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Direct shoot regeneration from leaf explants of *Digitalis lamarckii*, an endemic medicinal species

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Abstract: This study reports, for the first time, an efficient in vitro plant regeneration protocol for *Digitalis lamarckii* Ivan. (dwarf foxglove) via direct shoot organogenesis. Two sets of experiments were carried out; the first compared different concentrations of 6-benzylaminopurine (BAP), kinetin, thidiazuron (TDZ), and zeatin alone using leaf explants excised from in vitro germinated seedlings, while the second set tested the combinations of indole-3-butyric acid (IBA) with BAP, kinetin, TDZ, and zeatin for shoot multiplication from the leaf explants, which were already cultured and developed numerous shoots during the first set of experiments. For shoot regeneration (the first set of experiments), TDZ was the most effective at 1.0 mg L^{-1} concentration, producing a mean of 10.3 shoots per explant and was significantly more effective than BAP. For shoot multiplication (the second set of experiments), a combination of 0.2 mg L^{-1} IBA with 0.2 mg L^{-1} TDZ produced significantly more shoots per explant (16.5 shoots) than with BAP (11.0 shoots), zeatin (5.5 shoots), or kinetin (4.0 shoots). The regenerated shoots were readily rooted on medium containing 0.5 mg L^{-1} indole-3-acetic acid (IAA). Rooted regenerants were then transferred to the pots, where they grew well and attained maturity. The described protocol provides a simple way to regenerate plants through direct shoot organogenesis, which would be useful for a large-scale production of cardenolides, germplasm conservation, and genetic transformation studies in this medicinally important endemic species.

Key words: *Digitalis lamarckii*, dwarf foxglove, leaf explant, direct shoot regeneration, shoot multiplication

Endemik tıbbi bir tür olan *Digitalis lamarckii*'nin yaprak eksplantlarından direkt sürgün rejenerasyonu

Özet: *Digitalis lamarckii* (bodur yüksükotu) için direkt sürgün organogenezisi yolu ile etkili bir in vitro rejenerasyon protokolu ilk defa bu çalışma ile rapor edilmektedir. İki farklı deney seti kurulmuştur; ilk sette, in vitro koşullarda çimlendirilmiş fidelerden alınan yaprak eksplantları kullanılarak, 6-benzilaminopürin (BAP), kinetin, thidiazuron (TDZ) ve zeatinin farklı konsantrasyonları karşılaştırılmış, ikinci sette ise sürgün çoğaltımı amacıyla, birinci sette kültüre alınan ve çok sayıda sürgün meydana getirmiş olan eksplantlar kullanılarak, indol-3-butirik asidin (IBA), BAP, kinetin, TDZ ve zeatin ile kombinasyonları test edilmiştir. Sürgün rejenerasyonu (birinci deney seti) sonuçlarına bakıldığında, TDZ'nin, BAP'den istatistiki olarak çok daha etkili olduğu bulunmuş, 1,0 mg L^{-1} dozunda kullanıldığında ise eksplant başına ortalama 10,3 sürgün üreterek, en başarılı sonucu vermiştir. Sürgün çoğaltımı (ikinci deney seti)

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sonuçlarına bakıldığında ise, 0,2 mg L^{-1} IBA'nın 0,2 mg L^{-1} TDZ ile kombinasyonu, eksplant başına ortalama 16,5 sürgün üreterek, BAP (11,0 sürgün), zeatin (5,5 sürgün) veya kinetin (4,0 sürgün) ile kombinasyonuna göre istatistiki olarak çok daha başarılı sonuçlar vermiştir. Rejenere olan sürgünler, 0,5 mg L^{-1} indol-3-asetik asit (IAA) içeren ortamda kolaylıkla köklendirilmiştir. Köklendirilmiş rejenerantlar, daha sonra saksılara aktarılmış ve normal bir gelişme seyri göstererek olgunlaşma sürecini tamamlamışlardır. Bu çalışmada tanımlanan protokol ile, bitki rejenerasyonunun direkt sürgün organogenezisi yoluyla başarıldığı ortaya konulmuş, ve bunun, tıbbi önemi olan bu endemik türde büyük ölçekli kardenolit üretimi, germplasm korunması ve genetik transformasyon çalışmalarına katkı sağlaması beklenmektedir.

