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In vitro ADVENTITIOUS SHOOTS REGENERATION FROM LIGULATE FLORETS IN THE ASPECT OF APPLICATION IN CHRYSANTHEMUM BREEDING

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Abstract. Chrysanthemum mutants can be chimeras. The regeneration *in vitro* of adventitious shoots from ligulate florets can lead to the separation of chimera components, resulting in producing a new cultivar. There was determined the effect of various factors on the number and length of shoots regenerating *in vitro* from ligulate florets of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time'. The ligulate florets were inoculated on the MS [1962] medium supplemented with cytokinin (0; 4.44; 8.88; 13.32; 22.20 $\mu M \cdot dm^{-3}$ BAP; 4.65; 23.23; 46.47; 69.70 $\mu M \cdot dm^{-3}$ KIN) and auxin (0; 0.54; 1.08; 1.61; 2.69 $\mu M \cdot dm^{-3}$ NAA). Most shoots regenerate when 8.88; 13.32 $\mu M \cdot dm^{-3}$ BAP or 69.70 $\mu M \cdot dm^{-3}$ KIN and 2.69 $\mu M \cdot dm^{-3}$ NAA or 8.88 $\mu M \cdot dm^{-3}$ BAP and 1.61 $\mu M \cdot dm^{-3}$ NAA are supplemented. Adding 0.29; 1.44 or 2.89 $\mu M \cdot dm^{-3}$ GA_3 to the MS medium with 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA limits the shoot regeneration efficiency and does not stimulate their elongation. An increase in the shoot number and length is affected by the subculture of regenerating ligulate florets from the MS medium containing 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA on the medium with 2.89 $\mu M \cdot dm^{-3}$ GA_3 and 2.69 $\mu M \cdot dm^{-3}$ NAA. There were found no differences in the number and length of shoots regenerating on ligulate florets inoculated on solid or in liquid MS medium with 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA. The subculture of regenerating ligulate florets from the solid into liquid medium increases the number of regenerating shoots and stimulates their elongation growth, however these shoots are deformed.

Key words: *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam., petal, micropropagation, growth regulators

INTRODUCTION

Chrysanthemums show a high tendency to spontaneous [Malaure et al. 1991a] and induced mutations, which is commonly applied to chrysanthemum breeding [Zalewska

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et al. 2010, Zalewska et al. 2011]. Chimeras are created as a result of the occurrence of mutation within the meristem [Tilney-Bassett 1986]. In sectorial chimeras the tissue sector is genetically different, covering all the histogenic layers. In periclinal chimeras a different genotype is presented in one or two whole layers. In mericlinal chimeras a part of one or two layers is genetically different [Burge et al. 2002]. The emergence of chimeras is a large limitation for mutation breeding [Chakrabarty et al. 1999]. Many spontaneous or induced inflorescence-colour mutations cover only a few entire ligulate florets, a single ligulate floret, only its part or the mutation appears only as a spot or stripe from a single to a few thousand cells in size [Stewart and Dermen 1970, Malaure et al. 1991b, Chakrabarty and Datta 2010]. Due to a small area of tissues, such mutations are lost since one cannot preserve them with any chimera component separation methods available; hence the need to develop new methods of separating chimera component genotypes and thus producing new cultivars. Such a possibility is available through the regeneration of adventitious shoots from the entire ligulate florets or only their fragments changed in colour sampled from chimera inflorescences. In culture *in vitro* adventitious shoots, formed from single cells of histogenic layer L1 or L2 of ligulate floret, will present, respectively, the genotype of layer L1 or L2 and they will be organisms built up only from genetically homogenous tissues [Malaure et al. 1991b, Chakrabarty et al. 1999, Chakrabarty et al. 2000, Mandal et al. 2000a]. Besides, over the last few years, many chrysanthemum breeding programs have been focusing on rooted cuttings irradiation *in vivo* and then the separation of chimera components via the regeneration of adventitious shoots from ligulate florets [Chakrabarty and Datta 2010].

