Effect of polyploidy and pollination methods on capsule and seed set of pansies (*Viola* × *wittrockiana* Gams)

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Abstract

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Pansy, *Viola* × *wittrockiana*, is a popular ornamental plant. Effects of polyploidy on phenotype and four pollination methods on capsule and seed set were studied using ten octoploid ($2n = 8x$) and two hexadecaploid ($2n = 16x$) genotypes, originating from induced mutagenesis. Principal component analysis, using 19 phenotypic, phenological and physiological characteristics, revealed that hexadecaploids showed larger differences to the corresponding standard cultivars than octoploids. Number of seed per capsule was similar among genotypes. Capsule set with open pollination was 32–64 %, with self-pollination by hand 18–49% and with cross-pollination by hand 14–43%, while no plant successfully set capsules with seeds under isolators if not pollinated manually. Thus, *Viola* × *wittrockiana* is self-compatible but requires an agent-mediation for successful pollination. The induced phenotypes were found stable over four generations. Hexadecaploids had more attractive phenotypes but fewer seeds than octoploids. However, variation in seed set enabled selection of plants with high fertility, and average seed set increased over generations. Thus, new varieties, fulfilling aesthetic criteria as well as economic and agronomic traits, can be bred from induced mutagenesis.

Keywords: garden pansy; fertility; self-compatibility; flow cytometry; phenotype

Pansies *(Viola* × *wittrockiana* Gams) are popular bedding plants. Whilst perennial by nature they are usually grown as an annual or biennial in garden beds, pots, borders, in hanging baskets or in landscapes (Horn 1996; Bailey 1998). There is a seemingly unlimited need for new varieties. The planned pansy breeding through purposeful hybridization began in 1862 (Horn 1956). Breeding goals focused on selecting plants for unusual flower colours and increased flower size from the initial cross made between the small flowered heartsease (*V. tricolor* L.) and yellow large flowered *V. lutea* Huds. (Wittrock 1895). Hybridization between an alpine perennial, *V. cornuta* L., and *V. altaica* (Ker-Gwal) Pall. – a perennial with large and varied flower colours gave hundreds of pansy cultivars (Wittrock 1895; Horn 1956). Garden pansy (*V.* × *wittrockiana* Gams) is an octoploid ($n = 6$, $8x = 48$) which is thought to be derived from cross combinations among *V. tricolor* ($2n = 26$), *V. lutea* ($2n = 48$), *V. cornuta* ($2n = 22$) and *V. altaica* (Clausen 1927; Horn 1956; Yockteng et al. 2003). Garden pansy is larger than its ancestors in plant height and flower size (Wittrock 1895).

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Table 1. Genotypes of *Viola* × *wittrockiana* used for investigating the effect of pollination method on capsule and seed set

Groups	Genotype[1]	Original cultivar[2]
Standard cultivars	PW	–
	LB	–
Floral mutants	S1	LB
	S2	PW
	S3	LB
	S4	PW
	S5	LB
	S6	LB
	S7	LB
	S8	PW
Hexadecaploids	MPW	PW
	MLB	LB

[1]S1–S8: floral mutants originating from PW (Pure White) or LB (Light Blue)and selected (S) for flower colours after mutagenesis and selfed in two generations, numbering of the S genotypes follows Ajalin et al. (2003) MPW (hexadecaploid Pure White) and MLB (hexadecaploid Light Blue): hexadecaploid genotypes originating from PW and LB, respectively, and selfed in two generations after induced mutagenesis (M); [2]Original octoploid standard cultivar (PW or LB) used for polyploidy induction (Ajalin et al. 2002)

It can reach 15 to 25 cm in height and have single larger (up to 10 cm) single coloured, bicoloured, tricoloured or multicoloured flowers on longer peduncle (Horn 1996; Armitage 1997; Bailey 1998).

Spontaneous crossing and polyploidisation are common throughout the genus *Viola* and often lead to fertile derivates; many species originated from natural hybridization (Clausen 1927; Stebbins 1971). Utilization of polyploidy, either autopolyploidy from induced mutagenesis or allo-polyploidy from spontaneous or artificial interspecific hybridization, is a useful breeding method for creating new ornamental genotypes with desirable phenotypic traits such as plant vigour, compactness, larger flowers, novel flower colours, tolerance to pathogens and environmental stress (Dewey 1980; Lewis 1980; Zeven 1980; Levin 1983; Schum 2003).

