Chapter

In Vitro Propagation of *Digitalis trojana* Ivanina., an Endemic Medicinal Plant of Turkey

Nurşen Çördük and Cüneyt Aki

Abstract

Digitalis trojana Ivanina is a member of the Plantaginaceae family and known by its common name, Helen of Troy foxglove. It is perennial endemic to Çanakkale and Balıkesir, northwestern Turkey. In order to develop an efficient shoot regeneration protocol, the leaf explants of *D. trojana* were cultured on Murashige and Skoog (MS) medium containing 6-benzyl adenine (0.1, 0.5, 1.0, 3.0, 5.0 mg/L) and α-naphthalene acetic acid (0.1, 0.5, 1.0 mg/L), 3% (w/v) sucrose and 0.8% (w/v) agar. The highest number of regenerated shoots was obtained from leaf explants that were cultured on MS medium with 3.0 mg/L BA+0.1 mg/L NAA. Regenerated shoots were rooted on MS medium without plant growth regulators. Rooted plants (2–3 cm) were separately transferred to pots containing a mixture of peat and perlite (2:1 v/v) and acclimatized successfully in a growth chamber.

Keywords: endemic, foxglove, *in vitro*, propagation, regeneration

1. Introduction

Turkey has a rich biodiversity as a result of its location, its geological structure and different climatic zones. Turkey hosts three biogeographical regions: Mediterranean, Euro-Siberian and Irano-Turanian. As a result of located on the meeting point of these three different regions, Turkey is one of the most important areas in the world in terms of biological diversity. It is one of the world's richest countries with regard to diversity of plant species, hosting 167 families, 1320 genera and 9996 species [1]. This genetic diversity gains importance especially with the diversity of endemic, rare, medicinal and cultivated plants. A species, or other category of organism that is unique to a defined geographic location such as an island, nation, country, or habitat type is considered endemic to that location. The endemism rate of the Turkish flora is 31.8% and each year new such species are identified. Turkey has 3649 endemic plant species of flora with 31.82% rate of endemism [1–4]. Asteraceae, Lamiaceae, Fabaceae, Caryophyllaceae and Scrophulariaceae families constitute 50% of the distribution of endemic taxa in Turkey [5]. High levels of endemism within Turkey are concentrated in specific areas, such as the Amanos Mountains, the Ilgaz Mountains, the Central Taurus Mountains, the Taşeli Plateau, the Bolkar and Aladağlar Mountains, Kazdagi, Uludağ Mountain, the mountains between Gümüşhane and Erzincan, the Munzur Mountains, and Lake Tuz and its saline steppes [1].

Kazdagi Mountain (Mt. Ida) is in the north western of Turkey, at the transition area of the Euro-Siberian and Mediterranean regions. Kazdagi is the gene center of the west Anatolian region. Endemic and rare taxa have been preserved on the different geological massive and especially in the subalpine zone [6]. The Mountain is ecologically and floristically diverse, containing a number of plant species endemic to Turkey. It has a rich flora thanks to climatic conditions, geological structure and its location. There are about 800 taxa grown naturally in the area and 79 of them are endemic to Turkey. Thirty-one of the endemic taxa are grown only in this Mountain [7–10].

Due to exceptional amount of endemism that brings a huge responsibility to Turkey, it is to ensure that these species are adequately protected from threats or extinction, particularly for those which are related to the crops upon which much of the world depends. Endemic plant species may be endangered due to their distribution specific to a specific region and the decrease in the number of individuals in the population. Endemic plant species should be taken under protection in *ex situ* as well as *in situ* in order for the biodiversity of our country to be passed on to future generations, to prevent the destruction of gene resources, to prevent the loss of wild forms of many cultured species, and for the sustainability of vegetative production. Plant tissue culture is one of the methods used in *ex situ* conservation of plant gene resources. Tissue culture allows the production of new tissue, plant or herbal products by culturing parts of a plant such as cell, tissue, organ, meristem or embryo under aseptic conditions in an appropriate artificial nutrient medium [9, 11, 12]. *In vitro* culture technologies have been increasingly used for *ex situ* conservation of rare or endangered endemic plants [13, 14]. Besides, high amounts of production of medicinal plants and their metabolites can be achieved via *in vitro* culture [15–17].