The regeneration of adventitious shoots in chrysanthemums depends on the type and the concentration of growth regulators, especially auxins and cytokinins. In general, one can differentiate between three groups of cultivars in terms of regeneration: requiring auxins at a higher concentration than cytokinins, requiring cytokinins at a higher concentration than auxins as well as requiring auxins and cytokinins at similar concentrations, despite the general common tendency that a high concentration of cytokinins and a low concentration of auxins induces the formation of shoots and a high concentration of auxins and a low concentration of cytokinins induces rhizogenesis [Park et al. 2005, 2007]. The regeneration *in vitro* of adventitious shoots is also affected by e.g. the factors including the genotype [Park et al. 2005, 2007], the explant type [Lu et al. 1990], the level of endogenous growth regulators [Kumar and Kanwar 2006], the type of the substance used to solidify the medium [Zalewska 2010]. In liquid media the cell divisions as well as a further regeneration and growth occur faster and are more even. Shaking prevents the formation of the gradients of nutrition substances as well as growth regulators [Pierik 1987]. Gibberellins in cultures *in vitro* trigger the elongation of shoot internodes. Usually they inhibit adventitious shoots regeneration from callus tissue cells, although in some species they trigger their formation. However, if adventitious morphogenesis has already been initiated by other growth regulators, having added gibberellins, an increase in the number of structures being formed is observed [Omura and Hidaka 1992 in Paudyal and Haq 2000, Maggon and Singh 1995].

The aim of the present research has been determined as defining the effect of growth regulators on the regeneration of adventitious shoots from *in vitro* isolated ligulate florets of 'Cool Time' chrysanthemum. There was also investigated the effect of GA_3 and

liquid medium on the regeneration and elongation growth of adventitious shoots. The more shoots we obtain, the highest is the chance for breeding new cultivars.

MATERIAL AND METHODS

For the purpose of research, *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time' was selected. It is a pot cultivar, with half-full inflorescences with a green and yellow disk of tubular florets visible, ligulate florets white in colour. Right after planting, the generative development was induced by exposing the cuttings to the short day. Shading was applied from 6 pm do 8 am, shortening the natural day to 10 hours. After 5 days of growing under short day, chrysanthemums were pinched out over the 5th leaf, counting from the bottom of the shoot. All the buds set on branched shoots were brought to flowering.

The ligulate florets were sampled from inflorescences completely open in which two whorls of tubular florets produced pollen. They were rinsed under running water. Then they were placed in 5% detergent solution for 5 minutes. In sterile conditions in the laminar air-flow cabinet the ligulate florets were transferred into 70% ethanol solution for 5 seconds. They were then incubated in a 0.5% solution of sodium hypochlorite for 5 minutes and rinsed three times for 10 minutes in sterile distilled water. Prior to the inoculation onto the medium the explants were dried on sterile paper.

The MS [Murashige and Skoog 1962] medium was used modified by increasing the content of calcium and iron by half. The medium included 3% (w/v) of sucrose. The medium was solidified with 0.8% (w/v) Duchefa agar. Having added all the nutrients, prior to autoclaving, pH of the medium was set at the level of 5.8. The 350 ml jars and 100 ml laboratory flasks were poured with 30 ml of the medium each. One explant was inoculated into a jar/ laboratory flask.

Cultures *in vitro* were maintained in the growth room at the temperature of 24 ±2ºC, exposed to a 24-hour photoperiod (16 hours of light, 8 hours of dark), using fluorescent lamps Philips TLD 36W/54 emitting daylight. The quantum irradiation intensity was set up at 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The effect of the growth regulators on the regeneration of adventitious shoots

The solid medium was supplemented with cytokinin (0; 4.44; 8.88; 13.32; 22.20 $\mu M \cdot dm^{-3}$ BAP; 4.65; 23.23; 46.47; 69.70 $\mu M \cdot dm^{-3}$ KIN) and auxin (0; 0.54; 1.08; 1.61; 2.69 $\mu M \cdot dm^{-3}$ NAA). The horizontal inoculation with the abaxial side of entire ligulate floret onto the medium was applied. The experiment was set up in a completely randomised design, for two factors. For each of the 45 experimental treatments 12 replications were applied, one explant for each.

The effect of GA_3 and liquid medium on the regeneration and elongation growth of adventitious shoots

A. Adventitious shoots regeneration on the GA_3 containing media. Ligulate florets were inoculated onto solid medium supplemented with 8.88 $\mu M \cdot dm^{-3}$ BAP and

2.69 μM·dm^{-3} NAA, as well as GA_3 at the concentration of 0.29; 1.44; 2.89 μM·dm^{-3}. Each of the experimental treatments covered 30 replications, one explant for each.

B. Subculturing explants regenerating adventitious shoots on the media containing GA_3 instead of BAP. Culture *in vitro* was initiated on a solid medium the composition of which was supplemented with 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA. After 7 weeks the explants with shoots were subcultured onto solid medium containing 2.69 μM·dm^{-3} NAA as well as GA_3 at the concentration of 0.29; 1.44; 2.89 μM·dm^{-3}. Twelve explants were subcultured onto each medium, one per jar.

C. Regeneration of adventitious shoots in liquid medium. Ligulate florets were placed in liquid medium supplemented with 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA. There were used 30 replications, one explant for each. After 7 weeks ligulate florets were subcultured to fresh medium with the same composition.

