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IN VITRO PROPAGATION OF *CENTAUREA RUPESTRIS* L.

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A rapid clonal propagation method was developed for *Centaurea rupestris* L., a Balkan Apennine endemic which contains a flavonoid with strong antiphytoviral, antibacterial and antifungal activity. Shoots from aseptically germinated seeds were used for culture initiation. The highest multiplication rate, 11.88 shoots per explant, was achieved in 4 weeks-culture-period in the third subculture on MS medium supplemented with 1 μM 6-benzylaminopurine and 2.9 μM gibberellic acid. The best rooting of excised shoots was achieved on half-strength MS medium supplemented with 3 μM indole-3-butyric acid. Rooted plantlets were transferred to potting soil and acclimatized to outdoor conditions.

Key words: *Centaurea rupestris* L., endemic plant, flavonoid, micropropagation.

Abbreviations: BA – 6-benzylaminopurine; GA_3 – gibberellic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; K – kinetin; MS – Murashige and Skoog (1962) medium; NAA – α- naphthaleneacetic acid

INTRODUCTION

Centaurea rupestris L. is an endemic species which grows in a limited area of the Croatian coast, with exclaves in the Apennines and the Balkan Peninsula (Pavletić and Trinajstić, 1983). It is a perennial herb 15–70 cm high. The stem is erect, slender, fluted, unramified or poorly ramified from the middle, and covered with cobwebby hairs. The flowers are lemon- to golden-yellow (Hegi, 1931). Flower and leaf extracts of *Centaurea rupestris*, as well as the flavonoid quercetagetin 3'-methylether isolated from the flowers, have strong antiphytoviral activity, which is based on the flavonoid's interference with the initiation of viral infection (Rusak et al., 1997). This flavonoid also has antibacterial and antifungal activity (Rusak et al., 2002).

The naturally growing populations of *C. rupestris* L. used for isolation of this flavonoid are rare. This work attempts to develop methods for in vitro clonal multiplication, rooting and acclimatization of this endemic species. In vitro propagation can ensure the availability of plant material throughout the year.

MATERIALS AND METHODS

Seeds of *Centaurea rupestris* were collected from a natural habitat in Uvala Scott near Kraljevica (Croatian northern coastal region) in July 1999. Seeds were sterilized for 5 min with a 2% water solution of sodium dichloroisocyanurate dihydrate (Pliva, Zagreb) followed by three 5 min sterile distilled water rinses, another sterilization with a 6% solution of hydrogen peroxide, and three more 5 min rinses in sterile distilled water. The seeds were inoculated in test tubes (20 × 16 mm) filled with 15 ml MS (Murashige and Skoog, 1962) basal nutrient medium. Seedlings (2–4.5 cm) raised in this culture were separated from their roots and inoculated on shoot multiplication media containing MS mineral salts (full- or half-strength macroelements), 100 mg/l myo-inositol, 1 g/l casein hydrolyzate, 0.1 mg/l

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thiamine HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 2 mg/l glycine, 2.9 µM GA_3, 30 g/l sucrose, 9 g/l agar (Biolife, Milano) and 1 µM K or BA (0.5 or 1 µM). The multiplication rate, that is, the average number of shoots after 4 weeks in culture, was determined for 32 explants of different genotypes per medium through 3 subcultures (Tab. 1). Elongated shoots excised from *C. rupestris* multiple shoot cultures were rooted on half-strength MS medium supplemented with different auxins: IAA, IBA or NAA (3 µM each). Results were recorded for 48 explants per medium after 4 weeks in culture. The media pH was adjusted to 5.7 before autoclaving at 118 kPa and 120˚C for 20 min. The cultures were incubated at 22 ± 2˚C under a 16 h photoperiod (40W fluorescent light, 80 µmol $m^{-2}s^{-1}$). Plants were acclimatized in plastic containers filled with sterile soil, all enclosed in vented plastic bags. After 17 ± 3 days of growth in high humidity the plants were transferred to a greenhouse. To minimize the influence of the different *C. rupestris* genotypes used as initial material, relatively high numbers of explants (32 per shoot multiplication medium and 48 per rooting medium) were statistically analyzed. Mean values, standard deviations and Duncan's test (Duncan, 1955) were used for analysis and interpretation of the data. Shoot multiplication of *C. rupestris* on MS media containing different concentrations of macroelements and plant growth regulators was statistically analyzed globally by merging the results from all three subcultures, and also separately by comparing the results from the third subculture (Tab. 1).

