ACTA BIOLOGICA CRACOVIENSIA Series Botanica 47/1: 237–245, 2005

IS THE BLUE ZINC VIOLET (*VIOLA GUESTPHALICA* NAUENB.) A TAXON OF HYBRID ORIGIN? EVIDENCE FROM EMBRYOLOGY

ANETA SIUTA[1], MONIKA BOŻEK[1], MONIKA JĘDRZEJCZYK[2], ADAM ROSTAŃSKI[2], AND ELŻBIETA KUTA[1*]

[1]*Department of Plant Cytology and Embryology, Jagiellonian University, ul. Grodzka 52, 31–044 Cracow, Poland*
[2]*Department of Plant Systematics, University of Silesia, ul. Jagiellońska 28, 40–032 Katowice, Poland*

Received March 29, 2005; revision accepted May 10, 2005

Investigations of reproductive processes of the blue zinc violet (*Viola guestphalica* Nauenb.) from its natural location in Germany (Blankenrode) and from two sites of introduction in Poland (Wełnowiec zinc spoil and a private garden in Sosnowiec-Ostrowy Górnicze) showed significant disturbances resulting in reduced plant fertility. Pollen viability estimated by acetocarmine staining was relatively low, with 54% viable pollen grains in plants from Wełnowiec and 62% from the garden. Specimens from Blankenrode had 80% viable pollen but the pollen grains differed in size conspicuously. Giant abnormal pollen grains accompanied very small ones. Necrosis affected anthers and pistils, including degeneration of whole anthers and ovules, whole embryo sacs or embryo sac elements, and abortion of embryos. The pathway of female gametophyte and embryo development was normal in 61% of the ovules. Necrosis of somatic tissues and generative cells at different developmental stages was found in 28% of the ovules, and irregular development in 11% of them. As a consequence, embryo viability was reduced to 33%. Embryological data indicated that *V. guestphalica* is not a well-stabilized genotype. The possible origin of the blue zinc violet is discussed.

Key words: *Viola* L., zinc violets, pollen, megasporogenesis, female gametophyte, embryo, heavy metals.

INTRODUCTION

Two representatives of the section *Melanium* Ging. (pansies), called blue (*Viola guestphalica* Nauenb.) and yellow [*V. calaminaria* (Ging.) Lej.] zinc violets, have a limited area of distribution on metal soils in Central Europe. They represent very interesting examples of microevolutionary processes leading to the creation of metal-tolerant genotypes (for review: Ernst, 1999; Bone and Farres, 2001; Wierzbicka and Rostański, 2002). Yellow zinc violets have a wider area of distribution, occurring on heaps from abandoned zinc mines in Belgium, Germany and the Netherlands since Roman times, although they have declined severely since the beginning of the previous century (Valentine et al., 1968). The blue zinc violet is restricted to the small area of a former lead and zinc mine in Blankenrode near Paderborn (Westphalia, Western Germany). It occurs on heaps which arose in medieval times. They belong to heavy metal communities *Violetalia calaminariae* with vegetation very poor in species (Ellenberg, 1988). Ernst (1968) proposed separating the vegetation with the blue zinc violet in Blankenrode as *Violetum calaminariae westfalicum*. Both violets are very endangered endemic plants and are under legal protection in natural reserves.

The soil in the area occupied by zinc violets is rich in heavy metals such as Pb, Zn and Cd (Ernst 1974). Metal-tolerant plants have developed special mechanisms to allow them to avoid heavy metal toxicity (Ernst, 1990; Ernst et al., 1992; Antosiewicz, 1992). According to Ernst (1974), the shoots of the yellow zinc violet from Breinigerberg contained low amounts of heavy metals, indicating that the taxon does not employ the accumulation strategy. Jędrzejczyk et al. (2002) obtained similar results in an investigation of the effect of high

*e-mail: e.kuta@iphils.uj.edu.pl

PL ISSN 0001-5296

concentrations of Pb and Zn on biomass production and on the accumulation of these metals in roots and aboveground parts of yellow and blue zinc violets. On the other hand, Meyer (1995) estimated rather high Zn content ranging from 1,608 to 2,001 mg kg^{-1} dry weight in different parts of blue zinc violets from Blankenrode, with the highest concentration in leaves. Both zinc violets were colonized by arbuscular mycorrhizal fungi, organisms accumulating or binding heavy metals and therefore alleviating metal toxicity (Hildebrandt et al., 1999).

Controversy persists about the taxonomic status, age and origin of metal-tolerant pansies. They have been recognized as varieties, a subspecies, and also a separate species with *Viola lutea* or *V. tricolor* indicated as putative ancestors. In 1964, Ernst designated the blue form as a subspecies of the yellow zinc violet and named it *V. calaminaria* (DC.) Lej. subsp. *westfalica* (Lej.) Ernst. (cited after Ernst, 1968). On the basis of differences in morphological and cytological characters, Nauenburg (1986) separated the two zinc violets into different species, naming the blue zinc violet *V. guestphalica* Nauenb. Somatic chromosome numbers 2n = 52 for the blue form and 2n = 48 for the yellow violet were established by Kakes and Everards (1976) and confirmed by Nauenburg (1986), Rostański et al. (2003), and recently by Hildebrandt et al. (data unpubl.).

Zinc violets are very attractive ornamental perennials, and very good plant material to colonize heavy metal spoils. In 1996, blue and yellow zinc violets were introduced to the Wełnowiec zinc spoil in Upper Silesia, Poland (Jędrzejczyk and Rostański, 2001). Six years after introduction, the plant population was declining rather than expanding. Analysis of the reproductive processes undertaken in the current study provided an explanation of the unsuccessful transplantation.