D. Subculturing explants regenerating adventitious shoots into liquid medium. Ligulate florets were inoculated onto solid medium with 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA added. After 7 weeks of maintaining culture *in vitro* 12 explants together with shoots were transferred one by one to liquid medium with the same composition of growth regulators.

For all the experiments the control treatment was made up by cultures *in vitro* maintained on solid medium supplemented with 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA. There was placed a total of 30 ligulate florets, one per jar.

Ligulate florets were transversely cut into half and horizontally inoculated with the abaxial side onto the medium.

GA_3 was added to the media only after their sterilization using sterile filters Minisart RC-15 provided by Sartorius Stedim Biotech. GmbH with the pores 0.20 μm in diameter. Cultures on liquid media were shaken at the frequency of 100 rotations per minute. The experiments were set up in a completely randomised design for a single factor.

Counting all the shoots regenerated was possible only once the explants were taken out from the jars. First there were isolated shoots well-visible with a naked eye and then observations of explants were made under the stereoscopic microscope MS-Z TRI provided by Precoptic at magnification from 0.7 × 10 to 4.5 × 10. All the shoots regenerated were divided, for practical reasons, into two groups: ≥0.5 cm long (adequate for successive stages of micropropagation as multiplication or rooting) as well as <0.5 cm long (additionally requiring elongation prior to the stages of multiplication or rooting). The effect of growth regulators contained in the medium on the shoot regeneration was defined quantitatively by calculating the mean number of all the shoots per explant inoculated, as well as the mean number of shoots ≥ or <0.5 cm long per explant. There was also calculated the percentage share of the explants regenerating shoots.

As for subculture of the explants regenerating shoots onto solid media containing GA_3 or into liquid medium, the results verified statistically were as follows: mean number of all the shoots as well as shoots ≥ or <0.5 cm long per regenerating explant.

The real numerical data (x) for the mean number of shoots were transformed by $\log_{10}(x + 1)$, while for the data expressed as percentage, the Freeman-Tukey transformation was used. After the transformation, the results were statistically verified using the

method of the analysis of variance and means for the treatment were evaluated with the Newman-Keuls test at the significance level of P = 0.05. Tables with results provide real numerical data, while alphabet letters point to homogenous groups having made the statistical calculations based on transformed data.

RESULTS AND DISCUSSION

The effect of growth regulators on the regeneration of adventitious shoots from ligulate florets

In the first week after the initiation of culture *in vitro*, irrespective of adding growth regulators to the medium, ligulate florets got bigger, most clearly in the proximal part, in the region of the ovary. In the second week the explants started proliferating callus in the region of the ovary. Then, successively, starting from the third week, callus appeared on the surface of the petals. The explants inoculated onto the medium without growth regulators or containing only BAP, KIN or NAA did not proliferate the callus tissue. The first shoots were observed in the fourth week of culture. Regeneration occurred via the callus tissue.

The statistical analysis of the data suggests that the regeneration of shoots from ligulate florets in the cultivar investigated strongly depends on the qualitative and quantitative composition of the growth regulators added to the medium (tab. 1). The shoots are formed only when the medium contains both cytokinin and auxin at the right concentration. With a lack of cytokinin in the medium and when enriching the medium with 4.65 or 46.47 $\mu M \cdot dm^{-3}$ KIN, ligulate florets did not show a capacity for shoot organogenesis at all. Neither did the explants inoculated on the medium without NAA added form any shoots.

Most shoots in total and including those ≥0.5 cm long were regenerated by ligulate florets placed onto the media supplemented with 8.88 $\mu M \cdot dm^{-3}$ BAP and 1.61 $\mu M \cdot dm^{-3}$ NAA; 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA; 13.32 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA; 69.70 $\mu M \cdot dm^{-3}$ KIN and 2.69 $\mu M \cdot dm^{-3}$ NAA (tab. 1, fig. 1A).

Significantly most shoots <0.5 cm long were formed on the explants inoculated onto media with 4.44; 8.88; 13.32 $\mu M \cdot dm^{-3}$ BAP or 69.70 $\mu M \cdot dm^{-3}$ KIN and 2.69 $\mu M \cdot dm^{-3}$ NAA and on the media with 8.88 $\mu M \cdot dm^{-3}$ BAP and 1.08 or 1.61 $\mu M \cdot dm^{-3}$ NAA (tab. 1, fig. 1B).

When the medium was supplemented with 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA from a single explant, as many as more than 36 shoots regenerated. The medium was selected to perform further research.

The percentage share of explants initiating shoot regeneration was greatest when the medium was added with 8.88 $\mu M \cdot dm^{-3}$ BAP and 1.61 or 2.69 $\mu M \cdot dm^{-3}$ NAA (tab. 1).

