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Indirect somatic embryogenesis and shoot organogenesis from cotyledonary leaf segments of *Digitalis lamarckii* Ivan., an endemic medicinal species

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Abstract: This study describes, for the first time, an in vitro protocol for the development of somatic embryos and shoots from a callus derived from cotyledonary leaf segments excised from 3-week-old in vitro-germinated seedlings of *Digitalis lamarckii* Ivan. (dwarf foxglove), an endemic medicinal species of Turkey. The embryogenic callus was induced on Murashige and Skoog (MS) medium containing 0.54 µM α-naphthalene acetic acid (NAA) and 2.22 µM 6-benzylaminopurine (BAP). Somatic embryos developed readily when the embryogenic callus was transferred to MS medium containing BAP (4.44 or 8.87 µM) alone or BAP combined with NAA (1.34, 2.69, or 5.37 µM). The most effective hormonal combination for somatic embryogenesis was 1.34 µM NAA and 8.87 µM BAP, which produced a mean of 37.0 embryos per cotyledonary leaf explant. An organogenic callus was induced on MS medium containing 2.69 µM NAA and 2.22 µM BAP. Shoot development was observed when the organogenic callus was transferred to MS medium containing different concentrations of BAP alone (2.22, 4.44, or 8.87 µM) or BAP combined with NAA (1.34 or 2.69 µM). The highest mean number of shoots (5.67 shoots per explant) was obtained when the medium contained 8.87 µM BAP and 2.69 µM NAA. The regenerated shoots were readily rooted on MS medium containing 1.0, 2.5, or 5 µM indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA). Rooted regenerants were then transferred to the pots, where they grew well and attained maturity. Over 90% of the regenerants survived the hardening process. The protocol described could be useful for germplasm conservation, commercial cultivation, genetic improvement, and cardenolide production studies in *D. lamarckii*.

Key words: *Digitalis lamarckii* Ivan., dwarf foxglove, cotyledonary leaf segments, callus formation, somatic embryogenesis, shoot organogenesis

Endemik tıbbi tür *Digitalis lamarckii* Ivan.'ın kotiledon yaprağı segmentlerinden indirekt somatik embriyo ve sürgün oluşumu

Özet: Türkiye'nin endemik tıbbi türlerinden biri olan *Digitalis lamarckii* Ivan. (bodur yüksükotu)'nın in vitro koşullarda çimlendirilmiş üç haftalık fidelerinden izole edilen kotiledon yaprağı segmentlerinden elde edilen kallustan, in vitro somatik embriyo ve sürgün oluşumu protokolu ilk defa bu çalışmada tanımlanmıştır. Embriyonik kallus, 0,54 µM α-naftalen asetik asit (NAA) ile 2,22 µM 6-benzilaminopürin (BAP) içeren Murashige ve Skoog (MS) ortamında elde edilmiş, ve BAP'yi (4,44 veya 8,87 µM) tek başına veya NAA (1,34, 2,69 veya 5,37 µM) ile kombinasyon halinde içeren MS ortamında kültüre alındığında, somatik embriyolar kolaylıkla gelişmişlerdir. Embriyo rejenerasyonu için en

etkili hormonal kombinasyon, 1,34 µM NAA ile 8,87 µM BAP olmuş ve kotiledon yaprak eksplantı başına ortalama 37,0 embriyo elde edilmiştir. Organogenik kallus, 2,69 µM NAA ve 2,22 µM BAP içeren MS ortamında elde edilmiş, ve BAP'yi (2,22, 4,44 veya 8,87 µM) tek başına veya NAA (1,34 veya 2,69 µM) ile kombinasyon halinde içeren MS ortamında kültüre alındığında, sürgün oluşumu gözlenmiştir. En yüksek ortalama sürgün verimi (eksplant başına 5,67 sürgün), 8,87 µM BAP ve 2,69 µM NAA içeren ortamda elde edilmiştir. Sürgünler, indol-3-asetik asit (IAA) veya NAA (1,0, 2,5 veya 5,0 µM) içeren MS ortamında kolaylıkla köklendirilmiştir. Rejenere edilen bitkiler, saksılara aktarılarak, büyümeye ve olgunlaşmaya bırakılmışlardır. Rejenere edilen bitkilerin tamamına yakını, dış koşullara alıştırma sürecini, canlılıklarını koruyarak tamamlamışlardır. Tanımlanan bu protocol, *D. lamarckii*'nin germplasm depolanması, ticari üretim, genetik iyileştirme ve kardenolit üretimi çalışmalarına katkı sağlayabilecektir.