MATERIALS AND METHODS

SITES AND POPULATIONS

1. Blankenrode (western Germany). Two small populations of *Viola guestphalica*, a local endemic plant, occur in the area of Blankenrode near Paderborn in Westphalia. One population is strictly connected with a ditch in the area of a former zinc and lead mine. The second occupies a neighboring meadow flooded with a heavy metal-rich stream originating from the heaps surrounding the ditch. Soil in both sites is rich in Zn (9%) and Pb (0.3%) (Ernst, 1974).
2. Wełnowiec zinc spoil (Katowice, Upper Silesia, southern Poland). Seedlings were transferred to the zinc spoil in 1996. The concentration of heavy metals in the ground (basic content is furnace slag) depends on the age of particular parts of the heap (the older the part, the greater the concentration), ranging from 0.1 to 3.5% Zn and from 0.07 to 0.4% Pb (Jędrzejczyk and Rostański, 2001).
3. Private garden (Sosnowiec-Ostrowy Górnicze, Upper Silesia southern Poland). Seedlings were transferred in 1996 to soil with very low concentrations of Zn (0.02%) and Pb (0.005%) (Jędrzejczyk et al., 2002).

PLANT MATERIAL

The blue zinc violet seeds originated from several sources: (1) the Institute of Botany of the University of Cologne. Seeds were collected from plants grown from seeds harvested in the area of Blankenrode, outside a nature reserve; (2) Naturschutzzentrum – Biologische Station – Hochsauerlandkreis; (3) Björn Malkmus, Rare Seeds Company (www.rareplants.de); (4) from plants colonizing the Wełnowiec zinc spoil; and (5) from plants introduced to the private garden in Sosnowiec-Ostrowy Górnicze.

For embryological studies and pollen viability tests, flowers were collected and fixed in a mixture of 96% ethanol and glacial acetic acid (3:1) directly in the field. In the case of the material from Blankenrode, flowers were harvested from plants growing outside the protected area.

POLLEN VIABILITY TESTS

Pollen viability was estimated using acetocarmine or Alexander's dyes. Mature pollen grains were isolated from 43 flowers from 17 plants from all sites. Cytoplasm and nuclei of viable pollen grains stain red by acetocarmine, while nonviable, empty and shrunken pollen remain colorless (Singh, 2003). Alexander's dye contains malachite green, staining the cellulose of pollen walls green, and acid fuchsin, which stains the pollen protoplasm red (Alexander, 1969; Singh, 2003). Viable pollen grains are red with a green wall, and nonviable ones are entirely green.

OVARY ANALYSIS AND EMBRYO VIABILITY TESTS

Conspicuously enlarged ovaries (n = 46) of plants from all sites were analyzed to determine the frequency of normally developed ovules with embryo and endosperm. Dissected ovules were transversally cut with a razor blade and observed under a Zeiss Stemi SV microscope.

To estimate embryo viability, two quick viability tests completed within 24 h were applied. In the tetrazolium test, isolated embryos (n = 175) were incubated in a diluted (1%) solution of 2,3,5-triphenyltetrazolium chloride (Aldrich) according to Dorywalski et al. (1964). Initially the tetrazolium solution is colorless, but changes to red when it comes into contact with hydrogen (reduction) derived from enzymes in the respir-

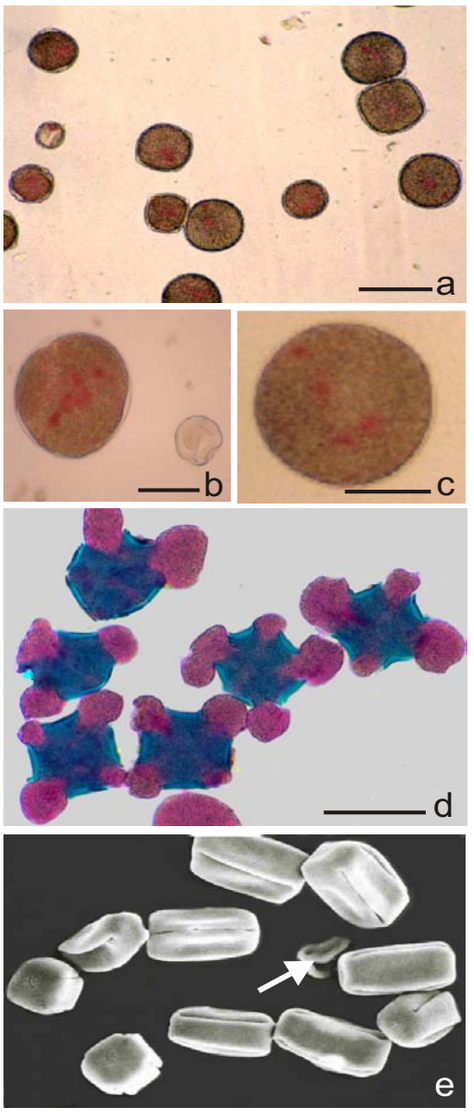

Fig. 1. Pollen of plants from all sites. (**a**) Pollen grains of one anther differing in size, (**b–c**) Abnormal giant pollen grains, (**d**) Pollen grains germinating inside the anther, (**e**) Four-aperturate pollen grains, degenerated pollen (arrow). (a-c) acetocarmine staining, (d) Alexander's staining, (e) SEM. Bars in (b,c) = 50 µm, in (d,e) = 100 µm, in (a) = 120 µm.

