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BIOLOGICAL FACTORS AFFECTING REGENERATION OF ADVENTITIOUS SHOOTS FROM *in vitro* ISOLATED LIGULATE FLORETS OF CHRYSANTHEMUM

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Abstract. In mutation breeding of chrysanthemum the regeneration *in vitro* of adventitious shoots from ligulate florets can lead to the separation of chimera components and, as a result, to producing a new original cultivar. The success of that method considerably depends on the result being the number of the shoots formed. The more is produced, the greater the chances for an effective separation of chimera components and creating a new stable cultivar. The present research defines the effect of such factors as the inflorescence development stage, the type of the explant as well as the position of its inoculation on the increase in the efficiency of adventitious shoots regeneration. The ligulate florets of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time' were inoculated on the Murashige and Skoog [1962] medium supplemented with 2 mg·dm^{-3} BAP and 0.5 mg·dm^{-3} NAA. There was shown no significant effect of the inflorescence development stage (incompletely open with a partially visible disk or with the entire visible disk in which tubular florets do not produce pollen or completely open in which two or half of the whorls of tubular florets produce pollen) on the shoot regeneration efficiency. Most shoots regenerate on transversely- or lengthwise-cut into half or on the entire pierced ligulate florets – horizontally inoculated, with the abaxial side on the medium.

Key words: *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam., ligulate florets, adventitious organogenesis, inflorescence, explant, inoculation

INTRODUCTION

In chrysanthemum breeding spontaneous and induced mutations, most frequently resulting in a change in the inflorescence colour, are essential. However, many cultivars produced as a result of mutagenesis are chimeras with tissues of a varied genetic composition. In chimeras the area with a changed colour in the entire inflorescence often covers only one or a few ligulate florets. It can be even limited to a narrow streak differ-

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ent in colour against a single ligulate floret. Then, due to the location of the mutation and a small area of mutated tissues, one cannot apply common chimera component genotype separation methods, which limits breeding considerably [Stewart and Dermen 1970, Malaure et al. 1991b, Chakrabarty et al. 1999, Mandal et al. 2000b]. The application of the adequate method of adventitious shoots regeneration *in vitro* from the entire ligulate florets or only from their inconsiderable fragments covered by the mutation can lead to the separation of chimera components and, as a result, to producing a new original cultivar (a stable mutant).

The explants derived from young organs proliferate organogenic callus tissue faster [Gahan and George 2008] and regenerate more shoots than the explants from older organs [Nakano et al. 1994, Lu et al. 1990, Nugent et al. 1991]. After the explant division, adventitious shoots regeneration is most often observed at the cutting places, which is connected with an increased supply of endogenous growth regulators into those regions and with an intensive uptake of exogenous growth regulators as well as the proliferation of the callus tissue. The effect of the inoculation position (the position of the explant placement on the medium) on the adventitious shoots regeneration comes from maintaining or not the natural polarity as well as ensuring the availability of nutrients and growth regulators from the medium to those parts of the explant in which competent cells are found [Gahan and George 2008].

For the purpose of research, 'Cool Time' chrysanthemum cultivar was selected. The aim was to determine the relationship between the inflorescence development stage and the efficiency of the process of adventitious shoots regeneration from ligulate florets, as well as defining the effect of selected types of the flower explant and the position of their inoculation on the regeneration of adventitious shoots.

MATERIAL AND METHODS

Culture of donor plants. The research involved the use of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time' pot cultivar with half-full inflorescences with a visible green and yellow disk of tubular florets, ligulate florets white in colour. Right after planting the generative development was induced exposing the cuttings to the short-day applying shading, thus shortening the natural long day to 10 hours. The plants were shaded from 6 pm to 8 am. After 5 days of growing under short day, chrysanthemums were pinched out over the 5^{th} leaf, from the bottom of the shoot. All the buds set on branched shoots were brought to flowering.

Disinfection of explants. The ligulate florets sampled for research into the regeneration of adventitious shoots were first 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.

