ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Hortorum Cultus 11(5) 2012, 81-90*

MICROPROPAGATION AND INFLUENCE OF *in vitro* CULTURE ON DEVELOPMENT OF *Cirsium pannonicum* (L. f.) LINK REGENERANTS

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Abstract. *Cirsium pannonicum* is a protected species in Poland. The sources of threats are both spontaneous successional changes in vegetation leading to overgrowth of xerothermic grasslands and human activity. Active methods of protection are therefore indispensable for preservation of the species. Micropropagation in an *in vitro* culture may be one of the useful tools to protect the species actively. The objective of present work was to develop an efficient system for *C. pannonicum in vitro* propagation and comparison of morphological traits and the ability to flower in plants obtained by micropropagation and from seeds. Isolated shoot tips from 10-day-old seedlings were cultured on MS medium supplemented with: 6-benzylaminopurine (BA), kinetin (KN) or zeatin (ZEA) at concentration of 1.0, 2.0 or 3.0 $mg \cdot L^{-1}$ in combination with naphthaleneacetic acid (NAA; 0.1 $mg \cdot L^{-1}$). The highest shooting frequency 93.6% and shoot multiplication rate 2.8 shoots/explant was obtained on medium supplemented with 2.0 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ NAA. In subsequent subcultures, average 3.3 axillary shoots per explant on MS with 3.0 $mg \cdot L^{-1}$ BA was recorded, the difference was not statistically significant. The highest rooting frequency 86.1% was observed on 1/2 MS medium. Regenerated plants produced leaf rosettes and inflorescence stems typical for this species. However, compared to plants developed from seeds, these were fewer, much shorter and contained a greater number of capitula on individual stems. In the first year after acclimatization into the field condition, approximately 64% of individuals flowered. During the next years, all plants flowered in a period typical for the species. The flowers were fertile and the seeds were viable.

Key words: shoot tip, rooting, acclimatization, flowering

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INTRODUCTION

Cirsium pannonicum is a herbaceous perennial growing up to 120 cm height, with long, thread-like roots. The stem is simple, unbranched, or has few elongated branches with a single capitulum at the top; it is somewhat outflanked in the lower part and leafless in the upper part. The leaves are undivided, lanceolate, with the top short-hairy, finely serrated edge, ending with cloves with thin, prickly setae. The stem and leaves at the bottom are slightly hairy. Capitula, from 14 to 25 mm in length, occur singly on top of the stem and branch ends. The scales are arranged in an overlapping coat, and are purple and sharp on top. Tubular flowers are purple. 3–4 mm long achenes bear several times longer papose. The plant flowers from June to July [Sychowa 1971, Werner 1980, Szafer et al. 1986]. *C. pannonicum* grows in direct sunlight, on a limestone base of slopes of hills or steep slopes of valleys, usually with southern exposure. It most often occurs in moderately warm and dry grassland from the *Cirsio-Brachypodion pinnati* community, for which it is a characteristic species [Matuszkiewicz 2001]. *C. pannonicum* in Poland is subject to strict protection [Piękoś-Mirkowa and Mirek 2006]. The range of *C. pnnonicum* covers mainly the south-eastern part of the country: Małopolska and Lublin Upland, and Roztocze [Zając and Zając 2001]. In Europe, its presence is limited to eastern and central parts of the continent [Münzbergová 2005]. The plant is threatened mainly due to succession changes in the plant communities where it grows. Since they are not used, low, loose grasslands transform into more compact and relatively high, and are then overgrown with bushes and trees, thus eliminating photophilous species. Therefore, active protection is required for preservation of this species. Reproduction in *in vitro* culture could be one element in the protection of this species.

The use of shoot tips has been very effective in micropropagation of many species of the Asteraceae family such as *Saussurea lappa* [Sudhakar Johnson et al. 1997], *Arnica chamissonis* [Cassells et al. 1999], *Centaurea rupestris* [Ćurković Perica 2003], *Santolina canescens* [Casado et al. 2002], *Artemisia vulgaris* [Sujatha and Ranjitha Kumari 2007], *Carlina acaulis* [Trejgell et al. 2009] and *Senecio macrophyllus* [Trejgell et al. 2010]. However, in the case of *Cirsium pannonicum* found low efficiency propagation of shoots [Trejgell and Tretyn 2010]. The aim of this study was to develop a protocol of efficient system *in vitro* micropropagation of *C. pannonicum* and comparative analysis of morphological traits and the ability to flower in plants obtained by micropropagation and grown from seeds in the field.

