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SOMACLONAL VARIATION OF CHRYSANTHEMUM PROPAGATED *in vitro* FROM DIFFERENT EXPLANTS TYPES

Natalia Miler, Małgorzata Zalewska
University of Technology and Life Sciences in Bydgoszcz

Abstract. In order to find a more efficient source of somaclones we performed a regeneration of adventitious shoots from two explant types, leaves and internodes, in eight chrysanthemum cultivars representing three groups consisted of original cultivars and their radiomutants. After the *in vitro* regeneration and growth, plants were transferred to the glasshouse and the phenotypes at anthesis were observed. Three new, attractive variants with changed inflorescence colours were obtained from two cultivars ('Albugo' and 'Alchimist Tubular') from leaf explants. The frequency of somaclones was 1.1% for 'Albugo' and 5.4% for 'Alchimist Tubular'. The analysis of pigment content in ligulate florets revealed the presence of carotenoids in somaclones, while in control plants they were lacking. The genetic distinctiveness of new somaclones was corroborated with the estimation of the genetic similarity coefficients based on RAPD-PCR technique. Somaclones retained their phenotypes during subsequent vegetative propagations.

Key words: chimerism, adventitious shoots, genetic similarity, RAPD

INTRODUCTION

Although *in vitro* cultures are known to be a source of unwanted variation during micropropagation, from the breeder's point of view any source of variation is proper if it leads to stable valuable variants. Larkin and Scowcroft [1981] proposed the use of the term 'somaclonal variation' to determine the total phenotypic changes in plants derived from *in vitro* cultures, with the origin of both genetic and epigenetic. The somaclonal variation based on genetic changes is a promising breeding tool especially for ornamental plants, since they are mostly vegetatively propagated crops.

Chrysanthemum, one of world most popular ornamental plant, is eagerly bred with the usage of mutagenic treatment and many cultivars obtained this way had been released worldwide [Schum 2003]. Unfortunately, physical mutagens are not commonly

Corresponding author: Natalia Miler, Department of Ornamental Plants and Vegetable Crops, Laboratory of Biotechnology, University of Technology and Life Sciences, Bernardyńska 6, 85-029, Bydgoszcz, Poland, e-mail: nmiler@utp.edu.pl

available and also chemical mutagenic agents may be harmful both for the environment and for the breeder. Additionally the application of aggressive mutagenic factors can cause changes of a wide scope in the plant genome, which often leads to the weakening of vigour and resistance [van Harten 1998]. Somaclonal variation may be an alternative way of creating new cultivars.

One of the problems with the somaclonal variation is that the effectiveness of forming new variants is low. The emergence of the somaclonal variation is enhanced by the usage of explants ontogenetically distant from the meristem, and adding plant growth regulators (PGRs) to the medium as well as the induction of indirect regeneration via callus [Karp 1995]. Several experiments had been conducted to estimate the usability of various plant tissue culture systems in the induction of somaclonal variation in chrysanthemum. Khalid et al. [1989] compared petals and leaf explants regeneration and stated that petals produce more variants. Also, the genetic distinctiveness of petal-induced somaclones had been corroborated [Kengkarj et al. 2008]. However, the application of petals as explants is not convenient due to the need of explant sterilization and the restricted availability of petals during the year.

The phenomenon of chimerism is another difficulty with the application of somaclonal variation as a breeding tool in chrysanthemums. Periclinal chimeras arise mostly as an effect of mutagenic treatment and commonly consist of a mutated epiderm and unmutated inner layers, which are genetically identical with the original cultivar exposed to mutagenesis [Jerzy and Zalewska 1997]. Although using explants without meristems enhances the probability of obtaining somaclonal variants, in chimeras it also leads to the separation of components, which does not provide any new variation [Broertjes and Keen 1980]. Since many chrysanthemums are periclinal chimeras with one histogenic layer genetically different from others, it is crucial to point whether the changes are a result of somaclonal variation or a result of the separation of chimera components.

In our experiment three groups of cultivars were used, each consisted of one original cultivar and its radiomutants to verify whether the alternations were novel or just an effect of the separation of chimera components. We compared the effectiveness of the induction of somaclonal variation across two explant types: leaves and internodes which performed the regeneration of adventitious shoots. Also, we corroborated the genetic distinctiveness of three novel somaclones and demonstrated their uniqueness.

MATERIAL AND METHODS

Eight cultivars of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. were used in this study: 'Albugo' and its mutant 'Albugo Sunny'; 'Alchmist' and its two mutants 'Alchimist Tubular' and 'Alchimist Golden Beet'; 'Satinbleu' and its two mutants 'Satinbleu Minty' and 'Satinbleu Honey'. All of the mutants were obtained in the Department of Ornamental Plants and Vegetable Crops at the University of Technology and Life Sciences, Bydgoszcz, Poland, as a result of the mutagenic treatment with gamma rays *in vitro* [Zalewska et al. 2011].