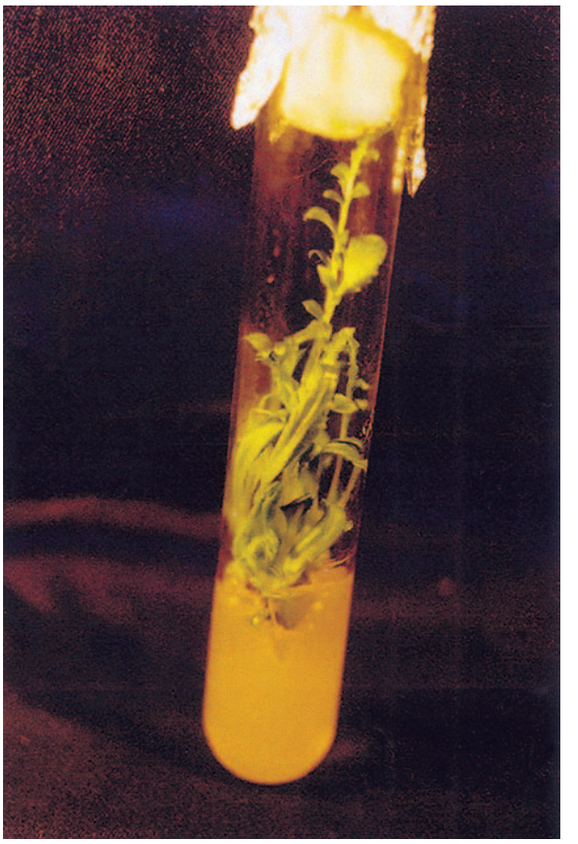

Fig. 1. Shoot multiplication of *Centaurea rupestris* after 3 weeks of cultivation in the third subculture, on MS medium supplemented with 100 mg/l myo-inositol, 1 g/l casein hydrolyzate, 0.1 mg/l thiamine HCl, 0.5 mg/l pyridoxine HCl, 0.5 mg/l nicotinic acid, 2 mg/l glycine, 30 g/l sucrose, 9 g/l agar, 2.9 µM GA_3 and 1 µM BA.

RESULTS

INITIAL CULTURE

The serial sterilization procedure with sodium dichloroisocyanurate dihydrate and hydrogen peroxide was successful; after 20 days on MS basal nutrient medium, 96% of the inoculated seeds were free of visible contamination and 46.3% of the seeds germinated. Seedlings reaching 2–4.5 cm in length were separated from their roots and used as primary explants.

MULTIPLICATION PHASE

Shoots elongated from axillary buds of each explant. The highest multiplication rate, 11.88 shoots per explant, was achieved in the third subculture on full-strength MS medium supplemented with 1 µM BA and 2.9 µM GA_3 (Fig. 1; Tab. 1). By Duncan's test the number of shoots induced on this medium in the third subculture was statistically higher than the number of shoots induced on the other media in the same subculture. However, when the results of all three subcultures were merged, the number of shoots per explant achieved on the same medium was 9.94; this was not statistically higher than the number of shoots induced on full-strength MS medium supplemented with 0.5 µM BA and 2.9 µM GA_3 (Tab. 1). When the same concentration of BA was supplemented, the multiplication rate was higher on media containing full-strength than on those containing half-strength MS macroelements. Shoot multiplication on media without cytokinins was not satisfactory.

