Generative reproduction of *Biscutella laevigata* L. from calamine waste heaps

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Introduction

During the individual development (ontogenesis) of angiosperms, a group of plants which *Biscutella laevigata* L. belongs to, alternation of generations occurs. This means that the diploid generation – sporophyte, and the haploid generation – gametophyte, occur successively. The sporophyte is a leafy plant that is responsible for vegetative functions such as growth, nutrition (as it photosynthesizes), uptake of water and mineral ions from the environment, etc. It is also responsible for the production of flowers in the generative phase. Flowers are directly involved in generative (sexual) reproduction. A perianth with sepals and petals is responsible for attracting pollinators with floral scent and color. The reproductive success of insect-pollinated flowers, including our species of interest, depends on the visitation frequency of pollen-carrying pollinators. The parts responsible for sexual

reproduction, i.e. generative structures, are stamens (male organ) and pistils (female organ). The stamen consists of a filament (although filamentless stamens exist) and anthers, each of which contains two pollen sacs. The pistil has a basal part (an ovary with ovules) and a style with a stigma on the end where pollen grains produce pollen tubes during germination. Most flowering plants (about 72%) develop bisexual flowers (so-called perfect flowers) – one flower contains both pistils and stamens. In unisexual flowers, only pistils (female flower) or stamens (male flower) develop. During ontogenesis, the reproductive cells are formed within pistils and stamens. Within somatic tissues of a parent plant, both male (in pollen sacs of anthers) and female (in ovules of the ovary) gametophytes are formed, along with gametes (sperm cells and egg cells). Fertilization is preceded by pollination: pollen grains germinate on the pistil's stigma, then the pollen tube grows through the style until it reaches an ovary and delivers two sperm cells into the ovule through the micropyle. A double fertilization

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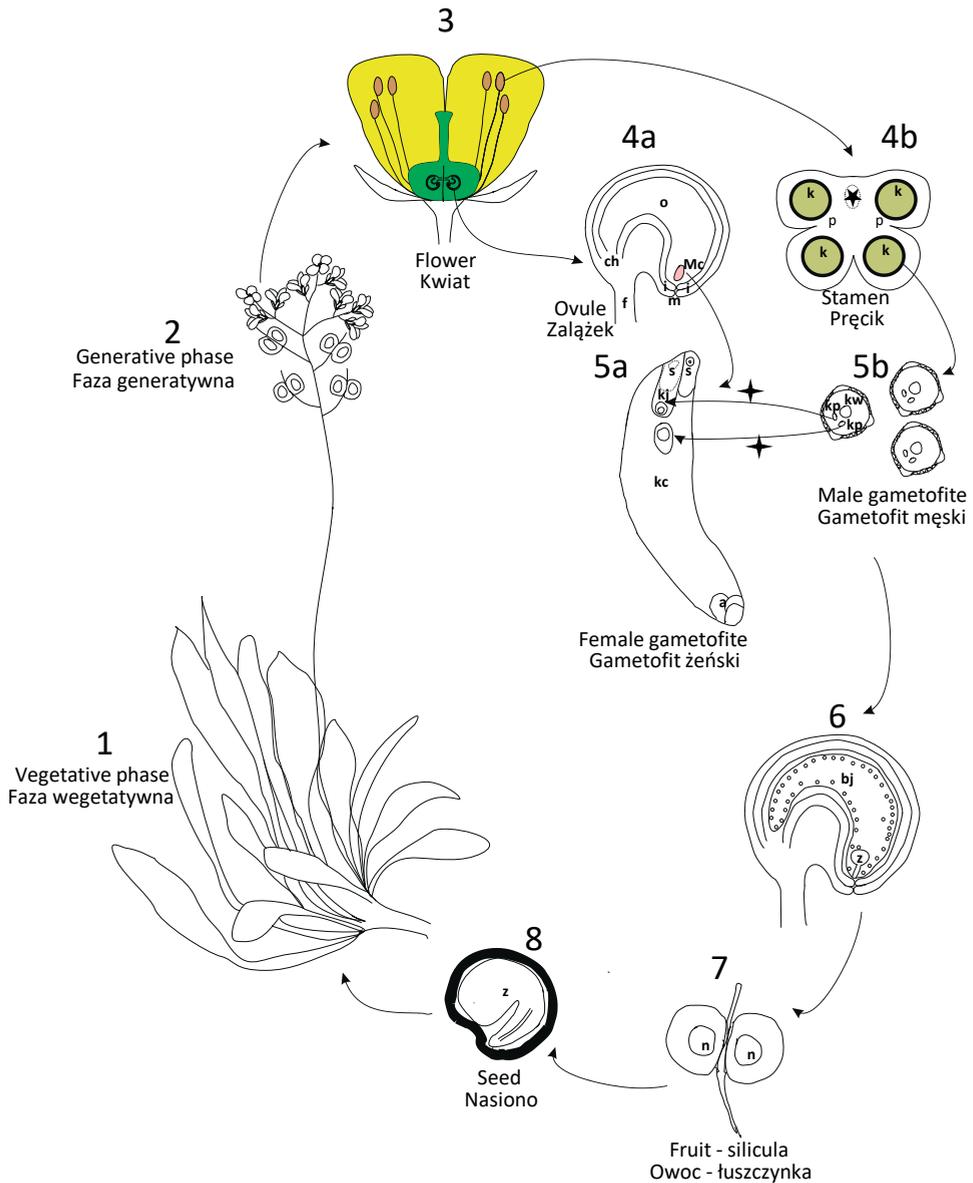
Fig. 1. The life cycle of *Biscutella laevigata*. Mature sporophyte: 1 – plant in the vegetative and 2 – generative phase. The structure of a flower: 3 – sepals and petals (perianth), pistil composed of two carpels, superior ovary with two ovules, tetradynamous stamens (4 long and 2 short). The generative structures of flower: 4a – camplyotropus ovule (characteristically curved) with megaspore mother cell visible (megasoprocyte, Mc) in the nucellus (o), f – funicle, ch – chalazal region, m – micropylar region, i – integuments (outer and inner), 4b – stamen with two anthers (p) connected by a connective (vascular bundle, asterisk). Microspore mother cells (microsporocytes) in pollen sacs (k). The stage of mature gametophyte: 5a – mature female gametophyte (embryo sac, 7-celled) formed from megasporocyte, as a result of two successive processes of megasporogenesis (meiotic division) and megagamethophytogenesis (3 mitoses). Egg cell (kj) and two synergids (s) forming egg apparatus in the micropylar region, 3 antipodal cells (a) in the chalazal region, central cell (kc) with diploid secondary nucleus (after fusion of 2 polar nuclei) in the central part of the female