Anahtar sözcükler: *Digitalis lamarckii*, bodur yüksükotu, yaprak eksplantı, direkt sürgün rejenerasyonu, sürgün çoğaltımı

Introduction

Digitalis lamarckii Ivan., commonly known as dwarf foxglove, is a least concern endemic species belonging to the family Scrophulariaceae (Davis, 1978; IUCN, 2001). Members of the genus *Digitalis* L. are medicinally and economically important plants as they contain cardiac glycosides, which increase the force of systolic contractions and regulate heart rhythm (Baytop, 1999). It is also used as diuretic and in the reduction of oedema due to its ability to improve cardiovascular circulation (Perez-Bermudez et al., 1990). 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 treatments (Yeh et al., 2001; Lopez-Lazaro, 2007; Newman et al., 2008).

Plant cell and tissue cultures have been widely used for the commercial propagation of a large number of plant species including many medicinal or ornamental plants (Rout et al., 1999; Sahai et al., 2010; Ahmadabadi & Bock, 2010). Because of continuous deforestation and extensive collection, in vitro conservation of medicinal plant germplasm is important to support chemical analysis and genetic improvement studies. As the cardenolides are of commercial interest, studies have focused on the in vitro culture of several *Digitalis* species including *D. lanata* Ehrh., *D. purpurea* L., *D. thapsii* L., *D. davisiana* and *D. obscura* L. over the last 3 decades (Perez-Bermudez et al., 1983; Arrillaga et al., 1986; Brisa & Segura, 1987; Perez-Bermudez et al., 1987; Herrera et al., 1990; Cacho et al., 1991; Pradel et al., 1997; Fatima et al., 2009; Gurel et al., 2011). However, during our literature search, no report concerning in vitro regeneration of *D. lamarckii* was found. In this report, we describe a simple and very effective protocol for direct shoot regeneration and multiplication of *D. lamarckii* using leaf explants excised from in vitro germinated seedlings and testing different hormonal combinations.

Materials and methods

Seed sterilisation and germination

Seeds of *D. lamarckii* were collected, in September 2009, from natural populations growing in Bolu, south-west part of the Black Sea region of Turkey (at the altitude of 1510 m, 40°37′71″N and 32°26′26″E). Seeds were surface sterilised by dipping into 50 mL of 20% commercial bleach with a few drops of Tween 20 in a 250 mL beaker and stirring at 250 rpm on a magnetic stirrer plate for 10 min, followed by rinsing with sterile distilled water several times. An average of 20-25 seeds were aseptically cultured in petri dishes (90 × 15 mm) containing 30 mL of Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 3% (w/v) sucrose. The medium was 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 were kept at 23 °C for the first 2 days in dark and then transferred to 16 h light:8 h dark photoperiod (provided by cool-white fluorescent light, irradiance 50 m mol^{-2} s^{-1}) at a relative humidity of 60%. The whole leaves (ca. 10 mm) were isolated from 1-month-old seedlings and used as explants for shoot regeneration and multiplication experiments.

Direct shoot regeneration from leaf explants isolated from seedlings

The whole leaf explants were cultured in petri dishes (90 × 15 mm) containing 30 mL of solid MS medium containing different concentrations (0.1,

0.5, 1.0, or 3.0 mg L^{-1}) of BAP, kinetin, TDZ, or zeatin alone (Table 1). Shoot regeneration experiments were repeated 4 times, each having 8 replicates (i.e. a total of 32 explants per treatment). The cultures were kept at 23 °C under 16/8 h light/dark photoperiod (provided by cool-white fluorescent light, irradiance 50 m mol^{-2} s^{-1}) at a relative humidity of 60%. Both the frequency (%) of explants developing shoots and mean numbers of shoots per explant were recorded after 2 months of culture.

Shoot multiplication from cultured leaf explants

The whole leaves, which were cultured on shoot regeneration media and developed numerous microshoots during the first set of experiments (Table 1, Figure), were used as explants for multiple shoot regeneration (Table 2, Figure). The whole leaf explants were transferred to petri dishes (90 × 15 mm) containing 30 mL of solid MS medium containing combinations of 0.2 mg L^{-1} IBA and 0.2 mg L^{-1} TDZ, BAP, kinetin, or zeatin (Table 2). Shoot multiplication experiments were performed 4 times, each having 8 replicates (i.e. a total of 32 explants per treatment). The cultures were kept at 23 °C under 16/8 h light/dark photoperiod (provided by cool-white fluorescent light, irradiance 50 m mol^{-2} s^{-1}) at a relative humidity of 60%. Both the frequency (%) of explants developing shoots and mean numbers of shoots per explant were recorded after a 4-month culture period.

