African Journal of Biotechnology Vol. 9(11), pp. 1587-1591, 15 March, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Direct shoot organogenesis of *Digitalis trojana* Ivan., an endemic medicinal herb of Turkey

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Accepted 8 February, 2010

An efficient protocol for *in vitro* propagation of *Digitalis trojana* Ivan. was developed via adventitious shoot regeneration. Leaf explants were cultured on MS which were supplemented with different concentrations of NAA (0.1, 0.5, 1.0 mg/ml) and BAP (0.1, 0.5, 1.0, 3.0, 5.0 mg/ml) for shoot formation. Adventitious shoots were formed on leaf explants within three weeks in culture. The best shoot proliferation was observed among explants cultured on MS medium with 0.1 mg/ml NAA + 3.0 mg/ml BAP. Regenerated shoots were multiplicated by subculture. Then they were cultured on MS with 0.1% (w/v) activated charcoal for root formation. All of the *in vitro* regenerated plantlets were successfully acclimatized *ex vitro* and then grown healthy.

Key words: *Digitalis trojana* Ivan., regeneration, *in vitro*, naphthalene acetic acid, 6-benzylaminopurine.

INTRODUCTION

In vitro culture technologies have been increasingly used for *ex situ* conservation of rare or endangered endemic plants (Sasaran et al., 2006; George et al., 2007). Besides, high amounts of production of medicinal plants and their metabolites can be achieved via *in vitro* culture (Constabel, 1990; Rout et al., 2000; Chaturvedi et al., 2007). *In vitro* culture technologies support a rapid propagation of plants and production of an economically viable amounts of plant secondary metabolites.

Digitalis trojana Ivan. (foxglove), in the family *Scrophulariaceae*, is an endemic plant of Ida Mountain, Canakkale, Turkey (Uysal and Öztürk, 1991). This plant has been marked as a vulnerable (VU) in Red Data Book of Turkish Plants (Ekim et al., 2000). On the other hand various species of the genus *Digitalis* are used as a source of cardiac glycosides which are important for their use in medicine (Herrera et al., 1990; Haux, 1999), moreover several interesting anticancer effects have been observed in *Digitalis* (Shiratori, 1967). It was reported that *D. trojana* contained the highest amount of cardiac glycosides in leaves among the endemic *Digitalis* species (Tanker et al., 1988).

There are lots of reports about *in vitro* cultures of other *Digitalis* species (Erdei et al., 1981; Perez-Bermudez et al., 1984; Matsimoto et al., 1987; Herrera et al., 1990; Vela et al., 1991; Sale et al., 2002) but though it is a valuable medicinal and endemic plant, there is no report on an *in vitro* culture protocol for *D. trojana*. The aim of this research is to carry out an efficient *in vitro* propagation of *D. trojana* for *ex situ* conservation and to obtain rich source of cardenolides from this species. In the present investigation, an efficient *in vitro* plant regeneration of *D. trojana* Ivan. via direct adventitious shoot organogenesis from leaves was reported.

MATERIALS AND METHODS

Explant source and sterilization

D. trojana Ivan. seeds were collected from the 807th m of the Ida Mountain in August 2008. Seeds were sterilized with 3% sodium hypochlorite and 0.1% Tween 20 for 20 min and then rinsed with sterile distilled water. After surface sterilization, seeds were germinated aseptically on MS medium (Murashige and Skoog, 1962).

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Abbreviations: BAP, 6-Benzylaminopurine; **IAA,** indole-3-acetic acid; **MS,** murashige and skoog medium; **2,4-D,** 2,4-dichloro-phenoxyacetic acid; **NAA,** α- naphthalene acetic acid.

