IUFS J Biol 2012, 71(2):121-127

In vitro propagation of endemic plant *Centaurea arifolia* Boiss. Taxa

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Abstract

Centaurea L. (Asteraceae) is one of the largest genera in the flora of Turkey. *Centaurea arifolia* belonging to the genus Centaurea is considered as DD (Data Deficient) according to IUCN category. There are no studies identified aiming to propagate this species *in vitro.* In this study, the seeds were germinated aseptically in Petri dishes containing growth regulator free Murashige and Skoog half-strength (MS ½) medium. Leaf explants of six-week-old grown seedlings were cultured on MS medium supplemented with 1 mg l^{-1} BAP plus 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP plus 0.2 mg l^{-1} NAA for indirect regeneration and after three weeks, actively growing calli appeared and proliferated. Induction of adventitious shoot regeneration from calli was achieved approximately 150% (MS1) and 120% (MS2), within three weeks. Regenerated adventitious shoots were best rooted on MS medium containing 1 mg l^{-1} IBA. This protocol provides a successful propagation technique through indirect in vitro organogenesis from leaf segments of *C. arifolia*.

Keywords: *C. arifolia*, endemic species, micropropagation
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(Received: 20.12.2010 Accepted: 09.12.2011)

Endemik *Centaurea arifolia* Boiss. bitkisinin *in Vitro* üretimi

Özet

Centaurea L. (Asteraceae) Türkiye florasının en geniş cinslerinden biridir. Centaurea cinsine ait olan *Centaurea arifolia* IUCN kategorisine göre DD (veri yetersiz) olarak değerlendirilmiştir. Bu tür ile ilgili herhangi bir in vitro propagasyon çalışmasına rastlanmamıştır. Bu çalışmada, tohumlar büyüme düzenleyicisi içermeyen 1\2 Murashige ve Skoog (MS ½) ortamında petri kaplarında aseptik koşullarda çimlendirilmiştir. Altı haftalık fidelerden alınan yaprak eksplantları dolaylı rejenerasyon için 1 mg l^{-1} BAP + 0.1 mg l^{-1} NAA ve 2 mg l^{-1} BAP + 0.2 mg l^{-1} NAA hormon kombinasyonlarını içeren MS ortamına transfer edilmişlerdir. Üç hafta sonra aktif olarak büyüyen kalluslar elde edilmiştir. Kalluslardan üç hafta sonra adventif gövde rejenerasyonunun teşviki yaklaşık olarak % 150 (MS1) ve % 120 (MS2) oranında başarı elde edilmiştir. Rejenere olmuş adventif gövdeler 1 mg l^{-1} IBA içeren MS ortamında köklendirilmiştir. Bu yöntem, *C. arifolia'*nın yaprak segmentlerinden in vitro dolaylı organogenesis aracılığı ile oluşturulmuş başarılı bir üretim tekniği sağlamaktadır.

Anahtar Kelimeler: *C. arifolia*, endemik tür, mikroçoğaltım

Introduction

Centaurea L. is the third largest genus in terms of the species number in Turkey (Wagenitz 1975). There are 197 species in 34 sections and about 60% is endemic (Wagenitz 1975; Davis et al. 1988; Güner et al. 2000; Özhatay and Kültür 2006; Özhatay et al. 2009). *C. arifolia* Boiss. belonging to this genus, is also an endemic species and considered as DD (Data Deficient) due to the lack of information regarding its dispersal and abundance according to IUCN category (Ekim et al. 2000; IUCN 2001).

C. arifolia Boiss.

Endemic. Biannual or perennial with thickened taproot. Stem erect, 1-2 m, with several capitula on long nearly naked branches. Leaves thin, papery when dry, with scattered septate hairs; basal and lower oblong with cordate to hastate base and long petiole, lower sometimes nearly triangular with a pair of linear-lanceolate lateral segments; median and upper lanceolate, not decurrent. Involucre 30-35 x 25-35 mm, nearly globose. Appendages large, totally concealing basal part of phyllaries, dark brown (lighter towards margin), nearly orbicular, margin ± regularly fringed, fringes 2-3 mm, appendage ending in a 4-5 mm spinule. Flowers are purple. Achenes c. 5 mm; pappus 7-8 mm.