Fig. 1. *Continued*

gametophyte, 5b – mature male gametophyte (pollen grain) formed from the microsporocyte, as a result of two successive processes of microsporogenesis (meiotic divisions) and microgametophytogenesis (2 mitotic divisions). At the pollination stage there are three cells: vegetative cell (kw), two sperm cells (kp). 6 – double fertilization, preceded by the transfer of pollen grains to the stigma (pollination) and delivery of two sperm cells to embryo sac (through pollen tubes growing in pistil's tissues). One sperm cell fuses with an egg cell to form a zygote, and an embryo (z) from it. The second sperm cell fuses with central cell, forming a primary endosperm cell, and a nuclear endosperm (bj) from it, a nutritive tissue for developing embryo (asterisk, a, b). The ovule develops into a seed, and integuments into seed coat. 7 – silicula, a fruit formed from the ovary, contains two seeds (n). 8 – seed. After germination, a seedling is formed from the embryo (z), and a new sporophyte from it. The cycle ends

Ryc. 1. Cykl rozwojowy *Biscutella laevigata*. Dojrzały sporofit: 1 – roślina w fazie wegetatywnej i 2 – generatywnej. Budowa kwiatu: 3 – działki kielicha i płatki korony (okwiat), słupek górny, dwuowocolistkowy, w zalążni dwa zalążki, 6 pręcików czterosilnych (4 dłuższe, 2 krótsze). Struktury generatywne kwiatu: 4a – zalążek kampylotropowy (charakterystycznie zagięty) z widoczną komórką macierzystą megaspor (megasporocytem, Mc) w ośrodku (o), f – sznureczek, ch – chalaza, m – mikropyle, i – integumenty (zewnętrzny i wewnętrzny), 4b – główka pręcika z dwoma pylnikami (p) połączonymi łącznikiem (wiązka przewodząca, gwiazdka). W komorach pyłkowych (k) komórki macierzyste mikrospor (mikrosporocyty). Stadium dojrzałych gametofitów: 5a – dojrzały gametofit żeński (woreczek zalążkowy, 7 komórkowy) powstały z megasporocytu, w wyniku dwóch następujących po sobie procesów megasporogenezy (podział mejotyczny) i megagametofitogenezy (3 mitozy). Komórka jajowa (kj) i dwie synergidy (s) tworzą aparat jajowy na biegunie mikropylarnym, 3 antypody (a) na biegunie chalazalnym, komórka centralna (kc) z diploidalnym jądrem wtórnym (po fuzji 2 jąder biegunowych) w centralnej części gametofitu żeńskiego, 5b – dojrzały gametofit męski (ziarno pyłku) powstały z mikrosporocytu, w wyniku dwu następujących po sobie procesów mikrosporogenezy (podział mejotyczny) i mikrogametofitogenezy (2 podziały mitotyczne). W stadium pylenia zbudowany z trzech komórek: komórki wegetatywnej (kw), dwóch komórek plemnikowych (kp). 6 – podwójne zapłodnienie, następuje po przeniesieniu pyłku na znamię słupka (zapylenie) i po wprowadzeniu dwóch komórek plemnikowych do woreczka zalążkowego (poprzez łagiewki pyłkowe rosnące w tkankach słupka). Jedna komórka plemnikowa łączy się z komórką jajową i tworzy się zygota, a z niej zarodek (z). Druga komórka plemnikowa, łączy się z komórką centralną, powstaje pierwotna komórka bielma, a z niej bielmo jądrowe (bj), tkanka odżywcza dla rozwijającego się zarodka (gwiazdka, a, b). Zalążek przekształca się w nasiono, integumenty w łupinę nasienną. 7 – łuszczynka, owoc powstaje w wyniku przekształcenia się zalążni słupka, zawiera dwa nasiona (n). 8 – nasiono. Po wykiełkowaniu, z zarodka (z) tworzy się siewka, z niej nowy sporofit. Cykl się zamyka

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event takes place. The process leads to the formation of an embryo[1] from a fertilized egg cell and endosperm[2] tissue from the central cell of female gametophyte. Ovules develop into seeds, and ovarian tissues form fruits that protect and spread seeds. The whole developmental cycle is shown in Figure 1. The haploid gametophytes which develop in flowers during the life cycle of angiosperm plants are unable to live independently. Given that they are associated with the parent/mother organism, they use its resources and are completely dependent on it. The formation of gametophytes is preceded by the specialized cell division (meiosis[3])

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[1] Embryo – the initial stage of a sporophyte (2n) which is formed after fertilization (the fusion of the sperm cell and the egg cell) in the process called embryogenesis. At the same time as the development of the embryo, the endosperm and the seed coat are formed.

[2] Endosperm – a nutritive tissue for an embryo (3n), it is formed after double fertilization (the fusion of the central cell of the female gametophyte (2n) and the sperm cell (n)).

[3] Meiosis – a type of cell division in which four haploid cells are formed from a diploid mother cell. It is the basis for sexual reproduction. In plants, it determines the transition of the diploid phase (sporophyte) into the haploid phase (gametophyte). In the first meiotic division, the number of chromosomes is reduced. As a result of the recombination of chromosomes and genes, haploid cells have a different gene combination from the stem cell, which ultimately leads to increased biodiversity. The second meiotic division is similar to mitotic division. However, it should be emphasized that the term 'meiosis' refers to cell nucleus changes. Most often it is accompanied by the process of cytoplasmic division – cytokinesis. However, in the world of plants, meiosis is not always accompanied by cytokinesis, e.g. in the tetrasporic type of embryo sac development.

of microspore[4] and megaspore[5] mother cells. During meiosis, recombination (the exchange of genetic material by crossing over) occurs. Recombination increases the genetic diversity of the next generation. It is particularly important for the adaptation of offspring to difficult environmental conditions and for the evolution of plants colonizing the post-industrial areas.

In disturbed post-mining areas (heaps, wastelands), conditions for plant growth are very difficult, with strong winds and insolation, poor soil structure, high concentrations of heavy metals, nutrient deficiency and water shortage (e.g. Baker et al. 2010). These areas have specific flora because only species that have developed tolerance to high concentrations of heavy metals grow in the soil there. The plants characterized by this high tolerance are called metallophytes and have been described in over 30 families of angiosperms, the most common of which are Asteraceae (the aster family), Brassicaceae (the mustard family), Plumbaginaceae (the leadwort family), Poaceae (the grass family), Violaceae (the violet family) (Wierzbicka and Rostański 2002, Siwek 2008b, Bothe and Słomka 2017, Sychta et al. 2018). Reproduction-related trade-offs vary as they depend on the time the species has had for adaptation. The shorter the time is (for example, only a few generations), the higher the level of disturbances and reduced fertility.

