**REGENERATION OF SOMATIC EMBRYOS FROM *IN VITRO* ISOLATED LIGULATE FLORETS OF CHRYSANTHEMUM**

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**ABSTRACT**

Chrysanthemum mutants obtained as a result of induced mutagenesis or those which occur spontaneously as sports can be chimeras built from tissues of a varied genetic composition. Regeneration *in vitro* of somatic embryos from the whole ligulate florets covered by mutation or only from their small fragments can lead to the separation of chimera components and, as a result, producing a new original cultivar. There was determined the effect of various factors on regeneration *in vitro* of somatic embryos from ligulate florets of *Chrysanthemum* x *grandiflorum /*Ramat./ Kitam. ‘Cool Time’. The somatic embryogenesis occurs on the ligulate florets inoculated onto the MS medium (Murashige and Skoog, 1962) with 4 mg l-1 2,4-D, as well as with this auxin and 1; 2 or 5 mg l-1 BAP or KIN. Most embryos regenerate on transversely-cut-into-half ligulate florets inoculated onto the medium with 1 mg l-1 KIN and 4 mg l-1 2,4-D.

**Key words:** *Chrysanthemum × grandiflorum* /Ramat./ Kitam.,ligulate floret, somatic embryos, *in vitro*, growth regulators, explant type

**INTRODUCTION**

*Chrysanthemum* x *grandiflorum* */*Ramat./ Kitam. is a species highly appreciated by ornamental plants producers and breeders. However, it is estimated that about half of chrysanthemum cultivars are periclinal chimeras. As for morphology, they do not differ from genetically homogenous cultivars, however, problems appear if their components separate from each other, which is of special importance in the laboratory production of microcuttings. It is so since chimeras are the plants built from genetically-different tissues (Broertjes and van Harten 1988) and to maintain their stability, it is necessary to apply propagation with the use of meristematic explants. Their formation also considerably limits mutation breeding (Chakrabarty et al. 1999). Many spontaneous or induced inflorescence colour mutations in *Chrysanthemum* x *grandiflorum* */*Ramat./ Kitam. cover only a few whole ligulate florets or appear only in a form of a spot or stripe from one to a few thousand cells in size (Stewart and Dermen 1970, Malaure et al. 1991, Chakrabarty and Datta 2010). Due to a small area of the tissues, such mutations are lost since they cannot get isolated and preserved using common methods of chimera components separation from leaf explants by adventitious shoot regeneration (Zalewska et al. 2007). For that reason it is necessary to use other kinds of explants which contain such a genetic change and more efficient regeneration methods which facilitate the restitution of the plants changed. Tanaka et al. (2000) confirmed the usefulness of floral tissues as an alternative source of explants for the regeneration of somatic embryos in chrysanthemum. It is the somatic embryogenesis, which is currently the most efficient method of plant regeneration, it allows for their restitution from single explant tissues (Gahan and George 2008). Somatic embryos, unlike the adventitious shoot, undergo successive development stages of embryogenesis, similar to the development of zygotic embryos (Dodeman et al. 1997).

The somatic embryo, similarly as the adventitious shoot, gets regenerated from a single somatic cell of the explant (Broertjes et al. 1976, Pavingerová et al. 1994, Gahan & George 2008). The separation of chimera components, especially the sectorial ones, could thus occur through the regeneration of somatic embryos from the whole ligulate florets or only from their fragments. The somatic embryos regenerating from single cells of histogenic layer L1 or L2 of the ligulate floret, will represent the genotype of layer L1 or L2, respectively, and they will be built only from genetically homogenous tissues.

The induction of somatic embryogenesis in chrysanthemums in cultures *in vitro* is affected e.g. by such factors as: growth regulators (May and Trigiano 1991, Tanaka et al. 2000, Mandal and Datta 2005) and the genotype (Pavingerová et al. 1994). The efficiency of regeneration is also enhanced by the division of the explant, which is connected with an intensified inflow, into the areas of the cutting places, of endogenous growth regulators as well as with an intensive uptake of exogenous growth regulators and the proliferation of the callus tissue (Gahan and George 2008). The genetic stability of chrysanthemums derived from somatic embryos regenerated on media with relatively high concentration of auxin 2,4-D (4 mg l-1 ) and different content of cytokinins (from 1 to 5 mg l-1 KIN or BAP) was confirmed with flow cytometry for ‘Richmond’ cultivar by Lema-Rumińska and Śliwińska (2009).

Both the regeneration through adventitious shoots and somatic embryos allows for the restitution of genetically homogenous plants with the phenotype changed as a result of breeding. However, a potential efficiency of somatic embryogenesis, facilitating the regeneration from each living somatic cell, gives advantage over the regeneration through adventitious shoots since it offers a chance for the mutations which cover only an inconsiderable area of tissues to get identified.

