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WAS REDUCED POLLEN VIABILITY IN *VIOLA TRICOLOR* L. THE RESULT OF HEAVY METAL POLLUTION OR RATHER THE TESTS APPLIED?

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We used different tests to assess the effect of high soil concentrations of heavy metals on pollen viability in plants from metallicolous (MET) and nonmetallicolous (NONMET) populations. The frequency of viable pollen depended on the test applied: MET plants showed no significant reduction of pollen viability by acetocarmine, Alexander, MTT and X-Gal dye testing, but a drastic reduction of pollen viability in MET flowers (MET 56% vs 72% NONMET) by the FDA test. There was no correlation between pollen viability estimated in histochemical tests and pollen germination in vitro or in vivo. We discuss the terminology used to describe pollen viability as determined by histochemical tests.

Key words: *Viola tricolor*, pollen viability tests, pollen germination, metalliferous sites, heavy metals, acetocarmine test, Alexander test, X-Gal test, FDA test, MTT test.

INTRODUCTION

Plants living in highly polluted environments have to complete their life cycle in order to survive and establish permanent populations. They must develop viable male gametophytes with sperm cells, female gametophytes with a functional egg cell and, after double fertilization, embryos and seeds able to germinate. Usually sexual reproduction is combined with vegetative propagation, allowing clones to be produced when sexual reproduction fails; in this way the genetic structure of the population is influenced by the two modes of reproduction. The switch to asexual reproduction itself is usually a selective disadvantage in adverse conditions but is sometimes maintained (Deng et al., 2007; Deschamps et al., 2007). Many authors have found various kinds of developmental disturbances and degenerative processes in anthers and ovules of pioneer colonizing plants, resulting in a high percentage of aborted ovules and anthers (Ostrolucká, 1989; Ostrolucká et al., 1995; Mičieta and Murin, 1996; Izmaiłow, 2000; Kościńska-Pająk, 2000; Czapik et al., 2002; Izmaiłow and Biskup, 2003; Biskup and Izmaiłow, 2004; Malayeri et al., 2005; Kłosowska et al., 2009; Yousefi et al., 2009), even though generative organs are highly protected regions of the plant organism (Ernst et al., 1992; Wierzbicka and Panufik, 1998; Sun et al., 2005). As the female gametophyte is a single few-celled structure enclosed in a small ovule, it is more convenient to examine microsporogenesis and male gametophyte development, which yield huge numbers of pollen grains. The various pollen viability tests are based on staining techniques that determine pollen enzymatic activity, membrane integrity, and the stainability of the cytoplasm and nucleus (Shivanna and Rangaswamy, 1992; Khatun and Flowers, 1995; Nepi and Franchi, 2000; Rodriguez-Riano and Dafni, 2000; Vižintin and Bohanec, 2004); they have been widely adopted as a simple indicator of plant tolerance and incorporated into environmental biomonitoring assays. The choice of viability test depends on the species, pollen state and staining conditions, since

Abbreviations: MET – metallicolous population; NONMET – nonmetallicolous population; FDA – fluorescein diacetate; MTT – 2,4 diphenyl tetrazolium bromide; X-Gal – 5-bromo-4-chloro-3-idolilo-α-galactoside.