The genus *Digitalis* L., commonly known as the foxglove, is a member of the Plantaginaceae and encompasses approximately 20 species. *Digitalis* species are biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are often crowded in basal rosettes [18, 19]. This genus is native to western and southwestern Europe, western and central Asia, Australasia and northwestern Africa. *Digitalis* species is represented by eight species and two subspecies, including four endemic, in Turkey. Among these species, *Digitalis trojana* Ivanina is known by its common name, Helen of Troy foxglove. It is perennial endemic to Kazdagi, northwestern Turkey [20]. According to the Red Data Book of Turkish Plants, conservation status of this species is declared as vulnerable (VU) [21]. Digitalis species are a medicinally important group because of the cardenolides or cardiac glycosides (CGs) applied in human medicine. The cardenolides have been used as the most effective heart drugs for treating several heart defects [22–24]. They also show a broad spectrum of biological activities, containing anti-carcinogenic, acaricidal and antibacterial properties. Recent studies are especially focused on the anticarcinogenic effects of digoxin and digitoxin particularly for cancer treatments [25, 26]. There are lots of reports about *in vitro* cultures of *Digitalis* species [22, 27–31]. The aim of this research is to carry out an efficient *in vitro* propagation of *D. trojana* for *ex situ* conservation.

2. Materials and methods

2.1 Plant materials

Plant seeds of *D. trojana* were collected from the Kazdagi National Park, Turkey (**Figure 1**). Plant samples were prepared as herbarium materials and voucher