The aim of the research was to determine the effect of growth regulators and the kind of the floret explant (the whole or a cut) on the regeneration of somatic embryos in *Chrysanthemum* x *grandiflorum* */*Ramat./ Kitam. ‘Cool Time’ in terms of using that regeneration method for breeding.

**MATERIAL AND METHODS**

The research involved *Chrysanthemum* x *grandiflorum /*Ramat./ Kitam. ‘Cool Time’ 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 completely open inflorescences 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 horizontal inoculation with the abaxial side of the explant onto the medium was applied. There were inoculated both whole and transversely-cut-into-half ligulate florets.

The MS (Murashige and Skoog 1962) medium, modified for the regeneration of somatic embryos by addition the PGRs by Lema-Rumińska (2012), supplemented with 4 mg l-1 2,4-dichlorophenoxyacetic acid (2,4-D), as well as with 4 mg l-1 2,4-D and 1; 2 or 5 mg l-1 kinetin (KIN) or 6-benzylaminopurine (BAP) was used to regenerate somatic embryos. The MS medium was additionally modified by increasing the content of calcium and iron by half. The medium included 3% (w/v) of sucrose and was solidified with 1.2 % (w/v) Purified Lab-AgarTM provided by Biocorp. Prior to autoclaving, pH of the medium was set at the level of 5.8. The 350 ml jars were poured with 30 ml of the medium. One ligulate floret was inoculated into a jar.

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 μmol m-2 s-1.

The experiment was set up in a completely randomised design. For each of the 14 experimental treatments 20 replications were applied, one explant for each.

For 10 successive weeks observations were made into the regeneration of callus tissue and adventitious roots. After this time under the stereoscopic microscope MS-Z TRI provided by Precoptic at magnification from 0.7×10 to 4.5×10 regenerated embryos were isolated from explant. There were defined the number of regenerated embryos and their development stages.

The effect of growth regulators contained in the medium and the explant type applied on the embryos regeneration was defined quantitatively by calculating the mean number of embryos per explant inoculated, as well as the mean number of embryos in the different development stages per explant. There was also calculated the percentage share of the explants regenerating embryos. Results for mean number of adventitious roots per explant inoculated and the percentage share of the explants regenerating adventitious roots were also statistically verified.

The real numerical data (x) for the mean number of embryos and roots were transformed by 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**

In the first week after the start of cultures *in vitro*, all the explants got bigger, especially in the region of the ovary. Starting from the second week, there was observed the regeneration of green callus tissue. The process occurred on the surface of all ligulate florets, both whole and transversely-cut-into-half, irrespective of the composition of growth regulators in the medium applied. Callus proliferation was most intensive around the ovary as well as in the cutting places. The first adventitious roots started regenerating via callus tissue in the third culture week. With the successive observation there was observed browning of the surface of the corolla. After yet another week, the callus was becoming cream-yellow-green in colour, and in some areas – even reddish.

Somatic embryogenesis occurred via the callus tissue regenerated around the ovary as well as on the abaxial and adaxial side of ligulate florets, applying each combination of the quantitative and qualitative composition of growth regulators, both on the whole and on transversely-cut-into-half ligulate florets (Table 1). Most embryos got regenerated on transversely-cut-into-half ligulate florets inoculated on the medium supplemented with 1 mg l-1 KIN as well as 4 mg l-1 2,4-D. The share of the explants initiating regeneration in that case accounted for 85%.