In the cultivar investigated on the media on which shoots regeneration took place, the concentration of cytokinin was always higher than the auxin concentration. Malaure et al. [1991a] report that on the fragments of petals in 16 chrysanthemum cultivars the adventitious shoots regeneration occurred first as affected by BAP in the combination with NAA. The optimal concentration of those growth regulators differed depending on

Table 1. Effect of the growth regulators added to the MS medium on adventitious shoots regeneration (mean ±SD)

Concentration of growth regulators (μM·dm^{-3})			Number of shoots per explant inoculated			Explants regenerating shoots (%)
BAP	KIN	NAA	All	≥0.5 cm long	<0.5 cm long	
0.00	0.00	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
0.00	–	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
0.00	–	1.08	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
0.00	–	1.61	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
0.00	–	2.69	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
4.44	–	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
4.44	–	0.54	0.50 ± 0.50^{b}	0.00 ± 0.00^{c}	0.50 ± 0.50^{b}	8.33^{c}
4.44	–	1.08	0.58 ± 0.50^{b}	0.33 ± 0.26^{c}	0.25 ± 0.25^{b}	16.67^{bc}
4.44	–	1.61	1.17 ± 0.77^{b}	0.67 ± 0.51^{c}	0.50 ± 0.34^{b}	25.00^{bc}
4.44	–	2.69	3.50 ± 3.50^{b}	1.25 ± 1.25^{c}	2.25 ± 2.25^{ab}	8.33^{c}
8.88	–	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
8.88	–	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
8.88	–	1.08	1.42 ± 1.08^{b}	0.50 ± 0.42^{c}	0.92 ± 0.69^{ab}	25.00^{bc}
8.88	–	1.61	8.83 ± 6.90^{a}	4.08 ± 2.56^{a}	4.75 ± 4.39^{ab}	58.33^{a}
8.88	–	2.69	36.09 ± 31.03^{a}	16.17 ± 14.30^{a}	19.92 ± 16.82^{a}	41.67^{ab}
13.32	–	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
13.32	–	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
13.32	–	1.08	0.08 ± 0.08^{c}	0.08 ± 0.08^{c}	0.00 ± 0.00^{b}	8.33^{c}
13.32	–	1.61	0.42 ± 0.42^{b}	0.42 ± 0.42^{c}	0.00 ± 0.00^{b}	8.33^{c}
13.32	–	2.69	14.15 ± 9.48^{a}	2.07 ± 1.34^{abc}	12.08 ± 8.14^{a}	33.33^{b}
22.20	–	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
22.20	–	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
22.20	–	1.08	0.17 ± 0.17^{c}	0.00 ± 0.00^{c}	0.17 ± 0.17^{b}	8.33^{c}
22.20	–	1.61	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
22.20	–	2.69	0.33 ± 0.26^{b}	0.00 ± 0.00^{c}	0.33 ± 0.26^{b}	16.67^{bc}
–	4.65	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	4.65	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	4.65	1.08	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	4.65	1.61	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	4.65	2.69	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	23.23	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	23.23	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	23.23	1.08	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	23.23	1.61	0.25 ± 0.25^{c}	0.25 ± 0.25^{c}	0.00 ± 0.00^{b}	8.33^{c}
–	23.23	2.69	0.42 ± 0.29^{b}	0.17 ± 0.17^{c}	0.25 ± 0.25^{b}	16.67^{bc}
–	46.47	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	46.47	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	46.47	1.08	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	46.47	1.61	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	46.47	2.69	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	69.70	0.00	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	69.70	0.54	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	69.70	1.08	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	69.70	1.61	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00^{c}
–	69.70	2.69	4.00 ± 2.19^{ab}	3.00 ±1.80^{abc}	1.00 ± 0.58^{ab}	25.00^{bc}

a, b, c… – means in columns followed by the same letter do not differ significantly at P = 0.05 (Newman-Keuls test)