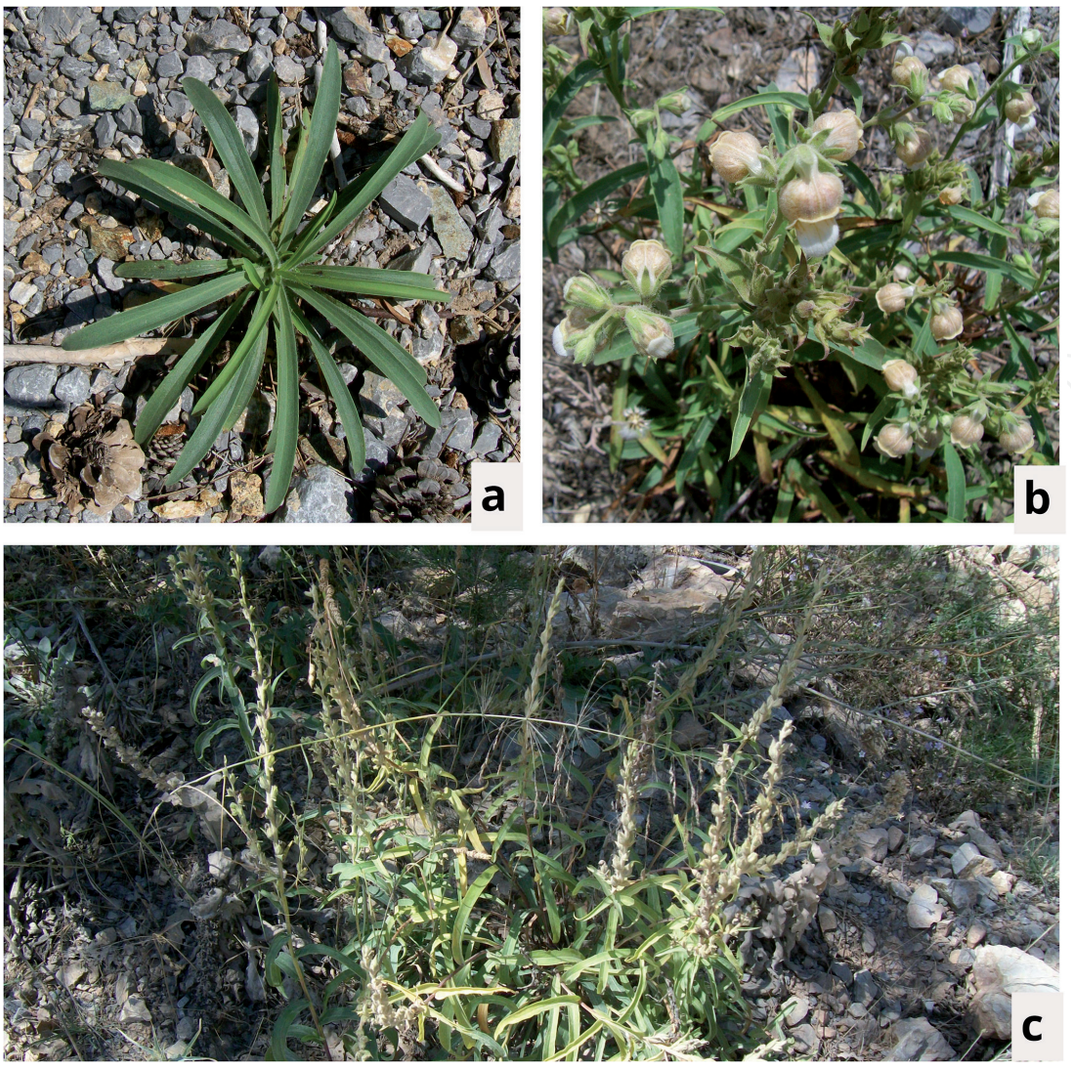

Figure 1.
Plant samples of Digitalis trojana (a, b, c).

specimens were deposited in the Herbarium of Çanakkale West Anatolia (CBB, Çanakkale, Turkey) under number 351, 352 and 353.

Seeds were surface-sterilized by stirring in 3% (v/v) solution of sodium hypochlorite containing two drops of 0.1% Tween 20 for 20 min. Under sterile conditions, followed by 5 times rinsed in sterile water. The surface-sterilized seeds were cultured on MS basal medium (MS: M0222, Duchefa Biochemie B.V., Haarlem, Netherlands) [32], containing 3% (w/v) sucrose and 0.8% (w/v) agar, and keep in the dark for a week for successful germination. Germinated seedlings were subsequently transferred to a growth chamber at 25 ± 2°C under a 16-h light and 8-h dark long day cycle with a light intensity of 72 μmol m^{-2} s^{-1}provided by cool-white fluorescent lamps.

2.2 Induction of adventitious shoots

Shoot cultures of *D. trojana* were established *in vitro* using leaf segments (5x5 mm) excised aseptically from 12-week-old *in vitro* germinated seedlings. The explants were cultured on MS medium supplemented with BA (0.1, 0.5, 1.0, 3.0, 5.0 mg/L) in combination with NAA (0.1, 0.5, 1.0 mg/L) and 3% (w/v) sucrose (S0809, Duchefa). All media were gelled with 0.8% (w/v) agar (P1001, Duchefa) and the pH was adjusted to 5.75 before autoclaving. All cultures were maintained

at 25 ± 2°C under the 16/8 h photoperiod with a light intensity of 72 μmol m^{-2} s^{-1}. Ten explants were cultured per petri dish for each type of explant, and at least five replicates were used for each treatment. The mean number of regenerated shoots per explant was recorded in each culture after twelve weeks.

2.3 Shoot multiplication, rooting and transfer of plantlets to soil

In order to multiply the shoots, the well-developed shoots (ca. 1 cm in length) grown for 8 weeks on shoot induction medium were excised and transferred to fresh MS medium containing the same PGRs as the shoot induction medium. For rooting, the shoots (2–3 cm in length) produced after multiplication were separately transferred to basal MS medium, 3% (w/v) sucrose and 0.8% (w/v) agar without plant growth regulators. All the plants with well-developed roots were taken out of the solid medium, gently washed in running water and shortly after transplanted into pots (5 mm in diameter) containing a mixture of peat and perlite (2:1 v/v). Plantlets were covered with plastic cups to maintain a high humidity and a few holes were opened on the cups. The diameter of the holes was gradually increased over the next 2 weeks. After one month, the plantlets were uncovered and were then transplanted to new pots (15 mm in diameter) containing the mixture of 2 peat:1 perlite.

2.4 Statistical analysis

The mean number of shoots and percent of explants forming shoots were analyzed after twelve weeks from initial inoculation for all explants. All data were evaluated by analysis of variance and mean values were compared using MINTAB. The interaction of plant growth regulators was analysis with MSTAT. The statistic model ($Yijk = \mu + Ai + Bj + AB\ ij + \Sigma\ ijk$) was used for assigning the effects of plant growth regulator's concentration on shoot regeneration.