The shoot tips of the cultivars were excised from greenhouse-cultivated plants. The explants were sterilized in 0.8% (v/v) sodium hypochloride solution for 10 minutes, rinsed three times with sterile distilled water and inoculated onto the MS [Murashige and Skoog 1962] standard medium, solidified with 0.8% (w/v) agar (Duchefa Biochemie B.V., Haarlem, The Netherlands) and supplemented with 3% (w/v) sucrose. The medium did not contain any plant growth regulators (PGRs). Once all the components were added, the pH was adjusted to 5.8 before the medium was sterilized in an autoclave at 121°C for 20 min. The explants were cultured in 350 ml glass jars, each containing 40 ml of the medium. *In vitro* cultures were maintained in a sterile growth room, at 24 ±2°C with a 16-h photoperiod. Light was supplied by Phillips TLD 36W/54 fluorescent tubes at a photon flux density of 35 μmol m^{-2} s^{-1}.

Sterile shoots were propagated *in vitro* using single-node explants on the MS medium without PGRs. Each propagation cycle lasted approximately 8 weeks, cycles were repeated four times to obtain the required number of plantlets.

For adventitious shoots regeneration, the leaves and internodes were excised from the middle part of plantlets of an approximate length of 8 cm, and were subcultured onto the MS medium supplemented with 2.0 mg dm^{-3} indole-acetic acid (IAA) and 0.6 mg dm^{-3} 6-benzylaminopurine (BAP). Culture-room conditions were established as described above. Leaf explants (consisted of an intact blade and a petiole) were inoculated vertically with a petiole stuck into the medium. The internodes were put horizontally onto the medium surface. There were 5 explants (leaves or internodes) in every culture jar. A total number of cultured explants is indicated in table 1. Adventitious shoots regeneration was conducted for 8 weeks on the same medium, without any additional subcultures. A mean number of regenerated shoots per cultured explants were estimated in order to compare the regeneration ability between leaves and internodes of the same cultivar. Data were statistically verified with t-Student test at different p levels (tab. 1).

Simultaneously with the adventitious shoots regeneration, 50 control shoots of each cultivar were grown *in vitro* from single-node explants on the MS medium without PGRs to establish control objects of a very high genetic stability. In this method a direct growth of a shoot from a lateral bud without any callus formation is performed.

The regenerated adventitious shoots, (at the most 100 plantlets from each cultivar and each explant type, shoots length varied from 1-3 cm) and 50 control plantlets from each cultivar were subcultured onto rooting medium (MS based, supplemented with 2.0 mg dm^{-3} IAA) for 10 days. Rooted microcuttings were then planted into plastic cuvettes filled with peat : perlite (3 : 1) substrate, covered with a perforated polyethylene foil and occasionally sprayed with water during the acclimatization for 2 weeks in a glass-house. After the acclimatization, the plants were transplanted into pots and cultured in the greenhouse for a month in a long-day conditions and, subsequently, in a short-day conditions in order to induce flowering.

At anthesis, the colours of inflorescences were determined with the Royal Horticultural Society Colour Chart [1966] at both, adaxial (inner) and abaxial (outer) surfaces of ligulate florets. The phenotypes of regenerates were compared with the phenotypes of control plants. The plants representing each phenotype were counted, and the frequencies of variation for each explant type were estimated as the percentage of plants differing from control plants for a cultivar.

Table 1. Number of cultured leaves and internodes, number of regenerating explants and a total and mean number of adventitious shoots formed from respective explant types in eight chrysanthemum cultivars. For the mean number of adventitious shoots, significantly lower mean number of shoots between two explant types in each cultivar are labeled with * at p ≤0.05, ** at p ≤ 0.01, and *** at p 0.001

Cultivar	Explant type	Cultured explants	Regeberatig explants (%)	Adventitious shoots	
				total	mean per cultured explant
ALB[1)]	leaf	115	93 (80.8)	395	3.43
	internode	100	93 (93.0)	272	2.72*
AS	leaf	110	2 (1.8)	7	0.06**
	internode	90	41 (45.5)	104	1.15
ALC	leaf	75	54 (72.0)	145	1.93*
	internode	120	59 (49.2)	373	3.11
AGB	leaf	30	3 (10.0)	35	1.16
	internode	65	45 (69.2)	135	2.07
AT	leaf	85	51 (60.0)	120	1.41
	internode	110	99 (90.0)	200	1.81
S	leaf	105	37 (35.2)	56	0.53***
	internode	105	88 (83.8)	216	2.05
SH	leaf	95	35 (36.8)	83	0.87***
	internode	120	109 (90.8)	221	1.84
SM	leaf	90	2 (2.2)	5	0.05***
	internode	95	76 (80.0)	136	1.43

[1)] Abbreviations of cultivar names: ALB – 'Albugo', AS – 'Albugo Sunny', ALC –'Alchimist', AGB – 'Alchimist Golden Beet', AT – 'Alchimist Tubular', S – 'Satinbleu', SH – 'Satinbleu Honey', SM – 'Satinbleu Minty'

In order to confirm the stability of the changed and the true-to type phenotypes, the plants were vegetatively propagated in the greenhouse using shoot-tip cuttings. From each phenotype the final number of 25 plants were cultivated up to anthesis (the second vegetative generation) to verify their inflorescence colour.

