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Micropropagation from inflorescence stems of the Spanish endemic plant *Centaurea paui* Loscos ex Willk. (Compositae)

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Abstract Tissue culture techniques have been established as a useful approach for ex situ conservation of rare, endemic or threatened plant species. This report describes the micropropagation of *Centaurea paui* Loscos ex Willk (Compositae), an extremely endangered plant species endemic to the Valencia Community (eastern Spain), as a conservation measure which does not cause damage to the wild plants used as explant source. Inflorescence nodal segments of *C. paui* were selected as explants for in vitro establishment. The best rate of shoot proliferation was obtained on Murashige and Skoog (MS) mineral medium supplemented with 0.5 mg/l 6-benzyladenine or with 2 mg/l kinetin. Maximum shoot elongation was achieved without growth regulators, and the addition of cytokinins significantly decreased their size. In vitro rooting of shoots was difficult after 6 weeks on rooting media. The combination of 2 mg/l indole-3-acetic acid plus 2 mg/l indole-3-butyric acid on MS medium yielded the best results. In this medium, 40% of shoots rooted before 30 days of culture. About 70% of the rooted plants were successfully transferred to pots and acclimatized to ex vitro conditions.

Key words *Centaurea paui* · Endemic species · Micropropagation · Plant conservation

Abbreviations *BA* 6-Benzyladenine · *IAA* Indole-3-acetic acid · *IBA* Indole-3-butyric acid · *2iP* 6-γ-γ-Dimethylallylaminopurine · *Kin* Kinetin · *MS medium* Murashige and Skoog medium · *NAA* α-Naphthaleneacetic acid

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Introduction

A variety of geographical, orographical and geological factors combine in eastern Spain to give rise to a great diversity of environments and landscapes with a specially rich flora (Aguilella et al. 1994). Many species of this flora are described as rare, endemic or threatened, and proper management of this plant diversity is required, due to strong anthropogenic pressures that endanger a great number of species in most of these environments (Gómez-Campo 1987).

In addition to conventional in situ methods, various strategies of ex situ preservation are available for the conservation programs of the endemic or threatened plants (Fay 1992). In vitro culture is an efficient method for ex situ conservation of plant diversity (Krogstrup et al. 1992; Fay 1994), because with this technology many endangered species can be quickly propagated and preserved from a minimum of plant material, and with low impact on wild populations. Some rare, endemic and endangered plants from the Iberian Peninsula have already been successfully micropropagated (Iriondo and Pérez 1990, 1996; Lledó et al. 1995; Martín and Pérez 1995; Amo-Marco and Lledó 1996; Amo-Marco and Ibáñez 1998).

Centaurea paui Loscos ex Willk (Compositae) is an endemic species growing wild only in Sierra de Espadán (Castellón province, eastern Spain), an area of the Valencia Community under strong anthropogenic pressure (Aguilella et al. 1994). During the vegetative period, this plant grows as a perennial herb with leaves and buds arranged in a basal rosette, and during the flowering season, several inflorescence stems develop from the rosette (Fig. 1A). Each flowering stem gives rise to leaves, axillary buds and a single head of purple flowers at the apex (Fig. 1B). This species yields many seeds, but often hybridizes with other species of *Centaurea* (Aguilella et al. 1994). For this reason, a practical micropropagation procedure to conserve this species, avoiding the hybrid material, would be the use of axillary buds, located in the inner