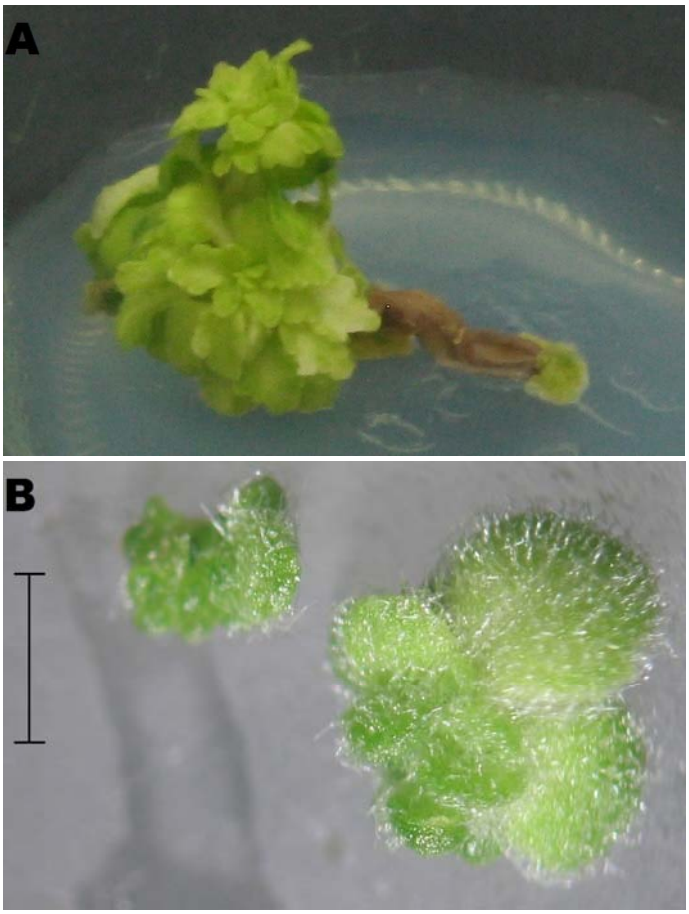

Fig 1. Regeneration of adventitious shoots from the ligulate florets of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time' on the MS medium supplemented with 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA; A shoots regenerating during culture; B shoots <0.5 cm long isolated from the explant; bar 1 mm

the genotype. The cultivars did not form shoots on the media with KIN and IAA added. The best medium for the regeneration of shoots from the explants from ligulate florets in chrysanthemums 'Biarizte', 'Yellow Biarizte', 'Storika', 'Pinkgin', 'Linker Pink', 'Dark Linker Salmon' and 'Bari' contained 6.66 $\mu M \cdot dm^3$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA [Kengkarj et al. 2008], in chrysanthemum 'PKV Shubhra' 8.88 $\mu M \cdot dm^{-3}$ BAP and 8.56 $\mu M \cdot dm^3$ IAA [Lakshmi et al. 2006], and in chrysanthemum 'Shiroyamate' 13.32 $\mu M \cdot dm^3$ BAP and 53.74 $\mu M \cdot dm^3$ NAA [Matsumura et al. 2010]. Mandal et al. [2000a], for chrysanthemum 'Maghi', received the best regeneration result on the medium supplemented with 23.23 $\mu M \cdot dm^3$ KIN and 5.37 $\mu M \cdot dm^3$ NAA. Unlike in the present research, the shoots in that experiment were being formed on the media containing 4.65; 23.23 or 46.47 $\mu M \cdot dm^3$ KIN and 1.08 $\mu M \cdot dm^3$ NAA.

The capacity for shoot regeneration in chrysanthemum depends strongly on the cultivar [Park et al. 2005, Nahid et al. 2007, Park et al. 2007]. The same growth regulators can show a different effect not only on the explants from the groups distant in terms of taxonomy but also on the cultivars and clones of the same species. To receive a satisfactory regeneration result in a given genotype, it is indispensable to define a genotype-specific composition and concentration of growth regulators. That effect can be due to the genotypic differences in terms of the capacity for uptaking respective growth regulators from the medium and then metabolizing those growth regulators [Nahid et al. 2007]. The cultivar-specificity in that aspect should be considered especially in breeding programs when the number of regenerated shoots should be possibly the highest to increase the chances for receiving a new cultivar at the same time.

Chakrabarty et al. [1999] in 'Colchi Bahar' chrysanthemum and Datta et al. [2001] in 'Puja' chrysanthemum report on intensive regeneration at the edge of the proximal part of the entire ligulate florets. The organogenesis of shoots in the proximal part of leaf explants was also observed in *Gypsophila paniculata* L. [Ahroni et al. 1997, Zuker et al. 1997]. Differentiating leaf cells in dicotyledonous plants usually starts in the distal part and progresses towards the proximal part. As a result, the cells from the proximal part are least varied and most frequently participate in adventitious organogenesis. Petals being transformed leaves show a similarity in that aspect [Messeguer et al. 1993], which could also be observed in the present experiments.

The effect of GA_3 and liquid medium on the regeneration and elongation growth of adventitious shoots

The first week after the start of cultures *in vitro* ligulate florets got bigger – ovaries most considerably. Starting from the third week the callus regeneration started, first around the ovary and then on the surface of petals, especially at cutting regions. Callus was being formed on all the explants inoculated in the research. Shoot regeneration occurred via the callus, starting from the fourth week of cultures.

