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In vitro propagation and *ex situ* conservation of the vulnerable Greek endemic *Centaurea paxorum* Phitos & Georgiadis (Asteraceae): A plant of high medicinal value

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Abstract
Centaurea L. is a large genus which comprised of several species, many of which are used in folk medicine. *Centaurea paxorum* Phitos & Georgiadis (Asteraceae) is a range-restricted Greek endemic only to a single island (Paxoi-Antipaxoi) and is assessed as vulnerable according to the IUCN criteria. 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 *C. paxorum*, an extremely rare-endangered indigenous plant species endemic to the Greek flora, as a conservation measure which does not cause damage to the wild plants used as explant source. There are no studies identified aiming to propagate this species *in vitro.* The effect of the cytokinin; BA applied at 0.1 mg/l in combination with 3 different auxins (IBA, NAA, IAA) each applied at 0.025 mg/l on multiple shoot induction potential was studied. Induction of adventitious shoot regeneration from shoot tips was achieved approximately 100% with the production of 4.83 shoots per explant of average mean length of 15.51 mm after 30 days of culture in MS medium supplemented with 0.1 mg/l BA+ 0.025 mg/l IBA. In the subsequent rooting stage, the individual effect of the 3 auxins (IBA, NAA, IAA) each applied at 0.1 mg/l was studied. The elongation of the initial shoot tips was best enhanced (41.67 mm) with the application of 0.1 mg/l NAA to the MS rooting medium, being increased approximately by 1.5 cm compared to the control explants (auxins-free). Regenerated adventitious shoots were best rooted on MS medium containing 0.1 mg/l IAA (30 days) exhibiting 100% rooting with the formation of 8 roots per rooted microplant 43.26 mm long. The present study shows the results on the regeneration capability of *C. paxorum* in tissue culture. This study is the first to record an efficient protocol to micropropagate *C. paxorum* using shoot-tip explants.

Keywords: *Centaurea paxorum, ex situ* conservation, greek flora, medicinal plant, micropropagation protocol, plant growth regulators

1. Introduction

Centaurea L. is a large genus which comprised of several species (spp.), many of which are used in folk medicine (Nacer *et al*., 2006) [1]. Several bioactive chemicals have been isolated and purified from different species of *Centaurea* including sesquiterpene lactones (Bruno *et al.,* 1996; Youssef, 1998) [2, 3], volatile constituents (Lazari *et al.,* 2000) [4], essential oils (Dural *et al.,* 2003) [5], flavonoid C-glycosides and other biologically active constituents (Ribeiro *et al.,* 2002) [6], exhibiting anti-inflammatory and immunological effects (Garbacki *et al.,* 1999) [7], cytotoxic/ cytostatic effects (Koukoulista *et al.,* 2002) [8], antioxidant (Kumarasamy *et al.,* 2002) [9] and antibacterial activities (Kumarasamy *et al.,* 2003) [10].

Centaurea paxorum is assessed as Vulnerable [IUCN criteria B1a, b(ii), C2a(i), D2]. Factors that limit its distribution are the small population size (<5000 individuals), the fact that is endemic only to a single island (Paxoi-Antipaxoi), the chasmophytic life-form, the high probability of habitat alteration /destruction due to natural phenomena such as earthquakes and rock erosion as well as human activities including tourist development, construction works, overgrazing and land use changes (Krigas, 2009) [11]. According to Dimopoulos *et al.* (2013, 2016) [12, 13], *C. paxorum* is a range-restricted Greek endemic hemicryptophyte that grows on coastal habitats and is distributed in Ionian islands. Linnaeus gave to this plant the name *Centaurea* due to its resemblance to the mythological monster "Centaur" with a horse-shaped human head as was proposed by Hippocrates (Ekim, 1994) [14]. The majority of studies in *Centaurea* species are primarily based on early flowering by increasing the number of flowers and suitable sowing period for *C. montana* (Cox, 1988) [15] and *C. moschata* var. *imperials* (Selaru and Draghici, 1989) [16], and on seed storage times for *C. masculosa* (Davis *et al.*, 1993) [17], whereas propagation studies are limited.