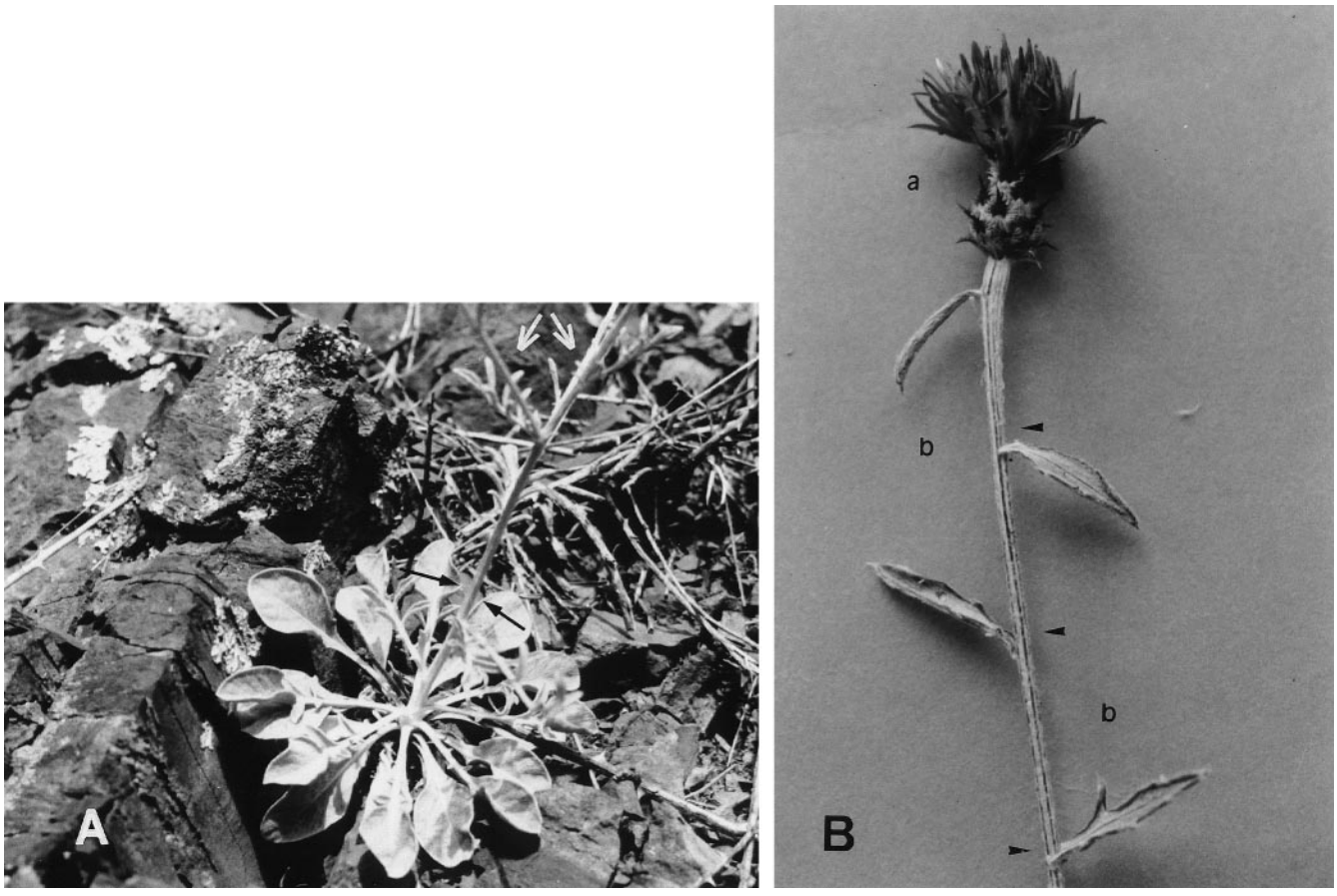

Fig. 1 A, B The threatened and endemic *Centaurea paui* in the field. **A** A wild plant in the flowering season, with the characteristic form of leaves in a rosette, and various inflorescence stems (*arrows*). **B** Detail of an inflorescence stem of *C. paui*, with a terminal "head of flowers" instead of "flower" (*a*), and the stem sections (*b*) with leaves and axillary buds (*arrows*) used as explants for in vitro establishment

base of the rosette and, in the flowering season, along the inflorescence stems.

