Ploidy identification of doubled chromosome number plants in *Viola* × *wittrockiana* Gams. M 1-generation

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ABSTRACT: The aim of this study was to develop a protocol for production of polyploid M1-generation plants of *Viola* × *wittrockiana* Gams. Two variants of colchicine treatment were compared for their efficiency. Early detection of novel ploidy levels was achieved by screening of stomata size, leaf index value (leaf blade length/width), and other morphological characteristics of the M1-generation. Secondary screening for novel ploidy levels was performed by flow cytometry (FCM). Hexadecaploid, aneuploid, and mixoploid plants were successfully identified by FCM.

Keywords: flow cytometry; polyploidisation; colchicine; hexadecaploids; polyploidisation efficiency; morphological characteristics; *Viola* × *wittrockiana* Gams.

Garden pansy *Viola* × *wittrockiana* Gams. is a popular perennial in cold climates, whereas they are grown as an annual or biennial bedding plants (SMRŽ 1932). The modern garden pansy is thought to have originated from extensive cross breeding between *V. altaica* Pall. (n = ?), *V. cornuta* L. (n = 11), *V. lutea* Huds. (n = 24) and *V. tricolor* L. (n = 13). NOVOTNÁ (1977) and HORN (1956) mentioned that CLAUSEN (1931) determined the chromosome amount of *V.* × *wittrockiana* Gams. (2n = 48) and considered the basic chromosome number x = 6. Therefore the known garden pansy varieties are octoploid. The polyploid plants usually have gigantic characteristics, such as thicker, wider and greener leaves with greater stomata size and larger flowers (UHLÍK 1981) and are attractive ornamentals.

Polyploidy can be induced by treatment with mitotic spindle poisons such as colchicine. However, a treatment of multicellular tissues leads to mixoploidy. Therefore, the ploidy of individuals obtained from treated tissues must be established. This has been traditionally done by chromosome counting which is time-consuming and laborious. Furthermore, the method is not suitable for detection of mixoploidy in tissues with low proportion of dividing cells such as leaves (UHLÍK 1981). Recently, stomata size, leaf index value and other changes in plant morphology were found useful indicators in the primary screening for new ploidy levels in M1-generation of *V.* × *wittrockiana* Gams. (AJALIN, KOBZA 2001).

Flow cytometry (FCM) would appear to offer advantages for many genetic and plant breeding and might be used in association with *in vitro* culture, especially as a very rapid and easy marker for ploidy manipulations such as polyploidisation by colchicine, haploidisation or somatic fusions (OLLITRAULT, MICHAUX-FERRIERE 1992). Also, FCM might to be suitable for the detection of mixoploid plants or for the control of ploidy level in progenies of conventional interspecific hybrids (OLLITRAULT-SAMMARCELLI et al. 1994).

FCM analysis of the nuclear DNA content is based on the analysis of the relative fluorescence intensity of nuclei stained with a DNA fluorochrome. In most plants, analysis of relative DNA content of nuclei isolated from young leaf tissue yields a histogram showing a dominant peak corresponding to nuclei at the G_1 phase of the cell cycle (DOLEŽEL 1991). Ploidy level can be deduced by comparing peak position of G1 nuclei of a plant with known ploidy with that of unknown sample. FCM assay has some important advantages over chromosome counting. It is convenient (sample preparation is easy), rapid (several hundreds of samples can be analyzed in one working day), it does not require dividing cells, it is non-destructive (one sample can be prepared, e.g., from a few milligrams of leaf tissue), and can detect mixoploidy (DOLEŽEL 1997).

MATERIALS AND METHODS

Starting material and colchicine treatment methods were evaluated and described previously in AJALIN and KOBZA (2001). Whereas two treatment methods were used for chromosome number doubling with two cultivars of *Viola*

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× *wittrockiana* Gams. – group of Pirna. The first method was achieved by soaking or dipping swollen seeds in colchicine aqueous solution (0.1%, 0.3%, 0.5%), for three treatment periods (7, 14 and 21 hours). The second one by treatment of the apex of the young seedlings. One drop of colchicine aqueous solution (1%, 1.5%, 2%) was applied to the apex of the seedling every day during the treatment period (3, 4 and 5 days). 5,400 of germinable seeds and 1,800 of young seedlings were treated in the experiment which was replicated three times. Immediately after the treatment, the plants were checked for the presence of different morphological characteristics. Some plants of the second treatment (swollen seeds) were different, compared to other seedlings in the same variant and to control. The cotyledons leaves were thicker, greener and smaller. The hypocotyls were thicker and shorted. The true leaves of these plants also, were thicker, greener and relatively were rounded. Also, most plants of the first treatment (of the apex) were different. The true leaves were thicker, greener and relatively were rounded. These different plants of both methods were selected and sampled for stomata size, leaf index value and hypocotyls length measurement.

