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ANALYSIS OF FLOWERING ABILITY OF REGENERATED *CARLINA ACAULIS* SUBSP. *SIMPLEX* PLANTS.

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S u m m a r y

Regenerated plants of *Carlina acaulis* subsp. *simplex* induced on shoot tips and fragments of hypocotyls, cotyledons and roots were used as an experimental material. Explants were isolated from 10-day-old, sterile seedlings and were put on growth media supplemented with BA (3 mg×dm^{-3}), and NAA (0,1 mg×dm^{-3}). Plantlets were acclimatized to *ex vitro* conditions and planted to the field. Analysis of flowering ability, inflorescence stem morphology, and survival level was the objective of the study.

The plants regenerated from shoot tips and cotyledons were able to flower in the first year after acclimatization, however no vital seeds were found, while in the case of hypocotyl- and root-regenerated plants flowering appeared in the second year after acclimatization. Number of flowering-able plants grew in time, reaching 100% level. Few percent of inflorescence stems displayed branches ending with additional capitula. The number of this type of plants decreased in successive years, while the average length of inflorescence stem increased. In the case of intensively flowering plants, the survival rate decreased in 3 consecutive years.

Key words: *Carlina acaulis,* seedling explants, inflorescence stem morphology, survival plants *in vivo*

INTRODUCTION

In most of perennial plants, the process of reproduction may take two forms: vegetative reproduction and generative reproduction (E c k e r t , 2002). Vegetative reproduction means the production of novel individuals off the maternal organism without zygote stadium. In this case, specimens obtained (clones) are genetically identical to the maternal organism, and, as a rule, they develop and flower more quickly than the ones obtained from seeds. The plants created in this way may stay connected with the maternal plant or may separate (L e w a k , 2002). Generative reproduction increases genetic diversity in a given population. The generative phase begins with a "ready to flower" state, which leads to changes of differentiation pattern and activity of apical meristem. Within the shoot apical meristem, flower buds develop instead of leaves and axillary buds primordials (B e r n i e r et al. 1993). In rosette forming plants, the subapical part of meristem is activated, which brings about inflorescence stem development. Flowering is a complex morphogenetic process, controlled by both endogenous (genetic) and exogenous (light, temperature) factors (B e r n i e r , 1988; B u r n et al. 1993; K i n e t , 1993; H a y a m a and C o u p l a n d , 2003; M o u r a d o v et al. 2002). Flowering may also be initiated by stress conditions, such as drought, nutrient deficit, or plant density (L e a v y and D e a n , 1998). Readiness to flower is connected with reaching some minimal size by a plant. The size may be displayed as an individual weight or number of leaves (M i t k a and T u m i d a j e w i c z , 1993; K l i n k h a m e r et al. 1991). The generative phase ends with the production of seeds. The seed, and consequently embryo, development is linked to the inflow of many organic and inorganic compounds to the seed and accumulation of these compounds therein. All these processes are a considerable energetic burden, and require efficient photosynthetic systems.

Analysis of flowering ability of regenerated *Carlina acaulis* subsp. *simplex* plants was the objective of the work. The development of an efficient regeneration system and obtaining flowering-competent and viable seeds-producing plants may be one of the key elements in the protection of this species. The northern border of *Carlina acaulis* incidence runs through Poland. *C. acaulis* is a photophilous plant, tolerating occasional shade, and preferring dry soils. The flowering period occurs in late July-early August. In the past, this species was frequently harvested for use in traditional medicine and as a decoration. This plant contains tannins, inuline, etheric oils and resin, which made it useful in medicine. Harvesting decreased the population size, and in certain locations the plant became totally extinct. Economic activity (such as marble mining) and plant succession on meadows are further dangers for *C. acaulis* (P i ę k o ś - M i r k o w a and M i r e k , 2003).

