ACTA BIOLOGICA CRACOVIENSIA Series Botanica 53/2: 68–72, 2011
DOI: 10.2478/v10182-011-0026-z

SHOOT MULTIPLICATION AND IN VITRO ROOTING OF *CARLINA ONOPORDIFOLIA* BASSER

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Received January 21, 2011; revision accepted April 15, 2011

This paper is the first published report describing micropropagation of *Carlina onopordifolia*, using shoot tip and hypocotyl explants. The explants were excised from 10-day-old seedlings and transferred to proliferation medium supplemented with 6-benzylaminopurine (BA; 1.0 or 3.0 mg l^{-1}), kinetin (Kn; 1.0 or 3.0 mg l^{-1}) or zeatin (ZEA; 1.0 or 3.0 mg l^{-1}) in combination with naphthaleneacetic acid (NAA; 0.1 mg l^{-1}). The shoot tips were significantly better than hypocotyls as initial material for shoot regeneration. For shoot multiplication, MS medium supplemented with BA proved superior to the other cytokinins tested. Medium supplemented with 1.0 mg l^{-1} BA gave the highest shoot propagation frequency (66.9%) and number of shoots per explant (2.5). Single shoots were separated from each other and rooted on MS supplemented with IBA for the whole period of culture, with long- or short-pulse IBA application. The highest rooting frequency (84.8%) and root number (18.8) were for short-pulse (1 min) 1000 mg l^{-1} IBA solution. The higher IBA concentration stimulated callus formation and the development of short roots. The shoots were transferred to MS medium without growth regulators. Survival was highest (54.4%) for the plants from the short-pulse 100 mg l^{-1} IBA treatment. After 8 weeks of acclimatization the plantlets were removed to field conditions and grew normally.

Key words: *Carlina onopordifolia*, micropropagation, cytokinins, rooting, auxin.

INTRODUCTION

The extinction of Asteraceae populations and whole species began to accelerate rapidly in the latter part of the 20th century as a result of increasing transformation of the environment. The Asteraceae family includes species that are protected and red-listed as declining (critically endangered), vulnerable at isolated localities, and rare, potentially endangered (Zarzycki and Szeląg, 2006).

Carlina onopordifolia (Asteraceae) is an endemic species of Central and Eastern Europe. In Northern Europe it appears in only a few isolated stands. In southeastern Poland and eastern Ukraine, *C. onopordifolia* is one of the rarest species of flora, representing Pannonian elements (Poznańska and Kaźmierczakowa, 2001). It is listed in the *Red List of Plants and Fungi in Poland* as a vulnerable, endangered species believed likely to move into the declining – critically endangered category in the near future if the causal factors continue to operate (Zarzycki and Szeląg, 2006). A direct threat to it is harvesting of inflorescences, which limits its propagation and lowers its abundance. Natural processes of succession occurring in xerothermic grasslands (growth of shrubs and trees) create shade, preventing the plants from entering the generative stage, leading to population extinction. Developing an effective system of regeneration would be a useful tool for conservation of this species. Here we present a protocol for in vitro micropropagation of *C. onopordifolia*. To our knowledge it is the first. Earlier studies of regeneration in the genus *Carlina* used *C. acaulis*. In that case, propagation was reported to be most effective from zygotic embryos (Grubisić et al., 2004) and shoot tips (Trejgell et al., 2009).

MATERIAL AND METHODS

PROPAGATION CULTURE

Seeds were surface-disinfected with 70% (v/v) EtOH for 30 sec and in 20% (v/v) commercial solution of NaOCl (Domestos) for 30 min, followed by 4 or 5 rinses in sterile distilled water. The seeds were germinated on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg l^{-1} GA_3. Shoot tips