Statistical analysis

Data were statistically analyzed using SPSS, Version 17.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to calculate statistical significance, and mean ± SD (standard deviation) differing significantly were determined using Duncan's multiple range test at $P < 0.05$ level.

Results and discussion

This study reports, for the first time, an efficient in vitro plant regeneration protocol for *D. lamarckii* via direct shoot organogenesis. To achieve this, 2 sets of experiments were carried out; the first compared different concentrations of BAP, kinetin, TDZ, and zeatin alone using leaf explants excised from in vitro germinated seedlings (Table 1), while the second set tested the combinations of IBA with the same

Table 1. Direct shoot regeneration from leaf explants of in vitro grown seedlings of *D. lamarckii* cultured on MS medium containing different concentrations of BAP, kinetin, TDZ, or zeatin. Data were collected after 2 months of culture initiation.

PGRs (mg L^{-1})		Mean frequency (%) of explants developing shoots*	Mean number of shoots per leaf explant*
Control		0	0
BAP	0.1	0	0
	0.5	6.3^{d}	2.4 ± 0.7^{d}
	1.0	6.3^{d}	3.5 ± 0.5^{cd}
	3.0	0	0
Kinetin	0.1	0	0
	0.5	0	0
	1.0	0	0
	3.0	0	0
TDZ	0.1	12.5^{c}	6.6 ± 1.2^{bc}
	0.5	25.0^{b}	7.5 ± 1.0^{b}
	1.0	37.5^{a}	10.3 ± 1.5^{a}
	3.0	0	0
Zeatin	0.1	0	0
	0.5	0	0
	1.0	0	0
	3.0	0	0

* Means ± SD (standard deviation) with the same letter within columns are not significantly different according to Duncan's multiple range test at $P < 0.05$.