MATERIALS AND METHODS

Cirsium pannonicum (L. f.) Link seeds were obtained from the collection of the Botanical Garden of Maria Curie-Skłodowska University in Lublin (Poland).

In vitro propagation of plants. The seeds were surface-sterilized with 70% (v/v) EtOH for 30 s, then in 20% (v/v) commercial solution of NaOCl (Domestos) for 30 min, and finally they were rinsed 4 times with sterile distilled water. The seeds were transferred onto Petri dishes (6 cm in diameter) containing 10 ml of the MS medium [Murashige and Skoog 1962], which was supplemented with 1 $mg \cdot L^{-1}$ gibberellic acid (GA3).

In experiment was used 500 seeds, 10–12 seeds were cultured in each Petri dish. Shoot tips were isolated from 10-days-old seedlings of *C. pannonicum* and cultured in 100 ml Erlenmeyer flasks with 30 ml of MS propagation medium supplemented with 3% sucrose , 0.7% plant agar (Duchefa) and combination of cytokinins: BA, KN or ZEA at concentration of 1.0, 2.0 and 3.0 $mg \cdot L^{-1}$ with NAA (0.1 $mg \cdot L^{-1}$), pH 5.7. Explants (5 in each flask) were cultivated in continuous white fluorescent light (45 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and at 26±1°C. Shoot tips exposed on MS without growth regulators were the control variant. The shoots were subcultured 3 times, at 4 weeks intervals. The percentage of explants producing shoots, the number of shoots per explant, and axillary shoot length were recorded after each subculture.

Rooting and acclimatization. Axillary shoots cultured on MS supplemented with combination of BA and NAA from the 3rd subculture were excised and transferred into 300 ml glass jars with 50 ml of full-strength or half-strength MS medium, without or with IBA (0.01 or 0.1 $mg \cdot L^{-1}$). The percentage of rooted shoots, number of roots per shoot, and the longest root length were recorded after 6 weeks of culture. The plantlets were removed from the *in vitro* cultures, washed gently with sterile water, transferred to plastic pots containing a sterile mixture of vermiculite and sand (1:1 v/v and acclimatized to the *ex vitro* conditions for 4 weeks in a greenhouse. After 8 weeks, the plantlets were transferred to field conditions. In May, the plants were replanted to the field.

Analysis of morphological characteristics and flowering ability. The ability of plants to flower, leaves and inflorescence stem morphology were compared and the survival level was analyzed for plants from the *in vitro* cultures and plants growing from seeds in three consecutive vegetation cycles. The analyses were performed in the second half of June each year. 10 plants from the collection in the Botanical Garden of UMCS and 30 plants from *in vitro* culture grown on an experimental plot at NCU were used. Biometric observations and those of insects visiting flowers were made between 10.00 and 1.00 p.m., when all the capitula were fully open.

Seeds vitality was analyzed by the tetrazolium chloride test (TZ) [Gosling 2004] and ability to germinate on the MS medium supplemented with GA_3 (1 $mg \cdot L^{-1}$) was recorded when the radicle was at least 2 mm long. Germination was monitored for 3 weeks.

Analysis of the C, N and P content in soil. Soil samples were taken from the Nicolaus Copernicus University in Toruń experimental field and the Botanical Garden of Maria Curie-Skłodowska University in Lublin. The content of carbon was determined using Tiurin's method, nitrogen (organic and ammonium) – Kjeldahl's method [Bednarek et al. 2004], and phosphorus – Bleck's method with Gebhardt's modification [Gebhardt 1982].

