IN VITRO SHOOT REGENERATION OF A *CENTAUREA AMAENA* BOISS. & BALANSA – A CRITICALLY ENDANGERED AND ENDEMIC PLANT

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Abstract. This study was conducted to investigate in vitro regeneration potential of *Centaurea amaena*, a critically endangered and an endemic plant in Turkey. For this purpose, cotyledon, leaf and cotyledon node explants were cultured in Murashige and Skoog (MS) media supplemented with different concentrations of 6-benzylaminopurine (BAP; 1-4 mg L^{-1}), thidiazuron (TDZ; 0.3-1.2 mg L^{-1}) or *meta*-Topolin (*m*T; 0.5-4 mg L^{-1}) with or without 0.5 mg L^{-1} α-naphthalene acetic acid (NAA). In axillary shoot regeneration experiments from cotyledon node explants, the greatest number of shoots per explant (9.975) was obtained from 4 mg L^{-1} *m*T-containing MS media, which yielded a shoot regeneration frequency of 70.83%. In indirect organogenesis experiments, the greatest number of shoots per explant in cotyledons (4.152 shoots/explant with the shoot regeneration frequency of 55.00%) was obtained from 1 mg L^{-1} *m*T-containing media and the greatest number of shoots per explant in leaves (4.132 shoots/explant with the shoot regeneration frequency of 50.00%) was obtained from 4 mg L^{-1} *m*T-containing media. Only callus induction was observed in TDZ-containing media or combinations of TDZ concentrations with NAA. About 50.00% root formation was achieved from half-strength MS medium containing 2.0 mg L^{-1} indole-3-butyric acid.
Keywords: *axillary shoot regeneration, BAP, mT, organogenesis, TDZ*

Introduction

Centaurea amaena Boiss. & Balansa belonging to the *Phalolepis* section of the Compositae family is classified as CR B2ab (i,iii) based on IUCN criteria (Atasagun et al., 2013; Atasagun and Aksoy, 2018). The species occurs over the stony slopes of Yılanlı Mountain, located between 38° 38' - 38° 41' N longitudes and 35° 30' - 35° 35' S latitudes to the west of Kayseri, Turkey and naturally grows in arid environments at altitudes of between 1.170-2.300 m (Atasagun and Aksoy, 2018). The species exists in two localities in Kayseri with distribution area of about 0.55 km^2. Number of individuals was identified by Atasagun and Aksoy (2018) as 5672. Atasagun and Aksoy (2018) indicated that the species had a quite isolated distribution between Erciyes and Yılanlı mountains of Kayseri; it was an endemic species under the threat of extinction mostly because of anthropogenic effects, animal grazing, stone quarry activities, Erciyes Mountain master plan, negative impacts of seasonal conditions based on altitude of spread zones, use of plant seeds by *Oxycarenus sp.* of Heteroptera suborder as nutrient and all these issues reduced formation of new individuals and negatively influenced the growth and development of the population.

C. amaena is rich in phenolics and flavonoids, thus has a strong antioxidant activity. Plants can be used as raw and processed food preservers and natural additives in

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pharmaceuticals, alternative medicine and natural treatment (Albayrak et al., 2017). *C. amaena* is under the threat of extinction and thus urgent measures should be taken to prevent extinction and for propagation of the species. Conservation is the effective storage of the diversity in the gene pool until its actual or potential use, and the introduction of this genetic diversity into the use of humanity. There are two primary conservation systems including in situ and ex situ conservation (Şehirali et al., 2015). In situ conservation means preservation of natural resources in their own habitats. However, ex situ conservation has become the most common practice for the conservation of plant genetic sources (Şehirali et al., 2015). Within the scope of ex situ conservation, in vitro tissue culture techniques are used. In recent years, several endangered and endemic species have been propagated and preserved from quite small quantity of plant material through these techniques without any significant impacts on wild populations (Erdağ and Emek, 2005a, b).

