ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS
SBORNÍK MENDELOVY UNIVERZITY V BRNĚ

Volume LVIII 11 Number 1, 2010

POLYPLOIDY INDUCTION IN *Phlox paniculata* L. UNDER *in vitro* CONDITIONS

P. Matiska, H. Vejsadová

Received: September 30, 2009

Abstract

MATISKA, P., VEJSADOVÁ, H.: *Polyploidy induction in Phlox paniculata L. under in vitro conditions.* Acta univ. agric. et silvic. Mendel. Brun., 2010, LVIII, No. 1, pp. 101–106

The objective of this work was to find an effective method of polyploidy induction using chemomutagens, colchicine and oryzalin, in diploid cultivar of *Phlox paniculata* 'Fujiyama' (syn. Mt. Fuji, Fuji). Ploidy level was determined by the flow cytometry method (FCM). Two methods of treating the explants (*in vitro* regenerated shoots) were tested; chemomutagen infiltration from nutrient media ("the infiltration method") and dipping of the explants in a chemical mutagen solution ("the dip method"). The highest values of tetraploid (5%), mixoploid (1.67%) frequency and polyploidization efficiency (1.25) were found in explants treated with 0.2% colchicine for 24 h in the dip method. Concentrations of 10 µM oryzalin and 0.2% colchicine for 14 d were the most effective for obtaining tetraploids in the infiltration method. The results will be exploited to other *P. paniculata* cultivars for breeding of this species.

Phlox, *in vitro* culture, colchicine, oryzalin, polyploidization, flow cytometry method (FCM)

Perennials, including cultivars of the species *Phlox paniculata* L., are plants of the temperate zone. They are widely used as ornamental plants in orchard designs with their relatively easy cultivation requirements. On a worldwide scale, the assortment of varieties is colourfully interesting, however on the other hand, these varieties lack habitat adaptability including disease and pest resistance. Frost resistance but sensitivity to pathogens such as mildew, septoria leaf spot and nematodes are characteristic of *P. paniculata* cultivars (ŠAFRÁNKOVÁ, 2006).

P. paniculata occurs as diploid, that is 2n = 14; the basic number of chromosomes is n = 7 (FLORY, 1934; LEVIN, 1963; SMITH and LEVIN, 1967). The first methods (GAGANOW, 1961) that were based on enriching the existing assortment with varieties adapted to the environment have developed into modern techniques such as polyploidization with aim to obtain parent breeding material for further hybridization. Various techniques of polyploidy induction are described in the literature for some plant species (PETERSEN et al., 2002; GREPLOVÁ et al., 2003; KUCHTOVÁ, 2008). In *Phlox drummondii,* DHILLON (1970) found polyploidy induction under *ex vitro* conditions using application of colchicine on seedling apical meristems. In *P. paniculata,* experiments with polyploidization under *ex vitro* conditions were not successful (unpublished results). *In vitro* methods provide for isolating polyploid cells and growing up the new plants and *in vitro* polyploidy induction has never been described nor published in *P. paniculata*.

The objective of this work was to find an effective method of polyploidy induction using two polyploidizating agents, colchicine and oryzalin, in diploid cultivar of *Phlox paniculata* 'Fujiyama' (syn. Mt. Fuji, Fuji) under *in vitro* conditions. The regeneration rate and polyploidization efficiency were studied in two methods of mutagen application.

MATERIALS AND METHODS

Plant material

Intact leaves of plants cultivated for 6 months in a greenhouse were used as primary explants. Explants were surface sterilized with 1% sodium hypochlorite (20% commercial bleach Savo) for 20 min and than rinsed three times in sterile distilled water. The explants were cultivated in Petri dishes for 4 weeks in a growth room maintained at a temperature

of 23 / 19 °C (day / night) with 16 h photoperiod and a photon fluence rate of 55 µmol.m^{-2}.s^{-1} provided by cool white fluorescent tubes.