A. Adventitious shoots regeneration on the media containing GA_3. Additional adding of gibberellic acid to the medium containing 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA decreased the efficiency of adventitious shoots regeneration. Neither did it affect the stimulation of shoots elongation growth (tab. 2). On the media containing GA_3 there was also observed a less intensive appearance of shoots <0.5 cm long. The percentage share of the explants initiating shoots regeneration on the media with GA_3 was much lower than on the medium without growth regulator.

B. Subculturing explants regenerating adventitious shoots on the media containing GA_3 instead of BAP. Most shoots, 10 on average, was produced from ligulate florets subcultured from the medium containing 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA onto the medium with 2.89 $\mu M \cdot dm^{-3}$ GA_3 and 2.69 $\mu M \cdot dm^{-3}$ NAA added (tab. 3). Subculturing explants onto that medium also affected mostly the stimulation of shoots elongation growth. None of the combinations significantly increased the average number of shoots <0.5 cm long.

Table 2. Effect of the concentration of GA_3 added to the MS medium + 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA on adventitious shoots regeneration (mean ±SD)

Concentration of GA_3 ($\mu M \cdot dm^{-3}$)	Number of shoots per explant inoculated			Explants regenerating shoots (%)
	All	≥0.5 cm long	<0.5 cm long	
0.00	1.42 ± 0.25^{a}	0.29 ± 0.09^{a}	1.13 ± 0.25^{a}	75.00^{a}
0.29	0.33 ± 0.23^{b}	0.00 ± 0.00^{b}	0.33 ± 0.23^{b}	18.52^{b}
1.44	0.11 ± 0.23^{b}	0.00 ± 0.00^{b}	0.11 ± 0.23^{b}	7.41^{b}
2.89	0.36 ± 0.23^{b}	0.11 ± 0.08^{b}	0.25 ± 0.23^{b}	17.86^{b}

a, b, c… – means in columns followed by the same letter do not differ significantly at P = 0.05 (Newman-Keuls test)

Table 3. Effect of the concentration of GA_3 added into the MS medium with 2.69 $\mu M \cdot dm^{-3}$ NAA used for the subculture of explants regenerating adventitious shoots (mean ±SD)

Concentration of GA_3 ($\mu M \cdot dm^{-3}$)	Number of shoots per explant regenerating		
	all	≥0.5 cm long	<0.5 cm long
0.00[1)]	1.89 ± 1.11^{b}	0.39 ± 0.94^{c}	1.50 ± 0.50^{a}
0.29	3.00 ± 1.41^{b}	2.55 ± 1.26^{bc}	0.45 ± 0.64^{a}
1.44	4.55 ± 1.56^{b}	3.33 ± 1.39^{b}	1.22 ± 0.71^{a}
2.89	10.00 ± 1.77^{a}	8.86 ± 1.58^{a}	1.14 ± 0.80^{a}

[1)] control – MS medium + 8.88 $\mu M \cdot dm^{-3}$ BAP and 2.69 $\mu M \cdot dm^{-3}$ NAA without subculturing
a, b, c… – means in columns followed by the same letter do not differ significantly at P = 0.05 (Newman-Keuls test)

C. Regeneration of adventitious shoots in liquid medium. The application of liquid medium, as compared with solid medium with the same composition, does not increase the efficiency of shoot regeneration significantly (tab. 4). On the solid and in liquid media there were produced both shoots ≥0.5 cm and <0.5 cm long. However, there were shown no differences in their number. On the solid medium the percentage share of explants initiating shoot regeneration accounted for as much as 75% and it was definitely higher than in the liquid medium.

D. Subculturing explants regenerating adventitious shoots into the liquid medium. Ligulate florets subcultured from the solid medium into liquid medium supplemented with the same growth regulators formed 3.5-fold more shoots (tab. 5). In the liquid medium there were formed on average more than fourteen-times more shoots ≥0.5 cm long than in the solid medium. There was shown no significant effect on the mean number of shoots <0.5 cm long. However, all the shoots isolated from ligulate florets subcultured into liquid medium were deformed and grown with callus tissue, which ruined their application potential for further stages of micropropagation completely and, finally, for growing in conditions *in vivo*.