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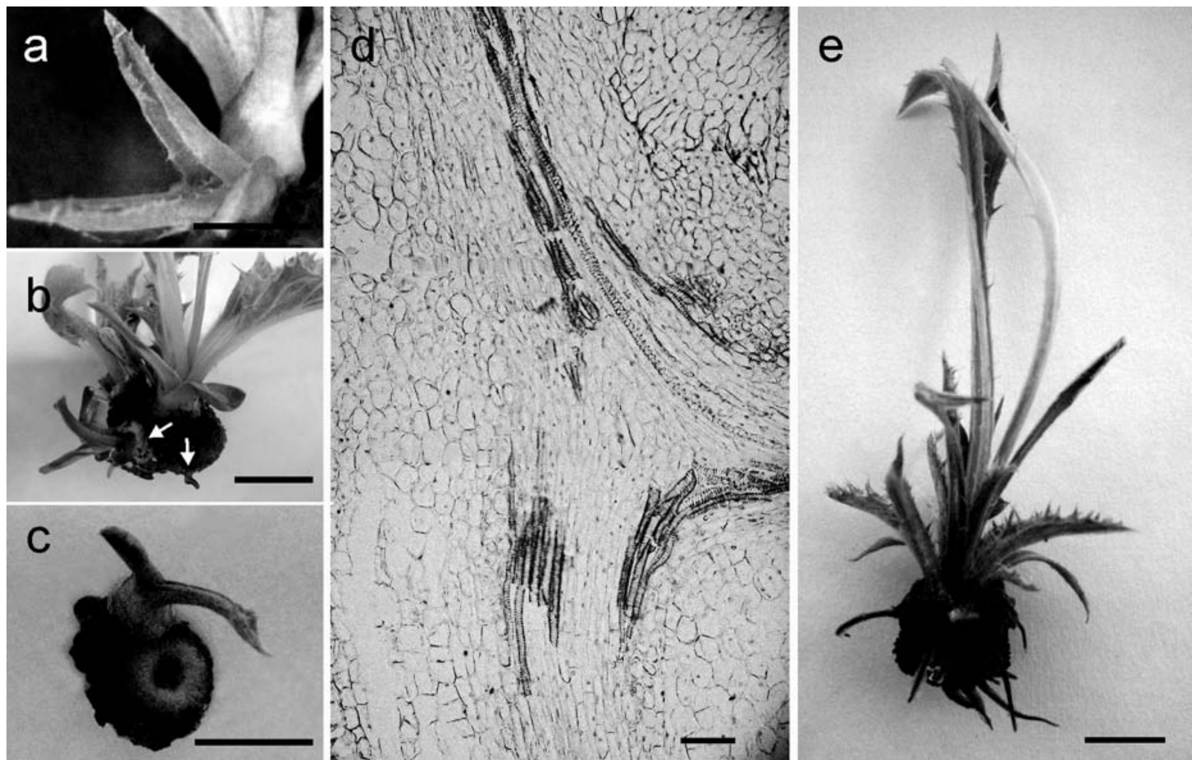

Fig 1. Micropropagation of *Carlina onopordifolia*. (**a**) Development of axillary shoot on medium supplemented with 3 mg l^{-1} BA after 4 weeks of culture, (**b**) Formation of adventitious buds (arrows) within callus, (**c**) Adventitious bud on hypocotyl explant, (**d**) Differentiation of vascular tissue inside callus, (e) Rooted microshoot after 1 min treatment with 1 g l^{-1} IBA solution and cultured on MS medium without auxin. Bar = 2 mm (a), 10 mm (b, e), 0.1 mm (d).

with cotyledonary nodes (3–4 mm long), fragments of hypocotyls (1 mm long, cut under the node) were isolated from the seedlings. Hypocotyl explants were positioned vertically with normal polarity. Explants were transferred to Erlenmeyer flasks containing 30 ml proliferation medium based on MS supplemented with 30 g l^{-1} sucrose, 0.7% agar and growth regulators. Different types of cytokinins were tested in combination with NAA (0.1 mg l^{-1}): BA, KN or ZEA at concentrations of 1.0 or 3 mg l^{-1}. Medium pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Twenty explants (5 explants/flask) were used per treatment. These were subcultured at intervals of 4 weeks, maintained at 26±1°C under continuous white light (45 μmol $m^{-2}.s^{-1}$). After 8 weeks the percentage of explants producing shoots, the number of shoots per explant, and axillary/adventitious shoot length were recorded.

ROOTING AND ACCLIMATIZATION

Single shoots obtained in subcultures (at least 20 mm long) were excised from shoot clusters and transferred to glass jars filled with 50 ml MS basal medium or medium supplemented with IBA at a concentrations of 0.1 mg l^{-1} (2 or 4 weeks) or 1.0 mg l^{-1} (2 weeks). In addition, a two-step rooting procedure was used. After 1 min incubation in a sterile solution of IBA (10, 100 or 1000 mg l^{-1}) the shoots were placed on growth regulator-free medium.