Anahtar sözcükler: *Digitalis lamarckii* Ivan., bodur yüksükotu, kotiledon yaprak segmentleri, kallus oluşumu, somatik embriyogenesis, sürgün organogenesisi

Introduction

Digitalis lamarckii Ivan., commonly known as foxglove, is an endemic species of the Turkish flora belonging to the family Plantaginaceae. This species has been marked as vulnerable (VU) in the *Red Data Book of Turkish Plants* (1). Members of the genus *Digitalis* are medicinally and economically important plants as they contain cardiac glycosides that strengthen cardiac diffusion and regulate the heart rhythm (2). In addition to the cardiotonic effects of lanatoside C, digoxin, and digitoxin, preliminary studies have revealed that digoxin and digitoxin are also effective agents in cancer chemotherapy, in particular for prostate and breast cancer treatment (3-5). The crude drug of *D. lamarckii* is used to treat heart diseases, including myocardial infarction, edema, angina, cardiac dysfunction, hypertrophy, and arterial hypertension (2,6,7). As the cardenolides are of commercial interest, studies have focused on the in vitro culture of several *Digitalis* species over the last 2 decades (8-16).

In vitro regeneration of plants via somatic embryogenesis has some distinct features, such as single-cell origin, the consequent low frequency of chimeras, and the production of a high number of regenerants (17). Somatic embryos are also an important alternative for achieving the production of cardenolides in vitro (9,18). Plant regeneration via somatic embryogenesis and organogenesis has been reported in several *Digitalis* species, including *D. lanata*, *D. purpurea*, *D. thapsi*, and *D. obscura* (19-24). However, in a literature survey, no report concerning the in vitro regeneration of *D. lamarckii* was found. The aims of this study were, therefore, to develop an efficient regeneration system for *D. lamarckii* via indirect somatic embryogenesis and shoot organogenesis using cotyledonary leaf explants excised from in vitro-germinated seedlings, and to test different hormonal combinations.

Materials and methods

Plant material

Seeds of *D. lamarckii* were collected from natural populations growing in different localities of Bolu, in the northwestern part of the Black Sea region of Turkey (N40°37.71′, E32°26.26′), at an altitude of 1510 m, in September 2008. Identification of the species was done according to work of Davis (25), and voucher specimens (IEker-1726) were deposited at the Abant İzzet Baysal University Herbarium (Bolu, Turkey).

Surface sterilization and culture medium

Seeds of *D. lamarckii* were surface disinfected with 20% commercial bleach with a few drops of Tween-20 for 10 min using a sonicator, and finally rinsed with sterile distilled water several times. An average of 20-25 seeds were aseptically germinated on Murashige and Skoog (MS) medium (26). The MS medium used throughout the study for seed germination, callus induction from cotyledonary leaf segments, somatic embryo and shoot development from the callus, and rooting of the regenerated shoots was supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar and autoclaved at 121 °C and 1.06 kg cm^{-2} pressure for 15 min after adjusting the pH to 5.8 with 0.1 N HCl or 0.1 N KOH. The cultures (except rooting) were grown in petri dishes (9 × 1.5 cm) and were kept at 23 ± 1 °C under a 16-h light, 8-h dark photoperiod provided by cool-white fluorescent light with an irradiance of 50 μmol^{-2} s^{-1} at a relative humidity of 60%.

Callus induction, somatic embryogenesis, and shoot organogenesis

Embryogenic and organogenic calli were induced within 4 weeks when cotyledonary leaf segments (5-8 mm) excised from 3-week-old in vitro-germinated seedlings were cultured on MS medium containing 0.54 µM NAA and 2.22 µM BAP or 2.69 µM NAA and 2.22 µM BAP, respectively. To obtain somatic embryos, the embryogenic callus was transferred to MS medium containing combinations of BAP (4.44 or 8.87 µM) and NAA (1.34, 2.69, or 5.37 µM) (Table 1). To obtain adventitious shoots, the organogenic callus was transferred to MS medium containing different concentrations of BAP (2.22, 4.44, or 8.87 µM) alone or BAP combined with NAA (1.34 or 2.69 µM) (Table 2).