Screening of mentioned characteristics (primary selection) revealed that 232 plants (135 plants of the cv. Pure White and 97 of the cv. Light Blue) were presumably polyploids with higher ploidy-levels, and they were selected for FCM analysis. The chromosome number of the starting material was determined (2n = 48) in root-tips of germinated seeds as follows: root-tips were pre-treated for 4 hours at room temperature (RT) in 8-oxyquinoline 0.002 M solution, fixed in acetic acid-ethanol (1:3) and kept in the refrigerator (5°C) for 24 hours. Afterwards, they were hydrolyzed in 5N HCl for 30 min in RT, stained by Schiff reagent for 60 min in RT, macerated in 45% acetic acid for 2 min, and squashed in a drop of 45% acetic acid.

FLOW CYTOMETRIC PLOIDY SCREENING

Samples were prepared according to GALBRAITH et al. (1998). Briefly, a small portion of leaf-stalk tissue (about 20 mg) was chopped with a sharp razor blade in a Petri dish containing 0.5 ml of Otto I buffer (0.1M citric acid, 0.5% Tween 20). The homogenate was filtered through a 50-µm nylon mesh to remove fragments and large tissue debris. Then 1 ml of Otto II buffer (0.4 M Na_2HPO_4) containing 4 µg/ml DAPI (4.6-diamidino-2-phenylindole) was added to stain nuclear DNA.

The relative fluorescence intensity of DAPI-stained nuclei was measured by a Partec PAS flow cytometer. The instrument was equipped with high-pressure mercury arc lamp HBO 100W/L and with a standard optical filter set for the analysis of DAPI fluorescence. Prior to analysis, the signal gain of the instrument was adjusted so that the peak of corresponding G_1-phase nuclei isolated from the leaf-stalk tissue of the control plants (2n = 8x = 48) was located on the channel 100. This setting was kept constant and periodically was checked. To estimate ploidy level, the position of G_1 peak of the sample on a histogram was compared to that of a reference plant with known ploidy. The comparison was made between individual histograms obtained under identical conditions (external standardization) (Figs. 4A and B). For some samples only, the comparison was made within a single histogram, comparing G_1 peak position of two samples (reference + specimen), processed in one tube (internal standardization) (Fig. 4B).

RESULTS AND CONCLUSION

Changes in the morphological characteristics such as leaf index value, hypocotyls length and thickness as well as changes in stomata sizes were important indicators for the detection of new ploidy levels in M1-generation of *Viola* × *wittrockiana* Gams. Stomata sizes of selected plants were ranging from 52–64 µm while the stomata sizes of control plants were ranging from 41–50 µm (Fig. 1). The leaf index value of selected plants was ranging from 0.91–1.20 while the mean index value with the control plants was ranging from 1.41–1.57 (Table 1.). Also, the hypocotyls were shorter (Fig. 2). Primary selection of doubled chromosome number plants was successful 75.5% in the cv. Pure White (66%

Table 1. The effect of colchicine treatment of the apex on the leaf index value in *Viola* × *wittrockiana* Gams. (November 10, 2000)

cv. Light Blue		cv. Pure White	
Variant	Mean value	Variant	Mean value
Control	1.57	Control	1.41
1%–3 days	1.20	1%–3 days	1.04
1%–4 days	1.05	1%–4 days	1.07
1%–5 days	1.02	1%–5 days	0.91
1.5%–3 days	0.97	1.5%–3 days	0.98
1.5%–4 days	0.97	1.5%–4 days	1.10
1.5%–5 days	1.11	1.5%–5 days	1.20
2%–3 days	0.96	2%–3 days	1.08
2%–4 days	1.10	2%–4 days	1.10
2%–5 days	1.20	2%–5 days	1.10

