Industrial Crops and Products 40 (2012) 76–80

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Direct somatic embryogenesis from hypocotyl segments of *Digitalis trojana* Ivan and subsequent plant regeneration

Sandeep Kumar Verma[a], Gunce Sahin[a], Buhara Yucesan[a], Ismail Eker[a], Nevin Sahbaz[a], Songul Gurel[b], Ekrem Gurel[a,*]

[a] *Abant Izzet Baysal University, Department of Biology, 14280 Bolu, Turkey*
[b] *Sugar Institute, Department of Plant Breeding, 06790 Etimesgut, Ankara, Turkey*

ARTICLE INFO

Article history:
Received 17 November 2011
Received in revised form 22 February 2012
Accepted 23 February 2012

Keywords:
Digitalis trojana
Helen of troy foxglove
Hypocotyl segments
Direct somatic embryogenesis
Tissue culture

ABSTRACT

This study describes, for the first time, an *in vitro* protocol for the direct development of somatic embryos and subsequent plant regeneration from hypocotyl segments excised from 21-days-old *in vitro*-germinated seedlings of *Digitalis trojana* Ivan (Helen of troy foxglove). Two sets of experiments were carried out, the first compared different concentrations of four cytokinins: N6-benzyladenine [BAP], 6-furfurylaminopurine [kinetin], 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea [TDZ], and 6-(4-hydroxy-3-methylbut-2-enylamino) purine [zeatin] alone, while the second set tested TDZ or BAP combinations with IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) or NAA (α-naphthalene acetic acid). In the first set of experiments, TDZ was found the most effective at 1.0 mg/l concentration, producing a mean of 10.7 somatic embryos per explant. In the second set, a combination of 1.0 mg/l TDZ with 0.5 mg/l IAA produced significantly more somatic embryos per explant (13.8 embryos) than with BAP (8.8 embryos). During subculture on growth regulator-free half-strength MS medium, somatic embryos gradually developed into plantlets. Regenerated plantlets were successfully transplanted and grown in a greenhouse environment. The efficient regeneration protocol reported here provides an important method of micropropagation of this plant. Furthermore, this protocol may be used for a large-scale production of cardenolides and genetic transformation of this valuable medicinal plant for its further improvement.

1. Introduction

Digitalis, belonging to the family Plantaginaceae, is a genus consisting of about 20 species of herbaceous perennials, shrubs and biennials that are commonly called foxgloves. *Digitalis trojana* Ivan, commonly known as Helen of troy foxglove, is an endemic and medicinally important species and has been marked as vulnerable (VU) in the Red Data Book of Turkish Plants (Ekim et al., 2000). This species is also the most widespread member of the nine *Digitalis* taxa (eight species and one subspecies) grown in Turkey [*D. ferruginea* L. subsp. *ferruginea*, *D. ferruginea* L. subsp. *schischkinii* (Ivanina) K. Werner, *D. davisiana* Heywood, *D. grandiflora* Mill., *D. viridiflora* Lindl., *D. cariensis* Boiss., *D. trojana* Ivanina, *D. lamarckii* Ivanina, *D. lanata* Ehrh.] (Davis, 1978). In addition, *D. trojana* contains the highest amount of total cardiac glycoside in leaves among the other endemic *Digitalis* species of Turkey [*D. davisiana*, *D. cariensis*, *D. lamarckii*] (Tanker et al., 1988).

Several *Digitalis* species have been used therapeutically as they are a source of cardiac glycosides. In India (Ayurvedic medicine), it is used as an ointment containing *Digitalis* glycosides to treat wounds and burns. *Digitalis* glycosides (cardenolides) are also well recognized drugs that have been used for myocardial infarction, edema, angina, cardiac dysfunction, hypertropy and arterial hypertension (Grieve, 1982; Chevallier, 1996). Recently, cancer chemotherapy effects were also reported for cardenolides of different *Digitalis* species (Yeh et al., 2001; Lopez-Lazaro et al., 2005; Newman et al., 2008; Platz et al., 2011).

The cardenolides of *Digitalis* species including *D. trojana* have a high economical value, and due to a large scale and unrestricted exploitation to meet their ever increasing demand by pharmaceutical industries, coupled with limited cultivation and insufficient attempts to replenishment it in the wild, the population of such important plant species has been markedly depleted. Natural propagation of *D. trojana* through seeds is possible, but this method is not effective in producing a sufficient number of planting stock as the germination frequency of the seeds is rather poor. Therefore, many *in vitro* culture protocols for the members of the genus

Abbreviations: BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, α-naphthalene acetic acid; TDZ, thidiazuron; 2,4-D, 2,4-dichlorophenoxy acetic acid; MS, Murashige and Skoog.

* Corresponding author. Tel.: +90 374 254 12 20; fax: +90 374 253 46 42.
E-mail addresses: gurel_e@ibu.edu.tr, gurel_e@yahoo.com, gurel.ekrem@gmail.com (E. Gurel).

0926-6690/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.indcrop.2012.02.034

Digitalis have been developed (Tewes et al., 1982; Arrillaga et al., 1986, 1987; Diettrich et al., 1986; Reinbothe et al., 1990; Lapena and Brisa, 1995; Gurel et al., 2011; Verma et al., 2011a,b). However, based on a literature survey, there is only one report concerning direct shoot organogenesis from leaf explants of *D. trojana* (Corduk and Aki, 2010), but to date, there is no published work on direct somatic embryogenesis of *D. trojana*. In this communication, we describe a simple and an effective protocol for the induction of direct somatic embryos from hypocotyl segments and subsequent plant regeneration in *D. trojana* Ivan.

