

IN VITRO PROPAGATION AND CARDIAC GLYCOSIDE PRODUCTION
IN ENDEMIC *DIGITALIS* L. SPECIES OF ANATOLIA

by
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THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE ABANT IZZET BAYSAL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
IN
THE DEPARTMENT OF BIOLOGY

DECEMBER-2011

Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

IN VITRO PROPAGATION AND CARDIAC GLYCOSIDE PRODUCTION IN ENDEMIC *DIGITALIS* L. SPECIES OF ANATOLIA

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December 2011, 99 pages

Members of the genus *Digitalis* L. are medicinally and economically important plants as they contain cardiac glycosides that increase contractions of the heart and regulate heart rhythms. Several methods for high-frequency *in vitro* regeneration of endemic *Digitalis* species of Anatolia were established testing different types of basal medium and explants excised from *in vitro* germinated seedlings. Of those tested medium formulations, Linsmaier and Skoog (1965), supplemented with 0.5 mg/L thidiazuron (TDZ) and 0.25 mg/L indole-3-acetic acid (IAA), was selected mainly for the optimization of later tissue culture protocols of *Digitalis davisina* Heywood, *D. cariensis* Jaub. et Spach em. Werner and *D. trojana* Ivan. For *D. lamarckii* Ivan., benzyl-amino-purine (BA) and naphthylene-acetic-acid (NAA) were more productive in terms of shoot formation.

As an explant source, flamingo-bill type (FBT) explants in general were more productive than other tested hypocotyl, leaf or root explants. In addition to shoot regeneration, callus induction was employed in some species (*D. cariensis* and *D. lamarckii*) on LS or Murashige and Skoog medium (1962) containing various concentrations of BA and NAA. Callus cultures obtained from *D. lamarckii* only was able to produce multiple shoots. Regeneration frequency varied from species to species producing a range of 1 to 9 shoots per explant within 6 or 8 weeks of *in vitro* cultivation periods. Cardenolide profiles of leaf materials of endemic Anatolian *Digitalis* collected from different locations at various vegetation periods (budding, flowering or seed setting) and of tissue culture samples (leaf, complete shoots, etc.) produced *in vitro* were also investigated using two instrumental analysis; thin layer chromatography (TLC) and reverse phase high performance liquid chromatography (RP-HPLC). TLC analysis provides us new insights as a fingerprint pattern of the cardenolides depending on various biotic and/or abiotic factors (physiological ages, different locations or vegetation periods). HPLC analysis was substantiated in terms of quantitative analysis of cardenolides detected by TLC. In that, glucogitorosid content, a major cardenolide, found in our endemic species, was directly correlated with plant growth and development by means of exogenously added growth regulators or seasonal factors as well as the geography where they distributed.

Keywords: *Digitalis davisiana*, *Digitalis cariensis*, *Digitalis trojana*, *Digitalis lamarckii*, plant regeneration, natural populations, cardenolides, TLC, HPLC.

ÖZET

ANADOLU ENDEMİK *DIGITALIS L.* TÜRLERİNİN *IN VITRO* ÇOĞALTIMI VE KARDİYAK GLİKOZİTLERİNİN ÜRETİLMESİ

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Aralık 2011, 99 sayfa

Digitalis L. türleri içeriğindeki kalp glikozitlerinin kalp kası kasılma ve kalp ritmini düzenlemesinden ötürü tıbbi ve ekonomik değeri yüksek olan bitkilerdir. Bu çalışmada birçok doku kültürü uygulaması çoklu sürgün üretme maksadıyla farklı besi ortamları, büyüme düzenleyicileri ve *in vitro* koşullarda çimlendirilen fidelerden izole edilen çeşitli eksplantlar ile yapılmıştır. Sürgün rejenerasyonu, farklı besi ortamları arasından 0.5 mg/L thidiazuron (TDZ) ve 0.25 mg/L indol-3-asetik asit (IAA) içeren Linsmaier and Skoog (1965) besi ortamı, *Digitalis davisina* Heywood, *D. cariensis* Jaub. Et Spach em. Werner ve *D. trojana* Ivan türlerinde başarıyla optimize edilmiştir. *D. lamarckii* Ivan.'da ise büyüme düzenleyicilerinden benzil-amino-pürin (BA) ve naftalen-asetik-asit (NAA)'nın daha başarılı olduğu gözlenmiştir.

Eksplant kaynağı olarak çoğunlukla filamingo gagası tipi eksplantları, kullanılan diğer hipokotil, yaprak veya kök eksplanlarına göre daha başarılı bulunmuştur. Sürgün rejenerasyonuna ek olarak bazı türlerde (*D. cariensis* ve *D. lamarckii*) kallus üretimi çeşitli konsantrasyonlarda BA ve/veya NAA içeren LS veya Murashige ve Skoog (1962) besi ortamlarında çalışılmıştır. Bunlardan sadece *D. lamarckii*'den elde edilen kalluslardan çoklu sürgün üretilebilmiştir. Çalışmalarımızda sürgün rejenerasyonu, türden türe değişmekle beraber, 6 veya 8 haftalık bir kültür sürecinde, eksplant başına 1 ila 9 arasında sürgün üretimi ile farklılıklar göstermiştir. Kardenolit analizleri çeşitli lokasyon ve vejetasyonlardan (tomurcuk, çiçeklenme veya olgun meyve) toplanan yaprak örnekleri ile beraber çeşitli *in vitro* materyaller (yaprak, sürgün vs.) ince tabaka kromatografisi (İTK) ve ters faz yüksek performanslı sıvı kromatografisi (TF-YPSK) kullanılarak tamamlanmıştır. Bu analizlerden İTK analizleri, kardenolitlerin çeşitli biyotik ve/veya abiyotik faktörlere (fizyolojik yaş, farklı lokasyon ve vejetasyon dönemleri) bağlı olarak türler arasında parmak izine benzer sistematik ilişki sergilediğini göstermiştir. YPSK analizleri ile İTK de saptanan kardenolitler nicel olarak da tespit edilmiştir. Bu noktada glukogitorosit miktarı, Anadolu'da yetişen endemik yüksükotlarında en sık rastlanan kardenolit olarak, bitkilerin büyüme ve gelişmesine; yetiştiği coğrafyaya veya doku kültürü ortamına eklenen büyüme düzenleyicilerine bağlı olarak farklılıklar göstermiştir.

Anahtar Kelimeler: *Digitalis davisiana*, *Digitalis cariensis*, *Digitalis trojana*, *Digitalis lamarckii*, bitki rejenerasyonu, doğal populasyonlar, kardenolitler, İTK, YPSK.

This thesis is dedicated to the following scientists for their great contributions to Digitalis research: Klaus Werner, Martin Luckner, Max Wichtl and Mekin Tanker.

ACKNOWLEDGEMENTS

I should convey my heartfelt gratitude to everyone who helped me in the preparation and completion of this research.

I owe a great deal to my supervisor, Prof. Dr. Ekrem Gürel, for his guidance and supervision throughout this study.

I am grateful to my Chefs: Prof. Dr. Wolfgang Kreis and Dr. Frieder Müller-Uri from Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Department of Pharmaceutical Biology for their guidance and technical supports.

Also special thanks go to my wife Sibel Ülkeröglü, Dr. İsmail Eker, Associate. Prof. Dr. Arzu Türker, Dr. Cevher Altuğ and Dr. Muhammet Yıldırım, Dr. Sandeep K. Verma, Dr. Emine Akyüz and Associate. Prof. Dr. Serpil Y. Karakaş for lending a hand wherever they could and for all the wonderful support they gave me.

I hereby deeply appreciate the financial support of TÜBİTAK (The Scientific and Technological Research Council of Turkey) for the project TOVAG-106O470 and the doctoral research program (2214-Fellowship programs).

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CHAPTER 1.

INTRODUCTION

Turkey is one of the most important countries in terms of flora richness intersecting three important phytogeographical regions wherein about 10.000 ferns and seeded plants are present. The significance of this number becomes evident if we compare it with Europe as a whole, containing about 24% more species (about 11500), distributed over a thirteen times larger area. Endemism rate reflected in many countries of Europe are almost equal to the number of species in whole Turkey (Table 1). Currently the genus *Digitalis* L. comprises 23 species including the four species of the former genus *Isoplexis* (Lindl.) Benth. (Bräuchler et al. 2004; Herl et al. 2007; Clemente et al. 2011) (Table 2). A detailed discussion about molecular phylogeny of the genera *Digitalis* was published by Bräuchler et al. (2004). Starting from morphological and bio-geographical data for several relationships of the genera in connection with the molecular aspects were also investigated in the last two decades (Carvalho and Culham 1997, 1998; Nebauer et al. 2000).

Table 1. The number of endemic species distributed in different plant families in flora of Turkey (Avci 2005).

Family Names	Endemic species
Compositae	430
Fabaceae	375
Scrophulariaceae	241
Labiatae	240
Cruciferae	194
Caryophyllaceae	187
Liliaceae	118
Umbelliferae	117
Boraginaceae	108
Rubiaceae	74

In Flora of Turkey (Davis 1978), genus *Digitalis* was represented with 9 taxa belonging to 8 species as follows; (1) *Digitalis cariensis* Boiss. ex Jaub. et Spach, (2) *Digitalis davisiana* Heywood, (3) *Digitalis ferruginea* L. subsp. *ferruginea*, (4) *Digitalis ferruginea* L. subsp. *schischkinii* (Ivan.) Werner, (5) *Digitalis grandiflora* Miller, (6) *Digitalis lamarckii* Ivan, (7) *Digitalis lanata* Ehrh., (8) *Digitalis trojana* Ivan and (9) *Digitalis viridiflora* Lindl. Four of these species used in this study (*D. trojana* Ivan., *D. davisiana* Heywood, *D. cariensis* Boiss. ex Jaub. et Spach ve *D. lamarckii* Ivan.) are endemic to Anatolia (Figure 2).

Table 2. Species of the genus *Digitalis* according to Werner (1965) and Bräuchler (2004).

WERNER (1965)	BRÄUCHLER (2004)	Subspecies.
<i>D. atlantica</i> Pomel	<i>D. atlantica</i>	
<i>D. cariensis</i> Boiss.	<i>D. cariensis</i>	<i>lamarckii</i> <i>trojana</i> <i>cariensis</i>
<i>D. ciliata</i> Trautv.	<i>D. ciliata</i>	
<i>D. davisiana</i> Heyw.	<i>D. davisiana</i>	
<i>D. ferruginea</i> L.	<i>D. ferruginea</i>	<i>ferruginea</i> <i>schischkinii</i>
<i>D. grandiflora</i> Mill.	<i>D. grandiflora</i> Mill.	
<i>D. heywoodii</i> P. et M. Silva	<i>D. heywoodii</i>	<i>mariana</i>
<i>D. laevigata</i> Waldst.	<i>D. laevigata</i> Waldst.	<i>laevigata</i> .
<i>D. lanata</i> . Ehrh.		
<i>D. lutea</i> L.	<i>D. lutea</i> L.	<i>lutea</i> <i>australis</i>
<i>D. mariana</i> Boiss.	<i>D. mariana</i>	
<i>D. dubia</i> Rodr.	<i>D. minor</i>	
<i>D. nervosa</i> Steud.	<i>D. nervosa</i>	
<i>D. obscura</i> L.	<i>D. obscura</i>	<i>obscura</i> <i>laciniata</i>
<i>D. parviflora</i> Jacq.	<i>D. parviflora</i>	
<i>D. purpurea</i> L.	<i>D. purpurea</i> L.	Several ssp.
<i>D. subalpina</i> Br.-Bl.	<i>D. subalpina</i>	Several ssp.
<i>D. thapsi</i>	<i>D. thapsi</i> L.	
<i>D. viridiflora</i> Lindl.	<i>D. viridiflora</i> Lindl.	
<i>I. isabelliana</i> (Webb & Berthel.) Morris	<i>I. isabelliana</i> (Webb) linding	
<i>I. canariensis</i> (L.) Loudon	<i>I. canariensis</i> L.	Several ssp.
<i>I. chalcantha</i> Svent. & O'Shan.	<i>D. chalcantha</i> (Svent. & O'Shan.) Albach, Bräuchler & Heubl	
<i>I. sceptrum</i> (L.) Loudon	<i>I. sceptrum</i> L.	

Digitalis species are either biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are often crowded as basal rosettes. Flowers are zygomorphic and arranged in terminal, bracteate racemes. The calyx is equally five-lobed and shorter than the corolla tube. The corolla, with a cylindrical-tubular to globose tube, is often constricted at the base and the limb is more or less two-lipped. The upper lip is usually shorter than the lower, which is spotted or veined inside. The species are native throughout northern Africa, Europe and parts of Asia (Werner 1965) with main centers of diversity in the western and eastern Mediterranean (Bräuchler 2004).

Members of the genus *Digitalis* are medicinally and economically important medicinal plants since they contain cardiac glycosides, which increase the force of systolic contractions and regulate heart rhythm (Baytop 1999; Kreis and Müller-Uri 2010). Cardiac glycosides are also used as diuretic and in the reduction of oedema due to its ability to improve cardiovascular circulation (Clemente et al. 2011). In addition to the cardiotonic effects of lanatoside C, digoxin and digitoxin, preliminary studies have revealed that digoxin and digitoxin are also effective agents in cancer chemotherapy, in particular for prostate and breast cancer treatments (Yeh et al. 2001; Lopez-Lazaro, 2007; Newman et al. 2008).

Cardenolides are composed of two distinct structures; aglycone and sugar portion. For naming of the compounds, sugar portion is important. As the cardenolides are of commercial interest, studies have focused on the *in vitro* culture of several *Digitalis* species including mainly *D. lanata* and *D. purpurea* (Clemente et al. 2011). In this study for the first time, Anatolian endemic species and their cardenolide profiles using chromatographic techniques (TLC and/or HPLC) in *in vitro* samples and natural populations were investigated (Figure 1 and 2).

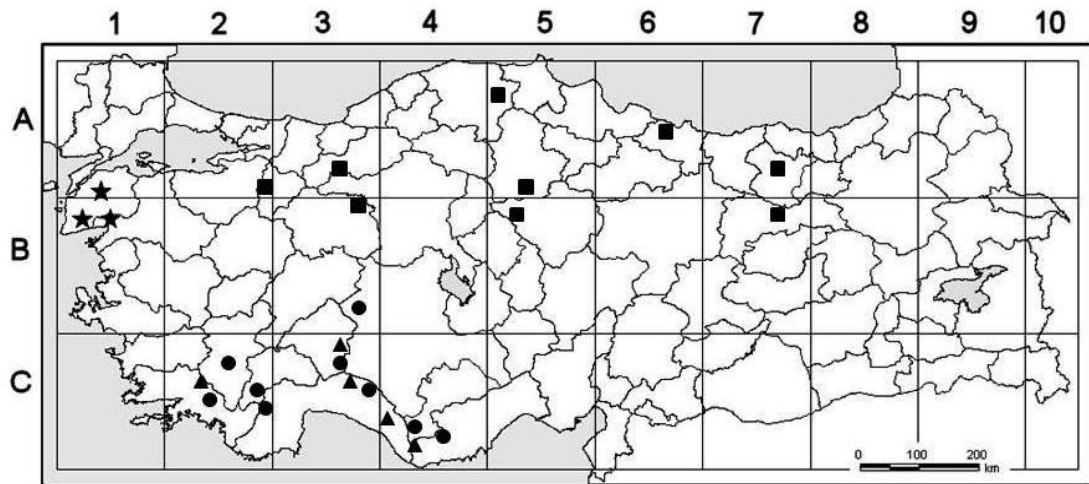


Figure 1. Natural populations of endemic species distributed in Anatolia: *D. trojana* (★), *D. davisiana* (▲), *D. cariensis* (●), *D. lamarckii* (■).



Figure 2. Endemic *Digitalis* species from Anatolia: A) *D. trojana* A1: General morphology, A2: Flowers, A3: Fruits; B) *D. davisiana* B1: General morphology, B2: Flowers, B3: Fruits; C) *D. cariensis* C1: General morphology, C2: Flowers, C3: Fruits; D) *D. lamarckii* D1: General morphology, D2: Flowers, D3: Fruits.

The steps of the cardenolide pathway to the extent of progesterone formation are similar to cholesterol metabolism in animals that do not accumulate cardenolides. However, progesterone reduction is a divergent intersection point of distinct metabolic routes in both animals and plants. There is a need for progesterone double bond reduction in providing the required 5β -configured pregnanes for cardenolide formation. The *Digitalis* enzyme, progesterone 5β -reductase (P5 β R), catalyses the stereo specific NADPH-dependent reduction of the Δ^4 -double bond in progesterone to 5β -pregnane-3,20-dione (Kreis et al. 1998).

During the last two decades, many studies have been carried out on the characterisation of *Digitalis* enzymes that take a role in the biosynthesis of cardenolides (Kreis and Müller-Uri 2010; Müller-Uri and Kreis 2010). Several lines of research could be followed to answer questions about evolution of the cardenolide biosynthetic pathway (Figure 3).

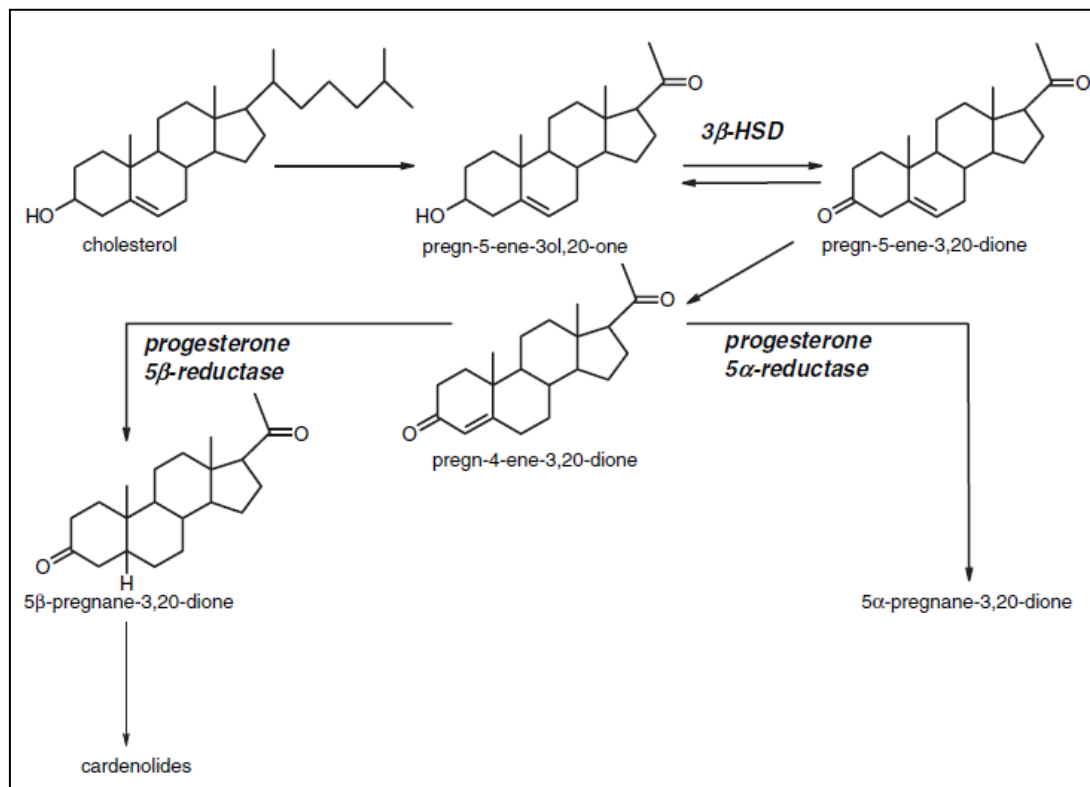


Figure 3. Cardenolide biosynthesis through progesterone 5 β -reductase (P5 β R), according to Kreis et al. (1998).

The *Digitalis* cardenolides are characterized by a steroid nucleus with its rings connected cis-trans-cis, possessing a 14 β -hydroxyl group, and substituted at C-17 β with an unsaturated five-membered lactone ring. At position 3 β a sugar side chain with up to five carbohydrate units is attached, containing glucose and various rare 6-deoxy, 2,6-dideoxy and 6-deoxy-3-methoxy sugars, such as D-fucose, D-digitoxose or D-digitalose. Since many of those sugars are the members of deoxy-metal pentose, they are hardly found in plants. The sugar groups in *Digitalis* are D-position and some are found as acetylated and their glycosides are found at β -configuration. Thus, having these chemical properties provide an inhibition effect on ATPase enzyme

which leads Na^+ and K^+ pumping in heart muscles and regulate heart rhythms (Baytop 1999).

On the other hand, the presence of cardenolides in herbivorous insects for defensive purposes has been reported by several researchers (Daloze and Pasteels 1979; Malcolm et al. 1989; Dobler et al. 2011). For example, monarch butterfly (*Danaus plexippus*) whose caterpillars sequester cardenolides from its apocynaceous host plants (mostly *Asclepias* species) and transfer them into the adult stage. Dobler et al. (2011) also reported that several other aposematic insects feeding on cardenolide bearing apocynaceous species sequester these compounds, e.g. the lygaeid bugs *Oncopeltus fasciatus*. On the otherhand, several clinical researches showed significant observations which led to the search for the presence of digitalis-like compounds (DLC) in mammals originated from sodium homeostasis as natriuretic hormone which act as an endogenous inhibitor of Na^+ , K^+ -ATPase (Nesher et al. 2007).

Plant cell and tissue cultures have been widely used for the commercial propagation of a large number of plant species including many medicinal or ornamental plants (Rao and Ravishankar 2002; Yoshimatzu 2008). Because of continuous deforestation and extensive collection, *in vitro* conservation of medicinal plant germplasm is important to support chemical analysis and genetic improvement studies. As the cardenolides are of commercial interest, studies have focused on the *in vitro* culture of several *Digitalis* species including *D. lanata*, *D. purpurea*, *D. thapsii*, *D. davisiana* and *D. obscura* over the last three decades (Perez-Bermudez et al. 1983; Arrillaga et al. 1986; Brisa and Segura, 1987; Cacho et al. 1991; Pradel et al. 1997; Fatima et al. 2009; Gurel et al. 2010; Verma et al. 2011a, 2011b).

Clinical studies with some *Digitalis* species grown in Turkey show high toxic effects on animal treatments (Table 4). Of those, *D. lamarckii* showed the highest toxicity to the mammals and very recently antimicrobial studies using leaf extracts were investigated in this species (Benli et al. 2009). Among all known *Digitalis* species, *D. lanata* is a main source of glycosides for pharmaceutical industry. Table 3 represents some important cardenolides isolated from *D. lanata* and their lethal dosages. Due to the commercial importance of *D. lanata* in industrial level, many agricultural and biotechnological companies in Germany have investments on large-scale production for cardenolides (Figure 4). Some earlier studies on economic importance of *Digitalis* species grown in Turkey were reported (Tanker et al. 1981; Imre et al. 1982). However, there has been no attempt in terms of the production or breeding of *Digitalis* species in Turkey.

Table 3. Lethal dosages of some commercially important cardenolides.

Cardenolide	Structure	LD₅₀ (mg/kg; lethal dose for Cats)
Neo-glucodigifucosid	Gluc(1→2)-Fuc-A	0.38
Neo-odorobiosid G	Gluc(1→2)-Dtl-A	0.97
Gluco-verodoxin	Gluc-Dtl-E	0.15
Digoxin	Dx-Dx-Dx-C	0.68
Digitoxin	Dx-Dx-Dx-A	0.32
Strospesid	Dtl-B	0.41

Table 4. *Digitalis* species grown in Turkey and their clinical use on cats (from Baytop, 2002).

Species	LD ₅₀ (g/kg; lethal dose for Cats)
<i>D. davisiana</i>	0.99
<i>D. ferruginea</i>	0.053
<i>D. grandiflora</i>	0.033
<i>D. lamarckii</i>	0.025
<i>D. schishkinii</i>	0.072
<i>D. vidriflora</i>	0.087



Figure 4. Harvesting *D. lanata* for large-scale cardenolide production (from Luckner and Wichtl 2000).