Rooting and hardening off

All of the shoots (2-3 cm long) that developed from the callus were transferred to Magenta vessels containing MS medium supplemented with 1.0, 2.5, or 5.0 µM IAA or IBA for root formation for 6 weeks (Table 3). The shoots that rooted were transferred to pots containing an autoclaved mixture of soil, manure, moss, and sand at a ratio of 1:2:2:1, and were kept under growth-room conditions at 20-22 °C with low humidity (25%-35%).

Data collection and statistical analysis

Each treatment of somatic embryogenesis and organogenesis used 7 explants and the experiments were repeated in triplicate, thus having a total of 21 replicates per treatment. Somatic embryos at different developmental stages and developed adventitious shoots were counted under a microscope. The mean number of somatic embryos per explant was recorded after 8 weeks of culture, while the mean numbers of shoots and roots and the lengths of roots were recorded after 6 weeks of culture. The data were statistically analyzed using Microsoft Excel. Analysis of variance (ANOVA) was used to calculate statistical significance and, using a free internet-based program, the significance of difference among the means was determined using Tukey's test at $P < 0.05$.

Results and discussion

Somatic embryogenesis from callus

Embryogenic calli were observed when cotyledonary leaf segments excised from 3-week-old seedlings were cultured on MS medium supplemented with 0.54 µM NAA and 2.22 µM BAP. Within as early as 1 week, small amounts of friable callus developed on the cut surfaces of the explants and subsequently covered the entire surface of the explant. When transferred to an embryo-induction medium containing different hormonal combinations (Table 1), part of the callus was able to develop into somatic

Table 1. Effects of different concentrations and combinations of NAA and BAP on the development of somatic embryos from callus derived from cotyledonary leaf segments of *D. lamarckii*. Data were collected after 8 weeks of culture.

Concentrations of NAA + BAP (µM)	Mean number of somatic embryos at different stages per cotyledonary leaf explant*				
	Globular	Heart	Torpedo	Cotyledonary	Total
1.34 + 4.44	12.7 ± 1.9^{bc}	7.5 ± 1.3^{b}	6.2 ± 0.5^{b}	1.8 ± 03.^{bc}	28.2 ± 0.9^{b}
1.34 + 8.87	15.2 ± 2.7^{a}	10.0 ± 2.5^{a}	8.8 ± 0.7^{a}	3.0 ± 0.2^{a}	37.0 ± 1.3^{a}
2.69 + 4.44	11.5 ± 1.7^{bc}	6.5 ± 1.2^{b}	5.0 ± 0.6^{bc}	2.0 ± 0.2^{b}	25.0 ± 1.1^{bc}
2.69 + 8.87	10.3 ± 1.8^{bcd}	6.8 ± 1.4^{b}	5.3 ± 0.4^{bc}	1.0 ± 0.2^{d}	23.4 ± 1.1^{c}
5.37 + 4.44	8.2 ± 1.5^{cd}	4.8 ± 1.3^{c}	4.2 ± 0.3^{c}	2.8 ± 0.3^{a}	20.0 ± 1.0^{d}
5.37 + 8.87	9.2 ± 1.1^{cd}	5.8 ± 1.1^{bc}	4.3 ± 0.6^{c}	1.7 ± 0.2^{c}	21.0 ± 0.8^{bc}

*Values are the mean ± standard error (SE), and means with the same letter within a column are not significantly different at $P < 0.05$ according to Tukey's test.

embryos. Within 4 to 6 weeks of culture initiation, numerous globular somatic embryos were visible all around the surface of the embryogenic callus. All of these globular embryos first appeared as protrusions on the surface of the tissue, and then individual embryos enlarged into distinct bipolar structures and passed through each of the typical stages of embryo development, namely the globular, heart, torpedo, and cotyledonary stages (Figures 1a-1d).

