



MICROPROPAGATION OF *CARLINA ACAULIS* L.

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An efficient shoot propagation system for *Carlina acaulis* was developed in this study. The experimental material consisted of shoot tips and fragments of hypocotyls excised from 10-day-old seedlings. The explants were transferred to proliferation medium supplemented with different types of cytokinins: 6-benzylaminopurine (BA, 4.4 or 13.3 μM), kinetin (Kn, 4.7 or 13.9 μM) and zeatin (Zea, 4.6 or 13.7 μM) in combination with naphthaleneacetic acid (0.54 μM NAA). The morphogenetic response was best in culture on medium supplemented with 13.3 μM BA, and shoot organogenesis frequency was highest for shoot tips (100%). On average, 7.5 shoots were induced per explant of the initial material, and the multiplication rate in five subsequent subcultures was 6.1. Shoot length was lower in culture with BA in the medium than with Kn or Zea. Plantlets rooted with 60% frequency in vitro on full-strength MS medium and with 55.3% frequency ex vitro. Reduction of the mineral salt concentration (1/2MS) stimulated rhizogenesis. Addition of auxins stimulated both the frequency and number of roots per shoot, but only in combination with full-strength MS medium. Regenerated plants were able to flower and gave viable seeds.

Key words: *Carlina*, shoot tip, hypocotyl, benzylaminopurine, ex-vitro rooting.

INTRODUCTION

Stemless carline thistle, *Carlina acaulis* L., of the *Asteraceae* family, is a monocarpic perennial plant which grows in dry and rocky habitats up to 2000 m a.s.l. in Central and Southern Europe. It is most frequent in southern Poland in the Carpathian and Sudeten Mts., Lubelska Upland, and in northeastern Poland in the Warmia and Mazury Lake District (Zajac and Zajac, 2001). It is considered a medicinal plant for the inulin, essential oil and tannins that it contains. The rhizome is massive and vertically set in the ground. The leaves are elliptic-oblong, pinnatifid to pinnatisect, up to 30 cm long, and set in a rosette. The stem rarely reaches 15 cm height, and usually the plants are acaulescent. The capitula (25–50 mm diameter) are surrounded by silvery white or pale pink bracts. Flowering occurs from July to September. The flowers are hermaphrodites. The fruits are achenes 5 mm long (Tutin et al., 1976).

Encroachment by trees and bushes leads to shading of *C. acaulis* habitats and consequently decreased flowering, posing a threat to this generatively reproducing species. Its inflorescences have been massively harvested for decorative use, signif-

icantly reducing the population, and the species has been placed under protection in Poland (Zarzycki and Szelag, 2006). In situ protection is insufficient in areas highly transformed by anthropogenic impacts or natural succession. Active methods of protection employing biotechnological methods should prove useful for conservation of *C. acaulis*.

Among the important biotechnological methods are in vitro vegetative propagation and protection of the morphogenetic potential of cultured cells, tissues and organs (Rybczyński and Mikula, 2006). In earlier studies the donor material for regeneration of *C. acaulis* has been immature zygotic embryos, shoots, leaves and inflorescence fragments. Shoot organogenesis was observed only on zygotic embryos (Grubisić et al., 2004) and shoot fragments (Galek and Kukułczanka, 2000). Our study aimed to develop an efficient micropropagation system with seedlings as the initial material, which could serve as a tool for active protection of the species, but also as a starting point for research on obtaining biologically active compounds synthesized by *Carlina acaulis* without the need to harvest plants.

Abbreviations: BA – 6-benzylaminopurine; GA₃ – gibberellic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; Kn – kinetin; NAA – naphthaleneacetic acid; MS – Murashige and Skoog medium; Zea – zeatin.