Besides conventional methods of propagation, endemic and threatened plants could efficiently be conserved with various *ex vitro* strategies (Fay, 1992) [18], including in *vitro* cultural methods, which have low impact on wild populations with a minimum of plant material (Cuenca *et al.*, 1999; Krogstrup *et al.*, 1992; Fay, 1994) [19, 20, 21]. 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, 2000; Okay and Demir, 2010) [19, 22-25]. Tissue culture studies were conducted on *C. junaniana* (Hammatt and Evans, 1985) [22], *C. paui* (Cuenca *et al.*, 1999) [19], *C. cyanus* (Kakegawa *et al.*, 1991; Tanimoto and Ishioka, 1991) [26, 27], *C. macrocephala* (Hosoki and Kimura, 1997; Takashi and Daisuke, 1997) [28, 29], *C. spacchii* (Cuenco and Marco, 2000) [24], *C. rupestris* (Perica, 2003) [30], *C. zeybekii* (Kurt and Erdag, 2009) [31] and *C. ultreiae* (Mallon *et al.*, 2010)

[32], and the sterilization applications, rooting and shoot formation mediums were studied (Okay and Demir, 2010) [25]. In addition, factors affecting *in vitro* plant regeneration of the critically endangered *C. tchihatcheffii* Fisch et. Mey have been investigated (Özel *et al.*, 2006) [33]. Micropropagation of *C. zeybekii* Wagenitz (Kurt and Erdağ, 2009) [31] and adventitious shoot regeneration of *C. depressa* Bieb. have also been reported (Özel *et al.*, 2008) [34]. However, no tissue culture studies on C. *paxorum* Phitos & Georgiadis were performed up to date. Therefore, this paper reports a simple protocol for micropropagation from shoot tip explants of endemic *C. paxorum*.

The study, as an aid to conservation, can lead to other advanced tissue culture studies within this and other threatened or endangered flora of this species. This study is a step forward towards the regeneration, sustainable utilization, and conservation of this critically endangered indigenous species.

2. Materials and Methods

2.1 Plant material and *in vitro* culture conditions

For the initial establishment of the plant material *in vitro*, juvenile shoot tips of *C. paxorum*, 1-2.5 cm long were dissected and removed from mother plants maintained in a peat: perlite (1:1) substrate in pots in external environmental conditions in the nursery area of the laboratory on 10/10/2017. For the disinfection of plant material, n=40 shoot tip explants were soaked in 70% ethanol for 1 min followed by 2% NaOCl solution for 17 min with agitation and rinsed for 4–5 times with sterile distilled water. The basal culture medium used for the initial establishment phase was the Murashige and Skoog (MS) (Murashige and Skoog, 1962) [35] supplemented with 30 g/l sucrose (Duchefa, The Netherlants), 0.25 mg/l benzyladenine (BA), 0.1 mg/l indole-3-butyric acid (IBA), 0.1 mg/l gibberrelic acid (GA_3) and solidified with 6 g/l Plant Agar (Duchefa, The Netherlands). After 15 days of culture, the percentage of explants with no infections (fungi- and bacteria-free) was recorded. After a period of 6 weeks, the proliferated shoots were divided and sub-cultured in two different culture media, MS and Woody Plant Medium (WPM) (McCown and Lloyd, 1981) [36] PGRs-free for the alleviation and/ or inhibition of the vitrification problem. Both culture media (MS, WPM) were supplemented with 20 g/l sucrose as a carbon source and solidified with 6 g/l Plant Agar (pH: 5.8). The proliferated plant material derived from both culture media (absence of vitrified explants) was transferred after 3 weeks of culture into a hormone-free MS culture medium for 4 weeks prior to experimentation.

The experimental material used was shoot-tip explants of 1.5-2 cm long obtained from previous *in vitro* cultures. Two experiments were conducted. In the first experiment, the effect of the cytokinin; BA applied at 0.1 mg/l in combination with 3 different auxins; IBA, α-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA), each applied at 0.025 mg/l on multiple shoot induction potential was studied. The control treatment was BA- and auxins-free. In the second experiment, at the rooting stage, the single effect of 3 different auxins (IBA, NAA, IAA) applied at 0.1 mg/l was studied. The control treatment was auxins-free. The basal culture medium used for both experiments was the MS supplemented with 20 g/l sucrose and solidified with 6 g/l Plant Agar. The pH value of the culture media was adjusted to 5.8 before adding the gelling agent and afterwards the media were sterilized in autoclave at 121 °C for 20 min. Shoot-tip explants were transferred into Magenta vessels containing 35 ml of MS medium. All cultures were maintained in a growth chamber with a 16-h light duration (40 μmol/m^2/s) supplied by cool white fluorescent lamps and a constant temperature of 22 ± 2 °C. After 30 days of culture for both experiments, the following measurements were recorded: shoot number/explant, shoot length (mm), shoot multiplication percentage (%), root number/rooted microplant, root length (mm) and rooting percentage (%).