Studies concerning the reproductive biology of specimens inhabiting these harsh environments after metal ore mining indicate that flower structures (both perianth and generative organs) and embryological processes at all stages of development (gametophytes, gametes, embryos) show disturbances and degenerations in comparison with these processes in individuals from reference populations (areas uncontaminated by heavy metals) (Izmaiłow 2000, Słomka et al. 2012, Kwiatkowska and Izmaiłow 2014). The most frequent alterations include: necrosis of flower buds, flowers, male and female generative lines; degenerations or lack of gametophytes; necrotic cells in gametophytes; disturbances in embryo development; embryos with single or group of necrotic cells; disturbances in the formation of nutritive tissue (endosperm). The irregularities observed during sexual reproduction reduce the seed set and progeny counts. This research shows that the development of generative structures and generative processes may be used to evaluate the degree of species adaptation to such extreme environments. A lower frequency of disturbances and degenerations indicates that a population is more adapted to the environment. If a population of an investigated taxon is at an early stage of colonization, disturbances in reproduction are more frequent than in well-adapted populations of metallophytes that have already developed adaptations in the sporophyte phase (Czapik 2002, Przedpełska and Wierzbicka 2007, Siwek 2008a, Słomka et al. 2012, Kwiatkowska and Izmaiłow 2014, Izmaiłow et al. 2015). Therefore, it can be assumed that the level of adaptation of an individual can be measured by the frequency of disturbances and degenerations in the structures responsible for sexual reproduction, as well as in the reproductive processes.

Ecological embryology deals with the analysis of reproduction processes in individual plants from anthropogenically disturbed areas (Czapik 2002). It is a good tool for estimating the degree of adaptation of individuals to the environment. The study of plant adaptations in the course of sexual processes appears to be

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[4] Microspore – a haploid cell (n) formed after meiotic division, as a result the tetrad of microspores is formed. The arrangement of microspores in a tetrad may vary.

[5] Megaspore – a haploid cell (n) formed after meiotic division, as a result the tetrad of megaspores is formed, mostly arranged in a linear type.

complementary to the study on the degree of their adaptation at the level of sporophytes. This approach in regard to *B. laevigata* studies is presented in this chapter.

Reproductive biology of *B. laevigata* from the calamine waste heap

Data on the sporophyte phase of *B. laevigata* from the post-mining heaps in the Olkusz region (with calamine soils strongly contaminated with zinc, lead and cadmium) in relation to the life strategy and genetic structure of the population of this species (Bemowska-Kałabun et al. – Chapter 5 and 6 of this volume) shows that these individuals are diploid ($2n = 2x = 18$, i.e. they have two sets of 9 chromosomes). Thus, in the description of the embryological processes of *B. laevigata*, disorders resulting from hybridity and/or polyploidization can be excluded. The disturbances and degenerative processes found during embryological processes reflect only the impact of the environment.

The analysis of the environmental impact on the reproduction of *B. laevigata* presented in this chapter includes a description of the following structures and embryological processes: anthers and ovules in which microspore and megaspore mother cells arise (microsporocytes and megasporocytes), the course of meiotic division and the development of the male and female gametophytes (pollen grain and embryo sac respectively) with the formation of male and female germ units, the course of double fertilization, and the development of the seeds containing the embryo in the process of embryogenesis. The study involved individuals from the *B. laevigata* population from old calamine mine heaps in Bolesław together with the reference population from the Jaworzynka Valley in the Tatra Mountains.

An organ that is directly involved in sexual reproduction is the flower. The flower of *B. laevigata* is bisexual. The superior ovary, composed of two carpels, contains two camplyotropus ovules. Stamens are tetradynamous, i.e. they have four long and two short stamens (Fig. 1: 2, 3). In stamens, inside pollen sacs, surrounded by tapetum, male gametophytes (pollen grains) are formed in two consecutive processes: microsporogenesis[6], which includes reductive division, meiosis of microspore mother cell and recombination, and microgametophytogenesis[7]. Mature pollen grains, at the pollination stage, are three-celled and contain a vegetative cell and two sperm cells (Fig. 1: 4b, 5b). In the ovules in the ovary, just like in the male lineage, a female gametophyte (embryo sac) is also formed in two consecutive processes: megasporogenesis[8] (which includes reductive division, meiosis of megaspore mother cell, and recombination) and megagametophytogenesis[9]. In *B. laevigata*, the embryo sac develops according to the Polygonum of

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[6] Microsporogenesis – meiotic division occurring in the microspore mother cell. n – a haploid number of chromosomes. Half of the somatic number of chromosomes (2n). The haploid number of chromosomes occurs in cells after a meiotic division. Haploid cells in plants are megaspores, microspores (of which gametophytes are formed) and their derivatives, gametes – egg cell, sperm cells.