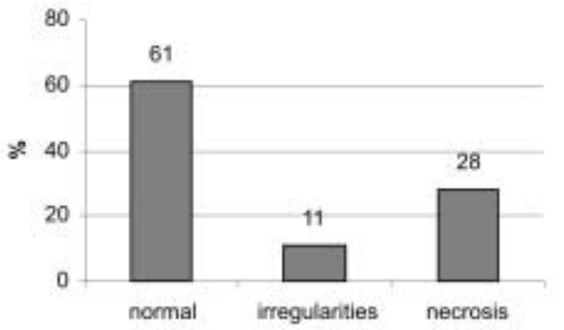

Fig. 2. Frequency of necrosis and irregularities in the development of female gametophyte, embryo and endosperm in plants from all sites.

ation process. Embryos showing active respiration turn red and are considered viable. The darker the color, the greater the respiratory activity in the seed. Light pink indicates an embryo with less viability than an embryo staining dark red. Depending on the location of the stainless parts, embryos were arbitrarily classified into seven classes and deemed viable or nonviable: Class I – embryos stainless; Class II – embryos stained 10%, Class III – embryos stained 30%; Class IV – embryos stained 50%; Class V – embryos stained 70%; Class VI – embryos stained 90%; Class VII – embryos stained 100%. Viable embryos represented the color patterns of classes VI and VII.

In the indigo carmine test, isolated embryos (n = 10–20) were incubated in a 0.1% solution of indigo carmine for 2 h at 30˚C. Nonviable embryos or parts of embryos stained dark blue, whereas viable embryos remain unstained (Hendry and Grime, 1993). Embryo stainability methods were followed by a seed germination test (n = 50). Seeds were germinated on wet filter paper in Petri dishes for six weeks. The sample was small because *V. guestphalica* is a protected endangered species.

For SEM studies, pollen grains isolated from opened flowers were sputter-coated with gold (Pelco) and analyzed in a Tesla BS 340 scanning electron microscope.

SLIDE PREPARATION FOR EMBRYOLOGICAL STUDIES

The embryological processes were analyzed in 135 flowers at different stages of development (buds, semi- and fully opened flowers) of plants growing in Blankenrode (44), on the Wełnowiec zinc spoil (39) and in the garden (52). Flowers and young capsules were fixed in a mixture of 96% ethanol and glacial acetic acid (3:1) for 24–48 h, then stored in 70% ethanol at 4˚C until used. Material dehydrated in an ethanol series was embedded in paraffin and sectioned 10 µm thick on a rotary microtome (Reichert), transferred to glass slides, stained with Heidenhain's hematoxylin combined with alcian blue, and mounted in Canada Balsam (Aldrich).

The results were statistically analyzed with the *t*-test for independent samples (Statistica ver. 5, 1997, Statsoft, Inc.).

Microscope sections were photographed with a Zeiss Axio Cam MRe digital camera with Zeiss Axio Vision 3.0 software. Photographs of capsules and isolated embryos were taken under a Zeiss Stemi SV 11 stereomicroscope equipped with an MC80 microphotography attachment on Kodak film.

