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Research Article/Araştırma Makalesi

In vitro CONSERVATION OF CRITICALLY ENDANGERED *Dianthus ingoldbyi* Turrill UNDER SLOW GROWTH CONDITIONS

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Abstract: This study was performed in order to micropropagate the critically endangered Balkan endemic *Dianthus ingoldbyi* and to investigate the anatomical and morphological characteristic of shoots following the slowing down of the growth of *in vitro* shoots under cold and dark conditions. For this purpose, axillary buds isolated from aseptic seedlings were cultured on Murashige and Skoog (MS) medium including 1mg/L Benzylaminopurine (BAP) and 0.3mg/L Naphthaleneacetic acid (NAA) and a regeneration ratio of 84.8% was obtained. The number of shoots per explant was 5.9 after 30 days from culture initial and the mean shoot length was 3.6cm. These shoots were transferred to MS medium including 0.5mg/L NAA and stored at 4 ^{o}C in a refrigerator in total darkness for 1-6 months without subculturing. 58% of shoots survived after 6 months of cold storage conditions. Growth of shoots was significantly slowed down and there was no anatomical or morphological deformation at the end of the experimental period. Shoots transferred to normal conditions from cold storage developed better than the control group. Well rooted plants were acclimatized to outdoor conditions with a survival rate of 48%. In conclusion, the cold storage technique used in this study is suggested as an effective method for *in vitro* conservation of the critically endangered *D. ingoldbyi*.

Key words: Cold storage, micropropagation, anatomy, morphology, Balkan endemic.

Kritik Tehlikedeki *Dianthus ingoldbyi* Turrill'in Yavaş Büyüme Koşulları Altında *In vitro* Korunması

Özet: Bu çalışma kritik biçimde tehlike altındaki Balkan endemiği *Dianthus ingoldbyi*'nin mikroçoğaltımı ve düşük sıcaklık ve karanlık koşullar altında *in vitro* sürgünlerin büyümesinin yavaşlatılmasını takiben sürgünlerin anatomik ve morfolojik karakterlerinin incelenmesi amacıyla yapılmıştır. Bu amaçla, aseptik fidelerden elde edilen aksillar tomurcuklar 1mg/L Benzilaminopürin (BAP) ve 0,3mg/L Naftalen asetik asit (NAA) içeren Murashige and Skoog (MS) besi yerinde kültüre alınarak %84,8 oranında rejenerasyon elde edilmiştir. Kültürün 30. gününde eksplant başına sürgün oranı 5,9 iken ortalama sürgün uzunluğu 3,6cm olmuştur. Bu sürgünler 0,5mg/L NAA içeren MS besi yerine aktarılarak buzdolabında sürekli karanlık koşullarda ve 4^{o}C sıcaklıkta alt kültüre almaksızın 1-6 ay süresince depolanmıştır. Soğukta depolamanın 6. ayı sonunda sürgünlerin %58'i hayatta kalmıştır. Deneylerin sonunda hayatta kalan sürgünlerin büyümesinin anlamlı düzeyde yavaşladığı, anatomik ve morfolojik açıdan herhangi bir deformasyonun bulunmadığı tespit edilmiştir. Soğukta depolamadan normal koşullara aktarılan sürgünler kontrol grubundan daha iyi gelişmişlerdir. İyice köklenen fidelerin %48'i başarılı biçimde dış koşullara şaşırtılmıştır. Sonuç olarak bu çalışmada kullanılan soğukta depolama tekniğinin kritik tehlikedeki *D. ingoldbyi*'nin *in vitro* korunmasında etkili bir metot olduğu öne sürülmektedir.

Anahtar kelimeler: Soğuk depolama, mikroçoğaltım, anatomi, morfoloji, Balkan endemiği.