1.1. The objectives of the study

This study provides a new insight for plant production from endemic Anatolian *Digitalis* species via tissue culture techniques for large-scale production as well as determination of their cardenolide profiles. Within this framework, eastern *Digitalis* species including *D. cariensis*, *D. lamarckii*, *D. trojana* and *D. davisiana*, for the first time, were studied, with a special reference with the discovery of cardenolide pathway(s) in *Digitalis*. In this view of point, we aimed at (1) establishing efficient tissue culture protocols for each species, which would enable a large scale production of cardenolides without disturbing their natural populations; (2) determining their cardenolide profiles; (3) understanding the cardenolide biosynthesis in relation to certain physiological and environmental factors (biotic and abiotic) under both *in vitro* and *ex vitro* conditions and finally (4) providing a new way of understanding the taxonomy of the species underlying their cardenolide patterns so as to make them chemotaxonomical markers.

In addition to endemic *Digitalis* species of Anatolia, *D. ferruginea* subsp. *ferruginea*, *D. ferruginea* subsp. *schischkinii* and *D. lanata* were also studied for biochemical analyses, and their cardenolide contents were compared with the endemics.

CHAPTER 2.

MATERIAL AND METHODS

3.1. Field trips

All endemic *Digitalis* species given below were collected according to the Flora of Turkey (Davis 1978) from different locations and vegetation periods (budding, flowering and fruit setting) in 2007 and 2009. Voucher specimen of the species were deposited in herbarium of AIBU.

3.1.1. Collecting *D. davisiana*

We could find only one location in Antalya for natural populations of *D. davisiana*, commonly known as Alanya foxglove, between Alanya and Hadim, after 17.5 km Hadim at 1100 m, N 36° 31.915' E 032° 14.383', Eker 1729, B. Yücesan, E. Gürel.

3.1.2. Collecting *D. cariensis*

Two different locations for *D. cariensis* commonly known as Mugla foxglove, were found in Antalya.

Location 1: Between Alanya and Taşkent, 12 km after Mahmutlar, roadside, nearby pinus populations at 981 m altitute, İ. Eker 1727, B. Yücesan, E. Gürel (fruit); ibid., 16 km after Mahmutlar, 1041 m, N 36⁰ 31.541¹ E 032⁰ 13.367¹, Eker 1888, B.Yücesan (flowers); Between Gülnar and Aydıncık, after 2 km through Aydıncık, roadside, nearby pinus populations, at 670 m, N 36⁰ 16.752¹ E 033⁰ 23.355¹, 23.04.2008, Eker 2027 (budding).

Location 2: Between Manavgat-Akseki, after 80 km from Manavgat, roadside, at 1299 m, N 37⁰ 05.38¹ E 031⁰ 46.07 (fruit), B. Yücesan.

3.1.3. Collecting *D. trojana*

Two different locations of *Digitalis trojana* Ivan., commonly known as Helen of Troy, were found in Çanakkale and Balıkesir.

Location 1: Balıkesir, at Ida Mountains, between Mehmetalan Köy and Yayala, at 500 m, I.Eker 1730, (fruit); ibid. 5 km after Zeytinli through Mehmetalan, roadside, open forest, at 250 m altitute, N 39⁰ 38.615¹ E 026⁰ 57.552¹, 08.06.2007, Eker 1905, B. Yücesan (flowers); ibid., National Park of Ida Mountains, at 345 m, N 39⁰ 38.885¹ E 026⁰ 57.402¹, Eker 1907, B.Yücesan (flower); ibid., 435 m, N 39⁰ 39.650¹ E 026⁰ 57.578¹, Eker 2435 (flower).

3.1.4. Collecting *D. lamarckii*

Digitalis lamarckii Ivan. (Syn. *D. orientalis*), known as dwarf foxglove, is a least concern endemic species. Plant materials were collected from three different locations.

Location 1: Bolu, between Mudurnu and Nallıhan, 8 km through Nallıhan, pinus trees, roadside, Eker 1726, B. Yücesan, E. Gürel (fruit).

Location 2: Ankara, between Gerede and Kızılcahamam, Akyarma, 33 km through Kızılcahamam, nearby pinus populations, roadside, Eker 1790, B. Yücesan, E. Gürel (budding, flowering and fruits), at 1520 m altitute, N 40⁰ 32.71¹ E 032⁰26.026¹.

Location 3: Konya, Between Beysehir and Derbucak, Huglu, N 37⁰ 27.894¹ E 031⁰33.808¹ at 1353 m (budding), Eker 2611

3.2. Growth media preparation and tissue culture studies

All tissue culture studies described below were conducted in *in vitro* under sterile conditions as follows: surface sterilization and germination of seeds, preparation of regeneration media, explant choice, transferring explants to regeneration media and non-sterile hardening off studies (*ex vitro*).

3.2.1. Laboratory facilities

Surface sterilization, explant choice and preparation, transferring explants to the suitable basal media were carried out in a laminar flow. Culture tubes or Petri plates were kept in a growth chamber for incubation; the room was equipped with an air conditioner to keep the ambient air at 23 °C and a humidifier to keep a constant humidity at 55-60%. For the light regime, a 16 hours light and 8 hours dark photoperiod provided by cool-white fluorescent light irradiance ($50 \text{ m mol}^{-2} \text{ s}^{-1}$) was used. For greenhouse conditions, different sizes of pots filled with commercial compost were used after autoclaving for further plant growth and development at unstable temperatures varying between 20 and 25 °C at low humidities (20-30%) under solar light (day light).

3.2.2. Seed sterilization

Seeds were surface sterilised by dipping into 50 mL of 20% commercial bleach (Domestos ®) with a few drops of Tween 20 in a 250 mL beaker and stirring at 250 rpm on a magnetic stirrer plate for 10 min, followed by rinsing with sterile distilled water several times. For germination, an average of 20-25 seeds were aseptically cultured in Petri dishes (90 × 15 mm) containing 30 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose. The medium was solidified with 0.8% (w/v) agar and autoclaved at 121 °C and 1.06

kg/cm² pressure for 15 min after adjusting the pH to 5.8 with 0.1 N HCl or 0.1 N KOH.

3.2.3. Medium preparation

In vitro studies were carried out using up to six different basal medium formulations as follows; **MS** (Murashige and Skoog, 1962), **LS** (Linsmaier and Skoog, 1965), **B5** (Gamborg et al. 1968), **SH** (Schenk and Hildebrandt 1972), **CP** (Chee and Pool 1987) and **MM** (Huang and Murashige 1976). Of those, LS, MS or B5 were frequently used for *in vitro* studies of *D. lamarckii*, *D. trojana* and *D. cariensis* and other media were restricted to the some experiments with *D. davisiana* only.

In addition to the medium types, different plant growth regulators (PGRs; auxin and/or cytokinin) were also tested. Thidiazuron (TDZ), one of the most useful cytokinin-like PGR, was used at various concentrations (ranging from 0.1 to 1.0 mg/L) in regeneration experiments. Additionally, benzyl-amino purine (BA) was selected as an alternative cytokinin (ranging from 0.5 to 2.0 mg/L) for some experiments. Cytokinins were either used alone or in combination with auxins, including indole acetic acid (IAA) or naphtylene acetic acid (NAA) at 0.25 to 1.0 mg/L concentrations.

3.2.4. Preparation of explants

For *in vitro* studies, cotyledonary leaf (25 mm²), mature leaf (30 mm²), hypocotyl (0.5-1.0 cm in length), root (0.5-1.5 cm in length) and/or flamingo bill-type (FBT) of explants were used. Latter explant (FBT) was prepared as described by earlier reports (Pozueta-Romea et al. 2001) root tissue, one cotyledon leaf and the shoot-tip meristem of the seedling were removed; remaining hypocotyl with one cotyledon was used as an explant (Figure 5). All tested explants throughout the studies were excised from *in vitro* germinated seedlings whose ages up to 6 weeks old. For cardenolide analysis, seeds of all tested endemic *Digitalis* species were planted into the pots, and were kept in greenhouse conditions for 8 months.



Figure 5. A general view of a flamingo-bill type (FBT) explant.

3.3. Transferring the explants to regeneration media

The explants excised from *in vitro* germinated seedlings were transferred to either callus or regeneration media. Tissue culture protocols were described below in detail for each species. Experimental data were evaluated in the Results and Discussion part.

3.3.1. Shoot regeneration and rooting of *D. davisiana*

Following germination *in vitro*, four different types of explants (hypocotyl, leaf, root and FBT explants) excised from 2- to 3-week-old seedlings were used in two sets of experiments. The first set was designed to determine the best hormonal composition for rapid regeneration, using full-strength B5 medium, supplemented with different concentrations (0.1, 0.25 or 0.5 mg/L) of TDZ alone or in combination with 0.25 or 0.50 mg/L IAA (Table 6). In the second set, different basal medium formulations were compared at full-strength, supplemented with 0.5 mg/L TDZ and 0.25 mg/L IAA: 3.16 g/L B5 (Gamborg et al. 1968), 4.40 g/L MS (Murashige and Skoog 1962), 4.53 g/L MM (Huang and Murashige 1976), 4.40 g/L LS (Linsmaier and Skoog 1965), 4.46 g/L CP (Chee and Pool 1987), and 4.19 g/L SH (Schenk and Hildebrandt 1972). All media formulations and plant growth regulators (PGRs) were purchased from Duchefa Biochemie (Netherlands). Mean number of shoots per explant and frequency of organogenic explants (%) were recorded after 6 weeks of incubation. Culture tubes (175 x 25 mm) or Petri plates (90 x 15 mm) were used for regeneration. Each treatment used 10 explants; experiments were carried out in triplicate with 30 replicates per treatment.

For rooting and hardening of regenerants prior to transferring to rooting medium, regenerated shoots (approximately 2 cm) were cultured for an additional 2 weeks in

Magenta vessels (GA-7; Sigma), containing full-strength LS medium without PGRs. Shoots were removed singly and placed in Magenta vessels with full-strength LS medium supplemented with IAA (0.1 or 0.5 mg/L) or indole-3-butyric acid (IBA) (0.1 or 0.5 mg/L). Frequency (%) of shoots that rooted and the number of roots per shoot were recorded after 4 weeks in culture. Each rooting treatment used 10 shoots; experiments were performed in triplicate with a total of 30 replicates per treatment. To remove agar, rooted plantlets (5–6 cm) were washed with distilled water and then either (1) transferred to sterile compost for hardening or (2) cultured for an additional 2 or 4 months on full- or half-strength LS medium with no PGRs (for more details see Table 24). The latter group of material was used for cardenolide extractions.

3.3.2. Shoot regeneration and rooting of *D. cariensis*

After a longer germination period (4-6 weeks) compared to the other species, regeneration studies were carried out using different explant types either from young plantlets (2-3 week old) or from mature ones (six-weeks old or more). For adventitious shoot regeneration, explants were transferred to the LS medium containing 0.5 mg/L TDZ alone or in combination with 0.25 mg/L IAA for six-week incubation. On the other hand, axillary parts of seedlings were also used in some experiments wherein TDZ (0.1-1.0 mg/L) alone or in combination with IAA (0.25 or 0.50 mg/L) were treated. Regenerated shoots were kept in Magenta boxes containing LS media without a growth regulator for additional two weeks prior to rooting stage.

Rooting stage of the shoots were carried out using various concentrations of IAA or IBA (0.1, 0.5 or 1.0 mg/L) or control group without PGR. After 5-6 weeks, some of the rooted shoots, namely regenerants, were transferred to the pots containing compost for acclimatization process, while remaining shoots were subcultured in

different strengths (half- or full-strength) of LS medium for ongoing experiments *in vitro*. For the callus culture, various concentrations of BA (0.5, 1.0, 1.5 or 2.0 mg/L) and/or NAA (0.1, 0.5 or 1.0 mg/L) were used in MS medium. In this experiment, only leaf explants were treated for callusing for an eight-week cultivation period.

For regeneration studies, 10 explants per Petri plate (9 x 25 cm) were used, and each was repeated thrice (i.e., 10x3=30 explants per treatment). For rooting experiment, two shoots were used for each Magenta or jar and repeated 10 times (i.e., a total 20 shoots per treatment).

3.3.3. Shoot regeneration and rooting of *D. trojana*

Following germination *in vitro*, two different types of explants (hypocotyl or FBT) excised from 2- to 3-week-old seedlings were used in two sets of experiments. The first set of experiments were designed to determine the best hormonal composition for rapid regeneration of explants tested, using full-strength LS medium supplemented with different concentrations (0.1, 0.25, 0.5 or 1.0 mg/L) of TDZ alone or in combination with IAA (0.25 or 0.50 mg/L). In the second set, three different basal medium formulations (B5, MS or LS) supplemented with 0.5 mg/L TDZ alone or in combination with 0.25 mg/L IAA were compared. Mean number of shoots per explant and frequency of organogenic explants (%) were recorded after six weeks of culture. Plastic Petri plates (9.0 cm x 2.5 cm) were used for regeneration. Each treatment used 10 explants; experiments were carried out in triplicate (i.e., a total of 30 replicates per treatment).

Before transferring to rooting medium, regenerated shoots (*ca.* 2 cm in length) were cultured for an additional two weeks in Magenta boxes containing LS medium containing no PGRs. Shoots were singled out and placed in Magenta boxes

containing LS medium supplemented with IAA (0.1 or 0.5 mg/L) or IBA (0.1 or 0.5 mg/L). Frequency (%) of shoots that rooted and the mean number of roots per shoot were recorded after six weeks in culture (Table 14). Each rooting treatment used 10 shoots; experiments were performed in triplicate. To remove agar, rooted plantlets (5-6 cm) were washed with distilled water and transferred to Magenta boxes containing vermiculate under sterile conditions for four weeks, then transferred to pots containing compost.

3.3.4. Shoot regeneration and rooting of *D. lamarckii*

After germination, FBT and hypocotyl explants excised from 2-3 week-old seedling were used in several experiments using LS media containing various concentrations of BA (0.5, 1.0 or 2.0 mg/L) alone or in combination with NAA (0.5, 1.0 or 2.0 mg/L). When the regenerants were 6 -7 week old and looked healthy, they were moved to rooting media (with or without PGR) as described in *D. trojana*. There was only one exception that NAA was used as an auxin instead of IBA at the same concentrations as that of IAA (1.0, 2.5 or 5.0 mg/L). The effects of high concentration of PGR were also tested in one experiment in which BA (1.0, 3.0 or 5.0 mg/L) and NAA (1.0, 3.0 or 5.0 mg/L) were used for callus induction from hypocotyl and cotyledonary leaves after six weeks of culture. In that experiment, some organogenic callus that developed was transferred to the LS medium containing various concentrations of BA (1.0, 3.0 or 5.0 mg/L) and/or GA₃ (0.25 or 0.50 mg/L).

Regeneration studies were conducted using five cotyledonary leaves, and repeated thrice. Callus that produced in the last experiment where a higher concentration of BA and NAA used was weighed around 250-300 mg and transferred to the Petri plates (BA and/or GA₃) for indirect shoot regeneration (i.e. four massive callus for each plate; experiment was repeated thrice). Rooting process was the same as described above for *D. trojana*.

3.3.5. Calculations for tissue culture studies

Data were statistically analyzed using Microsoft Excel (Office 2007, ToolPack Analyser) and SPSS ,Version 17.0 (SPSS Inc., Chicago, IL, USA). One- or two-way analysis of variance was used to calculate statistical significance and the significance of difference among the means was determined using Tukey's test at 0.05 probability.

3.4. Plant extractions and analytical experiments

In this study, cardenolide extraction and their acid hydrolysis were described in detail. Optimization of TLC and HPLC analysis, various reference substances of cardenolides are shown as below.

3.4.1. Cardenolide extraction

Microextraction of cardenolides was slightly modified from a previously published protocol (Wiegrebe and Wichtl 1993), in which leaf materials (fresh or dried) were ground to fine powder (fresh leaves were ground in liquid nitrogen) with a pestle and mortar. After that about 300 mg fresh leaf powder (or 50 mg dry material) was transferred to the centrifuge tube containing 1 ml of 70% Methanol. After 30 min treatment in an ultrasonic cleaning bath at 65–70 °C, the extract was rapidly cooled

on ice for 3 min and then centrifuged for 10 min at 13,000 rpm. The supernatant was thoroughly mixed with 0.25 ml of 15% lead acetate solution (w/v) and centrifuged. After elucidating the lead acetate residue, 0.5 ml of 4% monosodium phosphate was added and centrifuged. The supernatant was transferred to 2 ml centrifuge tubes, diluted to a final volume of 2 ml with water and then centrifuged at room temperature (RT) for 8 min at 13,000 rpm. The supernatant was mixed with 0.5 ml chloroform:isopropanol (3:2) and centrifuged for 5 min as before. The lower phase was transferred to 2 ml centrifuge tubes as the first extraction. The remaining methanolic solution was used for the second extraction by adding chloroform:isopropanol and centrifuging at RT for 5 min at 13,000 rpm. Both of extractions were mixed and evaporated under an air flow chamber for 3 hours and finally dissolved in 500 μ l methanol (HPLC grade). Thin layer chromatography (TLC) was performed on TLC plates (10x20 cm silica gel 60W, Merck, Germany) after being loaded 50 μ l of each sample. TLC was run in a glass chamber containing mobile phase as follows chloroform: metanol: water (81:18:1). After 10-25 min, plate was taken out from the chamber and sprayed with Jensen-Kny's reagent to identify the cardenolide groups depending on coloration of the bands at 120 °C for 10 min (Jork et al. 1990). The spots appeared on the chromatogram at 366 nm UV light as yellow, green or blue bands refer to A, B or C-type of cardenolides (Figure 6).

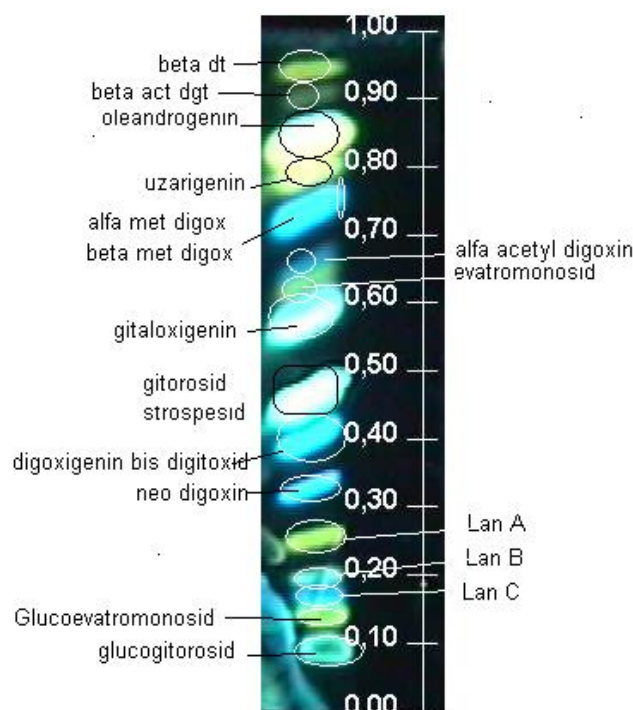


Figure 6. List of reference substances according to their R_F values (ranging from 0.0 to 1.0) given at the right margin. Mobile phase was chloroform:methanol:water (81:18:1).

3.4.2. Acid hydrolysis

Acid hydrolysis is used for better understanding for the aglycone portion of the cardenolides. Following steps, in terms of acid hydrolysis, were done according to Eissenbeiß (1987): After an efficient cardenolide extraction (method mentioned above), final residue dried in vacuo in centrifuge tubes was dissolved in a 240 µl of acetone: HCl (1:100) solution (HCl must be concentrated as 32%). After 1 min shaking in vortex, samples were put in an ultrasonic bath for 5 minutes and then followed by centrifugation at 13,000 rpm for 10 minutes. Then, they were kept in darkness at RT for about 12 hours. After that, 102 µl of saturated sodiumhydrogencarbonate (NaHCO₃) solution was added to each tube (this step stops the hydrolysis of genin). Vortex and centrifugation steps were followed as

before, the supernatant was pipetted out, and then saved in a new tube. Remaining residue washed with 1 ml acetone followed by vortex and centrifuge again. After repeating the last two steps, all three supernatants were collected together in larger centrifuge tubes (15 ml). The solution including 4 ml distilled water and 5 ml chloroform was added to each of these large centrifuge tubes. Then, the samples were vortexed and centrifuged again at highest speed (13,000 rpm). Finally, organic phase was evaporated into dryness prior to addition of 500 μ l HPLC grade MeOH.

3.4.3. HPLC determination

Two different HPLC systems were carried out using a previously published protocol (Wiegrebe and Wichtl, 1993), which was modified for HPLC determination.

System 1: Chromatography was at a flow rate of 1.2 ml/min with a binary pump solvent delivery system, a dual λ absorbance detector operating at 220 nm and 350 nm, an autosampler (Waters Autosampler 717 Plus) injecting 20 μ l of each sample. For column, Reprosil-Pur C18 AQ, 5 μ m, 250 mm x 4 mm for reverse phase (RP)-HPLC were used. Cardenolides were eluted with acetonitrile (A) and water (B) gradients as follows: 0–20 min 20% (A), 80 % (B); 20–27 min 32% (A), 68% (B); 27-35 min 58% (A), 42 % (B); 35-40 min 60% (A), 40% (B); 40-60 min 0% (A), 100% (B); 60-65 min 20% (A), %80 (B).

System 2: Chromatography was at a flow rate of 1.2 ml/min with a column temperature of 40 °C on an HPLC with a binary pump solvent delivery system, a UV-DAD detector operating at 220 nm, a microvacuum degasser, a manual injector and an Hewlett Packard model integrator (Agilent 1100 series; Germany) were used. For columns, ZORBAX Eclipse XDB-C18 (4.6 x 150 mm) for reverse phase (RP)-HPLC was used throughout. A 20 µl aliquot of pretreated extract (as described above) was used for analysis. Cardenolides were eluted with acetonitrile (A) and water (B) gradients as follows: 0–20 min 20% (A), 80% (B); 20–30 min 30% (A), 70% (B); and 30–40 min 40% (A), 60% (B). Average peak area of the glycoside in samples were automatically calculated and monitored by ChemStation LC/MS software against that of standards. HPLC runs were repeated three times for every sample group.

3.4.4. Mass spectrometric determination of cardenolides

This system was used to verify standard substances which did not exist in our cardenolide stock for plant extracts. In this case, neo-digitalinum verum, neo-glucodigifucosid, glucolanadoxin, neo-odorobiosid, purpurea glycosid B and glucoevatromonosid were detected by LS-MS analysis. In order to find certain retention times which corresponds with our HPLC system-1, plant extracts were fractionated every 2 minutes into 2 mL centrifuge tubes. Every fractions corresponded to the retention time versus molecular weight under different HPLC system as below. Cardenolides after detection with LS-MS was also confronted with TLC analysis which had been already done before by Tanker et al. 1981 and, HPLC analysis reported by Wiegrebe and Wichtl 1993.

LC-MS/MS analyses (liquid chromatography and mass spectrophotometric analysis) were carried out on a Dionex (Idstein, Germany) Ultimate 3000 UPLC system, comprising a SRD-3400 degasser, an HPG-3400 RS binary pump, a WPS-3000 TRS autosampler and a TCC-3000 RS column compartment. The UPLC was connected to a 4000 QTrap mass spectrometer from ABSciex (Darmstadt, Germany). LC parameters were determined as follows: The plant extract (in methanol) was diluted twenty fold in water. An aliquot of 5 μ l was injected and separated on a YMC Ultra HAT Pro C18 column (2 x 50 mm, 2 μ m), running the following gradient: 0 min 20 % B; 7 min 25 % B; 13 min 60 % B; 13.5 min 95 % B; 15 min 95 % B, using 0.1 % formic acid as solvent A and acetonitrile as solvent B. Before each injection, the system was equilibrated for 5 min with 20 % B. Flow was set at 0.5 ml/min.

MS parameters were set as follows: temperature 300 °C, gas 160 psi, gas 275 psi, curtain gas 40 psi, ion spray voltage 5500 V, declustering potential 50 V, entrance potential 10 V; scan range m/z 350-750 or 750-1150 Da, cycle time: 2 sec. Q1 resolution was set at unit resolution.