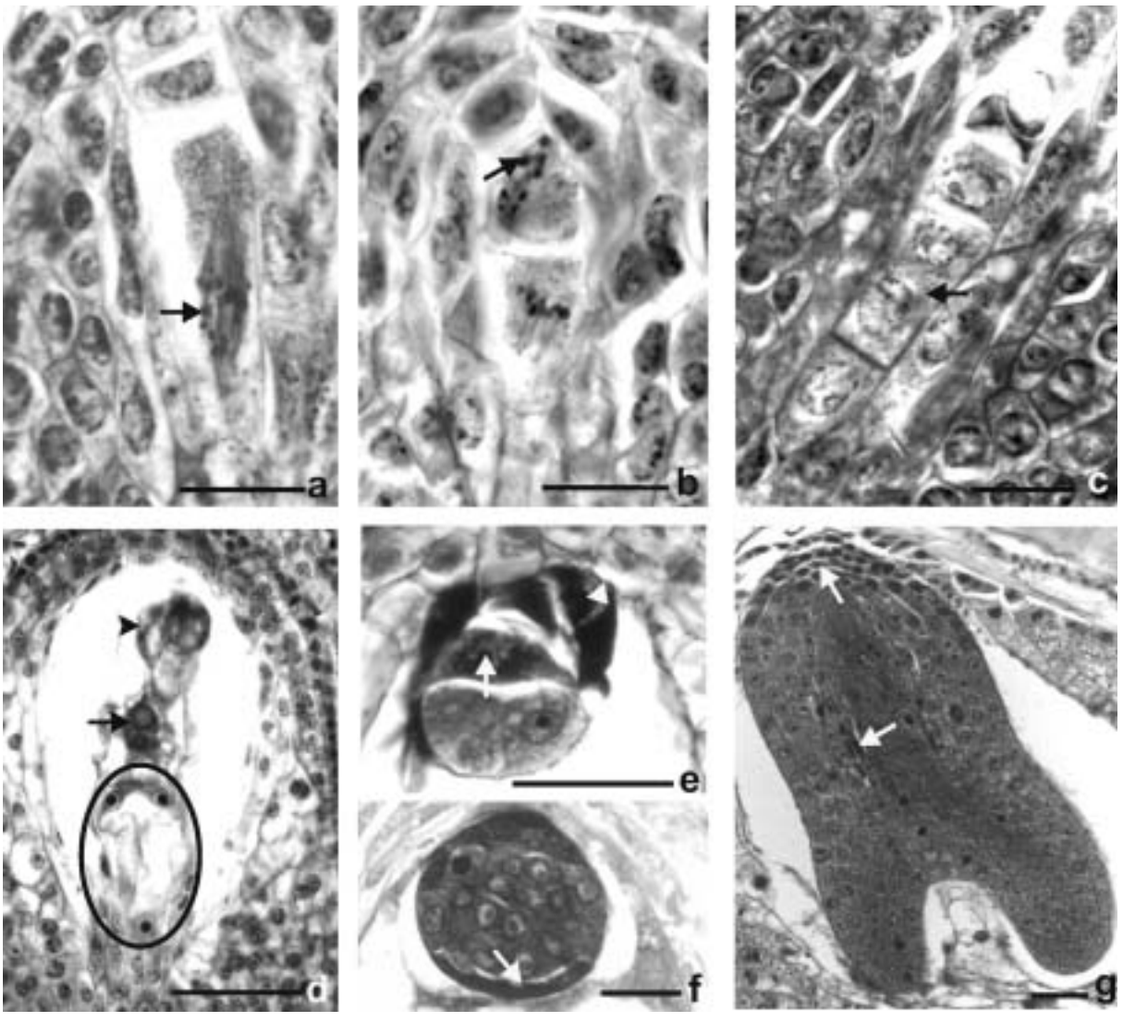

Fig. 3. Irregularities and necrosis in the development of female gametophyte, embryo and endosperm in plants from all sites. (**a**) Disturbed metaphase I with univalents (arrow) scattered along the spindle, (**b**) Disturbed metaphase II, chromosomes scattered along one spindle (arrow), (**c**) Tetrad stage with one 2-nucleate megaspore (arrow), (**d**) Double embryo sac in one ovule; 4-nucleate, 3 nuclei visible (circle), and mature embryo sac with 2 polar nuclei (arrow) and 2 synergids (arrowhead), (**e**) Few-celled proembryo with necrotic cells (arrow) and endosperm (arrowhead), (**f,g**) Embryos showing symptoms of necrosis (arrows): (**f**) Globular-stage embryo, (**g**) Torpedo-stage embryo. Bars in (a–c) = 20 μm, in (d–g) = 50 μm.

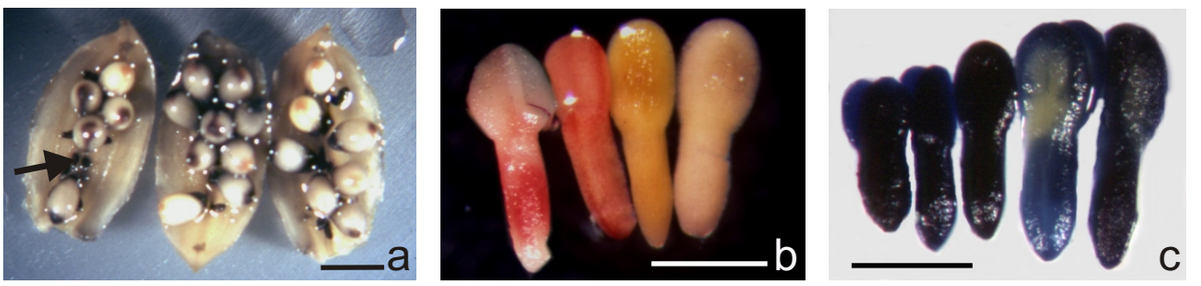

Fig. 4. Capsule and embryos. (**a**) Capsule with normal and degenerated (arrow) ovules, (**b,c**) Isolated embryos tested for viability: (**b**) Tetrazolium test, (**c**) Indigo carmine staining. Bars in (b,c) = 1 mm, in (a) = 2 mm.

TABLE 1. Pollen viability estimated by acetocarmine staining

Locality	No. of plants	No. of flowers (n)	No. of pollen grains analyzed	% of stainable pollen grains ± SD
Blankenrode	4	10	1777	80 ± 8
Wełnowiec zinc spoil	6	16	2124	54 ± 10
Garden	7	17	2362	62 ± 20
Total	17	43	6263	65 ± 18

Standard deviations were calculated from the percentages of stainable pollen grains in anthers of analyzed flowers.

TABLE 2. Necrosis and irregularities in development of female gametophyte, embryo and endosperm in plants from Blankenrode (B) and the garden (G) [%]

Stage of development	No. of ovules analyzed		Normal		Irregularities		Necrosis	
	B	G	B	G	B	G	B	G
Early stages[1]	254	215	149 [59]	155 [72]	34 [13]	52 [24]	71 [28]	8 [4]
Young ES2 and mature ES[3]	394	212	258 [66]	170 [80]	44 [11]	-	92 [23]	42 [20]
Embryo and endosperm[4]	79	171	19 [24]	60 [35]	13 [17]	9 [5]	47 [59]	102 [60]
Total	727	598	426 [58]	385 [65]	91 [13]	61 [10]	210 [29]	152 [25]

[1]archespore, megaspore mother cell (MMC), meiosis, tetrad stage
[2]1- to 4-nucleate embryo sac (ES)
[3]ES with egg apparatus, central cell with two polar nuclei or secondary nucleus
[4]zygote + nuclear endosperm; proembryo (few-celled to globular stage), heart- and torpedo-shaped embryos

RESULTS

POLLEN VIABILITY

Average pollen viability of plants from all sites was 65%, as estimated by the acetocarmine test (Tab. 1). The frequency of stainable pollen grains was lower in flowers from the areas of introduction (54% at Wełnowiec and 62% in the garden) than from the natural population in Blankenrode (80%). Pollen grains of flowers from Blankenrode, despite their relatively high stainability, differed conspicuously in size. Very small and very big pollen grains occurred together within one anther and even within one pollen sac (Fig. 1a) in several flowers. Such giant pollen had abnormal, multinucleate structure (Fig. 1b,c), with divided nuclei and partly formed cell walls. Additionally, mature, normally shaped pollen grains started to germinate inside the anthers before anthesis (Fig. 1d). The majority of pollen grains had four apertures (Fig. 1e).