Culture initiation. The research involved the use of the MS medium [Murashige and Skoog 1962], supplemented with 2 $mg \cdot dm^{-3}$ BAP and 0.5 $mg \cdot dm^{-3}$ NAA

(8.88 μM·dm^{-3} BAP and 2.69 μM·dm^{-3} NAA), with pH set prior to autoclaving as 5.8. The medium was modified by increasing the content of calcium and iron by half and contained 30 g·dm^{-3} of saccharose. To solidify it, Duchefa agar was applied at the amount of 8 g·dm^{-3}. The medium was poured 30 ml each per 350 ml jar. Each jar contained a singe ligulate floret. The composition and concentration of growth regulators added to the MS medium were chosen based on the best results of previous research. It was demonstrated that the regeneration of shoots in chrysanthemum 'Cool Time' occurs when the medium contains the adequate concentration of both BAP and NAA [Tymoszuk and Zalewska, in print].

Culture conditions. 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}.

Research into the effect of the inflorescence development stage on the regeneration of adventitious shoots. To initiate culture *in vitro*, ligulate florets were sampled at the following four inflorescence development stages:

– inflorescence incompletely open with a partially visible disk, tubular florets do not produce pollen;

– inflorescence incompletely open with the entire visible disk, tubular florets do not produce pollen;

– inflorescence completely open, two of the whorls of tubular florets produce pollen;

– inflorescence completely open, half of the whorls of tubular florets produce pollen.

The experiment was set up in a completely randomised design, for each of the 4 experimental treatments 60 replications were applied, one explant for each.

Research into the effect of the explant type and the inoculation position on the regeneration of adventitious shoots. The following explant types and inoculation positions were used:

– entire ligulate floret horizontally inoculated with the abaxial side;

– transversely-cut into half ligulate floret horizontally inoculated with the abaxial side;

– lengthwise-cut into half ligulate floret horizontally inoculated with the abaxial side;

– entire pirced ligulate floret horizontally inoculated with the abaxial side;

– entire ligulate floret polarly inoculated, proximal part put into the medium;

– transversely-cut into half ligulate floret, distal part polarly inoculated, proximal part apolarly inoculated.

Ligulate florets were sampled from inflorescences completely open in which two whorls of tubular florets produced pollen. Ligulate florets were cut and pierced (interchangeably in 6 places), using the scalpel, after sterilization, prior to being inoculated on the medium.

The experiment was set up in a completely randomised design, for each of the 6 experimental treatments 40 replications were used, one explant for each.

Observations and the method of the analysis of variance. In the present experiments for 12 successive weeks observations were made into the dynamics of the regeneration of shoots visible with a naked eye through the jar glass. 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 last group included also the shoots whose accurate isolation and counting was possible only at an adequate magnification using the stereoscopic microscope. There was calculated the average number of all the shoots per explant inoculated, mean number of shoots ≥ or < 0.5 cm long per explant inoculated as well as the percentage share of the explants regenerating shoots.

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. Besides, to receive normal distribution of the features investigated, one had to reject their extreme values, clearly different from the others. The results were statistically verified using the method of the analysis of variance for a single-factor experiment with a different number of replications and means for the treatment were evaluated with the Newman-Keuls test at the significance level of $\alpha = 0.05$. Tables with results provide real numerical data (not transformed), while alphabet letters point to homogenous groups of transformed means for which the significance test was made.

RESULTS AND DISCUSSION

Research into the effect of the inflorescence development stage on the regeneration of adventitious shoots. In the first week of culture *in vitro* all the explants got bigger, most intensively in the region of the ovary. Starting from the third week, callus regeneration started; first around the ovary and then on the surface of petals.

Adventitious shoots regeneration occurred via callus tissue. The first single shoots were noted in the fifth week of culture on the explants sampled from inflorescences completely open in which two or half of the whorls of tubular florets produced pollen (fig. 1). On the ligulate florets derived from the inflorescences incompletely open, with the entire visible disk, without producing pollen and from the inflorescences completely open in which half of the whorls of tubular florets produced pollen, new shoots appeared successively starting from the beginning of regeneration, in the sixth week, until the end of the culture (fig. 3 A).

Deriving ligulate florets from inflorescences incompletely open with a partially visible disk or the entire visible disk, in which tubular florets did not produce pollen, as well as inflorescences completely open in which two or half of the whorls of tubular florets produced pollen, did not show a significant effect on the shoot regeneration efficiency (tab. 1). Neither was there proven that the mean number of shoots ≥ 0.5 cm and < 0.5 cm long getting formed on the explant depended on the inflorescence development stage. On the explants derived from inflorescences completely open with two whorls of tubular florets produced pollen there was observed no formation of shoots

≥ 0.5 cm long. Neither was it found that the explants sampled from the inflorescences at respective development stages showed differences in the capacity for shoot regeneration. In practise such results point to the possibility of starting cultures at various dates, with no fear of lowering the result of shoots regeneration.