The colour of an inflorescence in chrysanthemum is primarily a result of ability of synthesis of anthocyanins and carotenoids, which is genetically managed. In order to determine the presence or absence of these main pigments in inflorescences of the studied chrysanthemums, the spectrophotometric analysis of extracts from ligulate florets was conducted. From the plants obtained from single-nodes, leaves as well as from internodes, three inflorescences representing each colour (true-to-type as well as changed phenotype) were chosen for pigments extraction. In case of a light yellow somaclone of 'Albugo' and a golden red somaclone of 'Alchimist Tubular', represented only by a single plant, three samples for carotenoid and anthocyanins extraction were

taken from the only one inflorescence. Florets from the middle whorl of inflorescences were collected, proximal parts of florets (which are usually poorly coloured) were discarded, only their distal parts were taken for measurements. Carotenoids extraction was conducted according to Wettstein [1957] in 100% acetone. Anthocyanins were extracted according to Harborne's [1967] in 1% (v/v) HCl in methanol. The absorbance measurement of extracts was conducted with the UV-VIS 1601-PC SHIMADZU spectrophotometer, from 330 nm to 500 nm wavelength for carotenoids detection and from 500 nm to 600 nm for anthocyanins. A pigment presence was stated if a definite peak appeared in an absorbance graph at a wavelength characteristic for denominated pigment.

The genetic diversity analysis was performed with a RAPD-PCR technique [Welsh and McClelland 1990, Williams et al. 1990]. The analysis was conducted for the control plants representing cultivars of Albugo and Alchimist groups and the three somaclones representing new phenotypes, unprecedented previously in these two groups, namely the golden red, the golden brown as well as the light yellow somaclone. Satinbleu group was excluded from genetic analysis since any new variants were not obtained within it.

DNA was extracted from young fresh leaves from a single plant representing studied phenotype: either true-to-type or variant. For the extraction of DNA the Genomic Mini AX Plant kit (A&A Biotechnology, Gdynia, Poland) was used. Ten primers were selected on the basis of the references on the genetic diversity estimation in chrysanthemum, namely primers A: 5'-GGG AAT TCG G-3', B: 5'-GAC CGC TTG T-3' and C: 5'-GGA CTG GAG T-3' from Lema-Rumińska et al. [2004], primers D: 5'-GCT GCC TCA GG-3' and E: 5'-TAC CCA GGA GCG-3' from Shibata et al. [1998], primers F: 5'-CAA TCG CCG T-3' and G: 5'-GGT GAC GCA G-3' from Wolff [1996], primers H: 5'-CCC AGT CAC T-3' and I: 5'-TGG CGT CCT T-3' from Martin and Gonzalez-Benito [2005] and primer J: 5'-AGC GTG TCT G-3' from Chattarjee et al. [2006]. The reactions of DNA amplification were performed in a volume of 25 µl, containing 20 ng template DNA, 0.5 unit of *Taq* RUN polymerase (A&A Biotechnology, Gdynia, Poland) with the final concentration of 0.2 mM of each dNTP, 1 µM of a single primer and 2 mM of $MgSO_4$. The amplification was performed in a NyxTechnik ATC 401 thermocycler with the heated lid. The PCR conditions were programmed as follows: one cycle of 4 min at 94°C; 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, followed by one cycle of 4 min at 72°C and the storage at 4°C. The amplification products were resolved by electrophoresis in 1.8% agarose gel stained with ethidium bromide, in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH = 8.0) at 120 V. DNA GeneRuler Express DNA Ladder (Fermentas, Lithuania) served as the DNA size standard. After the separation the bands were visualized under UV light and photographed using the Gel Doc system (Bio-Rad, USA). All clear repeatable bands were scored and a matrix of 01 code was elaborated for each genotype with the usage of the QuantityOne System (Bio-Rad, USA).

The genetic similarity coefficient [Nei and Li 1979] was estimated using TREECON 1.3 computer program [van de Peer and de Watcher 1994] and a dendrogram was created using the unweighted-pair group method with an arithmetic mean (UPGMA). In order to evaluate the credibility of phylogenetic tree branching, a bootstrap analysis with 1000 sampling was applied.

RESULTS

Adventitious shoots in all the cultivars and in both types of explants started to form after approximately three weeks of callus proliferation. The structure of calli formed from leaves as well as from internodes were similar: abundant, not very thogh, nodular, coloured light green. Numbers of obtained shoots varied among cultivars and explant types (tab. 1). In most cultivars leaves produced less shoots than internodes, it was especially visible in cultivars representing Satinbleu Group. There were no differences in mean shoots numbers between two explant types in 'Alchimist Golden Beet' as well as in 'Alchimist Tubular'.

For control plants each single-node explant produced a single shoot from a lateral bud without forming of callus.