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TABLE 1. Effect of different cytokinins in combination with 0.54 μM NAA on regeneration of *Carlina acaulis* from shoot tips and hypocotyl fragments (donor material) and following 5 subcultures

Explant	Cytokinin [μM]	Donor material					Average for 5 subcultures			
		% explants produ- cing roots	Root number per explant*	Longest root length (mm)* (CV)	% explants produ- cing shoots	Shoot number per explant*	Shoot length (mm)* (CV)	Shoot number per explant*	Shoot length (mm)* (CV)	
Shoot tip	BA	4.4	0	-	-	95	7.9 \pm 0.4 a (17)	41.1 \pm 4.4 bc (26)	5.6 \pm 0.4 ab (57)	30.3 \pm 1.2 c (20)
		13.3	0	-	-	100	7.5 \pm 0.8 a (24)	37.2 \pm 4.4 c (16)	6.1 \pm 0.2 a (42)	26.3 \pm 0.9 c (23)
	Kn	4.7	47.5	1.8 \pm 0.4a (47)	12.3 \pm 6.1 b (85)	65	2.6 \pm 0.2 cd (26)	47.0 \pm 4.6 ab (33)	4.3 \pm 0.3 bc (56)	39.9 \pm 2.5 b (23)
		13.9	25.6	2.8 \pm 0.9a (75)	23.0 \pm 11.5a (99)	76.4	3.6 \pm 0.2 bc (22)	43.0 \pm 2.8 bc (21)	3.9 \pm 0.2 bc (54)	40.7 \pm 1.3 b (20)
	Zea	4.6	61.3	1.4 \pm 0.5a (43)	10.2 \pm 1.9 b (41)	64.3	1.5 \pm 0.1 d (33)	56.1 \pm 3.2 a (17)	2.7 \pm 0.3 cd (66)	52.8 \pm 2.3 a (24)
		13.7	37.3	3.0 \pm 0.9a (70)	11.8 \pm 3.2 b (41)	75	2.1 \pm 0.1 d (29)	59.9 \pm 5.3 a (28)	3.5 \pm 0.3 cd (55)	49.1 \pm 2.2 ab (26)
Hypocotyl	BA	4.4	0	-	-	10	1.5 \pm 0.3 d (40)	37.2 \pm 1.3 c (11)	4.0 \pm 0.3 bc (60)	29.6 \pm 2.0 c (26)
		13.3	0	-	-	63.3	4.6 \pm 0.6 b (50)	31.2 \pm 2.0 cd (17)	6.1 \pm 0.4 a (55)	30.0 \pm 1.7 c (22)
	Kn	4.7	12.5	2.5 \pm 0.5a (28)	13.0 \pm 3.6 b (47)	20.8	1.0 \pm 0.0 d (0)	26.0 \pm 1.8 d (19)	2.7 \pm 0.4 cd (58)	30.7 \pm 2.7 c (27)
		13.9	10.0	1.7 \pm 0.7a (70)	7.0 \pm 2.1 b (60)	0	-	-	-	-
	Zea	4.6	25.0	2.2 \pm 0.4a (38)	9.0 \pm 2.3 b (67)	33.3	1.5 \pm 0.2 d (28)	36.0 \pm 3.9 c (28)	1.6 \pm 0.4 d (69)	50.1 \pm 3.9 a (24)
		13.7	10.0	1.3 \pm 0.6a (46)	8.0 \pm 2.1 b (45)	0	-	-	-	-

* means \pm standard error; means with the same letter within columns do not significantly differ by ANOVA followed by Tukey's test ($p < 0.05$); CV – coefficient of variation (%)

MATERIAL AND METHODS

PROPAGATION CULTURE

Seeds of *Carlina acaulis* L. were obtained from the Botanical Garden of Adam Mickiewicz University in Poznań (Poland), Maria Curie-Skłodowska University in Lublin (Poland), and the Alpine Botanical Garden in Rezia (Italy). Seeds were surface-sterilized with 70% (v/v) EtOH for 30 s, then in 20% (v/v) commercial bleach (Domestos) for 30 min and finally rinsed four times with sterile distilled water. The seeds were transferred to Petri dishes (6 cm diameter) containing 10 ml MS medium (Murashige and Skoog, 1962) supplemented with 2.9 μM GA_3 . Shoot tips and hypocotyl fragments (1 mm long, cut out under the node) were isolated from 10-day-old *C. acaulis* seedlings.

