Industrial Crops & Products 121 (2018) 313–319

Contents lists available at ScienceDirect

Industrial Crops & Products

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

In vitro propagation of *Digitalis lanata* Ehrh. through direct shoot regeneration – A source of cardiotonic glycosides

Bhushan P. Bhusare[a], C.K. John[b], V.P. Bhatt[c], T.D. Nikam[a,*]

[a] Department of Botany, Savitribai Phule Pune University, Pune, 411007, Maharashtra, India
[b] Plant Tissue Culture Division, CSIR-National Chemical Laboratory, Pune, 411008, Maharashtra, India
[c] Herbal Research and Development Institute (HRDI), Mandal-Gopeshwar, 246401, Chamoli, Uttarakhand, India

ARTICLE INFO

Keywords:
Digitoxin
Digoxin
Direct organogenesis

ABSTRACT

An efficient method for the *in vitro* propagation of *Digitalis lanata* Ehrh. through direct organogenesis from leaf and petiole explants has been standardised. MS basal medium supplemented with cytokinins BAP, KIN, TDZ either alone or along with auxins IAA and NAA at different concentrations were tried. TDZ at 4.54 and 6.81 µmol/l were optimum for direct regeneration of shoots from leaf (4.4 ± 0.6 shoots/explant), and petiole (3.0 ± 0.8 shoots/explant) explants respectively. Among the various concentrations of auxins IAA, IBA and NAA tried for rooting, the best response occurred on MS basal medium supplemented with 17.13 µmol/l IAA. On greenhouse transfer about 60% of the plantlets survived. *In vitro* raised plantlets were morphologically similar to mother plants. Cardiotonic glycosides digoxin and digitoxin were extracted by modified methods and estimated by HPLC. There were no significant differences in digoxin and digitoxin content in leaves of naturally grown and *in vitro* raised plants. The method for *in vitro* propagation of *D. lanata* through direct organogenesis from leaf and petiole explants reported here will be of great use for the rapid and large scale clonal propagation, production of biomass for extraction of cardiotonic glycosides, *ex situ* conservation, and improvement through conventional plant breeding and transgenic methods.

1. Introduction

Digitalis lanata Ehrh. ("*Woolly Foxglove*"/"*Grecian Foxglove*", family: Plantaginaceae) is a biennial or perennial herb widely distributed in temperate parts of Hungary, Romania and Balkan Peninsula in Europe, North Africa and West Asia. It is cultivated in the Netherlands (Li et al., 2014), in some parts of the United States (Hollman, 1985) and the Himalayan region of India (Negi et al., 2012). The leaves of the plant are exclusively used for the isolation of important cardiotonic glycosides digoxin and digitoxin which are used in the treatment of congestive heart failure (CHF) and atrial arrhythmia (Somberg et al., 1986; Rahimtoola and Tak, 1996; Ehle et al., 2011). For oral use, *Digitalis* glycoside is still the only safe drug treatment for improving hemodynamics in patients through balancing the cardiac activity (Schwinger et al., 2003; Pérez-Alonso et al., 2009; Patel, 2016; Kreis, 2017). As per recent research findings *Digitalis* glycosides digoxin, digitoxin and lanatoside C are effective in treating viral infections (Hoffmann et al., 2008; Cheung et al., 2014; Zhyvoloup et al., 2017) and different types of cancer (Prassas and Diamandis, 2008; Newman et al., 2008; Sharma and Purkait, 2012; Wu et al., 2012a). Chemical synthesis of these glycosides is possible, but too expensive to be cost effective (Clemente et al., 2011). Although the medicinal glycoside content is low, the pharmaceutical industry still relies on natural source. *D. lanata* is preferred over *D. purpurea* and other species of *Digitalis* as the content of cardiotonic glycosides is higher (Bown, 1995; Duke, 2002). Traditional method of propagation of *D. lanata* is through seeds. However, *D. lanata* being native of temperate regions, seeds need to be stored at 4 °C. In warmer climates, low seed germination, and rapid loss of seed viability, when stored at room temperatures, pose limitations on the use of seeds for large scale planting (Verma et al., 2016). At present there is destructive over-exploitation of *D. lanata* from wild to meet the ever-increasing demands of both allopathic and traditional medicine industries. Collection of plant material before seeding results in rapid disappearance of natural populations. Although there were attempts to produce *D. lanata* varieties having high cardenolide contents through conventional breeding methods (*i.e.* in-breeding and crossing), but offsprings were not stable (Verma et al., 2012). To fix the character, cumbersome long-term breeding programmes are required which can