3. Results and discussion

The explants cultured on MS medium without plant growth regulators were only slightly expanded callus and shoot formation wasn't observed in this medium. Adventitious shoots were formed out of the cut edges of the explants, cultured on MS medium containing BA in combination with NAA within three weeks in the culture (**Figure 2**). 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 3.0 mg/L BA+0.1 mg/L NAA and 5.0 mg/L BA+1.0 mg/L NAA. Although the number of regenerated shoots are higher on media containing 5.0 mg/L BA+1.0 mg/L NAA, the vitrification was occurred in the shoots in this medium and the shoots were not multiplicated. As a result of analysis of variance, it was shown that the effect of NAA varied depending on BA concentration for shoot regeneration at 12th week of culture ($P < 0.01$).

BA was promoted to shoot formation by interaction with NAA (**Figure 3**). The ratio of NAA to BA was also very significant. Especially, 3.0 or 5.0 mg/L concentration of BA was effective for shoot formation from *D. trojana* leaf explants. Our results are consistent with previous report on *D. thapsi* L. [30], which reported that the presence of high concentrations of BA (3, 4, 5 mg/L) 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 *D. minor* L. [33]. The best shoot proliferation was observed among

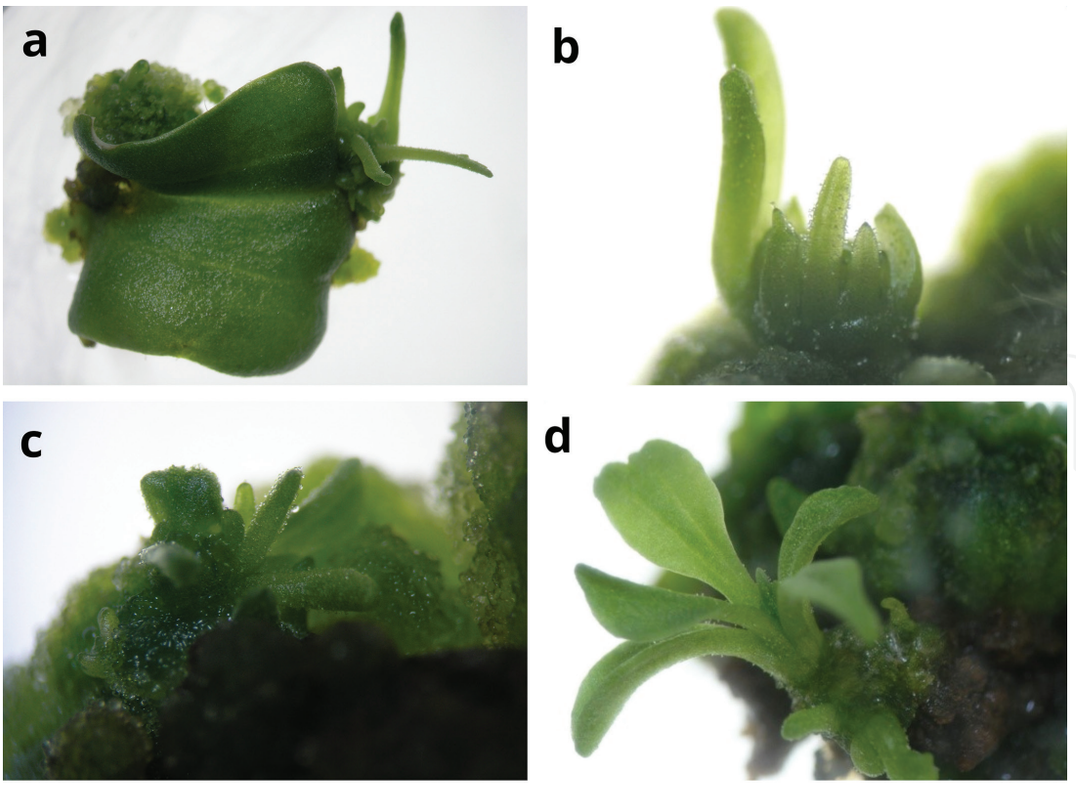

Figure 2.
Morphogenic callus induction and shoot regeneration in D. trojana from leaf explant culture (a), multiple adventitious shoot regeneration on MS medium with 3.0 mg/L BA +0.1 mg/L NAA (b, c, d).