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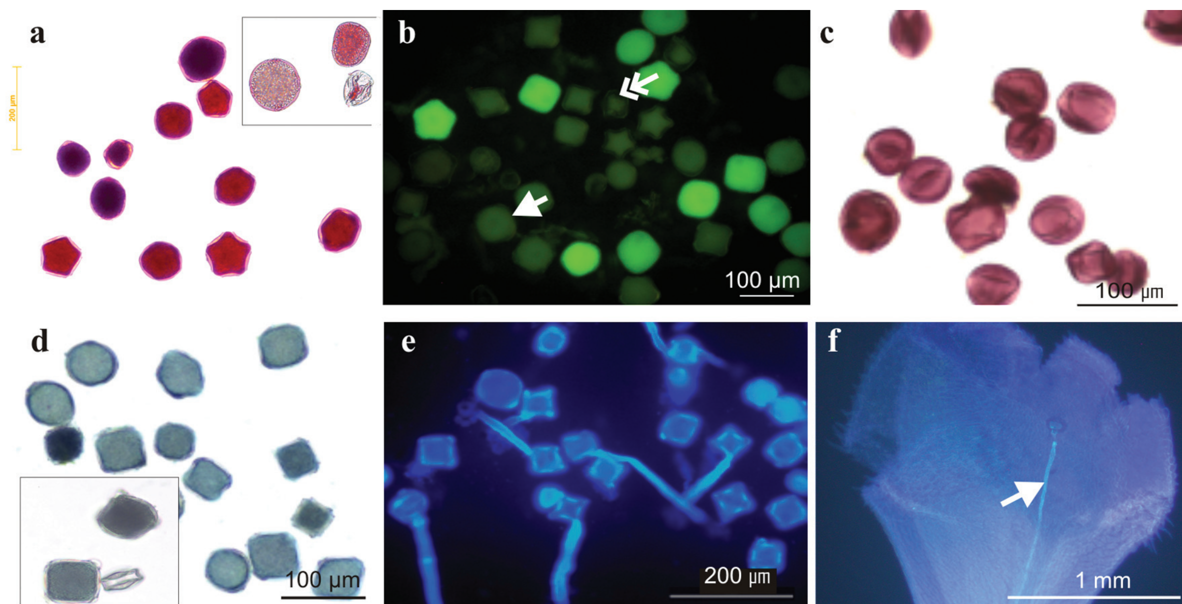

Fig. 1. Stainability **(a–d)** and germination **(e–f)** of *V. tricolor* pollen from metallicolous **(b, d)** and nonmetallicolous **(a, c, e, f)** populations. **(a)** Acetocarmine staining; empty nonstained pollen grain magnified; differences in size visible, **(b)** Fluorescein diacetate (FDA) staining; degenerated pollen grains without shrinking (arrow) and with shrinking (double arrow) of cytoplasm, **(c)** Tetrazolium salt (MTT) staining, **(d)** X-Gal staining; empty nonstained pollen grain magnified, **(e)** Pollen grains germinating on saccharose medium; pollen tubes visible, **(f)** Pollen grains germinating on stigma; pollen tube penetrating style (arrow).

results can differ between staining techniques. In vitro germination assays are commonly used to determine the actual germinability of pollen under artificial conditions, though they are more laborious and do not necessary indicate germinability reliably (Khatun and Flowers, 1995; Rodriguez-Riano and Dafni, 2000).

In this study we applied several tests to determine pollen viability in heartsease, *Viola tricolor* (sect. *Melanium*, Violaceae), a metal-tolerant species (pseudometallophyte) which grows abundantly on metal-contaminated (Zn, Pb, Cd, Cu) waste heaps and noncontaminated sites in Central and Western Europe (Słomka et al., 2008, and literature cited therein). We wanted to find out (1) whether an environment polluted with heavy metals affects the pollen viability of heartsease, (2) whether pollen viability estimates depend on the test applied, (3) whether histochemical tests indicate pollen viability or rather pollen stainability, and (4) whether stainable pollen grains are cytologically balanced.

MATERIALS AND METHODS

PLANT MATERIAL

Plants from two populations, in the Botanical Garden (BG) in Cracow (nonmetallicolous – NONMET) and the Bukowno waste heap (BH) near Olkusz (metallicolous – MET), were collected in the field in 2009 and transported to the laboratory. The soil at BG contains only trace amounts of heavy metals (mean 533 mg/kg Zn, 105 mg/kg Pb, 4 mg/kg Cd; Słomka, unpublished); the soil at BH contains high levels of heavy metals (mean 7271 mg/kg Zn, 1491 mg/kg Pb, 85 mg/kg Cd; Słomka et al., 2008). One opened flower was taken from each of 91 plants and examined for pollen viability or germination; 4–9 of those flowers were used depending on the test.

POLLEN VIABILITY

For acetocarmine and Alexander tests, freshly opened flowers were fixed in 96% ethanol and glacial acetic acid (v/v 3:1). For FDA, MTT and X-Gal tests, pollen from nonfixed newly opened flowers was used immediately.

Acetocarmine test – 1% acetocarmine was used for staining (Singh, 2003). Cytoplasm stains red in viable pollen and remains transparent in nonviable pollen.

Alexander test – Alexander's dye is a mixture of malachite green staining the cellulose of pollen walls green, and acid fuchsin staining the pollen protoplast red (Singh, 2003). Viable pollen grains appear red, and nonviable pollen grains stain green.

