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Chrysanthemum plants regenerated from ovaries: a study on genetic and phenotypic variation

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Abstract: Chrysanthemum (*Chrysanthemum* × *grandiflorum* /Ramat./Kitam.) is the second most popular ornamental plant in the global flower industry, and there is still a demand for novelty, which forces breeders to search for new sources of variation. The aim of the present study was the evaluation of phenotypic as well as genetic variation of chrysanthemum plants regenerated from ovaries in vitro. In the first vegetative season of plants evaluated in the glasshouse, nine phenotypic variants (16.36%) and 46 (83.6%) true-to-type plants were observed. The variation included variegated, marble-like, and lighter-green leaves, and changes in the morphology of inflorescences and ligulate florets, as well as changes in the shape of corymb. Variants with variegated and marble-like leaves were unstable. All 55 regenerants had the same ploidy level (2n = 6x) as control plants, estimated by flow cytometry. Genetic analysis based on RAPD-PCR revealed genetic distances ranging from 0.93% to 7.69% between variants and control plants. It was concluded that variable regenerants did not originate from the gynogenic pathway, but they regenerated from somatic tissue and underwent somaclonal variation.

Key words: Genetic distance, ploidy level, RAPD, somaclonal variation

1. Introduction

Chrysanthemum is one of the world's most popular floricultural plants. The National Chrysanthemum Society of Britain lists over 6000 cultivars of this plant (Datta, 2013). Although there is an abundance of chrysanthemum cultivars, rapidly changing demands from chrysanthemum consumers force breeders to continuously create new cultivars. In the production of new cultivars, biotechnological methods are commonly applied, with the extensive application of in vitro techniques involving mutation breeding and utilization of somaclonal variation (Jain, 2001; Schum, 2003).

Conventional methods of breeding are still important at the starting phases of breeding programs, although cross-breeding in chrysanthemum is hindered for many reasons (Anderson, 2007). Recent studies revealed that chrysanthemum is a segmental allohexaploid with an ambiguous pattern of inheritance (Klie et al., 2014). Moreover, self-incompatibility (SI) resulting from the existence of a sporophytic system complicates inheritance studies, as well as homozygous plant production, which is crucial in the breeding process (Drewlow et al., 1973; Wang et al., 2014b). The application of haploids and doubled haploids can solve many problems related to cross breeding, as well as genetic studies on chrysanthemum. Moreover, in other ornamentals, haploids and doubled haploids may serve as a source of new variation for creation of new cultivars, since appearance, not only yielding and resistant traits, is a main goal in breeding of this group of plants (Ferrie, 2012).

Gynogenesis, which is sporophytic development of a female gametophyte, is a method to produce haploid plants (Chen et al., 2011). For gynogenesis, in vitro isolated ovules, ovaries, or whole flowers are usually used as explants. Regeneration from ovules is very low in chrysanthemum, while an abundance of plantlets are obtained from ovaries (Miler and Muszczyk, 2015). The first efforts to produce haploids in chrysanthemum were by Watanabe (1977) with in vitro anther culture of native Japanese species, but without success. In research conducted by Wang et al. (2014a) not more than three plantlets were regenerated from 2579 of inoculated ovules, and only one plant was a haploid. To date, no routine protocols for production of

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haploids or doubled haploids from ovary or ovule culture in chrysanthemum have been reported.

The excision of an ovary is much easier than the isolation of an ovule, which requires microscopic procedures, involves much time, and increases the risk of contamination. Practical aspects, as well as good regeneration of shoots, make ovaries desirable explants for gynogenesis. The problem is that an ovary is a complex organ consisting of an ovule containing an embryo sac, which can be a source of haploid plants, as well as an ovary wall built of somatic cells, which can also regenerate nonhaploid shoots. Thus, one cannot be sure whether the plantlets obtained from ovaries originated from the cells of an embryo sac. Flow cytometry (FCM) is a method commonly used for determination of regenerants' ploidy levels and for screening haploid plants (Leal et al., 2006; Olszewska et al., 2015). As for diploid plants, there is a suspicion of spontaneous diploidization of regenerants, but more likely the regeneration occurs from somatic tissues (Bohanec, 2009). Since it is not possible to distinguish doubled haploids from shoots originated from somatic tissues by flow cytometry, molecular markers may serve as a helpful tool (Wang et al., 2014a).