Viola flowers are either cleistogamous or chasmogamous. They have five unequal sepals and petals; the bottom petal is enlarged, probably serving as a landing strip for insect pollinators (Armitage 1997; Durka 2000; Yockteng et al. 2003). The cultivated pansies have diverse pollination conditions and the flowers may exhibit cleistogamy (Clausen 1927). *V.* × *wittrockiana* is reported to be (*i*) highly fertile but self-incompatible (Horn 1956; Eminno, Sink 1968), (*ii*) usually cross-pollinated even though self-compatible (Novotná 1977), or (*iii*) self-compatible and usually cross-pollinated due to occurrence of protandrous type of dichogamy (Jhon et al. 1988). Thus, optimal pollination approaches need to be disclosed for efficient breeding and commercialisation.

We hypothesize that there should be effects of polyploidy as well as of pollination methods on capsule and seed set success and also on phenotypic characteristics.

MATERIAL AND METHODS

Twelve garden pansy genotypes (Table 1) from the Faculty of Horticulture, Mendel University in Brno, Lednice, Czech Republic were studied. Fourth generation (M_4) self-pollinated seeds of two hexadecaploid ($2n = 16x$) and eight octoploid ($2n = 8x$, floral mutants) were the derivatives of polypoid induced mutagenesis (Ajalin et al. 2003; Dalbato 2005). In addition, seeds of two standard cultivars, initially used for the polyploidy induction (Ajalin et al. 2002), were included; they are both octoploid ($2n = 8x$) and belong to Pirna series – cv. Pure White and cv. Light Blue.

Seeds, 150–300 for each genotype, were sown on August 15, 2003, for flowering the following spring. They were sown in 30 × 20 cm plastic flats filled with standard substrate (Horti B, AGRO CS a.s., Říkov, Czech Republic); 100 seeds per flat were sown. The flats were placed in a greenhouse (18–22°C daytime, 12–16°C night time). A chemical pesticide (Previcur fungicide, Bayer CropScience AG, Monheim am Rhein, Germany) was used regularly according to recommendation for pansies. Seedlings with 2 to 3 true leaves were transplanted into multi-cell trays. On the second week of October, seedlings with 4 to 7 leaves were transplanted into 9-cm diameter pots and watered when needed. On the second week of November, after 12 weeks of sowing, well established seedlings with 8 to12 leaves were transferred to cold frames in which they overwintered outside under glass coverings. Plant materials used for experiment were collected in the following spring and summer.

Ploidy levels were analysed using a flow cytometer (Partec PAS, Münster, Germany). The procedures for sample preparation and measuring of relative DNA content followed Galbraith et al. (1983) and Doležel (1997). The signal corresponding to the G_1 phase of nuclei from standard octoploid cultivars (reference) was set to channel 100. At least 2,000 nuclei were analysed for each sample and peaks representing G_1 phase of nuclei (dominant peaks) were applied to determine ploidy levels of genotypes.

Four pollination methods: hand cross-pollination, self-pollination by hand, natural self-pollination and open-pollination were evaluated using 20 plants of each genotype for each method. Plants for open-pollination were grown in the experiment field using distance (≥ 200 m) isolation between genotypes and all other plants were grown in insect-protected isolators. All newly opening flowers and flower buds likely to open within a day were removed at transplanting to avoid contamination of other flowers. The cultural practices were conducted according to commonly accepted recommendation for garden pansy.

For self-pollination by hand, the lower petal with pollen was carefully bent by hand toward the stigma to ensure sufficient pollen deposition. Crossings were performed according to Clausen (1926), Novotná (1977) and Jhon et al. (1988) by taking the lower petal with pollen from newly opening flower of the perspective father plant, and then gently rolled and brushed on the castrated stigma of the mother plant flower, carefully removing and depositing the pollen. Flowers almost or newly opening were castrated and pollinated at the same time.