2.2 *Ex vitro* acclimatization of rooted *in vitro* plants

In late-April 2018, obtained rooted microplants with well-developed shoots were washed with running tap water and transferred to a peat moss (Terrahum): perlite (Geoflor) (1:1 v/v) substrate. The n=27 in total plantlets were transferred onto 84-position trays and placed on a bench plastic tunnel with adjustable relative humidity (internal mist system), with 18-21°C base temperature, 15-25°C air temperature and 70-85% relative humidity for 18 days, under unheated greenhouse conditions of reduced light intensity provided by shading net. Afterwards, on 14/5/2018 the plants were taken out of the mist and placed on a greenhouse table bench in conditions of reduced relative humidity (35-45% RH) and increased light intensity (absence of shading net) where they remained for 15 days. The results were evaluated after 5 weeks (26/4/2018-31/5/2018). On 31/5/2018, the 26 out of the 27 finally survived plants were transplanted in larger volume pots of 0.33Lt (8x8x7 cm) on a soil substrate containing peat (TS2, Klassmann): perlite (Geoflor): soil, in a ratio of 2: ½: ½ v/v respectively, for the further growth and development of the young plantlets. Following that, the plants were transferred into the greenhouse and watered by hand once per day each for 1 min. After another 5 weeks, in early July (3/7/2018), the developed plants were transplanted into 2.5 Lt larger volume pots consisted of a mixture of peat (TS2): perlite: soil (2:1:1 v/v) and transferred to the external environment outside greenhouse under automatic sprinkling irrigation system and shading net until the end of the summer (31/8/2018) where the acclimatization process was successfully completed without additional losses.

2.3 Statistical analysis

In the first experiment at the shoot proliferation stage, the experimental design was completely randomized and included 5 treatments with 6 repetitions/treatment (3 explants/vessel x 2 vessels/ treatment). In the second experiment at the subsequent rooting stage, the experimental design was completely randomized and included 4 treatments with 6 repetitions/treatment (3 explants/vessel x 2 vessels/treatment). Both experiments were completely randomized and analyzed with ANOVA (Analysis of Variance) using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA) at $P \leq 0.05$, according to Duncan's multiple range test ± standard error (S.E.) in order significant differences among treatments to be established.

3. Results

After a 15-day period from disinfection and initial establishment of *in vitro*, the success of decontamination rate was high, 85% i.e. 34 out of the 40 in total explants were fungi- and bacteria-free, only the 15% of explants (6

out of 40) was contaminated by bacteria.

In the first experiment, multiple shoot induction to the 100% of the explants was obtained in the following two PGR's combination treatments: (A) 0.1 mg/l BA + 0.025 mg/l IBA and (B) 0.1 mg/l BA + 0.025 mg/l IAA. The number of produced shoots was higher (7.5 shoots/ explant) in the combined effect 0.1 mg/l BA + 0.025 mg/l IAA. In the control treatment (BA- and auxins-free) no shoot formation occurred. The length of shoots was greater (26.67 and 23.2 mm) respectfully in the case of the control and by adding 0.1 mg/l BA + 0.025 mg/l NAA to the culture medium. In all treatments, with the exception of the control mild hyperhydricity symptoms were observed especially in the new draft multiple shoots induced in the base of the initial explant. The effect of vitrification was more pronounced with the simultaneous appearance of a universal mild browning on the shoot stem with the application of 0.1 mg/l BA + 0.025 mg/l IAA. BA applied alone and simultaneously with auxins led to the callus formation (83.33-100%) with respect to the control where no callusing was evident (0%). In particular, the combined application of BA with auxins, irrespective of type (IBA, NAA, IAA) resulted in 100% callus induction, causing an increase of 16.67% in relation to the individual effect of the cytokinin (83.33%). Consequently, the second most appropriate treatment for the proliferation stage (4.83 shoots /explant, 100% shoot multiplication) with the effect of over-hydration being significantly reduced was the 0.1 mg/l BA + 0.025 mg/l IBA (Table 1, Fig. 1a-1e).