In previous studies, successful in vitro regeneration of *Centaurea* species were achieved from leaf, hypocotyl, cotyledon, node, soot tips, inflorescence stem parts-like explants with the aid of different cytokinin and auxin sources (Mallon et al., 2010; Aydoğan and Erdağ, 2015; Atalay and Erişen, 2017; Türkoğlu et al., 2018). In the different studies related to *Centaurea* species, the most commonly used cytokinins were BAP, kinetin, zeatin, TDZ and N6-(2-isopentyl) adenine (2-iP). Generally, BAP has been reported as the most efficient source of cytokinins for in vitro propagation of *Centaurea* species (Cuenca and Amo-Marco, 2000; Curkovic-Perica, 2003; Mallon et al., 2010; Atalay and Erişen, 2017). While the lowest response was obtained from zeatin, 2-iP and kinetin-containing media in *C. ultreiae* and from TDZ and kinetin-containing media in *C. lycaonica*, the use of kinetin was successful in *C. paui* (Cuenca et al., 1999) and *C. tchihatcheffii* (Ozel et al., 2006). In recent years, meta-topolin has been used to promote in vitro shoot regeneration as an alternative cytokinin source (Dimitrova et al., 2016). The use of *m*T as a cytokinin source in *Centaurea* species have not been reported before and there are no studies about in vitro regeneration of *Centaurea amaena*. Therefore, this study was conducted to produce an efficient in vitro regeneration method for *Centaurea amaena* and the effectiveness of BAP, TDZ and *m*T as a source of cytokinins was examined.

Materials and methods

Plant material and achene sterilization

Centaurea amaena Boiss & Balansa was used as the plant material of the present study. Mature seeds of the species were collected in limited numbers from the natural habitat, Yılanlı Mountain (Kayseri-Turkey), and seeds were preserved at +4 °C for later uses in further analyses. All experimental procedures were conducted at Tissue Culture Laboratory of Field Crops Department at Erciyes University Agricultural Faculty. Sterile distilled water and 50% diluted H_2SO_4 were used for surface sterilization of the seeds. Seeds were initially kept in this solution for a minute, rinsed through sterile distilled water, kept again in 50% diluted commercial bleach (ACE) in a magnetic stirrer for 10 min and finally rinsed through sterile distilled water three times and were germinated in sterile petri dishes with MSO media containing MS mineral salt and vitamins, 3% sucrose and 0.7% agar. About 15-20 seeds were placed into each magenta vessel (Sigma-Aldrich, 77 × 77 × 97).

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Indirect organogenesis

The cotyledon obtained from 2-weeks-old seedlings and leaves from about 1-month-old seedlings were used as explants for indirect organogenesis. Explants were cultured in MSO media containing different concentrations of TDZ (0.3-1.2 mg/l), BAP (1-4 mg L^{-1}) or *m*T (0.5-4 mg L^{-1}) alone or in combination with NAA (0.5 mg L^{-1}). About 7-8 weeks after the initiation of culture processes, callus induction ratio (%), shoot regeneration frequency (%) and number of shoots per explant were determined (*Fig. 1A*).