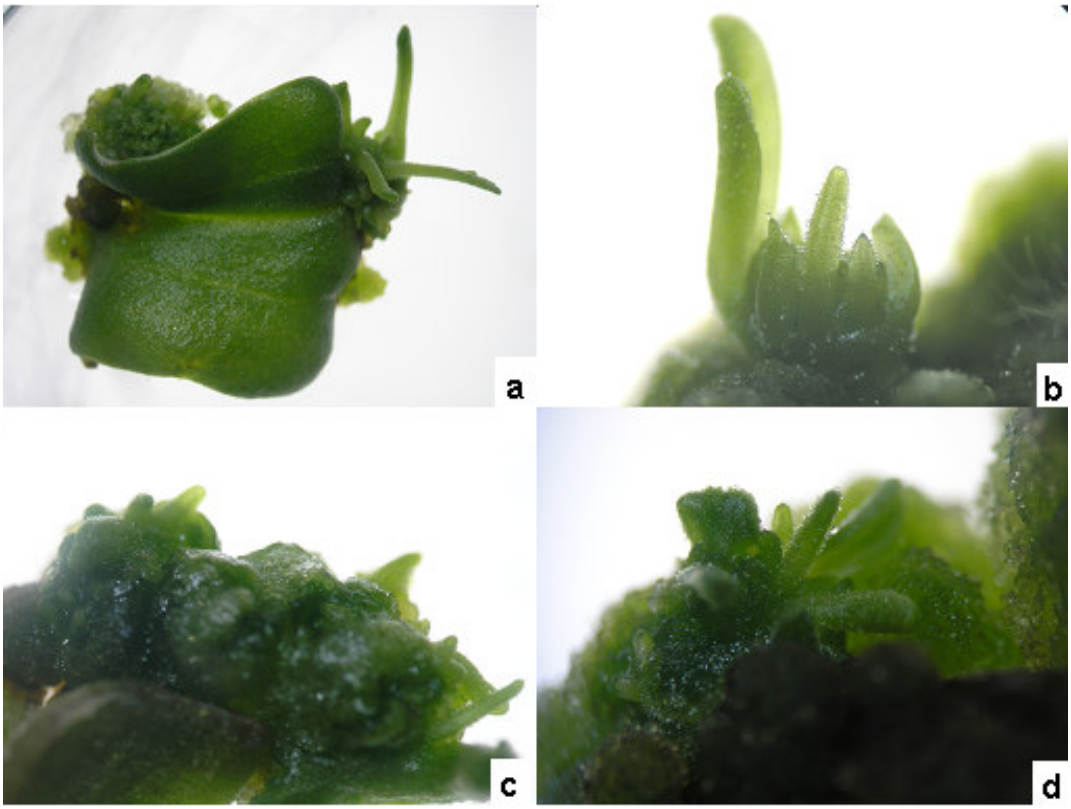

Figure 1. The leaf explants that cultured on MS medium which containing BAP combination with NAA (a), adventitious shoots that formed on leaf explants cultured on MS media containing 0.1 mg/ml NAA+3.0 mg/ml BAP (b, c and d).

Cultivation conditions

MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar were used for *in vitro* experiments. The pH of medium was adjusted 5.75 before adding agar, then autoclaved at 121°C for 15 min. All of the cultures were kept in the growth chambers at 25 ± 2°C under 16/8 h photoperiod with 72 µmol m^{-2} s^{-1}.

Culture initiation

Leaf explants were excised from twelve weeks old seedlings and cultured on MS containing different concentrations of NAA (0.1, 0.5, 1.0 mg/ml) and BAP (0.1, 0.5, 1.0, 3.0, 5.0 mg/ml) for direct shoot regeneration. Five explants per petri dishes were used for each trial with five replications. The percentage of shoot formation and the mean number of shoots per explant were recorded after twelve weeks. Regenerated shoots were subcultured on MS medium containing initial hormone levels for shoot multiplication. For elongation, shoots were separated individually and transferred to the glass culture vessels which contained MS basal medium.

***In vitro* rooting and acclimization**

Rooting occurred from shoots cultured on MS containing 0.1% (w/v) activated charcoal. At the end of the experimental series of our research, plantlets were adapted to *ex vitro* conditions and then transplanted to soil.