The percentage of rooted shoots, number of roots per shoot, and root length were recorded after 8 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 covered with transparent boxes to maintain humidity (60–70%). After 8 weeks of acclimatization the survival rates were noted, and then the plantlets were transferred to field conditions.

HISTOLOGICAL ANALYSIS

The basal part of rooted shoots was fixed in FAA solution (35% paraformaldehyde/glacial acetic acid/70% ethanol (5:5:90, v/v/v) after 4 weeks of shoot culture on rooting medium. Afterwards, tissues were dehydrated in a graded ethanol series and then embedded in paraplast (Ruzin, 1999). Sections 10 μm thick were prepared using a rotary microtome (MPS-2). Tissues were observed and micrographed using a light microscope (Nikon N-180M).

TABLE 1. Effect of different cytokinins in combination with 0.1 mg l^{-1} NAA on regeneration of *Carlina onopordifolia* from shoot tips and hypocotyl explants after 4 weeks of culture

Cytokinin [mg·L^{-1}]		Shoot tip			Hypocotyl		
		% explants producing shoots	Shoot number per explant*	Shoot length (mm)*	% explants producing shoots	Shoot number per explant*	Shoot length (mm)*
BA	1.0	66.9	2.5±0.31 a	50.8±2.5 de	17.2	0.6±0.26 ab	31.9±2.0 cd
	3.0	64.2	2.4±0.32 a	47.3±1.7 e	21.7	0.8±0.33 a	30.3±0.9 d
KN	1.0	25.3	0.7±0.14 b	77.3±3.2 a	2.2	0.1±0.04 b	38.5±3.5 bc
	3.0	47.4	1.1±0.18 b	58.1±2.2 cd	7.2	0.1±0.10 b	37.5±1.9 bc
ZEA	1.0	19.4	0.9±0.23 b	70.2±1.8 ab	2.2	0.2±0.19 b	43.0±3.1 abc
	3.0	48.7	1.3±0.28 b	66.5±0.8 bc	6.1	0.1±0.06 b	48.0±3.6 ab

*means ± standard error; means with different letters within columns differ significantly by ANOVA followed by Tukey's test at P<0.05.

TABLE 2. Analysis of the interaction between explant type and cytokinin treatment, by two-way ANOVA

Source of variation	Number of shoots /explant*			Shoot length		
	MS	F	P-value	MS	F	P-value
Explant type (A)	200.8859	95.5226	<0.0001	16450,21	254,5049	<0.0001
Treatment (B)	29.23225	13.90013	<0.0001	1523,99	23,57797	<0.0001
A x B	8.394565	3.991673	0.0015	389,3483	6,023697	0.0001

STATISTICAL ANALYSIS

All experiments were done in triplicate. Each treatment consisted of 20 explants or individual shoots. The data are expressed as means ± SE and were analyzed by ANOVA. Tukey's multiple range test was used to check the significance of differences between treatments at P<0.05.

RESULTS AND DISCUSSION

SHOOT MULTIPLICATION

Shoot induction occurred on *C. onopordifolia* shoot tips and hypocotyls on all the medium variants after 4 weeks of culture (Tab. 1). Two-way interaction between explant type and cytokinin and auxin treatment significantly affected shoot induction (Tab. 2). Shoot tips were significantly better than hypocotyls as initial material. The percentage of explants producing shoots and the multiplication rate were significantly higher from shoot tips in all treatments using cytokinins (Tab. 1). Development of axillary buds was observed (Fig. 1a), and sporadic adventitious buds were initiated within callus on the basal part of shoot tips (Fig. 1b). Adventitious buds also developed on the apical surface of hypocotyl explants (Fig. 1c). After 4 weeks of culture on medium supplemented with 3 mg l^{-1} BA the frequency of adventitious shoot organogenesis was 21.7%, with 0.8 shoots per hypocotyl explant Medium supplemented with BA was significantly more effective for shoot proliferation. On shoot tips an average 2.4–2.5 axillary shoots were formed on over 64% of shoot tips, independent of the BA concentration (Tab. 1). The frequency of shoot organogenesis (over 60%) and the proliferation rate (2.2–2.7 shoots per explant) in 5 subsequent subcultures remained at a similar level. This cytokinin plays a significant role in shoot formation as it overcomes apical dominance and promotes axillary bud development (Xu, 2008). BA has been commonly used to induce shoot organogenesis in many plants, including species of the Asteraceae (Orlikowska et al., 1999; Sujata and Ranjitha Kumari, 2007; Chakrabarty and Datta, 2008). Our results confirm the findings of an earlier study comparing the effectiveness of BA, KN and ZEA, in which BA was found to be the most and zeatin the least effective (Shiva Pakash et al., 1994).

