In vitro colchicine induction of tetraploids in *Pelargonium rapaceum*

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Abstract

The aim of this study was to produce polyploid plants in section *Hoarea* of *Pelargonium* as materials for breeding new commercial cultivars with desirable characteristics. We selected *Pelargonium rapaceum* (section *Hoarea*) as the starting material. Leaf explants of in vitro plantlets and regenerated diploid calli of *P. rapaceum* were treated with colchicine at different concentrations for various times, and then cultured in vitro to obtain regenerated plantlets. The DNA ploidy level of individual plants was determined by flow cytometry, and the plants were classified as diploid, mixoploid, or tetraploid. Three putative tetraploid plants were induced when in vitro leaf explants were immersed for 48 h in liquid Murashige and Skoog medium supplemented with 1000 mg L^{-1} colchicine, and when diploid calli were cultured on Murashige and Skoog medium supplemented with 1 mg L^{-1} colchicine for 30 days. The putative tetraploid plants were propagated using the same protocol, and their characteristics were evaluated. The guard cells and fertile pollen grains of regenerated plants were significantly larger than those of the original diploid plants. However, the flowers of the regenerated plants were the same size and color as those of the original diploid plants, and the pollen fertility of the regenerated plants was considerably lower than that of original diploid plants. Furthermore, when the regenerated plants were self-pollinated, there was an incomplete fruit and seed set. Consequently, no large flowers were produced by three tetraploid strains. Nevertheless, we propose that the colchicine treatment should be used to induce *P. rapaceum* tetraploid plants, and anticipate that when many more explants are treated with colchicine at optimal concentrations and times, this will induce tetraploid plants with larger flowers that could be useful for breeding new cultivars in *Pelargonium* section *Hoarea*.

Keywords: *Geraniaceae*, section *Hoarea*, yellow flower, flow cytometry, α-naphthalene acetic acid, 6-benzylaminopurine

INTRODUCTION

The genus *Pelargonium* (*Geraniaceae*) contains approximately 300 species in 13 sections (van der Walt and Voster, 1988). The large section *Hoarea* contains more than 70 species, including some that have great potential as breeding materials. These species are stemless plants with dormant tubers, some have bright yellow flowers, and some produce pea-shaped flowers, for example, *Pelargonium rapaceum* (L.) 'L'Hérit'. Species in the section *Hoarea*, however, are not among the ancestors of existing cultivars, possibly because species in this section are difficult to cultivate and have long periods of dormancy. *P. rapaceum* flowers resemble legume flowers, are pink or yellow, and often have red stripes on the lower part of the upper petals. One of the main aims of *Pelargonium* breeding is to develop cultivars with new flower colors and shapes, as well as early and continuously flowering cultivars. Previously, we successfully obtained hybrids between *P. rapaceum* and *Pelargonium crispum* (section *Pelargonium*) via ovule culture (Kakihara et al., 2012). The hybrids showed variations in several characteristics such as flower color, flower shape, and fragrance of the leaf. Besides hybridization, polyploidization represents another useful method for the commercial breeding of *Pelargonium* section *Hoarea*. Therefore, to develop valuable breeding materials for *Pelargonium* section *Hoarea*, we used colchicine to induce

polyploid plants, using *P. rapaceum* as the starting material.

MATERIALS AND METHODS

Plant materials and culture conditions

Plants of *P. rapaceum* were obtained from the University of Stellenbosch, South Africa. We used the protocol described by Sukhumpinij et al. (2010) to regenerate in vitro plantlets from explants of mature leaves of *P. rapaceum*. To induce callus formation, explants (5×5 mm) from mature leaves were placed adaxial side down on the surface of callus induction medium (Murashige and Skoog (MS) medium supplemented with 0.1 mg L^{-1} α-naphthalene acetic acid (NAA), 0.1 mg L^{-1} 6-benzylaminopurine, and 30 g L^{-1} sucrose, solidified with 6.5 g L^{-1} agar). These explants were then cultured in continuous darkness. The regenerated calli were subcultured on fresh MS medium with the same composition under a 16-h light/8-h dark photoperiod until shoots and plantlets regenerated. These calli and the leaves of in vitro plantlets were treated with colchicine. For all media, the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Colchicine treatment

