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EVALUATION OF THE GENETIC STABILITY OF PLANTS OBTAINED VIA SOMATIC EMBRYOGENESIS IN *Chrysanthemum* × *grandiflorum* (Ramat./Kitam.)

Justyna Lema-Rumińska[1], Elwira Śliwińska[2]
University of Technology and Life Sciences in Bydgoszcz

Abstract. Genetic and phenotypic stability of plants obtained via somatic embryogenesis may be disrupted. The reason can be an indirect regeneration of somatic embryos via callus or a high concentration of growth regulators added at the induction stage of somatic embryos. Somatic embryogenesis of 'Lady Salmon' (chimeric) and 'Lady Vitroflora' (non-chimeric) cultivars was induced on modified Murashige and Skoog (MS) media, supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) or 6-benzylaminopurine (BAP). Flow cytometry (FCM) revealed that the plants derived from somatic embryos of both cultivars maintained the ploidy of control plants obtained from the meristem. The colour and pigment content of the inflorescence of plants derived from somatic embryos of 'Lady Vitroflora' were similar to the original control plants. However, the ray florets of clones of 'Lady Salmon' did not contain carotenoids, characteristic for this cultivar, and consequently produced flowers of different colours. Thus, somatic embryogenesis in chrysanthemums can be applied for separating periclinal chimera components for chimeric cultivars and for receiving an additional source of variation in the breeding of cultivars. In the case of genetically homogeneous cultivars it can be used in production laboratories for cloning plants *in vitro* via somatic embryogenesis.

Key words: chimera, chrysanthemum, flow cytometry, pigments, somatic embryo

INTRODUCTION

Chrysanthemum is one of the most economically important cut-flower and pot species. This genus contains many hybrids and thousands of cultivars developed for horticultural purposes [Teixeira da Silva 2003]. Some of them are periclinal chimeras with changed external, L1, layer of cells [Mandal et al. 2000]. Such cultivars morphologically do not differ from homogeneous ones; however, when propagated *in vitro* by ad-

Corresponding author: Justyna Lema-Rumińska, Laboratory of Biotechnology, Department of Ornamental Plants and Vegetable Crops, UTP University of Technology and Life Sciences, Bernardyńska 6, 85-029 Bydgoszcz, Poland, , e-mail: lem-rum@utp.edu.pl

ventitious shoots they can provide segregated progenies [Zalewska et al. 2007]. However, in production laboratories the genetic stability and cultivar distinctiveness must stay unchanged. With that in mind, the selection of an adequate micropropagation method, adjusted to a specific chrysanthemum cultivar, is essential.

Somatic embryogenesis is one of the most efficient methods of micropropagation. Somatic embryogenesis in chrysanthemums is usually induced on MS [Murashige and Skoog 1962] medium supplemented with auxins, mostly 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthaleneacetic acid (NAA) as well as cytokinins, 6-benzylaminopurine (BAP) or kinetin (KIN) [May and Trigiano 1991, Oka et al. 1999, Shinoyama et al. 2004, Naing et al. 2013a, b, Lema-Rumińska and Niedojadło 2014]. Addition of growth regulators, however, can disturb the genetic stability of plants produced from embryos, especially indirect regenerated embryos and can lead to changes in ploidy [Karp 1995, Prado et al. 2010]; therefore the nuclear DNA content of somatic embryo-derived plants should be examined. Flow cytometry is one of the most effective and fast methods for plant DNA content estimation [Doležel and Bartos 2005]. This method was applied to evaluate genome size stability of plants produced via somatic embryogenesis in number of species [Pinto et al. 2010, Prado et al. 2010, Konieczny et al. 2012, Currais et al. 2013, Escobedo-GraciaMedrano et al. 2014] but only in one cultivar of chrysanthemum [Naing et al. 2013b]. As a result of somatic embryogenesis may be changed also phenotypic characteristics of the plant. One of the most visible characteristic in ornamental plants is the colour of inflorescence. Traditionally, the colour of inflorescences is compared with the catalog RHSCC [1966]. A more objective and precise method of assessing the colour of plants is measurement the absorbance of pigments by a spetrophotometer. This method was applied to evaluate the content of carotenoids and anthocyanins in ray florets and leaves in chrysanthemum and in *Lamiaceae* family [Lema-Rumińska and Zalewska 2005, Czajka et al. 2009, Lema-Rumińska et al. 2013].