Table 2. Phenotype effects in plants representing Albugo group obtained *in vitro* from different explant types. For abbreviation of cultiv ars names see tab. 1

Cultivar	Explant type	Inflorescence colour	Colour code		Presence of pigments[2)]	Number of plants (percentage)	Frequency of variants for explant type (%)
			adaxial	abaxial			
ALB	node[1)]	white	155 A	155 A	–	40 (100.0)	0.0
	leaf	white	155 A	155 A	–	90 (98.9)	1.1
		light yellow	10 C	9 D	C	1 (1.1)	
	internode	white	155 A	155 A	–	87 (100.0)	0.0
AS	node[1)]	yellow	7 C	7 D	C	45 (100.0)	0.0
	leaf	yellow	7 C	7 D	C	5 (100.0)	0.0
	internode	yellow	7 C	7 D	C	90 (100.0)	0.0

[1)] control
[2)] C – carotenoids

There were observed novel phenotypes among the plants propagated with leaf explants in two cultivars: 'Albugo' and 'Alchimist Tubular'. In 'Albugo' among most regenerates with a white, cultivar-specific, inflorescence colour, there appeared a single plant with a light yellow inflorescence colour (tab. 2, fig. 1). Among 'Alchimist Tubular' regenerates there were observed, apart from the abundance of typical silver violet inflorescences, three plants with inflorescences colours of golden brown and a single plant with a golden red inflorescence colour (tab. 3, fig. 2). Changes of colours were a result of emerging of carotenoids in inflorescences, which were not present in control plants. The absorbance at a 440 nm wavelength, which is used for carotenoids content estimation, was lower in golden red somaclone of 'Alchimist Tubular' than in golden brown somaclone. In these two cultivars, 'Albugo' and 'Alchimist Tubular', plants regenerated from internodes showed only true-to-type phenotypes, similar to the control and no changes in the presence or absence of anthocyanins were observed.

Fig. 1. Inflorescences of a control plant grown from nodal explant (a) and a light yellow somaclone (b) regenerated from leaf explant of 'Albugo' cultivar

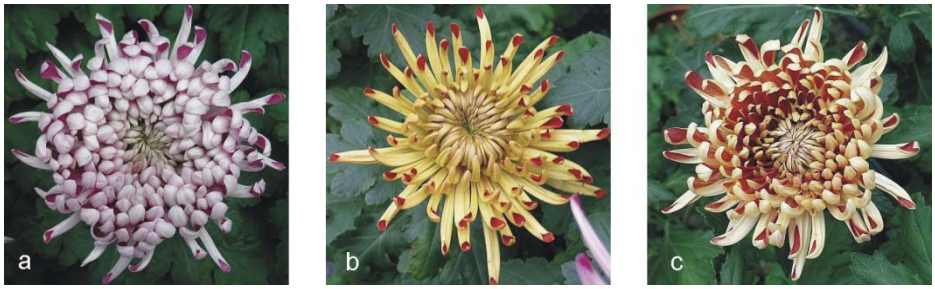

Fig. 2. Inflorescences of a control plant grown from nodal explant (a), a golden brown somaclone (b) and a golden red somaclone (c) regenerated from leaf explants of 'Alchimist Tubular' cultivar

High frequencies of variations were observed in plants regenerated from internodes and leaves in 'Alchimist Golden Beet' and 'Satinbleu Honey', as compared with the control plants (tab. 3, tab. 4). In both cultivars the frequency of variants formed from leaves reached 100%, which means that none of the plants regenerated from leaf showed the true-to-type phenotype in these cultivars. Nonetheless, these variants did not provide any new trait within groups as the colours of their inflorescences resembled of control plants of the original cultivars: 'Alchimist' and 'Satinbleu', respectively. Moreover, the inflorescences of these variants contained only anthocyanins, while control plants of 'Alchimist Golden Beet' and 'Satinbleu Honey' contained both anthocyanins and carotenoids. A very high percentage of plants originated as adventitious shoots with phenotypes resembling original cultivars, led us to the conclusion that 'Alchimist Golden Beet' and 'Satinbleu Honey' are very probably periclinal chimeras with only external histogenic layer changed in terms of inflorescence colour. Probably, inner tissues of these cultivars are genetically able for the synthesis of only anthocyanins and were not changed during the process of mutagenesis in which these cultivars were developed in 2006 [Zalewska et al. 2011].

Table 3. Phenotype effects in plants representing Alchimist group obtained *in vitro* from different explant types. For abbreviation of cultivars names see tab. 1

Cultivar	Explant type	Inflorescence colour	Colour code		Presence of pigments[2)]	Number of plants (percentage)	Frequency of variants for explant type (%)
			adaxial	abaxial			
ALC	node[1)]	dark violet	72 A	69 C	A	39 (100.0)	0.0
	leaf	dark violet	72 A	69 C	A	87 (100.0)	0.0
	internode	dark violet	72 A	69 C	A	85 (100.0)	0.0
AGB	node[1)]	golden beet	185 B	160 D	A C	49 (100.0)	0.0
	leaf	dark violet	72 A	69 C	A	33 (100.0)	100.0
	internode	golden beet	185 B	160 D	A C	7 (7.2)	92.8
		dark violet	72 A	69 C	A	90 (92.8)	
AT	node[1)]	silver violet	70 A	85 D	A	45 (100.0)	0.0
	leaf	silver violet	70 A	85 D	A	71 (94.6)	5.4
		golden brown	172 B	162 C	A C	3 (4.0)	
		golden red	184 A	160 D	A C	1 (1.4)	
	internode	silver violet	70 A	85 D	A	88 (100.0)	0.0