Table 2. Result of flow cytometric analysis of DNA ploidy levels, and description of plants of *Viola* × *wittrockiana* genotypes

Genotype	*CV* (%)	TFF	Flower colour	Flower diameter (mm)	Growth habit
PW	2.56	Early	pure white	30–55	LC
LB	2.59	Early	light blue	35–55	LC
S1	2.62	5–7	light blue	40–57	C
S2	2.56	5–7	bicolour	35–57	C
S3	2.69	5–7	black	40–60	C
S4	2.52	8–10	bicolour	35–55	C
S5	2.57	12	blue with face	35–57	C
S6	2.67	5–7	blue	40–57	C
S7	2.62	8–10	light blue	40–57	C
S8	2.60	8–10	bicolour with blotch	35–55	C
MPW	2.30	5–8	pure white	40–65	V + C
MLB	2.29	5–8	light blue	40–65	V + C

PW – Pure White, LB – Light Blue: standard cultivars, S1–S8: floral mutants, MPW – hexadecaploids Pure White, MLB – hexadecaploids Light Blue: hexadecaploids; CV – coefficient of variation obtained from flow cytometric analysis of DAPI-stained nuclei; TFF – time to first flowering in spring (time for floral mutants and hexadecaploid genotypes are given as time (days) after standard cultivars); LC – less compact, V + C – vigour and compact, C – compact

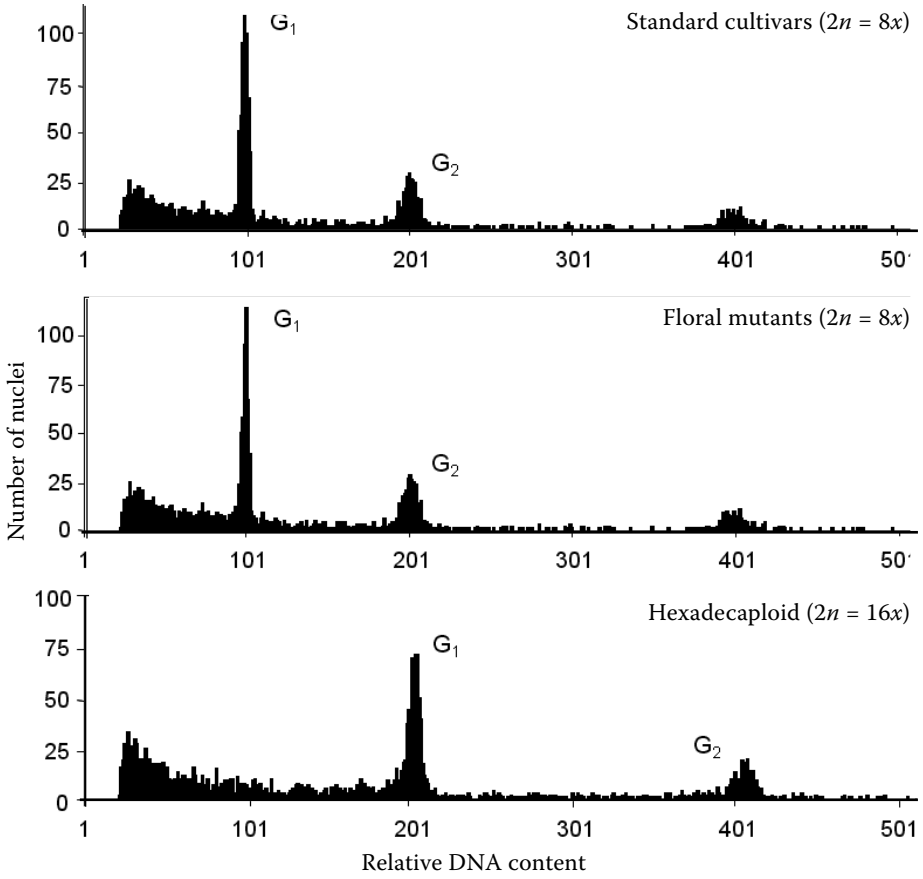

Fig. 1. Flow cytometric DNA histograms obtained from DAPI-stained nuclei in plants of *Viola* × *wittrockiana*

For natural self-pollination, un-manipulated flowers were marked and observed daily, using plants growing in isolators. Flowers for open-pollination in the field were marked and pollination was left to the natural agents. Self-pollination and cross pollination by hand were performed for 30 consecutive days from the second week of May, when the plants were in peak flowering.