Nutrient medium composition

A basic medium according to JAIN et al. (2001) was used for organogenesis initiation. This medium, modified by MATISKA and VEJSADOVÁ (2006), contained thidiazuron (TDZ) at concentration 6 µM and 11 µM 3-Indolelacetic acid (IAA) with 30 g.l^{-1} sucrose, vitamins B5 and 7.5 g.l^{-1} agar (Cell Culture Tested, Sigma). After 4 weeks, the adventitious shoots were transferred onto MS (MURASHIGE and SKOOG, 1962) growth medium with full salt concentration without the addition of TDZ.

Polyploidy induction

Two chemomutagens, colchicine and oryzalin (SIGMA-ALDRICH) were used in various concentrations for polyploidy induction. Two methods of treating the explants (*in vitro* regenerated shoot nodal segments) were tested; polyploidizating agent infiltration from nutrient media ("the infiltration method") and dipping of the explants in a chemical mutagen solution ("the dip method"). Colchicine (0.05, 0.1, 0.2, 0.4%) was dissolved directly in DMSO (dimethyl sulfoxide); while a stock solution was used for oryzalin (5, 10, 20, 40 µM). Under sterile conditions, the mutagens were quantitively transferred to a hot sterile medium or to sterile water. Polyploidizating agent infiltration from the culture medium took place over periods of 7 and 14 d. The dip method was based on dipping of 5 explants for 12 and 24 h in the chemomutagen solution (50 ml), then the explants were placed three times into sterile distilled water for 15 min. Shoot formation occurred on MS nutrient medium with addition of B5 vitamins and sucrose (30 g.l^{-1}) without growth regulators in Erlenmayer flasks.

The flow cytometric method (FCM) was used for detection of ploidy level. According to FCM method of DOLEŽEL (1997), leaf pieces of regenerated shoots were homogenized (chopped with a blade) in Petri dishes with the Otto I buffer (water solution of 0.1 M citric acid monohydrate and 0.5% Tween 20) to free the nuclei from cells. After filtration through 50 µm filter (to remove solid remains of the tissues), 1 ml of Otto II (0.4M $Na_2HPO_4.12H_2O$) containing fluorescent colouring matter binding to the DNA was added in specified amount to the suspension: 4 µl.ml^{-1} 4´,6-diamino-2-phenylindole (DAPI) – 4 µl.ml^{-1} of Otto II or with propinium iodide 50 µg.ml^{-1} with adding of RNAs 50 µg.ml^{-1} because propinium iodide is also binding the RNA.

Explant survival rate and the number of obtained polyploid (tetraploid and mixoploid) plants were assessed during shoot regeneration. A polyploidization efficiency (E) value was applied which expresses the effectivity of both tested methods. It is a relative value taking into account the percentage of obtained polyploids in the tested treatments, as well the treated explant survival rate (HANZELKA, 2002). The tetraploid plants obtained, were cultivated on MS multiplication medium with 4-week subcultivations. The plants were successfully transferred to *ex vitro* conditions.

Statistical analysis

Data were subjected to analysis of variance (two-way ANOVA) and means were separated using Duncan's test at p = 0.05. Each treatment with 48–60 explants was repeated three times, the values represent the means of three repeat experiments.

RESULTS AND DISCUSSION

After a 4-week cultivation of explants, shoot regeneration was induced. The shoots multiplied and rooted in a sufficient rate (MATISKA and VEJSADOVÁ, 2006). Tab. I as showed, in the dip method using colchicine, the explant survival rate was significantly different in the combinations 0.1% for 12 h (69%) and 0.4% for 24 h (16%). The treatments 0.1% for 24 h and 0.2% for 12 h or 24 h were not significantly different. The highest survival rate (64%) was found in explants treated with 10 µM oryzalin for 12 h. In the treatments with 40 µM oryzalin, all explants became necrotic. Values of tetraploid (5%), mixoploid (1.67%) frequency and polyploidization efficiency (E = 1.25) were the highest in explants treated with 0.2% colchicine for 24 h.

In the infiltration method using colchicine (Tab. II), significant difference in survival rate was only found between treatments 0.05% for 7 d (74%) and 0.2% for 14 d (36%) with 2.08% tetraploidy frequency and 1.30 polyploidization efficiency (E = PP /percentage of polyploids x SR/ survival rate is expressed as number of growing plants of total explant number). In 75% of surviving explants (treatment 10 µM for 14 d), 2.08% tetraploid, 6.25% mixoploid frequency and 1.61 polyploidization efficiency were found.