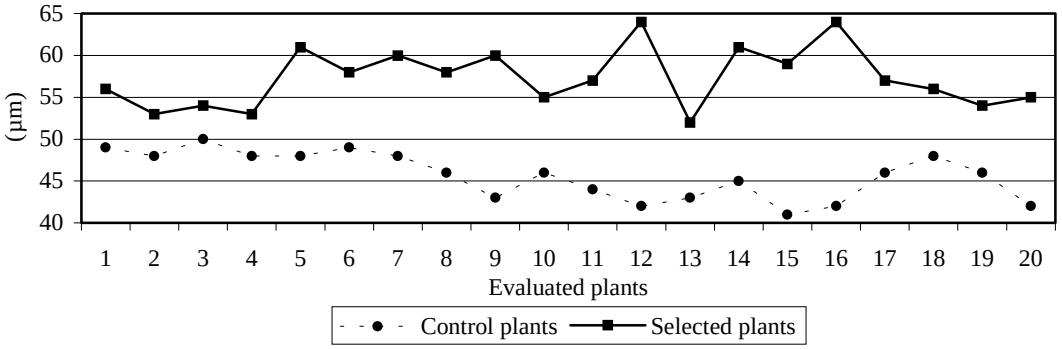


Fig. 1. Stomatal sizes for a sample of selected plants (presumably with higher ploidy level) of *Viola* × *wittrockiana* M1-generation, which were selected on the basis of leaf index value that was close to 1

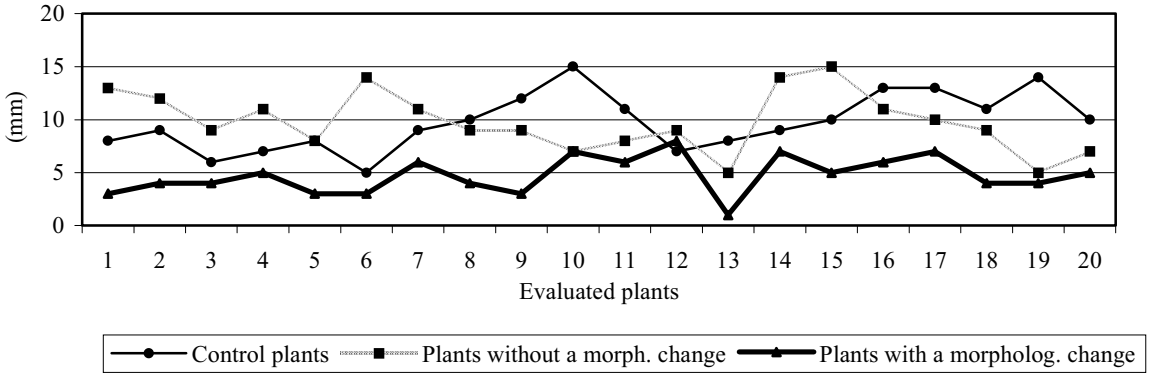


Fig. 2. The effect of colchicine treatment of swollen seeds on the hypocotyl length (mm) in *Viola* × *wittrockiana* Gams. – cv. Pure White

with the seed treatment and 85% with the apex treatment) and 67.5% in the cv. Light Blue (48% with the seed treatment and 87% with the apex treatment) (Tables 2 and 3).