TABLE 1. Effect of plant growth regulators and concentration of macroelements in MS medium on shoot multiplication of *Centaurea rupestris* L. during three subcultures

Medium	Cytokinin (μM)	No. of shoots per explant*			
		1st subculture	2nd subculture	3rd subculture	1st + 2nd + 3rd subculture
MS	–	1.13 ± 0.33	1.88 ± 1.05	2.00 ± 0.70 f	1.67 ± 0.85 g
	BA 0.5	8.88 ± 2.93	9.13 ± 3.50	9.38 ± 2.69 b	9.13 ± 3.07 ab
	BA 1.0	8.38 ± 3.74	9.56 ± 2.78	11.88 ± 3.55 a	9.94 ± 3.68 a
	K 1.0	4.75 ± 2.17	5.06 ± 1.48	5.88 ± 3.66 de	5.23 ± 2.64 ef
1/2 MS	–	1.25 ± 0.43	1.44 ± 0.50	1.56 ± 0.61 fg	1.42 ± 0.53 gh
	BA 0.5	5.81 ± 1.81	7.44 ± 1.50	7.50 ± 2.12 cd	6.92 ± 1.99 cd
	BA 1.0	7.25 ± 3.03	7.63 ± 2.18	8.13 ± 2.15 bc	7.67 ± 2.51 c
	K 1.0	4.88 ± 2.15	5.81 ± 2.10	5.88 ± 1.80 de	5.52 ± 2.07 e

*Mean ± standard deviation.
Means with same letters do not significantly differ at the 95% level of confidence (Duncan's multiple range test).

ROOTING AND ACCLIMATIZATION

Rooting of excised elongated shoots on half-strength MS medium supplemented with 3 μM IAA, 3 μM IBA or 3 μM NAA was 29%, 58% and 25%, respectively. Shoots inoculated on half-strength MS medium containing NAA developed callus on their bases and therefore were not acclimatized to outdoor conditions. Forty-two plantlets with developed roots (from media supplemented with IAA and IBA) were transferred to ex vitro conditions. The survival rate was high (86%), and 36 normal-looking plants were successfully acclimatized.

DISCUSSION

This study investigated the possibility of in vitro propagation of *Centaurea rupestris* L., a Balkan Apennine endemic species which contains a flavonoid with strong antiphytoviral, antibacterial and antifungal activity. Media containing MS macro- and microelements were used for all stages of *C. rupestris* culture. MS medium has been recommended for the culture of most Asteraceae species (George and Sherrington, 1984). The highest multiplication rate, 11.88 shoots per explant, was achieved in the third subculture on MS medium supplemented with 1 μM 6-benzylaminopurine and 2.9 μM gibberellic acid. However, when the results of all three subcultures were merged, the difference between the number of shoots achieved on full-strength MS medium supplemented with 1.0 μM BA (9.94) or 0.5 μM BA (9.13) was not statistically significant. Statistically nonsignificant differences in shoot multiplication on media supplemented with 1.0 μM or 0.5 μM BA have been reported for *Centaurea ragusina* (Pevalek-Kozlina, 1998) and *Fibigia triquetra* (Pevalek-Kozlina et al., 1997); unlike *C. rupestris*, whose multiplication was better on full-strength MS medium, those two species grew better on half-strength MS medium.

To completely eliminate the influence of variation in genetic material on the experimental results, especially on shoot multiplication, it would be necessary to inoculate the same genotype on all shoot multiplication media. Since that was impossible, I used a relatively high number of explants in the statistical analysis to minimize the influence of different genotypes.

Indole-3-butyric acid was the best root-inducing auxin for *C. rupestris*. The rooting percentage achieved on half-strength MS medium supplemented with 3 μM indole-3-butyric acid was 58%. In this experiment, calli developed on the bases of shoots inoculated on root-inducing medium supplemented with NAA, a phenomenon found in another root induction experiment, in *Fibigia triquetra* (Pevalek-Kozlina et al., 1997). In that research, adding activated charcoal to the NAA-supplemented medium reduced callus formation but also decreased root formation. As in the case of *C. rupestris*, IBA proved to be the best root-inducing auxin for *Fibigia triquetra* (Pevalek-Kozlina et al., 1997) and *Centaurea ragusina* (Pevalek-Kozlina, 1998). All three species were successfully acclimatized after rooting on half-strength MS medium supplemented with IBA or IAA.

This is the first report on clonal propagation of *C. rupestris*, a Balkan-Apennine endemic species. Propagating *C. rupestris* in vitro can ensure a continuous supply of enough plant material for further experiments on flavonoid activity.

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