Table 1: Effect of BA in combination with 3 different auxins (IBA, NAA, IAA) on *in vitro* shoot proliferation of *Centaurea paxorum*, after 30 days of culture

Treatments (mg/l)	Shoot number/ explant	Shoot length (mm)	Shoot multiplication (%)	Callus induction (%)
Control	1.00 ± 0.00 a	26.67 ± 2.32 b	0 a	0 a
0.1 BA	5.50 ± 0.65 b	15.92 ± 0.63 a	83.33 b	83.33 b
0.1 BA + 0.025 IBA	4.83 ± 0.46 b	15.51 ± 0.61 a	100 c	100 c
0.1 BA + 0.025 NAA	4.33 ± 0.66 b	23.20 ± 2.13 b	83.33 b	100 c
0.1 BA + 0.025 IAA	7.50 ± 0.68 c	17.36 ± 1.03 a	100 c	100 c
p-values	0.000***	0.000***	0.000***	0.000***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. *** $P \leq 0.001$

Fig 1: *In vitro* shoot proliferation of *Centaurea paxorum* shoot-tip explants after 30 days of culture in MS medium supplemented with different combinations of PGRs (mg/L): (a) Control (BA- and auxins-free); (b) 0.1 BA; (c) 0.1 BA + 0.025 IBA; (d) 0.1 BA + 0.025 NAA; (e) 0.1 BA + 0.025 IAA

Among the 3 auxins, IAA applied at 0.1 mg/l gave 100% rooting and the highest number of roots (8 roots/ rooted microplant) at the same time. Root length was greater in the case of the control (50.21 mm) and in the presence of 0.1 mg/l IAA (43.26 mm). All 3 auxins led to callus induction (33.33-100%) in contrast to the control (0%). No multiple shoot induction was observed in any of the 4 treatments. However, a significant increase in the length of the initial explant by 1.5 cm, from 26.67 mm (control) to 41.67 mm was recorded by incorporating 0.1 mg/l NAA into the MS medium (Table 2, Fig. 2a-2d).

Table 2: Effect of auxin type (IBA, NAA, IAA), each applied at 0.1 mg/l on *in vitro* rooting of *Centaurea paxorum* after 30 days of culture

Treatments (mg/l)	Root number / rooted microplant	Root length (mm)	Rooting (%)	Shoot length (mm)	Callus induction (%)
Control	4.50 ± 0.49 a	50.51 ± 6.97 b	100 b	26.67 ± 2.32 a	0 a
0.1 IBA	9.00 ± 0.77 b	35.75 ± 3.48 ab	83.33 a	34.17 ± 3.28 ab	83.33 c
0.1 NAA	9.80 ± 0.52 b	21.77 ± 1.38 a	83.33 a	41.67 ± 2.32 b	100 b
0.1 IAA	8.00 ± 0.95 b	43.26 ± 6.63 b	100 b	33.33 ± 2.95 a	33.33 b
p-values	0.000***	0.003**	0.000***	0.005**	0.000***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$. ** $P \leq 0.01$, *** $P \leq 0.001$

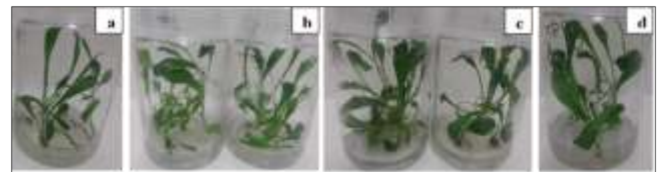

Fig 2: *In vitro* rooting of *Centaurea paxorum* shoot-tip explants after 30 days of culture in MS medium supplemented with 3 different auxin types (mg/l): (a) Control (auxins-free); (b) 0.1 IBA; (c) 0.1 NAA; (d) 0.1 IAA

Therefore, the 0.1 mg/l BA + 0.025 mg/l IBA treatment appears to be the most appropriate for the initial shoot induction stage and the single effect of auxin IAA (0.1 mg/l) for the *in vitro* rooting stage of *C. paxorum*. For the subsequent elongation stages of both produced shoots (proliferation stage) and formed roots (rooting stage), the MS culture medium without PGR's proved to be more effective.