[7] Microgametophytogenesis – development of a male gametophyte. After the first mitotic division of microspore, a generative cell and a vegetative cell are formed. After the second mitotic division, that is restricted to the generative cell only, 2 sperm cells are formed.

[8] Megasporogenesis – meiotic division occurring in the megaspore mother cell.

[9] Megagametophytogenesis – development of a female gametophyte. After the first mitosis of the functional megaspore, a 2-nucleate female gametophyte (embryo sac, ES) is formed. After the second mitosis a 4-nucleate ES is formed, and after the third mitosis a 8-nuclete ES is formed that converts into a 7-cell mature ES. In the micropylar region, an egg apparatus (egg cell + 2 synergids), centrally located a central cell, 3 antipodals at the chalazal region. All nuclei in ES are haploid (n) with the exception of the diploid, secondary nucleus of the central cell (2n) formed after the fusion of two polar nuclei (each n).

the monospore-type from chalazal megaspore, which becomes 8-nucleate after three mitoses. A mature female gametophyte is a seven-celled structure consisting of an egg apparatus (an egg cell with two synergids) in the micropylar region, and a central cell and three ephemeral antipodals in the chalazal region (Fig. 1: 4a, 5a). The embryo develops according to the Onagrad-type, one of the patterns of embryo development distinguished based on the origin of the part of the embryo from the apical and basal cell after the first zygote division, and the direction of divisions. Here, endosperm is of the nuclear type (Fig. 1: 6). The fruit is the two-seed silicula (Fig. 1: 7, 8). Comparative analyses of individuals from the calamine and Tatra Mountain populations show that the female gametophyte pattern type, and embryo and endosperm development are conservative features that do not change under the influence of environmental conditions. Similarly to other tested species from heaps (e.g. *Arabidopsis arenosa* (L.) Lawalrée, *Lotus corniculatus* L., *Vicia cracca* L. (Izmaiłow i in. 2015)). *B. laevigata* did not show the high frequency of necrosis of whole flower buds and flowers, degenerations of gametophytes, nor highly reduced pollen viability, which are characteristic features of plants at the early stages of colonization of heavy metal-enriched habitats (Kościńska-Pająk 2000, Czapik 2002, Izmaiłow and Biskup 2003, Siwek 2007). The specimens of *B. laevigata* formed siliculas, fruits in which seeds developed. The question of whether contaminated environment has a negative impact on reproductive processes of *B. laevigata* was investigated next.

The flower buds of *B. laevigata* were observed at various developmental stages. It was found that in some of them degenerations occur in parts of the stamens, in others only one was necrotic, or both developed properly (Fig. 2A). All microspores in the anther could also degenerate (Fig. 2B). Such partially necrotic stamens were detected in about half of the studied flower buds in specimens from the calamine population. The frequency of stamen degenerations in flower buds of plants from the calamine population was statistically significantly higher than that of the Tatra population. Thus, in plants from the calamine population, the elimination of some flower structures, caused by a limited amount of resources (e.g. nutritional elements), was observed. This way, others could develop properly and produce the correct male gametophytes. As a consequence, viable pollen produced by a single flower and the whole plant was reduced. A similar phenomenon was observed in another calamine species, *Armeria maritima* (Mill.) Willd. (own observations).