Data analysis

The mean number of shoots and the percentage of explants forming shoots were determinated after twelve weeks from initial inoculation for all explants. All data were evaluated by an analysis of variance and mean values were compared using MINTAB. The interaction of plant growth regulators was analysed with MSTAT. The statistic model (Yijk = µ + Ai + Bj+ AB ij + Σ ijk) was used to assign the effects of plant growth regulator's concentration on shoot regeneration.

RESULTS and DISCUSSION

The explants cultured on MS medium without hormones were only slightly expanded and no calli and shoots was observed on this medium. Adventitious buds were formed out of the cut edges of the explants, cultured on MS medium containing BAP in combination with NAA within three weeks in the culture (Figure 1). Mean number of shoots per explants and percent of explants forming shoots were recorded (Table 1). The highest shoot formation was obtained on media containing 0.1 mg/ml NAA+ 3.0 mg/ml BAP and 1.0 mg/ml NAA + 5.0 mg/ml BAP. Although the number of regenerated shoots are higher on media containing 1.0 mg/ml NAA + 5.0 mg/ml

Table 1. Shoot regeneration from leaf explants which cultured on MS medium containing different concentration of NAA and BAP.

Plant Growth Regulators (mg/ml)		Percent of explants forming shoots	Mean number of shoots per explants
NAA	BAP		
-	-	0.0	0.0
0.1	0.1	4.0	0.3 ± 0.1 $^{D a}$
0.1	0.5	16.0 ± 0.3	2.3 ± 0.5 $^{CD a}$
0.1	1.0	20.0 ± 1.4	10.3 ± 0.3 $^{BC a}$
0.1	3.0	32.0 ± 0.1	28.0 ± 1.8 $^{A a}$
0.1	5.0	45.0 ± 0.5	16.3 ± 0.1 $^{B b}$
0.5	0.1	0.0	0.0 $^{B a}$
0.5	0.5	0.0	0.0 $^{B a}$
0.5	1.0	0.0	0.0 $^{B b}$
0.5	3.0	32.0 ± 0.9	17.3 ± 0.3 $^{A b}$
0.5	5.0	12.0 ± 0.7	2.3 ± 0.6 $^{B c}$
1.0	0.1	0.0	0.0 $^{B a}$
1.0	0.5	13.3 ± 1.6	0.6 ± 0.1 $^{B a}$
1.0	1.0	12.0 ± 1.8	1.6 ± 0.5 $^{B b}$
1.0	3.0	4.0 ± 0.2	0.3 ± 0.1 $^{B c}$
1.0	5.0	14.6 ± 0.5	26.3 ± 3.0 $^{A a}$

*Variety of BAP concentration is significant in every NAA concentration and variety of NAA concentration is significant in every BAP concentration (means with the same *letter*s are not significantly different at P < 0.01).