Capsule set (with seeds) of observed/treated flowers was recorded from all genotypes and pollination methods. Number of flowers per plant, flower size, seeds per capsule and per plant, seed weight and pollen grain size (micrometer on a light microscope, 500× magnification) were recorded for all genotypes after self-pollination by hand.

For all characteristics investigated, each octoploid floral mutant and each hexadecaploid genotype was pair-wise compared with the corresponding octoploid standard cultivar using one-way analysis of variance (ANOVA, Table 1). Overall comparison of the twelve studied genotypes was done by principal component analysis (PCA) of 19 characteristics: capsule set (selfing by hand), capsule set (crossing by hand), seeds per capsule, seed yield per plant, pollen grain size, number of flowers, seed weight, leaf length-width ratio, leaf area, leaf per plant, stomata size, number of chloroplasts/guard cell pair, stomata density, plant height, plant dimension, flower diameter, peduncle length, plant dry weight and photochemical efficiency [data for the latter twelve characteristics from Dalbato (2005)].

RESULTS AND DISCUSSION

Within each genotype (Table 1), the variation in relative nuclear DNA was low; coefficient of variation ranging from 2.29 to 2.69% (Table 2). The histograms obtained from flow cytometric analysis were similar within each of the three groups (*i*) standard octoploid cultivars, (*ii*) octoploid floral mutants and (*iii*) hexadecaploid genotypes. Therefore, one flow cytometry histogram per group is shown (Fig. 1). All floral mutants were confirmed to be octoploids, as the corresponding standard cultivars (Table 2, Fig. 1), thus, no changes in poly-

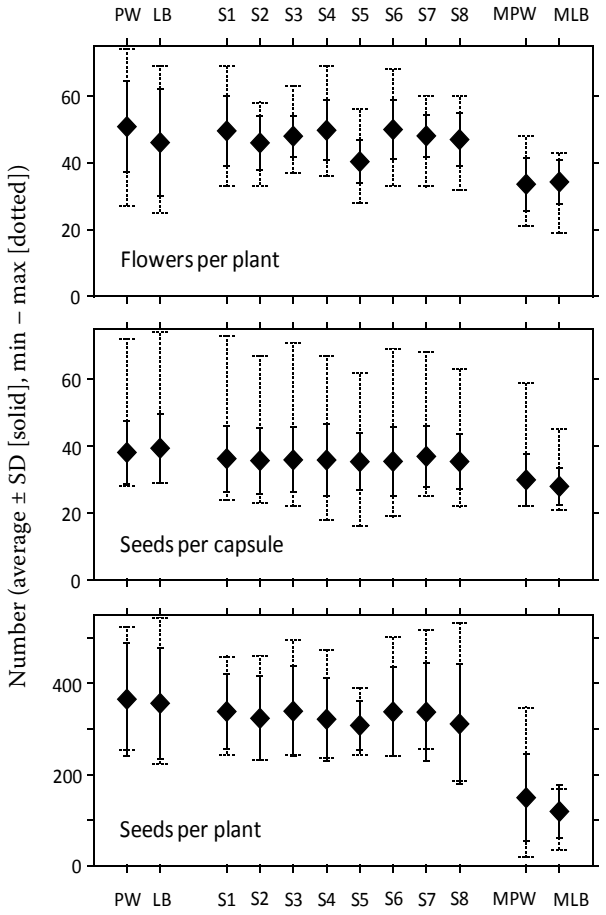

Fig. 2. Number of flowers and seeds set per capsule and plant of *Viola* × *wittrockiana* [the fourth (M_4) generation after polyploidy induction] after self pollination by hand. PW and LB: standard cultivars (2*n* = 8*x*), S1–S8: floral mutants (2*n* = 8*x*), MPW and MLB: hexadecaploid (2*n* = 16*x*)

ploidy level occurred during mutagenesis. In agreement with Gatt et al. (1998), Mable (2003) and Doležel and Bartoš (2005), flow cytometry was a suitable tool and provided a rapid and accurate confirmation of the ploidy levels reported for M_1 to M_4 generation plants of *V.* × *wittrockiana* (Ajalin et al. 2003; Dalbato 2005).