The partial elimination of generative structures in specimens from the calamine population was also observed in the female lineage. In the ovary, the development of one of two ovules was inhibited (usually at the stage of gametophytogenesis or mature gametophyte), while in the neighboring ovule a mature, normal female gametophyte was developing (Fig. 3A). This strategy allows for the maturation of a smaller number of ovules with female gametophytes that are properly developed and have a chance of being fertilized and transformed into viable seeds in an environment with limited resources (De Jong and Klinkhamer 2005). In a small percentage of ovules, from calamine populations only, shortened embryo sacs were observed. This indicates the cell's entry into the maturity phase of female gametophyte before the size and the shape of the embryo sac reaches that typical for this taxon (Fig. 3B). Entering the generative phase prematurely is also a characteristic feature of other plants from calaminarian grassland (Wierzbicka and Rostański 2002). Degenerations of cells in the female lineage
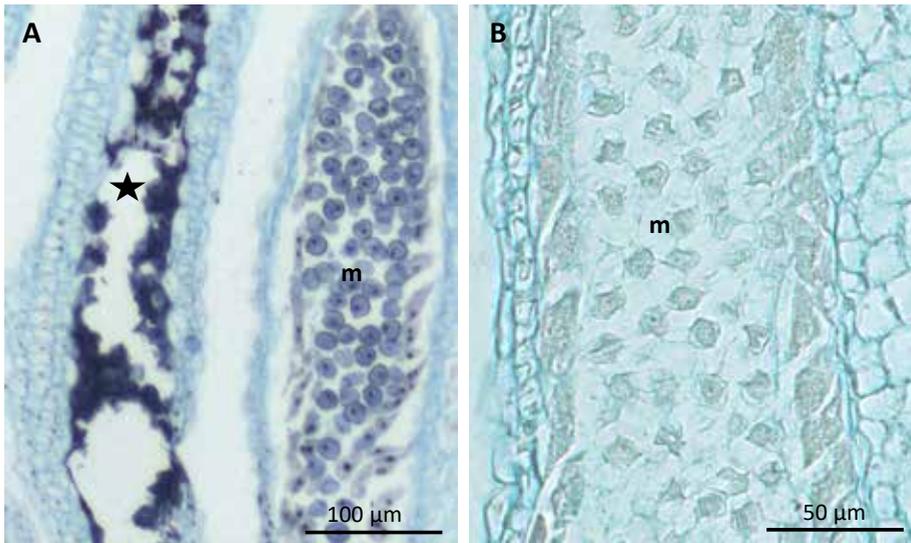
Fig. 2. Degeneration of cells of the male lineage in the anthers of flower buds of *Biscutella laevigata* from the waste heap population in Bolesław. A – degeneration of all microspores in the anthers (asterisk), normally developed microspores (m) in the neighboring anther, B – microspore degeneration (m) at an early stage of development. Photographs of slides under a light microscope

Ryc. 2. Degeneracja komórek linii męskiej w pręcikach pąków kwiatowych *Biscutella laevigata* z populacji hałdowej w Bolesławiu. A – degeneracja wszystkich mikrospor w komorze pylnika (gwiazdka), prawidłowo rozwinięte mikrospory (m) w sąsiednim pręciku, B – degeneracja mikrospor (m) na wczesnym etapie rozwoju. Fotografie preparatów spod mikroskopu świetlnego

were observed in several percent of the ovules studied in plants from the calamine population (Fig. 3C). Additionally, a small percentage of the analyzed ovules with mature, normal female gametophytes were degenerating as a result of the absence of fertilization. It is probable that the fertility of plants was limited due to the lack of pollinators.

In specimens studied from the calamine population, degenerations also occurred in ovules at a later stage of development. Necrotic ovules were observed (Fig. 3C) next to the normally developing embryos and endosperm (Fig. 4A, B). The degenerations in female lineage cells corresponded with the frequency of one-seeded siliculas, and those in which both seeds were not formed but which were present next to normal two-seeded siliculas (Fig. 4D, E). The latter was

probably a consequence of eliminating a portion of ovules, or unsuccessful fertilization of the correct embryo sacs.

In the seeds, or more precisely in the embryos of individuals from the calamine population, changes in the composition of the cell wall were detected. The fraction of pectins, highly esterified homogalacturonan, which is considered to bind and detoxify metals (Krzesłowska 2011), was identified. This indicates that at the seed level, i.e. in the next generation of the *B. laevigata*, adaptations to the environment can be observed, such as binding metals in the apoplast (cell wall complex). The current research of the authors of this chapter is focused on the analysis of the composition of cell walls of generative line cells. They are trying to identify the stage of development of haploid line cells (megasporocytes, megaspores, cells in