3.4.5. Calculations for analytical studies

In system 1, HPLC calibration was made according to different internal standard (IS, β -methyl-digitoxin) concentrations (ranging from 1 to 100 ppm). Regression (R^2) was determined as 0.97 and method recovery with spike method (IS added to plant material) was calculated 82% (n=10, 100 mg/L IS, Table 4). Since β -methyl-digitoxin was not found in *Digitalis* samples, and it has the highest lipophilic compound among the cardenolides, β -methyl-digitoxin was used as internal standard in many analytical experiments (Table 5).

Table 4. Different concentrations of internal standard (IS) with corresponding areas and standard deviations (SD).

IS Concentration	Area	SD
1 ppm	9988.2	975.8
5 ppm	52746.9	5625.2
10 ppm	106723.2	9958.1
25 ppm	268892.7	23451.8
50 ppm	526489.2	514254.7
100 ppm*	1054918.3	895651.5

*Method and injection recovery were done at this concentration, method recovery was 82% and injection recovery 96% (n=10).

Concentrations of tested cardenolides were calculated using the molecular weight ratio ($MW_{IS}/MW_{\text{cardenolide}}$) which was multiplied by the area obtained from that of particular cardenolide. Then, concentrations were formulated according to *R* value.

In system 2, cardiac glycoside contents were calculated separately using calibration curves of pure standards of jointly estimated lanatoside C and digoxin. For the calibration curves, concentrations of 2.5, 5.0, 10, 20, 30, 40 and 50 mg/L lanatoside C and digoxin were used (n=3 for each concentration, R values were 0.98 and 0.99 for lanatoside C and digoxin, respectively). Table 5 shows retention times (RT), respective peak areas and limit of detection (LOD) values.

Table 5. Some analytical calculations for cardenolides. Retention time (RT), peak area and limit of detection (LOD). (n=10, SD: standart deviation)

Cardenolide	RT±SD	Area±SD	LOD (ppm)
Digoxigenin	5.412±0.035	163.988±10.030	0.20
Gitoxigenin	13.497±0.070	151.394±14.879	0.30
Lanatoside C*	18.483±0.105	58.978±7.262	0.40
Digoxin	19.099±0.106	86.953±10.542	0.38
Digitoxin	31.454±0.057	92.823±14.329	0.50

*Recovery calculated was 85% using Lan C at 10 ppm in plant extracts (n=3).

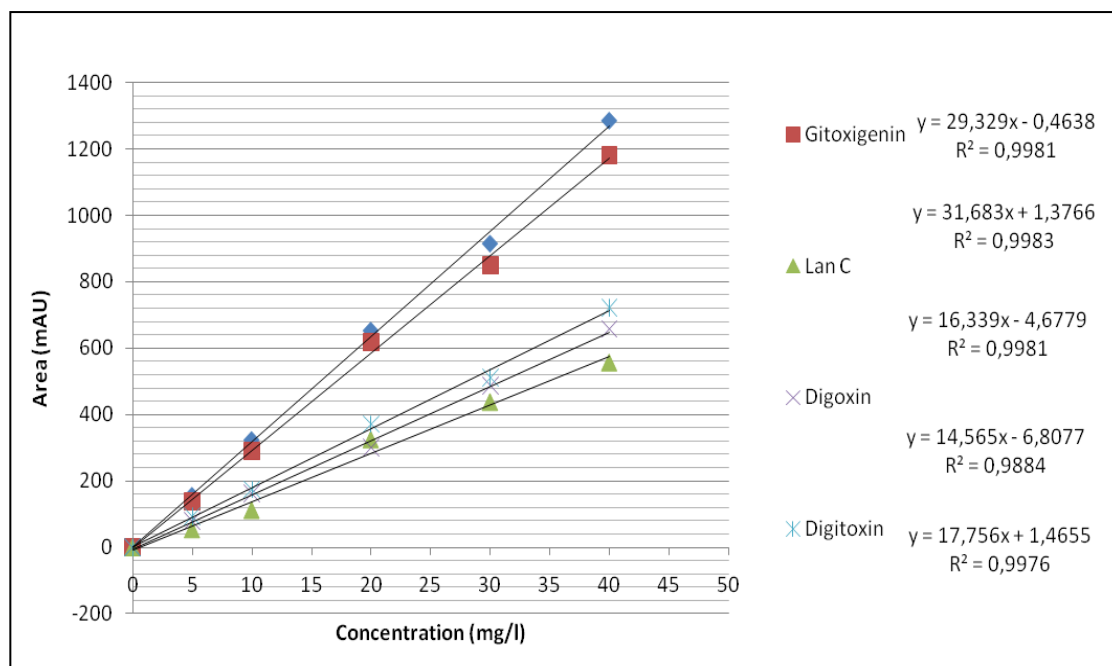


Figure 7. Regression formulas of various cardenolides tested in system 2.

CHAPTER 3.

RESULTS

5.1. *In Vitro* Regeneration Studies

5.1.1. Regeneration of *D. davisiana* and rooting

For the first time, this study reports a high-frequency *in vitro* plant regeneration protocol for *D. davisiana* and an assessment of cardiogenic glycoside production in *in vitro* produced materials (regenerated or germinated), as well as in leaves of natural populations. To develop an efficient *in vitro* regeneration protocol, two sets of experiments were carried out. In the first, different concentrations and combinations of TDZ and IAA were compared using B5 medium (Table 6), while in the second set, comparisons were made with six different media formulations (B5, MS, MM, CP, LS and SH) that were supplemented with the optimal PGR combinations (0.5 mg/L TDZ and 0.25 mg/L IAA) identified in the first set of experiments (Table 7). For both sets of experiments, four different types of explants (hypocotyl, leaf, root and FBT) were excised from 2- to 3-week-old seedlings germinated *in vitro*. There was a significant variation in shoot formation capacities between explant types, hormonal combinations and basal medium formulations. When the mean number of shoots per explant is considered, PGR-free medium and those media supplemented with TDZ alone or combined with lower TDZ (0.1 or 0.25) and IAA (0.25 or 0.5 mg/L) were found to be somewhat less effective in supporting shoot induction (Table 6).

When medium containing 0.5 mg/L TDZ and 0.25 mg/L IAA was employed, higher frequencies of explants produced shoots with significantly greater mean numbers of shoots per explant. In this medium, 73.3% of hypocotyl and 76.6% of FBT explants produced shoots with a mean of 6.3 and 5.9 shoots per explant, respectively. In general, TDZ at 0.50 mg/L was considerably more effective than at lower concentrations (0.10 or 0.25 mg/L), irrespective of whether it was used alone or in combination with IAA. With respect to using different explant types to determine shoot regeneration capacity, hypocotyl and FBT explants were almost equally as effective and were much more productive than leaf or root explants (Table 6; Figure 8a, b). The highest frequency (86.6%) of organogenic explants was achieved with FBT explants, and the highest mean number of shoots per explant (6.3) with hypocotyl explants. Using four different types of explants, six different basal media (B5, MS, MM, CP, LS and SH), supplemented with 0.5 mg/L TDZ and 0.25 mg/L IAA, were compared as to their effects on shoot regeneration (Table 7). When the treatment means were compared, LS medium was significantly more effective than others. When cultured on LS medium, FBT explants produced as many as 8.1 shoots per explant with 80% frequency of organogenic capacity, while hypocotyl explants produced 7.8 shoots with 73.3% frequency. B5 medium ranked second in terms of both number of shoots per explant and frequency of organogenic explants. Of the other media tested, MS, MM and CP were moderately effective while SH medium was least, producing the lowest numbers of shoots per explant (from 0.1 to 2.3 shoots). As shown in Table 6, hypocotyl and FBT explants were almost equally as productive, with significantly more shoots per explant and higher frequencies of organogenic explants than both leaf and root explants.

Callus formation patterns also differed depending on the medium type. FBT or hypocotyl explants cultured on LS or B5 medium produced little or no callus while the same explants produced large amounts of callus when cultured on CP medium (data not provided). It is noteworthy that leaf explants produced shoots only on the petiole, not on the lamina.

It was clear that hormonal composition of the culture medium was a significant factor in the efficiency of shoot regeneration, irrespective of explant type (Table 6). When means of different explant types were considered, it was evident that increasing concentrations of TDZ, from 0.1 to 0.5 mg/L, steadily improved shoot numbers; however, considerably higher numbers of shoots were obtained when TDZ was combined with IAA. Of the combinations examined, a higher TDZ level (0.5 mg/L), combined with lower IAA levels (especially 0.25 mg/L), was most effective for shoot regeneration from all explant types, particularly those from hypocotyl and FBT explants, which produced a mean of 6.3 and 5.9 shoots per explant, respectively. In the present study, FBT explants were found to be more productive than leaf or root explants, and almost equally as effective as hypocotyl explants.

All regenerated shoots were rooted readily when cultured for 4 weeks on LS medium containing either no PGRs or two different concentrations (0.1 or 0.5 mg/L) of IAA or IBA (Figure 8c). Irrespective of concentration, IAA was more effective than IBA (Table 8); however, for both auxins, higher concentration (0.5 mg/L) produced more roots than the lower (0.1 mg/L); 8.1 roots per shoot versus 4.9 for IAA, and 6.1 roots per shoot compared to 3.2 for IBA. Considerable rooting (4.6 roots per shoot) was also observed on medium containing no PGRs. Throughout the hardening process, 75–80% of the regenerants survived (Figure

8d). There was no problem with rooting plantlets for this species as all regenerated shoots were rooted easily, even in the absence of PGRs (Table 8). However, in terms of mean number of roots per shoot, both auxins (IAA or IBA) tested were more effective when higher concentrations (0.5 mg/L vs. 0.1) were used; in general, IAA was more effective than IBA.

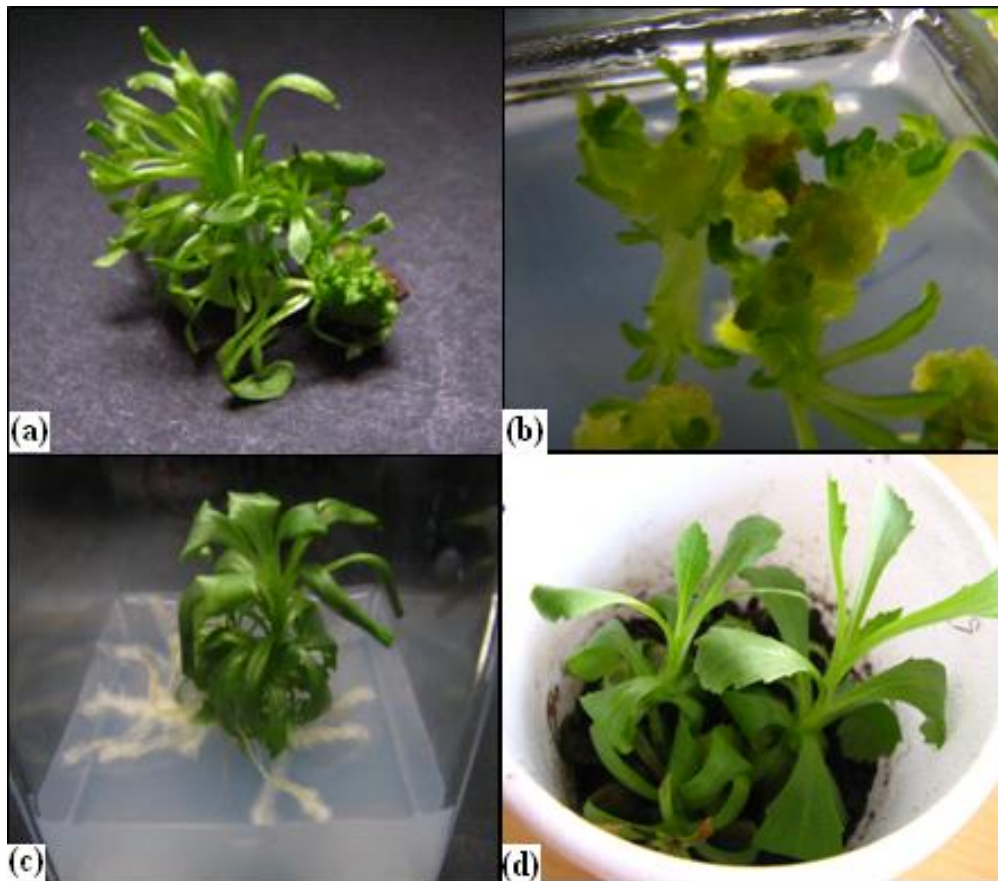


Figure 8. Steps of *in vitro* plant regeneration process of *D. davisiana* H.: (a) Multiple shoots developing from FBT explants or (b) from hypocotyl explants, (c) rooting of the shoots, and (d) acclimitization of the regenerants under non-axenic conditions.

Table 6. Effects of different TDZ or TDZ plus IAA concentrations (mg/L) on shoot formation from different explants excised from 2- to 3-week old *in vitro* germinated *D. davisiana* seedlings.

PGR(s)	EXPLANT TYPE							
	Hypocotyl		Cotyledonary leaf		Root		FBT	
	A	B	A	B	A	B	A	B
No PGRs	0	0	0	0	0	0	0.3±0.1 ^{fghi}	23.30
TDZ 0.10	0	0	0	0	0	0	0.9±0.1 ^c	86.6
TDZ 0.25	0.2±0.1 ^{ghij}	16.7	0.1±0.1 ^j	6.7	0	0	1.0±0.1 ^c	80.0
TDZ 0.50	0.5±0.1 ^{de}	50.0	0.1±0.1 ^j	10.0	0	0	1.1±0.2 ^c	80.0
TDZ 0.1+IAA 0.25	0.1±0.1 ^j	13.3	0	0	0	0	0.8±0.1 ^{cd}	73.3
TDZ 0.1+IAA 0.5	0	0	0	0	0.2±0.1 ^{ghij}	23.3	0	0
TDZ 0.25+IAA 0.25	0.8±0.2 ^c	43.3	0.2±0.1 ^{ghij}	4.0	0	0	1.2±0.2 ^c	80.0
TDZ 0.25+IAA 0.50	0.4±0.1 ^{efg}	33.3	0.2±0.1 ^{ghij}	4.0	0	0	0.5±0.1 ^d	53.3
TDZ 0.50+IAA 0.25	6.3±0.8 ^a	73.3	2.1±0.4 ^b	56.6	0.9±0.2 ^{cd}	40.0	5.9±0.7 ^a	76.6
TDZ 0.50+IAA 0.50	2.5±0.4 ^b	63.3	0.4±0.1 ^{efg}	43.3	0.3±0.1 ^{efgh}	33.3	2.7±0.4 ^b	76.6

Explants were cultured on B5 medium and the data were collected after 6 weeks of culture.

A: Mean numbers ± SE (standard error) of shoots per explant. B: Frequency of organogenic explants (%), Means with same letter within the columns are not significantly different at 0.05 probability level.

Table 7. Effects of different basal media supplemented with 0.5 mg/L and 0.25 mg/L IAA on shoot formation from different explants of *D. davisiiana* excised from 2- to 3week- old *in vitro* seedlings.

MEDIUM TYPE	EXPLANT TYPE							
	Hypocotyl		Cotyledonary leaf		Root		FBT	
	A ^a	B ^b	A ^a	B ^b	A ^a	B ^b	A ^a	B ^b
B5	6.3±0.8 ^{abc}	73.3%	2.1±0.4 ^g	56.6%	0.9±0.2 ^h	40.0%	5.9±0.9 ^{bcd}	76.6%
MS	5.7±0.8 ^{cd}	73.3%	2.1±0.4 ^g	46.6%	0.9±0.3 ^h	23.3%	3.9±0.6 ^f	60.0%
MM	4.3±0.7 ^{ef}	66.6%	2.0±0.4 ^{gh}	40.0%	0.1±0.1 ⁱ	10.0%	4.4±0.7 ^{def}	60.0%
CP	4.2±0.8 ^{cf}	53.3%	2.9±0.5 ^{fg}	56.6%	0	0	4.2±0.7 ^{ef}	63.3%
LS	7.8±1.0 ^{ab}	73.3%	2.5±0.4 ^g	76.6%	1.1±0.2 ^h	33.3%	8.1±0.9 ^a	80.0%
SH	2.3±0.4 ^g	63.3%	2.2±0.4 ^g	60.0%	0.1±0.1 ⁱ	13.3%	2.3±0.5 ^g	50.0%

Data were collected after 6 weeks of culture

A: Mean number±SE (standard error) of shoots per explant; B: Frequency of organogenic explants (%) Means with the same letter within the columns are not significantly different at 0.05 probability level.

Table 8. Rooting of *in vitro* regenerated *D. davisiiana* shoots on LS medium containing IAA or IBA after four weeks in culture. Data were collected after a 4 week of culture.

Auxins (mg/L)	% of shoots that rooted	Mean number of roots per rooted shoot*	Overall Mean
No PGRs	100	4.6±0.7 ^c	-
IAA 0.1	100	4.9±0.4 ^{bc}	6.5±0.4
IAA 0.5	100	8.1±1.1 ^a	
IBA 0.1	100	3.2±0.3 ^d	4.6±0.6
IBA 0.5	100	6.1±1.2 ^b	

* Means with the same letter within the columns are not significantly different at 0.05 probability level.

5.1.2. Regeneration of *D. cariensis* Boiss. Ex Jaub. & Spach and rooting

Probably due to the seed dormancy, germination period of *D. cariensis* was longer than others employed in this study species tested. Additionally, germination frequency was found very low in this species (2 to 5%). Seedlings grown *in vitro* (2- to 3-week old) were used as explant source. Hypocotyl, cotyledonary leaf, root and FBT explants excised from *in vitro* seedlings were cultured on LS, B5 or MS medium containing 0.5 mg/L TDZ or 0.5 mg/L TDZ and 0.25 mg/L IAA (Table 9). Of those explants, only FBT explants were found productive in terms of shoot regeneration. Of all basal media tested, LS medium supplemented with 0.5 mg/L TDZ and 0.25 mg/L IAA was found to be the most effective. In contrast to FBT, hypocotyl explants were found to be ineffective for regeneration. Necrosis on tissue was observed as a predominant pattern. There was no significant difference between treatments containing MS or B5 medium supplemented with 0.5 mg/L TDZ or 0.5 mg/L TDZ and 0.25 mg/L IAA (i.e. the mean value was 1.8 shoots per explant in each). Similarly, no significant difference can be established between treatments wherein 0.5 mg/L TDZ was used alone or in combination with 0.25 mg/L IAA (1.6 to 1.8 shoots per explant). Additionally, stratified seeds by scratching from the surface using a blade were placed on LS regeneration medium containing 0.5 mg/L TDZ and 0.25 mg/L IAA, and only few seeds showed very high shoot regeneration response (up to 50 shoots per seed; Figure 9).

Table 9. Shoot regeneration from different explants excised from *D. cariensis* (2- to 3-week old) on LS medium containing 0.5 mg/L TDZ or 0.5 mg/L TDZ+0.25 mg/L

MEDIUM TYPE	EXPLANT TYPE							
	Hypocotyl		Leaf*		Root		FBT	
	A ^a	B ^b	A ^a	B ^b	A ^a	B ^b	A ^a	B ^b
0.5 TDZ	0	0	0	0	0	0	1.6±0.33	100
0.5 TDZ+0.25 IAA	0	0	0	0	0	0	1.8±0.2	100
0.5 TDZ	0	0	0	0	0	0	1.2±0.13	100
0.5 TDZ+0.25 IAA	0	0	0	0	0	0	1.8±0.13	100
0.5 TDZ	0	0	0	0	0	0	1.4±0.22	100
0.5 TDZ+0.25 IAA	0	0	0	0	0	0	3.9±0.18	100

*Cotyledonary (2-3 week old) or mature leaf (6 weeks or more).

A: Mean number±SE (standard error) of shoots per explant; B: Frequency of organogenic explants (%) Means with the same letter within the columns are not significantly different at 0.05 probability level.

For callus cultures, leaf explants were placed onto MS medium containing different BA and/or NAA concentrations for eight weeks. Necrosis was a predominant pattern on the leaf surface for this species. As seen in Table 10, increasing concentrations of NAA depending on BA concentrations increased the callus production. Callus formation was not initiated when BA or NAA was used alone. In contrast to callus formation, necrosis was observed at a 70% frequency when BA was used at 0.5 mg/L or 90% when NAA at 0.1 mg/L. As noted above, combination of PGRs was found most effective in callus formation. It is noteworthy that MS medium containing 2.0 mg/L BA and 1.0 mg/L NAA was found most effective callus-inducing medium (more than 90% of the leaf explants produced callus; Table 10).

Many experiments were carried out to optimize an efficient *in vitro* regeneration system for *D. cariensis*. Of those, micropropagation was preferred using axillary buds on LS medium containing various concentrations of TDZ (ranging from 0.1 to 1.0 mg/L) and/or IAA (ranging from 0.1 to 0.5 mg/L). After eight-week cultivation period, the highest mean number of shoots per explant (axillary bud) was 2.0. Yet, there was no significant difference between treatments (data not shown).

Rooting stage of micropropagation was carried out using various concentrations (0.1, 0.5 or 1.0 mg/L) of the auxins IAA or IBA (Table 11). Most effective rooting medium was LS medium containing 1.0 mg/L IAA, producing 5.6 roots per regenerated shoot. LS medium without PGR did not induce any roots at all. On the other hand, 0.1 mg/L IBA (the lowest concentration) produced fewer numbers of root, as compared to other concentrations tested. Increasing concentrations of auxins (IAA or IBA) was correlated to the mean number of roots per shoot (i.e., 0.1 or 1.0 mg/L produced 2.6 or 5.6 roots per explant at IAA or 0.9 or 3.6 roots per shoot when IBA was used at similar concentrations, respectively).

After a four-week rooting stage, while some regenerants were transferred to the additional growing culture medium containing LS medium (half- or full-strength) without PGR for HPLC analysis, the remainings were subjected to hardening off process. Survival rate was 70-80% for this species.

Table 10. Frequency (%) of callus formation and necrosis on the leaf tissues of *D. cariensis*.

Leaf Explants		
PGRs (mg/L)	Frequency of callus formation (%)	Frequency of Necrosis (%)
Control	0	40
0.5 BA	0	70
1.0 BA	0	80
1.5 BA	0	90
2.0 BA	0	70
0.1 NAA	0	100
0.5 NAA	0	0
1.0 NAA	0	50
0.5 BA+0.1 NAA	15	60
0.5 BA+1.0 NAA	30	45
1.0 BA+0.1 NAA	35	45
1.5 BA+0.1 NAA	0	10
2.0 BA+0.1 NAA	10	55
0.5 BA+0.5 NAA	40	30
1.0 BA+0.5 NAA	35	30
1.5 BA+0.5 NAA	80	0
2.0 BA+0.5 NAA	80	10
0.5 BA+1.0 NAA	70	0
1.0 BA+1.0 NAA	60	20
1.5 BA+1.0 NAA	70	10
2.0 BA+1.0 NAA	90	5

Table 11. Rooting of *in vitro* regenerated *D. cariensis* shoots on LS medium containing IAA or IBA after four weeks in culture (SE: Standard error).

Auxin (mg/L)	Mean number of roots±SE per shoot*
No PGRs	0
IAA 0.1	2.6±0.3 ^c
IAA 0.5	3.6±0.3 ^b
IAA 1.0	5.6±0.4 ^a
IBA 0.1	0.9±0.2 ^d
IBA 0.5	3.2±0.4 ^b
IBA 1.0	3.6±0.3 ^b

*Data were collected after 6 week's of incubation. Means with the same letter within columns are not significantly different at 0.05 probability level.