The aim of the present study was the evaluation of phenotypic as well as genetic variation of chrysanthemum plants (*Chrysanthemum* × *grandiflorum*/Ramat./ Kitam.) regenerated from ovary culture in vitro. We focused on the phenotypic traits of regenerants at the flowering stage, cultivated for two seasons. We also estimated their ploidy level with flow cytometry, as well as the genetic distance using RAPD markers. An effort to answer the question on the origin of variants was made.

2. Materials and methods

2.1. Plant material and cultivation

Plants used in the study were obtained by Miler and Muszczyk (2015) in an experiment aimed to establish a protocol for regeneration of shoots from ovules and ovaries in 'Capitola' chrysanthemum (*Chrysanthemum* × *grandiflorum*/Ramat./ Kitam.). Details on the protocol of disinfection of explants, media preparation culture conditions, and experimental design, as well as regeneration results were described in Miler and Muszczyk (2015). In that experiment a total of 103 regenerants were produced from ovaries in one- or two-step protocols on induction media varying in concentrations of plant growth regulators (PGRs), i.e. 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D). Since the present experiment corresponds to the previous one performed by Miler and Muszczyk (2015), details on PGRs' concentrations and relation to obtained regenerants are given in Table 1. From all regenerants produced, 55 viable shoots were cultivated in a glasshouse and subjected to flow cytometric analysis.

Table 1. The relationship between the number of variants in chrysanthemum 'Capitola' evaluated in the study and their in vitro origin (Miler and Muszczyk, 2015). Total numbers of viable, flowering plants, and the number of in vivo observed variants (with their symbols) regenerated from ovaries in vitro are present in a table, according to the protocols and composition of induction media.

Induction media	One-step protocol[1]			Two-step protocol[2]		
BAP + 2,4 D (mg dm^{-3})	Total	Variants	Symbol	Total	Variants	Symbol
1 + 1	8	1	v35	12	6	v9, v14, v16, v18, v19, v20
1 + 2	0	0		8	0	
1 + 3	2	1	v52	0	0	
2 + 1	1	0		5	1	v4
2 + 2	10	0		3	0	
2 + 3	1	0		3	0	
Total	24	2 (8.3%)		31	7 (22%)	

[1]One-step protocol: explants remained for 15 weeks on induction medium without subculture.
[2]Two-step protocol: after 5 weeks on induction medium, ovaries were subcultured onto regeneration medium (supplemented with 2 mg dm^{-3} kinetin, 1 mg dm^{-3} IAA, and 4 mg dm^{-3} glycine) for the next 10 weeks.

Regenerated shoots were cut off from callus and transferred onto MS (1962) medium supplemented with 2.0 mg dm^{-3} (11.4 µM) indole-3-acetic acid (IAA) in order to initiate root formation. After 10 days, microcuttings were taken out of the media and planted in plastic multiplates filled with peat and perlite (3:1 v/v) substrate. During the next 2 weeks of acclimatization, the microcuttings were covered with a perforated polyethylene film and sprayed with water.

Control plants were obtained from chrysanthemum 'Capitola' mother plants grown in the glasshouse. Shoot tips (approximately 5 cm in length) were dissected from greenhouse-grown mother plants and rooted simultaneously with acclimatization of regenerants conducted under the same conditions. After acclimatization and rooting of cuttings, the plants were transplanted into pots 15 cm in diameter and cultivated in the glasshouse under natural photoperiod until anthesis. At anthesis, the phenotypes of the regenerants were compared with the phenotypes of 25 control plants.

Following flowering, the plants were cut back and maintained as mother plants for the next year of second vegetative propagation. Next season, in the springtime, cuttings of the control and variant plants were taken, rooted, and subsequently grown under natural photoperiod up to full flowering, similar to the previous year. Then the comparison of phenotype traits between control plants and variants took place. There were visually evaluated shapes of the whole corymb and inflorescence, as well as ligulate florets. The color of inner side of ligulate florets as well as the color of leaves was estimated using the Royal Horticultural Colour Chart (RHSCC, 1967).