When polyploidy was measured by FCM, histograms detected diploids (control), tetraploids and mixoploids (Fig. 1). In cases of mixoploidy, chimeric tissues present in apical meristem selectively grew into tetraploid or diploid shoots. After repeated propagation, we received plants consisting only of one non-chimeric tissue type. Differences in leaf morphology of tetraploid and diploid plants after their growing up *ex situ*, are shown in Fig. 2. Dark green leaf colour was apparent in tetraploid plants, suggesting higher chlorophyll content. Leaf blade widening and distinct venation were very marked.

In our experiments, 0.2% colchicine stimulated polyploidy induction in the dip and infiltration methods. The tetraploidy was also detected at the highest colchicine concentration (0.4%) after 14 and 24-day application. Chimera plants (mixoploids) often appeared with oryzalin infiltration as opposed to tetraploids. In repeated experiments we did not find plants with multiple ploidy (hexaploid, octoploid) as is often the case with other species (KUCHTOVÁ, 2008). These results suggested

I: *Survival rate of explants and polyploidization efficiency (E) using the dip method in diploid cultivar (2n) of Phlox paniculata 'Fujiyama'*

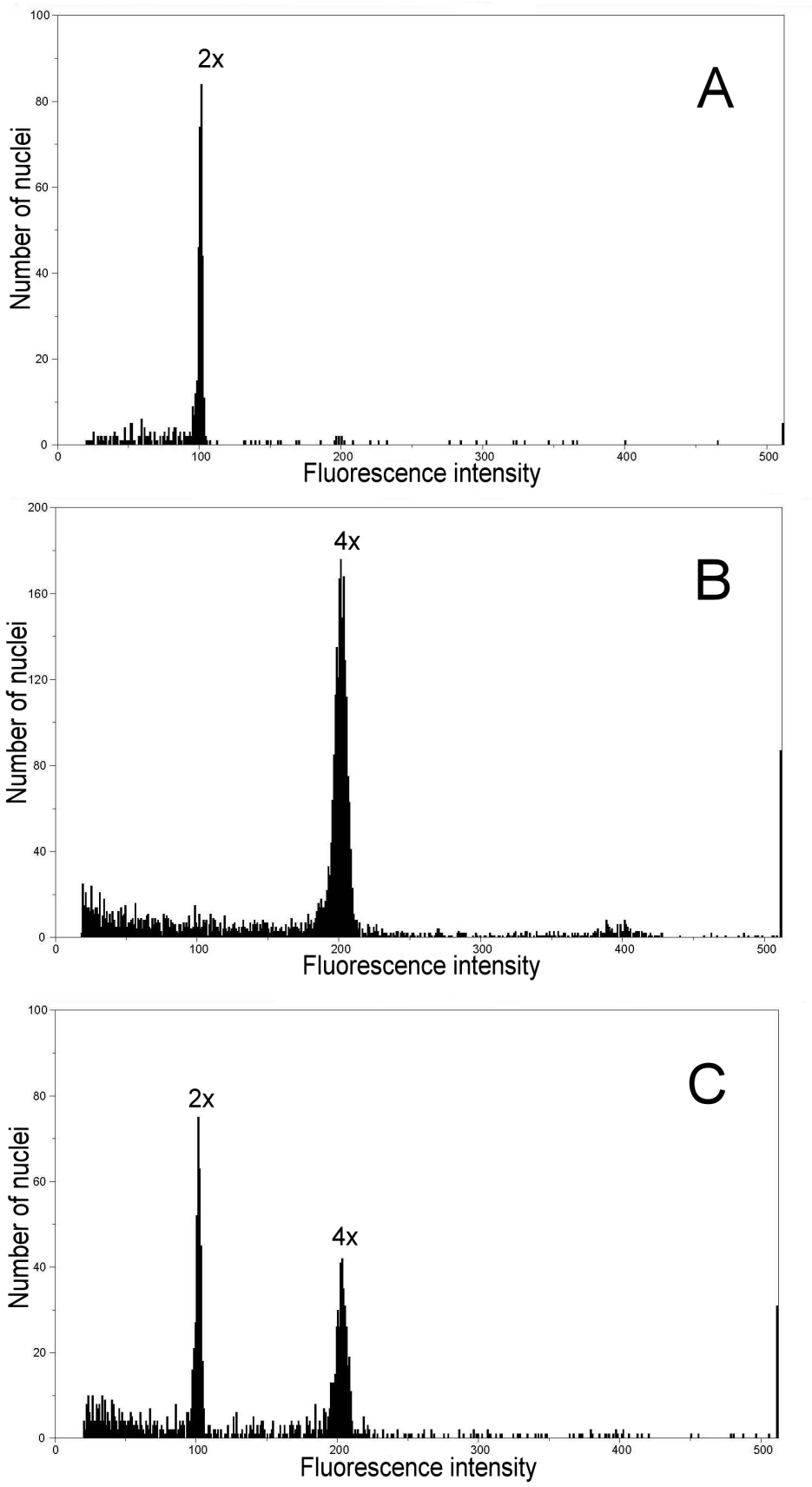
Treatments	Mean survival rate ± S.E. (%)	Tetraploid (4n) frequency (%)	Mixoploid (2n + 4n) frequency (%)	Polyploidization efficiency (E)*
Colchicine % / h				
–	96.67	0.00	0.00	0.00
0.1 / 12	69.40 ± 1.68d	0.00	0.00	0.00
0.1 / 24	39.48 ± 1.49a	0.00	1.67	0.00
0.2 / 12	43.42 ± 2.63a	0.00	0.00	0.00
0.2 / 24	35.00 ± 10.00ac	5.00	1.67	1.25
0.4 / 12	22.01 ± 4.23bc	1.67	0.00	0.33
0.4 / 24	15.92 ± 4.54b	1.67	1.67	0.19
Oryzalin µM / h				
–	100.00	0.00	0.00	0.00
10 / 12	63.61 ± 1.89c	0.00	0.00	0.00
10 / 24	25.35 ± 3.98b	0.00	0.00	0.00
20 / 12	23.64 ± 2.79b	0.00	0.00	0.00
20 / 24	3.34 ± 0.95a	1.67	0.00	0.06
40 / 12	0.00 ± 0a	–	–	–
40 / 24	0.00 ± 0a	–	–	–