Table 4. Effect of the application of the solid or liquid MS medium on adventitious shoots regeneration (mean ±SD)

MS medium + 8.88 μM·dm^{-3} BAP + 2.69 μM·dm^{-3} NAA	Number of shoots per explant inoculated			Explants regenerating shoots (%)
	all	≥0.5 cm long	<0.5 cm long	
Solid	1.42 ± 1.93[a]	0.29 ± 0.46[a]	1.13 ± 1.59[a]	75.00[a]
Liquid	4.50 ± 2.01[a]	1.23 ± 0.48[a]	3.27 ± 1.66[a]	13.64[b]

a, b, c… – means in columns followed by the same letter do not differ significantly at P = 0.05 (Newman-Keuls test)

Table 5. Effect of the application of the liquid MS medium for explant subculturing on adventitious shoots regeneration (mean ±SD)

MS medium + 8.88 μM·dm^{-3} BAP + 2.69 μM·dm^{-3} NAA	Number of adventitious shoots per explant regenerating		
	all	≥0.5 cm long	<0.5 cm long
Solid	1.89 ± 1.06[b]	0.39 ± 0.82[b]	1.50 ± 0.72[a]
Liquid	6.91 ± 1.29[a]	5.58 ± 1.0[a]	1.33 ± 0.88[a]

a, b, c… – means in columns followed by the same letter do not differ significantly at P = 0.05 (Newman-Keuls test)

In *Narcissus* 'Carlton' cultivar there was noted a relationship between the length of the shoots produced and the type of the explant they were derived from. As many as 97% of the shoots from the proximal part of the stem and 96.7% of the shoots derived from pedicles with ovaries did not reach the length of 0.5 cm [Kozak 1991]. The necessity of the stimulation of the elongation of shoots received from the entire ligulate florets or fragments of petals is reported by e.g. teamwork papers, including Chakrabarty et al. [1999] or Park et al. [2007]. Such situation limits the number of the shoots produced which are to be brought to flowering on purpose, and that does not remain without any effect on breeding success. For that reason in the present research GA_3 was added to the media.

GA_3 applied at the concentration of 0.12 or 1.16 μM·dm^3 as the only growth regulator or in combination with 8.88 μM·dm^{-3} BAP totally inhibited shoot regeneration from the fragments of epicotyl and hypocotyl in *Citrus sinensis* L. Osbeck. The elongation growth of the shoots received on the medium with 8.88 μM·dm^{-3} BAP and 0.76 μM·dm^3 ABA was possible once they were subcultured on the medium supplemented with 0.12 μM·dm^3 GA_3 [Maggon and Singh 1995]. Fragments of ligulate florets in 'Kasturba Gandhi' chrysanthemum, having been inoculated onto the medium with 4.44 μM·dm^3 BAP and 1.08 μM·dm^3 NAA proliferated the callus tissue, however, they showed little capacity for shoot regeneration. The callus was subcultured onto the medium supplemented, next to BAP and NAA, additionally with 0.58; 1.44; 2.89; 5.77 or

14.44 μM·dm^3 GA_3. After successive 2–3 weeks there was observed shoots organogenesis. The best results were recorded applying GA_3 at the concentration of 1.44 μM·dm^3. Subculturing the regenerated shoots onto the medium with 4.44 μM·dm^3 BAP and 1.08 μM·dm^3 NAA as well as 1.44 μM·dm^3 GA_3 stimulated both their elongation growth and further multiplication. In that case not only adding auxin and cytokinin but also gibberellin to the medium turned out to be the factor affecting the shoot regeneration [Chakrabarty et al. 2000]. The capacity for shoots regeneration with such medium supplemented with growth regulators is most probably cultivar-specific. The differences between inhibition and stimulation of shoots regeneration by GA_3 result from the time it is used in the culture. GA_3 usually inhibits the initiation of shoots regeneration, however, it is needed for further development [Omura and Hidaka 1992 in Paudyal and Haq 2000].

Vainstein et al. [1992] report that in *Dianthus caryophyllus* L. 'White Sim' the application of liquid medium, as compared with the solid medium, makes it possible to increase the percentage share of petals initiating regeneration as well as the number of shoots produced. Watad et al. [1996] placed the internodes of the same cultivar on the medium with 1.14 μM·dm^3 TDZ and 1.34 μM·dm^3 NAA: solid, liquid with continuous shaking at the frequency of 100 rotations per minute as well as liquid medium without shaking, with the floating membrane raft on the surface, for the explants to be placed. The best shoots regeneration results were reported applying liquid medium with the floating membrane raft. According to the authors, worse results of the regeneration in the liquid medium shaken can be due to damage as a result of shaking delicate forming adventitious shoots primordia. It could have also been the cause the present research failure.

Ligulate florets of six sports of 'Indianapolis' chrysanthemum after 2–3 months of culture when shoots regeneration started were transferred from the solid medium containing 46.47 μM·dm^3 KIN and 5.37 μM ·dm^3 NAA into the liquid medium supplemented with 9.29 μM·dm^3 KIN and 1.08 μM·dm^3 NAA. According to the authors, adding growth regulators at a higher concentration is needed only to initiate adventitious shoots regeneration. In the liquid medium intensive shoots multiplication was reported [Bush et al. 1976]. Maybe in 'Cool Time' one should have also decreased the concentration of growth regulators in the liquid medium.

