on the right side and an isolated embryo (on the left), B) isolated viable embryo (only the root stained in red), C) unviable unstained embryo (on the left side) and viable embryo, stained in dark red (on the right), D) unviable, unstained embryo (on the left) and two viable embryos, stained in red. Scale bars = 20 μm.

or unstained are unviable (Figs 4A and 4D). On basis of tetrazolium testing, the seeds (embryos) were differentiated in three classes (Fig. 5): Class I – viable embryos (whole embryo stained in red); Class II – unviable embryos (only the root of embryo stained in red); Class III – unviable embryos (unstained). It was established that the majority of embryos are viable (68.97% 63.46% for the Mt Vitosha and Rhodopi Mts populations, respectively (Fig. 5).

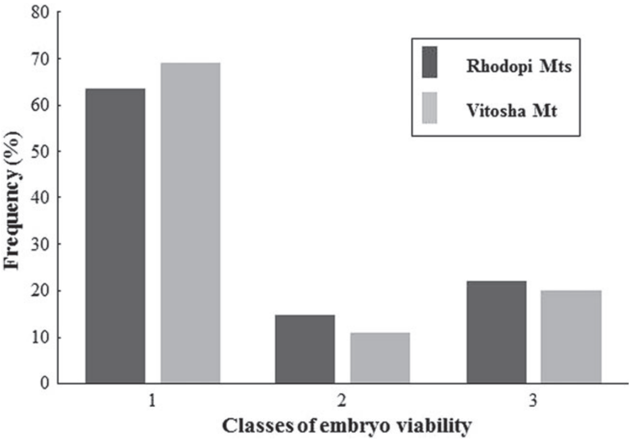

Fig. 5. Frequency of seeds (embryos) viability (%) assessed by tetrazolium test: 1 – Class I, viable embryos (stained in red); 2 – Class II, unviable embryos (only the root of embryo stained); 3 – Class III, unviable embryos (unstained).

Discussion

Embryological features

Pullaiah (1983) studied the embryology of the tribe *Senecioneae*, including the genus *Arnica*. The results of his investigation revealed only sexual reproduction in the studied representatives of this tribe. In the majority of the species in the genus *Arnica*, which are largely polyploids, gametophytic apomixis has found – diplospory and parthenogenetic development of the embryo (Afzelius 1936, Flovik 1940, Engell 1970). For that reason, Nordenstam (1977) excluded the genus *Arnica* from the tribe *Senecioneae*. In the new systematical classifications, this genus is included in the tribe *Madieae* (Baldwin et al. 2002, Noyes 2007).

The tapetum of the four-layered anther wall in *A. montana* is glandular and one-layered, consisting of multinucleate cells. After the meiosis in the MMCs it becomes ameboid (partial spreading of the cell walls occurs), as in most representatives of the family *Asteraceae* (Solntseva 1987).

The formation of microspore dyads, polyads and monads in the anthers, as result of deviations during the meiosis, was often reported for apomictic representatives of the family *Asteraceae* (some species of the genera *Crepis* L., *Taraxacum* Cass., *Rudbeckia* L., *Arnica* L., *Hieracium* L. (Solntseva 1987). In contrast with the apomictic species, in which a high percentage of sterile pollen grains is observed (Afzelius 1936, Gustafsson 1937, Babcock and Stebbins 1938, Fagerlind 1939, Battaglia 1947), in *Arnica montana* we found the formation of morphologically uniform and highly fertile pollen in the majority of the anthers.

The development of the female gametophyte in *A. montana* follows exclusively the *Polygonum* (monosporic)-type that begins from the chalazal megaspore of tetrad observed in the most *Asteraceae* (Solntseva 1987, Poddubnaya Arnoldi 1982), namely: *Chrysanthemum multicaule,(Deng at al. 2010);*, *Tugarinovia mongolica*, (Ma and Wang 2000), *Calendula officinalis,* (Ao 2007)**,** *Chrysanthemum grandiflorum*, (Deng et al. 2010). The Polygonum -type is not the only type for ES development in the *Asteraceae* family in which are observed**,** but more rarely, the *Adoxa*-type (Liu, 2001a), *Drusa*-type (Liu 2001b), *Oenothera*-type (Teng et al. 2008, Li et al. 2009) and *Pyrethrum cinerariaefolium* type (Hu 2005). The formation of a restitution nucleus in some ovules, a dyad of megaspores instead of tetrads as well as some other embryological peculiarities established during our study suggest that the female gametophyte development most likely follows the *Taraxacum*-type of meiotic diplospory (gametophytic apomixis). Consequently, we found indications that the embryo develops parthenogenetically and the proof of that is the preservation of the two synergids together with a multicellular globular embryo in the ES cavity. The same phenomenon we have observed in the species *Erigeron annuus* of the Asteraceae family (Yurukova-Grancharova at al. 2012)

In conclusion, on the grounds that during this study in *A. montana* both sexual and apomictic reproduction were established, we assume that this diploid species with 2n =

2x = 38 probably occupies an isolated position within the genus *Arnica*, in which polyploids with gametophytic apomixis (diplospory) predominate (Kao 2007, 2008; Noyes 2007).

The endosperm in the family Asteraceae was shown to be usually *ab initio* cellular by Davis (1966). Solntseva (1987) and Poddubnaya-Arnoldi (1982) described two types of endosperm (nuclear and cellular) in this family. It was established that in *Arnica montana* the endosperm is not *ab initio* cellular, but passes a free nuclear stage.

Pollen and seed viability

The estimated comparatively high viability of pollen (over 80%) and seeds (68.97% 63.46% for the Mt Vitosha and Rhodopi Mts populations, respectively) of *A. montana* in our study provides for the successful realization of its reproductive capacity, an important condition for the preservation of the size of its population. Pollen viability (potential fertility), seed production, viability and germination ability are closely connected to one another. In *Arnica montana* in particular, Strykstra et al. (1998) show that lighter seeds (achenes) are better dispersed by the wind but have a lower germination ability.

Conclusion

In the present study the main embryological features, mode of reproduction and reproductive capacity (pollen and seed viability) in *Arnica montana,* a threatened endemic species in Europe, were established. The study found a high plasticity of the female generative sphere compensating for the large number of sterile ovules and degenerating ES in the florets of capitula observed. Besides, this high plasticity has a significant importance for the better adaptation of the studied species. According to the results obtained, *A. montana* may be considered a facultative apomictic species in which sexual reproduction predominates (only 18% of apomictic embryos were observed). Thus, it may be concluded that sexual reproduction combined with vegetative reproduction determine the state of its populations rather than the diplospory (gametophytic apomixis), established during the study.

Acknowledgements

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