BA in the proliferation medium inhibited shoot growth. This inhibitory effect was significant at higher concentration for both types of explant (Tab. 1). Similar results have been reported from studies in *Carlina acaulis* (Trejgell et al., 2009) and other Asteraceae species (Joshi and Dhar, 2003; Baskaran and Jayabalan, 2005; Trejgell et al., 2010).

TABLE 3. Effect of IBA concentration and treatment duration on rooting of *Carlina onopordifolia* microshoots after 4 weeks of culture

Concentration (mg.l-1)	Time of treatment	Rooting frequency (%)	No. roots/ shoot*	Longest root length (mm)*	Survival rate (%)
0	4 weeks	0	-	-	-
0.1	4 weeks	18.9	1.4±0.2 a	15.8±2.0 c	30.0
0.1	2 weeks	29.0	1.3±0.2 a	47.4±4.5 a	36.8
1.0	2 weeks	24.1	1.5±0.1 a	35.2±4.7ab	43.5
10	1 min	70.0	3.7±0.7ab	36.8±4.8ab	52.6
100	1 min	52.7	5.5±0.8 b	24.3±3.0bc	54.4
1000	1 min	84.8	18.8±1.7 c	20.7±1.7 c	46.3

*means ± standard error; means with different letters within columns differ significantly by ANOVA followed by Tukey's test at P<0.05.

ROOTING AND ACCLIMATIZATION

The presence of auxin was necessary for induction of roots on *C. onopordifolia* shoots (Tab. 3). Earlier work showed IBA to be most effective for rhizogenesis from *C. onopordifolia* shoots (data not shown). IBA is an effective auxin for rooting in herbaceous plants (Faisal and Anis, 2003; Dhaka and Kothari, 2005). Using IBA at low concentration (0.1 mg l^{-1}) was ineffective, giving 18.9% rooting frequency and initiating only simple roots. Additional induction of callus on the cut end of shoots was observed. When applied for 14 days, IBA treatment (0.1 and 1.0 mg l^{-1}) slightly increased the frequency of rooting and stimulated root growth. We applied a two-step rooting procedure (Romano et al., 2002) to determine the most suitable concentration for root induction. The frequency of root initiation was highest (84.8%) and the number of roots reached maximum (18.8) under one-minute incubation in 1000 mg l^{-1} IBA solution (Tab. 3). A similar two-step rooting procedure has been used successfully for different species (Martin, 2003; Feyissa et al., 2005; Siddique and Anis, 2007; Husain et al., 2008; Conde et al., 2008). IBA at high concentration is known to suppress root elongation (Korach et al., 2002). In our studies, inhibition of root length was significant at 0.1 mg l^{-1} IBA with continued treatment, and at short-pulse 100 mg l^{-1} and 1000 mg l^{-1}. High IBA concentration also stimulated callus formation (Fig. 1e). Similar results have been reported in research on rooting of other species, for example *Artemisia judaica* (Liu et al., 2003) and *Hagenia abyssinica* (Feyissa et al., 2005). The presence of callus and inhibition of root growth may be associated with low survival of plants (Tab. 3) (Sudha et al., 2005). Histological analysis showed that vascular tissue differentiated inside the callus, but the connection between vascular bundles of shoots and roots was poorly formed (Fig. 1d). This restricted water uptake from the root into the shoot. Leaves that developed in culture deteriorated rapidly after transplanting to ex vitro conditions, and new leaves were formed in the second week of acclimatization. Hazarika (2006) suggested that these leaves may not have been photosynthetically competent ex vitro and were replaced with transitional leaves whose buds had been formed during in vitro culture. After 8 weeks of acclimatization the plantlets were removed to field conditions and looked healthy, with no visible phenotypic variation.

ACKNOWLEDGMENTS

We thank Dr. Anna Ponikierska (Department of Plant Taxonomy and Geography, UMK, Toruń) for help in preparing microscope slides.

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