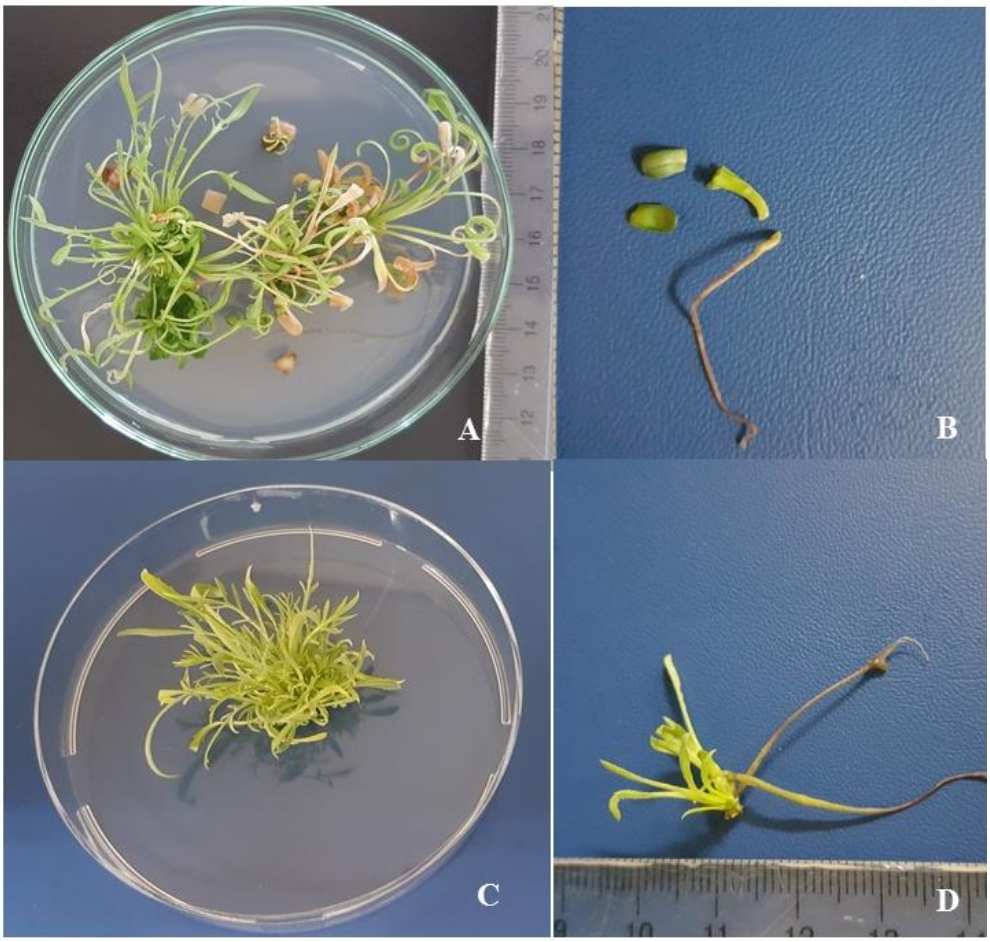

Figure 1. *In vitro propagation and rooting of Centaurea amaena. A - Indirect organogenesis from cotyledon explant on MSO medium supplemented with 4 mg L^{-1} mT, B - Isolation of cotyledon node explant, C - Axillary shoot regeneration form cotyledon node explant on MSO medium supplemented with 4 mg L^{-1} mT, D - Root formation of Centaurea amaena*

Axillary shoot regeneration

Cotyledon node explants were isolated from the plantlets about 15-20 days after initiation of germination processes and were cultured in Magenta Vessels with MSO media containing different concentrations of TDZ (0.3-1.2 mg L^{-1}), BAP (1-4 mg L^{-1}) or *m*T (0.5-4 mg L^{-1}) with or without 0.5 mg L^{-1} NAA (*Fig. 1B-C*). About 7-8 weeks after the initiation of culture processes, shoot regeneration frequency and number of shoots per explant parameters were determined.

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Rooting

Regenerated shoots were rooted in half-strength MS media containing 0.5, 1 or 2 mg L^{-1} IBA, 3% sucrose, solidified with 0.7% agar (*Fig. 1D*).

Experimental conditions

Entire sterile processes were conducted in a sterile cabin with a hepa-filter and horizontal air flow. Before the initiation of the sterile processes, the cabin was whipped with 96% ethyl alcohol, the cabin was then left open for 20 min and sterilized under UV light. All tools, equipment, distilled water and growth media used in this study were sterilized in an autoclave under standard conditions (1.2 atm pressure, 121 °C temperature, 20 min). Growth medium pH values were arranged as between 5.5-5.8 before agar supplementation. All cultures were grown under 16:8 light:dark photoperiod, 22 ± 2 °C temperature and 3000 lux light intensity.

Data analysis

Experiments were conducted at completely randomized design with 4 replications. Each replication had 6 (for cotyledon node) or 10 (leaf and cotyledon) explants. Before variance analysis, percentiles were subjected to "arcsine" transformation. Variance analysis was performed with SPSS software. Means were compared with the aid of Duncan's multiple range test.

Results and discussion

Indirect organogenesis

For plant regeneration through organogenesis in *C. amaena*, cotyledon and leaves taken from the plantlets obtained from sterile seeds were cultured in 20 different media containing different concentrations of BAP, TDZ or *m*T alone or in combination with NAA. About 7-8 weeks after the initiation of culture processes, callus induction ratio, shoot regeneration frequency and number of shoots per explant were determined. Callus induction ratios varied between 50 - 100%. While type of explant did not have any significant effects on callus induction ratios, PGR (plant growth regulator) concentrations and combinations had significant effects on callus induction ratios ($P < 0.01$). While the greatest callus induction ratios were obtained from BAP, BAP-NAA, *m*T-NAA, TDZ and TDZ-NAA-containing treatments, the lowest callus induction ratios were obtained from only *m*T-containing treatments (*Fig. 2*). Similarly, Atalay and Erişen (2017) also indicated that callus induction ratios in *Centaurea lycaonica* varied with the PGRs and reported high ratios for BAP-NAA and TDZ-NAA combinations. Researchers also identified differences in callus morphology of *C. lycaonica* based on PGR type and combinations.