Explants were transferred to Erlenmeyer flasks

containing 30 ml proliferation medium. Different cytokinins were tested in combination with 0.54 μM NAA: BA (4.4 or 13.3 μM), Kn (4.7 or 13.9 μM) and Zea (4.6 or 13.7 μM)

Each treatment consisted of 30 explants. The explants were cultivated at $26 \pm 1^\circ\text{C}$ under continuous white fluorescent light ($45 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) for 4 weeks. The cultures were subcultured five times. The frequency of shoot and root organogenesis, number of shoots and roots per explant, and adventitious shoot length (mm) were measured and analyzed.

ROOTING AND ACCLIMATIZATION

Shoots were rooted both in vitro and ex vitro. Individual shoots were excised from shoot clusters. Half of them were transferred to glass jars with 50 ml full-strength or half-strength MS medium, with or

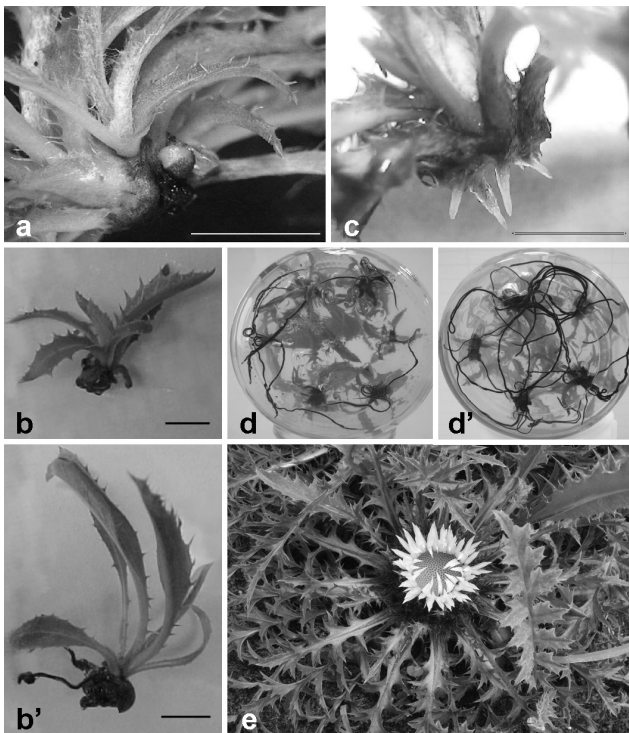


Fig. 1. Micropropagation of *Carlina acaulis*. (a) Shoot proliferation from shoot tips on medium supplemented with 13.3 μM BA after 4 weeks of culture. Bar = 5 mm, Shoot induction on MS with (b) 13.3 μM BA and (b') 13.7 μM Zea. Bar = 10 mm, (c) Rooted microshoot on MS with 0.54 μM NAA. Bar = 5 mm, Root induction on (d) MS and (d') 1/2 MS, (e) Flowering plantlets growing in field conditions.

without a selected auxin in one of two concentrations: NAA (0.054 or 0.54 μM), IAA (0.057 or 0.57 μM) or IBA (0.049 or 0.49 μM). The other half were transferred directly to ex vitro conditions (plastic pots containing a sterile mixture of vermiculite and sand), and their rooting ability was analyzed after 8 weeks.

Plantlets obtained after 8 weeks of culture on the rooting medium were removed from the in vitro cultures, washed gently in sterile water, transferred to plastic pots containing a sterile mixture of vermiculite and sand (1:1 v/v) and covered with transparent boxes to maintain humidity. After 8 weeks of acclimatization the survival rate was analyzed. Regenerated plants were transferred to field conditions in June. The morphology and flowering ability of the regenerated plants were analyzed in August-September of the next year. Viability of seeds was tested on MS medium supplemented with 2.9 μM GA₃.

STATISTICAL ANALYSIS

The experiments were done in three independent replicates. Each treatment consisted of 30 experimental units (explants or adventitious shoots), but

in the variants with zeatin and kinetin the number of shoots in subsequent subcultures depended on the extent of organogenesis in previous cultures. Data were statistically analyzed by ANOVA and the means were compared with the Tukey test ($P < 0.05$). Coefficients of variance (CV) were also calculated.