Introduction

The genus *Dianthus* of the family Caryophyllaceae is represented with 74 taxa in Turkey (İlçim et al. 2013). *Dianthus ingoldbyi* (Fig. 1A-B) is an endemic species of the genus growing only in some regions of the Balkan Peninsula, such as Çanakkale (Bozcaada-Sulubahçe and Eceabat-Arıburun, Turkey), Edirne (Keşan-Mecidiye, Turkey), and North-East of Greece (Reeve 1967, Uysal et al. 1992, Başak & Güler 2000, Trigas et al. 2007). It is a caespitose perennial 30 cm with a woody stock; stems puberulent below. Leaves 2mm wide, linear, acuminate, coriaceous. Flowers 2-5 together, subsessile. Epicalyx-scale 10-12, ovate, gradually acuminate. Calyx 15-17 x 2mm, tapering above the middle. Petal-limb 2-4mm, denticulate, whitish or greenish yellow (Tutin & Walters, 1993). According to the Red Data Book of Turkish Plants, the conservation status of *D. ingoldbyi* is listed as Critically Endangered (CR) (Ekim et al. 2000) and

no protection program for *D. ingoldbyi* is present at the moment. Therefore, if an effective conservation strategy is not developed as soon as possible, this species will most probably be extinct in nature in only a few decades.

Industrialization, urbanization, tourism, excessive pasturing, agricultural activities and pollution are the main contributing factors threatening and endangering the future of rare plants (Kargıoğlu et al. 2008). Therefore, protection of endangered plants has been one of the most important goals for botanists in recent years (Dayan et al. 2013). There are two distinct methods, *in situ* and *ex situ*, for germplasm conservation of plants. *In situ* conservation includes conservation in natural habitats (i.e. wilderness areas, natural parks) while *ex situ* conservation is a process of protecting an endangered plant species outside of its natural habitat (Withers & Engelmann 1998). *Ex situ* conservation includes classical methods like seed storage in seed banks, field gene banks and botanical gardens. Radhika Ramya et al. (2014) reported that when the biological material (organs, seeds) cannot be stored in a traditional manner, biotechnological methods like *in vitro* storage and cryopreservation are alternative approaches for *ex situ* conservation of such plant material. According to Fay (1994), the appropriate strategy for protection of endemic or nonendemic endangered species varies from species to species. Restoration of wild populations by individuals obtained *ex situ* techniques is an important approach for conservations of extinction-prone species. Thus, *in situ* protection techniques could be supported by *in vitro* methods that allow rapid propagations of threatened species (Touchell & Dixon 1999). Today, biotechnological approaches can effectively contribute to the conservation of germplasm with innovative techniques for a medium and long-term storage and these *in vitro* cultures have a great potential for a rapid cloning of a lot of individuals and transferring them to the nature when necessary.

A valid alternative for medium-term preservation of species is offered by *in vitro* slow growth storage conditions. Advantages of slow growth procedure are that contaminations and genetic modifications are reduced (Engelmann 1997, Reed 1999). *In vitro* cultures conserved by slow growth storage are maintained under modified conditions to reduce the growth rate. In general, the range of conservation period in cold storage is from a couple of months to one year and different factors, such as the presence/absence of light, the age, the size and the physiological state of shoot cultures can affect the maximum time of storage (Özüdoğru et al. 2010), and it is necessary to define these factors for each species when optimizing an *in vitro* conservation procedure (Benelli et al. 2012). The growth rate can be limited by reducing temperature and/or light intensity, adding osmotic compounds such as mannitol or sucrose in culture medium and by growth retardants (Grout 1995). Among these, the most commonly used procedures are the decrease of temperature and light intensity. These factors have physiological consequences to induce a significant reduction of cell metabolism and as a consequence the shoot growth. By this technique, the intervals between the subcultures can be extended, reducing also the cost of stock plant's maintenance as well as the risk of contamination of lines during subculturing (Benelli et al. 2012).

Despite all these advantages of *in vitro* cultures, some culture conditions other than media may affect the growth and development of plant material in culture. Hughes (1981) reported that (1981) light (wave length, intensity and photoperiod), temperature and the gas phase which develops above the culture system are the factors that affect the growth and development of plant cell and tissues in culture and these factors are not independent but interact in often complex ways. Therefore, it is important to make anatomical and morphological investigation on *in vitro* cultures for successful medium and/or long-term storage.