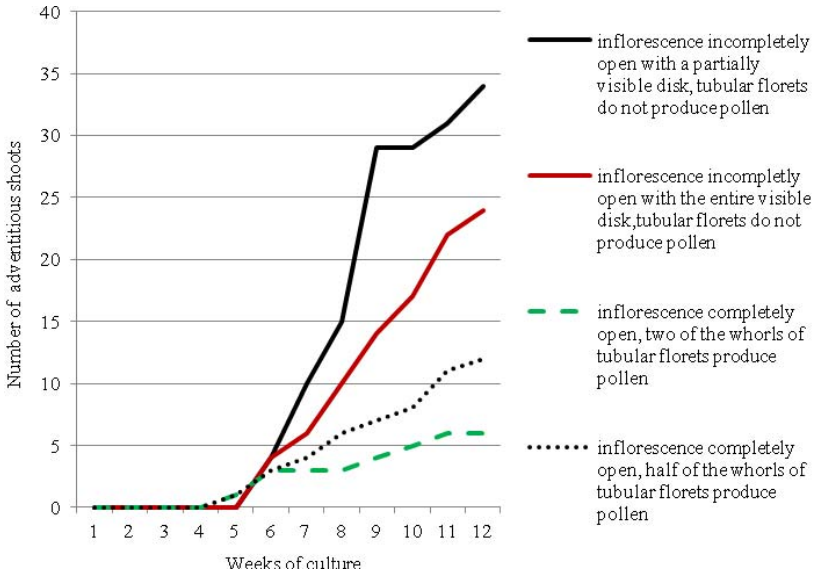

Fig. 1. Dynamics of adventitious shoots regeneration depending on inflorescence development stage

In 'Orlando', 'Pink Pixie Time' as well as 'Klondike' chrysanthemums the fragments of petals derived from inflorescences 2–3 days prior to full opening of ligulate florets regenerated many more shoots than those sampled 7–8 days after the opening of tubular florets [Park et al. 2007]. In *Chelidonium majus* L. Ono and Uehara [1982] initiated cultures from petals at three development stages distinguished as: 1 (petals 0.1–0.2 cm long light green in colour), 2 (petals 0.2–0.4 cm long and light yellow in colour) as well as 3 (petals 0.4–0.8 cm long and yellow in colour). The shoot regeneration was most often initiated by petals representing stage 1 and so the youngest ones. The differences recorded in the present research can be due to the comparison of different development stages or due to the specific genotype. To separate the components of chimeras in chrysanthemum, one must consider that to observe all the changed ligulate florets, the inflorescence must be already open and coloured.

Chakrabarty et al. [1999] sampled ligulate florets from 'Colchi Bahar' chrysanthemum at three dates, counting since planting the cuttings out: 90–95 days (I); 110–115 days (II) as well as 125–130 days (III). Adventitious shoots regeneration was most successful on the explants sampled at date II. Ligulate florets sampled from inflorescences at the other dates did not initiate shoot regeneration at all on some of the media used in the research. However, it is difficult to refer to the above results since the authors did

not describe in detail the stage of development of the inflorescence in 'Colchi Bahar' cultivar at the dates defined. Besides, in that cultivar the inflorescence is full and flat – unlike in 'Cool Time'.

Table 1. Mean number of adventitious shoots per explant inoculated and percentage share of explants regenerating adventitious shoots depending on inflorescence development stage

Inflorescence development stage	Number of adventitious shoots			Percentage share of explants regenerating adventitious shoots
	All	≥ 0.5 cm long	< 0.5 cm long	
Inflorescence incompletely open with a partially visible disk, tubular florets do not produce pollen	0.98 a	0.07 a	0.91 a	5.08 a
Inflorescence incompletely open with the entire visible disk, tubular florets do not produce pollen	0.97 a	0.15 a	0.82 a	13.56 a
Inflorescence completely open, two of the whorls of tubular florets produce pollen	0.41 a	0.00 a	0.41 a	7.41 a
Inflorescence completely open, half of the whorls of tubular florets produce pollen	0.24 a	0.06 a	0.18 a	6.00 a

a, b, c… – means in columns followed by the same letter do not differ significantly at $\alpha = 0.05$