After a total period of 5 weeks (26/4/2018-31/5/2018) being planted in the greenhouse under mist (26/4/2018-14/5/2018) and after gradually reduction of RH in the greenhouse bench outside mist (14/5/2018-31/5/2018) the rooted plantlets survived up to 90%, in specific 96.3% (i.e. 26 out of the 27 in total plantlets within 100 ml volume pots were remained alive). The raised plants were maintained in normal greenhouse conditions (RH 35-45%, increased light density without shade nets) and were transferred in bigger pots (0.33 Lt) allowing further growth without any losses (100% survival). On 3/7/2018, when plants reached the height of 10 cm (another 5 week-period) after transplantation they were ready to continue growing in bigger (2.5 Lt) pots in typical outdoor environmental conditions, or readily available for cultivation in the field. After another 8 weeks of vegetative growth and root system development during summer, on 31/8/2018, all the plants were successfully acclimatized, exhibiting 100% survival rate.

Fig 3: *Ex vitro* acclimatization and hardening of *in vitro* rooted plantlets of *Centaurea paxorum*: (a) microplants derived from the auxins-free culture medium; (b, c, d) plants originated from MS medium supplemented with IBA, NAA and IAA each at 0.1 mg/l, respectfully; (e) microplants within multi-seat discs enriched with peat: perlite (1:1 v/v) soil substrate in internal mist system; (f) transplantation of plants in 2.5 Lt pots with peat: perlite: sand (2:1:1 v/v) soil substrate, maintenance in the greenhouse bench wherein watered by sprinkling; (g, h) vegetative growth and development of plants outdoors the nursery 4 and 8 weeks from transplanting, respectively during summer period

4. Discussion

For the majority of the species that belong to the Asteraceae family the MS medium has been recommended for their culture under *in vitro* conditions (George and Sherrington, 1984) [37]. Similarly, the MS basal culture medium was chosen for the *in vitro* propagation of the studied *Centaurea* species (*C. paxorum*) with absolute success (100% shoot proliferation and rooting)

The most efficient cytokinin type for shoot proliferation of *C. junoniana* (Hammatt and Evans, 1985) [22] and *C. rigualii* (Iriondo and Pérez, 1996) [23] was proved to be the BA. In the present study with *C. paxorum*, the application of BA alone and in combination with all 3 auxin types (IBA, NAA, IAA) led after 30 days of culture to the production of multiple shoots with respect to the control explants (PGR's-free), raising the respective number of shoots per explant to 4.33-7.5 and exhibiting 83.33-100% shoot multiplication percentage. An explanation for the increase in shoot number due to BA application alone and in combination with auxins might be the suppression of apical dominance during subculture that induced basal dormant meristematic cells to form new shoots (Shukla *et al*., 2008) [38]. In accordance with our findings, different explant types; roots, stem nodes and cotyledons of *C. arifolia* Boiss. when cultured in MS medium fortified with 1 mg/L BA + 0.1 mg/L NAA resulted after 4 weeks in 50%, 40% and 80% shoot regeneration rates, respectively (Yüzbaşıoğlu *et al*., 2012) [39]. The optimum combination treatment for shoot proliferation of *C. paxorum* under study was found to be 0.1 mg/l BA + 0.025 mg/l IBA. Our results are partly in consistency with those presented in *C. macrocephala* (Hosoki and Kimura, 1997) [28], in *C. paui* Loscos ex Willk. (Cuenca *et al*., 1999) [19] and in *C. spachii* (Cuenca and Amo-Marco, 2000) [24] where higher shoot proliferation was performed by culturing of explants on MS medium supplemented with 0.1, 0.5 and 1mg/L BA, accordingly, without an auxin type.