RESULTS AND DISCUSSION

SHOOT MULTIPLICATION

Medium supplemented with BA in combination with NAA proved to be the best one for shoot organogenesis in *C. acaulis* explants (Tab. 1). The caulogenic role of BA has been shown in other plants of the *Asteraceae* family (Misra and Dutta, 1999; Emmanuel et al., 2000; Korach et al., 2002; Dhaka and Kothari, 2005). Shoots developed from 95% of the shoot tips exposed to 4.4 μM BA, and 100% of those exposed to 13.3 μM BA. Shoots were initiated in callus developing on the basal part of explants (Fig. 1a). On average, 7.9 shoots per explant were induced on medium with 4.4 μM BA, and 7.5 with 13.3 μM BA. The number of shoots in the 3rd subculture was higher on medium with 4.4 μM BA (Fig. 2a). A similar effect has been reported in *Carlina acaulis* subsp. *simplex* (Trejgell et al., 2009) and *Centaurea rupestris* (Ćurković Perica, 2003). On medium with 13.3 μM BA the multiplication rate was 6.1 shoots per explant during 5 subcultures (Tab. 1, Fig. 2b). Grubišić (2004) reported similar multiplication rates. Shoot organogenesis was lower in hypocotyls but rose during subsequent subcultures to an average 4.0 (4.4 μM BA) and 6.1 (13.3 μM BA) shoots per explant (Tab. 1, Fig. 3a,b). The difference in shoot formation between shoot tips and hypocotyls in response to BA could be related to the levels of endogenous cytokinin in vivo (Kim et al., 2001; Yucesan et al., 2007) or to differences in tissue sensitivity to plant growth regulators (Lisowska and Wysokinska, 2000).

The presence of kinetin and particularly zeatin in combination with NAA resulted in much poorer shoot organogenesis and lower proliferation rates (Tab. 1). With kinetin the results were 2.6 (4.7 μM Kn) and 3.6 (13.9 μM Kn) shoots per shoot tip on average, and in successive passages proliferation rates of 4.3 and 3.9, respectively (Tab. 1, Fig. 2c,d). Shoot organogenesis occurred in hypocotyls only on medium supplemented with 4.7 μM Kn, and the proliferation rate was 1.0 (per donor material), but in subsequent subcultures both the shoot organogenesis frequency and the proliferation rate increased (average 2.7 shoots/explant) (Tab. 1, Fig. 3c). The initial morphogenetic response was poorest when the explants were cultured on medium containing Zea. With 4.6 μM Zea shoot organogenesis frequen-