Don Palmer and Keller [2011] in *Hypericum perforatum* L. 'Topas' sampled four types of buds: unopend buds (stage A), buds with petals just visible above the sepals (stage B), buds with petals half protruding from the sepals but closed (stage C) as well as buds with petals fully opened (stage D). The regeneration of shoots occurred on all the petal explants at stage B and C. A lower capacity for adventitious shoot regeneration was noted for petals at stage D, with the lowest – at stage A. Earlier development stages, therefore, do not guarantee a high regeneration capacity.

Research into the effect of the explant type and the inoculation position on the regeneration of adventitious shoots. Prior to the end of the first week after the start of cultures *in vitro,* all the explants inoculated onto the medium in the experiment got bigger in size, especially in the proximal part, in the region of the ovary. In the successive week, callus regeneration started; initially around the ovary and then on the surface of petals. The callus tissue proliferated intensively at the places of piercing and transverse and lengthwise cutting of ligulate florets. As for the polar inoculation of the entire ligulate florets, callus was mostly formed in the proximal part put directly into the medium. While applying the transverse cut into half of the ligulate flowers and polar inoculation of the distal part and apolar of the proximal part, the callus tissue was mostly formed in the region of the cut, put in the medium, sporadically around the ovary.

Shoot organogenesis occurred in the region of the callus regenerated around the ovary (except for the transverse cut of ligulate florets into half, polar inoculation of the distal part and apolar – of the proximal part) as well as on the surface of the petals.

The appearance of the first single shoots was noted in the fourth week of culture on transversely-cut or lengthwise-cut into half ligulate florets horizontally inoculated with the abaxial side on the medium (fig. 2). The formation of successive shoots in the case of those two experimental treatments was observed starting from the sixth to the eleventh week (fig. 3 B), while when horizontal inoculation was used with the abaxial side of the entire ligulate florets and pierced entire ligulate florets, the shoot regeneration was visible from the sixth week and it continued similarly to the end of the culture. As for polar inoculation of the entire ligulate florets, the first shoot appeared in the seventh week and the successive ones were noted only in the tenth culture week. As for transverse cut of ligulate florets with polar inoculation of the distal part and apolar – of the proximal part, starting from the seventh as long as to the last week of culture, there was observed one shoot only.

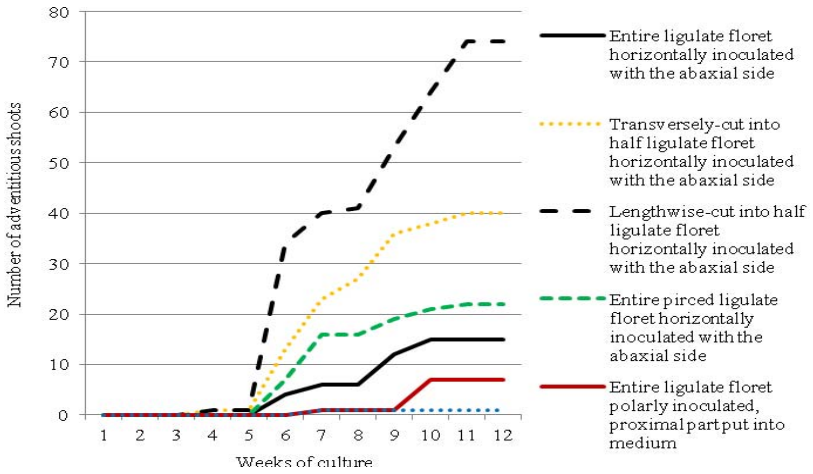

Fig. 2. Dynamics of adventitious shoots regeneration depending on explant type and inoculation position

Shoot regeneration was most efficient for transversely-cut or lengthwise-cut into half and entire pierced ligulate florets horizontally inoculated with the abaxial side (tab. 2). As for these three types of explants in combination with horizontal inoculation onto the medium, there were also formed significantly more shoots < 0.5 cm long than in the case of the other experimental treatments. The shoots ≥ 0.5 cm long emerged in biggest numbers on ligulate florets horizontally inoculated with the abaxial side, having got divided with the transverse or lengthwise cut into half. No such shoots were observed when applying transverse cut of ligulate floret into half and then the polar inoculation of the distal part or apolar inoculation of the proximal part. Almost 47% of the

explants transversely-cut into half and almost 64% of the explants lengthwise-cut into half and then horizontally inoculated with the abaxial side on the medium initiated shoots regeneration. The values were highest of the whole experiment. The lowest capacity for the shoots formation was seen for the ligulate florets which were transversely-cut into half, however their distal part was polarly inoculated in and the proximal part – apolarly.