Octoploid floral mutants and hexadecaploid genotypes delayed first flowering in spring 5–12 days compared to standard cultivars (Table 2). However, once flowering begun, full bloom was achieved within two weeks, as for standard cultivars. Effect of ploidy levels on flowering time was also reported in *Sedum pulchllum* (Smith 1946) and *Ocimum klimandscharicum* (Bose, Choudhury 1962). Thus, this effect indicates that it is to develop new varieties with modified flowering time through induced mutagenesis. The *V.* × *wittrockiana* standard cultivars had small to medium flowers and were less compact, whereas the hexadecaploids were high vigour plants with larger flowers and more compact in their growth habit (Table 2). The floral mutants produced attractive flowers, differing in size, colour and colour combinations between genotypes (Table 2).

Number of flowers per plant was significantly ($P < 0.01$) lower in hexadecaploids than in standard cultivars and floral mutants (Fig. 2). Flowers of octoploid floral mutants and hexadecaploids were generally larger than flowers of standard cultivars (Table 2).

Regarding numbers of flowers, capsules and seeds per plant, number of seeds per capsule, seed weight and pollen grain size, the genotypes within each of the three groups (*i*) octoploid standard cultivars, (*ii*) octoploid floral mutants and (*iii*) hexadecaploids were similar to each other (Figs 2–4). There were large differences between octoploid floral mutants and their corresponding standard cultivar (Table 1) regarding flower colour and growth habit

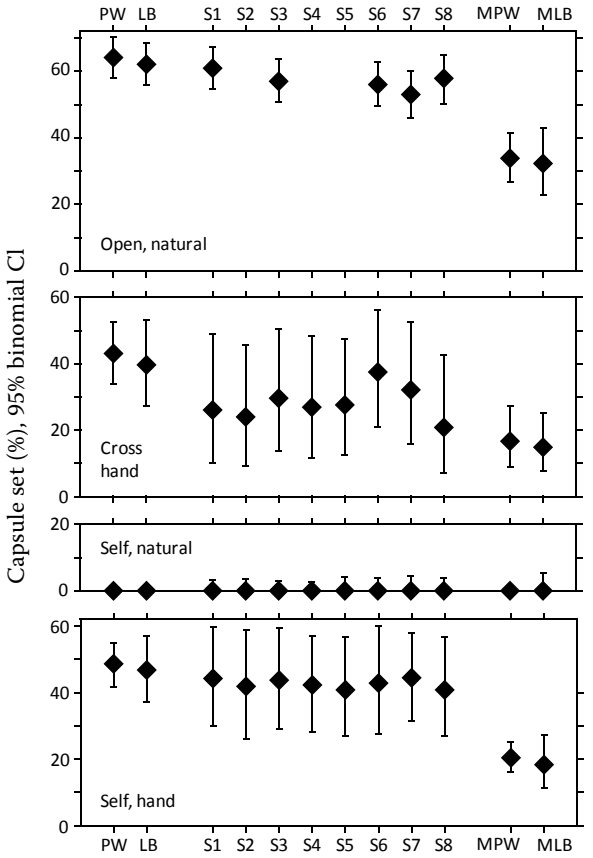

Fig. 3. Capsule set of *Viola* × *wittrockiana* after four different pollination methods. PW and LB: standard cultivars (2*n* = 8*x*), S1–S8: floral mutants (2*n* = 8*x*, S2, S4 and S5 were not included in the open pollination treatment), MPW and MLB: hexadecaploids (2*n* = 16*x*)