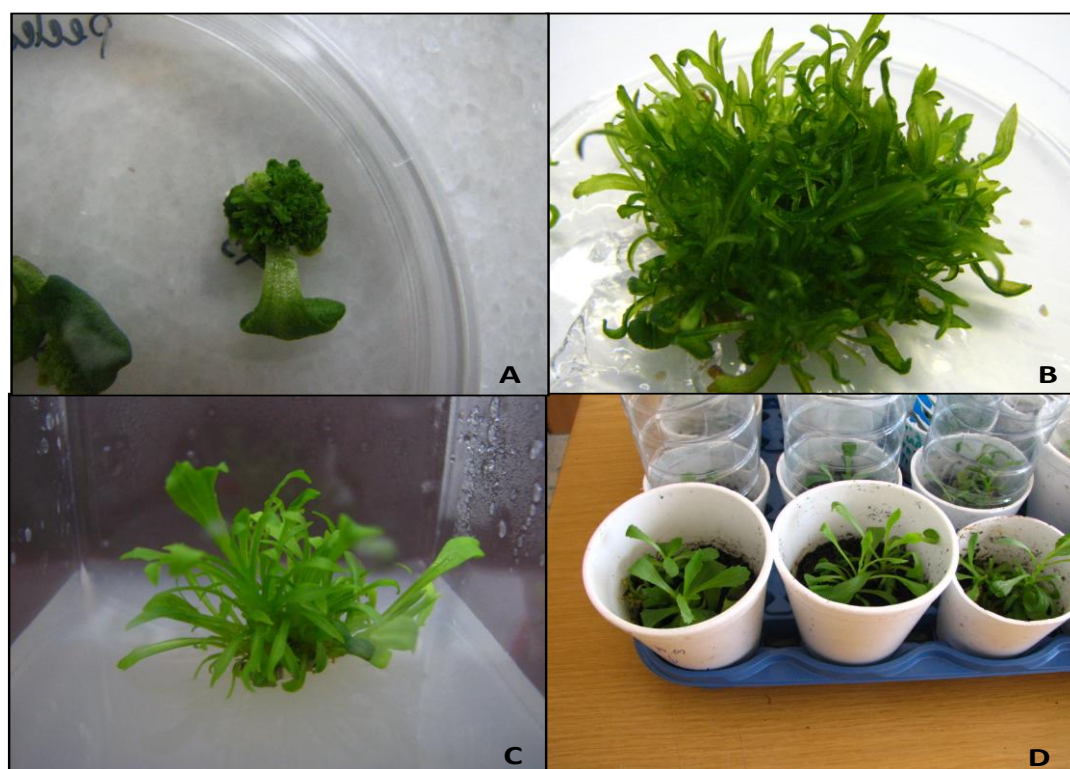


Figure 9. Reeneration stages of *D. cariensis* within 10 or 12 weeks of cultivation. Adventitious shoot regeneration from FBT (A), shoot regeneration from seeds (B), rooting stage (C) acclimitization (D).

5.1.3. Regeneration of *D. trojana* and rooting

To develop an efficient *in vitro* regeneration protocol, two sets of experiments were carried out. In the first, different concentrations and combinations of TDZ and IAA were compared using LS medium (Table 12), while in the second set, comparisons were made with three different media formulations (MS, B5 and LS) that were supplemented with either 0.5 mg/L TDZ alone or in combination with 0.25 mg/L IAA (Table 13).

In this study we clearly described a shoot regeneration that shows a great variability depending on the explant type, hormonal combinations as well as different basal media tested. The explants excised from 2-3 week old *in vitro* germinated seeds of *D. trojana* were cultured on LS medium with various concentrations of TDZ alone or in combination with different concentrations of IAA for shoot regeneration. In terms of overall means of the treatments, LS with 0.5 mg/L TDZ plus 0.25 mg/L IAA was selected as the best promotive medium, producing 2.2 shoots per explant (Figure 10a). Furthermore, on the same medium, the highest mean number of shoots was produced by FBT explants (6.6 shoots; Table 12). As for hypocotyl explants, regeneration capacity was found moderate, mean numbers of shoots per hypocotyl explant ranging from 1.7 to 3.0, with an overall mean of 2.3 shoots throughout the experiments. In addition to the medium formulation, regeneration response of the treatments involving low concentrations of TDZ (0.1 or 0.25 mg/L) alone was found more effective than the higher concentrations of TDZ (0.5 or 1.0 mg/L), producing an overall mean of 2.8 or 3.0 versus 1.9 or 2.1 shoots per explant, respectively (Table 12). However, when combined with 0.25 IAA, TDZ at 0.5 or 1.0 mg/L was found much more productive, producing an overall mean of 4.3 or 2.9 shoots per explant,

respectively. High concentration of IAA (0.50 mg/L) combined with low concentrations of the TDZ dramatically decreased the overall means of shooting from 3.1 to 1.7 shoots per explant, also forming intervening massive green and compact callus formation at the cut edges of the explants. Although hypocotyl explants produced the highest amount of shoots on LS medium containing 0.5 mg/L TDZ and IAA (roughly 3 shoots per explant), a massive callus formation was a predominant pattern over shoot regeneration as well as the growth and development (Figure 10b). Moreover, callusing pattern of the hypocotyl was dependent on medium formulation. When TDZ was used alone, a slight callus formation was initiated. However, on the other hand, higher concentrations of TDZ with any concentration of IAA produced more callus on the explant surface. It is well-known that the composition of basal medium has a significant effect on regeneration as well as growth and development of explants in culture. In this view of point, different basal media (MS, B5 or LS) containing 0.5 mg/L TDZ alone or in combination with 0.25 mg/L IAA were compared for their effects on shoot formation (Table 13). When the overall means of the treatments were taken into account, LS medium was found more effective than others, producing 3.4 shoots per explant on medium containing combinations of TDZ and IAA after 4 weeks of incubation. Of the other media tested (B5 or MS), there was no significant difference between the mean numbers containing either media type (MS or B5) supplemented with 0.5 mg/L TDZ alone (overall mean was 1.1 shoots per explant for MS or B5) or in combination with 0.25 mg/L IAA (overall mean was 1.9 or 2.2 shoots per explant; Table 13).

Rooting was readily achieved when shoots obtained from FBT or hypocotyl explants were singled out and cultured on LS medium containing with or without different concentrations of IAA or IBA. The data presented in Table 14 clearly indicates that

rooting was even available on the basal medium which produced a mean of 0.5 roots per shoot explant. However, adding IAA at 0.5 mg/L concentration not only increased the mean number of roots per shoot explant significantly but also produced longer, thicker and healthier shoots consequently (Figure 10c).

The rooted shoots were then transferred to Magenta boxes or glass jars containing vermiculite and subsequently moved to the pots in the growth chamber (Figure 10d). Hardening and acclimatization of regenerated plantlets were successful. Finally, 80% of the regenerants survived through the hardening off process.

Table 12. Mean numbers of shoots per hypocotyl and FBT explants of *D. trojana* cultured on LS medium containing four different concentrations of TDZ alone or combined with two different concentrations of IAA.

PGRs	EXPLANT TYPE		Overall Mean
	Hypocotyl	FBT	
0.1 TDZ	1.7±0.3 ^{ghij}	3.9±0.4 ^c	2.8±0.3 ^{BCDE}
0.25 TDZ	2.3±0.3 ^{efghi}	3.6±0.5 ^{cde}	3.0±0.3 ^{BCD}
0.5 TDZ	1.1±0.2 ^{ijkl}	2.6±0.4 ^{defgh}	1.9±0.2 ^{DE}
1.0 TDZ	0.5±0.1 ^{kl}	3.8±0.7 ^{cd}	2.1±0.5 ^{CDE}
0.1 TDZ+0.25 IAA	1.9±0.3 ^{fghij}	3.5±0.4 ^{cde}	2.7±0.3 ^{CDE}
0.25 TDZ+0.25 IAA	2.7±0.5 ^{cdefgh}	5.1±0.6 ^b	3.9±0.5 ^{AB}
0.5 TDZ+0.25 IAA	2.1±0.4 ^{fghij}	6.6±0.6 ^a	4.3±0.6 ^A
1.0 TDZ+0.25 IAA	0.1±0.1 ^l	5.7±0.4 ^{ab}	2.90±0.6 ^{BCDE}
0.1 TDZ+0.5 IAA	1.9±0.4 ^{fghij}	1.5±0.2 ^{hijk}	1.7±0.2 ^E
0.25 TDZ+0.5 IAA	1.5±0.3 ^{hijk}	2.7±0.3 ^{cdefgh}	2.1±0.2 ^{CDE}
0.5 TDZ+0.5 IAA	3.0±0.5 ^{cdefg}	3.1±0.3 ^{cdef}	3.1±1.5 ^{BC}
1.0 TDZ+0.5 IAA	1.0±0.3 ^{ijkl}	2.7±0.1 ^{cdefgh}	3.9±0.5 ^{AB}

Data were collected after 6 weeks of culture

Means±SE (standard error) with the same letter within the columns are not significantly different at 0.05 probability level.

Table 13. Comparison of different shoot regeneration media containing 0.5 mg/L TDZ alone or combined with 0.25 mg/L IAA by using hypocotyl and FBT explants excised from seedlings of *D. trojana* germinated *in vitro*.

MEDIUM TYPE	EXPLANT TYPE				Overall Mean
	Hypocotyl		FBT		
	A	B	A	B	
0.5 TDZ+MS	1.0±0.3 ^e	73	1.3±0.3 ^{de}	100	1.1±0.2^D
0.5 TDZ+0.25 IAA+MS	1.2±0.2 ^{de}	87	3.1±0.4 ^b	100	2.2±0.3^B
0.5 TDZ+B5	1.0±0.3 ^e	60	1.2±0.2 ^{de}	80	1.1±1.0^E
0.5 TDZ+0.25 IAA+B5	1.7±0.8 ^{cde}	93	2.0±0.4 ^{cd}	93	1.9±0.2^{CD}
0.5 TDZ+LS	1.3±0.2 ^{cde}	87	2.4±0.27 ^{bc}	100	1.9±0.2^{CD}
0.5 TDZ+0.25 IAA+LS	1.7±0.6 ^{cde}	93	5.1±0.4 ^a	100	3.4±0.4^A

Data were collected after 6 weeks of culture

A: Mean number±SE (standard error) of shoots per explant; B: Frequency of organogenic explants (%). Means with the same letter within the columns are not significantly different at 0.05 probability level

Table 14. Rooting of *in vitro* regenerated shoots of *D. trojana* cultured on LS medium containing different concentrations of IAA or IBA.

Auxins (mg/L)	Mean number of roots per rooted shoot*
No PGRs	0.54±0.23 ^d
0.1 IAA	1.02±0.40 ^c
0.5 IAA	3.78±0.44 ^a
0.1 IBA	0.84±0.12 ^{cd}
0.5 IBA	2.44±0.48 ^b

Data were collected after 6 weeks of culture

* Mean number ± SE (standard error) with the same letters being not significantly different at 0.05 probability level.

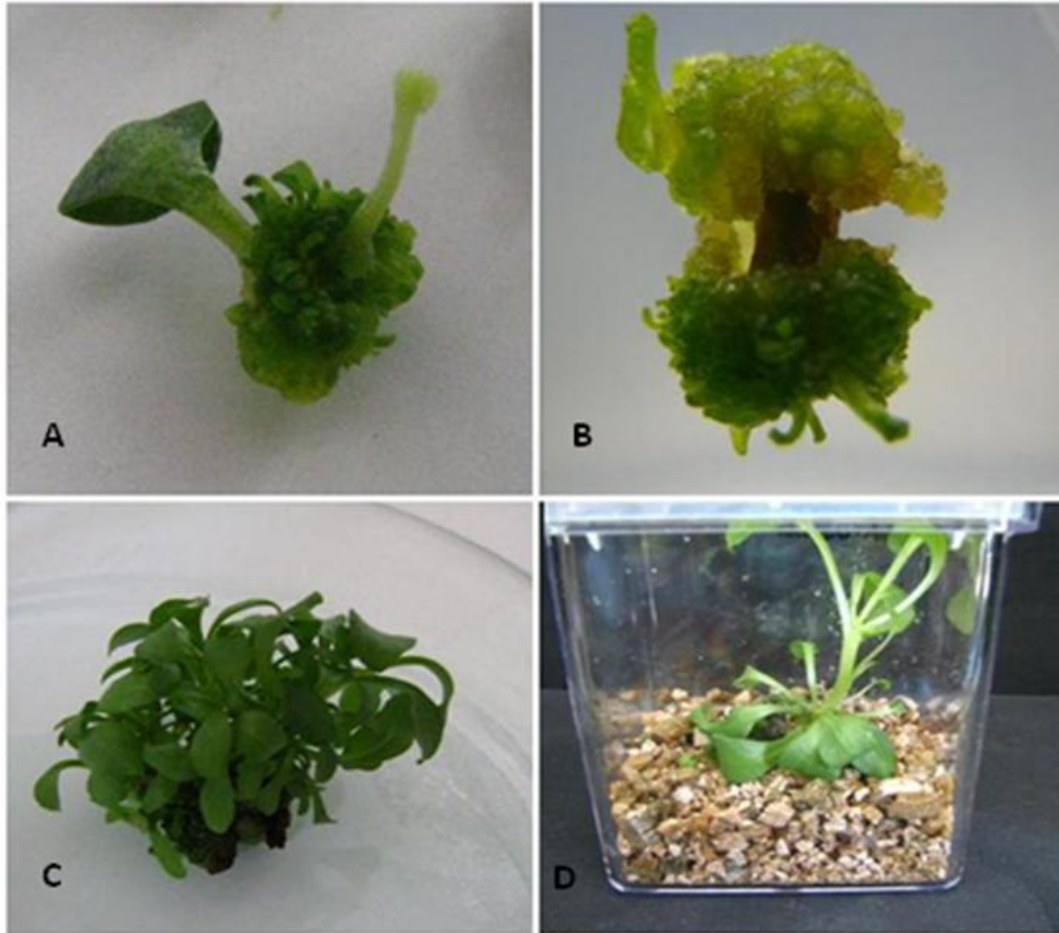


Figure 10. Steps of *in vitro* regeneration process of *D. trojana* during 10-12 weeks. Multiple shoots from FBT explants (a), or from hypocotyl explants (b), multiple shoot formation (c) acclimitization under non-axenic conditions in Magenta boxes containing vermiculate (d).

5.1.4. Regeneration of *D. lamarckii* and rooting

In vitro regeneration of *D. lamarckii* was carried out using 2- to 3-week old *in vitro* germinated seedlings. Various types of explants (FBT or cotyledonary leaf) were placed onto LS medium containing various concentrations of BA (0.5, 1.0 or 2.0 mg/L) alone or in combination with NAA (0.5, 1.0 or 2.0 mg/L) for a six-week cultivation period (Table 15; Figure 11). In terms of overall means of the experiments, cotyledonary leaf explants produced a higher number of shoots than FBT explants, but there was no significant difference between them in terms of the mean numbers of shoots per explant (4.9 shoots compared to 4.7). On the other hand, regeneration frequencies showed that all FBT explants produced shoots, while 55% of the hypocotyl explants only were able to do so. When BA was used alone at any concentration in LS medium, there was no significant difference in terms of shoot regeneration ($p < 0.05$). Fewer shoots per explant (3.5 shoots per cotyledonary leaf or 3.0 shoots per FBT explant) were produced on LS medium containing 0.5 mg/L BA only. On the other hand, gradual increase in BA concentrations (1.0 or 2.0 mg/L) raised the mean number of shoots moderately (4.4 or 4.2 shoots per cotyledonary leaf, 3.4 or 4.0 shoots per FBT explant, respectively). The lowest concentration of BA (0.5 mg/L) in combination with various dosages of NAA (0.5, 1.0 or 2.0 mg/L) did not show a significant difference in terms of shoot regeneration when FBT and cotyledonary leaf explants were compared. In this case, cotyledonary leaf explants produced shoots with a mean ranging from 4.4 to 5.2 shoots per explant, similarly FBT produced a mean of shoots ranging from 4.1 to 4.5. On the other hand, the mean number shoots obtained from cotyledonary leaf explants on LS medium containing 0.5 mg/L BA and 0.5 mg/L NAA was found almost 20% higher than that obtained at 0.5 mg/L BA and 2.0 mg/L NAA (5.2 to 4.1 shoots per explant).

When 0.5 mg/L or 2.0 mg/L BA concentrations were taken into account, it had the promotive effect on regeneration with FBT explants. Especially, mean number of shoots produced on the medium containing 0.5 mg/L BA and 2.0 mg/L NAA increased from 4.1 to 5.6 shoots per explant when BA concentration was doubled.

The highest mean number of shoots was 8.4 in cotyledonary leaf explants cultured on LS medium containing 2.0 mg/L BA and 0.5 mg/L NAA (compared to 7.1 shoots per FBT explant). When explant types were statistically compared, there was no significant difference between them ($p < 0.05$). It is noteworthy that cotyledonary leaf explants produced shoots at the highest frequency (93%; Figure 11). It was also observed that regeneration frequency decreased depending on decreasing concentrations of NAA (Figure 11). For example, mean number was fluctuated in cotyledonary leaf explants (from 8.4 to 4.7 and then 5.7 shoots per explant), and similarly, it was a gradual decrease in case of FBT (from 7.1 to 5.5 then 4.0 shoots per explant). Therefore, cotyledonary leaf explants were worth of using for later experiments.

Rooting of the shoots was readily achieved on LS medium supplemented with various concentrations of IAA or IBA. However, IAA was a considerably more effective type of auxin than IBA in terms of the mean number of roots per shoot, as well as the mean root length (Table 16). IAA at 2.5 mg/L produced 4.33 roots per shoot (90% of the shoots produced roots) and at this concentration root length measured after six weeks' cultivation on rooting media was highest (7.2 cm).

Table 15. Effects of various BA or BA plus NAA concentrations on regeneration from FBT and cotyledonary leaf explants of *D. lamarckii* after 6 weeks of cultivation on regeneration medium (\pm SE; standard error).

PGRs (mg/L)	Cotyledonary Leaf Explants		FBT explants	
	Mean number of shoots \pm SE per explant*	Regeneration frequency (%)	Mean number of shoots \pm SE per explant*	Regeneration frequency (%)
0.5 BA	3.5 \pm 1.1 ^{cd}	40	3.0 \pm 1.1 ^{cd}	100
0.5 BA+0.5 NAA	5.2 \pm 1.5 ^{bc}	47	4.5 \pm 1.1 ^{bcd}	100
0.5 BA+1.0 NAA	4.5 \pm 1.1 ^{bcd}	47	4.3 \pm 1.0 ^{bcd}	100
0.5 BA+2.0 NAA	4.4 \pm 1.2 ^{bcd}	47	4.1 \pm 1.1 ^{cd}	100
1.0 BA	4.4 \pm 0.9 ^{bcd}	47	3.4 \pm 1.2 ^{cd}	100
1.0 BA+0.5 NAA	4.3 \pm 0.8 ^{bcd}	47	5.2 \pm 1.8 ^{bc}	100
1.0 BA+1.0 NAA	5.3 \pm 1.1 ^{bc}	80	5.0 \pm 1.7 ^{bcd}	100
1.0 BA+2.0 NAA	4.7 \pm 1.0 ^{bcd}	47	5.6 \pm 1.5 ^{bc}	100
2.0 BA	4.2 \pm 0.9 ^{bcd}	47	4.0 \pm 1.0 ^{cd}	100
2.0 BA+0.5 NAA	8.4 \pm 1.1 ^a	93	7.1 \pm 2.1 ^{ab}	100
2.0 BA+1.0 NAA	4.7 \pm 1.1 ^{bcd}	53	5.5 \pm 1.9 ^{bc}	100
2.0 BA+2.0 NAA	5.7 \pm 1.7 ^{bc}	60	4.0 \pm 1.3 ^{cd}	100
Overall Mean	4.9\pm1.3	55	4.7\pm1.2	100

*Means with the same letter within the columns are not significantly different at 0.05 probability level.

Table 16. Effects of IAA and IBA on rooting of the regenerated shoots of *D. lamarckii* (\pm SE; standard error).

Auxin (mg/L)	% of shoots that rooted*	Mean number of roots per rooted shoot*	Mean root length (cm)*
No PGRs	66	2.11 \pm 0.4 ^{cd}	3.1 \pm 0.2 ³
1.0 IAA	85	3.00 \pm 0.5 ^{bc}	3.5 \pm 0.3 ^e
2.5 IAA	90	4.33 \pm 0.47 ^a	7.2 \pm 0.1 ^a
5.0 IAA	80	3.20 \pm 0.5 ^b	6.1 \pm 0.3 ^b
1.0 NAA	66	2.71 \pm 0.5 ^c	3.2 \pm 0.2 ^e
2.5 NAA	75	2.52 \pm 0.3 ^{cd}	5.6 \pm 0.1 ^{cd}
5.0 NAA	60	2.61 \pm 0.4 ^{cd}	5.3 \pm 0.3 ^d

*Data were collected after 6 weeks of culture. Means with the same letter within the columns are not significantly different at 0.05 probability level.

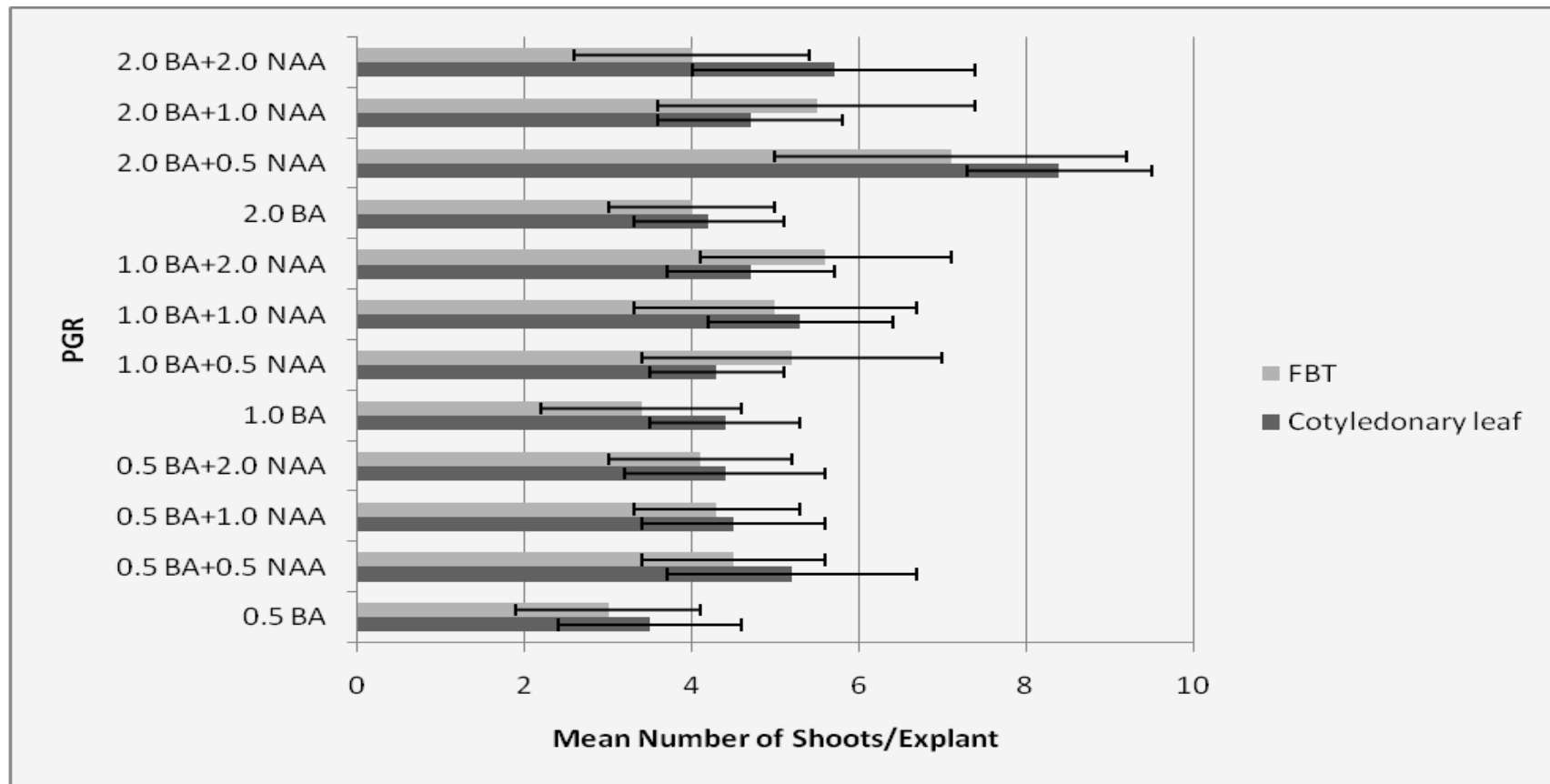


Figure 11. Comparison of explant types excised from *D. lamarckii* in vitro seedlings for their shoot regeneration capacities on LS medium containing various concentrations of BA and/or NAA.