Statistical analysis. The all experiment was repeated three times. Each treatment consisted in 30 experimental units – explants or individual shoots (5 per Erlenmeyer flask/glass jar). The results were expressed as percentage (organogenesis, *ability to germinate,* flowering ability, survival rate) or mean values and standard errors (stem or root length and diameter of capitula). The data were analyzed using the ANOVA method, and comparisons of the mean values of treatments were made by Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Presence of cytokinins was necessary to stimulate the development of axillary buds. The results obtained indicate that the BA supplemented medium was the best for multiple shoot formation of *C. pannonicum* (tab. 1). The maximum of shoots per explant, average 2.8 with shooting frequency 93.6% was recorded on shoot tips (initial material) cultured on the medium supplemented with BA at a concentration 2 $mg \cdot L^{-1}$ (fig. 1b). In the subsequent three subcultures, the maximum number of shoots per explant was 3.3 (on 3 $mg \cdot L^{-1}$ BA), the difference was not statistically significant (tab. 1). However, the presence of BA inhibited the growth of shoots, and the differences were statistically significant. Cytokinin BA has been commonly used for induction of shoot organogenesis in many plants including the species of the family Asteraceae [Sujatha and Ranjitha Kumari 2007]. A comparison of the effectiveness of different cytokinins for multiple shoot formation revealed in order of effectiveness BA > KN > ZEA [Shiva Prakash et

Table 1. Effect of different cytokinins in combination with 0.1 $mg \cdot L^{-1}$ NAA on regeneration of *Cirsium pannonicum* from shoot tips after 4 weeks of *in vitro* culture.

Tabela 1. Wpływ różnych cytokinin w kompinacji z 0,1 $mg \cdot L^{-1}$ NAA na regenerację *Cirsium pannonicum* z wierzchołków pędów po 4 tygodniach kultury *in vitro*.

Cytokinin Cytokininy		Concentration Stężenie $mg \cdot L^{-1}$	% explants producing shoots % eksplantatów tworzących pędy	Shoots numer per explant* Liczba pędów na eksplantat*	Shoots length Długość pędów mm *
Initial stage Etap inicjalny	-	-	3.3	0.1 ± 0.04 e	52.0 ± 5.3 a
	BA	1	84.5	1.9 ± 0.18 b	18.8 ± 1.5 cd
		2	93.6	2.8 ± 0.20 a	17.3 ± 0.8 cd
		3	73.3	2.4 ± 0.24 ab	15.9 ± 0.8 d
	KN	1	54.0	1.2 ± 0.18 c	35.1 ± 2.7 b
		2	65.0	1.1 ± 0.15 c	27.7 ± 1.1 bc
		3	56.5	1.0 ± 0.19 cd	25.6 ± 1.1 bc
	ZEA	1	35.0	0.5 ± 0.12 de	50.3 ± 4.2 a
		2	18.9	0.2 ± 0.08 e	34.8 ± 2.1 b
		3	20.6	0.4 ± 0.10 de	34.7 ± 1.7 b
Average from 3 subcultures Średnia z 3 pasaży	BA	1	95.5	2.6 ± 0.22 ab	18.7 ± 1.0 cd
		2	96,5	2.6 ± 0.35 ab	18.5 ± 0.9 cd
		3	92.7	3.3 ± 0.28 a	15.1 ± 0.6 d
	KN	1	0	0 d	-
		2	87.5	1.4 ± 0.22 bc	35.4 ± 3.4 b
		3	60.5	1.6 ± 0.31 bc	26.0 ± 2.3 c
	ZEA	1	0	0 d	-
		2	16.7	0.3 ± 0.18 cd	44.7 ± 2.8 b
		3	6.3	0.1 ± 0.08 d	41.1 ± 3.5 b

* means ± standard error, means with the different letter indicate significantly differences at $P < 0.05$;
* wartość średnia ± błąd standardowy, różne litery oznaczają różnice statystycznie istotne przy $P < 0,05$

al. 1994]. The results obtained in our experiment confirm previous assessment of the effectiveness of cytokinins, where KN was less effective than BA, and the results obtained on the medium with addition of ZEA were comparable to the control variant (medium without growth regulators) (tab. 1). In subsequent subcultures, proliferation of shoots on medium supplemented with KN and ZEA at the lowest concentrations was not reported (tab. 1). Similar results were recorded for *Eclipa alba* [Baskaran and Jayabalan 2005], *Artemisia vulgaris* [Sujatha and Ranjitha Kumari 2007] and *Carlina acaulis* [Trejgell et al. 2009]. In subsequent subcultures, a similar trend was observed both in proliferation of shoots and their length. Presence of BA in the medium most strongly stimulated the development of axillary shoots. The effectiveness of KN and ZEA was much smaller, and even in the case of the lowest levels of both growth regulators, shoot multiplication was not observed (tab. 1).