Plant growth regulators (mg/L)		Percent of explants forming shoots	Mean number of shoots per explants
BA	NAA		
—	—	0.0	0.0
0.1	0.1	4.0	$0.3 \pm 0.1^{D\,a}$
0.5	0.1	16.0 ± 0.3	$2.3 \pm 0.5^{CD\,a}$
1.0	0.1	20.0 ± 1.4	$10.3 \pm 0.3^{BC\,a}$
3.0	0.1	32.0 ± 0.1	28.0 ± 1.8^{Aa}
5.0	0.1	45.0 ± 0.5	$16.3 \pm 0.1^{B\,b}$
0.1	0.5	0.0	$0.0^{B\,a}$
0.5	0.5	0.0	$0.0^{B\,a}$
1.0	0.5	0.0	$0.0^{B\,b}$
3.0	0.5	32.0 ± 0.9	$17.3 \pm 0.3^{A\,b}$
5.0	0.5	12.0 ± 0.7	$2.3 \pm 0.6^{B\,c}$
0.1	1.0	0.0	$0.0^{B\,a}$
0.5	1.0	13.3 ± 1.6	$0.6 \pm 0.1^{B\,a}$
1.0	1.0	12.0 ± 1.8	$1.6 \pm 0.5^{B\,b}$
3.0	1.0	4.0 ± 0.2	$0.3 \pm 0.1^{B\,c}$
5.0	1.0	14.6 ± 0.5	$26.3 \pm 3.0^{A\,a}$

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

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

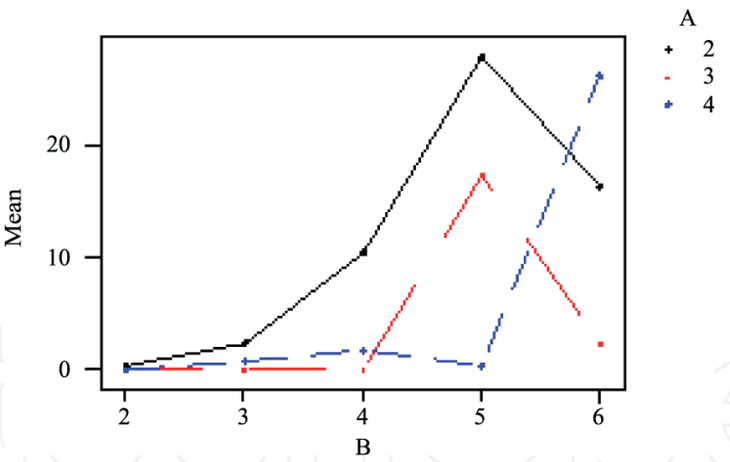

Figure 3.
Interaction of BA and NAA (A2; 0.1 mg/L NAA, A3; 0.5 mg/L NAA, A4; 1.0 mg/L NAA; B2; 0.1 mg/L BA; B3; 0.5 mg/L BA; B4; 1.0 mg/L BA; B5; 3.0 mg/L BAP; B6; 5.0 mg/L BA) (Y axis: The mean number of shoots).

explants cultured on MS medium with 3.0 mg/L BA +0.1 mg/L NAA (**Figure 4**). These 2–3 cm long elongated shoots were transferred to root formation medium, MS without plant growth regulators within 1 week. MS medium containing 0.5 mg/L NAA was also used as root forming medium but on this medium roots were not induced.