[1)] control
[2)]A – anthocyanins, C – carotenoids

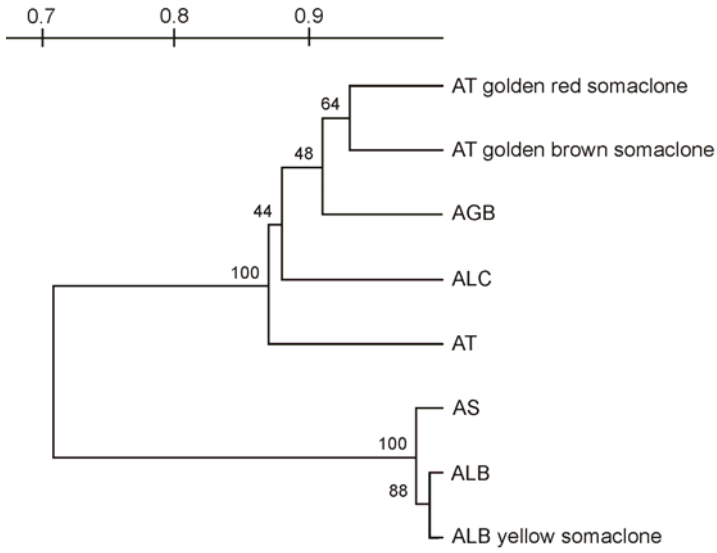

Fig. 3. Dendrogram based on the estimation of genetic similarity coefficients and UPGMA clustering presenting the relationships between original cultivars, mutant cultivars and somaclones. Numbers in nodes indicates bootstrap values for 1000 sampling. For abbreviations of cultivar names see tab. 1

Table 4. Phenotype effects in plants representing Satinbleu group obtained *in vitro* from different explant types. For abbreviation of cultivars names see tab. 1.

Cultivar	Explant type	Inflorescence colour	Colour code		Presence of pigments[2)]	Number of plants (percentage)	Frequency of variants for explant type (%)
			adaxial	abaxial			
S	node[1)]	dark pink	84 C	76 C	A	44 (100.0)	0.0
	leaf	dark pink	84 C	76 C	A	39 (100.0)	0.0
	internode	dark pink	84 C	76 C	A	94 (100.0)	0.0
SH	node[1)]	salmon	168 D	12 D	A C	41 (93.2)	6.8
		dark pink	84 C	76 C	A	3 (6.8)	
	leaf	dark pink	84 C	76 C	A	81 (100.0)	100.0
	internode	salmon	168 D	12 D	A C	1 (1.1)	98.9
		dark pink	84 C	76 C	A	93 (98.9)	
SM	node[1)]	pale pink	76 D	155 D	A	47 (100.0)	0.0
	leaf	pale pink	76 D	155 D	A	5 (100.0)	0.0
	internode	pale pink	76 D	155 D	A	99 (100.0)	0.0

[1)] control
[2)]A – anthocyanins, C – carotenoids

In four out of eight cultivars no changes were noted in the colour of inflorescences irrespective of the method of propagation *in vitro*: 'Albugo Sunny', 'Alchimist', 'Satinbleu' and 'Satinbleu Minty'. The control plants propagated *via* single-nodes of seven cultivars demonstrated the cultivar-specific phenotype. Control plants of 'Satinbleu Honey' were the only ones which were not even in terms of the colour of the inflorescence. Except for the salmon inflorescence colour, typical for this cultivar and observed in most plants, two plants were bearing dark pink inflorescences, and a single plant had the inflorescence double-coloured: pink and salmon.

In the successive year the stability of phenotypes was confirmed. 'Satinbleu Honey' was the only one for which again there appeared a single plant with the dark pink inflorescence, untypical for the cultivar. All novel variants repeated their phenotypes.

In the Albugo Group, seven out of the ten screened primers, namely B, C, D, E, F, H, I, applied in the RAPD analysis gave polymorphic DNA fragments. A total of 80 fragments were produced, while 22 of them were polymorphic. In the Alchimist Group all of the primers, except for primer G, gave polymorphic bands. A total number of bands was 112; 72 of them were polymorphic. In both cultivar groups primers B and E generated the highest number of polymorphic bands; for the Albugo Group there were six polymorphic bands produced with primer B and six produced with primer E, and for the Alchimist Group – 12 and 14, respectively.