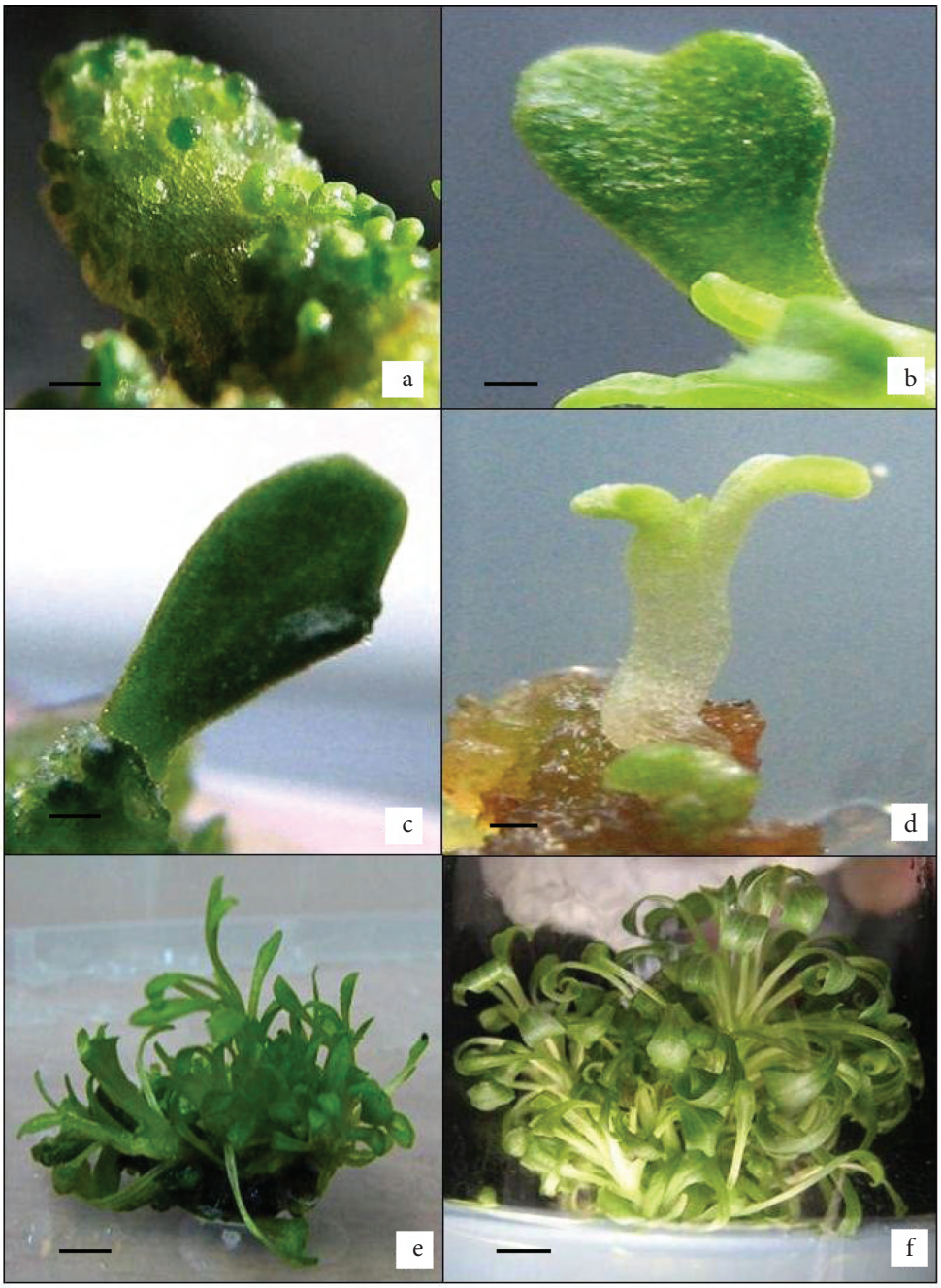

Figure 1. Somatic embryogenesis (**a-d**) and shoot organogenesis (**e**, **f**) from callus derived from cotyledonary leaf segments of *D. lamarckii*. Somatic embryos at different stages: **a)** globular (bar = 0.8 mm), **b)** heart (bar = 1 mm), **c)** torpedo (bar = 1 mm), and **d)** cotyledonary (bar = 1 mm), developed from embryogenic callus on MS medium supplemented with 1.34 µM NAA and 8.87 µM BAP; **e and f)** shoots regenerated from organogenic callus on MS medium containing 2.69 µM NAA and 8.87 µM BAP (bar = 1 cm).