*E (polyploidization efficiency) = PP (percentage of polyploids) x SR (survival rate is expressed as number of growing plants of total explant number).
Means in columns with the same letter are not significantly different at the p = 0.05 level by Duncan's test. Each value represents the means of three repeat experiments with 60 replicates each. S.E. – Standard Error.

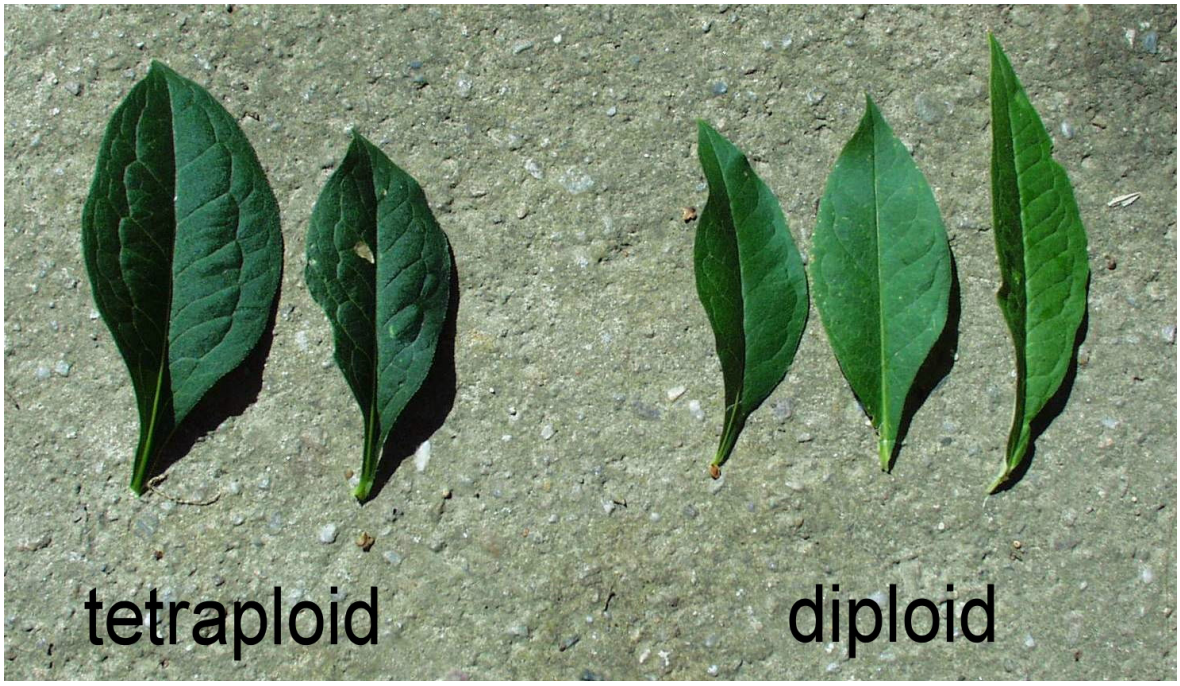
II: *Survival rate of explants and polyploidization efficiency (E) using the infiltration method in diploid cultivar (2n) of Phlox paniculata 'Fujiyama'*

Treatments	Mean survival rate ± S.E. (%)	Tetraploid (4n) frequency (%)	Mixoploid (2n + 4n) frequency (%)	Polyploidization efficiency (E)
Colchicine % / d				
–	100.00	0.00	0.00	0.00
0.05 / 7	74.17 ± 14.60b	0.00	0.00	0.00
0.05 / 14	51.83 ± 15.47ab	0.00	0.00	0.00
0.1 / 7	66.81 ± 6.60ab	0.00	0.00	0.00
0.1 / 14	39.58 ± 8.36ab	0.00	0.00	0.00
0.2 / 7	53.72 ± 1.18ab	0.00	0.00	0.00
0.2 / 14	35.73 ± 13.78a	2.08	2.08	1.30
Oryzalin µM / d				
–	100.00	0.00	0.00	0.00
5 / 7	94.25 ± 1.83c	0.00	0.00	0.00
5 / 14	82.76 ± 2.43abc	0.00	0.00	0.00
10 / 7	90.56 ± 0.56bc	0.00	0.00	0.00
10 / 14	74.69 ± 3.94ab	2.08	6.25	1.61
20 / 7	70.57 ± 10.82a	0.00	0.00	0.00
20 / 14	47.22 ± 7.35d	0.00	4.17	0.00

Means in columns with the same letter are not significantly different at the p = 0.05 level by Duncan's test. Each value represents the means of three repeat experiments with 48 replicates each. S.E. – Standard Error.



1: *Representative flow cytometric histograms of nuclei isolated from P. paniculata 'Fujiyama' microshoots treated by dipping 0.4% colchicine for 24 h (A. Diploid 2n. B. Tetraploid 4n. C. Mixoploid 2n+4n)*



2: *Morphology differences of leaves in tetraploid and diploid P. paniculata 'Fujiyama' plants*

an indirect correlation between increased chemomutagen concentration, the treatment duration and reduced explant survival which resulted in lower polyploidization efficiency.

The marked change in leaf shape of tetraploid plants is an interesting phenomenon. E.g., the tetraploid individuals of the green-leaved 'Gizela F_1' are characterized with more robust growth and richer flowers and in the 'Black Velvet Scarlet F_1' tetraploid plants, the genes for the unique dark brown leaf blades have been preserved (VEJSADOVÁ and KUCHTOVÁ, 2008). However, there are no mentions of changes in the shape of the leaf blade and distinct venation as we observed in tetraploid plants of the studied cultivar. The obtained results will be exploited to other *P. paniculata* cultivars for breeding of this species.