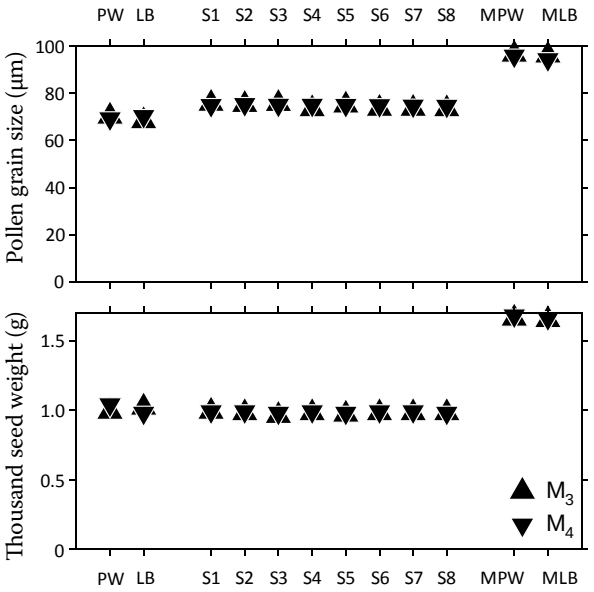

Fig. 4. Average pollen grain size and seed weight of standard cultivars PW and LB ($2n = 8x$), floral mutants S1–S8 ($2n = 8x$) and hexadecaploid MPW and MLB ($2n = 16x$) *Viola* × *wittrockiana* third (M_3, data from Dalbato 2005) and fourth (M_4) generation after polyploidy induction

(Table 2); the induced new phenotypes remained stable over the M_1 to M_4 generations (Ajalin et al. 2003; Dalbato 2005).

The extent of capsule set was dependent on pollination method and genotype (Fig. 3). Open-pollination gave the highest capsule set for all genotypes (32–64%) followed by hand self-pollination (18–49%) and crossing by hand (15–43%); no plant set capsules after natural self-pollination. ANOVA revealed a significant ($P \leq 0.05$) difference between PW and MPW as well as between LB and MLB regarding capsule set after open natural pollination, crossing by hand and selfing by hand, while the floral mutants did not differ significantly from corresponding standard cultivar ($P > 0.05$). The slightly lowered fertility level of the octoploid floral mutants and the low capsule set of hexadecaploid (Fig. 3) could be explained by the effect of induced mutagenesis, which is usually associated with decreased fertility level.

We conclude that capsule set in *V.* × *wittrockiana* is possible only when the pollen deposition on stigma is accomplished by pollination agent, either natural agents or by hand (Fig. 3; Novotná 1977; Jhon et al. 1988), thus, despite being self-compatible, they are usually cross-pollinated in nature. Such a pattern is also reported from other species, e.g. *Chamaecrista keyensis* (Liu, Koptur 2003), *Impatiens reptans* (Tian et al. 2004), *Dianthus guliae* (Gargano et al. 2009), *Pultenaea villosa* (Ogilvie et al. 2009) and *Anathallis* species (Gontijo et al. 2010).

Octoploid plants, standard cultivars as well as floral mutants, produced more seeds than hexadecaploids (Fig. 2), ANOVA revealed a significant ($P \leq 0.05$) difference between PW and MPW as well as between LB and MLB. However, the average number of seeds per capsule was relatively even among the three groups (Fig. 2). The lower number of seeds per plant for hexadecaploids (Fig. 2) is mainly a result of fewer capsules per plant than octoploids, a result that appeared for all pollination methods (Fig. 3). Induced polyploids have low fertility because of meiotic abnormalities (Lewis 1980; Levin 1983), allelopathic action (Zeven 1980), or unbalanced disjunction of univalents in meiosis (Callaway, Callaway 2000).

The seed set of hexadecaploids increased from M_1 to M_4 generation [(Ajalin et al. 2003; Dalbato 2005; Fig. 2), analogous to *Amaranthus* spp. (Pal, Pandey 1982)]. By the M_4 generation, 150–300 seeds per plants were obtained (Fig. 2) and 50–80% germinated (Dalbato 2005). The relative delay of flowering for hexadecaploids decreased from M_1 to M_4 generation (Ajalin et al. 2002; Dalbato 2005; Table 2). Thus, plants with high fertility level and reasonable early flowering can be selected for fur-

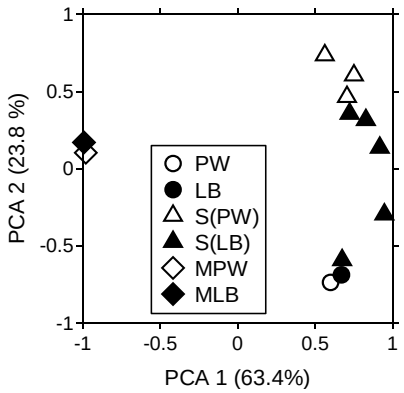

Fig. 5. Principal component analysis (PCA) of 12 genotypes of *Viola* × *wittrockiana*, using 19 different recorded characteristics (genotype average, $N = 20$) of phenology and physiology

The genotypes were two standard cultivars PW and LB ($2n = 8x$), eight floral mutants (S, $2n = 8x$) and two hexadecaploids (MPW and MLB, $2n = 16x$); floral mutants and hexadecaploids originated from either PW (open symbols) or LB (filled symbols). The two first axes, PCA 1 and PCA 2, of the multi-dimensional analysis are shown; these axes explain together 87.2 % of the analyzed data

ther breeding; intense selection for increased fertility combined with overall agronomic traits was recommended (Dudley, Alexander 1969).