In another regeneration study on *D. lamarckii*, we tried high concentrations of BA (ranging from 1.0 to 5.0 mg/L) and NAA (from 0.5 to 2.0 mg/L) using LS medium. In this experiment, cotyledonary leaf and hypocotyl explants were used for callus culture for a six-week cultivation period (Table 17; Figure 12a). Then, the tissues that formed friable and yellowish calli were transferred to LS medium containing lower concentrations of BA (0.1, 0.25 or 0.5 mg/L) and/or GA₃ (0.25 or 0.5 mg/L). After a six week cultivation, the number of shoots regenerated via indirect organogenesis were counted (see Table 10; Figure 12b, c). When taken callus formation into account, especially those developed on medium containing 3.0 mg/L and 0.5 or 2.0 mg/L NAA was selected for later experiments in which various concentrations of BA and/or GA₃ were tested for indirect shoot organogenesis (Figure 12c, d, e). Table 18 clearly indicates that the mean number of shoots increased from 2.8 to 6.2 shoots per callus (300~350 mg) when BA was used either alone or at decreasing concentrations from 0.5 to 0.1 mg/L. On the other hand, when 0.25 mg/L GA₃ was used in combination with various concentrations of BA (from 0.1 to 0.5 mg/L), shoot regeneration ability decreased from 6.1 to 2.5 shoots per callus. Also, GA₃ was not found any less effective for shoot induction when used alone. The most effective concentration of GA₃ for indirect shoot regeneration was 0.5 mg/L. In addition, while GA₃ at 0.5 mg/L produced 3.1 shoots, the mean number of shoots sharply increased up to 6.6 shoots per callus when combined with any concentrations of BAP (Table 18). However, shoot regeneration decreased gradually from 6.1 to 2.5 shoots per callus when 0.25 mg/L GA₃ was combined with 0.1, 0.25 or 0.5 mg/L BA. After rooting stage on LS medium containing 2.5 mg/L IAA, rooted shoots were transferred to the pots for further growth and development under greenhouse conditions (Figure 12f).

Table 17. Organogenic callus response (%) of explants (hypocotyl and cotyledonary leaf) on medium containing combinations of BA and NAA.

PGRs	Organogenic callus (%)
1.0 BA+0.5 NAA	83
3.0 BA+0.5 NAA	91
5.0 BA+0.5 NAA	95
1.0 BA+1.0 NAA	65
3.0 BA+1.0 NAA	75
5.0 BA+1.0 NAA	82
1.0 BA+2.0 NAA	79
3.0 BA+2.0 NAA	100
5.0 BA+2.0 NAA	66

Table 18. Shoot regeneration from callus of *D. lamarckii* cultured on LS medium containing BA or GA₃ alone or in combinations after a 6 weeks cultivation (\pm SE; standard error).

PGRs (mg/L)	Frequency(%) callus developing shoot	Mean number of shoots per callus
0.10 BA	83	6.2 \pm 2.1 ^{ab}
0.25 BA	83	4.1 \pm 1.0 ^b
0.5 BA	50	2.8 \pm 0.2 ^c
0.25 GA ₃	41	2.3 \pm 0.1 ^c
0.25 GA ₃ +0.10 BA	91	6.1 \pm 2.0 ^{ab}
0.25 GA ₃ +0.25 BA	58	4.3 \pm 1.1 ^b
0.25 GA ₃ +0.50 BA	80	2.5 \pm 0.9 ^{bc}
0.50 GA ₃	50	3.1 \pm 0.9 ^{bc}
0.50 GA ₃ +0.10 BA	75	5.7 \pm 1.5 ^{ab}
0.50 GA ₃ +0.25 BA	91	6.6 \pm 1.6 ^{ab}
0.50 GA ₃ +0.50 BA	83	6.0 \pm 1.5 ^{ab}

* Data were collected after 6 weeks of culture. Means with the same letter within the columns are not significantly different at 0.05 probability level.

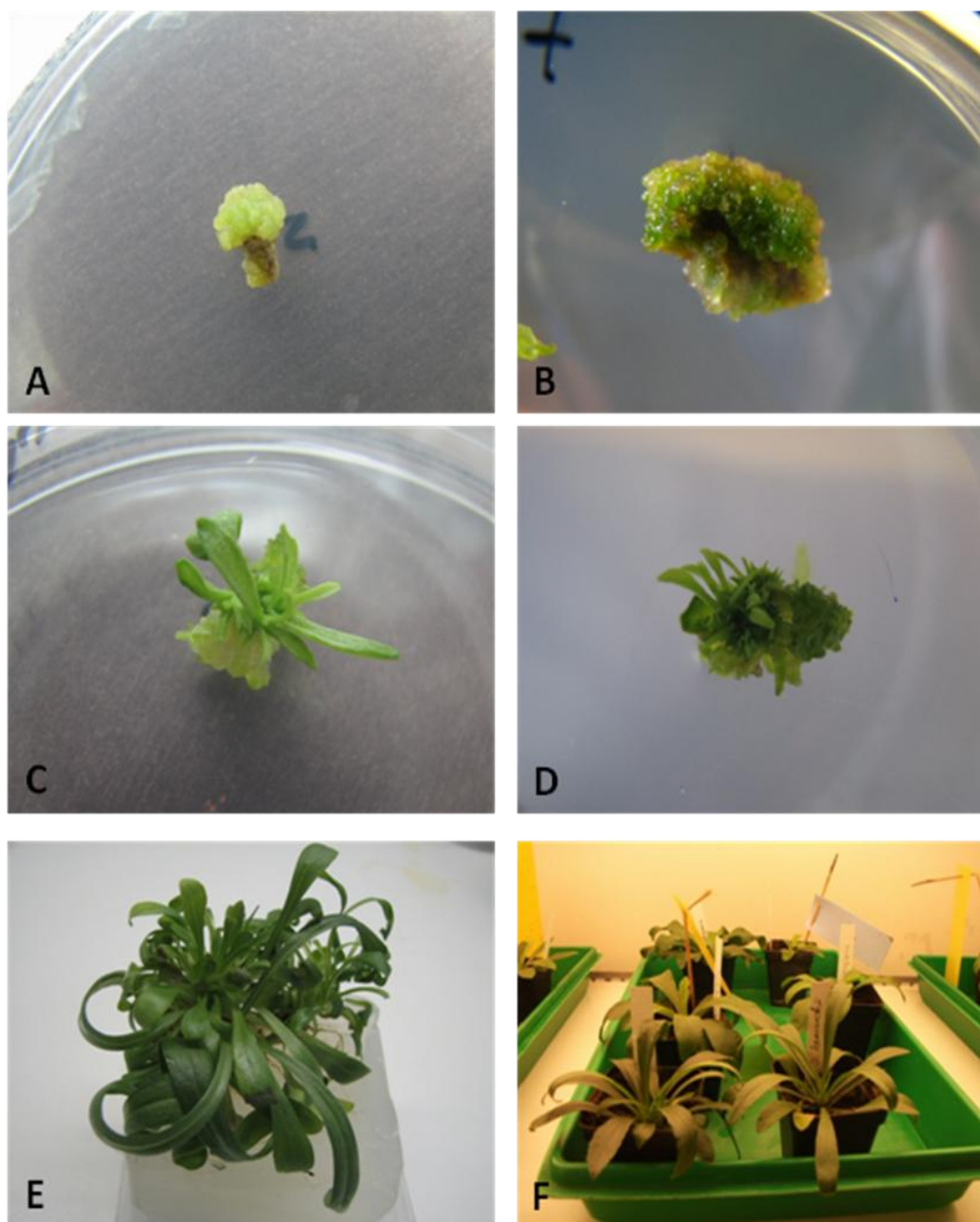


Figure 12. Steps of indirect organogenesis in *D. lamarckii*. Hypocotyl explants producing organogenic callus on medium containing 5.0 mg/BA and 1.0 mg/L NAA (A), organogenic callus on medium containing 0.5 mg/L BA and GA₃ (B), indirect shoot formation after a 6 weeks of cultivation (C, D), regenerants after rooting stage (E), regenerants being hardened off under growth chamber conditions.

In summary, tissue culture protocols proposed in the Results section are presented in Figure 13 through all stages.

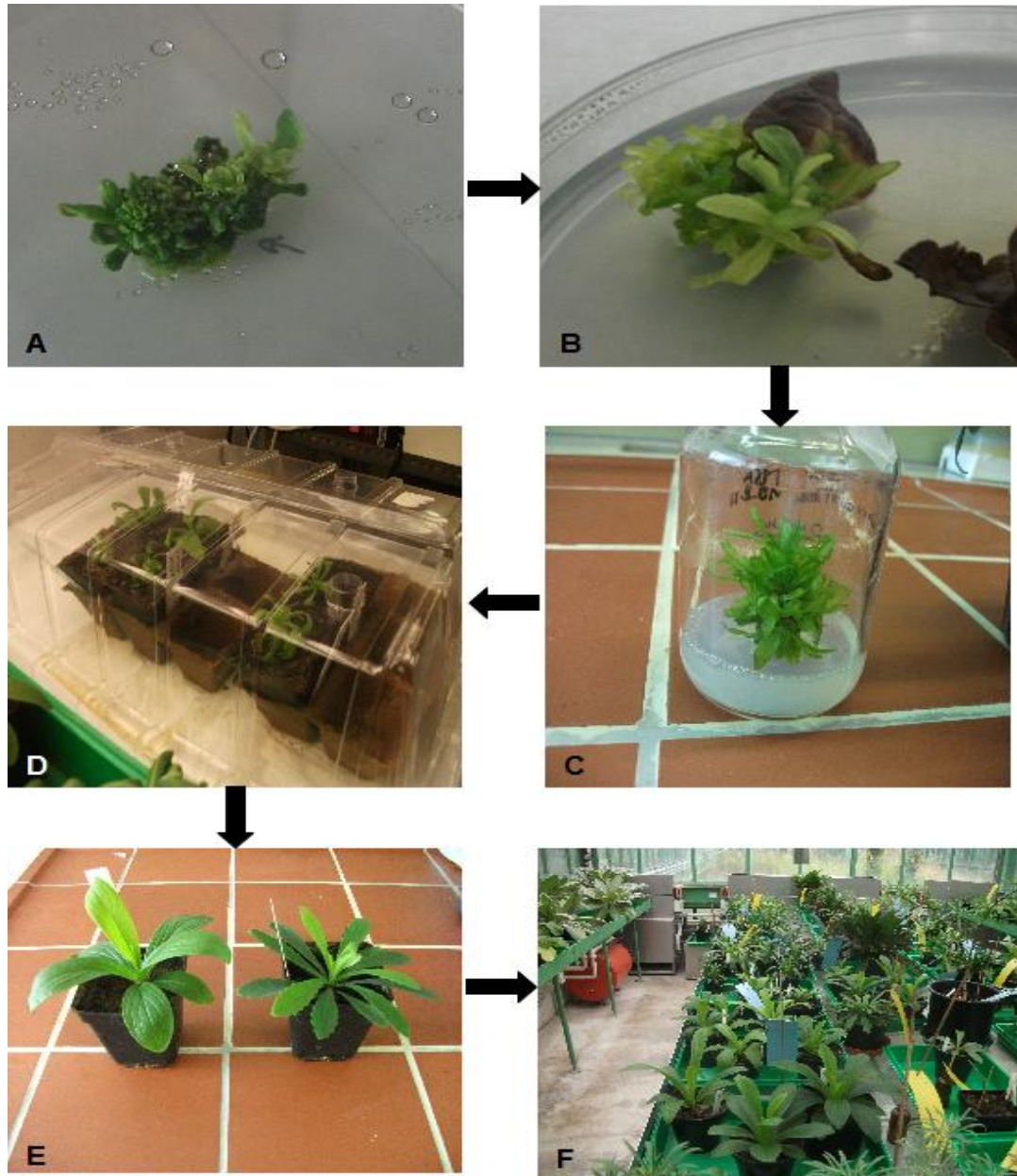


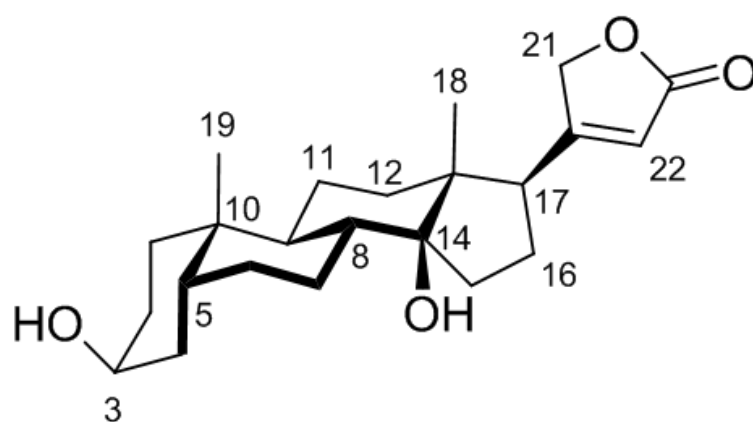
Figure 13. A general representation of *in vitro* multiplication of several *Digitalis* species. Adventitious shoot formation in *D. trojana* on MS medium containing 0.5 mg/L TDZ and 0.25 mg/L IAA (A), similar protocol for *D. cariensis* producing multiple shoots *in vitro* (B), rooting stage of *D. cariensis* on MS medium containing 0.5 mg/L IAA (C), hardening off stage for the rooted shoots from *D. trojana* samples (D), 8- months old healthy regenerants *D. schischkinii* (left one) and *D. davisiana* (right one) (E), *Digitalis* species grown under greenhouse conditions (F).

5.2. Phytochemical analysis

5.2.1. Cardenolide profiles of Anatolian *Digitalis* species

Commercially important derivatives of cardenolides (or genin groups) are presented in Figure 14. Depending on the presence of -OH (or -H) group in carbon number 12 and/or 16, different colorations (yellow, turquoise or blue) for each genine group appear on the TLC plate after spraying Jensen-Kny reagent (Jork et al. 1990). Theoretically, acid hydrolysis reveals the presence of major cardenolides after breaking the sugar groups attached to 3rd carbon atom. At the end of reaction, we can determine the concentration of the cardiac glycosides regarding to their pregnane structure as to whether they are either A (digitoxigenin), B (gitoxigenin) or C derivative (digoxigenin) of cardenolides. Table 19 represents cardenolide profiles of different *Digitalis* species, which are also distributed and naturally grown populations in Anatolia. Of those, *D. lanata*, *D. ferruginea* subsp. *ferruginea* and *D. ferruginea* subsp. *schischkinii* were compared with the endemics *D. davisiana*, *D. cariensis*, *D. lamarckii* and *D. trojana* for their cardenolide contents. For digoxigenin derivatives, *D. ferruginea* subsp. *ferruginea* has the highest content (0.73 mg/100 mg DW) followed by *D. lamarckii* (0.47 mg/100 mg DW). On the other hand, *D. cariensis*, *D. trojana* and *D. schischkinii* contained almost same amount of digoxigenin derivatives of cardenolides (0.38, 0.27 and 0.37 mg/100 mg DW). *D. lamarckii*, *D. cariensis* and *D. trojana* were found to contain higher gitoxigenin content (1.09, 0.84 and 0.89 mg/100 mg DW, respectively). Finally, the content of digitoxigenin-derivative of cardenolides was found predominantly in *D. lanata* (0.90 mg/100 mg DW) and followed by Ferruginea Alliance (0.49 or 0.50 mg/100 mg DW). When Anatolian endemics were taken into account, *D. trojana* was observed to have a very low content of glycosides. On the other hand, digoxigenin derivatives of

cardenolides were not detected in *D. davisiana*, *D. lamarckii* and *D. cariensis*. Among species, *D. davisiana*, for all groups of cardenolides, was found the poorest foxgloves containing only A and B groups at 0.26 and 0.15 mg/ 100 DW concentrations, respectively. Figure 15 demonstrates very clearly that the color densities of the lanes spotted on TLC plates were congruent with the mean numbers of cardenolides after HPLC analysis given in Table 19.



Genine Name	1	5	10	11	12	16
Digitoxigenin (A)	-H	-H	-CH ₃	-H	-H	-H
Gitoxigenin (B)	-H	-H	-CH ₃	-H	-H	-OH
Digoxigenin (C)	-H	-H	-CH ₃	-H	-OH	-H

Figure 14. Substitution of different positions in A, B and C groups of cardenolides and their appropriate colors represented in each row.

Table 19. Aglycon content (digitoxigenin, gitoxigenin and digitoxigenin) of the cardenolides extracted from leaf materials from different *Digitalis* species after acid hydrolysis (mg/100mg DW \pm SD).

Plant Species	Digitoxigenin (A)	Gitoxigenin (B)	Digoxigenin (C)
<i>D. lanata</i>	0.47 \pm 0.11	0.13 \pm 0.06	0.90 \pm 0.11
<i>D. lamarckii</i>	0.57 \pm 0.19	1.09 \pm 0.30	0.02 \pm 0.01
<i>D. cariensis</i>	0.38 \pm 0.09	0.84 \pm 0.21	0.03 \pm 0.01
<i>D. trojana</i>	0.27 \pm 0.10	0.89 \pm 0.28	0.08 \pm 0.01
<i>D. ferruginea</i> subsp. <i>ferruginea</i>	0.73 \pm 0.18	0.05 \pm 0.02	0.49 \pm 0.17
<i>D. ferruginea</i> subsp. <i>schischkinii</i>	0.37 \pm 0.09	0.20 \pm 0.09	0.50 \pm 0.19
<i>D. davisiana</i>	0.26 \pm 0.11	0.15 \pm 0.07	Not detected

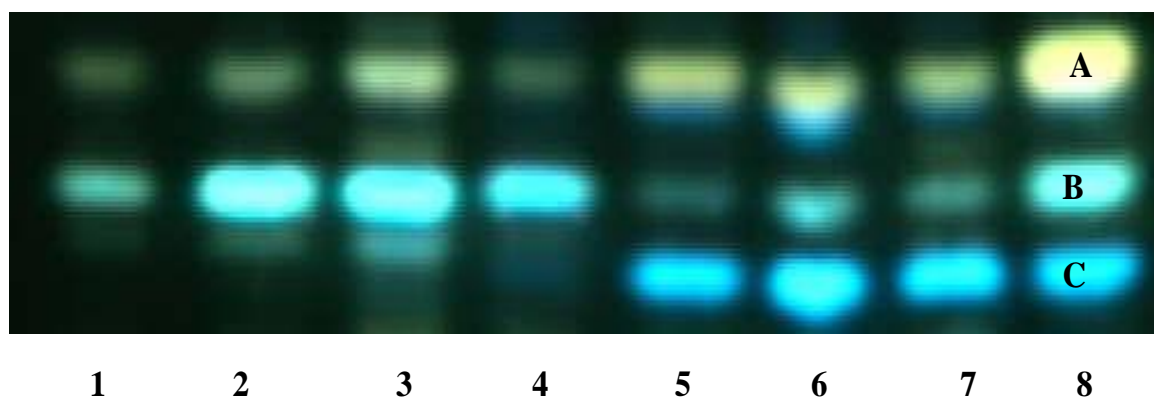


Figure 15. Acid hydrolysis of different *Digitalis* species collected from Anatolia: *D. davisiana* (1), *D. cariensis* (2), *D. lamarckii* (3), *D. trojana* (4), *D. ferruginea* (5), *D. schischkinii* (6), *D. lanata* from balkan species (7) and acid hydrolysis of lanatoside A, B and C as a marker.

When the percentages of cardenolide contents in dry leaf material of several *Digitalis* species were compared with data reported by Luckner and Wichtl (2000), there was no significant difference (Table 20.) among species. However, minimum values (lower bound) for cardenolide content of *D. cariensis*, *D. lamarckii* and *D. trojana* were estimated higher (30-40% more) than those of the literature. *D. davisiana* in both cases was found in similar ranges as reported by Luckner and Wichtl (2000).

Table 20. Comparison of cardenolide contents of A, B and C types (% range) in different *Digitalis* species with data reported by Luckner and Wichtl (2000).

Species name	Our Study	Luckner&Wichtl (2000)
<i>D. lanata</i>	1.3-1.6%	0.9-1.6%
<i>D. lamarckii</i>	1.3-1.9%	no data
<i>D. cariensis</i>	1.0-1.4%	0.6-1.2%
<i>D. trojana</i>	0.9-1.5%	no data
<i>D. davisiana</i>	0.1-0.2%	0.1-0.2%

In addition to TLC analysis of aglycone portions, it is noteworthy to show HPLC chromatograms of A, B and/or C-type cardenolides for each species as described clearly (Figure 16). As noted in Table 19 and 20, concentrations of A, B and C-type of cardenolides (mg/100 g DW) are shown for *D. lanata* (Figure 17), *D. trojana* (Figure 18), *D. cariensis* (Figure 19) and *D. lamarckii* samples (Figure 20). On the other hand, different vegetation periods (June, July and August) tested for *D. lamarckii* extracts shown on TLC plates revealed that C-type of cardenolides were not detected at all throughout the vegetation period. In June, budding or early-flowering stage, digitoxigenin derivatives of cardenolides (yellow) were detected slightly denser than that of July and August (Figure 21). It means that for the isolation or production of digitoxigenin derivative cardenolides, June is an available timing.

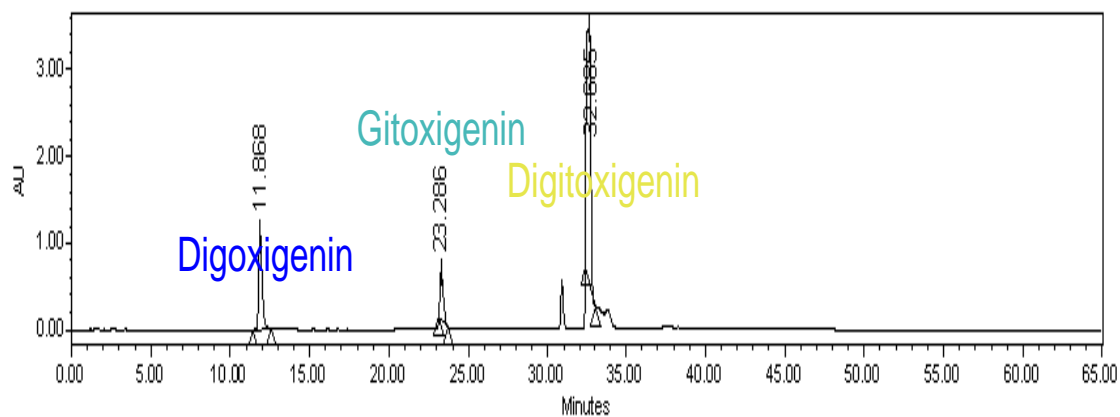


Figure 16 Aglycone portions (digitoxigenin, gitoxigenin and digoxigenin) of lanatoside A, B and C after acid hydrolysis with corresponding colors (letters of genine groups) of TLC analysis and retention times represented on the peaks.

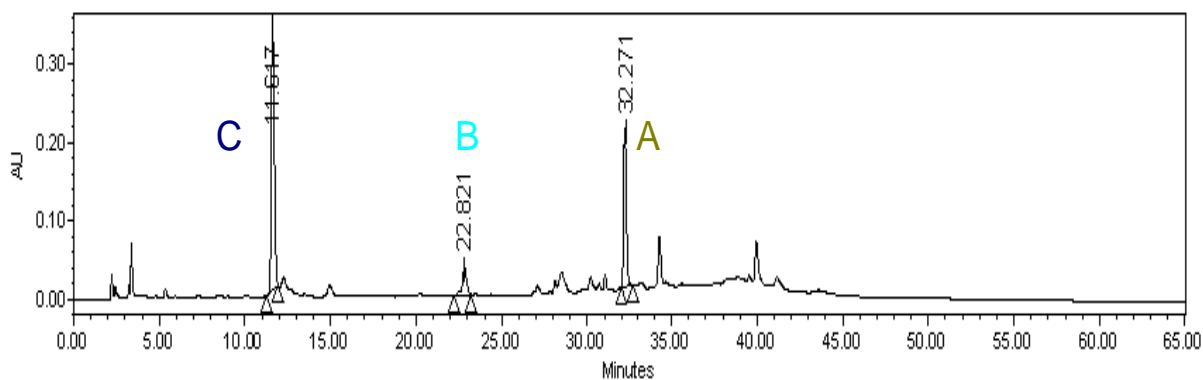


Figure 17. Aglycone portion of the cardiac glycosides represented A (digoxigenin), B (gitoxigenin) and C groups (digitoxigenin) after acid hydrolysis of *D. lanata* (control plant).