2.2. Estimation of ploidy level

The ploidy level of all regenerants produced, as well as of the control plants, was determined using a flow cytometry method. Leaves of hexaploid *C.* × *grandiflorum* 'Capitola' (2n = 6x = 54) were used as a reference standard with known ploidy. Samples were prepared according to Galbraith et al. (1983) with some modifications. Plant tissue was chopped with a sharp razor blade in a plastic petri dish containing 1 mL of nucleus-isolation buffer (0.1 M Tris, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1% (v/v) Triton X-100; pH 7.0) supplemented with 4,6'-diamidino-2-phenylindole (DAPI, 2 µg cm^{-3}) and modified by adding 1% (w/v) polyvinylpyrrolidone (PVP-10) to neutralize fluorochrome-staining inhibitors present in the cytosol of chrysanthemum. After chopping, the nuclei suspension was passed through a 50-µm mesh nylon filter. At least 5000 nuclei were measured using a Partec CCA flow cytometer (Münster, Germany), equipped with a mercury UV lamp. Histograms were analyzed using the Partec DPAC v.2.2 software (Münster, Germany).

2.3. Estimation of genetic diversity

Genetic diversity analysis was performed using a RAPD-PCR technique (Welsh and McClelland, 1990; Williams et al., 1990). The analysis was performed for two selected control plants and all seven stable variants that retain their traits after the second vegetative propagation (namely variants v9, v14, v16, v18, v19, v20, and v4).

Genomic DNA was extracted from 100 mg of young and fresh leaves of control plants and all variants using a Genomic Mini AX Plant Kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions. Quality and concentration of extracted genomic DNA were estimated by spectrophotometric method and only samples of high quality were used for RAPD-PCR.

Ten primers (DNA-Gdansk, Poland) were selected for the analysis on the basis of the references on the genetic diversity estimation in chrysanthemum (Miler and Zalewska, 2014); their names as well as sequences are given in Table 2. The DNA amplifications were performed in 25-µL reaction volumes, containing 20 ng of genomic template DNA and 0.5 units 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 using a C1000 Touch thermocycler with a heated lid (BioRad, USA) under the following conditions: one cycle of 4 min at 94 °C; 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 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 separated using 1.8% (w/v) agarose gel electrophoresis, stained with ethidium bromide, in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH

Table 2. The names and sequences of primers used for RAPD genetic analysis.

Primer name	Sequence 5'-3'	Primer name	Sequence 5'–3'
A	GGG AAT TCG G	F	CAA TCG CCG T
B	GAC CGC TTG T	G	GGT GAC GCA G
C	GGA CTG GAG T	H	CCC AGT CAC T
D	GCT GCC TCA GG	I	TGG CGT CCT T
E	TAC CCA GGA GCG	J	AGC GTG TCT G

8.0) at 120 V. A DNA GeneRuler Express DNA Ladder of 100–5000 bp (Thermo Fisher Scientific, USA) was used to determine the size of the fragments. The bands were visualized using the GelDoc system (BioRad, USA).

All RAPD bands obtained for each genotype were counted using a binary scoring system, which recorded the presence or absence of bands as 1 and 0, respectively. The genetic distances (%) were estimated according to Nei and Li (1979) using Treecon v. 1.3 program (van de Peer and de Watcher, 1994) and a dendrogram was constructed, using the unweighted pair group method with arithmetic average (UPGMA).

3. Results

Flow cytometric analysis proved that all samples of the studied chrysanthemum regenerants had the same ploidy level, compared to the reference standard of *C.* × *grandiflorum* 'Capitola', and were hexaploid. Modification of nucleus isolation buffer improved histogram quality, and the mean coefficient of variation (CV) for the regenerants was 3.6% (Figure 1).

