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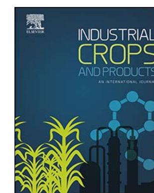

# Efficient direct shoot organogenesis, genetic stability and secondary metabolite production of micropropagated *Digitalis purpurea* L.

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ABSTRACT

Cardiovascular and cancer diseases are the first causes of death in the world. *Digitalis purpurea* L. is a medicinal plant that produces secondary metabolites, like digoxin and digitoxin, which are employed in therapies against heart failure. Moreover, anticancer and antiviral properties of these metabolites have recently been described. The present work details a method to obtain *in vitro* plants of *D. purpurea* from leaf segments through direct organogenesis. A reliable and efficient plant induction system was established by optimizing the concentration of naphthaleneacetic acid (NAA) and 6-benzylaminopurine (6-BAP). The highest frequency of shoot regeneration (98.5%) with an average number of shoots per leaf segment of 18.9 was achieved *via* direct organogenesis from leaf segment on MS medium containing 0.54 µM NAA + 13.2 µM 6-BAP. Additionally, Random Amplified Polymorphic DNA (RAPD) analysis showed 100% monomorphic bands, which indicated genetic stability of the obtained plants. Moreover, leaf powder of *de novo* regenerated plants fulfills the quality specifications of the British Pharmacopoeia and HPLC analysis revealed the presence of digoxin (22.6 µg $gDW^{-1}$) and digitoxin (220.7 µg $gDW^{-1}$) without significant differences in contents between *de novo* regenerated and mother plants. An efficient *in vitro* propagation protocol *via* direct organogenesis and genetic stability assessment of *D. purpurea* for obtaining leaf powder with quality for the use as raw material have thus been described. The protocol also provides an effective means for several approaches in biotechnology and breeding programs, in order to produce pharmaceutically interesting cardenolides.

## 1. Introduction

*Digitalis purpurea* L. is one of the most important medicinal plants, which has been used for many years. In 1785 the British physician William Withering described its pharmacological properties which make it very useful for the treatment of different cardiovascular diseases (Verma et al., 2016). *D. purpurea* plants produce cardenolides, mainly digoxin and digitoxin, which are a group of remarkable chemical compounds that are responsible for these pharmaceutical activities (Agrawal et al., 2012). Several pharmacological applications of these compounds have been reported, for instance, in chronic auricular fibrillation and cardiac insufficiency (Feussner and Feussner, 2010). For these purposes digoxin is the most used cardenolide, with total worldwide sales of US$ 142 million in 2012 (IARC Working Group, 2016). Moreover, antiproliferative and apoptotic effects were observed in several cancer cell lines (Rocha et al., 2014). These effects are related with the inhibition of the $Na^+/K^+$ ATPase that results in the later activation of the $Na^+/Ca^{2+}$ pump, the increase of intracellular concentration of $Ca^{2+}$ and the induction of apoptosis in cancer cells (reviewed in Elbaz et al., 2012). Nevertheless, other antiproliferative mechanisms have been proposed and are under investigation (Elbaz et al., 2012; Wei et al., 2013; Lin et al., 2015). Recently, antiviral

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properties against several human-infecting viruses like HIV, herpes, cytomegalovirus and adenovirus have been attributed to cardenolides (Bertol et al., 2011; Cai et al., 2014; Grosso et al., 2017; Zhyvoloup et al., 2017). In the case of HIV digoxin repressed viral gene expression by targeting the cellular $Na^+/K^+$ ATPase (Zhyvoloup et al., 2017). However, the use of *D. purpurea* for pharmacological purposes needs some further research.

Currently, the only source of cardenolides is the plant itself. Chemical synthesis of these compounds is unviable right now, due to their structural complexity (Verma et al., 2016). Nevertheless, multiple factors modulate cardenolide concentration in plants cultivated in the field, *e.g.* temperature, mineral soil composition, season, humidity and others (Sales et al., 2011). Furthermore, wild populations of *Digitalis* species are significantly affected by large-scale and uncontrolled exploitation in order to satisfy the pharmaceutical industry (Verma et al., 2016). Another important issue of *Digitalis* cultivation is the low germination rate of the seeds. In addition, there are some regions where the plant cannot be grown in open fields, like Cuba (basically because of the high temperature and humidity). As a consequence, several research groups have developed biotechnological strategies in order to reduce the excessive use of natural *Digitalis* populations, to conserve high yielding cardenolide producing plants or for genetic improvement. Such strategies include somatic embryogenesis (Lindemann and Luckner, 1997), temporary immersion systems (Pérez-Alonso et al., 2009, 2012), precursors addition, elicitation (Pérez-Alonso et al., 2014a; Patil et al., 2013) and organogenesis (Hagimori et al., 1982; Pérez-Bermúdez et al., 1984; Cacho et al., 1991; Fatima et al., 2009; Çördük and Aki, 2010; Gurel et al., 2011; Verma et al., 2011a,b; Karimi and Kazemitabar, 2013; Li et al., 2014; Pérez-Alonso et al., 2014b; Yücesan et al., 2014; Kreis et al., 2015; Mohammed et al., 2015). Organogenesis can be done directly or indirectly, direct organogenesis being the most successful for many species of the genus *Digitalis.* This morphogenetic process of plant regeneration allows the generation of entire plants, in a very easy, rapid, homogenized and continuous way during the whole year, without environmental restrictions.

Nevertheless, the scientific literature revealed the application of direct regeneration for *in vitro* production of *D. purpurea* only in studies by Patil et al. (2013) and Li et al. (2014). The latter protocol was used for *Agrobacterium tumefaciens*-mediated genetic transformation. However, neither analysis of leaf powder quality nor genetic fidelity of *in vitro* plantlets was assessed by these authors.