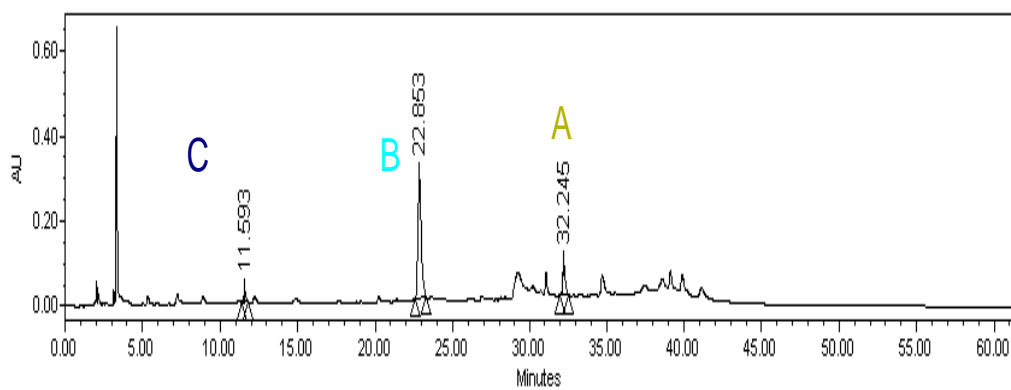


Figure 18. Aglycone portion of the cardiac glycosides represented A (digoxigenin), B (gitoxigenin) and C groups (digitoxigenin) after acid hydrolysis of *D. trojana*.

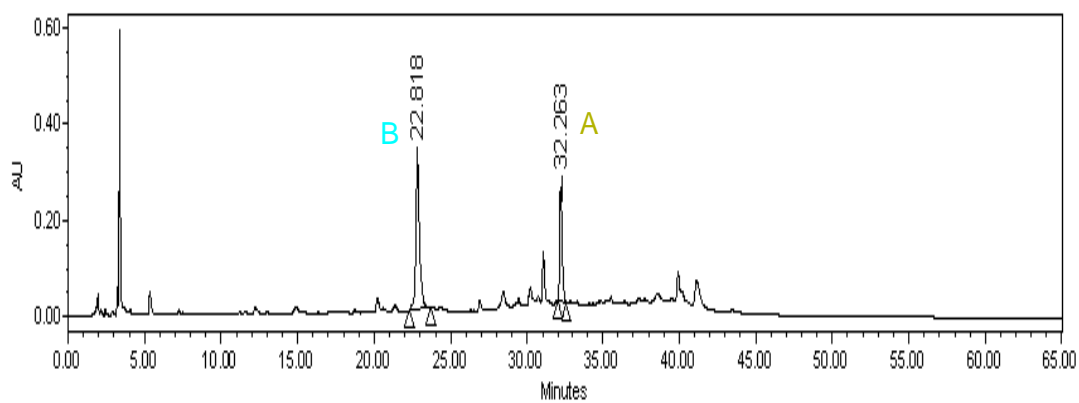


Figure 19. Aglycone portion of the cardiac glycosides represented A (digoxigenin), B (gitoxigenin) and C groups (digitoxigenin) after acid hydrolysis of *D. lamarckii*.

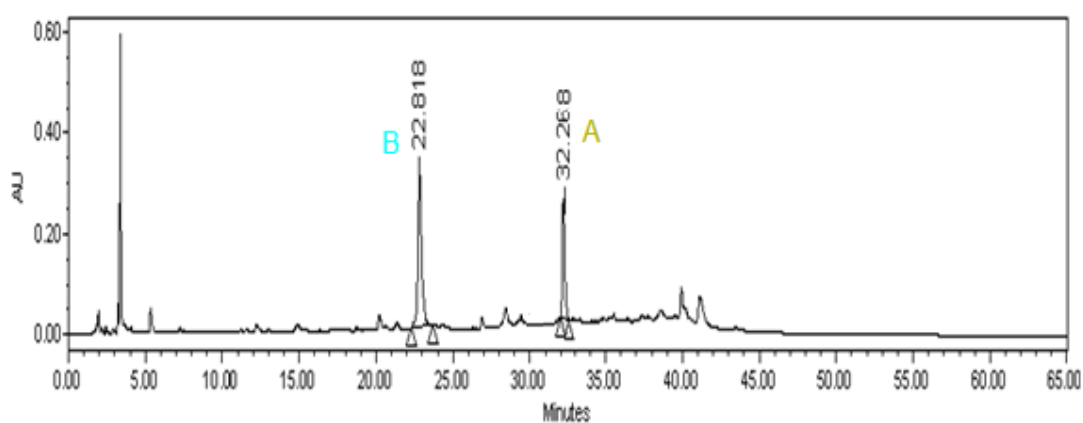


Figure 20. Aglycone portion of the cardiac glycosides represented A (digoxigenin), B (gitoxigenin) and C groups (digitoxigenin) after acid hydrolysis of *D. cariensis*.

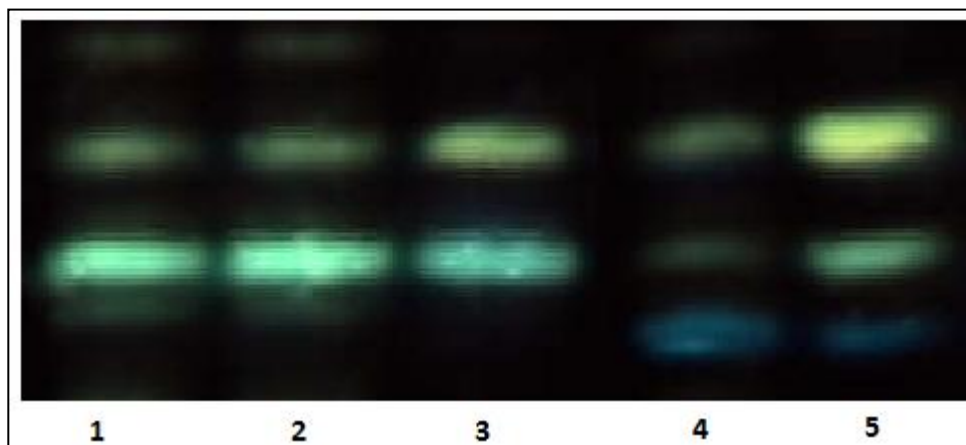


Figure 21. Cardenolide pattern (aglycons) of *D. lamarckii* collected in different vegetation period August (1), September (2), June (3) and *D. lanata* (4) corresponding to markers after acid hydrolysis (5).

In addition to the data given in TLC and HPLC analysis related to the aglycone portions of cardenolides, cardiac glycosides without acid hydrolysis were run on TLC plate. Depending on the polarity of the substances related to the sugar side attached at 3rd carbon atom, the lanes with different colors were visible in corresponding RF values (see Figure 22). Side chains of the cardiac glycosides were demonstrated in Figure 22. Commercially important genin groups depending on the presence of -OH (or -H) group in carbon number 12 and/or 16 different colors (yellow, turquoise or blue) for each genine group were clearly visible on the TLC plates. For cardenolides detected, glucodigifucosit, glucogitorosit, digitalinum verum, neo-digitalinum verum, purpurea glycosit B, neo-odorobiosit G, glucoverodoxin, glucolanadoxin, strosposit, gitoxigenin gluco metylosid and cardenolide AX (unknown cardenolide belonging to group A) were found in various amounts in *D. lanata* (HPLC data not shown), *D. cariensis*, *D. lamarckii* and *D. trojana*. Of those, C group cardenolides (digitoxigenin derivatives; i.e. lanatoside C and/or digoxin) were predominantly found (see blue lanes) in *D. lanata* leaf extracts

(Figure 17 and 21). However, other species (*D. cariensis*, *D. lamarckii* and *D. trojana*) were richer in terms of B groups (gitoxigenin derivative) than *D. lanata*. *D. davisiana* as shown in Table 20 was the poorest foxglove among all tested ones. In Figure 24, five different species followed by the numbers from 1 to 5 (*D. lanata*, *D. davisiana*, *D. cariensis*, *D. trojana* and *D. lamarckii*) were demonstrated in terms of their cardiac glycoside contents. Among the species, more lanes have been detected in *D. lanata* (at least 13 different lanes), while *D. davisiana* had 4, *D. cariensis* had 10, *D. trojana* had 9, *D. lamarckii* had 9 lanes on TLC plate. Some lane(s) was not reliable for identification of cardenolide groups detected between RF values 0.7 and 0.8. It was very clear that the lanes with turquoise color were predominantly found in *D. cariensis* and *D. lamarckii* (two lanes represented at RF 0.1 and 0.2, one lane at RF 0.5). On the other hand, cardenolide profile of *D. lanata* seemed to include all cardiac glycosides detected in our endemic species. TLC lanes in Figure 24 were concentrated (almost 3 folds) in Figure 25 to show similarity in terms of cardenolides as a fingerprint pattern among *D. cariensis*, *D. trojana* and *D. lamarckii*.

Our findings also proved that greenhouse samples, in this case *D. trojana*, grown under same conditions (from germination to harvesting) show different cardenolide patterns comparing to the natural populations shown in Figure 25. Comparing to Figure 26, the number of detectable cardiac glycosides on TLC plate seems higher than that of the lanes shown in Figure 24 and 25 for *D. trojana* samples (15 to 9 or 10 lanes). TLC analysis also shows that endemic species, except *D. davisiana*, seem richer than *D. lanata* in terms of gitoxigenin derivatives of cardenolides. In contrast to previous TLC analysis (Figure 24 and 25), *D. trojana* contained higher numbers of TLC lanes than *D. cariensis* and *D. lamarckii*. Of those, demonstrated in *D. trojana*

were 15 different cardiac glycosides. Similarly, *D. lanata* samples had 20 different lanes in greenhouse samples (Figure 26).

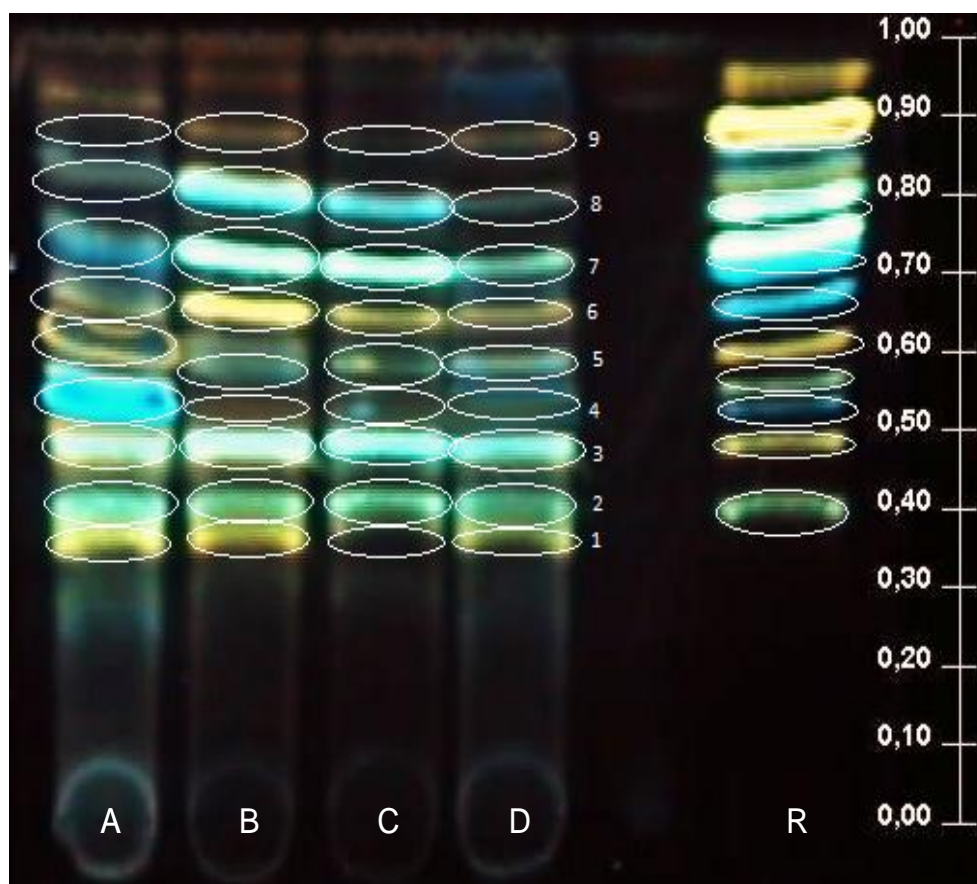


Figure 22. TLC analysis of cardenolides extracted from basal leaves with corresponding RF values as a fingerprint pattern for the comparison of closely related *Digitalis* species according to Werner's classification (1960, 1965): *D. lanata* (A), *D. cariensis* (B), *D. lamarckii* (C), and *D. trojana*(D) and some reference substances (R) as follows: Glucodigifucosid (1), glucogitorosid, Digitalinum verum, neo-digitalinum verum (2), Purpurea glycosid B+Neo-odorobiosid G (3), lanatoside C (4), Glucoverodoxin (5), Glucolanadoxin (6), Strospesit (7), gitoxigenin gluco-metylosid (8), A-X, an unknown digitoxigenin derivative cardenolide (9) (This plate was soaked twice in TLC chamber).

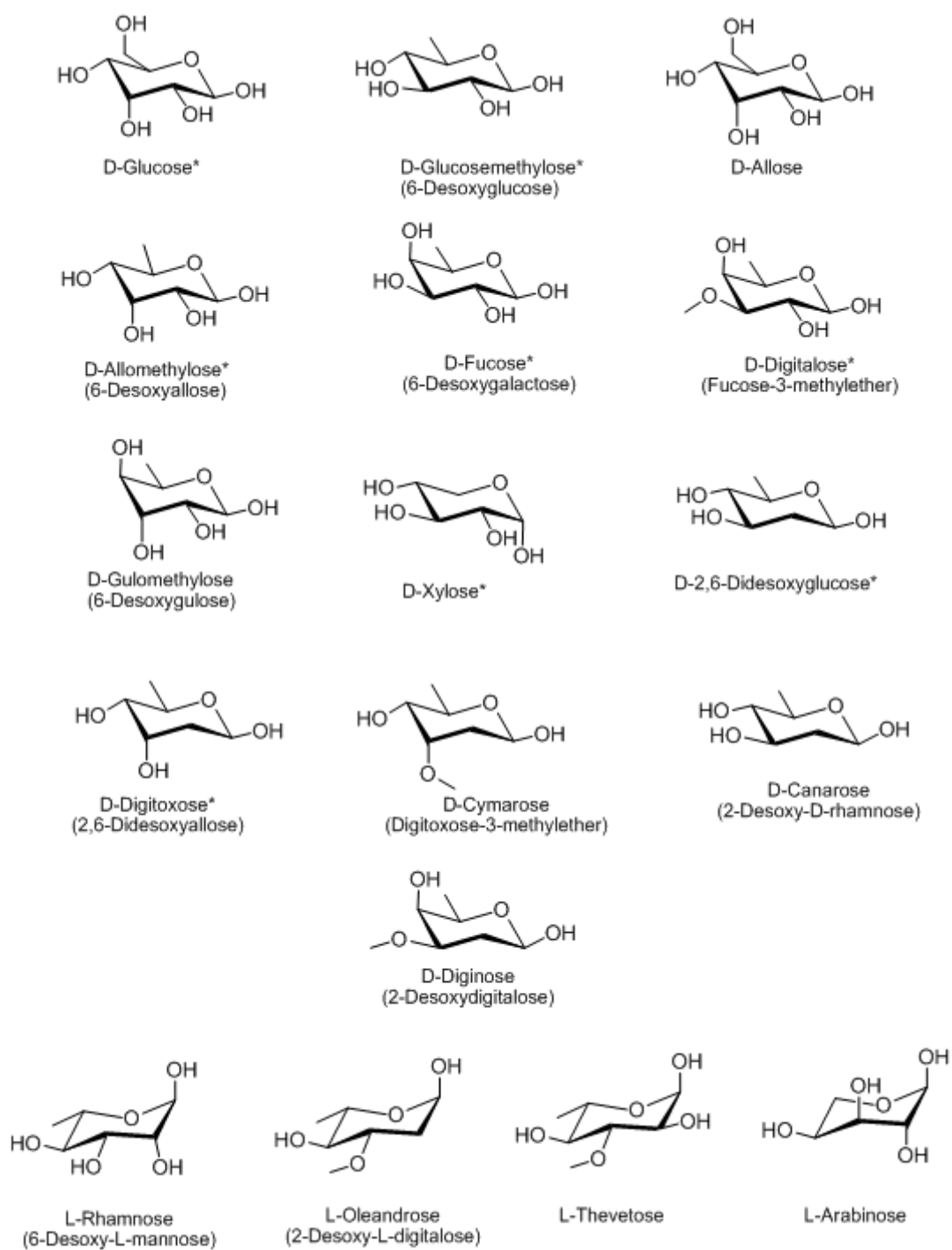


Figure 23. Sugar groups at side chain of cardiac glycosides (* represents main sugars) (from Luckner and Wichtl 2000).

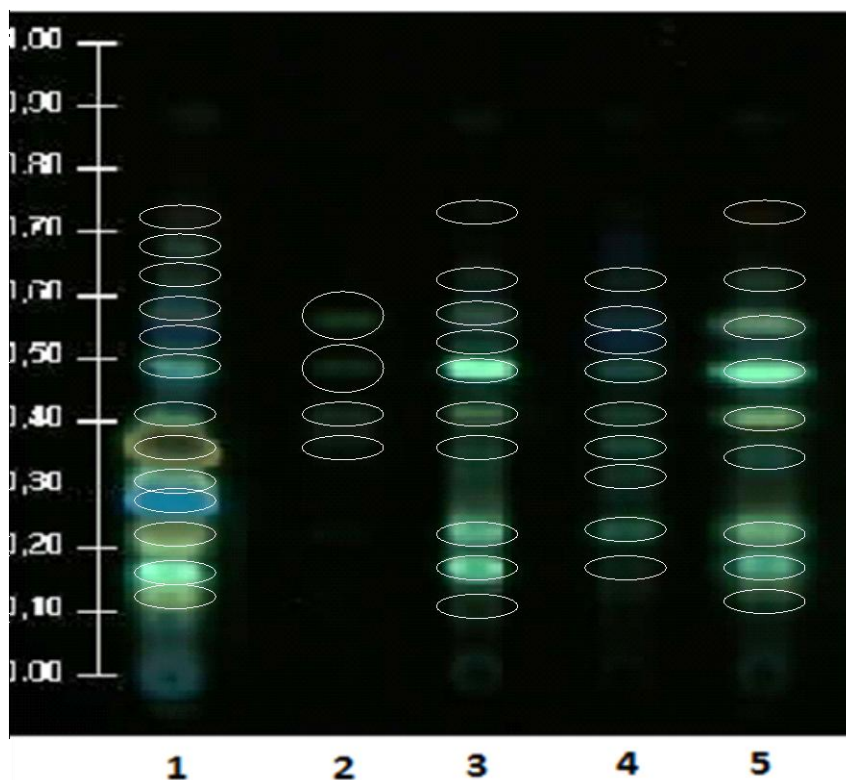


Figure 24. Cardiac glycosides belonging to different *Digitalis* species; *D. lanata* (1), *D. davisiana* (2), *D. cariensis* (3), *D. trojana* (4) and *D. lamarckii* (5).

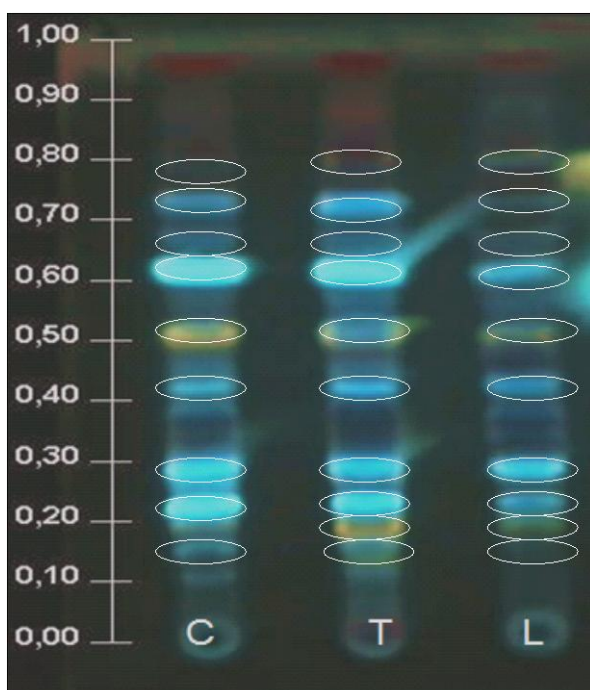


Figure 25. Repetition of Figure 24 with concentrated extracts that shows fingerprint patterns of *D. cariensis* (C), *D. trojana* (T) and *D. lamarckii* (L). Tourquoise lanes represent gitoxigenin; yellow lanes represents digitoxigenin derivatives of cardenolides.

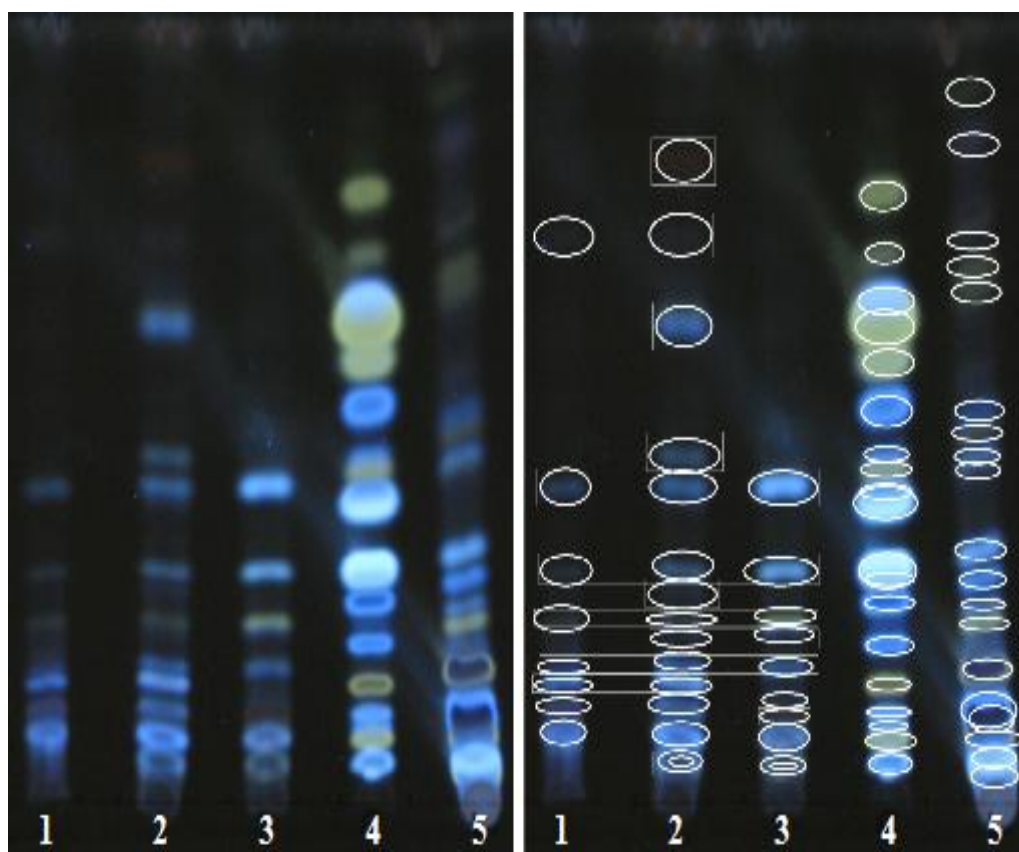


Figure 26. Cardenolide extracts (50 μ l for each spot) from 5 months old plants of different foxgloves endemic to Turkey. Lanes: 1- *D. cariensis*, 2- *D. trojana*, 3- *D. lamarckii*, 4- reference substances, 5- *D. lanata*. Both figures show the same TLC plate. The figure at the right identifies the cardenolides with circles with regard to their respective aglycone portions.