Some of the Europe's endemic and/or endangered *Dianthus* taxa which were previously studied in *in vitro* multiplication experiments are *D. giganteus* subsp. *croaticus* (Prolic et al. 2002), *D. gratianopolitanus* (Fraga et al. 2004), *D. pyrenaicus* (Marcu et al. 2006), *D. giganteus*, *D. alpinus*, *D. ferrugineus*, *D. gallicus* (Cristea et al. 2006), *D. petraeus* subsp. *simonkaianus* (Miclăuş et al. 2003), *D. nardiformis* (Holobiuc et al. 2009a), *D. henteri* (Cristea et al. 2010) and *D. giganteus* subsp. *banaticus* (Cristea et al. 2006, Jarda et al. 2014). On the other hand, there are some cryopreservation studies on carnation (*D. caryophyllus*) which is an ornamental plant (Seibert 1976, Seibert & Wetherbee 1977, Uemura & Sakai 1980, Halmagyi & Deliu 2007). In addition to these reports, there are some *in vitro* medium-term conservation studies on other endemic *Dianthus* taxa like *D. spiculifolius*, *D. glacialis* ssp. *gelidus* (Holobiuc & Blindu 2006, Holobiuc et al. 2009b, 2010), *D. tenuifolius, D. callizonus, D. superbus* L. ssp. *speciosus,* (Holobiuc & Blindu 2006) and *D. superbus* L. ssp. *alpestris* (Holobiuc et al. 2004-2005). These taxa were conserved under the minimal growth conditions. Although these *in vitro* multiplication, cryopreservation and *in vitro* medium-term conservation studies on *Dianthus* exist in literature, none of them addressed the cold storage conditions on this genus.

In the present study, anatomical and morphological effects of dark and cold storage periods on *in vitro* shoots of endangered *D. ingoldbyi* were investigated. The regrowth of cold storage shoots was also studied after transferring to normal culture conditions and it was discussed if this protection strategy is appropriate for *D. ingoldbyi* or not. Additionally, this paper gives the first protocol for micropropagation and *in vitro* conservation of *D. ingoldbyi.*

Materials and Methods

Dianthus ingoldbyi seeds collected from Mecidiye Coast of Edirne Province in 2006 were used in this study and the experiments were finished in 2009. Healthy seeds were soaked in commercial bleach of 20% for 20 minutes

Table 1. Growth results of *in vitro D. ingoldbyi* regenerants cultured on MS medium including 0.5mg/L NAA at NCC and CSC.

Culture Period (Days)	Mean Shoot Length (cm)		Number of Adventitious Shoots		Mean Rooting (%)		RER	RER
	NCC	CSC	NCC	CSC	NCC	CSC	NCC	CSC
Initial	3.6^{a}	3.5^{a}	1^{a}	1^{a}	0^{a}	0^{a}	-	-
30	5.2^{a}	3.9^{a}	6.5^{b}	2.3^{b}	65^{b}	17^{ab}	5.3	1.3
60	7.4^{b}	4.3^{a}	8.2^{bc}	2.3^{b}	75^{b}	33^{b}	6.3	1.3
90	8.5^{bc}	4.6^{a}	10.4^{bcd}	2.4^{b}	85^{b}	33^{b}	5.4	1.2
120	9.4^{cd}	4.7^{a}	12.1^{cd}	2.5^{b}	85^{b}	33^{b}	4.8	1.0
150	10.1^{cd}	4.8^{a}	13.6^{d}	2.5^{b}	88^{b}	33^{b}	4.3	0.8
180	10.7^{d}	4.8^{a}	14.8^{d}	2.5^{b}	88^{b}	33^{b}	3.9	0.7

- Values within each column followed by the same letters are not significantly different as revealed by the Student t test at 0.05% probability level.

and rinsed with sterile distilled water for three times. Then the seeds were immersed in 70% ethanol for 5 minutes and rinsed again with sterile distilled water for three times. Hormone free MS medium (Murashige & Skoog 1962) supplemented with 30g/L sucrose and 7g/L agar was used for germination. pH of the medium was adjusted to 5.6 and the media was autoclaved at 121°C for 15 minutes at 1 atm pressure. Ten seeds were placed in 200ml culture jars (25 jars in total) containing 50ml of culture medium. The culture jars were kept at 25 ± 2°C for 1 month under a 16-h photoperiod with a white fluorescent light (intensity: 3100 lux.).