The aim of the present study was to analyse the genetic stability and pigment content and colour of somatic-embryo-derived plants of two chrysanthemum radiomutants using flow cytometry, spectrophotometry and the catalogue RHSCC [1966]. To our knowledge this is the first report the use of somatic embryogenesis to separate components of the chimera in chrysanthemum radiomutants.

MATERIALS AND METHODS

Chrysanthemum (*Chrysanthemum* × *grandiflorum* Ramat./Kitam.) cultivars 'Lady Salmon' and 'Lady Vitroflora' obtained using radiomutations [Jerzy et al. 1991] were the source of explants. The research carried out by Zalewska et al. [2007] showed, that the cultivar 'Lady Salmon' is periclinal chimera while 'Lady Vitroflora' is stable cultivar with regard to colour.

To produce experimental material microcuttings, from the gene bank of the Laboratory of Biotechnology at the Department of Ornamental Plants and Vegetable Crops (UTP Bydgoszcz, Poland) were multiplied *in vitro* via the single-node method on modified MS [Murashige and Skoog 1962] medium with a half-fold increased content of iron

and calcium, without growth regulators, pH = 5.8. From the two youngest leaves of each microcutting rectangles of about 1 cm^2 were excised and used as explants for somatic embryogenesis.

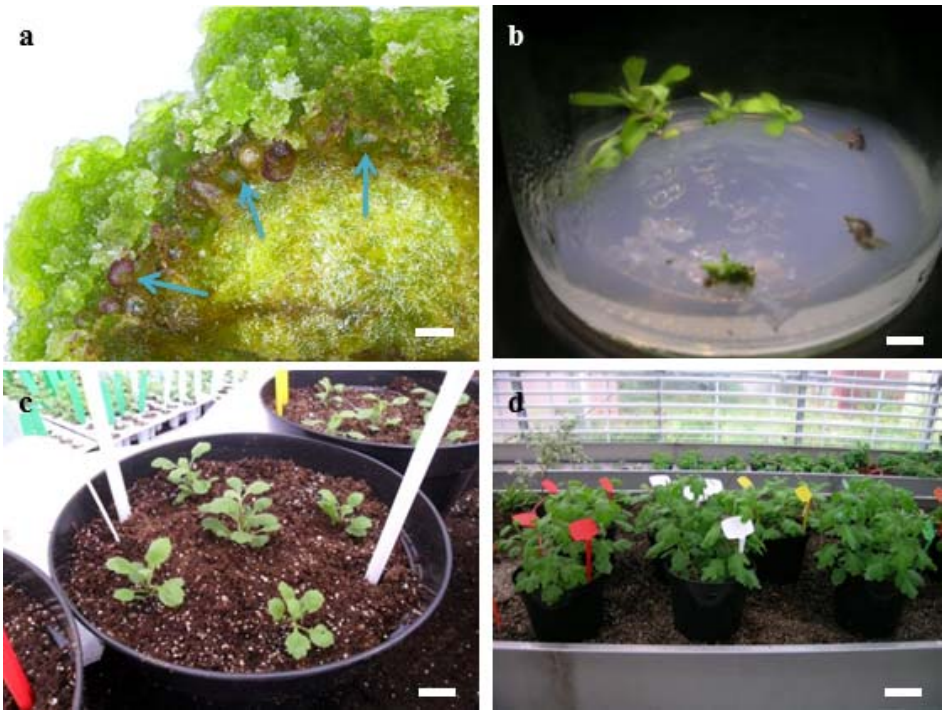

Fig. 1. Regeneration, conversion and growth of chrysanthemum plants from somatic embryos: a – induction of somatic embryos on a leaf explant (*bar* 1 mm), b – conversion of somatic embryos (*bar* 1 cm), c – microcuttings from somatic embryos (*bar* 2 cm), d – cultivation of the plants derived from somatic embryos in a greenhouse (*bar* 15 cm)