2. Materials and methods

2.1. Plant material

Seeds of *D. trojana* Ivan were collected in September 2008 from wild populations growing at Turkey: B1 Balıkesir, Edremit, Kazdağı (at the altitude of 250 m, N39°38.615, E026°57.552; at 345 m, N39°38.885 and E026°57.402; at 435 m, N39°39.650 E026°57.578). Identification of species was made according to Davis (1978), and voucher specimens (Eker-1905) were deposited at the Abant Izzet Baysal University Herbarium (Bolu, Turkey).

2.2. Surface sterilization and culture conditions

Seeds of *D. trojana* 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 for three times. An average of 20–25 seeds were aseptically cultured on 100 mm × 15 mm plastic Petri dishes containing 30 ml of Murashige and Skoog (1962) medium containing 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 ± 1 °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 at 50 μ mol^{-2} s^{-1}) at a relative humidity of 60%. Hypocotyl segments (5–8 mm) from 21-day-old seedlings were used as explants for culture initiation.

2.3. Hormonal composition of the culture media

The hypocotyl explants were cultured in plastic Petri dishes (90 mm × 15 mm) containing 30 ml solid MS medium containing different concentrations (0.1, 0.5, 1.0 mg/l) of BAP, kinetin, TDZ or zeatin alone (Table 1) or different combinations of TDZ (0.5, 1.0 or 2.0 mg/l) or BAP (1.0 mg/l) with varying concentrations of NAA, IAA or IBA (Table 2). Experiments were repeated three times, each using 20 replicates (*i.e.*, a total of 60 explants per treatment). Both the frequency (%) of explants developing direct somatic embryos and the mean numbers of somatic embryos per explant were recorded after a five weeks of culture.

2.4. Development of somatic embryos into plantlets

Hypocotyl-derived embryos were subcultured on half-strength MS medium containing no plant growth regulators for four weeks for the further growth of the embryos. After four subcultures, plantlets with 3–4 leaves (Fig. 1F) and 2–3 roots (Fig. 1G) were potted in mixture of soil:manure:moss:sand (1:2:2:1) for acclimatization in a 50% shaded greenhouse condition (Fig. 1H).

2.5. Statistical analysis

Data were statistically analyzed using a computer program (SPSS Statistics, version 17.0, SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to calculate statistical significance, and the mean ± SE (standard error) differing significantly were determined using Duncan's multiple range test at $p < 0.05$ level.

Table 1
Development of direct somatic embryos from hypocotyl explants excised from 21-day-old *in vitro* grown seedlings of *D. trojana* Ivan and cultured on MS medium containing different concentrations of BAP, kinetin, TDZ or zeatin alone. Data were collected after five weeks of culture.

PGRs (mg/l)		Mean frequency (%) of explants developing somatic embryos*	Mean number of somatic embryos per explant**
Control		0	0
BAP	0.1	0	0
	0.5	0	0
	1.0	0	0
Kinetin	0.1	40^{b}	6.3 ± 0.6^{b}
	0.5	40^{b}	9.3 ± 0.5^{a}
	1.0	20^{c}	4.3 ± 0.6^{cd}
TDZ	0.1	55^{a}	9.0 ± 0.4^{a}
	0.5	65^{a}	10.2 ± 0.9^{a}
	1.0	65^{a}	10.7 ± 0.9^{a}
Zeatin	0.1	35^{b}	2.5 ± 0.3^{d}
	0.5	40^{b}	3.8 ± 0.5^{d}
	1.0	40^{b}	6.0 ± 0.4^{bc}

* Mean frequencies of explants with the same letter within columns are not significantly different according to Duncan's multiple range test at $p < 0.05$.
** Mean numbers of somatic embryos ± SE (standard error) with the same letter within columns are not significantly different according to Duncan's multiple range test at $p < 0.05$.

3. Results and discussion

This study reports, for the first time, a simple and suitable protocol for the induction of direct somatic embryogenesis in *D. trojana* Ivan. To achieve this, two sets of experiments were carried out; the first compared different concentrations of BAP, kinetin, TDZ and zeatin alone (Table 1) while the second set tested different concentrations of TDZ or BAP combined with NAA, IAA or IBA (Table 2), both sets of experiments using hypocotyl segments excised from *in vitro* germinated seedlings.

3.1. Induction of direct somatic embryogenesis and plant regeneration

One week after the culture initiation, embryogenic clumps were clearly visible from the epidermal layers of the whole hypocotyl

Table 2
Development of direct somatic embryos from hypocotyl explants excised from 21-day-old *in vitro* grown seedlings of *D. trojana* Ivan and cultured on MS medium containing TDZ or BAP combined with different concentrations NAA, IAA or IBA. Data were collected after five weeks of culture.

PGRs (mg/l)	Mean frequency (%) of explants developing somatic embryos*	Mean number of somatic embryos per explant**
TDZ (0.5) + NAA (0.25)	30^{cdf}	7.4 ± 1.3^{c}
TDZ (0.5) + IAA (0.5)	50^{b}	11.4 ± 1.4^{ab}
TDZ (1.0) + IAA (0.5)	75^{a}	13.8 ± 1.2^{a}
TDZ (1.0) + IBA (0.25)	40^{bc}	6.2 ± 0.5^{cd}
TDZ (1.0) + IBA (0.5)	40^{bc}	7.8 ± 1.8^{bc}
BAP (1.0) + IAA (0.5)	20^{df}	8.8 ± 0.9^{bc}
TDZ (2.0) + IAA (0.1)	20^{df}	5.6 ± 0.9^{cd}

* Mean frequencies of explants with the same letter within columns are not significantly different according to Duncan's multiple range test at $p < 0.05$.
** Mean numbers of somatic embryos ± SE (standard error) with the same letter within columns are not significantly different according to Duncan's multiple range test at $p < 0.05$.