Axillary buds and leaf segments of 1 month old sterile seedlings were used as an explant and they were incubated on MS medium containing 1mg/L BAP and 0.3mg/L NAA for 1 month. Ten explants were transferred 200ml culture jars containing 100ml of culture medium and 3 jars were used for each explant type. Experiments were repeated 3 times.

At the end of 1 month, regeneration ratio was calculated and shoots were transferred to hormone free MS medium for further development. Fifteen days later, the lengths of the shoots were measured and well developed shoots were individually transferred to test tubes containing 25ml MS medium including 0.5mg/L NAA. All shoots were separated into seven groups each containing 30 shoots. One group was cultured at 25°C under a 16-h light photoperiod (light intensity: 3100 lux.) regime as Normal Culture Conditions (NCC). The other 6 groups were stored at 4°C under dark conditions as Cold Storage Conditions (CSC) without subculturing. This stage was repeated 3 times and at least 630 shoots were used throughout the experiment.

During the 6 months period shoot lengths, number of adventitious shoots and rooting and survival rate were recorded monthly. At the end of the 1st, 3rd and 6th months, leaf samples from all groups were fixed in FAA (Formaldehyde: Acetic Acid: Ethyl Alcohol; 5:10:85) solution and put in 70% ethyl alcohol for anatomical investigations. At the end of each month, a group including 30 shoots at CSC was transferred to NCC for 1 month without subculturing and their lengths, rooting and number of adventitious shoots were recorded. Relative Elongation Rate (RER) and Relative Re-elongation Rate (RER_r) of the shoots was calculated with a formula [(mean final length of shoots – mean initial length of shoots) ×100/days of culture] adapted from Benelli et al. (2012). RER_r means an elongation rate of shoots after being transferred to NCC from CSC. All *in vitro* plantlets were transferred to pots contain peat-perlite (1:1) mixture and covered with transparent jars for acclimatization.

For anatomical investigations, cross sections were prepared by hand from previously fixed leaves and their permanent slides were prepared by covering glycerin-gelatin. Preparations were investigated under an Olympus BX-50 microscope and photographed. Leaves of shoots cultured at NCC for 30 days were used as a control.

All data were statistically analyzed with one-way ANOVA using the SPSS (Statistical Package for the Social Sciences) software package. Differences between groups were determined with Student-t test at 0.05 significance level.

Results

Because of the limited number of the seeds, only 250 of them were used for *in vitro* germinations and 65% of these seeds were germinated (Fig. 1C). Leaf explants isolated from *in vitro* plantlets only showed necrosis and chlorosis at the end of 1 month. Regeneration rate was 84.8% for axillary buds. Shoot number per explant and mean length of the shoots were 5.9 and 3.6cm, respectively (Fig. 1D-E). There was no contamination in any culture stage.

Table 1 shows the mean shoot length, the number of adventitious shoots, mean rooting and RER of *D. ingoldbyi* cultured at CSC and NCC for 1-6 months. Based on the results at the end of the 180 days, the mean shoot length at NCC was 10.7 but it decreased to 4.8 at CSC. Similarly, the mean number of adventitious shoots at NCC (14,8) reduced at CSC (2,5). The mean rooting results also showed a similar decline pattern (88% at NCC to 33% at CSC). At the end of the experiments, RERs of shoots at NCC and CSC were 3.9 and 0.7, respectively. It is clear from the results (see also Table 1) that RERs of shoots at