MATERIALS AND METHODS

Regenerated plants of *C. acaulis* subsp. *simplex* were experimental material. The seeds (they come from the Botanical Garden of Lviv National Ivan Franko University) were surface sterilized in 70% EtOH for 30 s, then in 20% sodium hypochlorite (Domestos) for 20 minutes. After sterilization the seeds were rinsed four times with sterile water and placed on MS medium (Murashige and Skoog, 1962) supplemented with GA_3 (1 mg×dm^{-3}). Explants were isolated from 10-day old seedlings (shoot tips, fragments of hypocotyls, cotyledons, and basal part of roots), and exposed (30 explants of each type) on micropropagation medium MS supplemented with sucrose (3%), agar (0,6%) and growth regulators: BA (3 mg×dm^{-3}), and NAA (0.1 mg×dm^{-3}), pH 5,7. The explants were cultivated in continuous white fluorescent light (45 µmol×m^{-2}×s^{-1}) and at 26±1°C. After four weeks forming shoots were isolated and transferred to the fresh medium. Adventitious shoots from the 3rd subculture were rooted on the MS medium without growth regulators for four weeks. The plantlets were planted in pots filled with vermiculite and sand mixture (1:1) and acclimatized to the *ex vitro* conditions for 4 weeks in a greenhouse. In May the plants were replanted to the field and their flowering ability, inflorescence stem morphology, and survival level were analyzed in three consecutive vegetation cycles (30-40 plants regenerated from: shoot tips, hypocotyls, cotyledons, and 15-20 from roots). Analysis was performed when the youngest flowers in capitula were open (in 1st year in October, 2nd and 3rd in August (or November/December in case of the plants regenerated from hypocotyl and root in 2nd year). The vitality of seeds was analysed on the MS medium supplemented with GA_3 (1 mg×dm^{-3}) and in the field conditions. Additionally, there were analysed morphological traits and the flowering period of the F1 generation plants. Results were expressed as percentage (flowering ability) or mean values and standard errors (stem length and diameter of capitula).

RESULTS AND DISCUSSION

The plants originating from shoot tips and cotyledons grew intensively, a strong growth of maternal rosette and the development of 2 to 5 adventitious stems per individual were observed. Moreover, 54% of shoot tip-regenerants and 17% of cotyledons-regenerants were able to flower in October during the first year after acclimatization (Tab. 1). Among those, 69% plants displayed the typical inflorescence stem morphology (stem with a single capitula at the tip) (Fig. 1A), while 8% bore one or two additional capitula and 23 % carried three or more (Fig. 1B).The diameter of capitula fluctuated around 80 mm. The inflorescence stem length was in the range of 0 to 120 mm, with the mean value of 26 mm and 74 mm for plants regenerated from shoot tips and cotyledons, respectively. 53% of plants fell to the group with the stem length of 0-30 mm (Fig. 2). There were no viable seeds in harvested achenes, which could have been caused by the delayed flowering period (Pięk oś-Mirkowa and Mirek, 2003) and, consequently, a reduced number of pollinators. Alternatively, this might have been due to underdevelopment of seeds.

The plants regenerated from fragments of hypocotyls and roots did not flower in the first year after acclimatization. The hypocotyl-regenerants displayed a strongly developed, single, erect stem, while the root-regenerants grew much slower, produced a smaller number of leaves, and juvenile leaves (with undivided blade) lasted for a long time. One hundred percent of plants survived after four months acclimatization. (Tab. 1). Previous studies showed a similar survival level for other species of Asteraceae family, for example about 90% of plantles of *Echinacea purpurea* (Choffe et al. 2000) and *Achillea filipendulina* (Evenor and Reuveni, 2004) and 85% of rooted plant *Anthemis nobilis* survived (Echeverrigaray et al. 2000) The plants revealed no abnormal morphological traits (leaf and flower shape) and flowered in the typical period. In case of hardening plantlets of *Centaurea paui*, a lower survival rate (70%) was obtained, but the plants grew normally to maturity (Cuenca et al. 1999). Nevertheless, morphological differences are sometimes observed between regenerated and seed started plants. It was the case for *Eclipa alba* where the plants coming from seeds had a trailing habit with small leaves, while the *in vitro* cultured ones were erect and had larger leaves (Dhaka and Kothari, 2005).