Studies of different Centaurea species have focused on their taxonomy, morphology, anatomy, ecology, palynology, cytology, karyology, phytochemistry and molecular revision (Ertuğrul et al. 2004; Flamini et al. 2004; Çelik et al. 2005; Yaman 2005; Akan 2007; Bona and Aras 2008; Okay and Demir 2010). In addition, certain tissue culture methods have been developed in the production of the *Centaurea* species as well as for its *ex situ* conservation (Hammatt and Evans 1985; Iriondo and Perez 1996; Cuenca et al. 1999; Cuenca and Marco 2002; Okay and Demir 2010). Previous studies report propagation of this plant species through plant tissue culture (Özel 2002; Özel et al. 2006a,b). Tissue culture studies were conducted on *Centaurea junaniana* (Hammat and Evans 1985), *Centaurea paui* (Cuenca et al. 1999), *Centaurea cyanus* (Kakegawa et al. 1991; Tanimoto and Ishioka 1991), *Centaurea macrocephala* (Hosoki and Kimura 1997; Takashi and Daisuke 1997), *Centaurea spacchii* (Cuenco and Marco 2000), *Centaurea rupestris* (Perica 2003), *Centaurea zeybekii* (Kurt and Erdag 2009) and *Centaurea ultreiae* (Mallon et al. 2010), and the sterilization applications, rooting and shoot formation mediums were studied (Okay and Demir 2010). In addition, factors affecting in vitro plant regeneration of the critically endangered *Centaurea tchihatcheffii* Fisch et. Mey have been investigated (Özel et al. 2006a). Tıpırdamaz et al. (2006) emphasized that better propagation rates could be obtained through development of *in vitro* and *ex vitro* propagation techniques including propagation through seeds. *In vitro* germination and micropropagation of *Centaurea zeybekii* Wagenitz (Kurt and Erdağ 2009) and adventitious shoot regeneration of *Centaurea depressa* Bieb. have also been reported (Özel et al. 2008). However, no tissue culture studies on *C. arifolia* were performed up to date. Therefore, this paper reports a simple protocol for micropropagation from leaf explants of endemic *C. arifolia*.

Materials and methods

Plant material

The taxa *Centaurea arifolia* Boiss., from the genus Centaurea was employed as the experimental material in this study. Mature seeds (achene fruits) *Centaurea arifolia* were collected from a natural habitat during June in Hatay . They were dried at room temperature and the seeds collected from different plants were used for tissue culture applications The seeds were put into petri dishes and stratified for 1, 3 and 7 days at +4°C. In addition, the

seeds were also applied to sulfuric acid for breaking dormancy. Seeds were washed thoroughly under running tap water for 30 minutes. Subsequently, seeds were surface sterilized in 70% (w/v) ethanol for 10 minutes followed by 4.5% (w/v) sodium hypochloride solution for 15 min, then rinsed three times in sterile distilled water under laminar flow hood. Sterilized seeds were left to swell in distilled water for 1 hour. The seeds were implanted in petri dishes containing growth regulator free half-strength (MS ½) MS (Murashige and Skoog 1962) medium supplemented with 1% (w/v) sucrose and solidified with 0.8% agar (w/v). The pH of the MS medium was adjusted to 5.8 before sterilization by autoclaving at 121°C at 105 kPa for 20 min. The pH of the MS medium was confirmed after sterilization. The germination percentage was expressed as number of germinated seeds in percentages (%), For the subsequent treatments, germinated seeds were transferred to MS medium without hormones for seedling development. Well-developed seedlings were used as the starting material in the regeneration experiments. From approximately 6 week-old-plants, root, cotyledone, stem node and leaf explants were cut into 0.5 or 1 cm and cultured on various combinations of phytohormones in MS medium and subcultured twice (every 6 weeks). The plantlet was rooting in MS medium containing 1 mg/L IBA. Seed germination, seedling growth and subcultures were carried out in a growth chamber, illumination was provided by cool white fluorescent lamps at 300 µmol m^{-2} s^{-1} with a 12-h light period at 25 ± 2ºC. All experiments used a complete randomized design with three replications and 7 explant per replication. Data were recorded as number of multiplied shoots after 4 weeks, and percentage of shoots were calculated.