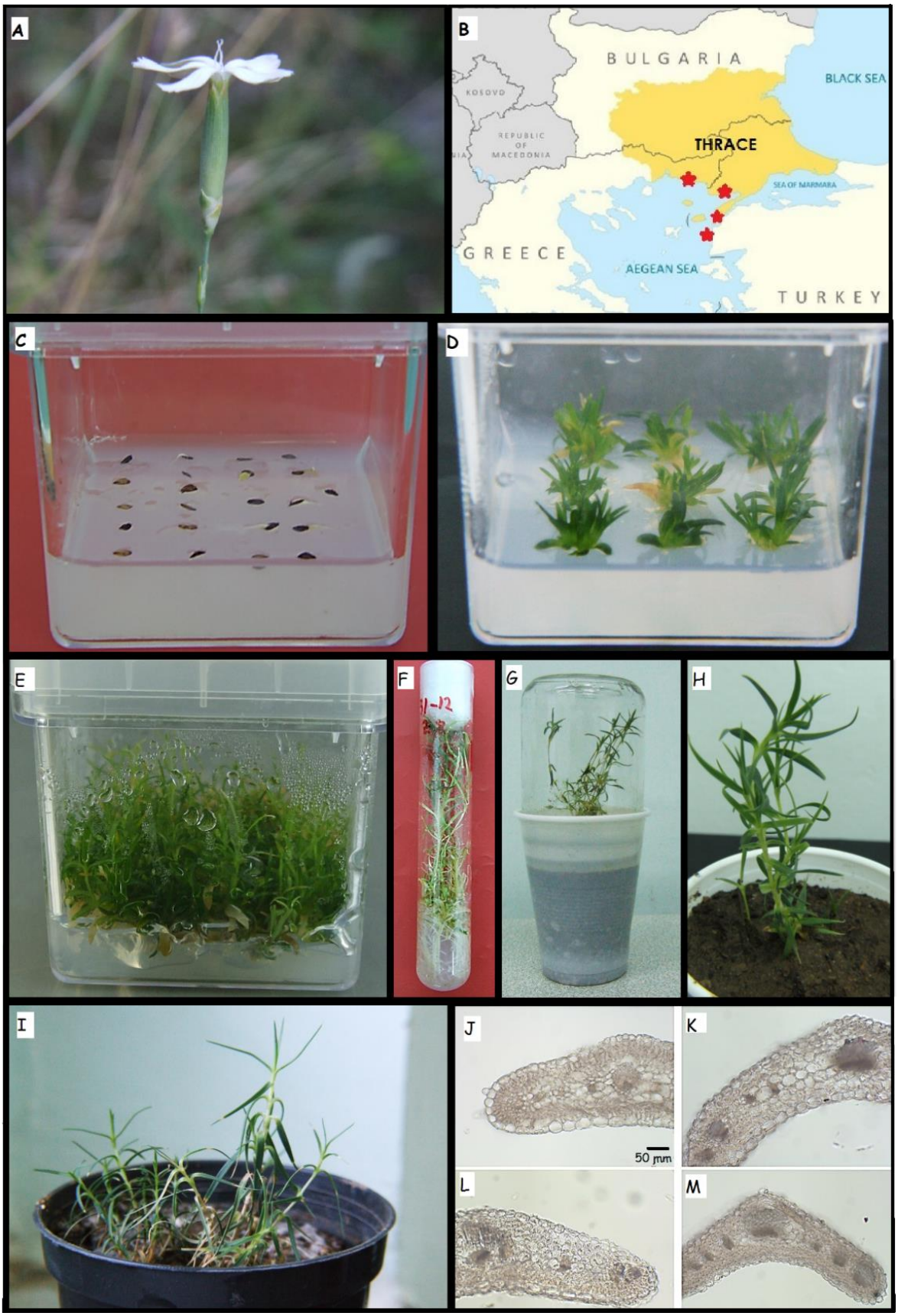

Fig. 1. *D. ingoldbyi* flower in natural habitat **(A)**, the distribution of the species in the Thrace Region **(B)**, *in vitro* seed germination **(C)**, shoot multiplication on MS medium containing 1mg/L BAP and 0.3mg/L NAA **(D,E)**, shoots which were stored at CSC for 6 months **(F)**, acclimatization of plantlets **(G,H)**, 6 months old plant which is acclimated **(I)**, anatomical observations on the leaf sections, control **(J)** and 1 **(K)**, 3 **(L)** and 6 months **(M)** at Cold Storage Conditions.

Table 2. Growth results of *D. ingoldbyi* shoots transferred to NCC for 30 days after cold storage.