FEMALE GAMETOPHYTE, EMBRYO AND ENDOSPERM DEVELOPMENT

Ovules of plants from the garden and from the natural site were investigated embryologically in detail (Tabs. 2, 3; Fig. 2). Specimens from the zinc spoil (Wełnowiec) were not included in the tables because only a few individuals of *V. guestphalica* survived six years after introduction there, limiting the amount of material.

In 61% of the ovules, the female gametophyte developed normally (Fig. 2) from the chalazal megaspore according to the monosporous Polygonum type. The embryo was formed following a suspensorless Asterad type, and was surrounded by the nuclear endosperm. The female archespore was multicellular, and in consequence more than one megaspore mother cell (MMC) occurred in ~1% of the ovules at the premeiotic stage. In the majority of ovules, only one MMC underwent meiosis. At the late tetrad stage, an intermediate megaspore(s) sporadically prolonged their viability, accompanying the chalazal megaspore developing in a 1-nucleate ES.

In the rest of the analyzed ovules (39%), necrosis of somatic and generative cells (28%) as well as irregularities (11%) accompanied female gametophyte development and further embryo and endosperm formation (Fig. 2). The term "necrosis" is used throughout the paper for dark-stained somatic or generative cells visible in the sectioned material, as no confirmation of programmed cell death (PCD) was provided by special methods visualizing PCD morphotypes.

Disturbed megasporogenesis (meiosis I and II) resulting in abnormal tetrad or polyad formation was observed in 7% of the ovules (Tab. 3). Univalents, multivalents and chromosomes scattered along the

TABLE 3. Irregularities in megasporogenesis, female gametophyte (ES), embryo and endosperm development in plants from Blankenrode (B) and the garden (G). Percentages calculated from the total number of ovules, 727 (B) and 598 (G)

Stage of ovule development	No. of ovules	
	B	G
Irregular meiosis (I and II divisions): multivalents, univalents, chromosomes scattered along the spindle	11	45
Tetrad with nuclei differing in size	8	-
Polyads	15	7
Unorganized 3–5-nucleate ES	17	-
Unorganized 6–8-nucleate ES	12	-
Unorganized 9–12-nucleate ES	2	-
Two egg cells in one ES	2	-
Doubled ESs	9	-
Egg cell + secondary nucleus + a few nuclei	2	-
Twin embryos	2	
Endosperm only	11	9
Total	91 [13%]	61 [10%]

spindle were most often observed (Fig. 3a,b). As a consequence, megaspores in tetrads were cytologically unbalanced, with nuclei differing in size, or else polyads (most frequently pentads) were produced (Fig. 3c).

In further stages of female gametophyte development, abnormalities were found mainly in the material from Blankenrode (Tabs. 2, 3). Unorganized, multinucleate (3–12 nuclei) embryo sacs (ES) represented 4% of the analyzed ovules. Double or even triple ESs were formed sporadically (1%), indicating that one megaspore was involved in the formation of the female gametophyte in the majority of ovules. Multiple ESs represented different stages of development; for example, a mature 7- or 8-nucleate ES was accompanied by a 1- or 4-nucleate ES (Fig. 3d). As a consequence, twin embryos were formed in 0.3% of the ovules.

At the stage of embryo and endosperm, only endosperm was noted in 1.5% of the ovules from both sites, with no visible sign of zygote and embryo necrosis (Tab. 3).

Necrosis affected not only the floral generative organs (anthers and pistils), including degeneration of whole anthers and ovaries, but also ovules, the female gametophyte or its elements, embryos and endosperm. Symptoms of necrosis were noted in ovules at different stages of development, starting from early stages (archespore, megaspore mother cells, megasporogenesis, tetrad stage) to embryo and endosperm formation (globular proembryo to torpedo-shaped embryos) at frequencies of 25% (garden) and 29% (Blankenrode). In plants from the garden and Blankenrode, the highest frequencies of ovules with necrosis were noted at the embryo (60%) and endosperm (59%) stages (Tab. 2). Embryos had dark-stained necrotic cells in the epidermis (Fig. 3e,f), but necrotic cells were also scattered in different parts of the embryo (Fig. 3g). Moreover, the compactness of cell connections between embryonic tissues was lost (Fig. 3e–g).

OVARY ANALYSIS AND ESTIMATION OF EMBRYO VIABILITY

Analysis of dissected ovaries and ovules showed that the frequency of ovules with normally formed embryo and endosperm was reduced, ranging from 25% to 50% depending on the locality, with the lowest from the Wełnowiec zinc spoil and the highest from Blankenrode (Tab. 4). On average, 45% of the ovules were small, undeveloped and degenerated (Fig. 4a), 13% were empty (without embryo and endosperm), and only 6% were large ovules with endosperm (Tab. 4).

Embryo viability may be a factor contributing to poor seed germination in *V. guestphalica*. Embryo viability was evaluated by the color pattern of embryos after tetrazolium treatment. On the basis of topographic analysis, the embryos were classified in seven arbitrarily defined classes (Fig. 5). The majority of embryos fell into two opposite classes: class I, representing completely unstained and therefore nonviable embryos (26%), and class VII with red-colored viable embryos (29%), as presented in Figure 4b. Embryos with intermediate color patterns were included in

TABLE 4. Ovary and ovule analysis [% ± std.dev.]

Locality	No. of plants	No. of flowers (n)	No. of ovules analyzed	Large ovules with embryo and endosperm	Large ovules with endosperm only	Large empty ovules	Small degenerated ovules
Blankenrode	4	11	437	232 [50 ± 25]	77 [17 ± 17]	-	128 [35 ± 29]
Wełnowiec zinc spoil	4	13	508	151 [25 ± 16]	41 [9 ± 13]	72 [15 ± 9]	244 [51 ± 10]
Garden	7	22	926	290 [30 ± 26]	-	166 [19 ± 30]	470 [51 ± 27]

Standard deviations were calculated from the percentages of ovules in analyzed flowers.