Explants (5×5 mm) from leaves of in vitro plantlets were immersed in liquid MS medium containing different concentrations of colchicine (0, 10, 100, or 1,000 mg L^{-1}) for 24, 48, or 72 h, in the dark with shaking at 100 rpm. Calli were inoculated onto solid MS medium supplemented with different concentrations of colchicine (0, 1, 5, 10, or 20 mg L^{-1}) and then cultured for 30 days in the dark. After these treatments, both leaf explants and calli were transferred to fresh medium with the same composition, except that it lacked colchicine, and subcultured to allow shoot elongation and plantlet regeneration. For root induction, healthy plantlets were transferred to MS medium supplemented with 0.1 mg L^{-1} NAA. The colchicine treatment for leaf explants consisted of nine replicates, with five explants in each culture bottle. Experiments were repeated twice. Statistical analysis was performed using Tukey-Kramer tests.

Characteristics of plants

The ploidy level (DNA count) of original diploid plants and regenerated plants of *P. rapaceum* was estimated as described by Jadrná et al. (2009) using an Accuri C6 flow cytometer (BD Biosciences, Tokyo, Japan). To reduce the amount of phenolic substances in the tissues, the leaves were stored in the refrigerator (5°C) for 7 days before the analysis. The propidium iodide (PI) dye was excited with a 488 nm (blue) laser, and PI emission was collected with a 585±20 nm and pass filter (FL2). The results were acquired using FlowJo vX 10.0.7r2 software (Tree Star Inc., Ashland, OR) for ploidy analyses. The ploidy level was determined by comparing with the major flow cytometry peak positions, using parsley as an internal standard. Morphological characteristics such as the length of guard cells, the size of fertile pollen grains, and flower size were also used to identify putative tetraploid plants. Regarding flower color, we used the Royal Horticulture Society color chart, which is based on measurement of human visual responses to color. The chromaticity of fresh petals was measured with a colorimeter (Konica Minolta CM-2600d, Tokyo, Japan). Chromaticity is expressed by the CIELAB (CIE 1976) method: L*, a*, and b* values indicate lightness, redness, and yellowness, respectively (Robertson, 1977). Pollen and seed fertilities were also measured in original diploid plants and regenerated plants. Pollen grains were stained with 1% aceto-carmine. The frequencies of stained (viable) and unstained (non-viable) pollen were determined after evaluating at least 1000 mature pollen grains. Original diploid plants and regenerated plants were self-pollinated, and the rates of fruit and seed set were determined.

RESULTS AND DISCUSSION

Regeneration of plants

The in vitro leaf explants treated with different concentrations of colchicine for various times gradually changed from green to brown on solid medium, and formed white calli after approximately 1 month in culture. These white calli immediately became green after transfer to light conditions. The calli were then subcultured for approximately 2 months to allow regeneration of shoots and plantlets. As shown in Table 1, the rate of callus regeneration ranged from 40-60%, whether or not the explants had been treated with colchicine. In contrast, both shoot and plantlet regeneration was significantly reduced by colchicine treatment. There was no clear relationship between colchicine treatment time/concentration and the number of shoots and plants regenerated. Many plants regenerated from in vitro leaf explants that were not treated with colchicine, and most of them were estimated to be diploid in the flow cytometry analysis. In the case of leaf explants treated with colchicine, there were a total of 13 surviving plants, of which nine were diploid, two were mixoploid (chimera), and two were tetraploid, as determined by flow cytometry analysis. Many plants were obtained when calli were treated with lower concentrations of colchicine (1 and 5 mg L^{-1}), but far fewer were obtained when calli were treated with higher concentrations of colchicine (10 and 20 mg L^{-1}; Table 2). According to the flow cytometry analysis, most regenerated plants were diploid. One mixoploid and two tetraploid plants were induced only when the calli were cultured on medium containing 1 mg L^{-1} colchicine.

Table 1. Effects of colchicine treatment on regeneration of calli, shoots, and plants from in vitro leaf explants of *Pelargonium rapaceum*.

Colchicine treatment		Callus	Shoot	No. of plants regenerated			
Concentration (mg L^{-1})	Time (h)	regeneration rate (%)	regeneration rate (%)	Total	Ploidy level[1]		
					Diploid	Mixoploid	Tetraploid
0	24	51.1	66.7	12	12	0	0
	48	46.7	40.0	12	11	1	0
	72	53.3	37.8	9	7	1	1
10	24	40.0	28.9	4	4	0	0
	48	44.4	20.0	0	0	0	0
	72	37.8	42.2	2	2	0	0
100	24	62.2	20.0	0	0	0	0
	48	48.9	15.6	1	1	0	0
	72	44.4	26.7	0	0	0	0
1000	24	57.8	24.4	0	0	0	0
	48	60.0	35.6	3	0	1	2
	72	40.0	62.2	3	2	1	0

[1]Estimation based on flow cytometry analysis.