The micropropagation of field plants growing as rosettes of leaves is often cumbersome due to fungal or bacterial contamination in the basal rosette buds surrounded by a cluster of leaves and located at the ground level which may be hard to sterilize. Moreover, the use of explants from the rosette implies the destruction of the mother plants (a great drawback to conservation of endangered species). For these reasons, in the present study we have used inflorescence stems with both leaves and axillary buds (Fig. 1 B) as the starting material for *C. paui* micropropagation. Inflorescence segments have also been selected for the micropropagation of other endemic species, for instance *Bowiea volubilis* (Hannweg et al. 1996), *Delphinium malabaricum* (Agrawal et al. 1991) and *Limonium cavanillesii* (Amo-Marco and Ibáñez 1998).

The present report describes a practical method for the rapid micropropagation of *C. paui* through shoot proliferation from inflorescence stems, as an aid for its conservation.

Materials and methods

Plant material and sterilization

The axenic shoot cultures were initiated from *C. paui* wild material harvested in the flowering season (early summer). The flowering stems (Fig. 1 B) were cut into segments (50 mm long) and thoroughly washed with soap and running tap water. The segments were then surface-sterilized by dipping them for 1 min in 70% (vol/vol) ethanol containing a wetting agent (three drops of Tween-80), followed by 20 min in 7% (wt/vol) calcium hypochlorite containing the same wetting agent. Afterwards, the segments were rinsed four times (5 min each) in sterile distilled water, and then cut into 20-mm-long nodal segments with one or two buds.

In vitro establishment and culture conditions

Sterilized explants were cultured initially on a medium containing only 3% (wt/vol) sucrose and 0.8% (wt/vol) agar (Probus, Badalona, Barcelona, Spain). After 7–10 days, contaminated or necrotic explants were discarded, and the remaining material was transferred to the establishment medium containing Murashige and Skoog (1962) MS mineral formulation with 2% (wt/vol) sucrose, 100 mg/l myo-inositol, 10 mg/l thiamine, 1.0 mg/l nicotinic acid, 1.0 mg/l pyridoxine and 0.5 mg/l kinetin (Kin).

The pH of all media was adjusted to 5.7 with KOH before adding agar, and they were sterilized in an autoclave at 121 °C for 20 min. The explants (five per vessel) were incubated in 55×75 mm glass flasks, containing 30 ml of culture medium, and sealed with two layers of aluminium foil. The cultures were maintained in a growth chamber at 25±1 °C during the light period and 20 °C during darkness, under a 16-h photoperiod provided by Osram cool white fluo-

Table 1 Effect of different cytokinins and concentration on the percentage of reactive explants, number of shoots, maximum shoot length, number of shoots longer than 10 mm and number of 10-mm segments obtained per explant of *Centaurea paui* after 4 weeks of culture on MS medium. The means ± SE are presented. For each column, means sharing at least one letter are not significantly different at the 0.05 level (Duncan's multiple-range test)

Cytokinin (mg/l)		Reactive explants (%)	Shoots per explant	Maximum shoot length (mm)	Shoots longer than 10 mm	Segments of 10 mm per explant
Control	0	96	1.8 ± 0.2 e	43.2 ± 3.9 a	1.2 ± 0.1 bcd	3.3 ± 0.2 bcd
Kin	0.5	100	2.8 ± 0.3 cde	27.4 ± 3.2 b	1.2 ± 0.1 bc	3.5 ± 0.3 bcd
	1	85	3.1 ± 0.4 bcde	20.7 ± 1.9 bcd	1.6 ± 0.2 b	3.4 ± 0.4 bcd
	2	92	4.2 ± 0.6 ab	19.2 ± 2.1 cd	1.5 ± 0.2 b	4.6 ± 0.6 ab
2iP	0.5	100	2.9 ± 0.3 cd	23.3 ± 3.6 bc	1.2 ± 0.2 bc	3.4 ± 0.4 bcd
	1	100	2.1 ± 0.2 de	19.8 ± 2.0 cd	1.2 ± 0.2 bc	2.6 ± 0.3 cd
	2	92	2.2 ± 0.2 de	12.2 ± 1.4 d	0.6 ± 0.2 d	2.3 ± 0.2 d
BA	0.5	92	4.8 ± 0.5 a	28.3 ± 2.6 b	2.7 ± 0.3 a	5.4 ± 0.5 a
	1	96	3.3 ± 0.3 bc	14.8 ± 1.3 d	0.9 ± 0.2 cd	3.5 ± 0.4 bcd
	2	92	3.4 ± 0.5 bc	15.5 ± 1.3 cd	1.3 ± 0.2 bc	3.6 ± 0.5 bc