PGRs and PGR × explant interactions had significant effects on shoot regeneration ratios and number of shoots per explant. Shoot regeneration frequencies varied between 0.00-57.50% in cotyledon explants and between 0.00-62.50% in leaf explant (*Table 1*). In both types of explants, callus induction was observed but shoot regeneration was not observed in TDZ-containing media. Similarly, Atalay and Erişen (2017) in *Centaurea lycaonica* and Aydoğan and Erdağ (2015) in *Centaurea zeybekii*, reported callus induction but no shoot regeneration in TDZ-containing media. In addition, Kazeroonian

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et al. (2018) indicated that type of cytokinin affected shoot regeneration and TDZ promoted callus induction rather than shoot regeneration in *Chrysanthemum morifolium* petiole explant. Dewir et al. (2018) reported that TDZ may result in various anomalies including inhibition of shoot proliferation, shoot elongation, etc., and indicated low TDZ concentrations, pulse treatment or short-duration TDZ treatments as efficient strategies to eliminate such anomalies. However, TDZ was also indicated as a strong synthetic cytokinin-like substance for shoot regeneration in several plant species (Erişen et al., 2011; Yorgancılar and Erişen, 2011; Uzun et al., 2014).

Table 1. *Effects of PGRs on adventitious shoot regeneration from cotyledon and leaf explants of Centaurea amaena*

PGRs	Shoot regeneration frequency (%)		Number of shoots per explant	
	Cotyledon	Leaf	Cotyledon	Leaf
1 mg L^{-1} BAP	40.00 ab*	40.00 bcd*	2.427 ef*	1.775 gh*
2 mg L^{-1} BAP	30.00 bc	50.00 ab	3.125 cd	3.997 ab
4 mg L^{-1} BAP	22.50 cd	47.50 ab	1.825 f	3.082 cd
1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	45.00 ab	62.50 a	2.052 f	3.930 ab
2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	25.00 cd	42.50 a-d	1.125 g	2.770 c-f
4 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	15.00 d	45.00 abc	1.042 g	2.875 cde
0.5 mg L^{-1} *m*T	40.00 ab	47.50 ab	3.265 bcd	1.415 h
1 mg L^{-1} *m*T	55.00 a	27.50 cd	4.152 a	3.375 bc
2 mg L^{-1} *m*T	37.50 ab	25.00 d	3.227 cd	2.832 cde
4 mg L^{-1} *m*T	55.00 a	50.00 ab	3.965a	4.132 a
0.5 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	55.00 a	25.00 d	2.902 de	2.290 efg
1 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	57.50 a	55.00ab	3.927 ab	3.117 cd
2 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	40.00 ab	27.50 cd	3.970 a	2.082 fgh
4 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	47.50 ab	57.50 ab	3.657 abc	2.392 d-g
0.3 mg L^{-1} TDZ	0 e	0 e	0 h	0 ı
0.6 mg L^{-1} TDZ	0 e	0 e	0 h	0 ı
1.2 mg L^{-1} TDZ	0 e	0 e	0 h	0 ı
0.3 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	0 e	0 e	0 h	0 ı
0.6 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	0 e	0 e	0 h	0 ı
1.2 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	0 e	0 e	0 h	0 ı

*Values within a column followed by different letters are significantly different at 0.05 significance level using Duncan's multiple range test

The greatest numbers of shoots per explant were obtained from 1 mg L^{-1} *m*T, 4 mg L^{-1} *m*T, 1-2 or 4 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA treatments in cotyledon explants and from 4 mg L^{-1} *m*T, 2 mg L^{-1} BAP and 1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA treatments in leaf explants (*Table 1*). Optimum PGR type and concentration varied with explant type. Similarly, Erişen et al. (2011) reported differences in shoot regeneration of explants based on PGR concentrations and combinations in *Astragalus cariensis*. Success of a culture is influenced by type and concentration of applied cytokinin since cytokinin uptake, transport and metabolism varied with the plant species and cytokinin interacted with endogenous cytokinin of the explants (Magyar-Tabori et al., 2010).