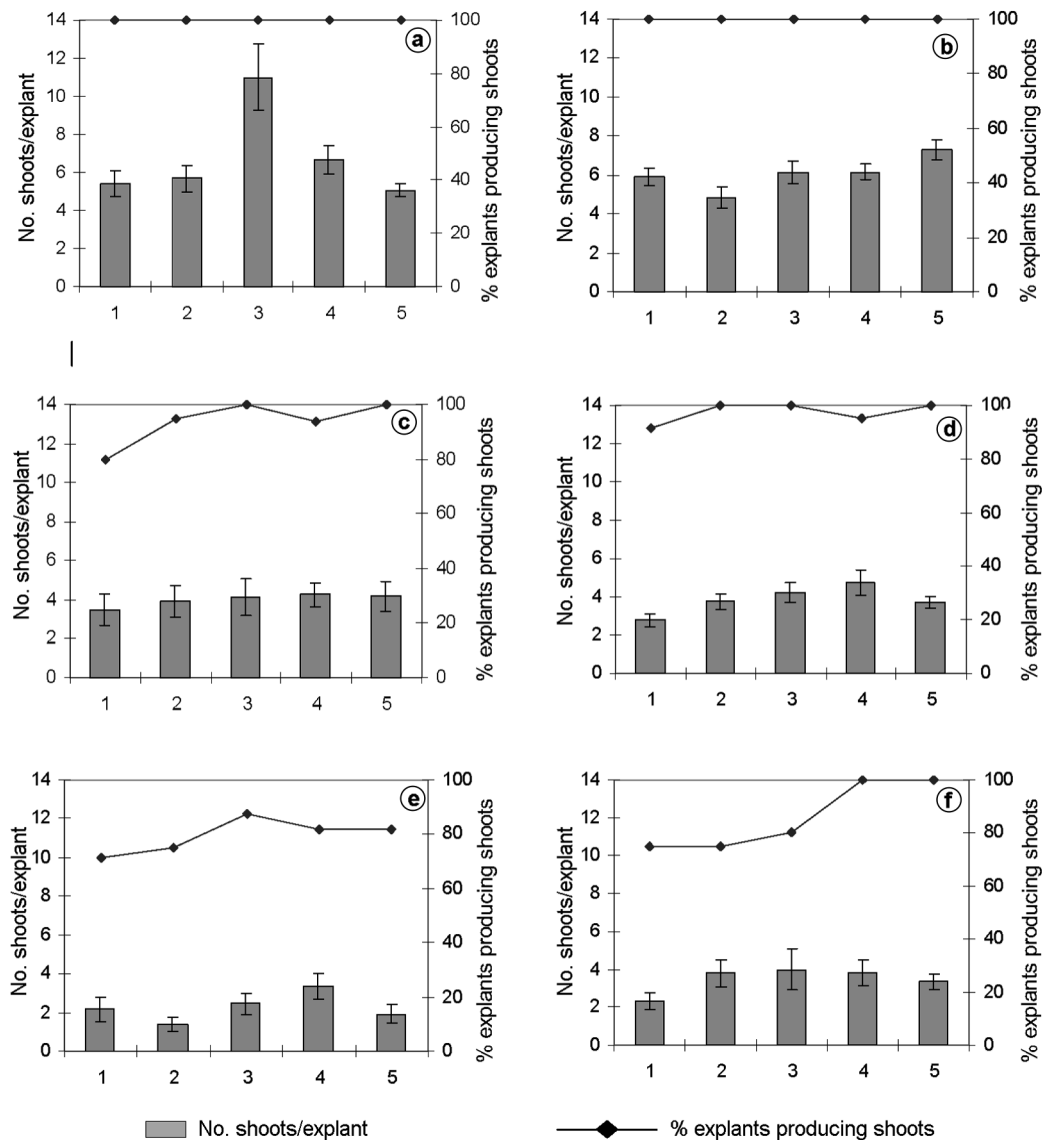


Fig. 2. Effect of different cytokinins in combination with 0.54 μ M NAA on multiplication rate and frequency of shoot organogenesis in 5 consecutive subcultures of shoots differentiating from shoot tips of *Carlina acaulis*. (a) 4.4 μ M BA, (b) 13.3 μ M BA, (c) 4.7 μ M Kn, (d) 13.9 μ M Kn, (e) 4.6 μ M Zea, (f) 13.7 μ M Zea.

cy was 64.3%, with 1.5 shoots per explant from shoot tips (Tab. 1), and it was stable over subsequent subcultures (average 2.7 shoots/explant) (Fig. 2e). The higher Zea concentration gave 75% frequency of shoot organogenesis and 2.1 shoots per donor material, and 3.5 shoots per explant in subsequent subcultures (Tab. 1, Fig. 2d). In hypocotyls, shoot organogenesis in consecutive subcultures was lower (Fig. 3d). The differential response to various cytokinins might be due to differences in their uptake, recognition by cells, and/or the mechanism of action of the cytokinins (Sujatha and Reddy, 1998; Kim et al., 2001).

The presence of BA in the medium strongly inhibited elongation of shoots (Fig. 1b) as compared to Kn and Zea (Fig. 1b'), as already seen during organogenesis from initial material (shoot tips), and the effect was even stronger in subsequent passages (Tab. 1). Inhibition of shoot elongation by BA was also reported in earlier studies on regeneration of different species of the *Asteraceae* family, for example in *Saussurea obvallata* (Joshi and Dhar, 2003) and *Eclipta alba* (Baskaran and Jayabalan, 2005). The presence of BA in regeneration media also inhibited root organogenesis in both the initial material and subsequent subcultures, while the use of