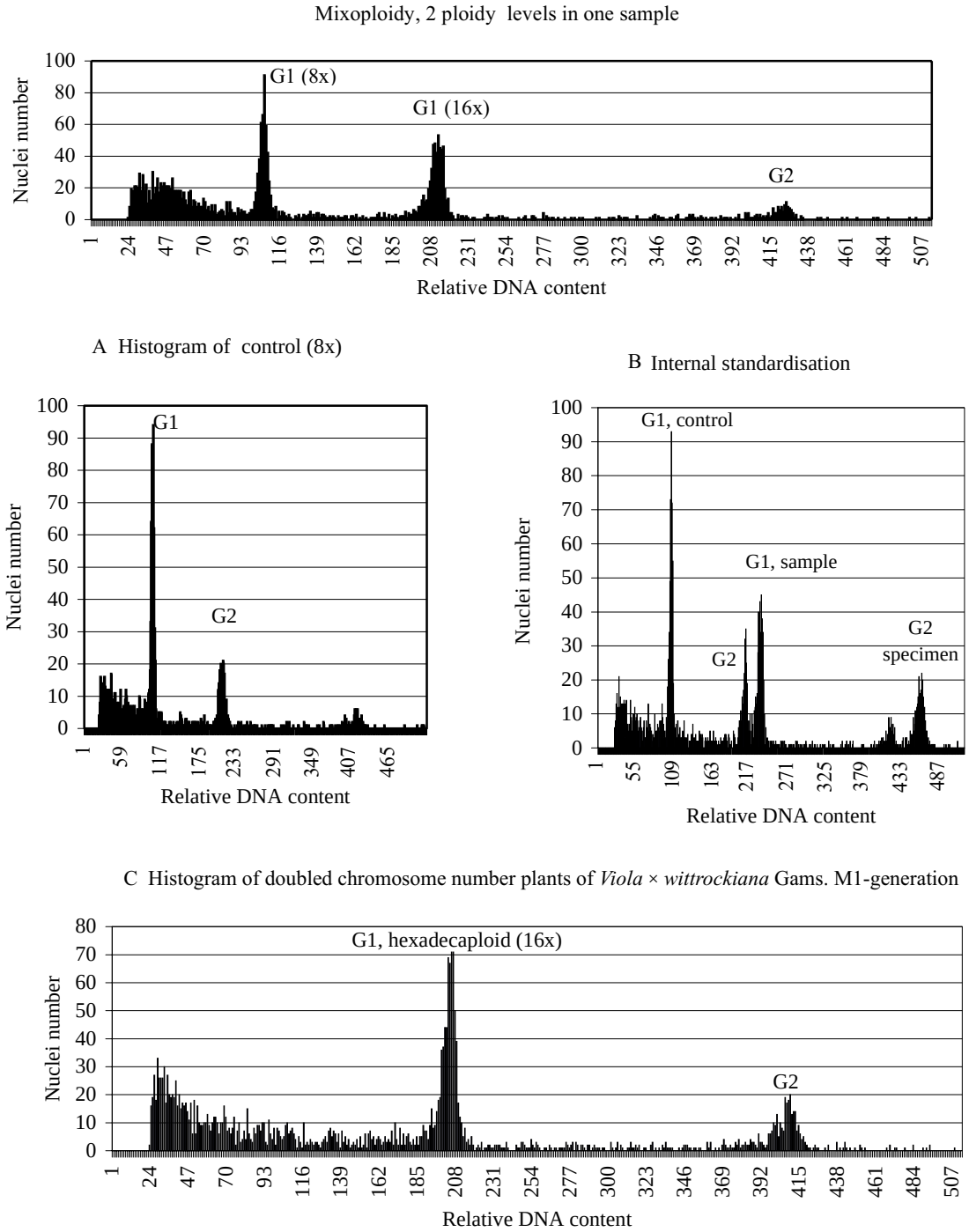
FCM analysis of the selected plants clearly revealed the existence of four groups of ploidy levels:

1. A group of individuals presented relative DNA content very similar to control (polyploidisation did not occur) (Fig. 4A).
2. A group of individuals presented relative DNA content superior by about 85% or 115% to control. Individuals of this group were considered aneuploid (Fig. 4B).
3. A group of individuals that had two or more ploidy levels in the same tissue, that means polyploidisation has not occurred in all cells of the treated tissues. These plants were classified as mixoploids (Fig. 3).