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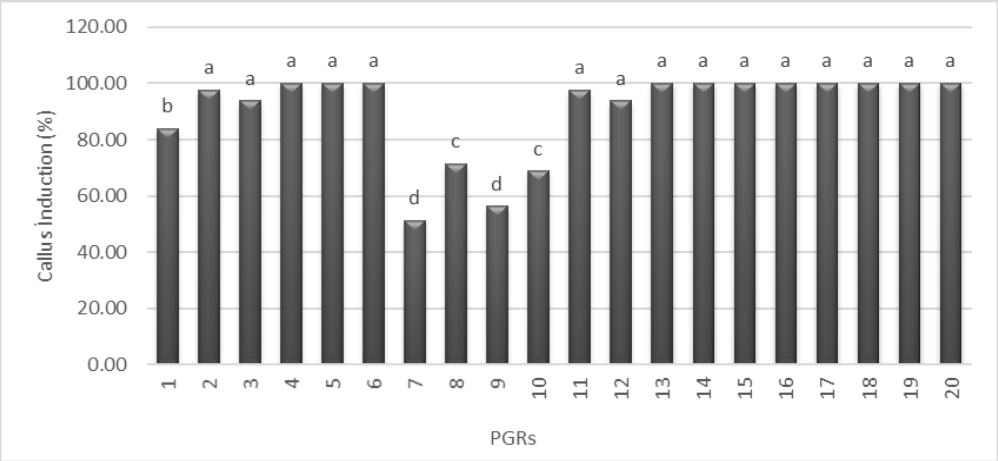

Figure 2. *Effects of different PGR concentrations and combinations on callus induction ratios. *Values within a column followed by different letters are significantly different at 0.05 significance level using Duncan's multiple range test.* ***1:*** *1 mg L^{-1} BAP;* ***2:*** *2 mg L^{-1} BAP;* ***3:*** *4 mg L^{-1} B AP;* ***4:*** *1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA;* ***5:*** *2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA,* ***6:*** *4 mg L^{-1} BAP + 0.5 mg L^{-1} NAA,* ***7:*** *0.5 mg L^{-1} mT;* ***8:*** *1 mg L^{-1} mT;* ***9:*** *2 mg L^{-1} mT;* ***10:*** *4 mg L^{-1} mT;* ***11:*** *0.5 mg L^{-1} mT + 0.5 mg L^{-1} NAA;* ***12:*** *1 mg L^{-1} mT + 0.5 mg L^{-1} NAA;* ***13:*** *2 mg L^{-1} mT + 0.5 mg L^{-1} NAA;* ***14:*** *4 mg L^{-1} mT + 0.5 mg L^{-1} NAA;* ***15:*** *0.3 mg L^{-1} TDZ;* ***16:*** *0.6 mg L^{-1} TDZ;* ***17:*** *1.2 mg L^{-1} TDZ;* ***18:*** *0.3 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA;* ***19:*** *0.6 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA;* ***20:****1.2 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA*

Axillary shoot regeneration

PGRs had significant effects on shoot regeneration frequency and number of shoots per explant ($P < 0.01$). Shoot regeneration frequency of cotyledon node explants varied between 12.50-87.50% and the greatest shoot regeneration frequency was obtained from BAP, BAP-NAA, *m*T and *m*T-NAA treatments (*Table* 2). The greatest number of shoots per explant (9.975 shoots) was obtained from 4 mg L^{-1} *m*T treatment and followed by 2 mg L^{-1} *m*T and 0.5-1 mg L^{-1} *m*T. Only *m*T-containing media had greater number of shoots per explant than BAP and TDZ-containing media. When the BAP-containing media were assessed in themselves, it was observed that the greatest number of shoots per explant (3.582 shoots) was obtained from 2 mg L^{-1} BAP treatment. BAP was also indicated as an appropriate cytokinin for axillary shoot regeneration in shoot apices of *Centaurea ultreia* (Mallon et al., 2010) and node explants of *Centaurea lycaonica* (Atalay and Erişen, 2017). Similarly, in present study, BAP-containing media were found to be more efficient than TDZ, however just *m*T-containing media were identified as the most efficient for axillary shoot regeneration from cotyledon node. There were not any studies in literature about the use of *m*T as a source of cytokinin in *Centaurea* species, but it was reported in studies on cassava, pelargonium and sweet basil that *m*T improved in vitro shoot proliferation and shoot quality (Wojtania, 2010; Köszeghi et al., 2014; Chauhan and Taylor, 2018). Fajinmi et al. (2014) demonstrated *m*T as a quite active and an alternative cytokinin to BAP and other cytokinins for shoot regeneration in shoot tip explant of *Coleonema album*. Such an efficiency of *m*T is mostly attributed to chemical structure allowing production of O-glucoside metabolites from hydroxyl group of side chain of *m*T and rapid translocation of it in plant tissues (Fajinmi et al., 2014). In only *m*T-containing media, number of shoots per explant increased with increasing *m*T doses. Dimitrova et al. (2016) also reported increasing