Table 2. Mean number of adventitious shoots per explant inoculated and percentage share of explants regenerating adventitious shoots depending on the explant type and inoculation position

Explant type and inoculation position	Number of adventitious shoots			Percentage share of explants regenerating adventitious shoots
	All	≥ 0.5 cm long	< 0.5 cm long	
Entire ligulate floret horizontally inoculated with the abaxial side	1.53 b	0.41 b	1.12 b	17.65 cd
Transversely-cut into half ligulate floret horizontally inoculated with the abaxial side	4.22 a	0.50 a	3.72 a	46.88 ab
Lengthwise-cut into half ligulate floret horizontally inoculated with the abaxial side	6.06 a	1.36 a	4.70 a	63.64 a
Entire pirced ligulate floret horizontally inoculated with the abaxial side	3.68 ab	0.41 b	3.26 ab	35.29 bc
Entire ligulate floret polarly inoculated, proximal part put into the medium	1.18 b	0.21 b	0.10 b	15.15 cd
Transversely-cut into half ligulate floret, distal part polarly inoculated, proximal part apolarly inoculated	0.33 b	0.00 b	0.33 b	7.41 d

a, b, c… – means in columns followed by the same letter do not differ significantly at $\alpha = 0.05$

The applicable literature provides information on the culture initiation from the entire ligulate florets in ‘Purima’ and ‘Colchi Bahar’ [Mandal et al. 2000b], ‘Flirt’, ‘Puja’, ‘Maghi’, ‘Sunil’ [Datta et al. 2005] or ‘Maghi’ chrysanthemums [Mandal et al. 2000a]. Malaure et al. [1991a] in sixteen chrysanthemum cultivars researched and Matsumura et al. [2010] in ‘Shiroyamate’ cultivar used fragments of petals 0.5 × 0.5 cm in size as explants. In ‘Kasturba Gandhi’ chrysanthemum Chakrabarty et al. [2000] cut fragments of petals 0.5 cm^2 in size. In ‘Orlando’, ‘Pink Pixie Time’ and ‘Klondike’ fragments of petals 0.5–0.6 cm long were placed [Park et al. 2005, Park et al. 2007]. Kengkarj et al. [2008] report on seven chrysanthemum cultivars, ‘Biarizte’, ‘Yellow Biarizte’, ‘Storika’, ‘Pinkgin’, ‘Linker Pink’, ‘Dark Linker Salmon’ and ‘Bari’ in which ligulate florets were cut into three parts: proximal, central and distal about 0.4 × 0.4 cm in size and then inoculated with the abaxial side.