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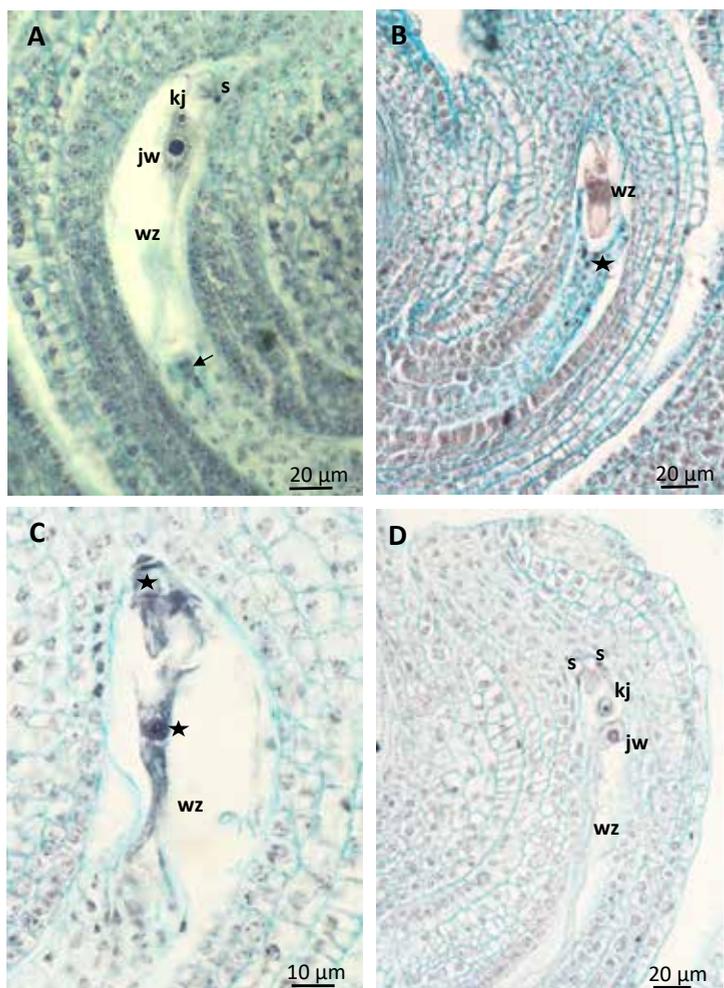
Fig. 3. Longitudinal sections of *Biscutella laevigata* ovules from: A – the Tatra population, B – from the heap population in Bolesław. Normal, elongated, slightly curved female gametophyte (embryo sac, wz), visible central cell with diploid secondary nucleus (jw) after the fusion of two polar nuclei, egg cell (kj) and one synergid (s) in the micropylar region, antipodals (arrow) in the chalazal region (A). Abnormal, strongly shortened, without clearly visible campylotropic shape, mature embryo sac (wz), visible degenerating antipodals (asterisk) (B). C – necrotic embryo sac (wz), visible degenerated cells (asterisks) surrounded by proper ovular tissues. D – mature, normal, but unfertilised embryo sac (wz), visible egg cell (kj) and two synergids (s), central cell with secondary nucleus (jw). Photographs of slides under a light microscope

Ryc. 3. Przekroje podłużne przez zalążki *Biscutella laevigata*: A – z populacji tatrzańskiej, B – z populacji hałdowej w Bolesławiu. Prawidłowy, wydłużony, lekko zagięty gametofit żeński (woreczek zalążkowy, wz), widoczna komórka centralna z diploidalnym jądrem wtórnym (jw) po fuzji dwóch jąder biegunowych, komórka jajowa (kj) i