rescent lamps, with a light intensity of 60 μmol m^{-2} s^{-1}. These conditions were also applied for the shoot multiplication and rooting experiments described below.

The vegetative shoots obtained in vitro from the axillary buds were excised and subcultured twice (every 6 weeks) on fresh establishment medium to provide a stock collection of shoots for micropropagation studies.

Shoot multiplication

Six-week-old shoots, obtained from the establishment medium, were cut into nodal segments (10 mm long) containing two axillary buds, and used as explants for multiplication experiments. Shoot propagation was performed on MS medium supplemented with the same organic compounds of the establishment medium and a cytokinin 6-benzyladenine (BA), Kin or 6-γ-γ-dimethylallylaminopurine (2iP), each one at three different concentrations (0.5, 1 or 2 mg/l). A control treatment without cytokinins was also included.

Data were recorded after 4 weeks, and the rate of shoot proliferation was determined by: (1) percentage of explants forming viable axillary shoots (reactive explants), (2) number of new shoots formed per explant, (3) length of the tallest shoot produced per explant, (4) number of shoots longer than 10 mm per explant, and (5) number of 10-mm-long segments (including shoot tips) that could be obtained per explant.

Shoot rooting and acclimatization of plantlets

Root induction was carried out with 10- to 12-mm-long individual shoots isolated from multiplication media. These explants were transferred to MS basal medium supplemented with the aforementioned organic compounds and the auxins indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or α-naphthaleneacetic acid (NAA) at various concentrations.

After 6 weeks of culture, the following data were recorded: (1) percentage of rooted shoots, (2) number of roots per rooted shoot, (3) length of the longest root per rooted shoot, and (4) length of the longest shoot per explant.

To control the time course of rooting, the percentage of rooted shoots was recorded weekly during the 6 weeks of culture.

Rooted plantlets were transplanted to 60×80 mm plastic pots containing vermiculite and perlite (3:1) and kept in the growth chamber under a day/night temperature regime of 28 ± 2 °C/25 ± 2 °C and a 16-h light photoperiod of 120 μmol m^{-2} s^{-1}, supplied by Osram fluorescent tubes. To maintain initially the plants at high humidity, pots were covered with clear plastic bags. The plantlets were acclimatized to reduced relative humidity by gradually opening the plastic cover, and after 4 weeks they were completely uncovered and hardened to greenhouse conditions.

Statistical design

Each treatment was applied to 25 explants (shoot multiplication experiments) or to 20 explants (rooting experiments) following a completely randomized design. The results were subjected to analysis of variance and the means compared using Duncan's multiple-range test at $P < 0.05$.

Results and discussion

Establishment of cultures and shoot multiplication

When the number of seeds that can be obtained from a threatened wild plant for ex situ propagation purposes is high, it could be argued that the use of seeds is an alternative for the establishment of axenic stocks of shoots without damaging the wild plants. However, when hybridization mechanisms take place, the seeds obtained are not an appropriate material for conservation programs (Lledó et al. 1993). Clearly, this is the case for *C. paui*. Hybridization of this species with *C. saguntina* Mateo & M. B. Crespo and *C. pinae* Pau has been reported (Aguilella et al. 1994). Thus collected seeds could originate from these interspecific crosses, and only the identification of adult plants by a specialist guarantees a reliable source of true-type plant material of *C. paui*. For this reason, we have selected axillary buds from testified adult genotypes and used in vitro culture techniques for the propagation and conservation of this endemic Spanish plant.