Days at CSC	Mean Shoot Length (cm)	RER_r	Number of Adventitious Shoots	Mean Rooting (%)	Survival Rate (%)
30	5.9	6.8^{b}	6.9^{a}	70.1^{ab}	100
60	6.9	8.6^{bc}	7.1^{a}	78.4^{b}	92
90	7.7	10.3^{cd}	7.1^{a}	80.3^{b}	83
120	8.3	11.9^{d}	7.5^{ab}	84.6^{c}	75
150	9.0	14.1^{e}	7.8^{b}	88.7^{cd}	67
180	9.6	16.0^{e}	7.9^{b}	90.0^{d}	58
Control*	5.2	5.3^{a}	6.5^{a}	65.0^{a}	100

*Control plants were cultured at NCC for 30 days. Values within each column followed by the same letters are not significantly different as revealed by the Student t test at 0.05% probability level.

NCC and CSC continuously decreased when the culture period was increased. But the decline of RER at CSC was more dramatic than at NCC. All these results show that the growth of *D. ingolbyi* shoots were significantly slowed down by cold storage.

Mean shoot length, RER_r, number of adventitious shoots, mean rooting and survival rate of *in vitro D. ingoldbyi* shoots which were transferred to NCC for 30 days after cold storage are shown in Table 2. The RER_r of the shoots increased when the culture period extended at CSC. RER_r of the shoots cultured at CSC for 180 days was maximum (16) and this value was extremely higher than the control shoots (5.3). Similarly, number of adventitious shoots and mean rooting increased when the culture period of shoots extended at CSC. These results show that shoots transferred to NCC after cold storage developed better than the control. On the other hand, survival rate of the shoots cultured at CSC decreased with extending culture period. All rooted plants obtained from the experiment were acclimatized and 48% of them survived (Fig. 1G-I).

Anatomical measurements of the leaves of *D. ingoldbyi* cultured at CSC and the control group can be seen in Table 3 and Fig. 1 (J-M). The outer tangential walls of epidermis cells and cuticle at CSC were thicker than control. There was no any other anatomical difference or abnormality. Morphologically, color of the shoots cultured at CSC were light green, but their color turned to dark green when they were transferred to NCC. The fast and healthy re-elongation and re-development of the shoots after cold storage shows that there was no permanent anatomical and/or morphological damage during the culture.

Table 3. Anatomical measurements of the leaves of *D. ingoldbyi* cultured at CSC and the control group.

	Cuticle thickness (µm)	Outer tangential walls of epidermis (µm)
Control	0.87 ± 0.04^{a}	2.27 ± 0.22^{a}
1 month	1.06 ± 0.09^{b}	3.26 ± 0.33^{b}
3 month	1.73 ± 0.13^{c}	4.69 ± 0.21^{c}
6 month	2.27 ± 0.26^{d}	5.35 ± 0.33^{d}

Discussions

In vitro slow growth storage techniques are being routinely used for short/medium-term conservation of numerous species including crop plants, e.g. potato, *Musa*, yam, cassava and rare and endangered species (Engelman 2011). The critically endangered species *Dianthus ingoldbyi* was propagated *in vitro* via shoot multiplication technique in the present study and the micropropagated shoots were conserved under slow growth conditions for 180 days. According to the anatomical and morphological investigations, it can be suggested that the cold storage is a useful technique for *in vitro* conservation of *D. ingoldbyi* without any permanent damage during the culture. This report is the first micropropagation and *in vitro* conservation report for *D. ingoldbyi*.

The *in vitro* germination was successful and sterile seedling usage was an advantageous choice for avoiding from contamination and obtaining explants with high regeneration ability. These results are in accordance with *in vitro* germinations of *D. mainensis* (Erst et al. 2014).