jedna synergida (s) na biegunie mikropylarnym, antypody (strzałka) na biegunie chalazalnym (A). Nieprawidłowy, silnie skrócony, bez wyraźnie zarysowanego kampylotropowego kształtu, dojrzały woreczek zalążkowy (wz), widoczne degenerujące antypody (gwiazdka) (B). C – obumarły woreczek zalążkowy (wz), widoczne nieżywotne komórki wz (gwiazdki) otoczone prawidłowymi tkankami zalążka. D – dojrzały, prawidłowy, ale niezapłodniony woreczek zalążkowy (wz), widoczna komórka jajowa (kj) i dwie synergidy (s), komórka centralna z jądrem wtórnym (jw). Światło woreczka obkurczone. Fotografie preparatów spod mikroskopu świetlnego
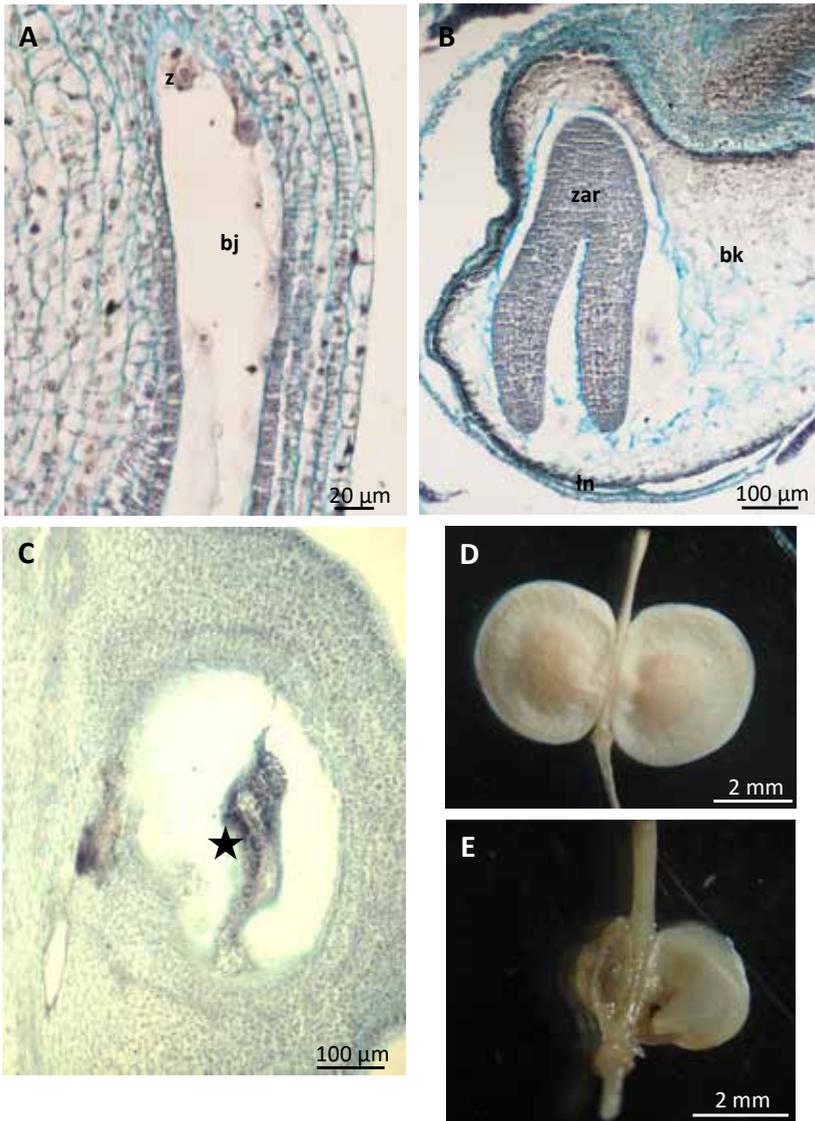
Fig. 4. Development of embryo, endosperm and seed setting in plants from the waste heap population in Bolesław (A, C, E) and the Tatra population (B, D). A – typical development, zygote (z) and nuclear endosperm (bj). B – Embryo (zar) at late torpedo stage with developing cotyledons surrounded by cellular endosperm (bk), the seed coat (łp) formed from integuments. C – part of ovary with degenerated ovule/developing seed (asterisk). D – normally developed fruit, two-seeded silicula. E – seedless siliclula. Photographs of slides under a light microscope (A–C). Photographs under a stereo microscope (D, E)