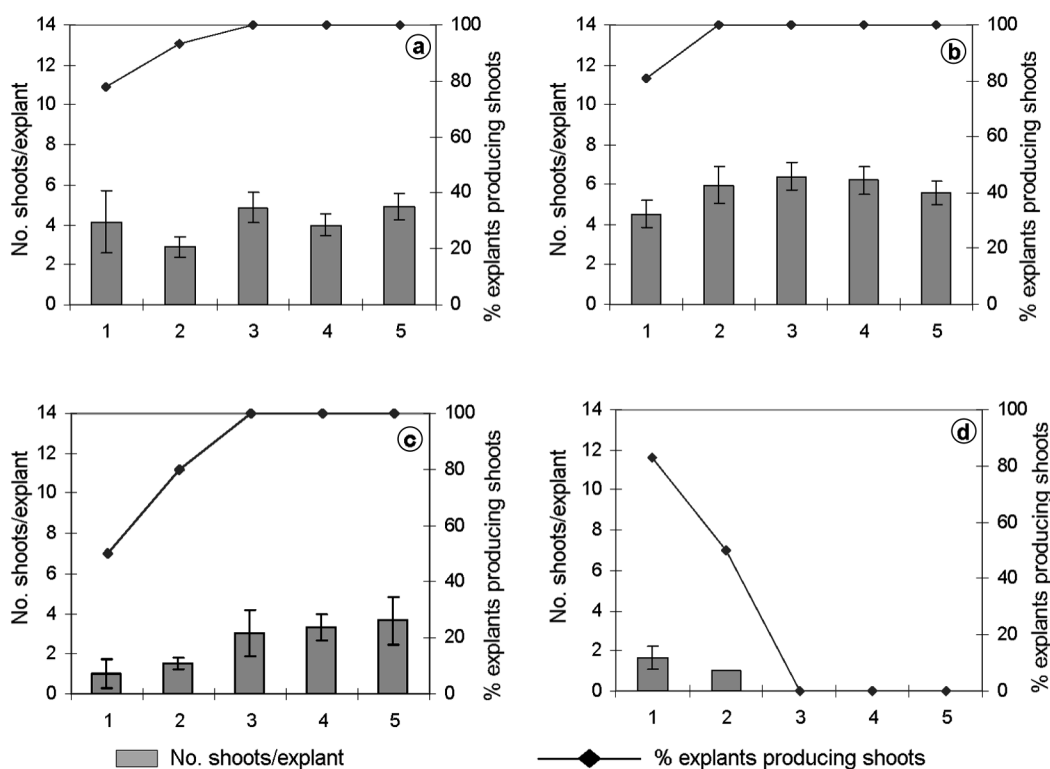


Fig. 3. Effect of different cytokinins in combination with 0.54 μ M NAA on multiplication rate and frequency of shoot organogenesis in 5 consecutive subcultures of shoots differentiating from hypocotyls of *Carlina acaulis*. (a) 4.4 μ M BA, (b) 13.3 μ M BA, (c) 4.7 μ M Kn, (d) 4.6 μ M Zea.

kinetin or zeatin stimulated rhizogenesis (Tab. 1). Similar effects were noted in a few species of the *Peaceae* family (Poonawala et al., 1999) and *Asclepiadaceae* family (Chaudhuri et al., 2004).

ROOTING AND SURVIVAL

Individual shoots were rhizogenesis-competent without auxin treatment *in vitro* and *ex vitro*, but with a lower frequency of rooted shoots (60% *in vitro*, 55.3% *ex vitro*) (Tab. 2). *Ex vitro* rooting has also been reported for *Myrtus communis* (Scarpa et al., 2000), *Camptotheca acuminata* (Liu and Li, 2001) and *Rotula aquatica* (Martin, 2003). Auxins in combination with full-strength MS medium increased both the frequency of rooted shoots and the number of roots per shoot, but IAA and IBA at the higher concentrations reduced the percentage of rooted shoots (Tab. 2). Similar effects were shown in *Eclipta alba* with the same types of auxins (Dhaka and Kothani, 2005). The roots formed directly on adventitious shoots, and callus was never induced at the base of shoots regardless of the medium used (Fig. 1c). The use of 0.54 μ M NAA inhibited root elongation, and the effect was statistically significant. Grubisić et al. (2004) recorded higher num-