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regenerations with increasing *m*T doses in *Pyrus communis*. As compared to only *m*T-containing media, number of shoots per explant decreased in *m*T-NAA combinations. But contrary to present findings, *m*T-NAA combinations increased the number of shoots per explant in *Coleonema album* and *Huernia hystrix* (Fajinmi et al., 2014; Amoo and Van Staden, 2013). Such differences were mainly attributed to synergic, antagonistic and additional interactions between auxins and cytokinin based on plant species and type of tissue (Coenen and Lomax, 1997).

Table 2. *Effects of PGRs on axillary shoot regeneration form cotyledon node explant of Centaurea amaena*

PGRs	Shoot regeneration frequency (%)	Number of shoots per explant
1 mg L^{-1} BAP	87.50 a*	2.867 ef*
2 mg L^{-1} BAP	87.50 ab	3.582 de
4 mg L^{-1} BAP	83.33 abc	2.445 f
1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	83.33 abc	3.000 ef
2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	83.33 abc	2.750 f
4 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	87.50 a	2.685 f
0.5 mg L^{-1} *m*T	87.50 ab	5.375 c
1 mg L^{-1} *m*T	87.50 ab	5.165 c
2 mg L^{-1} *m*T	83.33 ab	6.250 b
4 mg L^{-1} *m*T	70.83 a-d	9.975 a
0.5 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	66.66 b-e	2.750 f
1 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	74.99 abc	4.137 d
2 mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	83.33 abc	2.750 f
4 m mg L^{-1} *m*T + 0.5 mg L^{-1} NAA	58.33 cde	3.000 ef
0.3 mg L^{-1} TDZ	29.17 fg	0.875 g
0.6 mg L^{-1} l TDZ	37.49 ef	1.375 g
1.2 mg L^{-1} TDZ	41.67 def	1.083 g
0.3 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	12.50 g	0.750 g
0.6 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	12.50 g	0.750 g
1.2 mg L^{-1} + 0.5 mg L^{-1} NAA	25.00 fg	0.833 g

*Values within a column followed by different letters are significantly different at 0.05 significance level using Duncan's multiple range test

Rooting

Regenerated shoots were rooted in ½ MS media containing 0.5, 1 or 2 mg L^{-1} IBA. About 5-6 weeks after the initiation of culture processes, percentage of root-forming shoots were respectively identified as 33.33, 38.89 and 50.00%. IBA was also identified as an available auxin for promotion of rooting of regenerated shoots in *Centaurea rupestris* and *Centaurea arifolia* species (Curkovic-Perica, 2003; Yüzbaşıoğlu et al., 2012).

Conclusions

The primary target of the present study was to investigate regeneration potential of *Centaurea amaena*. In present experiments, 4 shoots per explant were obtained from

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cotyledons and leaves and 10 shoots per explant were obtained from the cotyledon nodes of *C. amaena*. It was concluded based on present findings that *m*T and BAP were efficient cytokinins for in vitro regeneration of *C. amaena*. Regenerated shoots were rooted in half-strength MS medium supplemented with IBA. A simple and efficient propagation procedure was developed in this study for the critically endangered species *C. amaena*. This procedure could be useful for the in situ and ex situ conservation of this valuable genetic source. However, the genetic stability of regenerated plants must be verified using molecular markers in further studies.

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