Table 2. Estimation of ploidy level of plants regenerated from colchicine-treated diploid calli of *Pelargonium rapaceum*.

Colchicine concentration (mg L^{-1})	No. of calli treated	No. of plants regenerated			
		Total	Ploidy level[1]		
			Diploid	Mixoploid	Tetraploid
0	-	6	6	0	0
1	30	18	15	1	2
5	30	14	14	0	0
10	30	2	2	0	0
20	30	4	4	0	0

[1]Estimation based on flow cytometry analysis.

Characteristics of regenerated plants

The length of guard cells was measured for the mixoploid and tetraploid plants (10 plants in total; Tables 3 and 4). The guard cells in three individual plants (A4, 7, and 11)

were significantly longer (1.2-1.3 times) than those of the original diploid plants. Therefore, we considered that these plants were tetraploid. These plants were propagated using the same protocol, and their characteristics were evaluated to assess their ornamental values (Table 5). The diameter of fertile pollen grains was significantly larger (1.4-1.6 times) in the three putative polyploid plants than in the original diploid plants. Their flower color, according to the RHS color chart, was 2D, the same as that of original diploid plants. Their flowers were also the same size as those of the original diploids. The pollen fertility ranged from 66 to 74%, which was considerably lower than that of the original diploid plants (94%). Furthermore, there was incomplete fruit and seed set when these three plants were self-pollinated. Thus, tetraploid regenerated plants appeared to possess no ornamentally desirable characteristics. However, it should be noted that their tubers were small and they were not yet fully grown. Indeed, a maximum of nine flowers was observed per peduncle in regenerated plants compared with over 15 in original plants, resulting in a smaller number of flowers pollinated (37-52 versus 127, respectively). All regenerated plants were observed to be growing normally growing, and we propose to reevaluate their characteristics when they have completed their growth in a few years' time. Furthermore, the absence of tetraploid plants with desirable characteristics may reflect the low rate of plant regeneration. Thus, it is likely that when many more explants are treated with colchicine at optimal concentrations and times, tetraploid plants with larger flowers will be induced.

Table 3. Length of guard cells in original diploid plants and plants regenerated from colchicine-treated in vitro leaf explants of *Pelargonium rapaceum*.

	Colchicine concentration (mg L^{-1})	Time (h)	Strain	Length of guard cells (µm) (Mean±SE)
Original plants				26.9±0.20 cd[1]
Regenerated Plants	0	48	A2	28.2±0.48 bc
	0	72	A1	26.4±0.29 cd
	0	72	B3	28.0±0.55 bc
	1000	48	A1	26.4±0.39 cd
	1000	48	A4	35.9±0.26 a
	1000	48	A5	29.3±0.72 b
	1000	72	B13	25.9±0.49 d

[1]Letters indicate significant difference (Tukey-Kramer test, $p<0.05$).

Table 4. Length of guard cells in original plants and plants regenerated from colchicine-treated calli of *Pelargonium rapaceum*.

	Colchicine concentration (mg L^{-1})	Strains	Length of guard cells (µm) (Mean±SE)
Original plants			26.9±0.20 b[1]
Regenerated plants	1	7	32.9±0.47 a
	1	11	34.5±0.47 a
	1	15	25.8±0.26 b

[1]Letters indicate significant difference (Tukey-Kramer test, $p<0.05$).

Table 5. Comparison of the characteristics between original plants and plants regenerated from colchicine-treated explants of *Pelargonium rapaceum*.

	Strains	Size of pollen (µm) (Mean±SE)	Pollen Fertility (%)	Seed[1] fertility	RHS[2] code	Chromaticity[3] L*	a*	b*
Original plants		66±14.6 c[4]	94.3	0.45	2D	70.4	-3.03	12.9
Regenerated plants	A4	99±11.3 b	71.8	0	2D	88.6	-2.12	23.3
	7	106±13.2 b	73.8	-	2D	88.0	-1.45	10.6
	11	95±11.8 a	66.4	0	2D	87.6	-1.87	15.0

[1]No. of viable seeds/No. of flowers pollinated.