In the second year after acclimatization, the number of flowering-competent plants among those regenerated from shoot tips and cotyledons increased to 85% and 100%, respectively (Tab. 1). Flowering occurred in August and September, which is the same as in naturally growing plants. Harvested seeds were viable. Moreover, the flowering of plants regenerated from hypocotyls and roots was observed in 26%, and 20% of them, respectively. However, in the latter case it was delayed and occurred in late autumn (late October and November), additionally the capitula of the root-regenerated plants were immature. The diameter of capitula fluctuated between 79 and 93 mm, but differences were not significant. The length of the inflorescence stem varied much more than in the first year (5-300 mm), with the average 84 and 92 mm for the plantlets from shoot tips and cotyledons, respectively. These values did not exceed those typical for the species. The most prominent group (27%) had the stem length between 31 and 60 mm (Fig. 1). In the case of hypocotyl- and root-regenerated plants that were flowering for the first time, the average inflorescence stem length was 50 mm and 5 mm, respectively. The number of plants

Table 1
Flowering ability and inflorescence stem morphology of *Carlina acaulis* subsp. *simplex* plantlets in three consecutive years after acclimatization.

Field cultured [in year]	Plant regenerated from:	Survival level [%]	% of flowering plants	Diameter of capitula [mm]	Average length of inflorescence stem [mm]	% of stems with branches	
						1-2	3 and more
1	S	100	54	82±6)*	26±9	8	23
	C	100	17	80±4	74±4	–	25
	H	100	0	–	–	–	–
2	R	100	0	–	–	-	–
	S	100	85	93±10	84±10	13	–
	C	100	100	84±7	92±7	9	–
	H	93,8	26	79±3	50±9	–	–
3	R	87,5	20	immature	5±0	–	–
	S	93	100	85±7	161±10	3	–
	C	90	100	81±6	172±5	1.5	–
	H	100	96	91±8	214±6	2	–
	R	100	100	95±11	183±15	2	–

S – shoot tip

C – cotyledon

H – hypocotyl

R – root

)* ±SE, standard error

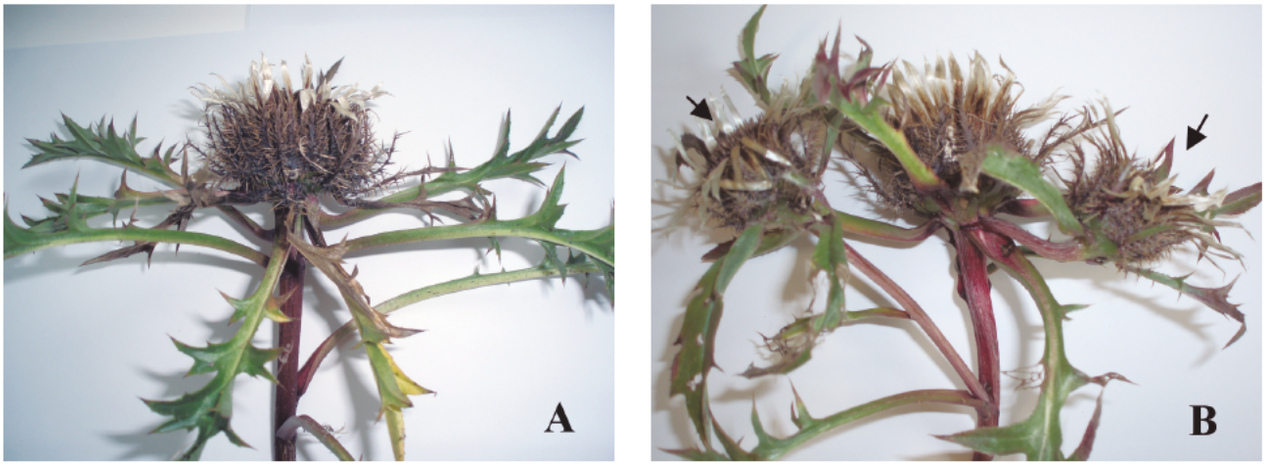

Fig. 1. Inflorescence stem *Carlina acaulis* subsp. *simplex*: unbranched (A) and branched (B) with additional capitula (arrows).