Tanaka et al. (2000) induced somatic embryogenesis in chrysanthemum ‘Aboukyu’ on, horizontally inoculated with the abaxial side, fragments of ligulate florets 1 cm long. The experiment involved the use of media containing 0.1 mg l-1 KIN as well as 10 mg l-1 IAA; 11.6 mg l-1 IBA; 12.6 mg l-1 2,4-D or 10.6 mg l-1 NAA. Somatic embryogenesis was initiated by the explants inoculated onto the medium with KIN and IAA as well as with KIN and NAA. IBA and 2,4-D did not induce embryogenesis. Successive research used the media supplemented only with KIN or IAA or the combination of KIN and IAA without or with BAP at various concentrations. Embryoids did not get formed on the media not containing growth regulators or supplemented only with auxin or cytokinin. The best results were reported again on the medium with 0.1 mg l-1 KIN and 10 mg l-1 IAA. Adding BAP decreased the percentage share of explants regenerating embryos. In *Fritillaria imperialis* L. petals were placed onto the media supplemented with three growth regulators at various concentrations - BAP (0; 0.1 or 1 mg l-1), NAA (0; 0.3 or 0.6 mg l-1), IAA (0; 0.4 or 0.8 mg l-1). Non-embryogenic callus was getting formed on the explants placed on the media containing auxin only (0.6 mg l-1 NAA or 0.6 mg l-1 NAA + 0.4 mg l-1 IAA) or BAP at a high concentration and auxin (1 mg l-1 BAP + 0.3 mg l-1 NAA + 0.4 mg l-1 IAA). Embryogenic callus tissue was received only on the medium with BAP at a low concentration and auxin at a higher concentration (0.1 BAP mg l-1 + 0.6 NAA mg l-1 + 0.4 mg l-1 IAA) (Mohammadi-Dehcheshmeh et al. 2007). In *Chamomilla recutita* L. the whole ligulate florets served as explants and parts of inflorescences 0.5×0.5cm in size containing tubular florets (Kintzios and Michaelakis 1999). The media were added with BAP, KIN, NAA, or 2,4-D in various quantitative and qualitative combinations. The indirect somatic embryogenesis occured only on the explants containing tubular florets, on the media with 2 mg l-1 BAP as well as 0.2 mg l-1 NAA or 2.5 mg l-1 KIN as well as 5 mg l-1 NAA added. However, only the embryoids on the medium with KIN and NAA were undergoing successive, after the globular one, development stages.

A comparison of our results with those reported by the above researchers suggests that the process of somatic embryogenesis depends heavily on the quantitative and qualitative composition of growth regulators, however, there is also found a very clear effect of the genotype as well as the kind of the explant. In chrysanthemums somatic embryogenesis is enhanced by the media containing both auxin and cytokinin throughout the culture period, which coincides with the report by Lema-Rumińska (2012). Similarly Naing et al. (2013) received very good results when somatic embryogenesis was induced from *in vivo* grown leaf explants of chrysanthemum ‘Euro’ inoculated on MS medium supplemented with 2.0 mg l-1 2,4-D and 2.0 mg l-1  KIN.

The present research did not show the relationship between the number of embryos at the globular stage and the heart stage and the concentration and the kind of growth regulators contained in the medium and the kind of the explant applied (Table 2, Fig 1). Most embryos at the torpedo stage regenerated on transversely-cut-into-half ligulate florets placed on the media supplemented with 1 mg l-1 KIN or BAP as well as 4 mg l-1 2,4-D as well as on the whole ligulate florets placed onto media with 1 or 2 mg l-1 KIN as well as 4 mg l-1 2,4-D added. On the ligulate florets transversely-cut-into-half placed onto the medium supplemented with 1 mg l-1 KIN as well as 4 mg l-1 2,4-D there were produced most embryoids at the cotyledonary stage, while most mature embryos were observed on the whole ligulate florets placed onto the medium with 1 mg l-1 KIN as well as 4 mg l-1 2,4-D as well as on the transversely-cut-into-half ligulate florets placed on the medium with 1 mg l-1 KIN or BAP as well as 4 mg l-1 2,4-D.

The presence of somatic embryos at various development stages after 30 days of culture on the surface of ligulate florets of chrysanthemum ‘Purima’ were also noted by Mandal and Datta (2005). According to May and Trigiano (1991), the capacity for the formation of mature embryos depends on the chrysanthemum genotype.

Most adventitious roots got regenerated on the whole ligulate florets placed onto the medium supplemented with 1 mg l-1 KIN as well as 4 mg l-1 2,4-D. In that experimental combination the capacity for initiating rhizogenesis was reported in more than 53% explants (Table 3).

Bhattacharya et al. (1990) on the medium with 1 or 2 mg l-1 2,4-D observed an intensive direct regeneration of roots from the fragments of laminas in ‘Birbal Sahni’ chrysanthemum. Tanaka et al. (2000) obtained adventitious roots regeneration from the fragments of ligulate florets in chrysanthemum ‘Aboukyu’ placed on the medium with 0.1 mg l-1 KIN as well as 10 mg l-1 IAA; 11.6 mg l-1 IBA; 12.6 mg l-1 2,4-D or 10.6 mg l-1 NAA. It is common knowledge that a high concentration of auxin and average/low of cytokinin enhance the root regeneration (Ilzuka et al. 1973). However, in the present research the capacity for rhizogenesis was also demonstrated by whole ligulate florets placed on the media with a higher concentration of cytokinin than auxin. The effect can be due to the genotype differences in the capacity for uptaking from the medium and then metabolizing respective growth regulators (Nahid et al., 2007).