The relatively fewer seeds for hexadecaploid genotypes were larger and heavier than those of standard cultivars and floral mutants. The thousand seed weight was 1.65 and 1.68 g for the genotypes MPW and MLB, respectively, and about 1 g for each of the octoploid standard cultivars and floral mutants (Fig. 4). Pollen grain size was significantly larger in hexadecaploids and octoploid floral mutants than in their corresponding octoploid standard cultivars ($P < 0.01$). Size of pollen grain and seed were stable over M_3 (data from Dalbato 2005) and M_4 generations (Fig. 4), as for ten generations of *Amaranthus* spp. (Pal, Pandey 1982).

Pollen grain (Bingham 1968) and seed size (Hanzelka, Kobza 2004) are used as indicators of ploidy level. However, pollen grain size may differ between plants of the same ploidy level (Fig. 4; Lewis 1980; Levin 1983), while seed size is a generally reliable indicator of ploidy level (Fig. 4; Sun et al. 1994; Bretagnolle, Lumaret 1995; Hanzelka, Kobza 2004).

Overall, the ten genotypes obtained from induced mutagenesis were separated from their original octoploid cultivars by PCA; the two hexadecaploids differed more than the octoploid floral mutants (Fig. 5) in which the genotype S6 was the closest to the standard cultivars. The high degree of explanation (63% of overall variation) for the first PCA axis (PCA 1, Fig. 5) is because the hexadecaploids (the negative extreme of axis 1) were markedly different from all octoploids (the positive extreme of axis 1) regarding 17 out of 19 included characteristics. The axis explaining the second largest amount of the overall variation, PCA 2 (Fig. 5) was most correlated to the characteristics "plant dimension" and "photochemical efficiency", in this regard the records of hexadecaploids were in between those of octoploid genotypes.

The genotypes obtained from induced mutagenesis had a number of attractive features such as more vigour, longer and thicker peduncle, larger flowers, compact growth habit and unique flower colours (Table 2; Ajalin et al. 2003; Dalbato 2005). These are important traits for aesthetic quality in pansy cultivars (Horn 1956; Bailey 1998). The most widespread effect of polyploidy is an increase in cell size, leading to a general vigour effect especially in flowers and seeds (Stebbins 1971; Lewis 1980; Levin 1983), and making the plants attractive for production of food, forage and flowers (Lewis 1980; Zeven 1980; Uhlík et al., 1981).

Currently, many garden pansy cultivars, including Pirna series, are treated by growth regulators with frequent application for obtaining compact plants. In view of the fact that the application of chemicals will be reduced to a minimum or even disappearing, breeding for compactness is of high importance. Exploiting induced mutagenesis in garden pansy can be an alternative to chemical enhancement of compact growth habit. Also Schum (2003) regarded mutagenesis induction as a suitable method for increased variation in ornamentals, and several induced auto-polyploids were successful (Dewey 1980).

CONCLUSIONS

Capsule set, and therefore seed set per plant, was dependent on pollination method, and a number of phenotypic characteristics were correlated to ploidy levels, thus confirming the hypotheses stated. There was no seed set under natural self-pollination in isolators, while there was a relatively high level of capsule set in flowers that were self-pollinated by hand. Thus, *V.* × *wittrockiana* is self-compatible but pollination requires facilitator (vector). Phenotypic characteristics, such as flower colour and size, were genotype-specific and stable over four generations, while seed set increased over generations for hexadecaploids, which had low fertility in the first generation after induced mutagenesis. Overall, there are great opportunities to breed new *V.* × *wittrockiana* varieties from induced mutagenesis, which can fulfil aesthetic criteria in addition to economic and agronomic traits.

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