Results and Discussion

Murashige and Skoog formulation is the most commonly used medium in plant tissue culture experiments (Molia 2000). In the present paper, in vitro germination of the plant seeds is achieved by use of basal salts medium containing growth regulator free half-strength (MS ½) MS. In vitro germination experiments, it was observed that the seeds of *C. arifolia* were very difficult to germinate. However, it has been observed that the seeds germinated in a time as short as three weeks. The seed were applied to cold and sulfuric acid for breaking dormancy but we did not observed any difference in seed germination. Germination frequency in *C. arifolia* seeds was around 25% after three weeks, but the germination percentage remained the same. The result is in agreement with Özel (2002), Özel et al. (2006a, b), and Okay and Günöz (2009), and showed that the germination percentage of *C. tchihatcheffii* seeds were quite low (Okay and Demir 2010). In the present study, six week old seedlings developed (Fig. 1A) on MS medium and were separated from primary roots, cotyledone, stem nodes and leaves, and transferred into MS basal medium with BAP (benzyl aminopurine), NAA (a-naphthaleneacetic acid), IBA (indole-3-butyric acid) and 2,4-D at different concentrations and combinations for indirect regeneration. Primarily, we used a wide range of hormone combination in basal medium salt and the most effective concentrations were selected. The effect of different hormone combinations on callus formation, adventitious shoot regeneration and then rooting using leaf segments of *C. arifolia* as explants for in vitro establishment, were studied (Table 1).

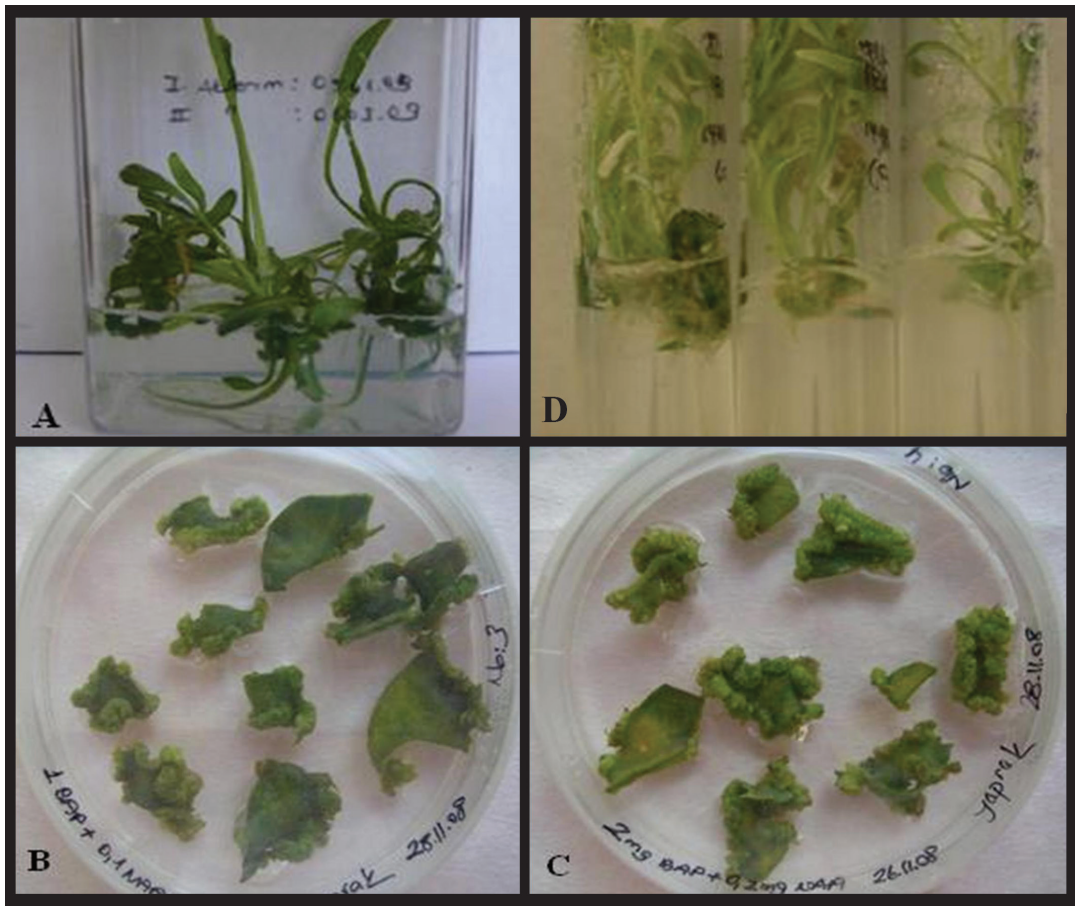

Figure 1. Micropropagation of *Centaurea arifolia*. **(A)** The seeds were implanted in dishes containing growth-regulator-free half-strength (MS ½). After six-week, a well-developed *Centaurea arifolia* seedlings onto MS medium. **(B)** Callus formation from leaf explants at MS1. **(C)** Callus formation from leaf explants at MS2. **(D)** Axillary shoot proliferation was obtained from callus which was developed in MS1 and MS2 and rooting on MS medium supplemented with 1mg/L IBA.

