IN VITRO REGENERATION AND ANALYSIS OF PHENOLICS IN *DIGITALIS FERRUGINEA* L. SUBSP. *SCHISCHKINII* (IVAN.) WERNER

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 MASTER OF SCIENCE
IN
THE DEPARTMENT OF BIOLOGY

JANUARY 2012

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

IN VITRO REGENERATION AND ANALYSIS OF PHENOLICS IN *DIGITALIS FERRUGINEA* L. SUBSP. *SCHISCHKINII* (IVAN.) WERNER

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January 2012, 53 pages

An efficient *in vitro* regeneration protocol was developed from cotyledonary leaf and hypocotyl explants of *Digitalis ferruginea* L. subsp. *schischkinii* (Ivan.) Werner. After sterilization, seeds were put on germination media, then seedlings were used as explant source. Explants were cultured on MS medium with different concentrations and combinations of plant growth regulators (TDZ, BA, KIN, TDZ+IAA, BA+IAA, KIN+IAA, TDZ+IAA+GA_3). The best shoot proliferation was obtained from hypocotyl explants cultured on medium containing 2.0 mg/l TDZ and 0.5 mg/l IAA, with a mean of 11.0 shoots per hypocotyl explant. Shoot formation was decreased to 6.5 shoots when the same concentration of TDZ and IAA was combined with 0.5 mg/l GA_3. 1.0 mg/l BA combined with 0.5 mg/l IAA produced a mean of 6.16 shoots per hypocotyl explant. The combinations of KIN and IAA were employed and 2.0 mg/l KIN combined with 0.5 mg/l IAA produced a mean of 7.0 shoots per hypocotyl explant, which was the highest shoot number achieved.

Consequently, hypocotyl explants were more effective for shoot production than cotyledonary leaf explants. Among all PGRs, the combination of TDZ and IAA was the most effective for shoot formation. Although not being as effective as the combinations with TDZ, different concentrations of KIN and BA also produced shoots, calli and roots. After shoot formation, healthy shoots were transferred to auxin-containing medium to induce roots. IAA was the most effective medium for the rooting of the regenerated shoots. Rooted shoots were transferred to pots containing soil and their acclimitization was achieved, 70% of plantlets being survived.

In the last part of the study involving total phenolic analysis, rooted shoots were dried and analyzed in accordance with absorbance values by UV-spectrophotometer. The results revealed that the total phenolic contents were not significantly different among the samples of different treatments. While the highest amount of total phenolic content was 1149.7 µg/g dw in samples regenerated on medium containing 0.2 mg/l TDZ combined with 1.0 mg/l IAA among TDZ and IAA combinations, for KIN and IAA combinations the highest total phenolic value was 1205.8 µg/g dw in 0.5 mg/l KIN combined with 1 mg/l IAA.

Keywords: *Digitalis ferruginea* subsp. *schischkinii*, *in vitro* culture, plant growth regulators, total phenolic contents, UV-spectrophotometer.

ÖZET

DIGITALIS FERRUGINEA L. SUBSP. *SCHISCHKINII* (IVAN.) WERNER BİTKİSİNİN *IN VITRO* ÇOĞALTIMI VE FENOLİK ANALİZİ

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Ocak 2012., 53 sayfa

Digitalis ferruginea L. subsp. *schischkinii* (Ivan.) Werner'in kotiledon yaprağı ve hipokotil eksplantlarından etkili bir *in vitro* çoğaltım protokolü geliştirilmiştir. Sterilizasyondan sonra tohumlar çimlendirme ortamına alınmış ve daha sonra çimlenen fidelerden eksplantlar izole edilerek, farklı bitki büyüme düzenleyicileri içeren MS ortamlarında (TDZ, BA, KIN, TDZ+IAA, BA+IAA, KIN+IAA, TDZ+IAA+GA_3) kültüre alınmıştır. En iyi sürgün oluşumu 2.0 mg/l TDZ ile 0.5 mg/l IAA içeren ortamdaki hipokotil eksplantından elde edilmiş ve eksplant başına ortalama 11.0 sürgün oluşumu gözlemlenmiştir. TDZ ve IAA'nın aynı konsantrasyonuna 0.5 mg/l GA_3 eklendiğinde ise sürgün oluşumu eksplant başına 6.5 sürgün vererek azalmıştır. 1.0 mg/l BA ile 0.5 mg/l IAA içeren ortamda, hipokotil explantı başına ortalama 6.16 sürgün elde edilmiştir. Sürgün oluşumu için KIN ve IAA kombinasyonları uygulanmış ve 2.0 mg/l KIN ve 0.5 mg/l IAA ortamı hipokotil explantı için ortalama 7.0 sürgün vermiştir.