MTT test – pollen was stained with 1% MTT dissolved in 5% saccharose (water solution) and heated until the dye evaporated completely (Khatun and Flowers, 1995). MTT is metabolized by mitochondr-

TABLE 1. Stainability and germination of *V. tricolor* pollen from metallicolous and nonmetallicolous populations; percentage are given in brackets

Test	Nonmetallicolous population (BG)		Metallicolous population (BH)		Statistics χ^2; P
	No. of flowers/ No. of pollen grains	No. of stainable or germinated pollen grains	No. of flowers/ No. of pollen grains	No. of stainable or germinated pollen grains	
Acetocarmine test	8/2515	2287[91][a]	4/861	850[99][b]	6.95; 0.01<P<0.001
Alexander test	8/2428	2403[98][a]	5/3061	2738[90][b]	6.25; 0.01<P<0.001
FDA test	9/1020	734[72][a]	7/953	534[56][b]	11.85; P<0.001
MTT test	4/297	252[85][a]	4/290	270[93][a]	0.7; 0.05<P<0.3
X-Gal test	7/820	738[90][a]	7/1007	806[80][a]	3.25; 0.02<P<0.05
Germination on medium	6/1952	390[20][a]	6/2158	410[19][a]	0.03; 0.5<P<0.9
Germination on stigma	8/220	n.d.*	8/197	n.d.*	–
Total	50/9252	6804[90]**	41/8527	5608[84]**	–

* only single pollen grains germinated, so no count is given; ** germination not included in total frequency of stainable pollen.
Values bearing different letters along one row differ significantly by the chi-square test

ial dehydrogenases, yielding a red formazan reaction product; viable pollen stains dark red (Nepi and Franchi, 2000).

X-Gal test – X-Gal indicates β-galactosidase activity catalyzing hydrolysis of α-galactosides to monosaccharides. Pollen was stained with 0.1% X-Gal in citrate buffer (pH 4.8) with several drops of 50 μl N,N-dimethyl formamide (Rodriguez-Riano and Dafni, 2000). Viable pollen stains blue-green.

FDA test (FCR technique, fluorochromatic reaction) – FDA dye was prepared according to Dafni (1992) [2 ml 20% saccharose in water with several drops of stock solution of FDA (2 mg FDA/1 ml acetone)]. Stained pollen was kept in a humid chamber for 30 min at 24°C and afterwards observed with a Nikon E80i microscope with a UV-2A filter. This technique is based on entry of FDA into the vegetative cell where it is hydrolyzed by esterase to a fluorescein. Viable pollen fluorescences yellow-green (Nepi and Franchi, 2000).

POLLEN GERMINATION

On stigma – 1, 3, 8 and 24 h after cross pollination of opened flowers by hand, pistils were fixed in 96% ethanol with glacial acetic acid (v/v 3:1). For clearing, samples were placed in 0.1 M NaOH for 30 min at 40°C. After rinsing with distilled water the pistils were treated with 0.01% toluidine blue for 15 min to suppress autofluorescence and then stained with 0.1% aniline blue diluted in 0.15 M K_2HPO_4 for 1 h as described by Wędzony (1996). Observations employed a Nikon E80i microscope with a UV-2A filter.

On medium – pollen was germinated for 3 h at 30°C in a humid chamber on medium containing 300 μg $Ca(NO_3)_2 \cdot 4H_2O$, 20 μg H_3BO_3, 2 g saccharose/1 ml distilled water as described by Hoekstra and Bruinsma (1975), a medium successfully used for *V. tricolor* previously (e.g., Lankinen, 2001). Only pollen grains with pollen tube length at least twice pollen diameter were deemed to be germinating. To visualize pollen tubes, pollen were stained with aniline blue as described above and observed with a Nikon E80i microscope with a UV-2A filter.

DATA ANALYSIS

The chi-square test in Statistica 7.0. was used to test the significance of differences between the MET and NONMET populations.

RESULTS AND DISCUSSION

Pollen features (aggregation and shape of grains, aperture number, shape and position, external wall layers, internal protoplasm) are very important for taxonomy and phylogeny (Walker and Dolye, 1975; Stuessy, 2009). Pollen size and viability are good markers of the course of microsporogenesis. Normal meiosis produces pollen grains regular in size and highly viable, and disturbed meiosis reduces pollen viability and causes variability of pollen grain size (very small and giant pollen are formed in addition to those normal in size); the latter can result from inbreeding depression, autopolyploidy, segmental allopolyploidy, hybridization, mutations, and also environmental effects (Stace, 1991).