With respect to *C. paxorum*, BA alone and with auxins led to the appearance of hyperhydricity symptoms being more severe in the combined effect of BA with IAA, thus resulting in mild browning from the base towards the apex of the explant. The hyperhydricity problem might take place during the axillary bud multiplication stage and has been correlated, among other factors, with the relatively high cytokinin level in the culture medium (Hazarika, 2006) [40]. On the contrary, in *C. montana* the higher number of adventitious shoots from leaf explants were obtained after 4 weeks of culture on MS medium fortified with 2 mg/L BA + 0.1 mg/L IAA (Aboualaiwi *et al*., 2011) [41]. However, in *C. rupestris* L., the highest shoot number per explant was achieved after 3 subsequent subcultures of 4 weeks-period each on MS medium, supplemented with 0.25 mg/L BA and 1 mg/L GA_3 (auxins-free) (Perika, 2003) [30].

In the current study employing *C. paxorum*, the application of BA alone and simultaneously with either IBA or IAA had an inhibitory effect on the elongation of produced multiple shoots approximately by 1 cm, where the combined application of BA with NAA did not differentiate shoot length to a substantial degree in comparison to the control (cytokinin- and auxin-free). In agreement up to a degree with our findings, the addition of 0.5 mg/L BA to the MS medium caused a decrease in shoot elongation of *C. paui* Loscos ex Willk. explants, therefore, the highest shoot length was achieved in the absence of plant growth regulators with the addition of cytokinins to have a negative impact (Cuenca *et al*., 1999) [19]. It is well known that cytokinins such as BA are responsible for the inhibition of apical dominance and the production of new shoots, however they can have a negative effect on shoot elongation due to the accumulation of these regulators in tissues (Costa *et al*., 2006) [42].

Addition of both plant growth regulators (cytokinins, auxins) to culture media is essential for callus induction, as cytokinin acts with the presence of auxin as a key to initiate cellular division. The response of explants differences due to the auxin/cytokinin ratio, may be due to differences in the content of these internal parts of the endogenous hormones, which in turn affects the optimum concentration in the callus induction from auxin or cytokinin or both when added to culture media (Taha, 2017) [43]. In the studied *C. paxorum*, BA applied alone and simultaneously with all 3 auxin types led to callus formation in the base of the explants (83.33-100%) in relation to the control in which no callogenesis occurred. According to Aydoğan and Erdağ (2015) [44], no callus formation was observed when *C. zeybekii* Wagenitz explants were cultured on MS medium in the absence of growth regulators. This is expected given that both auxins and cytokinins are paramount in promoting both cell division and cell growth, with auxins being necessary for both parameters while cytokinins only in cell division with callus induction to be the product of both processes, i.e. cell division and growth (Irene *et al*., 2019) [45]. Although auxins are known to be applied to induce callus, cytokinins can also induce in some other plants (Ikeuchi *et al*., 2013) [46]. Cölgecen *et al*. (2012) [47] reported that application of cytokinins were more effective on the callus induction in *C. tchihatcheffii* Fisch. & C.A. Mey than auxins. With respect to *C. paxorum* under study, the use of auxins irrespective of type (IBA, NAA, IAA) together with BA exhibited 100% callus induction being higher than that of BA alone (83.33%). An explanation for this attribute has been reported by Yang *et al*. (2008) [48], who supported that cytokinins facilitate the effect of auxins in callus induction. The reason is that cell division is a joint action that requires a synergistic relation between auxins and cytokinins (Varshney *et al*., 2013) [49]. The callus induction potential is depended on the present of types and concentration of

auxins in the culture medium, because different auxin types may give different effects (Ren *et al*., 2010) [50], however, this was not the case in our study with *C. paxorum* as the 3 auxin types of the same concentration exhibited the same maximum callusing percentage (100%). In tissue culture systems, callus formation is a common phenomenon (Bhattacharya and Bhattacharya, 2001) [51] and may be ascribed to the action of accumulated auxin at the basal cut end of the explants especially in the presence of cytokinins to the culture nutrient medium during cell proliferation (Marks and Simpson, 1994) [52].