Sonuç olarak, hipokotil eksplantı sürgün verimliliği açısından uygulanan bitki büyüme düzenleyicilerinde kotiledon yaprağı eksplantına göre daha etkili olmuştur. Sürgün oluşumunu artırmada en etkili bitki büyüme düzenleyicisi TDZ ve IAA kombinasyonu olmuştur. BA ve KIN'in oksinlerle kombinasyonunda TDZ kadar etkin olmamasına rağmen bu kombinasyonları içeren ortamlarda da sürgün, kallus ve kök oluşumu gözlemlenmiştir. Ortamlardan elde edilen sağlıklı sürgünler oksin içeren ortamlara kök oluşumu için aktarılmıştır. Sürgünlerde hızlı bir şekilde kök eldesi için en etkili oksin IAA olmuştur. Köklenen sürgünler plastik saksılara aktarılmış ve iklimlendirme işlemi yapılarak, bitkilerin %70'nin hayatta kalması sağlanmıştır.

Toplam fenolik miktarı analizlerini içeren çalışmanın son bölümünde ise, köklenen sürgünler kurutularak, örnekler absorbans değerlerine göre UV-spectrofotometrede analiz edilmiştir. Sonuçlar toplam fenolik miktarının farklı örnekler arasında önemli bir değişiklik göstermediğini ortaya koymuştur. TDZ ve IAA kombinasyonları arasında en yüksek toplam fenolik miktarı 0.2 mg/l TDZ'nin 1.0 mg/l IAA ile kombinasyonunda 1149.7 µg/g dw olurken, KIN ve IAA kombinasyonları için en yüksek toplam fenolik değeri 0.5 mg/l KIN'in 1.0 mg/l IAA ile kombinasyonunda 1205.8 µg/g dw olarak bulunmuştur.

Anahtar Kelimeler: *Digitalis ferruginea* subsp. *schischkinii*, *in vitro* çoğaltım, bitki büyüme düzenleyicileri, toplam fenolik miktarı, UV-spektrofotometre.

To My Family.

ACKNOWLEDGEMENTS

I express my sincere appreciation to my supervisor, Prof. Dr. Ekrem GÜREL, for his guidance and insight throughout the research. I would like to thank my laboratory friends B. Buhara YÜCESAN, Dr. Sandeep K. VERMA, Günce ŞAHİN, Arzu YILDIRIM for their help and suggestions during my study. I offer my gratefull thanks to Ass. Prof. Dr. İsmail Eker for his taxonomical contributions. I offer sincere thanks to my parents Ayşe and Halil ŞAHBAZ, my brother Cemil ŞAHBAZ, and my sister Esra ŞAHBAZ for their unshakable faith in me.

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ABBREVIATIONS

ANOVA	Analysis of variance
BA	Benzyladenine
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
KIN	Kinetin
MS	Murashige and Skoog's medium (1962)
NAA	1-α Naphthaleneacetic acid
SEM	Scanning electron microscopy
TDZ	Thidiazuron

CHAPTER I. INTRODUCTION

Throughout the ages humans have relied on nature for their basic needs including food, shelter, clothing, transportation, fertilizers, flavours, fragrances, and medicines (Cragg and Newman, 2005). Probably, the most important of all these needs are related to edible and medicinal usages.

Plants have long served as our major source of medicinal compounds. The earliest writings from ancient Babylonia, Egypt, China, and India teem with references to healing herbs, indicating a prehistoric origin for the use of plants as medicines. Although scientists have estimated that over 250.000 species of flowering plants exist on earth, most of these plants have yet to be explored for their medicinal properties. In general, humans have been using many species of plants medicinally for centuries. Through personal experience and knowledge passed down for generations, indigenous people have learned which species of plants may help alleviation of certain ailments, from relieving headache to treating heart disease. However, with the advent of synthetic chemistry, much of the past century has witnessed a decreased reliance on botanicals as sources of original therapeutic compounds, particularly in the Western world. Most of the large pharmaceutical companies had turned away from flowering plants in the mistaken belief that synthetic chemistry could cure the world's medical afflictions. Nevertheless, in recent years, there has been an explosion of interest regarding plants and their medicinal value, so that these firms realize that nature possesses answers to at least

some of the medical questions we face. The computerization of the laboratory makes it possible to find, analyze, and manipulate molecules faster than ever before. Contrary to popular belief, these revolutionary technologies make the plant kingdom more important as a source of potential medicines (Sumner, 2003).