To obtain somatic embryos, leaf explants were cultivated for 10 weeks on the same medium as described above, but supplemented with the auxin, 2,4-D and for 'Lady Salmon' contained additionally cytokinin KIN, or for 'Lady Vitroflora' BAP (fig. 1a). Optimum composition of the medium for each cultivar was established previously by Lema-Rumińska and Niedojadło [2014]. The pH of all media was adjusted to 5.8. Culturing of somatic embryos was performed in a growth room at 24 ±2ºC, air relative humidity 60%, and quantum irradiation intensity 42.22 $\mu mol \cdot m^{-2} \cdot s^{-1}$. TLD 54 lamps emitting daylight maintained a photoperiod of 16 h. To induce further development of somatic embryos, they were transferred to the medium containing 30 $g \cdot dm^{-3}$ sucrose (standard content) with the addition of 1 $mg \cdot dm^{-3}$ KIN. 50 somatic embryos (5 jars with 10 embryos each) were cultured on the medium. Shoots conversion was obtained after next 10 weeks (8 clones for 'Lady Salmon' with number from 1 to 8 and 7 clones for

'Lady Vitroflora' with number from 1 to 7; fig. 1b). Control plants were obtained *in vitro* from meristems. Shoots obtained from somatic embryos were proliferated using the single-node shoot method. From each clone 10 microcuttings were produced and rooted onto MS medium with half concentration of macronutrients and 2 $mg \cdot dm^{-3}$ of indole-3-acetic acid (IAA). Microcuttings acclimatized for 14 days were planted in a greenhouse in 29 cm diameter pots (5 plants of a clone per pot) containing Gramoflor GmbH & Co substrate mixed with perlite in the ratio 1:4 (fig. 1c). The plants were grown under the natural photoperiod, using the traditional cultivation method – a single shoot and top inflorescence per plant (fig. 1d).

The colour of the ray florets of fully developed inflorescences of control plants and clones was estimated using the Royal Horticultural Society Colour Chart [RHSCC 1966] in the light emitted by TLD 54 lamps.

Young, fully-developed leaves from microcuttings produced from meristems (control) and clones from somatic embryos were analysed by flow cytometry. The samples were prepared as previously described [Sliwinska and Lukaszewska 2005] using 4',6-diamidino-2-phenylindole (DAPI; 2 μg/ml) for DNA staining. For each sample, the relative DNA content was established in about 2000–3000 nuclei using a Partec CCA (Partec GmbH, Münster, Germany) flow cytometer. Analyses were performed on five replicates of each clone, using linear amplification. Histograms were analyzed using a DPAC v.2.2 computer programme (Partec GmbH, Münster, Germany). Coefficient of variation (CV) of the G_0/G_1 peak of *Chrysanthemum* × *grandiflorum* ranged between 1.98 and 5.12.

Spectrophotometric analysis was performed of plant pigments extracted from inflorescences of control plants (obtained *in vitro* from meristems) and clones derived from somatic embryos. Maximum absorption for carotenoids and anthocyanins was obtained using a two-beam spectrophotometer UV-VIS 1601-PC SHIMADZU, following the procedure described by Lema-Rumińska and Zalewska [2005]. Spectrophotometric analyses were performed on five replicates of each clone. The results were statistically verified using a one-way analysis of variance and a Student's t-test at the significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Ploidy of plants derived from somatic embryos. Flow cytometry analysis revealed that in both cultivars (chimeric and non-chimeric) the ploidy of plants derived from somatic embryos was the same as that of control plants produced from meristems (fig. 2). Thus somatic embryogenesis and further growth *in vitro* did not induce quantitative changes in DNA content and the utilised protocol assured genome size stability. Similarly, in previous reports on somatic embryogenesis in chrysanthemum cultivar 'Baeksun' no polyploidization occurred [Naing et al. 2013b]. This method of propagation seems to lead to the production of genetically stable progeny, as occurs in other species such as *Musa acuminata* or *Trifolium nigrescens* [Konieczny et al. 2012, Escobedo-GraciaMedrano et al. 2014].