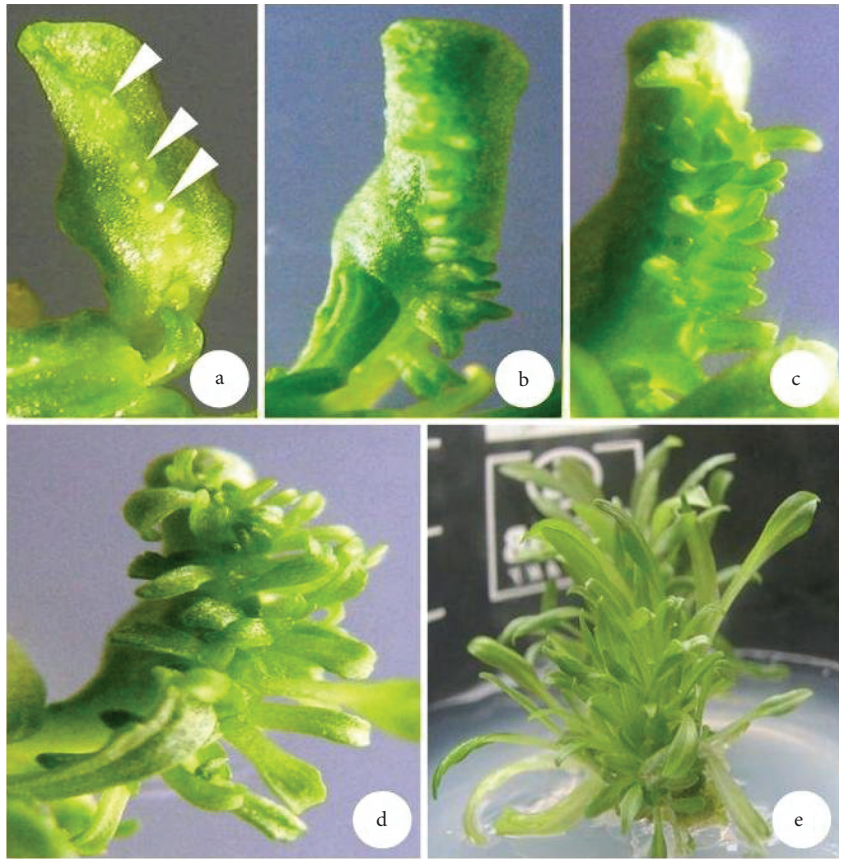

Figure. Direct shoot regeneration from leaf explants of *D. lamarckii*. a-nodular masses (arrowheads) protruding from the epidermal cell layers of leaf explants after 10 days of culture on MS medium containing 0.5 mg L^{-1} TDZ. b,c-development of shoots from the nodular masses after 20 or 30 days, respectively, of culture on MS medium containing 0.5 mg L^{-1} TDZ. d-multiple shoots developing from the main shoots after 2 months of culture on MS medium containing 0.5 mg L^{-1} TDZ, or e-after 4 months of culture on MS medium containing 0.2 mg L^{-1} TDZ and 0.2 mg L^{-1} IBA.

plant growth regulators as used in the first set of experiments (i.e. BAP, kinetin, TDZ, and zeatin) for shoot multiplication from the leaf explants, which were already cultured and developed numerous shoots during the first set of experiments (Table 2).

Direct shoot regeneration from leaf explants

A week to 10 days after the culture initiation, nodular masses and/or shoots were clearly visible as protuberances growing directly from the epidermal cell layers of the whole leaf explants cultured on MS medium supplemented with TDZ or BAP (Figure). After several weeks of culture on the same shoot regeneration media, the developed shoots further enlarged while more new shoots were developing from the explants (Figure). Frequencies of organogenic explants and mean numbers of shoots per explant are presented in Table 1. The hormone-free (control) medium as well as those containing several concentrations of kinetin and zeatin did not produce any shoots at all. Moreover, on media containing these concentrations of kinetin and zeatin, most of the leaf explants became necrotic after 2 months of culture initiation. On the other hand, explants cultured on medium containing higher concentration of kinetin and zeatin (1.0 or 3.0 mg L^{-1}) tended to induce callus formation. This observation agrees with the statement that kinetin has a relatively

Table 2. Multiple shoot formation from the leaf explants of *D. lamarckii* re-cultured on MS medium containing 0.2 mg L^{-1} IBA combined with 0.2 mg L^{-1} BAP, kinetin, TDZ, or zeatin. Data were collected after 4 months of culture initiation.

PGRs (mg L^{-1})	Mean frequency (%) of explants developing shoots*	Mean number of shoots per leaf explant*
IBA (0.2) + BAP (0.2)	75.0[a]	11.0 ± 0.9[b]
IBA (0.2) + Kinetin (0.2)	53.1[b]	4.0 ± 1.0 [c]
IBA (0.2) + TDZ (0.2)	84.4[a]	16.5 ± 1.1[a]
IBA (0.2) + Zeatin (0.2)	56.3[b]	5.5 ± 0.7[c]

* Means ± SD (standard deviation) with the same letter within columns are not significantly different according to Duncan's multiple range test at $P < 0.05$.

low biological activity in certain bio-assays (Bogaert et al., 2006).

Of the plant growth regulators tested, BAP at 0.5 and 1.0 mg L^{-1} was effective in inducing direct shoot organogenesis from leaf explants while the lowest (0.1 mg L^{-1}) and the highest (3.0 mg L^{-1}) concentrations produced no shoots at all (Table 1). The use of TDZ at 0.1, 0.5, or 1.0 mg L^{-1} was found highly effective for shoot regeneration, increasing concentrations resulting in a steady increase in both the frequency of explants producing shoots and mean number of shoots obtained per explant. However, 1.0 mg L^{-1} TDZ was the most effective, producing a mean of 10.3 shoots per explant at 37.5% frequency of organogenic explants, while 3.0 mg L^{-1} TDZ induced no shoot regeneration but formation of some callus. For an overall comparison, TDZ was significantly more effective for shoot regeneration than BAP (Table 1).