Ryc. 4. Rozwój zarodka, bielma, powstawanie nasion roślin z populacji hałdowej w Bolesławiu (A, C, E) oraz tatrzańskiej (B, D). A – prawidłowy rozwój, zygota (z) i bielmo jądrowe (bj). B – zarodek w stadium późnej torpedy (zar) z rozwijającymi się liścieniami otoczony bielmem komórkowym (bk), z integumentów zalążka tworzy się łupina nasienna (łp). C – fragment zalążni z obumarłym zalążkiem/rozwijającym się nasionem (gwiazdka). D – prawidłowo rozwinięty owoc, dwunasienna łuszczynka. E – brak nasion w łuszczynce. Fotografie preparatów spod mikroskopu świetlnego (A–C), fotografie spod lupy (D, E)

embryo sac before and after fertilization) in which there is a difference in the composition of cell walls, which affects the evolution of adaptations to the environment enriched in heavy metals.

Summary

In *B. laevigata* from calamine heaps, disturbances in embryological processes are observed. However, only partial degeneration of generative structures and cells (both male and female) can be the basis for developing an adaptation strategy involving the elimination of some of the flower structures in conditions of limited resources, so that the remaining structures can develop properly and the individual can achieve reproductive success (seed set). Thus, embryological observations of this metallophyte show that calamine populations living in extreme conditions (nutrient and water deficiencies with a large amount of metals in the substrate) have developed adaptive strategies at the gametophyte phase level over tens of generations.

Another adaptation seen in the development of the haploid phase is the accelerated maturation of the female gametophyte before it reaches the correct size and proper campylotropic shape (strong shortening of the embryonic sacs) to increase the chance of pollination and fertilization.

It was also found that the factor limiting the amount of seeds produced by *B. laevigata* may be the lack of a sufficient number of pollinators, which results in the death of parts of properly developed, non-fertilized gametophytes.

The adaptation of *B. laevigata* to the excess of heavy metals in soils of heaps seems to be a change in the chemical composition of the embryo's cell wall, with the synthesis of compounds immobilizing the metals reaching the seeds. Further research in this area will answer the questions about the developmental stage at which this adaptation arises. It might be inherited in the female lineage, and so the composition of the cell wall of all or one (egg) cell changes in the haploid phase, and thus the change is maintained by the zygote and later the embryo.

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