It is widely known that auxins are an essential prerequisite for root formation and development (Hartmann *et al*., 1997; George *et al*., 2008) [53, 54]. In the current study with *C. paxorum*, all 3 auxins (IBA, NAA, IAA) positively influenced rooting of shoot tip explants by raising the number of formed roots per rooted microcutting (x 1.78-2 times). In consistency with our findings up to a degree, root induction of the regenerated adventitious shoots in other *Centaurea* species (*C. rupestris* and *C. ragusina*) (Pevalek-Kozlina, 1998) [55] and *C. arifolia* Boiss. (Yüzbaşıoğlu *et al*., 2012) [39] was better enhanced by the exogenous application of IBA to the MS medium. With respect to the studied *C. paxorum*, 100% rooting was obtained with 0.1 mg/l IAA and in the control treatment (auxins-free). IAA is an endogenous auxin naturally produced in the leaves and buds transporting to the low part of the plant, increasing its concentration at the base of the cut, along with other naturally occurring substances, thus promoting rooting (Hartmann *et al*., 2010) [56]. In contrast with our results, low frequency of rooting was achieved in other *Centaurea* species including *C. tchihatcheffii* (Cölgecen *et al*., 2012) [47], *C. paui* (Cuenca *et al*., 1999) [19], *C. junoniana* (Hammatt and Evans, 1985) [22] and *C. macrocephala* (Hosoki and Kimura, 1997) [28]. In the current study with *C. paxorum*, all rooting parameters (root number, root length and rooting percentage) were simultaneously optimum in the presence of 0.1 mg/l IAA to the MS medium after 30 days of culture. In accordance with our results, in *C. spachii* the best rooting result (60%) was obtained on MS medium in the combined effect of 2 mg/l IAA with 2 mg/l IBA after a 3-week period of culture (Cuenca and Amo-Marco, 2000) [24]. Among the 3 auxin types applied, NAA negatively affected the elongation of roots causing a 3 cm – decrease and at the same time stimulated the elongation of the initial explant approximately by 1.5 cm compared to the auxin-untreated *C. paxorum* control shoot-tips. Aboualaiwi *et al*. (2011) [41] found in *C. montana* that the MS medium with either 1.5 mg/l NAA or 0.5 mg/l IBA gave better rooting performance for elongated shoots, taller than 1 cm whereas Perika (2003) [30] reported optimum rooting for excised *C. rupestris* shoots on half-strength MS medium enriched with 0.6 mg/l IBA. Rooted microcuttings with higher number of physiologically active roots together with a larger root system area have higher potential to be adapted under adverse conditions to soil environments due to their higher ability to absorb nutrients from the soil (Rima *et al*., 2011) [57].

An essential step of micropropagation process is the production of fully regenerated plantlets that have the ability to survive in the external environment, outside culture vessels. The most frequent and severe problem of the *in vitro* derived plantlets experience is to overcome the transplantation shock during their exit from the *in vitro* environment (Kozai, 1991) [58]. In the present study with *C. paxorum*, no negative effects were observed on the transplanted material, as rooted microshoots recovered in a short period of time under heated-mist system greenhouse conditions in pots enriched with a peat moss: perlite mixture at a 1:1 v/v ratio exhibiting 96.3% survival rate. A different substrate composed of a mixture of soil, sand and coconut fiber (2:1:1) proved to be suitable for the *ex vitro* adaptation of the *in vitro* regenerated *C. pseudaxillaris* plants (Traykova *et al*., 2015) [59]. Successful was also the acclimatization and gradual hardening of rooted microplants to *ex vitro* greenhouse conditions in other *Centaurea* species including *C. paui* Loscos ex Willk. which gave 70% survival rate (Cuenca *et al*., 1999) [19], *C. spachii* with high survival over 80% (Cuenca and Amo-Marco, 2000) [24] and *C. rupestris* L. with 86% survival (Perika, 2003) [30].

5. Conclusions

The micropropagation protocol established in this study provides a successful and rapid propagation technique to regenerate critically endangered *C. paxorum* from shoot tips for the first time. It avoids major destruction of the rare wild plant and can be utilized in future conservation programs to preserve the plant. Results of this study provide a practical method for improving the quality of *C. paxorum* micropropagation and may enhance commercial production and germplasm conservation of this endangered and medicinal important plant species. After this step, the multiplied material could be maintained *in vitro*, cryo-preserved or used in restoration programs. Besides, emphasizing optimization of propagation conditions through tissue culture, efforts should also be made to propagate the plant through seeds and/or cuttings which may become the most effective method for propagation of *C. paxorum* and conservation of germplasm of this critically endangered plant.

6. Ackowlegdments

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