SUMMARY

The objective of this work was to find an effective method of polyploidy induction using chemomutagens, colchicine and oryzalin, in diploid cultivar of *Phlox paniculata* 'Fujiyama' (syn. Mt. Fuji, Fuji). The regeneration rate and polyploidization efficiency (E) were studied in two methods of mutagen application.

Perennials, including cultivars of the species *Phlox paniculata* L., are plants of the temperate zone. Frost resistance but sensitivity to pathogens such as mildew, septoria leaf spot and nematodes are characteristic of *P. paniculata* cultivars. The first methods that were based on enriching the existing assortment with varieties adapted to the environment have developed into modern techniques (such as polyploidization) with aim to obtain parent breeding material for further hybridization. *P. paniculata*, in its basic diploid formation, has 14 chromosomes, n = 7. *In vitro* polyploidy induction has never been described nor published in *P. paniculata*.

Intact leaves of plants cultivated for 6 months in a greenhouse were used as primary explants. A modified MS medium was used for organogenesis initiation. This medium contained 6 µM thidiazuron (TDZ) and 3-Indolelacetic acid (IAA) at concentration 11 µM with 30 $g.l^{-1}$ sucrose, vitamins B5 and 7.5 $g.l^{-1}$ agar Sigma. The chemomutagens, colchicine and oryzalin were used in various concentrations for polyploidy induction. Two methods of treating the explants (*in vitro* regenerated shoot nodal segments) were tested; chemomutagen infiltration from nutrient media ("the infiltration method") and dipping of the explants in a chemical mutagen solution ("the dip method"). The highest values of tetraploid (5%), mixoploid (1.67%) frequency and polyploidization efficiency (1.25) were found in explants treated with 0.2% colchicine for 24 h in the dip method. Concentrations of 10 µM oryzalin and 0.2% colchicine for 14 d were the most effective for obtaining tetraploids in the infiltration method. The results will be exploited to other *P. paniculata* cultivars for breeding of this species.

SOUHRN

Indukce polyploidie u *Phlox paniculata* L. v *in vitro* podmínkách

Cílem této práce bylo zjistit účinnou metodu indukce polyploidie pomocí polyploidizačních činidel, kolchicinu a oryzalinu, u diploidního kultivaru *Phlox paniculata* 'Fujiyama' (syn. Mt. Fuji, Fuji). Míra regenerace a efektivita polyploidizace (E) byla studována u dvou metod aplikace chemomutagenů.
Phlox paniculata L. patří mezi trvalky, které jsou využívány v oblasti mírného pásma jako okrasné rostliny. Pro odrůdy *P. paniculata* je charakteristická jejich mrazuvzdornost, ale citlivost vůči patogenům jako je padlí, septoriová skvrnitost listů nebo háďátko. První metody založené na obohacení stávajícího sortimentu o odrůdy, které jsou přizpůsobivé vůči prostředí, se vyvinuly v moderní techniky (jakou je i polyploidizace) s cílem získat výchozí šlechtitelský materiál pro další křížení. *Phlox paniculata* má v základní diploidní sestavě 14 chromozomů (n = 7). *In vitro* indukce polyploidie nebyla u tohoto druhu zatím nikde popsána a publikována.
Jako primární explantáty byly použity intaktní listy z rostlin pěstovaných šest měsíců ve skleníku. Pro iniciaci organogeneze bylo použito modifikované MS médium. Toto médium obsahovalo 6 µM thidiazuronu (TDZ) a kyselinu indolyloctovou (IAA) v koncentraci 11 µM, 30 g.l^{-1} sacharózy, vitaminy B5 a 7,5 g.l^{-1} agaru Sigma. Pro indukci polyploidie byly použity chemomutageny, kolchicin a oryzalin v různých koncentracích. Byly testovány dvě metody ošetření explantátů (*in vitro* regenerované nodální segmenty výhonů); infiltrace chemomutagenu ze živného media ("infiltrační metoda") a máčení explantátů v chemickém roztoku mutagenu ("máčecí metoda"). Nejvyšší hodnoty frekvence tetraploidů (5 %), mixoploidů (1,67 %) a efektivity polyploidizace (1,25) byly zjištěny u explantátů ošetřených 0,2 % kolchicinem po dobu 24 hodin u "máčecí metody". Koncentrace 10 µM oryzalinu a 0,2% kolchicinu po dobu 14 dní byly nejúčinnější pro obdržení tetraploidů u "infiltrační metody". Výsledky budou využity pro šlechtění dalších kultivarů druhu *P. paniculata* L.