Abbreviations: BAP, N6-benzyladenine; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, kinetin; MS basal medium, Murashige and Skoog basal medium; NAA, α-naphthalene-acetic acid; PGRs, plant growth regulators; TDZ, 1-phenyl-3-(1 2 3-thiadiazol-5-yl)-urea

* Corresponding author.
E-mail address: tdnikam@unipune.ac.in (T.D. Nikam).

https://doi.org/10.1016/j.indcrop.2018.05.019
Received 25 January 2018; Received in revised form 4 May 2018; Accepted 7 May 2018

be prohibitively expensive. Hence, there is an urgent need for an efficient *in vitro* propagation method which can be of use in improving *D. lanata* through selection/transgenic methods. During the past three decades there have been many reports on biotechnological approaches for *in vitro* propagation and detection of cardiac glycosides in wild as well as cultivated *Digitalis* species (*D. cariensis, D. davisiana, D. ferruginea, D. lamarcki, D. minor, D. obscura, D. purpurea, D. thapsi* and *D. trojana*) as well as production of cardiac glycosides in tissue and cell culture (Patil et al., 2013; Verma et al., 2016). An efficient method for *in vitro* propagation through direct organogenesis is a prerequisite for the rapid and large-scale production of planting stocks, quick bulking of any high glycoside containing variants, and for improvement through genetic transformation. Hence it was decided to study the effects of various concentrations and combinations of PGRs on shoot regeneration from leaf and petiole explants of *D. lanata*, as well as rooting of these shoots to produce plantlets. The micropropagation method reported here will be of great use in rapid and large-scale propagation, and its introduction and exploitation in many places outside its native range without any climate/seasonality constraint and genetic improvement of this industrially important medicinal plant through conventional (selection) and modern (transgenic) methods.

2. Materials and methods

2.1. Source of explants

Seeds of *D. lanata* were obtained from Herbal Research and Development Institute, Mandal-Gopeshwar (coordinates: 30.4095°N, 79.3198°E), Chamoli, Uttarakhand, India. Seeds were washed under running tap water for 5 min followed by rinsing with sterile distilled water in a laminar air-flow cabinet. Then the seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 5 min and washed five times with sterile distilled water. The surface sterilized seeds were inoculated on MS (Murashige and Skoog, 1962) basal medium with 30 g l^{-1} sucrose and 8.0 g l^{-1} agar. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl prior to autoclaving at 121 °C, 103 kPa for 20 min. All cultures were incubated at 25 ± 2 °C for 8 h photoperiod with 36 µmol m^{-2} s^{-1} light intensity provided by cool white, fluorescent tubes (Philips, India). After germination, seedlings were transferred on fresh MS basal medium at four weeks interval. Apical buds, axillary buds, leaf and petioles from 12 week old seedlings were excised and used as a source of explants.

2.2. Direct shoot regeneration

MS medium supplemented with various concentrations of N6-benzyladenine (BAP), 6-furfurylaminopurine (KIN) and 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl)-urea (TDZ) and combinations of TDZ with indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) were used for shoot multiplication. These plant growth regulators (PGRs) were procured from Sigma-Aldrich, USA. Four types of explants were used initially *i.e.* apical buds (1 cm top segments containing apical buds), axillary buds (1.5–2 cm segments containing one axillary bud in the centre), leaf (1 cm squares) and petiole (1 cm long segments). Since the results obtained with leaf and petiole explants with higher number of shoots (3.0-4.4 shoots/explant), work was continued with these two types of explants. Results obtained with apical and axillary bud explants (1.0–2.0 shoots/explant), are not discussed in this communication.