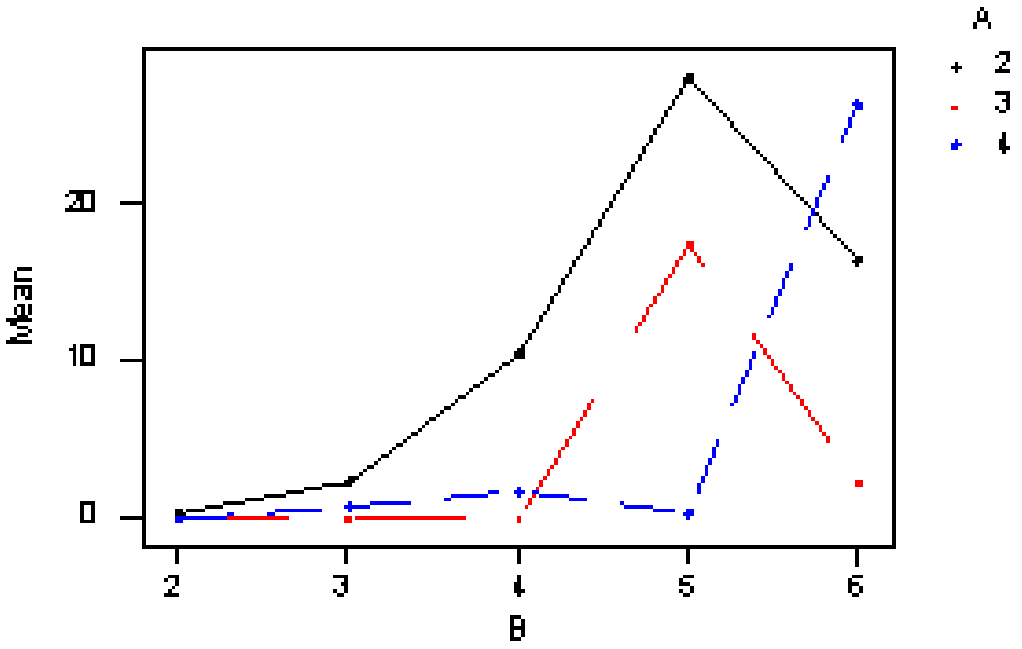

Figure 2. The mean number of shoots per explants depending on the interaction of NAA and BAP (A2 : 0.1 mg/ml NAA; A3, 0.5 mg/ml NAA; A4, 1.0 mg/ml NAA; B2, 0.1 mg/ml BAP; B3, 0.5 mg/ml BAP; B4, 1.0 mg/ml BAP; B5, 3.0 mg/ml BAP; B6, 5.0 mg/ml BAP).

BAP, the shoots were vitrificated and then not multiplicated.

For the results of analysis of variance, it was shown that the effect of NAA varied depending on BAP concentration for shoot regeneration at 12th week of culture (P < 0.01). BAP was promoted to shoot formation by interaction with NAA (Figure 2). The ratio of NAA to BAP was also very significant. Especially, 3.0 or 5.0

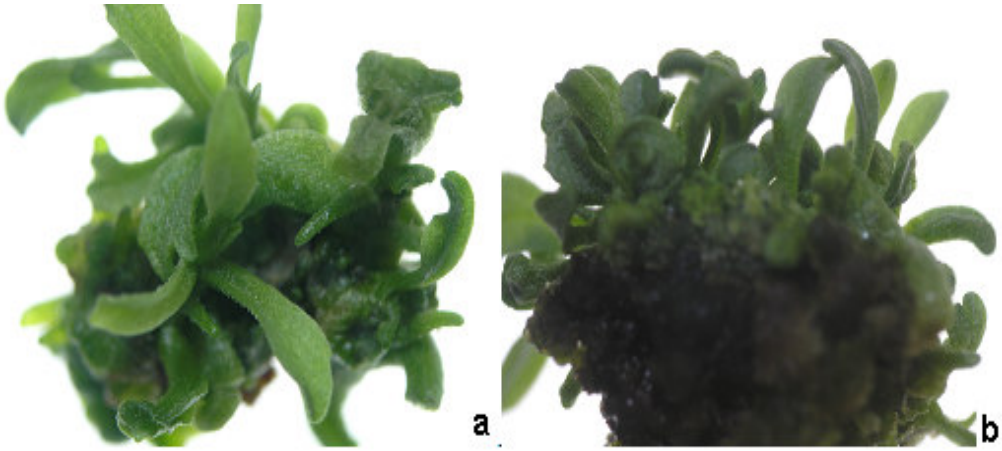

Figure 3. Multiplicated shoots which were cultured on MS media containing 0.1 mg/ml NAA+3.0 mg/ml BAP (a, b).