In this work, using inflorescence stems as explant and with the sterilization procedure described, only 20% of the cultured explants remained contaminated, while the mother plants used as explants source were preserved.

During the shoot multiplication phase, the percentage of reactive explants was consistently high (85–100%) in all media studied regardless of the cytokinin used and concentration added (Table 1). The unreactive explants showed hyperhydricity or apical necrosis.

Although shoot multiplication of *C. paui* was obtained on MS medium without cytokinins (Table 1), the analysis of variance showed a significant effect of the cytokinin treatment on all parameters measured: number of shoots

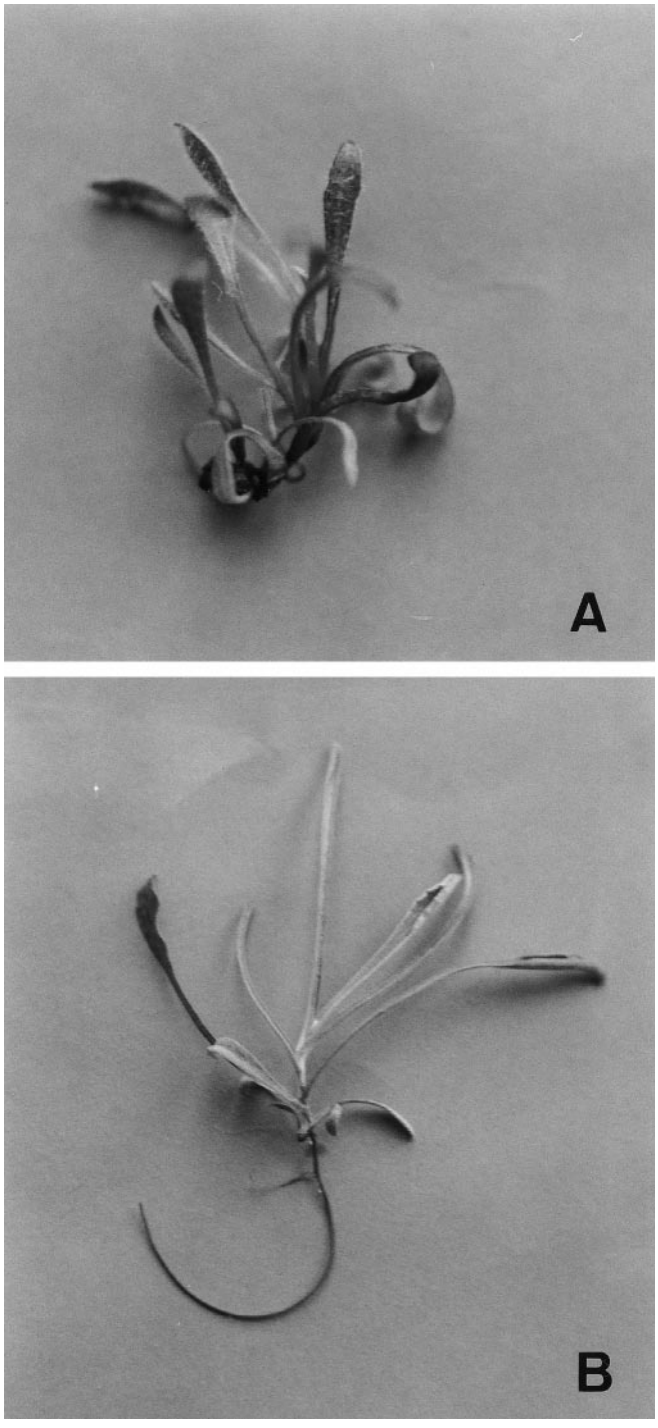

Fig. 2A, B Micropropagation of *C. paui* from axillary buds of inflorescence stems. **A** Shoot multiplication on MS medium + 0.5 mg/l BA. **B** A rooted axillary shoot obtained on MS medium with 2 mg/l IAA + 2 mg/l IBA, after 6 weeks of culture