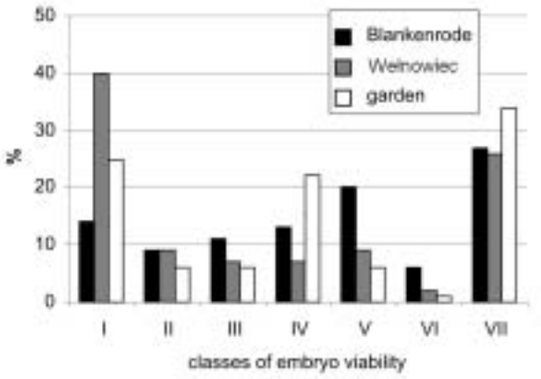

Fig. 5. Frequency of embryos in classes distinguished on the basis of stainability. Class I – embryos colorless; Class II – embryos stained 10%; Class III – embryos stained 30%; Class IV – embryos stained 50%; Class V – embryos stained 70%; Class VI – embryos stained 90%; Class VII – embryos stained 100%.

classes II-VI (45%). The lowest frequency of viable embryos was estimated in seeds from the Wełnowiec zinc spoil (29%), the highest from Blankenrode (35%), and almost the same from the garden (34%), making the average 33% (Tab. 5).

The indigo carmine test, in which, unlike in the tetrazolium test, viable embryos remain stainless (Fig. 5c), and the seed germination test confirmed the results from quick tetrazolium evaluation of embryo viability.

DISCUSSION

The disturbances in reproductive processes observed in *Viola guestphalica* from all investigated sites, resulting in reduced pollen and embryo viability, indicated that the taxon is not a well-stabilized genotype.

Two possible explanations could be proposed. First, the abnormalities in sexual reproduction, mainly necrosis affecting somatic and generative tissues, were similar to those described in non metal-tolerant taxa colonizing metalliferous soils, such as *Vicia cracca*, *Ranunculus repens*, *Capsella bursa-pastoris*, *Chondrilla juncea* and *Echium vulgare* (Czapik et al., 2002; Izmaiłow, 2000, 2002a,b; Izmaiłow and Biskup, 2003; Biskup and Izmaiłow, 2004; Kościńska-Pająk, 2000; 2002). Harsh conditions with physiological drought, low concentrations of nutrients and minerals, and high concentrations of heavy metals are very stressful for non metal-tolerant plants, harming their metabolic processes, and can therefore influence sexual reproduction, reducing plant fertility.

Second, the irregularities observed in the blue zinc violet perfectly fit the developmental abnormalities described in *Viola* hybrids. Hybridization plays a key

TABELE 5. Embryo viability assessed by tetrazolium test

Locality	No. of embryos analyzed	Viable embryos (%)
Blankenrode	55	35
Wełnowiec zinc spoil	55	29
Garden	65	34
Total	175	33

role in the evolution of the genus *Viola*. Interspecies natural hybrids have been described in the sections *Viola* (Valentine, 1962, 1976; Kuta 1978, 1981, 1988, 1989a,b, 1991; Neuffer et al., 1999) and *Melanium* (Clausen, 1931; Erben, 1996). Hybrids are fertile, partly fertile or sterile, depending solely on whether the genetic barriers between parental species are weak or strong and therefore on the genome homology. In the *Melanium* section, recognized as a young and still-evolving group (Yockteng et al., 2003), genetic barriers between species are notoriously weak. Species are separated mainly by ecological and geographical isolation. Pansies hybridize easily, forming hybrid swarms or populations of introgressive forms (Clausen, 1931; Fothergill, 1938; Kakes and Everards, 1976; Kakes, 1977, 1979; Erben 1996; Krahulcová et al. 1996).

The origin of *Viola guestphalica*, a metal-tolerant taxon, is still unsolved. Recent investigations of both zinc violets and related *Melanium* species using molecular markers (ITS1–5.8S rDNA–ITS2 regions) indicated that blue and yellow zinc violets form a clade with *V. lutea* (Hildebrandt et al., unpubl. data), the suggestion being that zinc violets evolved from *V. lutea* as ecotypes on contaminated sites and consequently were a subspecies of *V. lutea*, with the blue zinc violet being named *V. lutea* subsp. *westfalica*.

Strong evidence in favor of the hybrid origin hypothesis was provided by current embryological data. Representatives of the blue zinc violet from the natural site, Wełnowiec zinc spoil and the garden (with very low concentrations of heavy metals) showed abnormalities in sexual reproduction typical for hybrids. In male (Hildebrandt et al., unpubl. data) and female meiosis (this paper), multivalents and univalents instead of bivalents were formed in the first meiotic division. Additionally, chromosomes were scattered along the spindle(s) in both meiotic divisions. The presence of multivalents confirmed the high genome homology with the putative parental species, which is not surprising as the species of the *Melanium* section are closely related. The abnormalities accompanying male and female gametophyte development were found to be similar to those of other *Viola* hybrids. Pollen in the blue zinc violet varied conspicously in size and structure even within one pollen sac (e.g., giant

multinucleate or multicellular pollen grains, very small ones, empty and shrunken pollen, and regularly shaped four-aperturate pollen grains). Similar variability in pollen morphology, where the largest pollen grains were more than twice as big as the smallest ones, was observed in natural interspecific hybrids of the *Viola* section (Kuta, 1978, 1981, 1989a,b).