The explants disinfection method was highly effective. Depending on the experiments, the percentage share of the uninfected cultures accounted for 96–98%. There was observed no explant tissue damage by the disinfecting agents at the concentration and the time applied.

CONCLUSIONS

1. It was demonstrated that the regeneration of shoots occurs when the medium contains the adequate concentration of both BAP (or KIN) and NAA. Most shoots regenerate on the medium supplemented with 8.88 μM·dm^{-3} BAP and 1.61 μM·dm^{-3} NAA and on the media with 8.88 μM·dm^{-3}; 13.32 μM·dm^{-3} BAP or 69.70 μM·dm^{-3} KIN and 2.69 μM·dm^{-3} NAA.

2. Adding 0.29; 1.44 or 2.89 μM·dm^{-3} GA_3 to the medium with 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA limits the shoot regeneration efficiency. Neither does it stimulate shoot elongation. An increase in the shoot number and length is affected by the subculture of regenerating ligulate florets after 7 weeks from the medium containing 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA on the medium with 2.89 μM·dm^{-3} GA_3 and 2.69 μM·dm^{-3} NAA.

3. There were found no differences in the number of shoots regenerating from ligulate florets inoculated on the solid or into liquid medium with 8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA. The subculture of regenerating ligulate florets after 7 weeks from the solid into liquid medium increases the number of regenerating shoots and stimulates their elongation growth. The shoots, however, are deformed and grown with callus.

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REGENERACJA *in vitro* PĘDÓW PRZYBYSZOWYCH Z KWIATÓW JĘZYCZKOWATYCH W ASPEKCIE ZASTOSOWANIA W HODOWLI CHRYZANTEMY

Streszczenie. Mutanty chryzantem mogą być chimerami. Regeneracja *in vitro* pędów przybyszowych z kwiatów języczkowatych może doprowadzić do rozdzielenia komponentów składowych chimery i w rezultacie do uzyskania nowej odmiany. W badaniach określono wpływ różnych czynników na liczbę i długość pędów regenerujących *in vitro* z kwiatów języczkowatych *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time'. Kwiaty języczkowate inokulowano na pożywkę MS [1962] uzupełnioną cytokininą (0; 4,44; 8,88; 13,32; 22,20 $\mu M \cdot dm^{-3}$ BAP; 4,65; 23,23; 46,47; 69,70 $\mu M \cdot dm^{-3}$ KIN) oraz auksyną (0; 0,54; 1,08; 1,61; 2,69 $\mu M \cdot dm^{-3}$ NAA). Stwierdzono, że najwięcej pędów regeneruje przy dodatku do pożywki 8,88; 13,32 $\mu M \cdot dm^{-3}$ BAP lub 69,70 $\mu M \cdot dm^{-3}$ KIN i 2,69 $\mu M \cdot dm^{-3}$ NAA albo 8,88 $\mu M \cdot dm^{-3}$ BAP i 1,61 $\mu M \cdot dm^{-3}$ NAA. Dodatek 0,29; 1,44 lub 2,89 $\mu M \cdot dm^{-3}$ GA_3 do pożywki MS z 8,88 $\mu M \cdot dm^{-3}$ BAP i 2,69 $\mu M \cdot dm^{-3}$ NAA ogranicza wydajność regeneracji pędów i nie stymuluje ich elongacji. Na zwiększenie liczby i długości pędów wpływa przeniesienie regenerujących kwiatów języczkowatych z pożywki MS zawierającej 8,88 $\mu M \cdot dm^{-3}$ BAP i 2,69 $\mu M \cdot dm^{-3}$ NAA na pożywkę z 2,89 $\mu M \cdot dm^{-3}$ GA_3 i 2,69 $\mu M \cdot dm^{-3}$ NAA. Nie stwierdzono różnic w liczbie i długości pędów powstających na kwiatach języczkowatych umieszczonych na stałej lub w płynnej pożywce MS z 8,88 $\mu M \cdot dm^{-3}$ BAP i 2,69 $\mu M \cdot dm^{-3}$ NAA. Przeniesienie regenerujących kwiatów języczkowatych z pożywki stałej do płynnej zwiększa liczbę regenerujących pędów i stymuluje ich wzrost elongacyjny. Pędy te jednak są zdeformowane.

Słowa kluczowe: *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam., płatek, mikrorozmnażanie, regulatory wzrostu

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