Table 1: The effect of different hormone combinations on callus formation, adventitous shoot regeneration and rooting *of Centauria arifolia*.

Medium	Hormone Concentrations	Culture Development
MS 1	1 mg\L BAP+0.1 mg\L NAA	callus, shoot
MS2	2 mg\L BAP+0.2 mg\L NAA	callus, shoot
MS3	1 mg\L 2,4D	Callus
MS4	2 mg\L 2,4D	Callus
MS5	2 mg\L BAP+0.2 mg\L 2,4D	Callus
MS6	5 mg\L BAP+0.5 mg\L 2,4D	Callus
MS7	1 mg\L IBA	Root

Induction medium MS1-MS6, especially MS1 and MS2 (Fig. 1B-C), induced callusing from leaf explants in different frequencies and after three weeks, proliferated actively growing calli appeared. It was also found to be the most effective BAP and NAA combinations (1 mg l^{-1} BAP plus 0.1 mg l^{-1} NAA and 2 mg l^{-1} BAP plus 0.2 mg l^{-1} NAA) for indirect shoot regeneration as well as callus formation after three weeks. Calli developed from the roots, stem node and cotyledone explants were exposed to MS1 and MS2. After four weeks, shoot regeneration was obtained at the rates of approximately 50%, 40% and 80%, respectively, in MS1 (Table 2). It was found 42%, 20% and 75% in MS2 (Table 2).

Table 2. Frequency (%) of adventitious shoot regeneration from different part of plant on the MS1 and MS2. All experiments used a complete randomized design with three replications and 7 explant per replication. Data were recorded as number of multiplied shoots after 4 weeks, and percentage of shoots were calculated.

Medium	root	shoot	leaves	cotyledone	Petiol
MS1	50	40	150	80	-
MS2	42	20	120	75	-

Culture medium MS1 (150%) and MS2 (120%) were found to be the most efficient media for shoot regeneration from leaf explants (Figure 1D) (Table 1-2). Tıpırdamaz et al. (2006) reported that the shoot explants taken from *C. tchihatcheffii* seedlings were incubated in MS and ½ MS media containing 1 mg l^{-1} GA_3 + 0.225 mg l^{-1} BAP and the results showed 40.7% shoot regeneration frequency. In addition, Özel et al. (2006a), who worked on the micropropagation and shoot regeneration used immature zygotic embryos and stem nodes of *C. tchihatcheffii*. They observed no micropropagation from stem nodes. The immature zygotic embryos showed the highest shoot regeneration in the MS medium including 1 mg l^{-1} kinetin and 0.25 mg l^{-1} NAA. Cuenco et al. (1999) reported an efficient protocol to micropropagate the endemic species *C. paui* from nodal segments of inflorescence stems and performed on MS medium supplemented with a cytokinin 6-benzyladenine (BA), Kin or 6-g-g-dimethylallylaminopurine (2iP), each one at three different concentrations (0.5, 1 or 2 mg l^{-1}) for shoot propagation. The investigators determined that the best rate of shoot proliferation was obtained in MS medium supplemented with 0.5 mg l^{-1} 6-benzyladenine or with 2 mg l^{-1} kinetin. In addition, BA has been found to be an efficient cytokinin for shoot multiplication in *Centaurea junoniana* (Hammatt and Evans 1985) and *Centaurium rigualii* (Iriondo and Pérez 1996).

Root induction was carried out with four week old regenerated shoots obtained from MS1 culture medium. Regenerated shoots were best rooted in MS medium containing 1 mg l^{-1} IBA. However, the rooting process was slow and roots emerged after 4 weeks of culture. The result is in agreement with Cuenco et al. (1999), and showed that in vitro rooting of shoots was very slow and difficult after 6 weeks on rooting media (Figure 1D). The investigators showed that the combination of 2 mg l^{-1} indole-3-acetic acid plus 2 mg l^{-1} indole-3-butyric acid on MS medium yielded the best results. As in the case of *C. rupestris*, IBA also proved to be the best root-inducing auxin for *Centaurea ragusina* (Pevalek-Kozlina 1998).

The present study shows the preliminary results on the regeneration capability of *C. arifolia* in tissue culture. This study is the first to record an efficient protocol to micropropagate the endemic species *C. arifolia* using leaf explants.

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