Table 5. The coefficients of genetic similarity among original cultivars, mutant cultivars and somaclones of Albugo and Alchimist Groups estimated on the basis of the analysis of RAPD band profiles. For abbreviation of cultivars names see tab. 1

	ALB	ALB yellow somaclone	AS	ALC	AGB	AT	AT golden red somaclone	AT golden brown somaclone
ALB yellow somaclone	0.99	1.00						
AS	0.98	0.99	1.00					
ALC	0.73	0.72	0.73	1.00				
AGB	0.75	0.74	0.75	0.90	1.00			
AT	0.69	0.69	0.70	0.86	0.86	1.00		
AT golden red somaclone	0.73	0.72	0.73	0.87	0.92	0.89	1.00	
AT golden brown somaclone	0.68	0.68	0.69	0.88	0.90	0.86	0.93	1.00

The genetic analysis showed two main clusters complying with the cultivars allocated to the respective group (fig. 3). The relationships between the plants representing the Albugo Group were characterized by very high coefficients of genetic similarity. Among the light yellow somaclone and its initial cultivar 'Albugo' there was more than 99 % of genetic similarity (tab. 5). The highest genetic similarity in the Alchimist Group was observed between two new somaclones and it accounted for 93%. Surprisingly, a lower genetic similarity was stated among these somaclones and their initial cultivar 'Alchimist Tubular' than between the somaclones and 'Alchimist Golden Beet'.

DISCUSSION

Previously, plant tissue cultures were used as a source of variation in chrysanthemums by Bush et al. [1976] who performed regeneration on ligulate florets and Khalid et al. [1989], who claimed that the regeneration from ligulate florets led to a greater somaclonal variation than the regeneration from leaves. However, Zalewska et al. [2007] produced several new phenotypes of variants following the regeneration of adventitious shoots from leaves. Although in our experiment more adventitious shoots were formed from internodes, new variants were observed exclusively as a result of regeneration from leaves. One of the factors enhancing the somaclonal variation is a high distance from the organized meristem structure [Karp 1995]. We think that leaves, which are organs destined for a photosynthetic purposes, are in their anatomy and physiology further departed from the apical meristem structure then internodes, therefore they could be found to be more useful in the induction of somaclonal mutants. The use of explants bearing meristems by Miñano et al. [2009] during micropropagation of chrysanthemum on media supplemented with various PGRs, although resulted in some variants detected with a RAPD profiles, did not lead to any phenotypical variations.

The genetic evidence for the individuality of the produced somaclones came from the RAPD analysis of cultivar groups. The Alchimist Group was found to be more variable, since most of the generated bands were polymorphic and the genetic similarity exceeded from 86% to 94%. All of the three Albugo Group accessions were very closely related, with the lowest genetic distance between light yellow somaclone and its mother cultivar 'Albugo' being less than 1%. These results show that a wider range of changes occurred during the regeneration of 'Alchimist Tubular' somaclones. A very high genetic similarity between light yellow somaclone and 'Albugo' suggests that the change of inflorescence colour from white to yellow was a result of a point mutation or the new somaclone is being a periclinal chimera with epidermis only (L1 layer) varying from the rest of the plant body.

Our data confirm the applicability of the RAPD technique to the detection of somaclonal variation. Similarly, Kengkarj et al. [2008] successfully applied the RAPD technique to discriminate between somaclones obtained from ray florets of chrysanthemum, however they scored higher values of genetic distance – up to 52%. Screening for variations of chrysanthemums, tissue cultured under different conditions, was also undertaken applying the RAPD technique by Martin et al. [2002], and some variations were detected, however no data on subsequent phenotype observations were provided. Miñano et al. [2009] applied the RAPD technique in screening for somaclonal variation in chrysanthemum micropropagated with the usage of media containing different PGRs contents and recorded a low final frequency of variation 0.5%. The authors did not observe any morphological changes after plants acclimatization. Our data show that although the frequency of somaclonal variation is low, the changes induced during adventitious shoots regeneration have genetic background and may be essential. The somaclones produced in our experiment looked attractive, demonstrated good healthiness as well as stability during vegetative propagation commonly applied in chrysanthemum production and, as such, they can enrich the assortment of cultivars available on the market.

The chrysanthemum cultivars obtained as a result of somatic mutagenesis, induced or spontaneous, are frequently periclinal chimeras of only one external changed histogenic layer, especially if the explants containing meristems are irradiated [Langton 1980, Yamaguchi et al. 2009]. The 'Alchimist Golden Beet' and 'Satinbleu Honey' cultivars were produced via induced mutagenesis *in vitro* with the gamma irradiation of single-node explants and callus formed on leaves [Zalewska et al. 2011]. Drawing on the observations of phenotypes we have found that 'Alchimist Golden Beet' and 'Satinbleu Honey' were periclinal chimeras. In these cultivars, as a result of adventitious shoots regeneration, we reported a separation of chimeras components, namely the hidden phenotypes got revealed; no new variation occurred. It is seen from a high frequency of the variants and their appearance, not exceeding the pool of phenotypes already present in both cultivar groups.

The first experiment on the separation of chimera components in chrysanthemum was performed by Stewart and Dermen [1970], who induced *in vivo* adventitious buds on the chrysanthemum shoots deprived of the lateral buds. The phenomenon of the separation of chimeras components in chrysanthemums was also observed in plant tissue cultures following the application of the method of adventitious shoots regeneration from leaf explants [Jerzy and Zalewska 1997, Zalewska et al. 2007]. Also Malaure et al. [1991] used *in vitro* regeneration from the ligulate florets of chrysanthemums to separate

chimera components. So far there have been no reports on the phenomenon of the separation of chimeras components with the use of internodes. In chimeras, the existence of two different phenotypes derived from internodes means that the regeneration of adventitious shoots from internodes underwent from the tissues representing at least two histogenic layers.