The aim of this research was to carry out efficient *in vitro* plant regeneration of *Digitalis purpurea* L. *via* direct organogenesis and to evaluate the genetic stability of the *de novo* regenerated plants, in order to obtain metabolites with pharmaceutical quality specifications.

## 2. Materials and methods

### 2.1. In vitro morphogenesis of Digitalis purpurea

*Digitalis purpurea* cv. Berggold shoot cultures were initiated from *in vitro* germinated seeds, using only one line to avoid heterogeneity. *In vitro* plants, considered as mother or control plants were cultured on solid medium as previously described (Pérez-Alonso et al., 2009). Briefly, shoots were multiplied inducing multiple shoots from axillary buds in flasks containing MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg $l^{-1}$ thiamine HCl, 4.4 μM 6-benzylaminopurine (6-BAP), 0.57 μM indole acetic acid (IAA), 100 mg $l^{-1}$ *myo*-inositol, 30 g $l^{-1}$ sucrose and 3.0 g $l^{-1}$ Gelrite (Duchefa, Netherlands). The pH was adjusted to 5.8 with 0.5N KOH or 0.5N HCl before autoclaving at 1.1 kg $cm^{-2}$ and 121 °C for 20 min. The cultures were incubated in a growth chamber at 27 ± 2 °C under a 16 h photoperiod from cool white fluorescent lamps at a photosynthetic photon flux density of 70 μmol $m^{-2}$ $s^{-1}$. Mother plants were sub-cultured every 28 days as mentioned above.

For shoot induction, leaf segments (1.0 $cm^2$, adaxial surface to the medium) from *in vitro* plants (fourth-seventh subculture) were cultured on basal medium containing MS salts supplemented with 4.0 mg $l^{-1}$ thiamine HCl, 100 mg $l^{-1}$ *myo*-inositol, 30 g $l^{-1}$ sucrose and 3.0 g $l^{-1}$ Gelrite (Duchefa, Netherlands).

The effect of naphthaleneacetic acid (NAA) 0, 0.54 or 2.7 μM combined with 6-BAP 0, 4.4, 13.2 or 22.0 μM was tested on MS basal medium. The pH was adjusted to 5.8 with 0.5 N KOH or 0.5 N HCl prior to autoclaving at 1.1 kg $cm^{-2}$ and 121 °C for 20 min. This medium was called Shoots Induction Medium. Culture conditions were the same as mentioned above.

Evaluation of the percentage of leaf segments that produces adventitious roots or shoots, and the number of regenerated shoots per leaf segment were recorded after six weeks. Leaf segments with clearly differentiated shoots and leaves of approximately 1.0–2.0 cm in length were scored as leaf segments with shoots. The morphology of the formed shoots was also evaluated. Then, developed shoots were transferred to jar flasks containing 30 ml of MS medium supplemented with 1.0 mg $l^{-1}$ thiamine HCl, 4.4 μM 6-BAP, 0.57 μM IAA, 100 mg $l^{-1}$ *myo*-inositol, 30 g $l^{-1}$ sucrose and 3.0 g $l^{-1}$ Gelrite, pH 5.8; and subcultured every four weeks to the same fresh medium. Culture conditions were the same as mentioned above.

Ten replicates were done for each treatment (10 jar flasks with five explants each = 50 explants). The experiment was repeated four times.

### 2.2. Analysis of genetic stability using RAPD

Genetic homogeneity between the mother plant and selected *de novo* regenerated plantlets was assessed using RAPD (Random Amplified Polymorphic DNA). Twelve plantlets obtained from the best combination of growth regulators were randomly selected for this analysis after three subcultures. DNA was isolated from 100 mg of leaves of regenerated plants and the mother plant using the protocol described by Khayat et al. (2004). Genomic DNA integrity was analyzed through electrophoresis in 0.8% (w/v) of agarose gel 1X Tris–Borate–EDTA (TBE 1 X: 0.89 M Tris-HCl, 0.02 M EDTA and 0.89 M boric acid) and purity was analyzed in a Biophotometer (Eppendorf, Germany). Purified DNA was kept at −20 °C for further analysis.

A total of eight arbitrary decamer primers (Center of Genetic Engineering and Biotechnology, Havana, Cuba) and some combinations were tested to amplify fragments from genomic DNA of mother and *de novo* regenerated plants. Only primer combinations that produce distinct easily scorable amplification profiles were selected (Table 2). Amplification was performed using genomic DNA of each plant as a target. PCR was carried out in 30 μl total volume containing 100 ng of template DNA, 200 mM dNTPs, 1.5 mM $MgCl_2$, 1X TopTaq polymerase reaction buffer (QIAGEN, Germany), 1 unit of TopTaq DNA polymerase (QIAGEN, Germany) and 0.5 μM of primers. Amplification reaction was carried out using a MasterCycler ep Gradient (Ependorff, Germany) with an initial denaturation of DNA at 94 °C for 4 min, followed by 35 cycles consisting of 30 s denaturation at 94 °C, 30 s annealing at 30 °C and 2 min extension at 72 °C. These cycles were followed by a final extension of 15 min at 72 °C and hold temperature of 4 °C. Amplified fragments were analyzed by electrophoresis at 80 V for 2 h on 1.5% agarose gel in 1X TBE buffer, followed by staining in ethidium bromide (5 μg $ml^{-1}$).

After electrophoresis and staining, amplified bands were photographed under ultra-violet light using a Gel Documentation & Analysis System (WD-9413A). All the PCR reactions were performed at least twice to check the reproducibility.

### 2.3. Pharmacognostic analysis

#### 2.3.1. Microscopic analysis and numeric indexes determination

Samples of *Digitalis purpurea* leaf powder were produced from three randomly selected plants previously regenerated *via* direct organogenesis. Control was defined as one sample of leaf powder from a mother

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