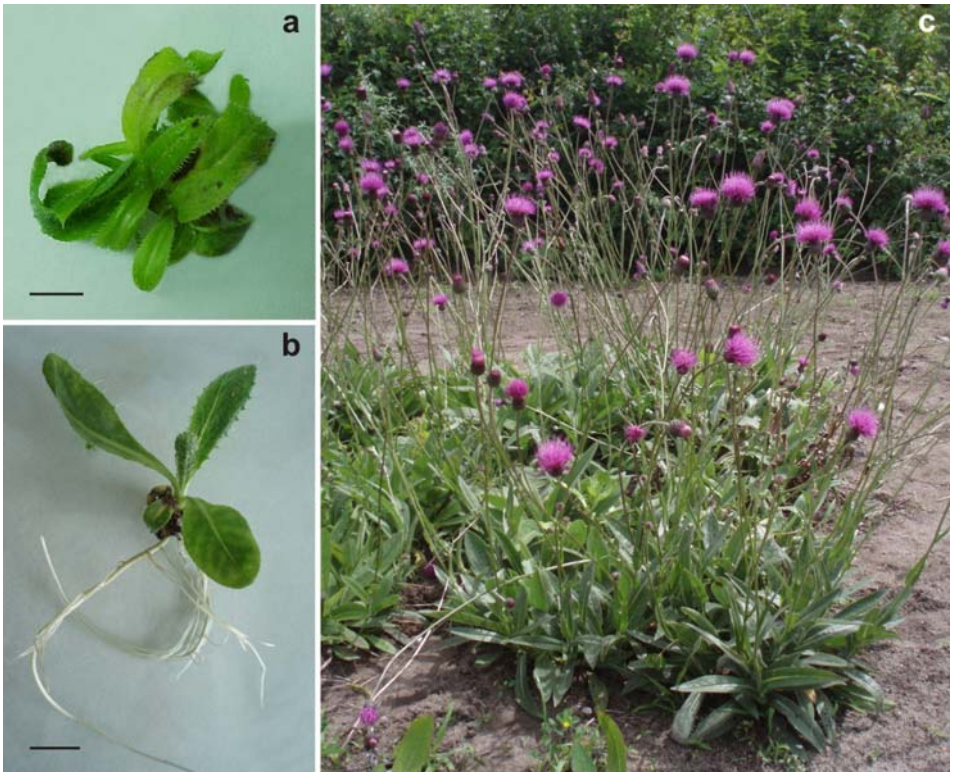

Fig. 1. Micropropagation of *Cirsium pannonicum*. (a) Multiple axillary shoot formation on MS with 2 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA after 4 weeks of *in vitro* culture Bar = 10 mm, (b) Rooted shoot on 1/2 MS Bar = 1 cm, (c) Flowering plantlets 2 years after acclimatization.

Ryc. 1. Mikrorozmnażanie *Cirsium pannonicum*. (a) pędy pachwinowe rozwijające się na pożywce MS z 2 mg·L^{-1} BA i 0,1 mg·L^{-1} NAA po 4 tygodniach kultury *in vitro* Odcinek = 10 mm, (b) ukorzeniony pęd na 1/2 MS Odcinek = 1 cm, (c) kwitnący regenerat 2 lata po aklimatyzacji.

Axillary shoots *C. pannonicum* did not require the presence of auxin for rooting, the average number of roots per shoot was 2.1 at rooting frequency 82.9% on MS medium (tab. 2). Reduction of mineral salts in the medium concentration (1/2MS) had no significant effect on either the percentage of rooted shoots (86.1%) or the number of roots (1.9) (tab. 2, fig. 1b). The presence of IBA at a concentration of 0.01 and 0.1 $mg \cdot L^{-1}$ in the rooting medium did not affect significantly the rooting of shoots of *C. pannonicum*, and an increased concentration of IBA in the medium lowered the percentage of rooted shoots. It was found in earlier studies that the use of auxins, especially IBA, and/or reduction of the amount of mineral salts in the medium clearly stimulated rooting in *Guzotia abyssinica* [Sujatha 1997], *Santolina canescens* [Casado et al. 2002], *Eupatorium triplinerve* [Martin 2003/4], and *Carlina acaulis* [Trejgell et al. 2009].