The meristems in lateral buds located on the single-node explants repeat the pattern of the histogenic layers characteristic for the apical meristem. The usage of explants of this type makes it possible to clone periclinal chimeras in which the exceptional look of the inflorescences is often a result of a varied distribution of pigments in the ligulate floret tissue layers [Stewart and Dermen 1970, Bush et al. 1976, Broertjes and van Harten 1988]. The control plants in seven out of eight cultivars in our experiment, propagated *in vitro* with the single-node explants, repeated the cultivar-specific phenotypes, with no variation being observed across them. In the experiment reported by Zalewska et al. [2007], 6 out of 11 cultivars were periclinal chimeras and all of them, after the propagation with the single-node explants *in vitro,* repeated their characters. In our experiment 'Satinbleu Honey', discriminated as one of the two chimeras, was unstable during propagation *in vitro* with the single-node explants as well as in the second vegetative generation, which resulted in a dark pink inflorescence colour of three control plants. This phenomenon of the rearrangement of the layers could have contributed to the instability in 'Satinbleu Honey' control plants. Cells of L2, which were "genetically dark pink" could have penetrated the "salmon" L1, which resulted in dark pink phenotype of ligulate florets of some of the control plants

The response to the question whether the cultivar is a chimera or not makes it possible to apply the adequate procedure during micropropagation. Periclinal chimeras should be propagated *in vitro* only with the use of the explants with buds and on the media without growth regulators. Pointing to the chimerism of a given cultivar also enforces the adequate procedure during cryopreservation used for genotype maintenance [Fukai et al. 1994]. Permanent monitoring of the genetic stability of chrysanthemum cultivars being chimeras is justifiable since even meeting the above requirements is not a hundred percent guarantee of maintaining the genotype, which is seen from our results for 'Satinbleu Honey' control plants.

CONCLUSIONS

1. Novel phenotypes of chrysanthemum arise as a result of the regeneration of adventitious shoots *in vitro* from leaves only. Although there was a variation among plants obtained from internodes in comparison with control plants in 'Albugo Golden Beet' and 'Satinbleu Honey', any new traits for the examined groups of cultivars were not observed in plants regenerated from internodes, only the recall of the mother cultivar phenotype occured.

2. The alternation in the phenotypes of inflorescences of the three obtained somaclones, as compared to control plants, was connected with the triggering of the synthesis of carotenoids in ligulate florets.

3. The molecular marker method based on RAPD-PCR technique is appropriate to confirm the genetic uniqueness of somaclones.

REFERENCES

Broertjes C., van Harten A.M., 1988. Applied mutation breeding for vegetatively propagated crops. Elsevier, Amsterdam.

Broertjes C., Keen A., 1980. Adventitous buds: do they develop from one cell? Euphytica 29, 73–87.

Bush S.R., Earle E.D., Langhans R.W., 1976. Plantlets from petal segments, petal epidermis, and shoot tips of the periclinal chimera *Chrysanthemum morifolium* 'Indianapolis'. Am. J. Bot. 63, 729–737.

Chattarjee J., Mandal A.K.A., Ranade S.A., Teixeira da Silva J.A., Datta S.K., 2006. Molecular systematic in *Chrysanthemum* × *grandiflorum* (Ramat.) Kitamura. Sci. Hortic. 110, 373–378.

Fukai S., Goi M., Tanaka M., 1994. The chimeric structure of the apical dome of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitam.) is affected by cryopreservation. Sci. Hortic. 57, 347–351.

Harborne J.B., 1967. Comparative biochemistry of the flavonoids. Academic Press, London.

Jerzy M., Zalewska M., 1997. Flower colour recurrence in chrysanthemum and gerbera mutants propagated *in vitro* with meristems and leaf explants. Acta Hortic. 417, 611–614.

Karp A., 1995. Somaclonal variation as a tool for crop improvement. Euphytica 85, 295–302.

Kengkarj P., Smitamana P., Fujime Y., 2008. Assessment of somaclonal variation in chrysanthemum (*Dendranthema grandiflora* Kitam.) using RAPD and morphological analysis. Plant. Tissue Cult. Biotechnol. 18, 139–149.

Khalid N., Davey R.D., Power J.B., 1989. An assessment of somaclonal variation in *Chrysanthemum morifolium:* the generation of plants of potential commercial value. Sci. Hortic. 38, 287–294.

Langton F.A., 1980. Chimerical structure and carotenoid inheritance in *Chrysanthemum morifolium* (Ramat.). Euphytica 29, 807–812.

Larkin P.J., Scowcroft W.R., 1981. Somaclonal variation – a novel source of variability from cell cultures for crop improvement. Theor. Appl. Genet. 60, 197–214.