Additional features characteristic for hybrids, such as unorganized, multinucleate female gametophytes, and multiple ESs, described previously in natural hybrids of *Viola* (e.g., *V. epipsila* × *V. palustris*, Kuta, 1989a,b; *V.* × *wittrockiana* – tripled hybrid *V. tricolor* × *V. lutea* × *V. altica*, Opoka, unpubl. data), were also found in the blue zinc violet, although with rather low frequency (1% and 4%, respectively).

The current embryological data strongly support the hypothesis (Kakes, 1979) that the blue zinc violet is a young species of hybrid origin, derived from two or more violet species. Since the blue zinc violet seems close to *V. lutea* (2n = 48), this taxon should be put forward as one of the putative parental species. The involvement of *V. tricolor* (2n = 26) as the other parent cannot be excluded, as natural hybrids of *V. lutea* × *V. tricolor* have been described from numerous European populations (Clausen, 1931; Fothergill, 1938; Krahulcová et al., 1996). It is worth mentioning that in such hybrid swarms, some individuals resembled the blue zinc violet in morphology and chromosome numbers.

The observed abnormalities in sexual reproduction and the reduced fertility of the blue zinc violet could be a consequence of its hybrid origin, coupled with the negative influence of extreme environmental conditions. To resolve the origin of zinc violets, advanced genome analysis of zinc violets and putative parental species using molecular methods as FISH and GISH is needed. Molecular markers such as AFLPs (or ISSRs) or allozymes could probably also be of help.

ACKNOWLEDGEMENTS

Seeds were kindly provided by the Institute of Botany of Cologne University. We are grateful to Dr. Józef Mitka of the Jagiellonian University for stimulating discussion on hybrid speciation. This work was supported by Jagiellonian University research grant DBN–414/CRBW/K-VI–1/2003.

REFERENCES

ANTOSIEWICZ DM. 1992. Adaptation of plants to an environment polluted with heavy metals. *Acta Societatis Botanicorum Poloniae* 61: 281–299.

ALEXANDER MP. 1969. Differential staining of aborted and non-aborted pollen. *Stain Technology* 44: 117–122.

BISKUP A, and IZMAIŁOW R. 2004. Endosperm development in seeds of *Echium vulgare* L. (Boraginaceae) from polluted sites. *Acta Biologica Cracoviensia Series Botanica* 46: 39–44.

BONE E, and FARRES A. 2001. Trends and rates of microevolution in plants. *Genetica* 112–113: 165–182.

CLAUSEN J. 1931. Cyto-genetic and taxonomic investigations on *Melanium* violets. *Hereditas* 15: 219–308.

CZAPIK R, IZMAIŁOW R, and KOŚCIŃSKA-PAJĄK M. 2002. Developmental disturbances and degeneration of plant embryo in polluted environment. *Polish Botanical Studies* 15: 39–48.

DORYWALSKI J, WOJCIECHOWICZ M, and BARTZ J. 1964. *Metodyka oceny nasion*. Państwowe Wydawnictwa Rolnicze i Leśne, Warszawa.

ELLENBERG H. 1988. *Vegetation ecology of Central Europe*. Cambridge University Press, Cambridge, New York, New Rochelle, Melbourne, Sydney.

ERBEN M. 1996. The significance of hybridization on the forming of species in the genus *Viola*. *Bocconea* 5: 113–118.

ERNST WHO. 1968. Das *Violetum calaminariae westfalicum*, eine Schwermetallpflanzen-gesellschaft bei Blankenrode in Westfalen. *Mitteilungen der Floristisch-soziologischen Arbeitsgemeinschaft* 13: 263–268.

ERNST WHO. 1974. *Schwermetallvegetation der Erde*. Gustaf Fischer Verlag, Stuttgart.

ERNST WHO. 1990. Mine vegetation in Europe. In: Shaw J [ed.], *Heavy metal tolerance in plants*, 21–35. CRC Press, Boca Raton, Florida.

ERNST WHO, VERKLEIJ JAC, and SCHAT H. 1992. Metal tolerance in plants. *Acta Botanica Neerlandica* 41: 229–248.

ERNST WHO. 1999. Evolution of plants on soils anthropogenically contaminated by heavy metals. In: Van Raamsdonk LWD, den Nijs JCM [eds.], *Plant evolution in man-made habitats*. Proceedings VIIth Symposium IOPB, Amsterdam. Hugo de Vries laboratory, Amsterdam.

FOTHERGILL PG. 1938. Studies in *Viola*, 1: The cytology of a naturally-occurring population of hybrids between *Viola tricolor* L. and *Viola lutea* Huds. *Genetica*: 159–186.

HENDRY GAF, and GRIME JP. 1993. *Methods in comparative ecology*. Chapman and Hall, London.

HILDEBRANDT U, KALDORF M, and BOTHE H. 1999. The zinc violet and its colonization by arbuscular mycorrhizal fungi. *Journal of Plant Physiology* 145: 709–71.

IZMAIŁOW R. 2000. Reproduction of *Vicia cracca* L. in the polluted environment of the Legnica-Głogów Copper Basin (Poland). *Acta Biologica Cracoviensia Series Botanica* 42: 125–133.

IZMAIŁOW R. 2002a. Embryogenesis in *Capsella bursa-pastoris* (*Brassicaceae*) in polluted and disturbed sites. *Polish Botanical Studies* 15: 11–19.

IZMAIŁOW R. 2002b. The effect of soil from polluted sites on reproductive success in *Ranunculus repens* (*Ranunculaceae*). *Polish Botanical Studies* 15: 5–10.