Table 2. Effect of IBA on rooting of shoots of *Cirsium pannonicum* after 6 of week *in vitro* culture

Tabela 2. Wpływ IBA na ukorzenianie pędów *Cirsium pannonicum* po 6 tygodniach kultury *in vitro*

Medium Pożywka	Concentration of IBA Stężenie IBA μM	Rooting Ukorzenianie %	Root number per shoot* Liczba korzeni na pęd*	Longest root length Długość najdłuższego korzenia, mm *
MS	0	82.9 ± 8.7	2.1 ± 0.3 abc	82.0 ± 4.8 a
	0.049	80.1 ± 12.5	3.6 ± 0.4 a	75.7 ± 3.5 a
	0.49	54.2 ± 4.2	2.9 ± 0.6 ab	88.3 ± 2.3 a
½ MS	0	86.1 ± 13.9	1.9 ± 0.3 bc	79.1 ± 8.3 a
	0.049	54.2 ± 10.8	1.7 ± 0.4 bc	76.9 ± 5.4 a
	0.49	37.5 ± 12.5	1.0 ± 0.2 c	42.8 ± 4.6 b

* means ± standard error, means with the different letter indicate significantly differences at $P < 0.05$;
* wartość średnia ± błąd standardowy, różne litery oznaczają różnice statystycznie istotne przy $P < 0,05$

After 12 weeks of acclimatization of regenerated plants, the survival rate was 93.3% (tab. 3). After planting in the soil, the regenerated plants produced a leaf rosette of lanceolate leaves with denticulate edges ended setae, typical for this species. In the first year after planting in the ground, only 14% of the individuals were able to bloom in June, but at the end of August, approximately 64% of individuals flowered (tab. 3). In the next two years, the beginning of flowering of all plants was recorded in the first days of June (fig. 1c); it ended in early July in the case of plants from *in vitro*, and in mid-July in plants obtained from seeds. The peak of flowering in both cases was due in the second half of June. Inflorescence stems were typical for this species and showed a characteristic arachnoid-hairy branching. However, compared to plants growing from seeds, they were fewer, much shorter and contained a greater number of capitula on individual stems. The capitula were developed properly, and their diameter was slightly larger than the plants derived from seeds in the first 2 years after acclimatization (tab. 3). The differences in inflorescence stem development may have resulted from

Table 3. Analysis of morphological characteristics and flowering ability of *Cirsium pannonicum in vitro* regenerated and obtained from seeds
Tabela 3. Analiza cech morfologicznych i zdolności do kwitnienia *Cirsium pannonicum* zregenerowanych *in vitro* i uzyskanych z nasion

Plant from: Rośliny z:	Field culture (in year) Uprawa polowa (w roku)	% of survival % przeżywalności	% of flowering plants % kwitnących roślin	Number of inflorescence stems/ individuals Liczba pędów kwiatostanowych/ osobnika	Length of inflorescence stem Długość pędu kwiatostanowego mm	Number of capitula on Liczba koszyczków na		Diameter of capitulum Średnica koszyczka mm*
						stem pęd	individual osobnika	
in vitro	1	93.3	64.3	2.6 ± 0.3 *i	807.4 ± 26.3	3.1 ± 0.2	7.7 ± 0.9 *i	35.2 ± 0.8 *s
	2	90.0	100	11.9 ± 1.5	764.1 ± 16.6	4.3 ± 0.2 *s	52.1 ± 9.8	36.8 ± 0.7 *s
	3	73.3	100	10.8 ± 0.7	706.9 ± 15.9	5.6 ± 0.4 *s	58.6 ± 14.2	32.5 ± 0.5
seeds nasion	1	100	100	14.0 ± 1.9	1093.5 ± 24.4 *s	3.4 ± 0.2	45.8 ± 9.3	29.0 ± 0.3
	2	100	100	18.8 ± 2.4 *s	1004.0 ± 18.4 *s	3.6 ± 0.1	68.1 ± 11.1	27.3 ± 0.8
	3	100	100	16.5 ± 1.8	1049.2 ± 13.7 *s	3.4 ± 0.1	54.9 ± 3.9	30.0 ± 0.4

means ± SE, * significant differences at $p \leq 0.05$, i – inhibition, s – stimulation
wartość ± SE, * różnice istotne przy $p \leq 0.05$, i – hamowanie, s – stymulacja

Table 4. Analysis of the carbon (C), nitrogen (N) and phosphorus (P) content in soil
Tabela 4. Analiza zawartości węgla (C),azotu (N) i fosforu (P) w glebie