bers of roots per shoot in *Carlina acaulis* in the presence of auxins. They used 8-day exposure to NAA, IAA, and IBA at concentrations four times lower than in our experiments. With NAA and IBA they found strong suppression of root elongation on microshoots of *C. acaulis*. IBA is known to be an inhibitor of root elongation (Korach et al., 2002), but in our study the inhibition caused by this auxin was not significant. Halving the mineral salts in the medium increased the frequency of rooted shoots to 94.1% and also the number of roots induced on shoots (Fig. 1d,d'). Auxins in combination with 1/2MS medium inhibited rhizogenesis, particularly root elongation (Tab. 2). The effect was similar in *Eclipta alba* with auxin-supplemented half-strength MS medium: reduced root formation and shorter roots (Baskaran and Jayabalan, 2005). In contrast, in *Guizotia abyssinica*, another member of *Asteraceae*, 1/2 MS with IBA yielded many more roots on microshoots (Sujatha, 1997).

The shoots rooted *in vitro* were easily acclimatized to *ex vitro* conditions, regardless of the medium used. After 8 weeks of greenhouse growth the survival rate ranged from 96 to 100%. When the unrooted shoots were transferred to the vermiculite/sand mixture, however, they developed roots

TABLE 2. Effect of culture conditions on rooting of *Carlina acaulis* plantlets after 8-week culture

MS salt	Auxin	Concentration (μM)	Rooting frequency (%)	No. roots/shoot* (CV)	Longest root length (mm)* (CV)
in vitro					
Full	–	–	60.0	3.0 \pm 0.3 c (50)	46.5 \pm 4.7 a (45)
	NAA	0.054	76.5	8.5 \pm 0.7 a (42)	46.6 \pm 4.5 a (45)
		0.54	70.0	8.7 \pm 0.6 a (38)	23.0 \pm 2.0 b (40)
	IAA	0.057	90.5	4.9 \pm 0.3 bc (38)	46.2 \pm 3.8 a (53)
		0.57	44.8	4.9 \pm 0.5 bc (41)	39.8 \pm 4.4 a (38)
	IBA	0.049	90.6	6.5 \pm 0.5 ab (41)	36.3 \pm 3.7 a (58)
		0.49	54.5	7.5 \pm 0.8 ab (44)	37.0 \pm 5.3 a (49)
Half	–	–	94.1	6.0 \pm 0.3 b (25)	43.8 \pm 4.3 a (52)
	NAA	0.054	90.0	7.1 \pm 0.7 ab (54)	25.1 \pm 4.6 b (64)
		0.54	60.0	5.7 \pm 0.8 bc (42)	18.7 \pm 6.3 b (96)
	IAA	0.057	70.0	3.7 \pm 0.5 cd (66)	24.1 \pm 3.9 b (58)
		0.57	50.0	5.8 \pm 0.8 bc (62)	22.2 \pm 3.6 b (60)
	IBA	0.049	77.8	4.7 \pm 0.5 bc (59)	31.7 \pm 5.9 ab (71)
		0.49	60.0	5.8 \pm 0.7 bc (53)	28.2 \pm 4.9 b (75)
ex vitro					
			55.3	2.5 \pm 0.2 d (37)	51.3 \pm 4.4 a (35)

*means \pm standard error; means with the same letter within columns do not significantly differ by ANOVA followed by Tukey's test ($p < 0.05$); CV – coefficient of variation (%)

within 8 weeks but the survival rate of those plantlets was only 53%. Low rooting frequency and root number per shoot may have been responsible for poor survival.

After acclimatization under greenhouse conditions the plantlets were transferred to the field, where they grew normally. The plants revealed no abnormal leaf morphology. In the next year after acclimatization, 46.5% of the plants flowered (Fig. 1e). The diameter of capitula with bracts (97.1 ± 3.4 mm) was typical for the species. The majority of the plants did not form a stem; 17% of them had a single unbranched stem 30–90 mm long. Flowering occurred in August and September, the same period as in naturally growing plants. The obtained achenes contained viable seeds, and 94% of them germinated on medium supplemented with GA_3 .

This study demonstrated that shoot regeneration from shoot tips can be a useful method for multiplication of *Carlina acaulis*.

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