The research was supported by the Ministry of the Environment of the Czech Republic (Project No. MZP0002707301).

REFERENCES

DHILLON, T. S., 1970: *Phlox drummondii*. I. cytogenetics of colchicine induced autotetraploids. *The Japanese Journal of Genetics,* 45: 305–312.

DOLEŽEL, J., 1997: Application of flow cytometry for the study of plant genomes. *Journal of Applied Genetics*, 38: 285–302.

FLORY, W. S., 1934: A cytological study of the genus *Phlox*. *Cytologia*, 6: 1–18.

GAGANOW, P. G., 1961: Staudenphlox. *VEB Deutscher Landwirtschaftsverlag*, Berlin, 191 pp.

GREPLOVÁ, M., FRČEK, J., REJLKOVÁ, M., KOPECKÝ, D., VAGERA, J. and DOLEŽEL, J., 2003: *In vitro* polyploidization of wild potato species. *Vědecké práce VÚB*, 14: 55–63.

HANZELKA, P., 2002: *Formation of gene resources using induced polyploidization in Callistephus chinensis Nees*. PhD Thesis, Faculty of Horticulture, Mendel University of Agriculture and Forestry in Brno. Lednice.

JAIN, A., ROUT, G. R. and RAINA, S. N., 2002: Somatic embryogenesis and plant regeneration from callus cultures of *Phlox paniculata* Linn. *Scientia Horticulturae*, 94: 137–143.

KUCHTOVÁ, P., 2008: *Induction of polyploid plants in Pelargonium × hortorum L. H. Bailey.* PhD Thesis, Faculty of Horticulture, Mendel University of Agriculture and Forestry in Brno, Lednice.

LEVIN, D. A., 1963: Natural hybridization between *Phlox mandata* and *Phlox glaberrima* and its evolutionary significance. *American Journal of Botany*, 50: 714–719.

MATISKA, P. and VEJSADOVÁ, H., 2006: Induction of morphogenesis in *Phlox paniculata* L. In: Proceedings of the XXII. International Symposium EUCARPIA, Sanremo, 2006. (Mercuri, A. and Schiva, T., Eds.), p. 34.

MURASHIGE, T. and SKOOG, F., 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 473–497.

PETERSEN, K. K., HAGBERG, P. and KRISTIANSEN, K., 2002: *In vitro* chromosome doubling of Miscanthus chinensis. Blackwell Verlag, Berlin. *Plant Breeding*, 121: 445–450.

SMITH, D. M. and LEVIN, D. A., 1967: Karyotypes of eastern north american *Phlox*. *American Journal of Botany*, 54: 324–334.

ŠAFRÁNKOVÁ, I., 2006: Pathogens of phlox (*Phlox paniculata*). *Zahradnictví*, 3: 36–37.

VEJSADOVÁ, H. and KUCHTOVÁ-JADRNÁ, P., 2008: Organogenesis induction in diploid cultivars of *Pelargonium × hortorum* L. H. Bailey. *Acta Pruhoniciana*, 89: 41–45.

Address

Ing. Pavel Matiska, Česká zemědělská univerzita v Praze, Fakulta agrobiologie, potravinových a přírodních zdrojů, Kamýcká 129, 165 21 Praha 6, Česká republika, e-mal: pamat@seznam.cz, RNDr. Hana Vejsadová, CSc., Výzkumný ústav Silva Taroucy pro krajinu a okrasné zahradnictví, v.v.i., Květnové nám. 391, 252 43 Průhonice, Česká republika, e-mail: vejsadova@vukoz.cz