When taken into account the cardenolide production of the samples collected from different places at different vegetations, it was clear that there was no significant differences in terms of cardenolide patterns among species. For instance, in Figure 27, when *D. lamarckii* samples collected from Ankara during budding (in June), fruit and seed-setting periods (in August and November) were compared, RF values of the lanes between 0 and 0.5 were similar. However, in June, one lane appearing at 0.6 which was also highest non-polar metabolite was found in budding stage only, not in August and November samples at all (Figure 27, see the white circle). Similarly,

different locations affect the cardenolide pattern of the species. For example, the samples collected in the same growth period (June) but in different locations (Konya and Ankara) were not stable for cardenolide accumulation when the density of lanes appeared were considered (see red and green circles in Figure 26). For example, sample 3 collected from Konya contained neo-odorobiosid G belonging to digitoxigenin derivative. On the other hand, *D. ferruginea* and *D. lanata* might be shown as distinct species having different patterns regarding to fingerprint assessment of cardenolides (see Discussion).

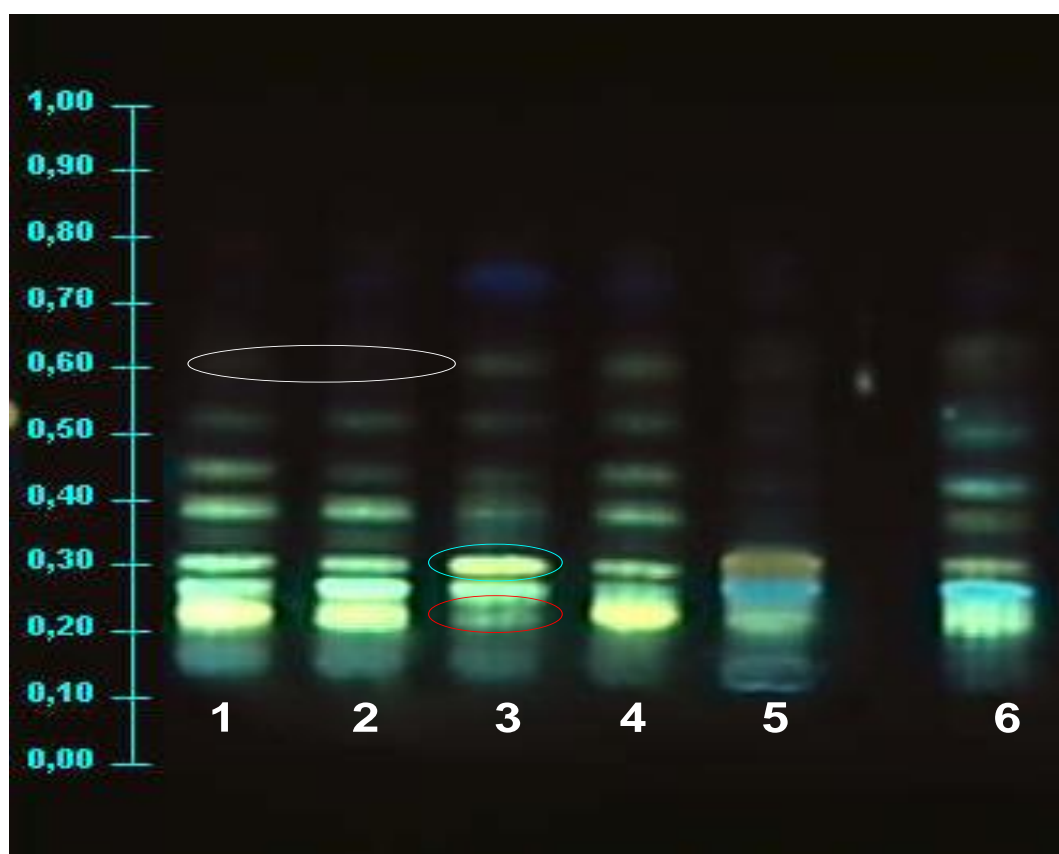


Figure 27. Cardiac glycosides of *D. lamarckii* collected from different locations in different vegetation periods as follows: August, flowering and/or fruit-setting; samples collected from Ankara (1), November; mature and dried fruit formation from Ankara (2), June; budding and/or flowering formation from Konya (3) June, budding and/or flowering formation from Ankara (4), *D. ferruginea*, flowering stage (5), *D. lanata* (flowering stage) (6). Circles in different colors represent presence or absence of cardiac glycosides on TLC plate.

5.2.2. Quantification of cardiac glycosides detected in natural populations

Some of the cardenolides detected on TLC plate were also quantified by using HPLC system (Figure 28). It was clearly shown that cardenolide content might fluctuate depending on physiological age, since they were not constantly found in vacuole of the cells. In terms of total cardenolides based on the content of A, B and C groups did not differ in *D. lamarckii*, *D. lanata* and *D. cariensis* significantly (Table 21 and Table 22). Of those cardenolides, glucolanodoxin, neo-glucodigifucosid, neo-odorobiosid G and glucogitorosid were predominantly found (see overall means of 58.8, 77.5, 82.5 and 90.3 mg/100 g DW, respectively in endemic species (Table 22). *D. lamarckii* was richest species in terms of neo-odorobiosid and glucogitorosid content when compared to other species (containing 121 and 112 mg/100 g DW, respectively (Figure 29). Higher strosposit was observed in *D. trojana* containing 61 mg/100 g DW, while *D. cariensis* and *D. lamarckii* containing 45 or 30 mg/100 g DW (Figure 28). Neo-digitalinum verum was detected at moderate amounts (ranging from 35 to 45 mg/100 g DW) in *D. trojana*, *D. cariensis* and *D. lamarckii*, whilst it was not detected in *D. davisiana* (<LOD). Similarly, strosposit, purpurea glycosid B and digitoxin were not detected in *D. davisiana* extracts. This finding was correlated with the qualitative analysis of cardenolides present in this species (see the lanes appearing very faint and Table 22).

Table 21. Cardenolide content (mg/100g DW) detected in some endemic Anatolian species (eighth months old) grown under greenhouse conditions (nd: not detected; \pm : SD).

Species	Neo-digit. Verum	Strospesit.	Glucogitorosid	Neo- glicodigifucosid	Gluco- lanadoxin	Neo-od.bio. G	Purpurea glycosid B	Gluco- evatromonosid	Digitoxin
<i>D. trojana</i>	39 \pm 7	61 \pm 17	95 \pm 11	78 \pm 18	79 \pm 20	87 \pm 17	26 \pm 9	19 \pm 10	5 \pm 2
<i>D. lamarckii</i>	45 \pm 12	45 \pm 11	112 \pm 19	80 \pm 22	96 \pm 14	121 \pm 25	25 \pm 6	18 \pm 7	nd
<i>D. cariensis</i>	35 \pm 10	30 \pm 12	100 \pm 15	65 \pm 18	94 \pm 13	98 \pm 13	20 \pm 7	10 \pm 6	nd
<i>D. davisiana</i>	nd	nd	39 \pm 10	12 \pm 6	41 \pm 11	24 \pm 8	nd	7 \pm 3	nd
Overall mean	29.8\pm17.1	34.0\pm23.5	90.3\pm28.8	58.8\pm34.2	77.5\pm29.7	82.5\pm33.9	17.8\pm12.9	13.5\pm13.9	1.3\pm2.0

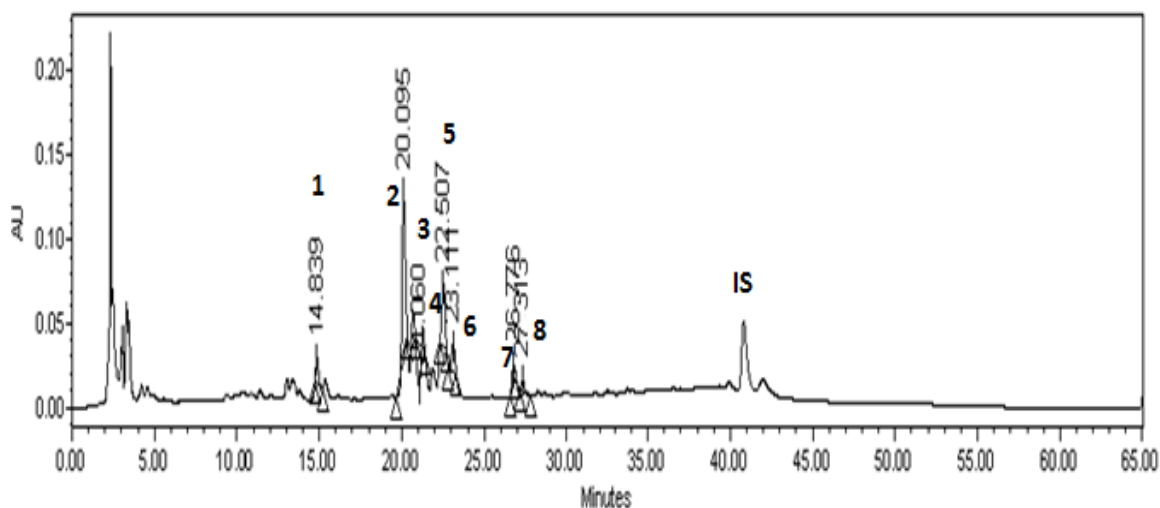


Figure 28. Cardenolides detected in *D. lamarckii*, *D. cariensis* and *D. trojana* samples as follow in numbers: Neo-digitalinum verum (1), glucogitorosid (2), strosposit (3), neo-glucodigifucosid (4), glucolanodoxin (5), neo-odorobiosid G (6), purpurea glycosid B (7) gluco-evatramonosid (8) and internal standart (IS). Chromatogram belongs to 8 months old *D. lamarckii* plants grown under greenhouse conditions.

Table 22. Glucogitorosid content of *D. davisiana* in different vegetation periods (System 2).

Period	Glucogitorosid (mg/100 g DW)
June	23.1±3.0
August	28.2±3.7
September	17.9±2.3

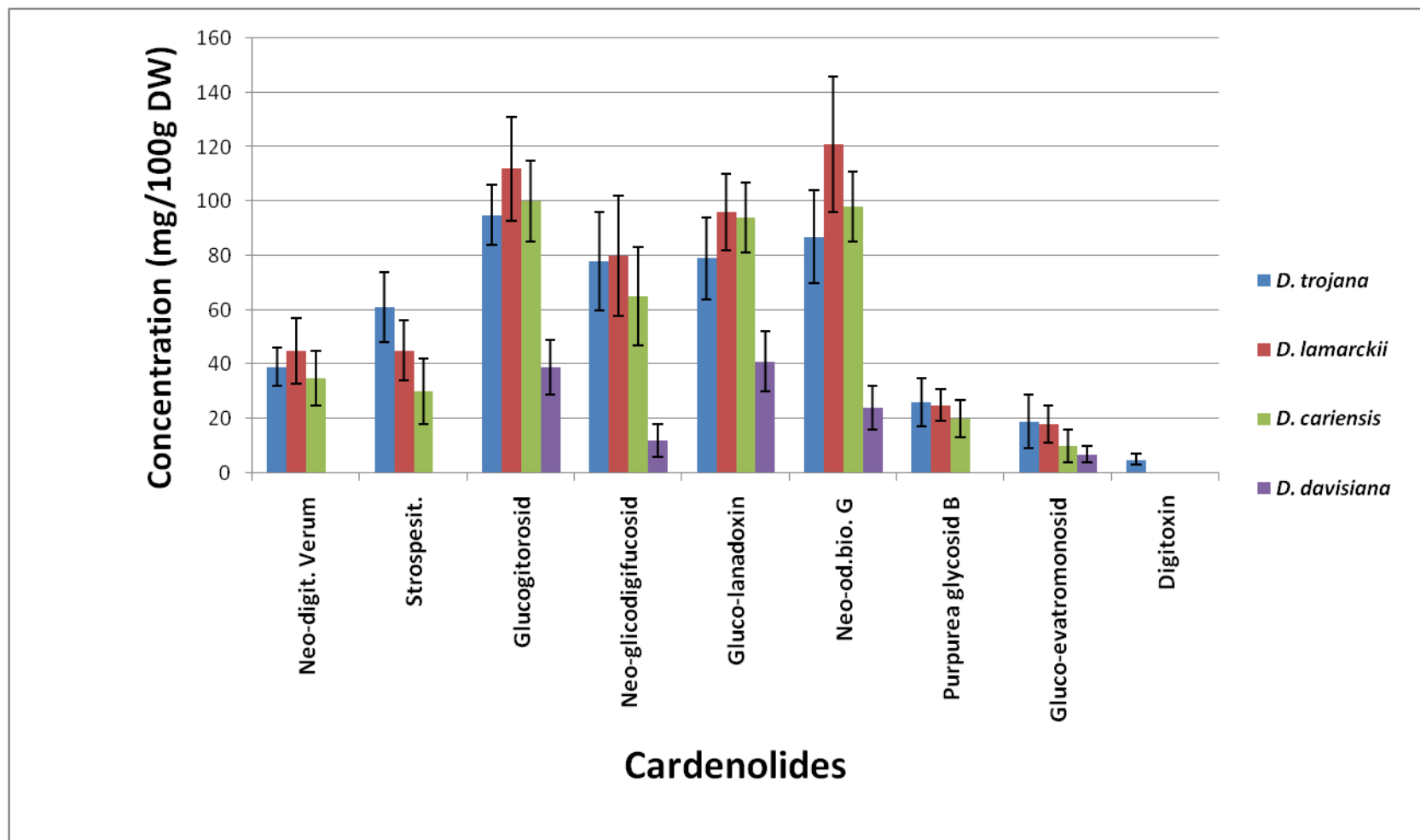


Figure 29. Contents of different cardenolides detected in endemic Anatolian foxgloves grown in greenhouse.

In some experiments using system 2 for HPLC analysis, cardenolide determination was carried out using glucogitorosid, a cardenolide mainly found in Anatolian endemics. In this case, different vegetation periods of *D. davisana* were evaluated in terms of glucogitorosid content in the leaves (Table 22, Figure 30). Samples collected in June (budding stage) or August (flowering stage) were appeared to have almost equal amounts of glucogitorosid in the leaf samples (23 or 28 mg/100 g DW). However, in September, samples had the lower amount of metabolite than other periods (17.9 mg/100 g, Figure 30). When compared greenhouse samples in Table 21, younger basal leaves (eight months old) had the highest amount of glucogitorosid (compare 39 with 28 mg/100 g DW).

Similarly, *D. cariensis* samples differ in glucogitorosid content when different locations were taken into account for natural populations (Table 23). For example, samples collected from Manavgat had almost 50% more glucogitorosid than Alanya samples (25.6 compared to 11.3 mg/100 g DW). On the other hand, glucogitorosid content in September for Alanya samples was higher than the samples collected in August at same location (compare 16 with 11.3 mg/100 g DW). When compared with greenhouse (younger samples), it was clear that cardenolide contents were significantly lower for natural populations collected in different periods of time.

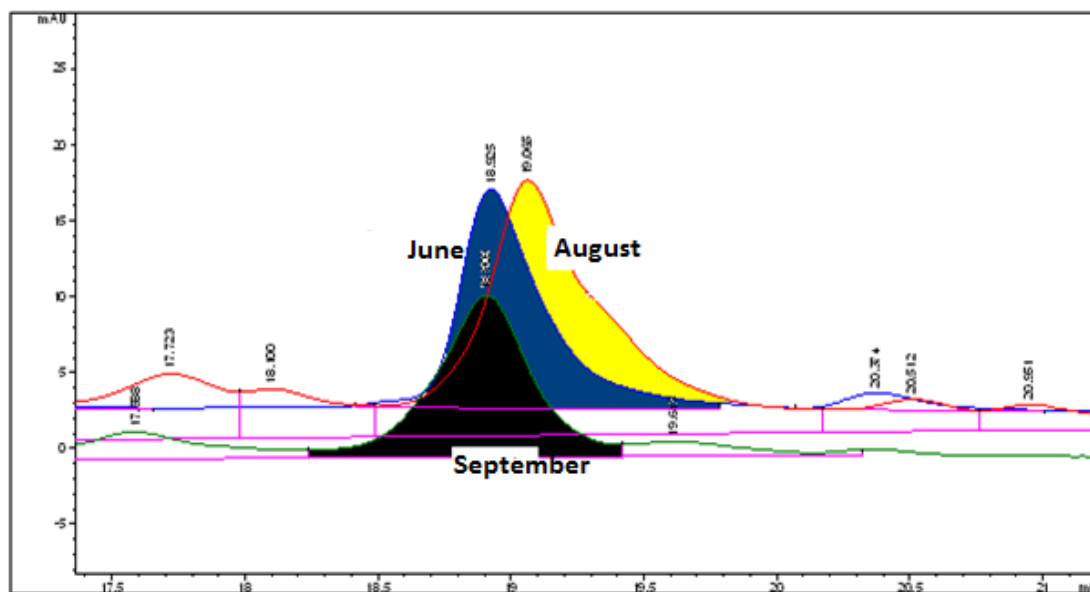


Figure 30. Chromatogram belonging to glucogitorosid pattern of *D. davisiana* in different vegetation periods (system 2).

Table 23. Glucogitorosid content in Alanya and Manavgat samples collected in August or September (\pm SD, standard deviation).

Location	Glucogitorosid content (mg/100 g)
Alanya	11.3 \pm 1.9
Alanya*	16.0 \pm 3.5
Manavgat	25.6 \pm 3.0

* collected in September

5.2.3. Quantification of cardiac glycosides detected in *in vitro* samples

HPLC was used to quantify the glucogitorosid content for *in vitro* materials, regenerated plantlets or germinated seedlings (Table 25). Glucogitorosid accumulation in *in vitro* regenerants was remarkably higher when shoots were regenerated on LS or B5 medium containing 0.5 mg/L TDZ and 0.25 mg/L IAA, producing 14.2 and 13.6 mg/kg DW glucogitorosid, respectively (Medium 1 and 2, Table 24). It is important to note that regenerants developed on LS medium containing either 0.5 mg/L TDZ and 0.25 mg/L IAA (Medium 4) or TDZ alone (Medium 5) produced only trace amounts of lanatoside C (data not shown), irrespective of 2- or 4-month culture duration in the final growth stage, glucogitorosid contents were dramatically reduced when TDZ was used alone during the first stage of regeneration (compare Medium 1 and 5, Table 25). For *in vitro* seedlings germinated on medium containing 0.5 mg/L GA₃ (Medium 6) produced considerably more glucogitorosid than those of the seedlings germinated on medium containing no PGRs (Medium 7), 16.1 compared to 7.4 mg/kg DW.

Glucogitorosid content of *D. cariensis* was also evaluated in *in vitro* samples (Table 26). Four months old seedlings or regenerants produced from either direct germination or via regeneration through FBT explants depending on medium formulation. It was shown that regenerants developed on LS medium containing 0.5 mg/L TDZ in combination with 0.25 mg/L IAA had the highest amount of glucogitorosid content (44.1 mg/kg DW). This finding was found three times higher than seedlings (44.1 to 14.2 mg/kg DW, Table 25).

Table 24. Glucogitorosid content of *D. davisiana* samples on different regeneration or germination medium (\pm SD, standard deviation).

Tissues	Medium	Amount (mg/kg DW)
Regenerants ^a	1	14.2 \pm 2.9
	2	13.6 \pm 2.3
	3	1.4 \pm 0.3
	4	5.8 \pm 1.4
	5	8.7 \pm 1.2
Seedlings ^b	6	16.1 \pm 3.3
	7	7.4 \pm 1.3

^aThe regenerated plantlets were obtained by regenerating shoots from FBT explants cultured on LS medium (1, 3, 4 and 5) or B5 medium (2) containing either 0.5 mg/L TDZ and 0.5 mg/L IAA (1, 2, 3 and 4) or 0.5 mg/L TDZ alone (5) after 6 weeks of culture, then followed by further growth of the shoots on LS medium containing no PGRs for 2 weeks before they were rooted on LS medium containing 0.5 mg/L IAA for 4 weeks, and finally grown on LS medium at full-strength (1, 2, 4 and 5) or half-strength (3) with no PGRs for 2 months (4) or 4 months (1, 2, 3 and 5).

^bThe germinated seedlings were obtained by germinating seeds and keeping them growing on full-strength LS medium containing 0.5 mg/L GA₃ (6) or no PGRs (7) for 6 months.

Table 25. Glucogitorosid content of 4-months old *D. cariensis* seedlings germinated from the seeds or regenerants produced from different media formulations (\pm SD, standart deviation).

Medium Type	Glucogitorosid (mg/kg DW)
LS (no PGR)	14.0 \pm 3.4
0.1 TDZ+0.1 IAA, LS	22.0 \pm 5.8
0.5 TDZ+0.25 IAA, LS	44.1 \pm 11.1
0.5 TDZ/0.25 IAA LS/2	27.2 \pm 3.2

12-weeks old regenerants were used for HPLC analysis to determine glucogitorosid contents in leaves or petiole parts of *D. lamarckii* (Table 26 and Figure 31). For 12 week-old samples, lamina or petiole parts were individually extracted. Seedlings germinated through seeds without any PGR were also evaluated in terms of their metabolite content. Of those samples, lamina had the highest amount of glucogitorosid content (143 mg/kg DW). On the other hand, petiole had almost 60% less glucogitorosid (53.4 mg/kg DW) than lamina samples. For the seedlings germinated *in vitro* through seeds, they had a significantly lower amount of cardenolide than lamina samples (compare 143.9 with 15.9 or 56.0 mg/kg DW). However, younger seedlings which were six-week old had the lowest amount in terms of glucogitorosid content among all tested materials (15.9 mg/kg DW). In this experiment, LS medium without PGR was found optimum for 12-week cultivation for regenerants after rooting stage. Without medium replenishment or subculturing, some experiments were continued until some extent to which tissues become brownish color. It was found that 18-week-old regenerants which were subjected to tissue-browning produced a quite low amount of glucogitorosid, compared to 12 week-old lamina samples (18 in contrast to 143 mg/kg DW). It is noteworthy that basal medium is critical for cardenolide accumulation as well as further plant growth and development. When cardenolide analysis in callus tissue belonging to all *Digitalis* species in this study were considered, none of the callus tissue produced cardenolide, at all.

Table 26. Comparison of glucogitorosid contents of various *D. lamarckii* samples excised from 6, 12 or 18-week-old regenerants or seedlings germinated through seeds (\pm SD, standart deviation).

Samples	Glucogitorosid
Lamina ¹	143.9 \pm 21.6
Petiol ¹	53.4 \pm 13.5
Regenerant ²	18.0 \pm 6.1
Seedling ²	15.9 \pm 4.6
Seedling ³	56.0 \pm 8.1

¹Lamina or petiole samples excised from 12-week-old regenerants, ² 18-week-old complete shoot without root ² 6- or ³ 18-week old seedlings without roots germinated through seeds.