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Table 1. Effect of the PGRs added to the MS medium and the explant type on the efficiency of somatic embryos regeneration

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Concentration [mg l-1] and type of PGRs | | | Ligulate floret | Number of embryos per inoculated explant | Explants regenerating embryos  (%) |
| 2,4-D | KIN | BAP |  |  |  |
| 4 | 0 | 0 | Whole | 1.77bc | 68.33a |
| Cut | 1.45bc | 65.00a |
| 4 | 1 | 0 | Whole | 3.27b | 68.33a |
| Cut | 5.70a | 85.00a |
| 4 | 2 | 0 | Whole | 2.80bc | 75.00a |
| Cut | 1.95bc | 60.00a |
| 4 | 5 | 0 | Whole | 0.91c | 60.00a |
| Cut | 1.35bc | 50.00a |
| 4 | 0 | 1 | Whole | 0.95c | 35.00a |
| Cut | 2.78bc | 63.33a |
| 4 | 0 | 2 | Whole | 0.55c | 30.00a |
| Cut | 1.55bc | 65.00a |
| 4 | 0 | 5 | Whole | 0.60c | 35.00a |
| Cut | 0.60c | 36.67a |

Table 2. Effect of the PGRs added to the MS medium and the explant type on the regeneration of somatic embryos in different development stages

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Concentration [mgl-1] and type of PGRs | | | | Ligulate floret | Development stage | | | | |
| globular | heart | torpedo | cotyledonary | mature |
| 2,4-D KIN | | BAP | |  | | | | |
| 4 | 0 | | 0 | Whole | 0.00a | 0.10a | 0.83bc | 0.62b | 0.22b |
| Cut | 0.05a | 0.00a | 0.80bc | 0.55b | 0.05b |
| 4 | 1 | | 0 | Whole | 0.20a | 0.45a | 1.07abc | 0.70b | 0.85a |
| Cut | 0.35a | 0.80a | 2.05a | 1.80a | 0.70ab |
| 4 | 2 | | 0 | Whole | 0.25a | 0.30a | 1.35ab | 0.70b | 0.20b |
| Cut | 0.05a | 0.25a | 0.65bc | 0.85b | 0.15b |
| 4 | 5 | | 0 | Whole | 0.07a | 0.10a | 0.22c | 0.30b | 0.22b |
| Cut | 0.05a | 0.10a | 0.80bc | 0.40b | 0.00b |
| 4 | 0 | | 1 | Whole | 0.15a | 0.10a | 0.25c | 0.20b | 0.25b |
| Cut | 0.25a | 0.22a | 1.10abc | 0.86b | 0.35ab |
| 4 | 0 | | 2 | Whole | 0.15a | 0.10a | 0.20c | 0.10b | 0.00b |
| Cut | 0.15a | 0.30a | 0.20c | 0.60b | 0.30b |
| 4 | 0 | | 5 | Whole | 0.20a | 0.25a | 0.15c | 0.00b | 0.00b |
| Cut | 0.00a | 0.05a | 0.40bc | 0.10b | 0.05b |

Table 3. Effect of the PGRs added to the MS medium and the explant type on the adventitious roots regeneration

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Concentration [mgl-1] and type of PGRs | | | Ligulate floret | | Number of roots per inoculated explant | Explants regenerating roots  (%) |
| 2,4-D | KIN | BAP |  |  | |  |
| 4 | 0 | 0 | Whole | | 0.37bc | 11.66c |
| Cut | | 0.10bc | 10.00c |
| 4 | 1 | 0 | Whole | | 1.40a | 53.33a |
| Cut | | 0.25bc | 5.00c |
| 4 | 2 | 0 | Whole | | 0.30bc | 15.00c |
| Cut | | 0.75b | 40.00b |
| 4 | 5 | 0 | Whole | | 0.20bc | 10.00c |
| Cut | | 0.00c | 0.00c |
| 4 | 0 | 1 | Whole | | 0.10bc | 10.00c |
| Cut | | 0.20bc | 5.00c |
| 4 | 0 | 2 | Whole | | 0.00c | 0.00c |
| Cut | | 0.30bc | 15.00c |
| 4 | 0 | 5 | Whole | | 0.10bc | 10.00c |
| Cut | | 0.00c | 0.00c |



**E**

**D**

**B**

**C**

**A**

Fig 1. Development stages of somatic embryos: A - globular (medium with 5 mg l-1 BAP and 4 mg l-1 2,4-D; magnification 1 × 10); B – heart (medium with 2 mg l-1 KIN and 4 mg l-1 2,4-D; magnification 1 × 10); C – torpedo (medium with 2 mg l-1 KIN and 4 mg l-1 2,4-D; magnification 1 × 10); D – cotyledonary (medium with 2 mg l-1 BAP and 4 mg l-1 2,4-D; magnification 1,5 × 10); E – mature (medium with 4 mg l-1 2,4-D; magnification 0,7 × 10); 1 bar = 1mm.