The hormonal composition of the culture medium was important for the development and maturation of the somatic embryos at various developmental stages (Table 1). The highest mean number of embryos for every type of morphogeny was obtained when the callus was cultured on MS medium containing a combination of 1.34 µM NAA and 8.87 µM BAP, producing means of 15.2, 10.0, 8.8, and 3.0 embryos per cotyledonary leaf explant at the globular, heart, torpedo, and cotyledonary stages, respectively. When the total numbers of embryos developed were taken into account, it was clear that increasing the NAA concentration from 1.34 to 5.37 µM steadily decreased the number of embryos (Table 1).

Shoot regeneration from callus, rooting, and hardening off

Shoot regeneration from the organogenic callus obtained on medium containing 2.69 µM NAA and 2.22 µM BAP was found to be strongly affected by the concentration and combination of plant growth regulators. When BAP was used alone at 2.22, 4.44, or 8.87 µM, there was no significant difference among the mean numbers of shoots per explant, with means of 0.33, 0.75, and 0.73 shoots, respectively (Table 2). In addition, shoot production efficiencies were considerably lower when BAP was used alone, as compared to combinations with NAA. The combination of the highest NAA (2.69 µM) and the highest BAP (8.87 µM) was found to be the most productive in terms of both the mean number of shoots per explant (5.67 shoots) and the frequency of explants producing shoots (100%). On the other hand, keeping the NAA concentration constant while increasing BAP steadily increased the mean number of shoots per explant, from 0.67 to 2.75 shoots at 1.34 µM NAA and from 0.58 to 5.67 shoots at 2.69 µM NAA, respectively (Figures 1e and 1f). Indirect shoot organogenesis with green nodular protrusions was observed after 10-15 days of culture initiation, and the shoot length reached 1.5-2.0 cm after 6 weeks of incubation (data not provided).

Regenerated shoots of 2-3 cm in length were isolated from the callus and transferred to MS medium containing 1.0, 2.5, or 5.0 µM IAA or IBA (Table 3). Roots started to emerge from the cut ends of the shoots within 2 weeks of transfer to the rooting medium. In general, IAA showed better response than IBA in terms of both the percentage of shoots that rooted and the mean number of roots per rooted shoot. However, 2.5 µM IAA was the most effective, producing a mean of 4.33 roots per shoot with 90% rooting frequency. No rooting was observed on medium containing no plant growth regulators. All of the emerged roots were noted as healthy, with many

Table 2. Effects of different concentrations and combinations of NAA and BAP on shoot regeneration from callus derived from cotyledonary leaf segments of *D. lamarckii*. Data were collected after 6 weeks of culture.

Plant growth regulators (µM)	Mean number of shoots per cotyledonary leaf explant*	Frequency (%) of explants producing shoots
2.22 BAP	0.33 ± 0.14^{c}	33.00
4.44 BAP	0.75 ± 0.25^{c}	50.00
8.87 BAP	0.72 ± 0.16^{c}	50.00
1.34 NAA + 2.22 BAP	0.67 ± 0.19^{c}	58.33
1.34 NAA + 4.44 BAP	2.17 ± 0.42^{b}	83.33
1.34 NAA + 8.87 BAP	2.75 ± 0.77^{b}	83.33
2.69 NAA + 2.22 BAP	0.58 ± 0.26^{c}	83.33
2.69 NAA + 4.44 BAP	2.41 ± 0.72^{b}	83.33
2.69 NAA + 8.87 BAP	5.67 ± 0.91^{a}	100.00

* Values are the mean ± SE, and means with the same letter within a column are not significantly different at $P < 0.05$ according to Tukey's test.

Table 3. Effects of IAA and IBA on rooting of the regenerated shoots of *D. lamarckii*. Data were collected after 6 weeks of culture.

PGRs (µM)	Percentage of shoots that rooted (%)	Mean number of roots per rooted shoot*	Mean root length (cm)*
No PGRs	0	0	-
1.0 IAA	85	3.00 ± 0.5^{bc}	3.5 ± 0.2^{e}
2.5 IAA	90	4.33 ± 0.47^{a}	7.2 ± 0.1^{a}
5.0 IAA	80	3.20 ± 0.5^{b}	6.1 ± 0.3^{b}
1.0 IBA	66	2.71 ± 0.5^{c}	3.2 ± 0.2^{e}
2.5 IBA	75	2.52 ± 0.3^{c}	5.6 ± 0.1^{cd}
5.0 IBA	60	2.61 ± 0.4^{c}	5.3 ± 0.3^{d}

* Values are the mean ± SE, and means with the same letter within a column are not significantly different at $P < 0.05$ according to Tukey's test. PGRs: plant growth regulators.

root hairs. The survival rate of the regenerants was over 90% when they were transferred to small plastic cups containing a mixture of soil, manure, moss, and sand and kept at approximately 25%-35% humidity. Under these conditions, the regenerants were able to grow further and produce new shoots until they were used for cardenolide extraction and estimation studies after a period of 6-8 months.