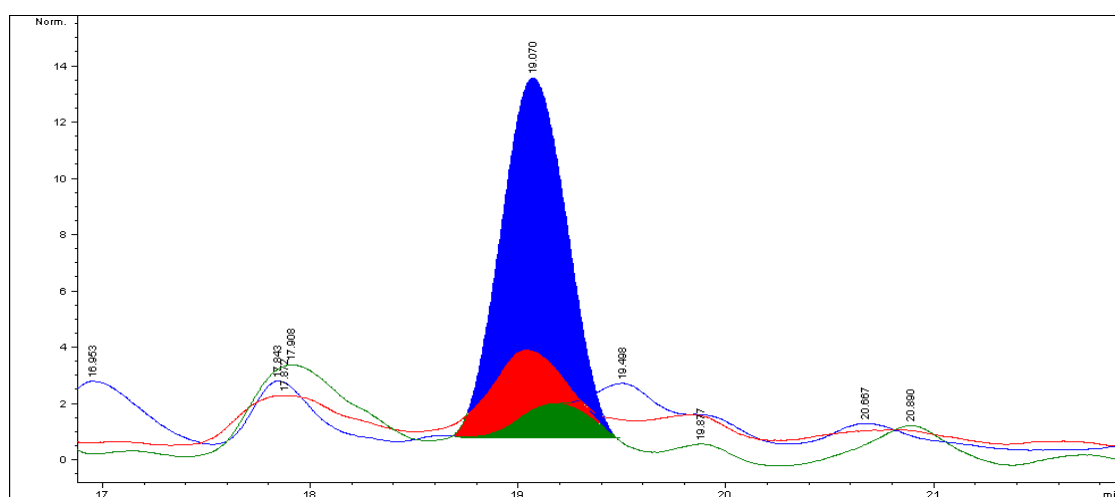


Figure 31. Comparison of glucogitorosid content of 12- or 18-week-old regenerants (Blue peaks represent 12-week-old lamina, red one represents petiole extracts. Green peaks represent 18-week-old green parts of germinated seedlings).

CHAPTER 4.

DISCUSSION

7.1. *In Vitro* Studies

There has been no report on *in vitro* regeneration together with cardenolide profiling of endemic Anatolian foxglove species. For the first time, this study reports a high-frequency plant regeneration protocol for *D. davisiana*, *D. cariensis*, *D. trojana* and *D. lamarckii* as well as an assessment of cardiotonic glycoside production in the leaves of natural populations and some *in vitro* produced materials (via regeneration or germination). To develop an efficient *in vitro* regeneration, once studies were initiated using *D. davisiana* for medium, growth regulator or explant selection to be employed for other *Digitalis* species. Two sets of experiments were carried out in the experiments of *D. davisiana*. In the first, different concentrations and combinations of TDZ and IAA were compared using B5 medium (Table 6), while in the second set, comparisons were made with six different media formulations (B5, MS, MM, CP, LS or SH) that were supplemented with the optimum PGR combination (0.5 mg/L TDZ and 0.25 mg/L IAA) that had been identified in the first set of experiments. For both sets of experiments, four different types of explants (hypocotyl, leaf, root and flamingo-bill type) were excised from 2- to 3-week-old seedlings germinated *in vitro*.

Irrespective of the explant type (Table 7), it was clear that PGR composition of the culture medium was a significant factor for the efficiency of shoot regeneration. When overall means of different explants excised from *D. davisiana* were considered, it was evident that increasing concentrations of TDZ, from 0.1 to 0.5 mg/L, steadily increased the shoot numbers; however, considerably higher numbers of shoots were produced when TDZ was combined with IAA. Of the combinations employed, a higher TDZ level (0.5 mg/L), combined with lower IAA levels (especially 0.25 mg/L), was the most effective combination for the shoot regeneration from all explant types, particularly those from hypocotyl and FBT explants, which produced a mean of 6.3 and 5.9 shoots per explant, respectively. In fact, these findings are consistent with later studies conducted with *D. cariensis* and *D. trojana*. Due to the high capacity for shoot induction, FBT explants induced adventitious shoots for many *Digitalis* species (*D. davisiana*, *D. cariensis* and *D. trojana*) 5–7 days earlier than with other types of explants, including hypocotyl explants (observed data). With FBT explants, it has been suggested that, once meristems are eliminated, shoot development occurs in the position of the medium where the regenerative callus would receive the nutrients and PGRs required for differentiation (Pozueta-Romea et al. 2001). Different basal medium formulations were also evaluated for regeneration efficiencies. Therefore, LS medium was found to be most effective (Table 8), which might be attributed to the higher thiamine content than other related basal media, as it was previously reported that presence of this vitamin increases vigorousness and longevity of tobacco tissues in culture (Linsmaier and Skoog 1965).

Therefore, LS medium was selected as a typical basal medium throughout the studies conducted for all *Digitalis* species involved in this study. As noted above, TDZ and IAA combinations (0.5 and 0.25 mg/L, respectively) was optimized to achieve a high efficiency *in vitro* regeneration of *D. cariensis* and *D. trojana*.

D. cariensis was one of the tested species, in which germination took not only longer than others but also frequency of germination was much lower (about 2 to 5% of all tested seeds; data not shown). There has also been one report on the difficulties (high dormancy) of germination as well as *in vitro* regeneration of *D. cariensis* (Yürekli et al. 1996). In spite of high dormancy, few numbers of seedlings were used to proceed shoot multiplication via either axillary or adventitious regeneration. Effective TDZ and IAA concentrations were found only successful with FBT explants of *D. cariensis* (Table 9). On the other hand, callus formation of *D. cariensis* was also achieved in leaf samples excised from older seedlings (8-week-old or more). Effects of different BA and NAA combinations were found critical for callusing. However, callus produced did not have an organogenic capability. Instead, necrosis was a predominant pattern when BA or NAA was used alone (Table 10). In order to overcome necrosis, in favor of callus production efficiency, it was clear that BA and NAA when combined at higher concentrations (higher than 0.5 mg/L BA or 0.1 mg/L NAA) were found effective. Similarly different TDZ or TDZ and IAA concentrations were tested on *D. trojana* using hypocotyl and FBT explants excised from 3-to 4-week-old seedlings. TDZ (0.5 mg/L) and IAA (0.25 mg/L), as noted optimal PGRs, were the most effective growth regulators for both types of explants examined (4.3 shoot per hypocotyl and FBT explant). In contrast to the low organogenic response of hypocotyl explants, FBT was found very successful, producing a mean of 6.6 shoots. Although hypocotyl explants produced considerably

few numbers of shoots in some treatments (see Table 12 and 13) than FBT explants, they are quite easier for excision than FBT explants which requires a great deal of care and effort for cutting prior to transferring onto the regeneration medium.

TDZ used alone initiated a slight callus formation on hypocotyl explants within 2-3 weeks. However, in combination with IAA (any concentration) produced more massive callus on the explants excised from *D. trojana* or *D. davisiana* (more callusing was observed on hypocotyl than FBT). This finding was also in accordance with Huetteman and Preece (1993). TDZ was reported to be stable for long term cultivation up to 33 days in *Phaseolus lunatus* callus (Mok and Mok 1985), thus it has been chosen for many *in vitro* regeneration systems of medicinal plants (Khawar et al. 2004; Linden and Riikonen 2006; Turker et al. 2009a, 2009b, Yucesan et al. 2009) and has been therefore regarded as a powerful inducer of adventitious shoot and somatic embryo formation in many ornamental plants (Lin et al. 1997; Mirici et al. 2005, Sanikhani et al. 2006). Saxena et al. (1992) suggested that TDZ helps to establish the optimal balance of cytokinin and auxin required for the induction and expression of embryonic response. On the other hand, LS medium as compared to other basal media was found effective for prolonged cultivations. This might be due to higher thiamine content of the medium used. Linsmaier and Skoog (1965) reported the effectiveness of thiamine as a growth factor on tobacco tissues during organ formation, increasing vigor and longevity of the targeted tissues. Our findings as noted before are consistent with this report in terms of vigorous indirect organogenesis and no longer need of subculturing during regeneration (up to eight weeks in all our experimented species).

Among all endemic species used in our tissue culture experiments, various concentrations of BA and BA plus NAA, instead of TDZ and IAA combinations, were used in *D. lamarckii*. Besides, efficiency of cotyledonary leaves comparing to other tested species (*D. davisiana*, *D.lamarckii* and *D. cariensis*) was found significantly higher than other types of explants. Since the highest number of shoot was produced with cotyledons (8.4 shoots per explant), this explant type was available for the regeneration system of *D. lamarckii*. In fact, our findings related to the regeneration capacity of cotyledonary explant are not significantly different from FBT explants but as mentioned before in case of hypocotyl explants, preparation of the cotyledonary leaf samples seem also quite easier than preparation of FBT explants. Callus formation was also studied in this species as done in *D. cariensis* samples. Herein one difference might be attributed to the callus production for organogenic response depending on PGR combinations, that the competency of the cells for regeneration is very successful for *D. lamarckii*. Within this framework, unless otherwise stated, among all tested species *D. lamarckii* was the most productive foxglove producing organogenic callus, frequencies ranging between 65 and 100% depending on BA and NAA concentrations. For instance, high concentrations of BA (3 or 5 mg/L) and NAA (1 or 2 mg/L) were found highly effective for callus production. Therefore, those of *D. lamarckii* callus after transferring to various concentrations of GA₃ and/or BA induced shoot formation efficiently. The mean number of shoots produced from callus on LS medium containing BA (0.1 mg/L) alone or in combination with GA₃ (0.25 or 0.5 mg/L) were about 6.0 shoots per callus (up to 6.7 shoots) after 6-week cultivation. According to literature survey, there has been only one report on effects of GA₃ on morphogenesis in which it was found that gibberellic acid by itself did not induce morphogenesis but

modified responses promoted by auxins and/or cytokinins (Gavidia et al. 1993). Our findings are strongly consistent with this report in terms of single usage of GA₃ (0.25 or 0.50 mg/L) producing 2.3 or 3.1 shoots while produced higher amounts when combined with BA (i.e. 0.5 mg/L GA₃ and 0.25 mg/L BA produced 6.6 shoots per callus with a frequency of 91% indirect regeneration; Table 18).

Following the shoot multiplication process, individual shoots were transferred to Magenta vessels containing LS medium so-called resting medium for 2-3 weeks for the selection of healthy regenerants for rooting stage. For root induction, LS medium supplemented with 0.5 mg/L IAA was found the most promotive auxin for all tested species. In our studies, the full development of the root system was achieved within 4- 6 weeks depending on species types. Some of the species subjected to cardenolide analysis (for example, *D. lamarckii*) were transferred to LS medium without PGR (resting medium as mentioned above) for additional weeks until they become 12- or 18-week-old. As for the hardening stage, upon transfer to small plastic pots containing commercial soil, about 80% of the rooted regenerants displayed new growth and normal morphological characteristics under greenhouse conditions (Figure 13).

In conclusion, the results mentioned above show that propagation of our four endemic Anatolian species by adventitious shoot regeneration *in vitro* makes possible (1) the rapid multiplication of elite plants with the formation of plant clones, and (2) long-term cultivation of plants which under normal growth conditions would have died at the end of the second vegetation period.

7.2. Biochemical studies

To our knowledge, cardenolide profiles of almost all known foxglove species have been established by many research groups (see review Clemente et al. 2011). In Turkey, Tanker et al. (1981) for the first time pointed out the establishment of possible infrastructures for large-scale cardenolide production in Anatolian foxgloves. However there has been no attempt for evaluation of this natural sources at an industrial level. Cardenolides have been of commercial interest and an increasing number of patients are suffering from cardiac insufficiency. In this study, natural populations of different endemic species were evaluated in terms of their cardenolide profiles, which provide us with new insight(s) through medicinal researches. Most of the recent studies concerning the biosynthesis of cardiac glycosides have been conducted with enzymes isolated from *Digitalis* plants and tissue cultures (Rhenius et al. 1997; Eisenbeiß 1999; Ernst et al. 2010). However, recent developments and the state of knowledge concerning the research on *Digitalis* (foxglove) cardenolides are still discussed (Müller-Uri and Kreis 2010). The *Digitalis* glycosides are cardenolides classified according to the substitution patterns of their steroid moieties. The A-type glycosides (digitoxigenin derivatives) are the most abundant, and the C-type glycosides (digoxigenin derivatives) are known as most important cardenolides. Biotransformation of cardiac glycosides was studied extensively using *D. lanata* cells and organ cultures (Kreis and Reinhard 1988; Kreis et al. 1989; Kreis et al. 1998; Theurer et al. 1998). In that, cultured *D. lanata* cells were found to have biotransformed cardenolide tridigitoxosides of the A-series into the respective 12 β -hydroxylated C series glycosides (Kreis et al. 1989). When taken into account these findings concerning cardenolide determination, biotransformation of the metabolites might be evaluated as adaptive variation during evolution of

Digitalis species. For example, when considered TLC analysis of Anatolian endemics, acid hydrolysis provides a basic knowledge as to whether pregnane structures depend on digitoxigenin, gitoxigenin or digoxigenin derivative. TLC bands were observed very clearly in several samples extracted from natural populations. Anatolian endemics (*D. davisiana*, *D. cariensis*, *D. trojana* and *D. lamarckii*) were found rich in gitoxigenin derivatives of cardenolide in contrast to other tested species (*D. lanata*, *D. ferruginea* or *D. schischkinii*). Interestingly, digoxigenin derivatives of cardenolides which were found in control groups were not detected TLC treatments in Anatolian endemics, at all. Of those, only *D. trojana* was found to have very low concentrations by means of HPLC analysis (Table 21). Our findings on the low content of the digoxigenin group (or trace amount) might be due to natural selection in favor of gitoxigenin derivatives being donated in natural populations of species. In other words, while digoxigenin-type (C-type) cardenolides was not detected (or detected trace amount) in *D. davisiana*, *D. trojana*, *D. cariensis* and *D. lamarckii*, they were abundant in *D. lanata*, *D. ferruginea* and *D. schischkinii*. As noted above, biotransformation pattern of cardenolides (A converted into C-type) were modeled by several research groups (Wichtl 1978; Kreis and Reinhard 1988, 1992; Rhenius et al. 1997). In our case from B to C-type seem more complicated than A to C, probably due to the need of an extra hydroxylation, in addition to 12 β -hydroxylation. We assume that another hydroxylation should occur at carbon 16 for this conversion (see Figure 3). The TLC lanes observed on cardenolide extracts of *D. davisiana* were relatively less than other species. That's why *D. davisiana* is one of the poorest foxglove among all known *Digitalis* species in terms of cardenolide content (see Tables 20 and 21). Depending on the physiological age, cardenolide contents within or among the species differ not only quantitatively but in some cases qualitatively (i.e., compare different lanes of *D. trojana* shown in Figure 24 or 25 with 26).

Similarly, seasonal variations differed in qualitatively for cardenolide accumulation (for example see TLC bands of *D. lamarckii* in Figure 27), the numbers of the TLC lanes in each row were not fitting correspondingly to the next one. This might be due to different collection time and/or location of the samples. It was shown that younger leaves in budding stage, June period for *D. lamarckii*, had some visible highly nonpolar cardenolides (see RF 0.6-0.7; Figure 27) while other periods (August or September) did not have them, at all. In addition to the seasonal differences, vegetation of the species may play a role for their cardenolide contents. For example, Konya samples collected in June show different patterns (qualitative) between RF values 0.1 and 0.3 comparing to Kizilcahamam samples (see Material and Method) collected at the same period. In addition to bioclimatic and geographical variations, this might be due to the high tendency of the genus *Digitalis* to hybridize in nature as reported by Chene (2010). Contrarily, greenhouse samples represented in Figure 26 did not differ qualitatively in natural populations of *D. lamarckii*.

As of the quantitative observation of the cardenolides, similar relationship was also observed on HPLC analysis of varying glucogitorosid contents depending on seasonal variations. In that, September was a month that was the weakest in terms of glucogitorosid accumulation for *D. davisiana* (see Table 23). Our finding was consistent with some earlier studies, in which seasonal changes account for fluctuations in metabolite accumulations (Gavidia 1996; Roca-Perez et al. 2004, 2005, 2006, Braga et al. 1997). Of those studies, Roca-Perez et al. (2004) reported that cardenolide content negatively correlated with plant phosphor (P) levels and copper (Cu) content in soils. On the other hand, cardenolide accumulation in leaves was negatively correlated with Zinc level (Zn) in younger leaves, but positively correlated with plant iron level (Fe). Besides mineral soil characteristics, Guiterez et

al. (2003) reported some *Bacillus* strains were able to induce cardenolide production in *D. lanata*.

Very interestingly, since cardenolide pathway ending with cardenolide production is restricted in a few unrelated families (*i.e.* Apocynaceae, Asclepiadaceae, Brassicaceae, Convallariaceae, Fabaceae, Hyacinthaceae, Ranunculaceae and Scrophulariaceae) similar cardenolide patterns of *D. cariensis*, *D. trojana* and *D. lamarckii* reflected on TLC plates (almost all lanes were qualitatively same among endemics; see Figure 22) might be evaluated as a fingerprint method in order to find closely related species in favour of chemotaxonomic approaches. That TLC and HPLC analysis (Table 19 and Figure 26) for endemic Anatolian species point out similar lanes shared by *D. cariensis*, *D. trojana* and *D. lamarckii* might be associated with morphological similarities of those as reported by Werner (1960, 1965). Therefore, disagreement with Davis and Werner's classification on endemic Anatolian *Digitalis*, in which Davis (1978) followed Ivanina (1955)'s approach that accepted *D.trojana*, *D. cariensis* and *D. lamarckii* as species level, but Werner (1960) evaluated them all under *Cariensis* alliance (see Table 2), might be revealed when either of views should be extended over a large distribution area with more sample collection. In order to clarify cardenolide patterns, we compared the percentages of glycone contents given in Table 21, A, B or C-type of cardenolides were ranging from 0.9 to 1.6% of a dried leaf material. Fluctuations in cardenolide content were found almost similar in *D. cariensis* and *D. lamarckii*. Since acid hydrolysis of the cardenolides avoids problems related to quantifications of highly hydrophilic glycosides, it might be useful to figure out for the representation of total cardenolide contents underlying A, B and/or C-types. Therefore, it is noteworthy to say that the likeness of the cardenolide pattern (with or without acid hydrolysis)

within these three species (*D. cariensis*, *D. trojana* and *D. lamarckii*) as noted before might refer cardenolides as a marker for taxonomy and might be improved for descriptive analysis to show similar taxon from different cardenolide containing plant families. In our literature survey, iridoid glycosides, products of the isoprenoid biosynthetic pathway, were reported useful markers for chemotaxonomy since they have a high structural diversity due to differing functional groups at the monoterpene skeleton like various sugars or epoxy groups (Taskova et al. 2005; Waterman 2007; Dobler et al. 2011). Similar to the iridoid glycoside, cardenolides as the products of the plant's terpenoid metabolism, can be suggested as a chemotaxonomical marker in at least Plantaginaceae family. In this view of point, *D. trojana* can be hypothesized as an intermediate species as Werner (1960) stated between *D. cariensis* and *D. lamarckii* alternatively to Davis' (1978) systematics in Flora of Turkey (for further reading Volume 6, page 686).

In this study, we showed, for the first time, new data related to the cardenolides separately for each species. *D. trojana* only differ in cardenolide pattern when 5-month-old basal leaves were harvested, since the numbers of lanes (15 lanes) were detected more than *D. cariensis* or *D. lamarckii* (9 TLC lanes corresponding to each other). Especially Figure 22, the samples collected when they were in flowering stage (July) shows that all TLC lanes detected in *D. cariensis*, *D. trojana* and *D. lamarckii* are alike. Thus, this similarity of cardenolides not only provides knowledge for taxonomic approach but also might be usefull for conservation of vulnerable species. For example, it does not seem necessary to collect *D. trojana* marked as vulnerable (Ekim et al. 2000), or *D. cariensis*, for isolation of many major cardenolides. Instead, Thracian *D. lanata*, *D. lamarckii* or Ferruginea alliance might be evaluated since they share similar patterns of some groups of cardenolides.

Moreover, they have been not in the risk group of plants for collection as yet. On the other hand, *in vitro* multiplication of *D. lamarckii* were faster and more effective than others. Thus, it can be alternatively propagated in *in vitro* conditions and transfer to the open fields for large scale investigations of many major compounds like glucogitorosid.

TLC prior to HPLC analysis is found very critical to determine cardenolides. Based on the mass spectractal analysis of cardenolides in LC-MS, many cardenolides detected in TLC were quantified in natural populations as follows glucodigifucosid, glucogitorosid, digitalinum verum, neo-digitalinum verum, purpurea glycosid B, neo-odorobiosid G, lanatoside C, glucoverodoxin, glucolanadoxin, strosposit, gitoxigenin gluco-metylosid and a digitoxigenin derivative cardenolide (A-X). In this experiment, we tried to minimize environmental changes which might alter the production of the cardenolides in eight months old samples grown in greenhouse conditions (Figure 29). Of those detected cardenolides belonging to A or B (mainly B) type were quantified by HPLC analysis using *D. davisiana*, *D. cariensis*, *D. lamarckii* and *D. trojana*. It was clearly shown that eight-month-old basal leaves had the highest amount of glucogitorosid content (up to 5 or 6 times higher than that of August or September samples). In any case, further research is required to establish whether the variation found in proportion of A or B-genins (types) is genetically determined or whether it can be due to climatic or other environmental factors.

For *in vitro* samples, glucogitorosid was found mainly in tissues and used for comparison of the species depending on medium formulations and/or tissue types extracted. Tissue culture samples from callus formation to redifferentiation show that cardenolide content or patterns depend on plant growth and development. It was observed that glucogitorosid content in *D. lamarckii*, *D. davisiana* and *D. cariensis* increased by time until some extent to which fruit formation was completed (August, September or later). However, cardenolide detected in tissue culture samples (up to 18-week-old regenerants) was significantly lower than that of natural populations. At the same time, glucogitorosid content when considered August or September samples was found almost same amount or 50% less than the values calculated from those collected species (compare 12-week-old regenerants with natural populations, in terms of glucogitorosid content). It is not easy to say directly different PGR combinations are effective on cardenolide accumulation, but when cardenolide content is considered parallel to the growth and development; various concentrations of PGRs together with a fresh basal medium might induce further growth and development. On the other hand, different epigenetic deviations might occur in terms of cardenolides content when regenerants compared with mother plants or natural populations. For example, this pattern can be well-understood in germinated seedlings when compared to the regenerants, they contained a lower amount of cardenolides, although physiological age of the all samples were the same (see Table 22 for *D. davisiana*; Table 25 for *D. cariensis*; Table 26 for *D. lamarckii*).

Digitalis leaves are not only the starting material for the isolation of commercial cardenolides but have also been shown to be the site of cardenolide biosynthesis. Several studies have reported a positive correlation between light, chlorophyll content and cardenolide production (see the studies in Hagimori et al. 1982).

However, chloroplast development only cannot be sufficient for expression of the cardenolide pathway. Cultivated shoots via *in vitro* regeneration techniques, on the other hand, accumulated significant amounts of some groups of cardenolides. From these observations, it may be concluded that for cardenolide formation tissue differentiation is at least as essential as the presence of active chloroplasts. For tissue culture, cardenolide accumulation might be stimulated by different PGRs indirectly underlying the growth and development of the species. Although suspension and/or callus cultures did not induce cardenolide formation in our experiments, some earlier experiments showed that exogenously added cardenolides were taken up by shoot or root cultures and thus modified into different cardiac glycosides (Kreis et al. 1998). In our studies, *in vitro* regeneration makes possible time-independent and large-scale production for *Digitalis* species. However, after greenhouse conditions regenerated plants should be moved to open fields to check the stability of the cultivars. Since we did not conducted our studies until the end of second year vegetation in terms of cardenolide content in *in vitro* regenerants, natural populations in contrast to regenerants seem to have significantly higher cardenolide contents in their leaves. However, selection of the best plant with respect to quality and quantity of cardenolides pave the way for a successful tissue culture studies for pharmaceutical industry. Since there has been no detected digoxigenin derivative cardenolides like digoxin most commonly known substance, two alternative ways can be proposed for future prospects as new affiliation to drug industry using *Digitalis* species. *In vitro* techniques can be improved to have hybrid forms among species or this step might be achieved by hand pollination (or other techniques) crossing of suitable inbred lines before tissue culture establishment. For example, closely related species according to Werner (1960), clones of *D. lanata* X *D. trojana*, *D. lanata* X *D. cariensis* or *D. lamarckii* could be compared in terms of their cardenolide contents after having new

progenies. Secondly, since there has been no detected digoxigenin derivative of cardenolides in Anatolian species in *in vitro* samples, exogenous addition of digoxigenin (or another type cardenolide) into basal media with appropriate PGR concentrations might account for production of C series of cardenolides. Elucidation is one of the best techniques for *in vitro* secondary metabolite production, thus it might be available in *Digitalis* species as tested in *D. lanata* before.

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