Lema-Rumińska J., Zalewska M., Sadoch M., 2004. Radiomutants of chrysanthemum (*Dendranthema grandiflora* Tzvelev) of the Lady group: RAPD analysis of the genetic diversity. Plant Breed. 123, 290–293.

Malaure R.S., Barclay G., Power J.B., Davey M.R., 1991. The production of novel plants from florets of *Chrysanthemum morifolium* using tissue culture. 1. Shoot regeneration from ray florets and somaclonal variation exhibited by the regenerated plants. J. Plant Physiol. 139, 8–13.

Martin C., Gonzales-Benito M.E., 2005. Survival and genetic stability of *Dendranthema grandiflora* Tzvelev shoot apices after cryopreservation by vitrification and encapsulation-dehydratation. Cryobiology 51, 281–289.

Martin C., Uberhuaga E., Perez C., 2002. Application of RAPD markers in the characterization of *Chrysanthemum* varieties and the assessment of somaclonal variation. Euphytica 127, 247–253.

Miñano H.S., Gonzalez-Benito M.E., Martin C., 2009. Molecular characterization and analysis of somaclonal variation in chrysanthemum cultivars using RAPD markers. Sci. Hortic. 122, 238–243.

Murashige T., Skoog F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Nei M., Li W.S., 1979. Mathematical model for studying genetic variation in terms of restriction nucleases. Proc. Natl. Sci. 76(10), 5269–5273.

RHSCC, 1966. Royal Horticultural Society Colour Chart, London.

Shibata M., Kishimoto S., Hirai M., Aida R., Ikeda I., 1998. Analysis of the periclinal structure of chrysanthemum sports by random amplified polymorphic DNA. Acta Hortic. 454, 347–353.

Stewart R.N., Dermen H., 1970. Somatic genetic analysis of the apical layers of chimera sports in chrysanthemum by experimental production of adventitious shoots. Am. J. Bot. 57, 1061–1071.

Schum A., 2003. Mutation breeding in ornamentals: an efficient breeding method? Acta Hortic. 612, 47–60.
Welsh J., McClelland M., 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. 18, 7213–7218.
Wettstein D., 1957. Chlorophyll-letale und der submikroskopische Formwechsel der Plastiden. Exp. Cell Res. 12, 427–487.
Williams J.G.K., Kubelik A.R., Livak J.L., Rafalski J.A., Tingey S.V., 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18, 6531–6535.
Wolff K., 1996. RAPD analysis of sporting and chimerism in chrysanthemum. Euphytica 89, 159–164.
Van de Peer Y., De Wachter Y., 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. App. Biosci. 10, 569–70.
Van Harten A.M., 1998. Mutation breeding. Theory and practical applications. Cambridge University Press, Cambridge.
Yamaguchi H., Shimizu A., Hase Y., Degi K., Tanaka A., Morishita T., 2009. Mutation induction with ion beam irradiation of lateral buds of chrysanthemum and analysis of chimeric structure of induced mutants. Euphytica 165, 97–103.
Zalewska M., Lema-Rumińska J., Miler N., 2007. *In vitro* propagation using adventitious buds technique as a source of new variability in chrysanthemum. Sci. Hortic. 113, 70–73.
Zalewska M., Tymoszuk A., Miler N., 2011. New chrysanthemum cultivars as a result of in vitro mutagenesis with the application of different explant types. Acta Sci. Pol., Hortorum Cultus 10(2), 109–123.

ZMIENNOŚĆ SOMAKLONALNA CHRYZANTEM ROZMNAŻANYCH *in vitro* Z WYKORZYSTANIEM RÓŻNYCH RODZAJÓW EKSPLANTATÓW

Streszczenie. Regenerację pędów przybyszowych *in vitro* z liści i międzywęźli ośmiu odmian chryzantem należących do trzech grup odmianowych złożonych z odmian wyjściowych i ich radiomutantów przeprowadzono po to, aby wskazać, który z eksplantatów może stanowić wydajniejsze źródło roślin będących somaklonami. Po regeneracji i wzroście *in vitro* rośliny były uprawiane w szklarni. W czasie pełni kwitnienia prowadzono obserwacje fenotypów. Z eksplantatów liściowych dwóch odmian 'Albugo' i 'Alchimist Tubular' uzyskano trzy nowe, atrakcyjne warianty o zmienionych barwach kwiatostanów. Frekwencja somaklonów u 'Albugo' wynosiła 1,1%, a u 'Alchmist Tubular' 5,4%. Po przeanalizowaniu zawartości barwników w kwiatach języczkowatych somaklonów wykazano obecność karotenoidów przy jednoczesnym ich braku w kwiatach roślin kontrolnych. Obliczenie współczynnika podobieństwa genetycznego na podstawie wzorów prążkowych uzyskanych za pomocą metody RAPD-PCR pozwoliło na wykazanie odrębności genetycznej uzyskanych somaklonów. Zmienione rośliny powtórzyły swoje cechy po kolejnym rozmnożeniu wegetatywnych.

Słowa kluczowe: chimeryzm, pędy przybyszowe, podobieństwo genetyczne, RAPD

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