(F = 6.9, P<0.0001), maximum shoot length (F = 12.4, P<0.0001), number of shoots longer than 10 mm (F = 8.3, P<0.0001) and number of 10-mm segments (F = 5.2, P<0.0001).

Thus, an average of 1.8±0.2 shoots per explant was obtained after 4 weeks of culture on MS medium without cytokinin. But this result was significantly improved by BA at all the concentrations tested, by 2 mg/l Kin or by 0.5 mg/l 2iP (Table 1). The maximum number of shoots (4.8±0.5) was obtained with 0.5 mg/l BA, followed by 2 mg/l Kin (4.2±0.6), without significant differences between them. In general, BA proved to be the most effective cytokinin for shoot production, especially at low concentrations, followed by Kin.

The greatest shoot elongation was obtained on MS medium without cytokinins (Table 1). In fact, shoot elongation decreased with increasing cytokinin concentration, especially for 2iP. Additionally, in culture media with 2iP, some flowers were formed, and at 2 mg/l 2iP, the appearance of the developed axillary shoots was also unsatisfactory. For Kin treatments, progressively higher concentrations induced more but smaller shoots, suggesting an inverse relationship between the number of shoots and shoot elongation.

The control medium without cytokinins yielded 1.2± 0.1 shoots longer than 10 mm per explant, and 3.3±0.2 segments of 10 mm per explant. The only cytokinin that significantly improved these results was again BA at 0.5 mg/l (Table 1). Thus, MS medium with 0.5 mg/l BA yielded 2.7±0.3 shoots longer than 10 mm and 5.4±0.5 segments of 10 mm per explant.

Therefore, 0.5 mg/l BA was the best growth regulator for shoot multiplication (Fig. 2A) in *C. paui*, since it yielded the highest number of shoots, the greatest number of segments and also the highest number of shoots longer than 10 mm for the rooting phase. BA has been found to be an efficient cytokinin for shoot multiplication in other endangered or endemic species: *Centaurea junoniana* (Hammatt and Evans 1985), *Vella lucentina* (Lledó et al. 1995), *Centaurium rigualii* (Iriondo and Pérez 1996) and *Syzygium alternifolium* (Sha Valli Khan et al. 1997).

Shoot rooting and acclimatization of plantlets

All auxins used promoted rhizogenesis in *C. paui*, but the percentage of rooted shoots was low in most media tested (Table 2). Moreover, the rooting process was very slow and most roots emerged after 4 weeks of culture. A low amount of callus growth was observed during rooting experiments in some media, but in all cases the root induction was observed directly at the base of shoots.

The percentage of shoot rooting achieved after 6 weeks of culture on MS medium with 2 mg/l NAA was 45%, 50% with 2 mg/l IBA and 45% with 2 mg/l IBA plus 2 mg/l IAA. This combination of two auxins was finally selected as the most effective for root induction (Fig. 2B) in *C. paui* shoots because on this culture medium the roots developed 2 weeks earlier than on the other media (Table 2) and also a vigorous root system was produced. This auxin combination seems to stimulate root initiation in *C. paui*, rapidly enhancing the process of root formation. IBA is more resistant than IAA to chemical degradation in tissue culture media, both during autoclaving and at room temperature (Dunlap and Robacker 1988; Nissen and Sutter 1990);

Table 2 Effect of auxins on the percentage of shoots of *C. paui* rooted weekly during the first 6 weeks of culture on MS medium [*w* = week(s)]