Table 2. Induced polyploidisation in *Viola* × *wittrockiana* Gams. through colchicine treatment of swollen seeds. Primary selection efficiency

Treatment	No. of primary selected plants		Plants obtained by FCM		**Primary selection (E) (%)**	
Variant	W	B	W	B	W	B
0.1%/7 hours	0	0	0	0		
0.1%/14 h.	0	0	0	0		
0.1%/21 h.	0	0	0	0		
0.3%/7 h.	0	0	0	0		
0.3%/14 h.	2	0	2	0	100	
0.3%/21 h.	2	2	1	2	50	100
0.5%/7 h.	0	0	0	0		
0.5%/14 h.	5	2	3	0	60	0
0.5%/21 h.	19	9	10	4	53	44
Total, mean No.	28	13	16	6	**66**	**48**

W: cv. Pure White B: cv. Light Blue



Figs. 3, 4. Ploidy analysis of M1-generation with *Viola* × *wittrockiana* Gams. by flow cytometry (FCM). The signal gain of the instrument was adjusted so that the peak of corresponding G1-phase nuclei isolated from the leaf-stalk tissue of the control plant was located on channel 100. To estimate ploidy level, the position of G1 peak of the sample on a histogram was compared to that of reference plant with known ploidy

4. A group of individuals presented relative DNA content superior by about 100% to control. This group was counted as a doubled chromosome number plants (hexadecaploids) (Fig. 4C).

Flow cytometry was a helpful method for the determination of ploidy levels including mixoploidy and aneuploidy. It was found convenient and rapid (about 100 samples were analyzed per one working day) and therefore it is recommended to be used for identifying of ploidy levels in plant breeding of polyploid *Viola* plants. An important advantage over other methods is the ability to identify mixoploids. For all the individuals studied, only leaf-stalk tissues were used. Leaf blade tissues were not convenient. Leaf blade has a greater content of slime,

Table 3. Induced polyploidisation in *Viola* × *wittrockiana* Gams. through colchicine treatment of the apex. Primary selection efficiency

Treatment	No. of primary selected plants		Plants obtained by FCM		Primary selection (E) (%)	
Variant	W	B	W	B	W	B
1%/3 days	36	26	27	22	75	85
1%/4 days	25	12	15	9	60	75
1%/5 days	9	7	8	7	89	100
1.5%/3 days	12	9	9	8	75	89
1.5%/4 days	10	10	9	6	90	60
1.5%/5 days	7	5	7	5	100	100
2%/3 days	3	5	3	5	100	100
2%/4 days	4	7	3	5	75	71
2%/5 days	1	3	1	3	100	100
Total, mean No.	107	84	82	70	**85**	**87**

W: cv. Pure White B: cv. Light Blue

Table 4. Induced polyploidisation in *Viola* × *wittrockiana* Gams. through colchicine treatment of swollen seeds. Polyploidisation efficiency (E)

Treatment	Survival rate (%)		Plants obtained by FCM		Polyploidisation (E) (%)	
Variant	W	B	W	B	W	B
0.1%/7 hours	95	85	0	0	0.00	0.00
0.1%/14 h.	82	72	0	0	0.00	0.00
0.1%/21 h.	78	80	0	0	0.00	0.00
0.3%/7 h.	90	79	0	0	0.00	0.00
0.3%/14 h.	83	64	2	0	0.18	0.00
0.3%/21 h.	75	45	1	2	0.08	0.10
0.5%/7 h.	97	78	0	0	0.00	0.00
0.5%/14 h.	88	51	3	0	0.29	0.00
0.5%/21 h.	66	39	10	4	0.73	0.17
Total No.			16	6		

W: cv. Pure White B: cv. Light Blue

Table 5. Induced polyploidisation in *Viola* × *wittrockiana* Gams. through colchicine treatment of the apex. Polyploidisation efficiency (E)

Treatment	Survival rate (%)		Plants obtained by FCM		Polyploidisation (E) (%)	
Variant	W	B	W	B	W	B
1%/3 days	24	18	27	22	2.16	1.32
1%/4 days	14	8	15	9	0.70	0.24
1%/5 days	7	3	8	7	0.19	0.07
1.5%/3 days	8	11	9	8	0.24	0.29
1.5%/4 days	8	6	9	6	0.24	0.12
1.5%/5 days	6	3	7	5	0.14	0.05
2%/3 days	9	6	3	5	0.09	0.10
2%/4 days	5	3	3	5	0.05	0.05
2%/5 days	3	2	1	3	0.01	0.02
Total No.			82	70		

W: cv. Pure White B: cv. Light Blue

which makes the filtration of the suspension very difficult.

Polyploidisation efficiency (E) was calculated according to the following equation:

E – polyploidisation = plants obtained by FCM (%) × survival rate (%).