Although rapid depletion of the resources taken from nature, human population have increased and, to ensure the basic needs alternative and more efficient production methods in a short time have been developed. In this connection, plant tissue culture techniques have gained a momentum to avoid starvation. Also, with the examination of chemical structures of plants, secondary metabolites have been used efficiently. Many pure substances obtained from plants may be used for making drugs in laboratory conditions. Among these substances known as secondary metabolites, morphine in 1816, quinine in 1820 and digitoxine in 1868 were isolated as pure from different plants (Atasü and Yenen, 1982; Mavituna,1992).

Secondary metabolites in the plants can be classified into three chemically distinct classes: terpenes, phenolics and nitrogen containing compounds that protect plants from herbivors and other animals damage. The phenolics are grouped into two classes on the basis of the number of phenol subunits: simple phenols and polyphenols. The group of simple phenols contains phenolic acids or phenols with carboxyl group underlying the specifity of their function. Polyphenols contain at laeast two phenol rings. Flavonoids, a subject of comperehensive studies in recent years, belong to the this group. More than 4000 flavonoids have been identified in different higher and lower plant species (Harborne and Turner, 1984. Marinova et al., 2005).

Some pehenolics such as anthocyanins may prevent leaf damage resulting from exposure to excessive light (Gould and Lee, 2002). Since the bulk of phenolics

remains present during leaf senescence and after death these compounds may also affect microbial decomposers (Harrison, 1971) and therefore delay microbial decomposition of plant litter (Zucker, 1988; Salusso, 2000). The amount of phenolics in plant tissues varies according to species, age and degree of decomposition (Graça et al., 2005).

Phenolic compounds with plant origin can contribute to the taste and aroma of many foods. They are especially the source of bitterness and sourness. The phenolics have natural antioxidant activity properties and many antioxidants exhibit beneficial effects including antibacterial, antiviral, antiallergic and antithrombotic. They also prevent cancer, heart and lung diseases by blocking reactions of free radicals (Nilsson et al., 2005; Nizamlıoğlu and Nas, 2010). However, certain studies have revealed that some synthetic antioxidants including hydroxytoluene and hydroxyanisole can be toxic and carcinogenic, and thus, in some countries their use have been prohibited and/or restricted (Koşar et al., 2002. Öztürk et al., 2002; Köksal, 2007; Yağcı et al., 2008). Therefore, it has been increased interest in fruits, vegetables, spices, herbal teas as a source of natural antioxidants (Tzia and Liadakis, 2003; Koca, 2007). Especially, the flavonoids, widely available in fruits and vegetables, have strong antioxidant activity (Roginsky and Lissi, 2005). Recently, determination of total phenolic compounds, flavonoids and antioxidant capacity in different fruit and vegetables have been introduced by different researchers (Cemeroğlu et al., 1994; Velioğlu et al., 1998; Zhishen et al., 1999; Tosun et al., 2003; Karadeniz et al., 2004; Kim and Padilla-Zakour, 2004; Del Caro et al., 2004; Uyan et al., 2004; Tsai et al., 2005; Oki et al., 2006; Velioğlu et al., 2006). However, *in vitro* determination of the amount of total phenolic compounds of many *Digitalis* L. species has not been found in the literature. In this thesis, for the first time, studies

on *Digitalis ferruginea* L. subsp. *schischkinii* (Ivan.) Werner are presented. We aimed to establish an *in vitro* regeneration protocol for the species *Digitalis ferruginea* subsp. *schischkinii* as well as determining the total phenolic contents.