All 55 regenerants achieved full development under the glasshouse conditions. In the first season of observations nine phenotypic variants (16.36%) were produced and 46 plants (83.6%) were true-to-type (Table 3). True-to-type regenerants, as well as the control plants, had full, middle-sized inflorescence with flat ligulate florets of dark pink color. As for the inflorescences, the 75 A symbol from the Royal Horticultural Society Colour Chart symbol was corresponding (RHSCC, 1967).

Seven out of the nine phenotypic variants were stable after the second vegetative propagation and observation of phenotypic traits. Two variants were unstable and lost their variation after the second vegetative propagation. Both of them had true-to-type inflorescence but showed chlorophyll defects: variant v52 had marble-like leaves and variant v35 had a white sector on leaves (Figure 2A).

Among the stable variants there were variations concerning ligulate flower shape, the form of the corymb, and leaf color. Variants v9 and v14 represented a compact corymb. In comparison to control plants, inflorescences on the corymbs of the variants were visually smaller and placed closer to the stem (more acute branching angle), and the stem was rigid and straight. The general visual impression was advantageous for plants representing corymb variants in comparison to the control (Figure 2B).

Four variants, i.e. v16, v18, v19, and v20, showed variations involving irregular semifull inflorescences with abnormal ligulate florets grown into tubes in 1/2 to 3/4 of their length, and were prongy at their ends (Figures 2C and 2D). Additionally, in the center of inflorescences there were disc florets present.

One plant had a lighter hue of leaf color; the original color of leaves in the control plants, as well as in true-to type regenerants, was 137 A (RHSCC, 1967), while the color of leaves in variant v4 was 141 C (RHSCC, 1967). This trait remained stable after the next several vegetative propagations.

RAPD primers produced 481 bands, while the total number of generated loci was 57 (Figure 2E). The approximate size of the amplified products ranged from 142 bp for G primer to 3164 bp for C primer. The highest number of loci (8) was produced with primer C, while the lowest number (3 loci) was produced with primer F. The

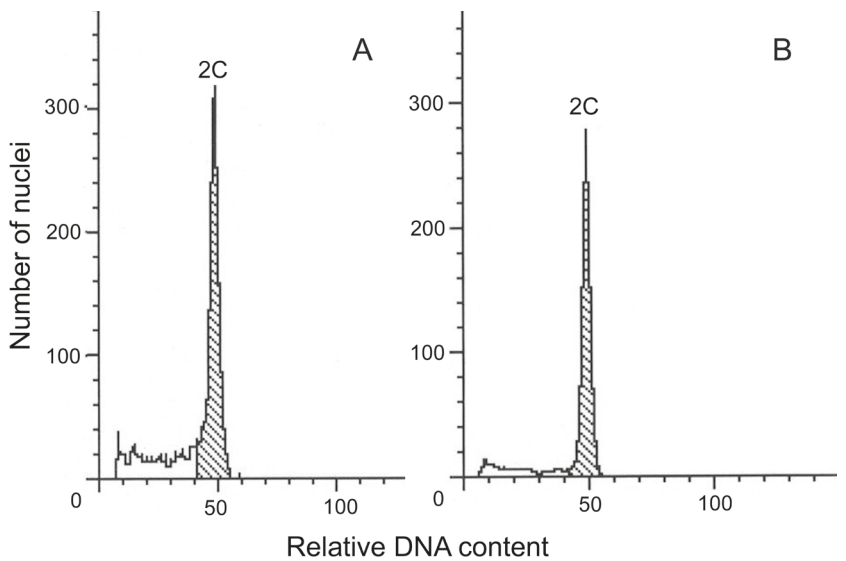

Figure 1. Histograms of *Chrysanthemum* × *grandiflorum* 'Capitola' ploidy level: A – control; B – regenerant.

Table 3. Phenotypic characteristics of chrysanthemum 'Capitola' plants regenerated in vitro from ovaries.