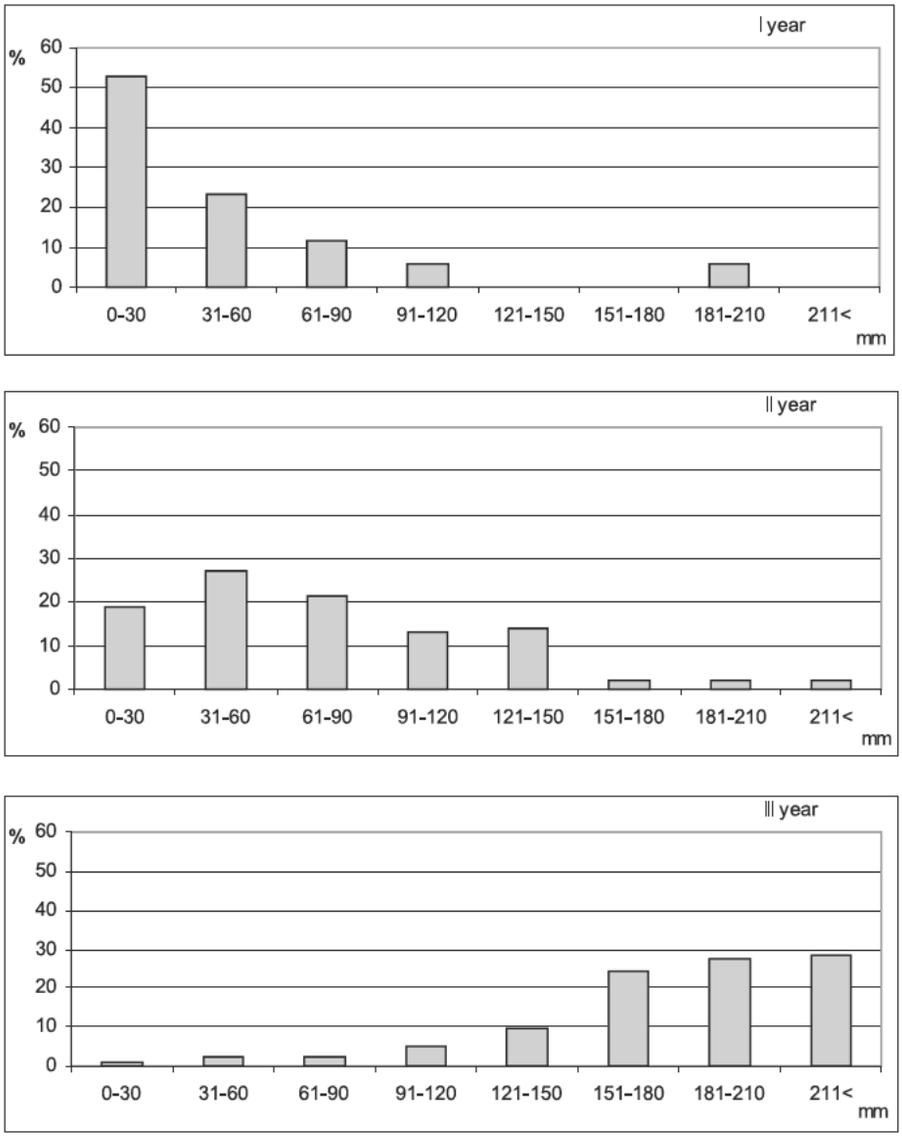

Fig. 2. Percentage of *Carlina acaulis* subsp. *simplex* individuals with flowering stem of different lengths [mm] in three successive year after acclimatization.

bearing branched stems was significantly reduced in comparison to the previous year (Tab. 1). The survival level for the second year after acclimatization was 100% in the case of the plants regenerated from shoot tips and cotyledons, while for the hypocotyl-, and root-regenerated ones it was 93.8% and 87.5%, respectively (Tab. 1).