IZMAIŁOW R, and BISKUP A. 2003. Reproduction of *Echium vulgare* L. (Boraginaceae) at contaminated sites. *Acta Biologica Cracoviensia Series Botanica* 45: 69–75.

JĘDRZEJCZYK M, and ROSTAŃSKI A. 2001. Zinc spoil heap – a habitat for introduced taxa *Viola guestphalica* Nauenb. and *Viola calaminaria* (Ging.) Lej. *Natura Silesiae Superiors*, Suppl.: 45–54.

JĘDRZEJCZYK M, ROSTAŃSKI A, and MAŁKOWSKI E. 2002. Accumulation of zinc and lead in selected taxa of the genus *Viola* L. *Acta Biologica Cracoviensia Series Botanica* 44: 49–55.

KAKES P. 1977. Genecological investigations on zinc plants II. Introgression in a small population of the zinc violet *Viola calaminaria* ssp. *westfalica* (Lej.) Ernst. *Acta Botanica Neerlandica* 26: 385–400.

KAKES P. 1979. Genecological investigations on zinc plants III. Cytology of hybrids between *Viola calaminaria* ssp. *westfalica* (Lej.) Ernst. and *Viola arvensis* Murr. *Genetica* 51: 135–142.

KAKES P, and EVERARDS K. 1976. Genecological investigations on zinc plants I. Genetics of flower colour in crosses between *Viola calaminaria* Lej. and its subspecies *westfalica* (Lej.) Ernst. *Acta Botanica Neerlandica* 25: 31–40.

KOŚCIŃSKA-PAJĄK M. 2000. Microspores and pollen grain in triploid *Chondrilla juncea* L. from polluted and unpolluted areas. *Acta Biologica Cracoviensia Series Botanica* 42: 135-140.

KOŚCIŃSKA-PAJĄK M. 2002. Embryo development and structure in the autonomous apomict *Chondrilla juncea* (*Asteraceae*) from a polluted area. *Polish Botanical Studies* 15: 21- 30.

KRAHULCOVÁ A, KRAHULEC F, and KIRSCHNER J. 1996. Introgressive hybridization between a native and an introduced species: *Viola lutea* subsp. *sudetica* versus *V. tricolor*. *Folia Geobotanica et Phytotaxonomica* 31: 219–244.

KUTA E. 1978. Cyto-embryological studies on the species of the *Viola* L. genus, *Nomimium* Ging. section from the territory of Poland. *Fragmenta Floristica et Geobotanica* 24: 23–91.

KUTA E. 1981. Further cyto-embryological studies on *Viola* L., section *Viola* L. *Acta Biologica Cracoviensia Series Botanica* 28: 69–82.

KUTA E. 1988. Embryological observations on two Canadian species of the *Viola* L. genus (Section Plagiostigma Godr.). *Acta Biologica Cracoviensia Series Botanica* 30: 39–50.

KUTA E. 1989a. Biosystematic studies on the genus *Viola* L., section *Plagiostigma* Godr. I. Karyological analysis of *V. epipsila* Ledeb., *V. palustris* L. and their hybrids from Poland. *Acta Biologica Cracoviensia Series Botanica* 31: 29–44.

KUTA E. 1989b. Biosystematic studies on the genus *Viola* L., section Plagiostigma Godr. II. Embryological analysis of *V. epipsila* Ledeb., *V. palustris* L. and their hybrids from Poland. *Acta Biologica Cracoviensia Series Botanica* 31: 45–62.

KUTA E. 1991. Biosystematic studies on *Viola* sect. *Plagiostigma*: III. Biometrical analysis of the Polish populations of *V. epipsila*, *V. palustris* and their spontaneous hybrids. *Fragmenta Floristica et Geobotanica* 35: 5–34.

MEYER J. 1995. Verteilung von Schwermetallen in Pflanzen und im Boden des Naturschutzgebietes "Waldwiese im Wäschebachtal". Masters dissertation. University of Münster, Münster.

NAUENBURG JD. 1986. Untersuchungen zur Variabilität, Ökologie und Systematik der *Viola tricolor* - Gruppe in Mitteleuropa. Ph.D. dissertation. Göttingen, Germany.

NEUFFER B, AUGE H, MESCH H, AMARELL U, and BRANDL R. 1999. Spread of violets in polluted pine forests: morphological and molecular evidence for the ecological importance of interspecific hybridization. *Molecular Ecology* 8: 356–377.

ROSTAŃSKI A, JĘDRZEJCZYK M, and JANAS L. 2003. Taxonomic problems within the section *Melanium* Ging. of the genus *Viola* L. *Genus International Journal of Invertebrate Taxonomy*, Suppl.: 65–74.

SINGH RJ. 2003. *Plant cytogenetics*. 2nd ed. CRC Press, Boca Raton, London, New York, Washington, D.C.

VALENTINE DH. 1962. Variation and evolution in the genus *Viola*. *Preslia* 34: 90–206.

VALENTINE DH. 1976. Patterns of variation in north temperate taxa with a wide distribution. *Taxon* 25: 225–231.

VALENTINE DH, MERXMÜLLER H, and SCHMIDT A. 1968. *Viola* L. In: Tutin et al. [eds.], *Flora Europaea*, vol. 2. Cambridge University Press, Cambridge.

WIERZBICKA M, and ROSTAŃSKI A. 2002. Microevolutionary changes in ecotypes of calamine waste heap vegetation near Olkusz, Poland. *Acta Biologica Cracoviensia Series Botanica* 44: 7–19.

YOCKTENG R, BALLARD Jr HE, MANSION G, DAJOZ I, and NADOT S. 2003. Relationship among pansies (*Viola* section Melanium) investigated using ITS and ISSR markers. *Plant Systematics and Evolution* 241: 153–170.