Phenotype name	Description	Number and share (%) of plants	Recurrence of traits after vegetative propagation	Symbol
True-to-type	Full, pink, middle-sized inflorescence; flat, open ligulate florets, no disc florets; loose corymb	46 (83.6)	yes	-
Marble-like leaves	Leaves with irregular, chlorophyll absent, marble-like sectors	1 (1.8)	no	v52
White sector	A sector of shoot with fully or partially white leaves, typical inflorescences and corymb	1 (1.8)	no	v35
Compact corymb	Inflorescences placed on a corymb closer to the axis, rigid and straight stem, smaller inflorescences	2 (3.6)	yes	v9 v14
Irregular ligulate florets	Semi-full irregular inflorescences, ligulate florets grown into tubes in ½ to ¾ of their length, prongy at their ends	4 (7.3)	yes	v16 v18 v19 v20
Lighter green leaves	Leaves representing lighter hue of green than in control plants	1 (1.8)	yes	v4

total share of polymorphic loci for the ten primers and nine genotypes was 19.3%.

The analysis of genetic distance values based on RAPD markers showed that all examined variants showed genetic distances from 0.93% (variants: v9, v14, and v19) to 7.69% (variant v16; Table 4), compared to the control plants. No genetic difference between two examined control plants or between variants v9 and v19 was found. The highest genetic distance was observed for variant v16, with the greatest difference among variants v16 and v4 (9.62%).

The UPGMA clustering algorithm grouped all analyzed plants into three clusters (Figure 3): the first cluster consisted of controls 1 and 2, as well as variants v4, v9, and v19; the second cluster included variants v20, v14, and v18, and the third with only a single genotype of variant v16. Although variants v20, v14, and v18 as well as variants v9 and v19 belonged to the same clusters, they presented phenotypic alternations.

4. Discussion

The chrysanthemum (*Chrysanthemum* × *grandiflorum*/ Ramat./Kitam.) inflorescence is composed of female ligulate florets and bisexual disc florets. The species ovaries contain a single anatropous ovule surrounded by an ovary wall. The application of ovaries as explants for successful gynogenesis was recorded for *Allium cepa* L., *Cucumis sativus* L., *Cucurbita pepo* L., *Hyoscyamus muticus* L., *Lilium longiflorum* L., *Morus alba* L., and *Spathiphyllum wallisii* Regel, as well as for cereal plants: *Zea mays* L., *Hordeum vulgare* L., *Panicum miliacem* L., *Oryza sativa* L., and *Triticum durum* Desf. (Bohanec, 2009). Ovaries may be potentially used for haploid induction since they are less labor-intensive and give good shoot yield, but the origin of regenerants remains ambiguous. There are several methods used to distinguish haploids from nonhaploids, including flow cytometry, chromosome counting, and chloroplast measurements, as well as phenotype observations; however, the distinction between spontaneously doubled haploids and somatic tissue-originated shoots is not so obvious.

In a previous study (Miler and Muszczyk, 2015), shoots regenerated from ovaries in one- or two-step protocols starting from various induction media. In the present study, the evaluation of phenotypic traits under full-flowering stage of the previously regenerated plants was performed. Nine variants, different from control plants, were observed and two of them were unstable after the second vegetative propagation. Corresponding to the previous study (Miler and Muszczyk, 2015), we can assume now that most of the variants (seven out of nine) were obtained in vitro from ovaries in the two-step protocol (Table 1). Frequency of variants (a percentage of not true-to-type plants) was 22.6% in regenerants obtained from ovaries subcultured on regeneration medium and 8.3% in regenerants nonsubcultured on regeneration medium (one-step protocol). Interestingly, the only unstable variants with chlorophyll defects (variants v35 and v52) were obtained from the one-step protocol. The highest frequency of variants was obtained from ovaries regenerated on induction medium containing 1 mg dm^{-3} BAP and 1 mg dm^{-3} 2,4-D in the two-step protocol.