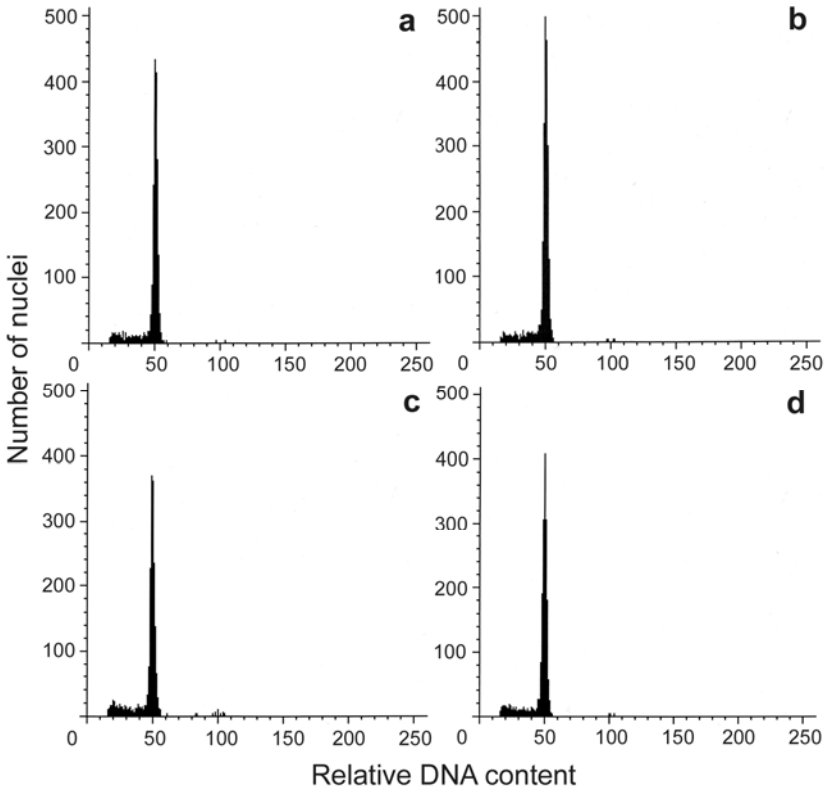

Fig. 2. Representative DNA histograms of nuclear preparations from leaves of chrysanthemum microcuttings produced from meristems (a, c) and clones from somatic embryos (b, d) of 'Lady Salmon' (a, b) and 'Lady Vitroflora' (c, d)

Pigments content and colour of inflorescences in plants derived from somatic embryos. In ray florets of 'Lady Salmon' plants produced from meristems, the concentration of carotenoids was high (10.60 ±1.74 mg·dm^{-3}), which resulted in a salmon colour of flowers, typical of this cultivar. Lema-Rumińska and Zalewska [2005] concluded that carotenoids content in 'Lady Salmon' plant propagated *in vivo* from meristems was differ (12.7 and 8.6 mg·dm^{-3}) in different growing conditions over successive study years. Another study conducted by Lema-Rumińska et al. [2013], suggested that differences in the concentration of pigments may result from different conditions of the real insolation and temperature throughout chrysanthemum growing, which affects the content of pigments. However, carotenoids were not detected in clones derived from somatic embryos in cultivar 'Lady Salmon' and the presence of anthocyanins was detected only in clones 2 and 3 (tab. 1). Consequently, flower colour in somatic-embryo-derived clones was different than of control plants (fig. 3a, b, c); it was either light pink (75C, 75D, and 76D; RHSCC 1966) or white (155 C and 155 D). These differences in flower

Table 1. Concentration of carotenoids and anthocyanins, established per 1 g of fresh weight of ray florets, and flower colour according to the Royal Horticultural Society Colour Chart (RHSCC 1966) in chrysanthemum plants derived from meristems (control) and somatic embryos of 'Lady Salmon' (LS) and 'Lady Vitroflora' (LV)

Symbol of clones	Concentration of pigments ($mg \cdot dm^{-3}$)		Colour code (% of plants)
	carotenoids 440 nm	anthocyanins 530 nm	
0 LS K (control)	10.60 ±1.74	–	16C(40), 15D(40), 16D(20)
1 LS	–	–	75D(40),76D(20),155D(40)
2 LS	–	2.55 ±0.75 [a*]	75D(60), 76D(20), 155C(20)
3 LS		1.95 ±1.09 [a]	76D(60), 155C(20),155D(20)
4 LS	–	–	155D(100)
5 LS	–	–	155D(50), 76D(50)
6 LS	–	–	155D(100)
7 LS	–		76D(50), 155C(25),155D(25)
8 LS	–	–	155B(80), 155D(20)
0 LV K (control)	–	2.83b ±1.83 [c]	75D(50),76D(50)
1 LV	–	2.48 ±0.64 [b]	75C(75), 75D(25)
2 LV	–	10.80 ±2.94 [a]	75B(60), 75C(40)
3 LV	–	8.31 ±4.16 [a]	75B(20), 75C(60)75D(20)
4 LV	–	5.19 ±2.92 [b]	75D(100)
5 LV	–	3.04 ±0.96 [b]	75D(60),75C(20),76D(20)
6 LV	–	1.60 ± 0.40 [c]	75C(50), 75D(50)
7 LV	–	2.50 ±0.58 [b]	75D(75),76D(25)