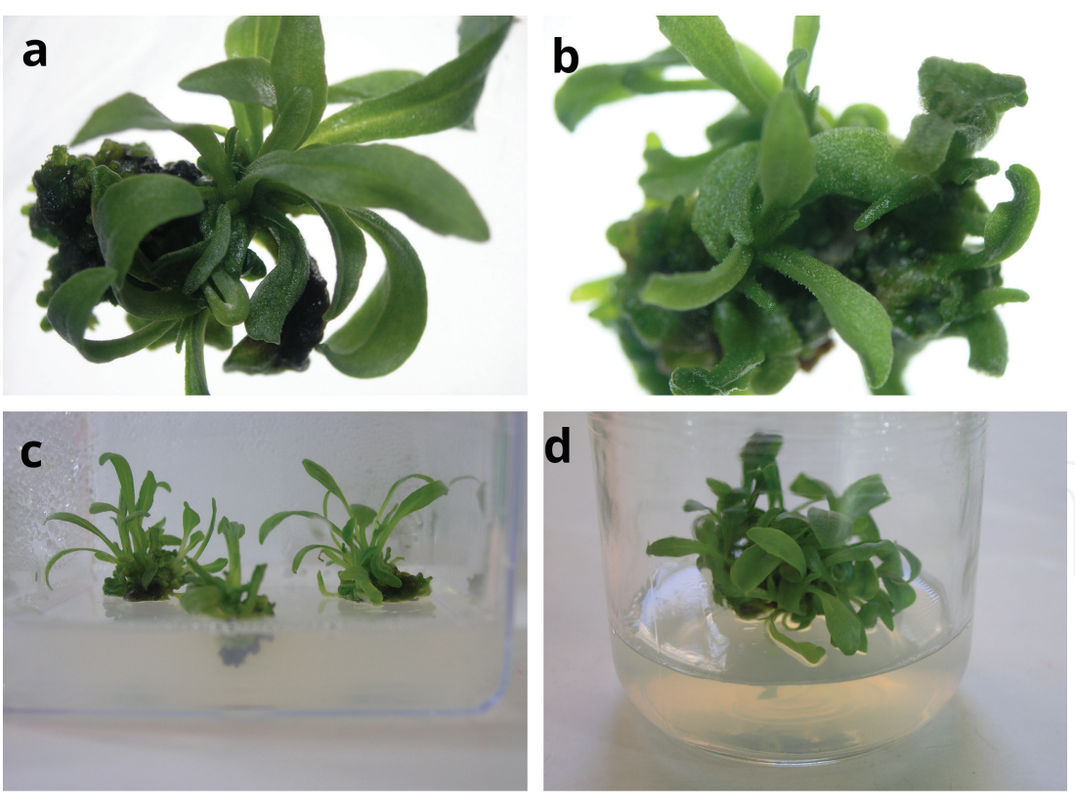

Figure 4.
Multiple adventitious shoots on MS medium with 3.0 mg/L BA + 0.1 mg/L NAA (a-d).

At the end of experimental series of our research, plantlets were adapted to *ex vitro* conditions and then transplanted to plastic flowerpots (**Figure 5**). Finally, plantlets were successfully acclimatized in the greenhouse after they were taken to the plastic cups containing 2 peat:1 perlite (**Figure 6**). All *in vitro* regenerated plantlets were grown healthy.