This study reports, for the first time, an efficient in vitro plant regeneration protocol for *D. lamarckii* via indirect somatic embryogenesis and shoot organogenesis. To achieve this, 2 sets of experiments were carried out. In the first set, different concentrations and combinations of NAA and BAP were compared using MS medium for indirect somatic embryos (Table 1), while the second set tested the effects of BAP alone or in combination with NAA on shoot organogenesis (Table 2). The majority of studies on somatic embryogenesis in *Digitalis* have been focused on 2 species, namely *D. lanata* and *D. obscura*. Arrillaga et al. (20,21) demonstrated that IAA is an effective auxin for promoting somatic embryogenesis in hypocotyl segments isolated from seedlings of *D. obscura*. Similar results were obtained when the root explants of the same species were cultured in vitro (10). These findings were further supported by Kuberski et al. (9), who reported that the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA triggered somatic embryogenesis and improved the later development of embryos in *D. lanata*. The triggering effect of plant growth regulators, especially auxins, on the stimulation of cell division and then the expression of embryogenesis has been already reported for a large number of plant species (27-32). Plant regeneration has also been reported from haploid (gametic) cells of *D. purpurea* (33), isolated mesophyll protoplasts of *D. lanata* (34) and *D. obscura* (12), and suspension cultures of *D. lanata* (22).

In the present study, combinations of auxin and cytokinin were found to be effective for somatic embryo development. The globular embryos first appeared as protrusions on the surface of the explants, and then individual embryos grew into distinct bipolar structures and passed through each of the typical stages of somatic embryo development, namely the globular, heart, torpedo, and cotyledonary stages. The description of such stages in *D. lamarckii* is similar to those already reported for several other plant species (35-38). A combination of lower auxin (1.34 µM NAA) and higher cytokinin (8.87 µM BAP) was found to be most effective for somatic embryogenesis from calli derived from cotyledonary leaf explants of *D. lamarckii* (Table 1). Similar results were obtained when the immature cotyledonary leaf explants of *Boechera holboellii* were cultured in vitro

(39). On the contrary, for shoot regeneration from calli derived from the cotyledonary leaf explants, a combination of higher auxin (2.69 µM NAA) and higher cytokinin (8.87 µM BAP) was the most effective (Table 2). These findings are consistent with earlier studies reporting that leaf explants excised from in vitro-germinated seedling of *D. trojana* were the most productive explants for shoot induction when cultured on medium supplemented with various concentrations of BAP and NAA (40). It appears that the limiting effect of a high concentration of NAA (2.69 µM) on embryo development disappears during shoot induction. Cacho et al. (41) investigated the effects of the auxins 2,4-D, NAA, and IAA used either alone or in combination with kinetin or BAP on the morphogenic potential of leaf, root, and hypocotyl explants of *D. thapsi*. In spite of testing 96 different hormonal combinations and using 3 different types of explants, they observed no somatic embryos. On the other hand, the promoting effect of NAA or BAP on callus induction and/or shoot regeneration has been reported for many plant species, including some medicinal endemics of Turkey (29-31).

Rooting of the shoots was readily achieved on MS medium supplemented with IAA or IBA. However, IAA was a considerably more effective type of auxin than IBA in terms of the mean number of roots per shoot, as well as the mean root length (Table 3). Shoot development and root development were observed to be concomitant in some of the treatments.

Many pharmaceutical companies depend, to a considerable extent, upon materials produced from natural plant populations of several important medicinal species. The lack of systematic efforts for the cultivation of *D. lamarckii*, a medicinally important endemic species of Turkey, is a known problem (1). Therefore, the protocol described here could contribute to the establishment of a large-scale production system through tissue culture, with specific reference to germplasm conservation, commercial cultivation, and genetic improvement studies, as well as cardenolide production, in *D. lamarckii*.

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