HORTSCIENCE 32(6):1124–1125. 1997.

Micropropagation of *Centaurea macrocephala* Pushk. ex Willd. by Shoot-axis Splitting

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Additional index words. vertical shoot-split, benzylaminopurine, indole-3-butyric acid, MS medium, Hyponex medium

Abstract. **Micropropagation of *Centaurea macrocephala* Pushk. ex Willd. was achieved by subculturing of vertically split shoots and division of axillary buds on MS-based medium with 0.44 μM BA. A proliferation rate of 2.0 per 16-day culture period was obtained. Seventy percent of microcuttings obtained through in vitro culture could be rooted on a modified Hyponex medium with 25 μM IBA. All plantlets were readily acclimatized and grown in a greenhouse. Chemical names used: benzylaminopurine (BA); indole-3-butyric acid (IBA).**

Centaurea macrocephala, native to the Caucasus, is a perennial plant that grows to a height of 1 m and bears yellow flowers (Bailey, 1976; Yoshimura and Tsukamoto, 1968). This ornamental plant is popular for its cut flowers and as a garden ornamental plant (Yoshimura and Tsukamoto, 1968). Although seed propagation is possible, conventional propagation of this species is by division to obtain uniform plants. The propagation rate by this method is four to eight per year. Some species can be efficiently propagated by division of axillary shoots or repeating nodal culture (Hosoki and Katahira, 1994; Hosoki and Tahara, 1993). In a preliminary experiment, we found these methods not to be applicable to *C. macrocephala*, because terminal and axillary shoots did not elongate during shoot-tip culture, indicating that transverse sectioning of internodes for nodal culture is impossible. Therefore, we used the split-shoot method in which the shoot axis was vertically split into halves so as to force growth of axillary buds for further multiplication.

Successful shoot-tip culture and organogenesis from vegetative organs of *Centaurea junoniana* Svent., a woody shrub, has been reported (Hammatt and Evans, 1985). However, the explants were all seedlings; subcultures, to estimate multiplication rate, were not attempted. The objective of our study was to evaluate the possibility of in vitro propagation of *C. macrocephala* using the split-shoot method.

Materials and Methods

Shoot tips (1 cm long) excised from greenhouse-grown plants at the four- to six-leaf stage were rinsed with tap water for 30 s, surface-disinfested with a solution of sodium hypochlorite (active chlorine, 0.5%) containing 0.1% Tween 20 (w/v) for 10 min, and then rinsed twice in sterile water. The basal portion and leaves were excised, resulting in ≈0.5-cm-long shoot-tip explants. These were placed into test tubes (2 cm in diameter, 15 cm tall) containing 15 mL of agar-gelled medium. The test tubes were covered with aluminum foil. The basal medium consisted of MS major salts and FeEDTA (Murashige and Skoog, 1962), Ringe and Nitsch microelements plus vitamins (Ringe and Nitsch, 1968), and supplemented with 2% (w/v) sucrose. BA was added at 0, 0.44, or 4.4 μM. The medium was solidified with 0.8% (m/v) agar (gel strength of 500–800 $g \cdot cm^{-2}$, Wako Chemical Co., Osaka) after the pH had been adjusted to 5.6 with NaOH. The cultures were incubated for 20 d at 25 ± 1 °C with a 16-h photoperiod provided by cool-white fluorescent lamps (FLR40SW, Mitsubishi Co., Tokyo) (52 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

In subcultures for multiplication, the shoot axis of explants with more than four expanded leaves were split vertically into halves so that one half included the shoot apex and axillary buds but the other half included only axillary buds. These split explants were subcultured onto fresh media containing BA at the same concentrations as in initial medium. Shoot splitting was not used for the explants with one to three expanded leaves because such small explants did not always include a shoot axis with nodes but included only leaves. These small explants were just transferred onto the fresh media without splitting. During subcultures, axillary buds that started growth were separated from the shoot axis and subcultured individually onto fresh medium. A total of four subcultures was conducted at ≈16-d intervals. Ten explants were used per BA concentration for initial cultures. The experiment was repeated three times; a total of 30 explants per each BA concentration was tested. Data on leaf growth, number of shoot apices and axillary buds with expanding leaves, and number of microcuttings (sum of vertical splits of shoots and divided growing-axillary buds) in the initial culture were pooled for four subcultures and three experiments, and effects of BA concentrations were analyzed by regression analysis and single degree-of-freedom contrast.