In the third year all the plants were able to flower, regardless of their origin. The number of individuals carrying branched inflorescence stems was less than 3% (Tab. 1). The diameter of capitula fluctuated between 81 and 95 mm. We did not observe significant differences during 3 years of observation. The stem length varied between 50 and 280 mm, and the group with the length over 210 mm was the biggest one (Fig. 1). In the third year of culture in soil, 93% of shoot tips plantlets, 90% of those regenerated from cotyledons and 100% of the remaining two groups survived.

The observed changes in inflorescence stem development (inhibition of growth on length and branching) in the first year after *in vitro* culture may be a result of exogenous benzylaminopurine in regeneration media and its elevated level in plant tissues. It is known that cytokinins cancel shoot tip domination, which manifests in the shortening of stems and activation of axillary buds (Kakimoto, 1996). In the fourth year of soil culture, only 40% of the plants regenerated from shoot tips survived. The obtained achenes contained viable seeds, 98% of seed germinated on medium supplemented with GA_3. At the same time, seedlings growing from seeds were observed in field conditions. Those plants revealed typical morphological traits: non-branched stems and a normal flowering period. The survival level decline is probably due to very intensive flowering in three consecutive vegetation seasons and the dying of maternal plants that do not produce adventitious stems in the last season. One may suppose that intensive flowering and seed production is a big energetic burden leading to senescence and dying. A similar phenomenon was reported by Czarnecka (2006) in a 15-year study of the flowering pattern of *Senecio macrophyllus* in nature. The absence of rosette growth was observed after a period of intensive flowering. It shows that the individuals under study entered a static phase or started dying.

CONCLUSIONS

All plants, regardless of explant source, were flowering-competent.

Plantlets coming from shoot tips and cotyledons were able to flower in the first year of soil culture.

Benzylaminopurine present in the regeneration medium could have been a factor causing inhibition of length growth and branching of inflorescence stems during the two initial vegetation seasons.

Intensive flowering might have been a cause of survival rate decrease.

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Analiza zdolności do kwitnienia regenerantów *Carlina acaulis* subsp. *simplex*

Streszczenie

Materiał eksperymentalny stanowiły regeneranty *Carlina acaulis* subsp. *simplex*, indukowane na wierzchołkach wzrostu oraz fragmentach hypokotyli, liścieni i korzeni. Eksplanty były izolowane z 10-dniowych sterylnych siewek i wykładane na pożywki uzupełnione w BA (3 mg×dm^{-3}) i NAA (0,1 mg×dm^{-3}). Regeneranty były aklimatyzowane do warunków *ex vitro* i wysadzane do gruntu. Celem badań była analiza: zdolności do kwitnienia, morfologii pędu kwiatostanowego oraz poziomu przeżywalności w 3 kolejnych sezonach wegetacyjnych.

Rośliny zregenerowane z wierzchołków wzrostu i liścieni były zdolne do kwitnienia już w pierwszym roku po aklimatyzacji, ale nie stwierdzono żywotnych nasion. Natomiast rośliny zregenerowane z fragmentów hypokotyli i korzeni były zdolne do kwitnienia w drugim roku po aklimatyzacji. Liczba roślin zdolnych do kwitnienia rosła w kolejnych latach osiągając poziom 100%. Niewielki odsetek roślin wykazywał rozgałęzione pędy kwiatostanowe zakończone dodatkowymi koszyczkami. Liczba tego typu roślin zmniejszała się w kolejnych latach, rosła natomiast średnia długość pędów kwiatostanowych. W przypadku roślin intensywnie kwitnących po 3 latach uprawy spadał wskaźnik przeżywalności.