Auxin (mg/l)		Percent rooting				
		1 w	2 w	3 w	4 w	6 w
Control	0	0	0	10	15	15
IAA	0.5	0	5	10	15	25
	1	0	0	0	10	15
	2	0	10	10	20	35
	5	0	0	0	5	15
IBA	0.5	0	0	0	15	20
	1	0	0	5	10	25
	2	0	5	5	10	50
	5	0	0	0	5	25
NAA	0.5	0	5	10	10	15
	1	0	5	5	10	20
	2	0	10	10	15	45
IBA 2+IAA 2		0	0	15	40	45

Table 3 Effect of auxins on the number of roots, maximum root length and shoot elongation obtained after 6 weeks of culture on MS medium. Due to the low percentage of shoots rooted in most media (see Table 2), the analysis of variance detected no effect of auxin treatment during the rooting phase on these parameters. The initial length of cultured explants was 10–12 mm

Auxin (mg/l)		Number of roots	Maximum root length (mm)	Shoot length (mm)
Control	0	1.0±0.0	27.3±3.7	18.0±2.0
IAA	0.5	1.0±0.0	14.6±3.9	22.7±2.8
	1	1.3±0.3	20.0±3.8	17.3±1.9
	2	2.1±0.5	27.9±12.1	25.0±2.6
	5	1.3±0.3	8.3±1.7	16.7±1.8
IBA	0.5	1.3±0.3	15.5±2.2	17.9±2.1
	1	1.8±0.4	14.4±2.1	16.6±2.0
	2	1.1±0.2	16.3±7.8	22.6±2.2
	5	2.2±0.4	15.4±6.2	22.2±1.6
NAA	0.5	1.0±0.0	5.0±1.2	23.1±2.7
	1	2.3±0.8	14.5±3.8	29.6±3.4
	2	2.0±0.1	13.1±2.9	39.0±16.7
IBA 2+IAA 2		1.8±0.4	39.8±16.4	17.2±1.8

however, the means by which IBA is more effective than IAA for the induction of rooting in vitro remain unclear (Epstein and Ludwing-Muller 1993).

The mean number of roots obtained per rooted shoot (Table 3) ranged from 1.0 to 2.3, and a wide range of means was obtained for the maximum root length (from 5.0 to 39.8 mm long) and for shoot length (from 16.6 to 39.0 mm). Nevertheless, and due to the low percentage of shoots rooted, the analysis of variance detected no effect of auxin treatment during the rooting phase on any of the parameters measured: number of roots per explant ($F = 1.5$, $P > 0.10$), maximum root length ($F = 0.9$, $P > 0.50$), and shoot elongation ($F = 1.5$, $P > 0.10$).

It is worth noting that during the rooting phase, the shoots elongated in all media tested, since the initial length of cultured explants was 10–12 mm (Table 3). This effect may be useful because we have observed that taller plants survive the transplantation and acclimatization phase better than smaller shoots not only in *C. paui* but also in other endemic species (Amo-Marco and Lledó 1996; Amo-Marco and Ibañez 1998).

When the rooted plantlets were transplanted to ex vitro conditions, most of them continued growing vigorously 1 month after the transfer. Under the hardening conditions used, 70% of the plants were successfully established in the greenhouse, without any sign of water stress after 4 weeks, when the plastic bags were finally removed. The appearance and growth of these plantlets were also normal.

To conclude, we report an efficient protocol to micropropagate the endemic species *C. paui* from nodal segments of inflorescence stems, avoiding both the destruction of the rare wild plants and the problems associated with interspecific hybridizations with other *Centaurea* species. This protocol provides a successful and rapid propagation technique for ex situ conservation of this endangered species, which complements the current in situ approaches for the preservation of biodiversity in the Iberian Peninsula. A plant stock is now maintained in vitro, and it can be utilized in the local conservation programs to preserve *C. paui*.

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