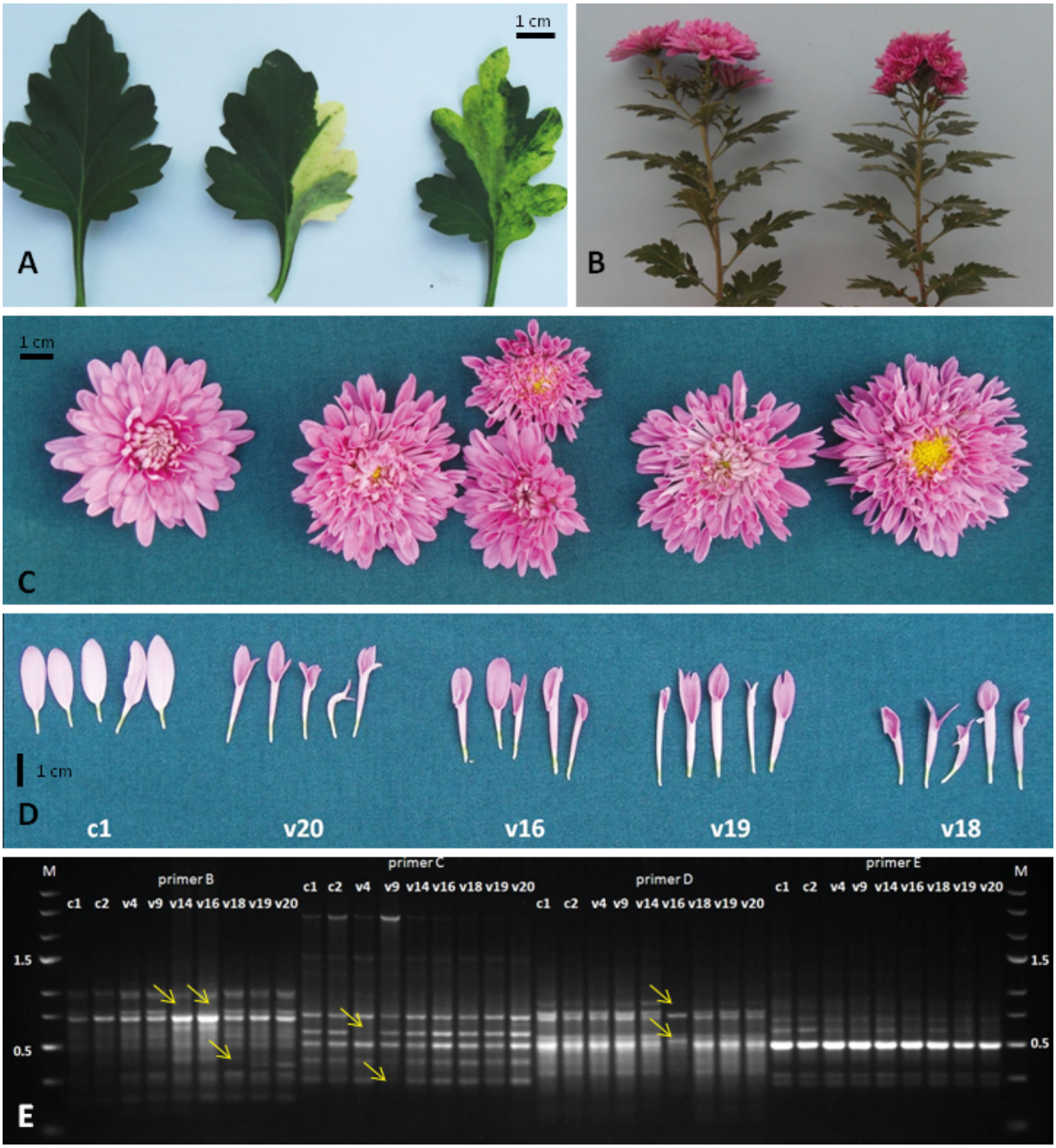

Figure 2. A. From the left: leaves of control plant, variant v35 with a white sector and a marble-like variant v52. B. Control 'Capitola' chrysanthemum with typical corymb (left) and a plant v9 representing variants with compact corymb (right). C and D. Inflorescences (C) and ligulate florets (D) of a control 'Capitola' chrysanthemum and variants with irregular florets, prongy, and grown into tubes. E. An example of banding pattern of RAPD markers for 'Capitola' chrysanthemum control plants (lines c1 and c2) and variants regenerated in vitro from ovaries generated with primers B, C, D, E. Arrows indicate variable bands; M – DNA ladder (kbp).