There exist some studies on the ornamental plant *D. caryophyllus* (carnation). For instance, Lubomski & Jerzy (1989) reported that the shoot formation response of *D. caryophyllus* was the best when a MS medium supplemented with BAP was used. However, Mangal et al. (2002) and Onamu et al. (2003) suggested that NAA and Kinetin combination is better for shoot induction from meristems. On the contrary, Ali et al. (2008) notified that addition of kinetin failed to stimulate shoot induction response and there was a decline in shoot induction with increase in the concentration of Kinetin. In the same study, when MS medium including 1.0mg/L BAP was used, shoot number per explant and mean shoot length were 25 and 5.2cm, respectively. In another study, Kovac (1995) reported high shoot multiplication rate on MS medium supplemented with 1.0mg/L BAP. Additionally, Van Altvorst (1992) and Yantcheva et al. (1998) reported high shoot number per explant on MS medium including 0.9mg/L BA and 0.3mg/L NAA. In the present study, when the axillary buds were cultured on MS medium supplemented with 1mg/L BAP and 0.3mg/L NAA for 30 days the regeneration response and shoot number per explants were 84.4% and 5.9, respectively. However, there

was no regeneration response from leaf segment explants. Although shoot number per explants seems low, it is almost in accordance with the above studies on *D. caryophyllus.* Additionally, it can be suggested that the reason of low shoot number per explants is adaptation of *D. ingoldbyi* to the special habitats unlike the carnation which is the cultivar plant. Thus, the results about endemic *D. giganteus* subsp. *banaticus* (18 shoots/explant at 110 days) are similar to our findings (Jarda et al. 2014). Erst et al. (2014) also reported 5.5 shoots/explant when they cultured *D. mainensis* on medium including 3µM BAP for 30 days.

Although Jarda et al. (2014) reported relatively long *in vitro* culture period (110–140 days) for *D. giganteus* subsp. *banaticus*, there was no clear information about subculture periods and that research was done at 22 °C. In our study, long culture period (180 days) without subculturing provides to conserve this species in a lesser volume (about 75 shoots per magenta, Fig. 1E) with minimal cost. Benelli et al. (2012) who made an investigation on some ornamental plants like *Anthurium andreanum, Ranunculus asiaticus* and *Carex hoshimensis* with slow growth technique reported that growth was slowed down during the continuous dark culture period (3, 6 and 9 months) and then regrowth rate was increased after transferring to normal culture conditions. The survival rate of the plants in these studies ranged from 100% to 56% depending on the increasing culture periods. Our present findings coherent with this result. On the other hand, shoots can also be cultured in the presence of light. Bhatt et al. (2004) reported that, 100% of *in vitro* shoots of *Brugmansia versicolor* which were cultured at 16h photoperiod for 6 months survived but survival rate was declined to 40% at continuous dark conditions. In the same study, the color of the shoots was observed to turn to yellowish. Likewise, in our study, color of *D. ingoldbyi* shoots cultured at CSC was a little yellowish but this symptom disappeared when the shoots were transferred to NCC.

It is a known fact that the healthy development of plantlets after cold storage depends on storage conditions and this can affect the morphological characteristic of plantlets (Hausman et al. 1994). In this study, the outer tangential walls of epidermis and cuticle of *D. ingoldbyi* leaves at CSC were thicker than control. These changes may be an adaptation to cold conditions. Similar results were observed for *Brassica napus* L. var. *oleifera* L., (Stefanowska et al. 1999) and *Rhododendron* (Wang et al. 2008). There was no other abnormality in *in vitro D. ingoldbyi* shoots meaning that the shoot development was not affected from the culture condition. On the contrary, shoots developed more efficiently than control when the shoots at CSC were transferred to NCC. Considering this latter finding, it can be suggested that cold storage of *D. ingoldbyi* shoots in constant darkness for 6 months does not affect the further development of plantlets. Bhatt et al. (2004) and Arda et al. (2012) published similar results for *Brugmansia versicolor* and *Helianthus annuus* respectively.

In conclusion, a micropropagation protocol and cold storage procedure for Balkan endemic and critically endangered *D. ingoldyi* was developed for the first time. It was determined that, *in vitro* shoots can be stored under dark and cold (4°C) conditions for 6 months without subculture. According to these results, it is suggested that the slow growth protocol developed for endemic and critically endangered *D. ingoldbyi* will contribute to germplasm conservation of this species via *in vitro* collecting techniques.

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