Soil – Gleba	Percentage content of minerals Procentowa zawartość pierwiastków		mg·kg^{-1}
	C	N	P
Garden of NCU Toruń Ogród UMK Toruń	1.30	0.08	525
Botanical Garden of UMCS Ogród Botaniczny UMCS Lublin	1.89	0.17	700

differences in the abundance of soil minerals. Soil abundance was examined in the experimental plot at the NCU and UMCS Botanical Garden, and it was found that the percentage of C and N is 1.45- and 2.1-fold higher, respectively, and P content is 1.3-fold higher (tab. 4). On the other hand, application of cytokinin to the culture medium may lead to cytokinin accumulation in plant tissues. It is known that cytokinins stimulate lateral bud growth by reducing dominance of the apical bud [Kotov and Kotova 2000, Shani et al. 2006]. Flowers of plants of both types of generations were often visited by various species of bumblebee – pollinators, 65.4% on average. The honey bee (24.7%) and diptera (9.9%) appeared on a slightly smaller number of flowers. In this study the flowers of regenerated plants were fertile, and the viability of seeds was 48%, as determined by the TZ test, while the percentage of germinated seeds in a medium supplemented with GA_3 was 42%.

CONCLUSIONS

1. BA was the most effective cytokinin in stimulation of development of axillary buds *Cirsium pannonicum.*

2. Presence of auxin in the medium was not necessary for rooting.

3. Regenerated plants grew normally and flowered in the same period as plants obtained from seeds, flowers were fertile and the seeds were viable.

This study demonstrated that axillary shoot regeneration from shoot tips of seedlings can be a useful method for multiplication of *Cirsium pannonicum.*

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MIKROROZMNAŻANIE I WPŁYW KULTUR *in vitro* NA ROZWÓJ REGENERANTÓW *Cirsium panonicum* (L. f.) LINK

Streszczenie. *Cirsium pannonicum* jest gatunkiem objętym ochroną w Polsce. Źródłem zagrożenia są zarówno spontaniczne sukcesyjne zmiany roślinności prowadzące do zarastania muraw kserotermicznych, jak i działalność człowieka. W celu zachowania gatunku wymagane jest stosowanie aktywnych metod ochrony. Mikrorozmnażanie kulturze *in vitro* może być jednym z przydatnych narzędzi aktywnej ochrony tego gatunku. Celem niniejszej pracy było opracowanie efektywnego systemu rozmnażania *in vitro Cirsium pannonicum* i porównanie cech morfologicznych i zdolności do kwitnienia roślin uzyskanych poprzez mikrorozmnażanie i z nasion. Izolowane z 10-dniowych siewek wierzchołki pędów były kultywowane na pożywce uzupełnionej o: 6-benzyloaminopurynę (BA), kinetynę (KN) lub zeatynę (ZEA) w stężeniu 1,0, 2,0 lub 3,0 $mg \cdot L^{-1}$ w kombinacji z kwasem naftalenooctowym (NAA; 0,1 $mg \cdot L^{-1}$). Najwyższy odsetek eksplantatów wykazujących organogenezę pędów (93,6%) i wskaźnik namnażania pędów 2,8 pędu/eksplantat uzyskano na pożywce zawierającej 2,0 $mg \cdot L^{-1}$ BA i 0,1 $mg \cdot L^{-1}$ NAA. W kolejnych pasażach odnotowano średnio 3,3 pędów pachwinowych na eksplantat na pożywce MS z 3,0 $mg \cdot L^{-1}$ BA, różnice nie były statystycznie istotne. Najlepsze ukorzenianie 86,1% zaobserwowano na pożywce 1/2 MS. Regeneranty tworzyły typowe dla gatunku rozety liściowe i pędy kwiatostanowe. Jednak, w porównaniu z roślinami kontrolnymi rozwijającymi się z nasion, pędów było mniej, były one krótsze i zawierały większą liczbę koszyczków na pojedynczym pędzie. W pierwszym roku po aklimatyzacji do warunków polowych zakwitło około 64% osobników. Podczas kolejnych lat kwitły wszystkie rośliny w typowym dla gatunku okresie. Kwiaty były płodne, a nasiona żywotne

Słowa kluczowe: wierzchołki pędów, ukorzenianie, aklimatyzacja, kwitnienie

Accepted for print – Zaakceptowano do druku: 29.05.2012