The efficiency has a maximum of 100, i.e. when all treated seedlings or seedlings from treated seeds have a doubled chromosome number and all have survived. Polyploidisation efficiency is based on the number of doubled plants and also on the survival rate (BOUVIER et al. 1994). By the treatment of swollen seeds, better polyploidisation efficiency could possibly be obtained by increasing the treatment period or colchicine concentration, but this would probably lead to higher mortality rate (Table 4). By the treatment of the apex, better polyploidisation efficiency could possibly be obtained by decreasing the treatment period or colchicine concentration. Survival rate has decreased with an increase of treatment period and colchicine concentration. Generally, the best doubling efficiencies of the apex treatment were obtained with the lowest concentration and for the shortest treatment period (Table 5), while the best doubling efficiencies of swollen seeds treatment were obtained with the highest colchicine concentration and for the longest treatment period (Table 4).

References

AJALIN I., KOBZA F., 2001. Chromosome number doubling of *Viola* × *wittrockiana* Gams. through colchicine treatment and its effect on early plant development. Hort. Sci. (Prague), *28*: 150–156.

BOUVIER J. et al., 1994. Oryzalin in chromosome doubling of apple shoots. Pl. Breed., *113,* Berlin, Blackwell Wissenschafts-Verlag: 347–352.

CLAUSEN J., 1931. Cytogenetic and taxonomic investigations in Melanium violets. Hereditas, *15*: 219–308.

DOLEŽEL J., 1997. Application of flow cytometry for the study of plant genomes. J. Appl. Genet., *38* (3): 285–302.

DOLEŽEL J., 1991. Flow cytometric analysis of nuclear DNA content in higher plants. Phytochem. Analysis, *2*: 143–154.

GALBRAITH D.W., LAMBERT G.M., MACAS J., DOLEŽEL J., 1998. Analysis of nuclear DNA content and ploidy in higher plants. In: ROBINSON J.P., DARZYNKIEWICZ Z., DEAN P.N., DRESSLER L.G., ORFAO A., RABINOVITCH P.S., STEWART C.C., TANKE H.J., WHEELESS L.L. (eds.), Current Protocols in Cytometry. New York, John Wiley & Sons, Inc.: 7.6.1–7.6.22.

NOVOTNÁ I., 1977. Výzkum zahradních macešek (*Viola* × *wittrockiana* Gams.). Sbor. mezinár. konf. Přínos okrasného zahradnictví k životnímu prostředí. Praha, Dům techniky: 58–69.

OLLITRAULT-SAMMARCELLI F. et al., 1994. Use of flow cytometry for rapid determination of ploidy level in the genus *Actinidia*. Sci. Hortic., *57*: 303–313.

OLLITRAULT P., MICHAUX-FERRIERE N., 1992. Etude critique de la technique de cytométrie in appliquée à l' amélioration des plantes, résultats obtenus pour quelques agrumes. Fruits, *47*: 195–203.

SMRŽ O., 1932. Macešky a jiné rostliny dvouleté či ozimé. Praha, Českoslov. Zahrad. Listy: 1–54.

UHLÍK J. et al., 1981. Kompendium pro postgraduální studium genetiky a šlechtění. Praha, VŠZ: 105–195.

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Identifikace úrovně ploidity rostlin se zmnoženým počtem chromozomů u *Viola* × *wittrockiana* Gams. M1-generace

ABSTRAKT: Ke zmnožení počtu chromozomů *Viola* × *wittrockiana* Gams. roztokem kolchicinu byly užity dvě metody, a to máčení nabobtnalých semen a aplikace na vegetační vrchol. Detekce polyploidů s novými úrovněmi ploidie byla provedena primární selekcí na základě měření a porovnání změněných morfologických znaků, jako je velikost průduchů, délka a šířka listové čepele (listový index), délka hypokotylu a dalších. Rozhodující selekce byla provedena průtokovou cytometrií, která identifikovala hexadekaploidní, aneuploidní a mixoploidní rostliny. Efektivita polyploidizace i primární selekce byly zhodnoceny.

Klíčová slova: průtoková cytometrie (FCM); polyploidizace; kolchicin; hexadekaploidy; efektivita polyploidizace; morfologické znaky; *Viola* × *wittrockiana* Gams.

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