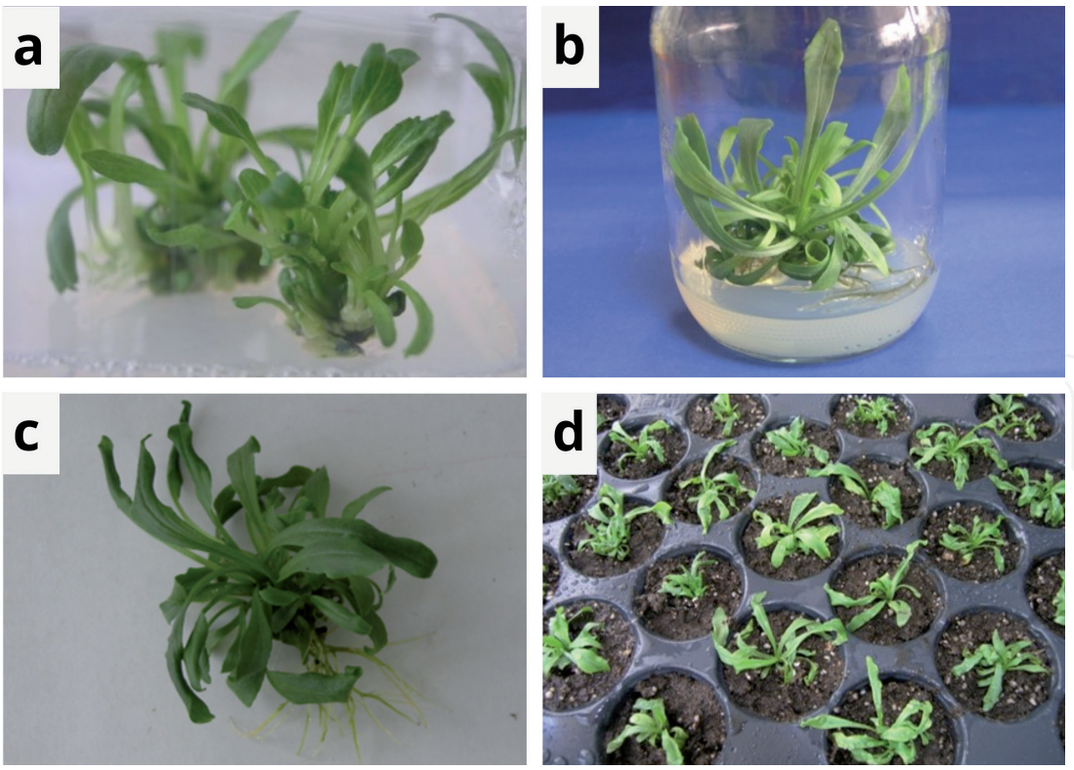

Figure 5.
Regenerated shoots in elongation medium (a), shoots developed their roots (b), regenerated plantlets (c), plantlets with well-developed roots transferred for acclimatization to pots (d).

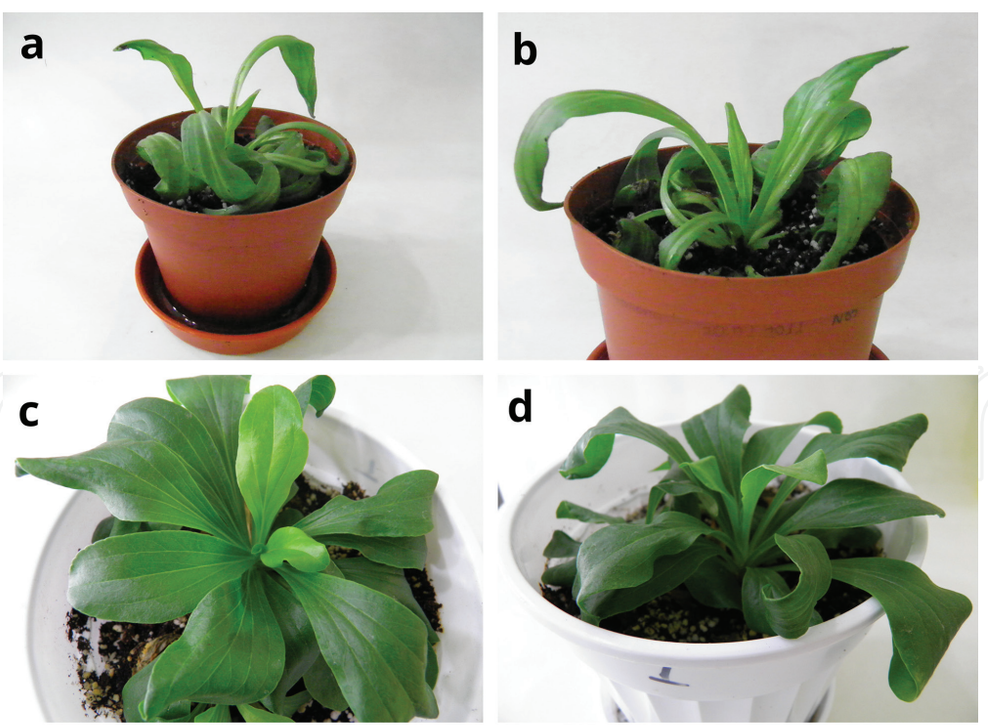

Figure 6.
Plantlets taken to the plastic cups containing 2 peat:1 perlite (a-d).

4. Conclusions

In conclusion, we attained an effective, rapid and high-efficient *in vitro* propagation protocol for mass production of *Digitalis trojana* plants. We showed that leaf

explants can be cultured on MS medium with 3.0 mg/L BA+0.1 mg/L NAA with a satisfactory frequency of plant regeneration of *Digitalis trojana*. The results of this study demonstrate that this protocol is a reliable method for future studies on this endemic species.

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