The explants were inoculated in horizontal and vertical positions on different culture media. All cultures were incubated at 25 ± 2 °C for 8 h photoperiod with 36 µmol m^{-2} s^{-1} light intensity. After four weeks of culture, the explants with regenerated shoots were sub-cultured on fresh media. The effect of different PGRs on shoot multiplication was recorded as number of explants responding (percentage), number of shoots per explant, and length of regenerated shoots.

2.3. Root induction and acclimatization

In vitro developed healthy shoots (2.0–2.5 cm long) were excised and placed on MS media supplemented with various concentrations of auxins such as IAA (1.42–34.26 µmol/l), IBA (1.22–14.70 µmol/l) and NAA (1.34–16.11 µmol/l). The effect of different auxin treatments were recorded as number of shoots responding as percentage, number of roots per shoot and length of roots after eight weeks. The rooted plantlets were carefully removed from the medium, gently washed under tap water to remove the adhering media, and transferred to plastic pots containing an autoclaved mixture of soil and sand (3:1). The potted plantlets were irrigated with half strength MS medium (only major and minor salts). Initially these pots were maintained in the culture room having 25 ± 2 °C temperature, 80–90% relative humidity, and 8 h photoperiod with 36 µmol m^{-2} s^{-1} light intensity for two weeks. Subsequently the plantlets were transferred to a greenhouse with 27 ± 2 °C temperature, 60–80% relative humidity, and natural light (60 µmol m^2 s^{-1}) and photoperiod (10–12 h). Percent plant survival, plant morphology, and cardiotonic glycoside content were recorded three months after greenhouse transfer.

2.4. Extraction and HPLC analysis of digoxin and digitoxin from leaves of *D. lanata*

To assess the effect of *in vitro* regeneration on secondary metabolite production, HPLC-DAD method was used to quantify the digoxin and digitoxin from leaves of *in vitro* regenerated plants of *D. lanata* and compared with those in leaves of naturally grown plants. A modified Pellati et al. (2009) protocol was used for cardenolides (digoxin and digitoxin) extraction. By the modified method digoxin and digitoxin were eluted in short time span (10 min/sample). In brief, basal leaves of *D. lanata* were excised from naturally grown and *in vitro* raised plantlets and dried at 40 °C in a hot air oven till constant weight. The dried leaves were powdered and 0.5 g powder was added to 20 ml of 75% (v/v) methanol in conical flask, shake extracted by placing on a rotary shaker for 12 h at 75 rpm, followed by ultrasonication for 15 min at 60 °C, cooling to room temperature, and centrifuging at 4000 rpm for 15 min. The supernatants were filtered using Whatman (grade 1) filter paper. The filtrates were concentrated under vacuum using a rotary evaporator at 30 °C. The residue was dissolved in 2 ml of 75% (v/v) methanol and filtered using 0.22 µm cellulose acetate filters. The filtrates were placed in HPLC vials and stored at −20 °C till use.

HPLC analyses for digoxin and digitoxin were carried out using an HPLC-1200 infinity series system (Agilent Technologies, Waldbronn, Germany), on a Symmetry C18 column (4.6 × 250 mm I.D., 5 µm, Waters, Dublin, Ireland). Digoxin and digitoxin content were calculated separately using calibration curves of pure standards (Sigma-Aldrich Chemie, Steinheim, Germany) of jointly estimated digoxin and digitoxin. For the calibration curves, concentrations of (12.5, 25, 50, 100, 200, 300 and 400 µg/ml) were used (n = 3 for each concentration, R values were 0.999 and 0.998 for digoxin and digitoxin respectively).

These cardenolides were eluted with an isocratic system of acetonitrile (ACN): water (H_2O): methanol (CH_3OH) at the proportion of 66:07:27 (flow rate: 0.4 ml/min, column temperature: 20 °C, sample injection volume: 10 µl, detector set at: 220 nm, and total running time: 10 min). Three HPLC runs were performed for each sample.

2.5. Experimental design and statistical analysis

All the experiments were organized in completely random design (CRD) and conducted three times. Data from each experiment of fourteen replicate (one explant per replicate) were analyzed by using ANOVA in SPSS software. The percentage data was arcsine transformed before subjecting to statistical analyses. The homogeneity of means/comparison of means was done using Duncan's Multiple Range Test (DMRT) at 5% probability level. Similarly, HPLC data were analysed