[2]According to Royal Horticulture Society color chart.

[3]Determined using CIELAB (CIE 1976) method.

[4]Letters indicate significant difference (Tukey-Kramer method, $p<0.05$).

CONCLUSIONS

Three strains were determined to be tetraploids based on flow cytometric analysis, the guard cell length, and the pollen diameter, although they did not produce large flowers. However, based on our results, we propose an initial protocol for colchicine treatment to produce polyploids of *P. rapaceum*.

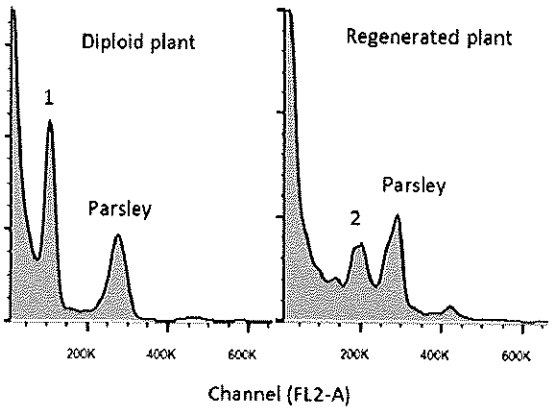
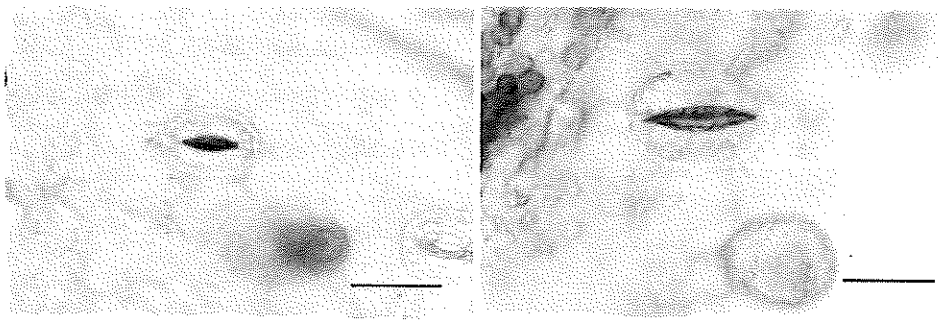
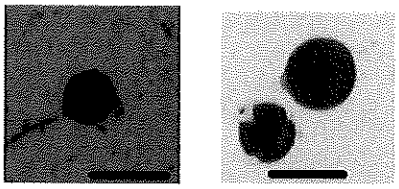


Figure 1. Histograms generated from flow cytometry analysis of diploid plants (Peak 1), regenerated plants (Peak 2) of *Pelargonium rapaceum*, and parsley.



Diplod plant Regenerated plant

Figure 2. Guard cells of original diploid plants and regenerated plants of *Pelargonium rapaceum* (scale bar, 20 µm)



Diploid plant Regenerated plant

Figure 3. Pollen grains of original diploid plants and regenerated plants of *Pelargonium rapaceum* (scale bar, 10 μm)

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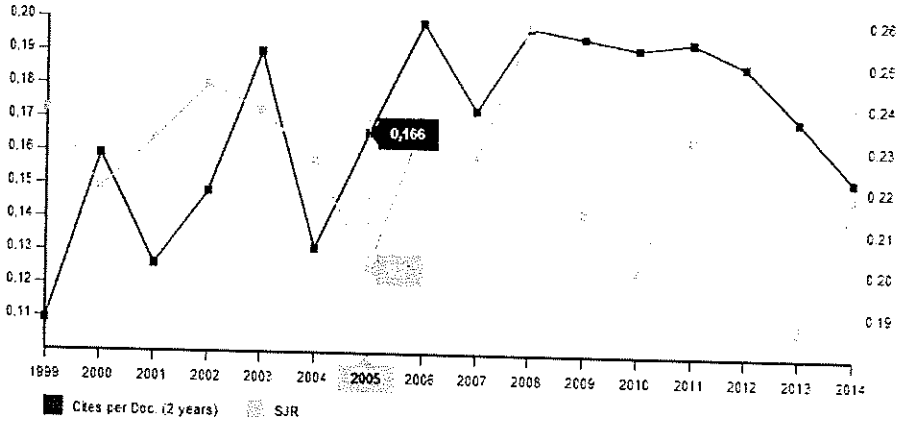

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