For rooting in vitro, 2- to 3-cm-long microcuttings were transferred to a modified Hyponex medium (Tada et al., 1978). Hyponex at 2 $g \cdot L^{-1}$ ($6.5N–6P_2O_5–19K_2O$, Hyponex Corp., Marysville, Ohio) supplemented with ($mg \cdot L^{-1}$) 100 NH_4NO_3 and 100 $MgSO_4$ were used as major salts, and the other components (FeEDTA, vitamins, microelements, sucrose, agar) and pH were the same as for the shoot multiplication medium. The modified Hyponex medium was supplemented with IBA at 0, 0.49, 4.9, or 25 μM. The culture conditions were identical to those for shoot multiplication. Twenty explants were tested for each IBA concentration. One month after culture, data on rooting frequency, root count per microcutting, and root length in the single experiment were analyzed as for the shoot multiplication experiment.

For acclimatization, rooted microcuttings were planted in a plastic tray containing sandy loam and covered with a plastic film to retain moisture (>90%). The ambient temperature was 20 °C and light conditions were the same as for in vitro culture. The plastic cover was opened gradually day by day during acclimatization, and it was removed on the 12th d.

Results and Discussion

Twenty days after shoot tip culture initiation, the shoot apex (terminal bud) had expanded to one to five leaves. About 1.6 more leaves were obtained on the medium with than without BA. The internodes of the shoot apex and axillary buds did not elongate, although there were nodes. Therefore, transverse sectioning of stems with nodes was impossible. After vertical shoot splitting, explants with apical shoots continued to grow; the shoot apex developed leaves and explants without apical shoots initiated growth of axillary buds, presumably by release of apical dominance. Leaf length decreased with increasing BA concentration (Table 1). Leaf count also increased with increasing BA concentration, but there was no significant effect of BA concentration. The number of shoot apices (terminal buds) and axillary buds that started leaf development and expansion during subcultures was significantly higher on BA-supplemented medium. The number of microshoots per shoot was also higher on BA-supplemented medium. Considering better leaf growth as measured by the longest leaf length, the basal medium supplemented with 0.44 μM BA appears to be suitable for shoot multiplication. If subcultures are conducted with this BA medium at 16-d intervals, 22 multiplications per year are possible if each explant produces 2.0 new microcuttings at each subculture. This multiplication rate is by far more rapid than conventional propagation through division that yields four to eight shoots per year.

Roots formed on 70% of the explants on

Received for publication 4 June 1996. Accepted for publication 3 Feb. 1997. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Table 1. Response of *Centaurea macrocephala* to in vitro multiplication by repeated splits of the shoot axis and division of axillary buds.[z]

BA concn (μm)	Shoot characteristic: Length of longest leaf (cm)	Leaves per leaf (no.)	Shoot apices and axillary buds with expanding leaves (no./shoot)	No microcuttings[y] obtained (no./shoot)
0	7.9 ± 0.1	3.2 ± 0.2	1.1 ± 0.02	1.4 ± 0.1
0.44	7.2 ± 0.1	4.4 ± 0.2	1.6 ± 0.1	2.0 ± 0.1
4.4	5.8 ± 0.2	5.1 ± 0.3	1.5 ± 0.1	2.1 ± 0.1
Significance				
Linear	**	**	NS	*
Quadratic	*	**	**	**
Contrast (μM)				
0 vs. 0.44	**	**	**	**
0 vs. 4.4	**	**	**	**
0.44 vs. 4.4	**	NS	NS	NS

[z]Mean ± SE of all the data of initial culture, four subcultures and three experiments.
[y]Sum of vertical splits of shoots and divided growing axillary buds.
[NS, *, **]Nonsignificant or significant at $P < 0.05$ or 0.01, respectively.

Table 2. Rooting of microcutting of *Centaurea macrocephala* during culture on modified Hyponex medium.

IBA concn (μM)	Rooting (%)	Roots of microcutting[z] (no.)	Length of longest root[z] (cm)
0	0	0	---
0.49	0	0	---
4.9	30	1.0 ± 0.4	3.5 ± 0.7
25.0	70	2.9 ± 0.7	2.3 ± 0.6
		Significance	
Linear		**	NS
Quadratic		NS	NS
Contrast (μM)			
0 vs. 4.9		NS	---
0 vs. 25.0		**	---
4.9 vs. 25.0		**	NS

[z]Mean ± SE of 20 microcuttings.
[NS, **]Nonsignificant or significant at $P < 0.01$.

the medium with 25 μM IBA, but rooting was less with the lower IBA concentrations (Table 2). The root count per explant on the medium with 25 μM IBA was significantly higher than that on the other media. Roots on the medium with 25 μM IBA were not significantly shorter than those on the medium with 4.9 μM. Thus, 25 μM IBA was suitable for rooting of explants. All rooted plantlets were established without any sign of water stress after acclimatization. Then they were transplanted to a wooden box containing 2 sandy loam : 1 bark (v/v) and grown in the greenhouse for 6 months.

In conclusion, in vitro propagation of *C. macrocephala* by vertical shoot-split may prove to be more efficient than the conventional propagation by division.

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