The presence of nontrue-to-type plants may be an effect of regeneration from generative cells as well as from vegetative cells that have undergone somaclonal variation. All regenerants represented the same ploidy level as control plants. No haploids were observed. The question we posed was whether they had been spontaneously doubled haploids or somaclonal variants.

Chrysanthemum ligulate florets with ovaries were previously used in many experiments related to in vitro mutagenesis, somatic embryo regeneration, and

Table 4. Genetic distance (%) between control plants (c1 and c2) and variants of 'Capitola' chrysanthemum regenerated in vitro from ovaries, estimated on the basis of RAPD markers generated with ten primers.

Symbol	c1	c2	v4	v9	v14	v16	v18	v19	v20
c1	0.00								
c2	0.00	0.00							
v4	1.87	1.87	0.00						
v9	0.93	0.93	0.93	0.00					
v14	0.93	0.93	2.80	1.85	0.00				
v16	7.69	7.69	9.62	8.57	6.67	0.00			
v18	1.85	1.85	1.85	0.92	0.92	7.55	0.00		
v19	0.93	0.93	0.93	0.00	1.85	8.57	0.92	0.00	
v20	2.80	2.80	2.80	1.85	1.85	8.57	0.92	1.85	0.00

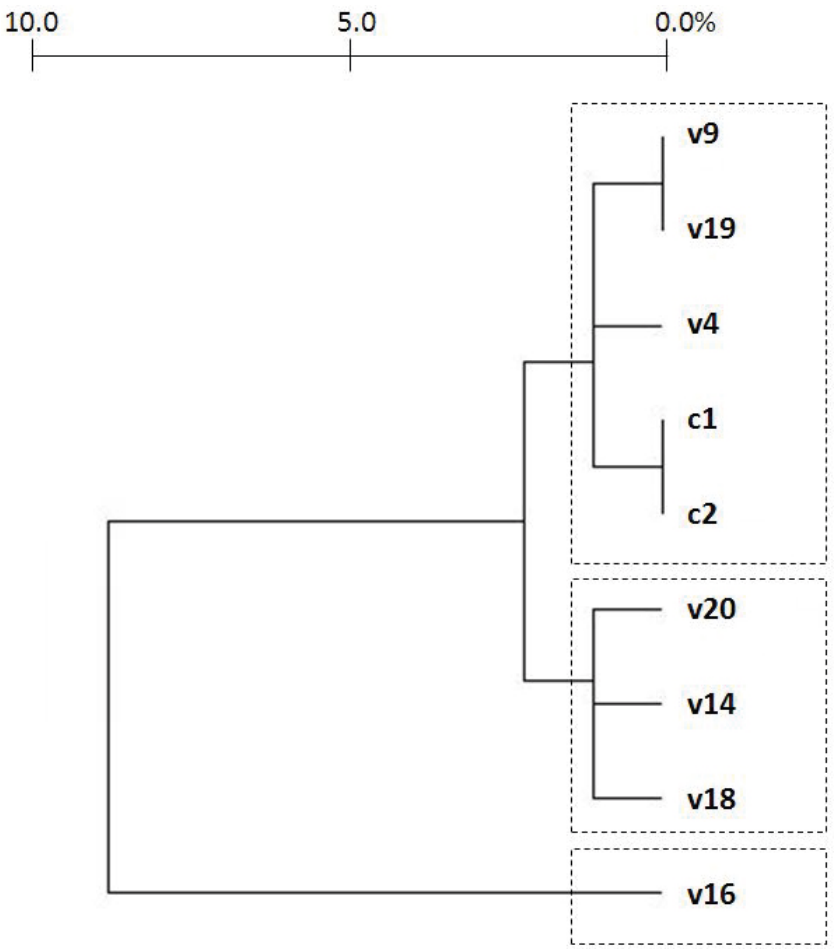

Figure 3. Dendrogram based on UPGMA cluster analysis estimated on the basis of RAPD markers illustrating genetic distances (%) between control plants (c1 and c2) of chrysanthemum 'Capitola' and of variants regenerated in vitro from ovaries.