TDZ has been widely used to induce shoot regeneration in different explants of various plant species (Ernst, 1994; Chen & Piluek, 1995; Chang & Chang, 1998; Bacchetta et al., 2003; Malabadi et al., 2004; Yucesan et al., 2007; Ling Fei et al., 2009; Turker et al., 2009a, 2009b; Gurel et al., 2011), and has been therefore regarded as a powerful inducer of adventitious shoots and somatic embryo formation in many ornamental plants (Lin et al., 1997; Mirici et al., 2005; Sanikhani et al., 2006). It was also reported that using TDZ at concentrations ranging from 0.2 to 2.5 mg L^{-1} was considerably more effective in inducing organogenesis or somatic embryogenesis than several other cytokinins, particularly in recalcitrant woody species (Huetteman & Preece, 1993). Saxena et al. (1992) suggested that TDZ helps to establish the optimal internal balance of cytokinin and auxin required for the induction and expression of somatic embryogenesis. This promoting effect of TDZ may also be attributed to the fact that TDZ is very stable in culture media and persistent in the plant tissue, as shown by a carbon isotope study (Mok & Mok, 1985).

In *Oncidium* spp. leaf cultures, although direct somatic embryogenesis and plant regeneration were promoted by several cytokinins (namely, 6-γ,γ-dimethylallylamino purine (2iP), BAP, kinetin, zeatin, and TDZ), TDZ was found the most effective (Chen et al., 1999; Chen & Chang, 2001; Chen & Chang, 2006). Working with *D. lamarckii*, both TDZ and BAP were highly effective for shoot regeneration but TDZ was able to induce significantly more shoots from leaf explants than other plant growth regulators tested (Table 1). Similar observations were also reported for regeneration of *Phalaenopsis* spp. and *Doritaenopsis* spp. (Ernst, 1994), and *Zingiber officinale* Rosc. (Lincy & Sasikumar, 2010) where TDZ was found much more efficient than many other plant growth regulators.

Multiple shoot formation from cultured leaf explants

Multiple shoot regeneration was achieved when the whole leaf explants, which had already cultured and developed numerous microshoots during the first set of experiments (Table 1), were transferred to shoot multiplication medium containing 0.2 mg L^{-1} IBA combined with 0.2 mg L^{-1} BAP, kinetin, zeatin, or TDZ (Table 2). These combinations of plant growth regulators were very effective for the induction of multiple shoots; the whole leaf explants developing a large number of shoots within 2 to 4

months of culture (Figure). When the leaf explants taken from the first experiment were cultured on the shoot multiplication medium, both the frequency of explants developing shoots and the mean number of shoots produced per explant increased significantly (Tables 1 & 2).

The highest mean number of regenerated shoots was obtained on medium containing 0.2 mg L^{-1} IBA combined with 0.2 mg L^{-1} TDZ; resulting in a mean of 16.5 shoots per cultured leaf explant (Table 2). This result was consistent with those of the first set of experiments (Table 1), in which TDZ was, in terms of both mean frequency of organogenic explants and mean number of shoots per explant, significantly more effective than BAP when they were used alone. On the other hand, the combination of 0.2 mg L^{-1} IBA with BAP was found more effective than with either kinetin or zeatin (Table 2), again being consistent with the results presented in Table 1 where none of the concentrations of both growth regulators was able to produce any shoots at all. Being in agreement with our results presented here for *D. lamarckii*, the promoting effect of TDZ in combination with IBA on shoot regeneration has been also reported for other species, such as *Jatropha curcas* L. (Khurana-Kaul et al., 2010). Variation in the activity of different cytokinins can be attributed to their differential uptake rates, as reported for some species (Blakesey, 1991), varied translocation rates to meristematic regions and certain metabolic processes in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds (Tran Thanh Van & Trinh, 1990). In addition, it was suggested that cytokinin efficiency may be affected by different affinities of cytokinin receptors involved in shoot induction process (Kaminek, 1992).

Following the shoot multiplication process, individual shoots were transferred to Magenta vessels containing a root induction medium (MS medium supplemented with 0.5 mg L^{-1} IAA). Over 90% of the shoots rooted and, upon transfer to small plastic pots containing a mixture of soil:manure:moss:sand (1:2:2:1), about 80% of the plantlets displayed new growth and normal morphological characteristics under greenhouse conditions.

In conclusion, the described protocol demonstrates that, within 2-4 months, one single leaf explant could produce a mean of more than 15 shoots directly developed from the cultured whole leaf explants, and would be expected to contribute to the future efforts for a large-scale production of cardenolides, germplasm conservation, and genetic transformation studies in this medicinally important endemic species.

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