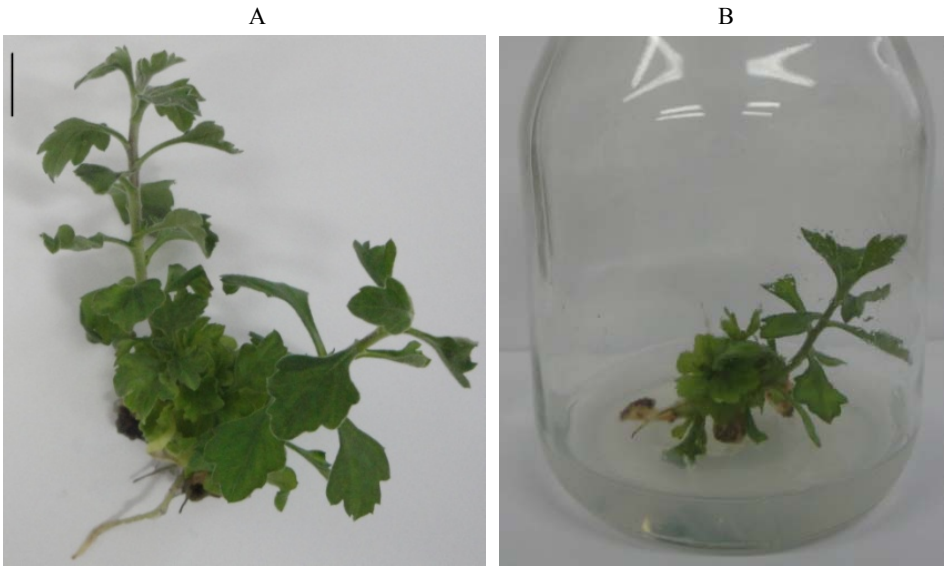

Fig. 3. Adventitious shoots in *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time': A –isolated from the ligulate florets sampled from inflorescence incompletely open with the entire visible disk in which tubular florets did not produce pollen; B – regenerating during culture from the ligulate florets transversely-cut into half and inoculated with the abaxial side on the medium; bar 1 cm

Similarly as in the present research, also the results reported by other authors demonstrate the effect of the type of the explant applied and the inoculation position on the result of adventitious shoots regeneration. Zalewska et al. [2011], in 'Satinbleu' chrysanthemum, applied polar and horizontal inoculation of leaf explants and internodes. A proximal part of internodes and leaf petioles were put into the medium. However, as for the horizontal inoculation, the abaxial side of the lamina remained in contact with the medium. Both explant types regenerated almost three-times more shoots when they were horizontally inoculated on the medium. Nugent et al. [1991] in *Dianthus caryophyllus* L. 'White Sim' compared the formation of shoots on the whole and lengthwise-cut receptacles. The cut explants regenerated twice as many shoots. In *Chelidonium majus* L. Ono and Uehara [1982] damaged petals, with the use of tweezers, in the proximal, central or distal part. The greatest capacity for initiating adventitious shoot regeneration was recorded for the petals damaged in the proximal part and the lowest – in their distal part.

CONCLUSIONS

1. The efficiency of adventitious shoots regeneration does not depend on the inflorescence development stage, which prolongs the period of sampling ligulate florets to initiate culture *in vitro* considerably.

2. Most shoots regenerate on transversely- or lengthwise-cut into half or on the entire pierced ligulate florets – horizontally inoculated, with the abaxial side on the medium.

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BIOLOGICZNE CZYNNIKI WPŁYWAJĄCE NA REGENERACJĘ PĘDÓW PRZYBYSZOWYCH Z IZOLOWANYCH *in vitro* KWIATÓW JĘZYCZKOWATYCH CHRYZANTEMY

Streszczenie. W hodowli mutacyjnej chryzantem regeneracja *in vitro* pędów przybyszowych z kwiatów języczkowatych doprowadzić może do rozdzielenia komponentów chimer i w rezultacie do uzyskania nowych, oryginalnych odmian. W tej metodzie o sukcesie w dużej mierze decyduje wynik w postaci liczby uzyskanych pędów. Im większa jest ich liczba, tym większe są szanse na skuteczną separację komponentów chimer i uzyskanie nowej stabilnej odmiany. W badaniach określono wpływ takich czynników jak stadium rozwoju kwiatostanu, rodzaj eksplantatu oraz pozycja jego inokulacji na zwiększenie wydajności kaulogenezy przybyszowej. Kwiaty języczkowate *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. 'Cool Time' wykładano na pożywkę Murashige i Skooga [1962] uzupełnioną 2 mg·dm^{-3} BAP i 0.5 mg·dm^{-3} NAA. Nie udowodniono wpływu stadium rozwoju kwiatostanu (niecałkowicie otwarty z widocznym częściowo oczkiem lub z widocznym całym oczkiem, w którym nie pylą kwiaty rurkowate albo całkowicie otwarty, w którym dwa lub połowa okółków kwiatów rurkowatych pyli) na wydajność regeneracji pędów. Najwięcej pędów powstaje na przeciętych poprzecznie lub podłużnie na pół albo na całych nakłuwanych kwiatach języczkowatych inokulowanych horyzontalnie stroną zewnętrzną.

Słowa kluczowe: *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam., kwiat języczkowaty, organogeneza przybyszowa, kwiatostan, eksplantat, inokulacja

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