organogenesis, as well as in separation of chimera components (Teixeira da Silva et al., 2015). Since many spontaneous inflorescence color mutations are visible as tiny spots or streaks on flowers, florets can be used as a source of variable tissue for breeding purposes (Chakrabarty and Datta, 2010). Regeneration of adventitious shoots from ligulate florets often occurs in the ovary region (Tymoszuk and Zalewska, 2014). Kengkarj et al. (2008) applied ligulate florets of seven chrysanthemum cultivars as explants in vitro and obtained 29 somaclones differing in inflorescence colors and shapes.

In our previous experiment (Miler and Muszczyk, 2015), we did not use whole florets but ovaries and although they were tiny (approx. 1–1.5 mm) they produced an abundance of callus tissue, from which shoots originated. Callus is thought to be a genetically unstable tissue, with a great potential for somaclonal variation (Karp, 1995). We assume that if regenerants are recovered from callus of somatic tissue-origin (e.g., from the ovary wall) they can show phenotypic as well as genetic variation resulting from somaclonal variation. It is important to emphasize that the shoots evaluated in the study cannot be a result of germination of self-pollinated embryos since: (i) most of chrysanthemums are self-incompatible (Wang et al., 2014b), (ii) ligulate florets were used, which do not produce pollen and (iii) the 'Capitola' cultivar has a full type of inflorescence, which means that only a few incidental disc florets (which produce pollen) could be present.

The genetic background of somaclonal changes in chrysanthemum can be confirmed by RAPD analysis (Newbury and Ford-Lloyd, 1993). In the research conducted by Kengkarj et al. (2008), the genetic distance evaluated on the basis of RAPD performed with ten primers ranged from 9% to 52% among somaclones obtained from ligulate florets and a mother cultivar; somaclones differed also phenotypically in terms of inflorescence color and shape. In addition, somaclonal variation was detected using RAPD markers in chrysanthemum regenerated from meristems and shoot culture, though no morphological changes in acclimatized plants were recorded (Miňano et al., 2009). RAPD analysis with application of the same set of primers as we used in the recent study also confirmed the genetic background of three somaclones differing in inflorescence color from two mother cultivars, obtained from in vitro culture of leaves (Miler and Zalewska, 2014).

In our experiment, all stable variants showed genetic distance higher than 0.0 compared to control plants. There was no genetic distance between variants v9 and v19 only, although the variants differed morphologically. The highest genetic distance was among variants v9 and v16. Variant v16 also showed the highest genetic distance to control plants. The coefficients of genetic distances in the whole group of studied plants did not exceed 10%. In research conducted by Miler and Zalewska (2014), genetic distances between related cultivars belonging to groups consisting of a mother cultivar and its mutants ranged from 2% to 31%, while between nonrelated cultivars the coefficients ranged from 30% to 50% (Kengkarj et al., 2008). Since chrysanthemums are highly heterozygous, one can expect that the genotype of gametophyte-derived plants should be considerably different than that of mother plants (Anderson, 2007). Thus, on the basis of RAPD markers analysis and relatively low coefficients of genetic distance, we can assume that variants obtained in the present study were the result of somaclonal variation rather than spontaneous diploidization. Perhaps the employment of a more sensitive method of genetic analysis, e.g., microsatellite fingerprinting or SCoT markers, would give more information on the origin of variants (Wang et al., 2014a; Feng et al., 2016).

Methods for enhancement of somaclonal variation were screened previously and it was found that the application of various explants (leaves and internodes as well as ligulate florets) for regeneration in chrysanthemum results in the development of novel variants (Kengkarj et al., 2008; Miler and Zalewska, 2014). Variants observed in the experiment present novel decorative values; especially v9 and v14 seem to be attractive due to their compact corymb, which is generally an advantage. From the breeder's point of view, although ovaries did not provide us with haploids or doubled haploids, they can still be efficiently used as explants performing somaclonal variation for breeding of chrysanthemum (*Chrysanthemum* × *grandiflorum* Ramat./Kitam.).

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