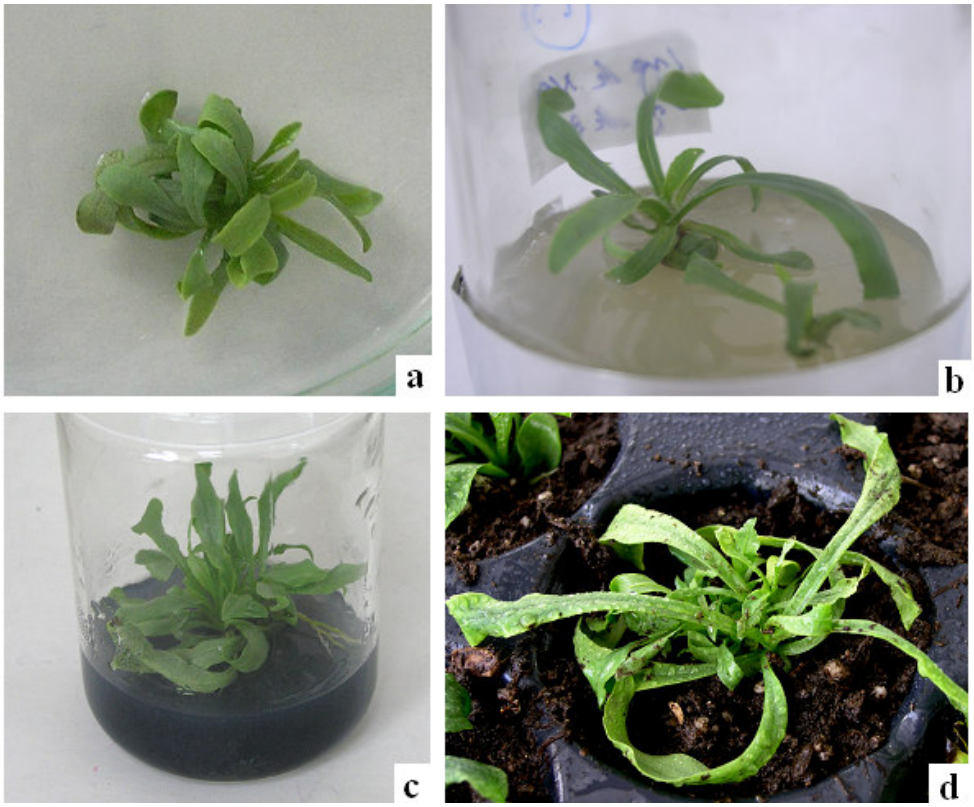

Figure 4. Elongated shoot was formed on MS with 0.1 mg/ml NAA+3.0 mg/ml BAP (a, b), root formation on MS medium containing 0.1% activated charcoal (c), and transferred plants to the soil (d).

mg/ml concentration of BAP was effective for shoot formation from *Digitalis trojana* leaf explants. Our results are consistent with previous report on *Digitalis thapsi* L. (Cacco et al., 1991), which reported that the presence of high concentrations of BAP (3, 4, 5 mg/ml) in combination with IAA or 2,4 D or NAA promoted callus formation and shoot organogenesis from leaf explants. In another research, BA promoted adventitious bud differentiation alone, but addition of auxin significantly increased the bud forming capacity of leaf explants of *Digitalis minor* L.

(Sales et al., 2002).

The best shoot proliferation was observed among explants cultured on MS medium with 0.1 mg/ml NAA + 3.0 mg/ml BAP (Figure 3). These 2 - 3 cm long elongated shoots were transferred to root formation medium, MS with 0.1% (w/v) activated charcoal with in 1 week. MS media containing 0.5 mg/ml NAA was also used as root forming media but on this media roots were not induced. At the end of experimental series of our research, plantlets were adapted to *ex vitro* conditions and then transplanted to viol containing soil (Figure 4). All of *in vitro* regenerated plantlets were grown healthy.

In vitro culture is provided *ex situ* conservation of endangered, endemic, medicinal plants and mass propagation of these plants (Sales et al., 2002). And it is also useful for production of high amount of secondary metabolites in medicinal plants. Efficient and rapid regeneration of these significant plants is the first and major stage of *in vitro* culture. A highly efficient and rapid direct shoot regeneration protocol was developed and optimized for *Digitalis trojana* which is a vulnerable endemic plant of Ida Mountain. Our results show that MS medium containing 0.1 mg/ml NAA + 3.0 mg/ml BAP, can be used as a regeneration medium for shoot formation from *Digitalis trojana* leaf explants. This regeneration protocol can be applied to micropropagation and also production of secondary metabolites of this species.

ACKNOWLEDGEMENT

This work was supported by Scientific Research Projects Commission of Çanakkale Onsekiz Mart University (2008/26).

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