Besides pollen diameter measurement (Kelly et al., 2002) the quickest and simplest methods of assessing viability rely on different tests. The results we obtained are not uniform: depending on the test,

pollen viability was lower or even higher in flowers from the MET than from the NONMET population (Tab. 1). Acetocarmine (Fig. 1a), Alexander and FDA (Fig. 1b) tests showed significant differences between the metallicolous and nonmetallicolous populations; the other ones did not (MTT, Fig. 1c; X-Gal, Fig. 1d; germination on medium, Fig. 1e; Tab. 1). The acetocarmine and MTT tests showed pollen viability even higher in the MET population, but the difference was significant only for the acetocarmine test (Tab. 1), which is considered the simplest and the less sensitive one because it only confirms the presence and stainability of cytoplasm (Dafni and Firmage, 2000). The FDA test indicated clearly lower pollen viability in the MET population, with up to a 16% difference between the NONMET and MET populations (Tab. 1). This test measures two pollen parameters: esterase activity and plasmalemma integrity. It distinguishes between nonstaining pollen grains that degenerated due to necrosis (pollen with shrunken cytoplasm) or to programmed cell death (pollen with cytoplasm sticking to the plasmalemma) (Shivanna and Rangaswamy, 1992; Nepi and Franchi, 2000). We observed both kinds of degenerated pollen in *V. tricolor* (Fig. 1b).

These results tell us that the term "pollen viability" should be used carefully and rather replaced by the more limited term "pollen stainability," as it depends strictly on the staining assay. A number of authors have discussed the terms used to describe the viability of pollen grains and their ability to germinate and fertilize ovules, and have recommended different terms (pollen sterility, stainability, viability, germinability, stigmatic germinability, fertilization ability, pollen quality) (for review see Dafni and Firmage, 2000; Klein, 2000). Stainable pollen grains may vary in size and thus be cytologically unbalanced and not viable. In this and earlier work, pollen grains conspicuously differing in size were produced in addition to those normal in size (Fig. 1a; Siuta et al., 2005). Pollen stainability rarely corresponds to pollen germination, which is the best index of pollen viability. In vitro germination, the success of which depends on medium composition (calcium and boron ions, sugar concentrations), temperature, humidity and other factors (Shivanna and Rangaswamy, 1992), was lower in both the MET and NONMET populations (20% vs. 19%; Fig. 1e, Tab. 1) than in other work on *V. tricolor* (e.g., Lankinen, 2001), suggesting that plant genotype could also be important. Similarly, in planta (in vivo) pollen germination, which depends closely on the physiological and biochemical characteristics of pollen and its interaction with stigma (Taylor and Hepler, 1997), was also very low here; only single pollen grains germinated (Fig. 1f). Other factors not studied here may have played a role, such as interaction between individual pollen grains, the temperature optima for different pollen grains, and nutrition (N, P) availability during flowering, which substantially influence pollen germination of *V. tricolor* in vivo (Skogsmyr and Lankinen, 2000; Lankinen 2001, 2008).

The number of ovules pollinated in natural conditions, which was very high in both the NONMET and the MET *V. tricolor* populations in situ (almost all ovules were fertilized; authors' unpublished data), suggests that the slightly reduced pollen stainability of plants from the MET population (not always statistically significant) does not reflect less tolerance to the metal-contaminated environment. Such is the case in other species well adapted to heavy metals (*Artemisia vulgaris*, *Chenopodium hybridum*, *Kochia scoparia*; Mičieta and Murin, 1996), made possible by surplus production of pollen, a strategy allowing some of it to be selectively aborted (Melser and Klinkhamer, 2001).

CONCLUSION

1. There was no evidence that the heavy metal pollution at the waste heap markedly reduced the pollen viability of heartsease.
2. The frequency of viable pollen depended on the test applied. One histochemical test (FDA) showed significantly reduced pollen viability in the MET population, but others showed pollen viability even higher than in NONMET flowers (acetocarmine, MTT).
3. Stainable pollen grains might be cytologically unbalanced because they vary conspicuously in size, and thus not viable.
4. Histochemical tests indicate pollen stainability rather than pollen viability.

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