* Means (n = 5) in columns followed by the same letter are not significantly different at $P < 0.05$ (Student's *t*-test)

Fig. 3. Inflorescences of cultivars: 'Lady Salmon' obtained from a – meristem (control) and from somatic embryos b – clone 1, c – clone 8 and 'Lady Vitroflora' from d – meristem (control), e – clone 3; (*bar* 2 cm)

colour in 'Lady Salmon' cultivar suggest that the embryos originated from different layers of this chimeric radiomutant. Similar effect in 'Lady Salmon' was obtained by Zalewska et al. [2007] but through adventitious shoots regeneration. Pink colour (75 C) was obtained from adventitious shoots in 93.7% flowering plants and white colour (155 B) in 6.3%.

Periclinal chimeras in chrysanthemum cannot be distinguished from genetically homogeneous cultivars based on morphological characters and, as suggested the current study also on the FCM analysis.

In contrast to 'Lady Salmon', in 'Lady Vitroflora' the same pigments, anthocyanins but not carotenoids, were present in the inflorescences of plants produced from meristems (control) and all seven clones (tab. 1). Although the concentration of anthocyanins was different in the various clones, their flowers had very similar colour (75B–76D) as control plants (75D–76D; fig. 3d, e). This suggests a non-chimeric structure of this cultivar. The higher content of anthocyanins in clones 2 and 3 in 'Lady Vitroflora' and 'Lady Salmon' could be the effect of the presence of 2,4-D in the regeneration medium. Similar effect in *Camptotheca acuminata* was previously observed by Pasqua et al. [2005].

CONCLUSIONS

Somatic embryogenesis in chrysanthemums can be applied for separating periclinal chimera components for chimeric cultivars and for receiving an additional source of variation in the breeding of cultivars. In the case of genetically homogeneous cultivars (non-chimeric) it can be used in production laboratories for cloning plants *in vitro* via somatic embryogenesis.

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OCENA STABILNOŚCI GENETYCZNEJ ROŚLIN UZYSKANYCH PRZEZ EMBRIOGENEZĘ SOMATYCZNĄ *Chrysanthemum* × *grandiflorum* (Ramat./Kitam.)

Streszczenie. Stabilność genetyczna i fenotypowa roślin uzyskanych przez embriogenezę somatyczną może zostać zachwiana. Powodem może być pośrednia regeneracja zarodków somatycznych poprzez kalus lub wysokie stężenie regulatorów wzrostu dodanych na etapie indukcji zarodków somatycznych. Embriogeneza somatyczna chryzantem odmian: 'Lady Salmon' (chimera) i 'Lady Vitroflora' (niechimera), była indukowana na zmodyfikowanych pożywkach Murashige i Skoog (MS) z dodatkiem 4 $mg \cdot dm^{-3}$ kwasu 2,4-dwuchlorofenoksyoctowego (2,4-D) i 1 $mg \cdot dm^{-3}$ kinetyny (KIN) lub 6-benzylaminopuryny (BAP). Cytometria przepływowa (FCM) wykazała, że rośliny pochodzące z zarodków somatycznych obu odmian utrzymują ploidalność roślin kontrolnych uzyskanych z merystemu. Barwa i zawartość barwników w kwiatostanach roślin pochodzących z zarodków somatycznych u 'Lady Vitroflora' były podobne do roślin kontrolnych. Jednak kwiaty języczkowate klonów 'Lady Salmon' nie zawierały karotenoidów, charakterystycznych dla tej odmiany, a w konsekwencji tworzyły kwiaty w różnych barwach. Tak więc embriogeneza somatyczna u chryzantem może być stosowana do oddzielania składników chimery peryklinalnej u odmian będących chimerami i otrzymania dodatkowego źródła zmienności w hodowli odmian. Natomiast u odmian jednorodnych genetycznie może być stosowana w laboratoriach produkcyjnych do klonowania roślin w warunkach *in vitro* poprzez embriogenezę somatyczną.

Słowa kluczowe: barwniki, chimera, cytometria przepływowa, chryzantema, zarodek somatyczny

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