1.1. The folkloric and medicinal history of *Digitalis* L.

The medicinal properties and beneficial effects of *Digitalis* have been recognized for centuries. *Digitalis purpurea* L. appeared as a remedy for the symptoms of heart disease, and controlled doses of its cardiac glycosides have long been used to improve heartbeat tone and rhythm. The use of *D. purpurea* extract for the treatment of heart conditions was first described by William Withering, an English botanist and physician, in his textbook describing the medical uses of foxglove in 1785. Withering described the ability of *Digitalis* to cause diuresis and slow the heart rate of patients with irregular pulse. Beginning in the twentieth century, many studies in animals and humans demonstrated positive inotropic properties of digitalis in normal as well as failing myocardium (Sumner, 2003; Rossi and Gheorghiade, 2007; Goldthorp, 2009). Afterwards, various species of the genus *Digitalis* (mainly *D. purpurea, D. obscura* and *D. lanata*) are used as a source of cardiac glycosides and in order to improve the economic return of the production of cardenolides numerous studies have been carried out with *in vitro* cultures of these species (Herrera et al., 1990).

Digitalis-based drugs, like digoxin, have been used for centuries to treat patients with irregular heart rhythms and heart failure and are still in use today. The clinical benefit of *Digitalis* for patients with heart disease is well-established. However, recent studies have also suggested that digitalis has antineoplastic activities at clinically relevant serum concentrations. Much of the early evidence

supporting the anticancer activity of *Digitalis* has been circumstantial. Observational studies suggest a protective benefit and improved outcomes in patients who develop cancer while they are taking *Digitalis*. Experiments to determine its mechanism of action have demonstrated that *Digitalis* inhibits cell growth and angiogenesis and induces apoptosis in multiple cancer cell lines. It is reasonable to expect that the addition of *Digitalis* to current cancer treatments will improve the clinical outcomes (Khan et al., 2009).

1.2. Taxonomy and geographical distribution of the genus *Digitalis* L.

The genus *Digitalis* L., commonly known as the "foxglove", was traditionally placed in the family Scrophulariaceae (the figwort family). But this genus is now located in the families Plantaginaceae (the plantain family) and Veronicaceae in the light of recent phylogenetic studies (Olmstead et al. 2001; Bräuchler et al., 2004). According to the most widely accepted treatment based on pyhtogeographical and morphological features, 19 species were recognized in the world and they naturally spread throughout the Mediterranean area, Europe, western and Central Asia, and north-western Africa (Werner, 1964; Bräuchler et al., 2004).

Turkey is one of the richest areas in the middle latitudes in terms of plant diversity. The main reasons for this are; climatic varieties, geomorphological and soil diversities, and the situation of the area at the junction of three flora region (Euro-Siberian, Mediterranean and Irano-Turanian) (Avcı, 2005). In this richness, the genus *Digitalis* are represented by 8 species and 1 subspecies (totally 9 taxa), and four species of all them (*D. trojana* Ivan., *D. davisiana* Heywood, *D. cariensis* Boiss. ex Jaub. et Spach and *D. lamarckii* Ivan.) are endemic to Turkey (Davis, 1978).

All *Digitalis* species are biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are crowded in basal rosettes. Flowers are zygomorphic and arranged in terminal, bracteate, and often second racemes. The calyx is equally deeply five-lobed and shorter than the corolla tube. The corolla is excerted, usually yellowish or whitish in Turkey; the tube is cylindrical-tubular, pouched or globo-tubular, constricted at the base; the limb is more or less two-lipped; the upper lip is usually shorter than the lower, which is spotted or veined inside; the lower lip with middle lobe slightly or much longer than anterio-lateral lobes. Stamens are 4, didynamous, included. Capsule ovoid, septicidal, beaked. Seeds many, oblong (Davis, 1978; Bräuchler et al., 2004) (Figure 1).

In this thesis, *Digitalis ferruginea* L. subsp. *schischkinii* (Ivan.) Werner has been studied. This subspecies is native throughout north-eastern Anatolia and western Caucasia (Davis, 1978; Demirkuş and Erik, 1994; Hügli, 2002; Özen and Kılınç, 2002; Töngel and Ayan, 2005) (Figure 2, 3).

Figure 1. *Digitalis ferruginea* subsp. *schischkinii* - a: Habit, b: Inflorescence, c: Flower.

Figure 2. Distribution of *Digitalis ferruginea* subsp. *schischkinii* in the world (blue dots represent natural range of the species).

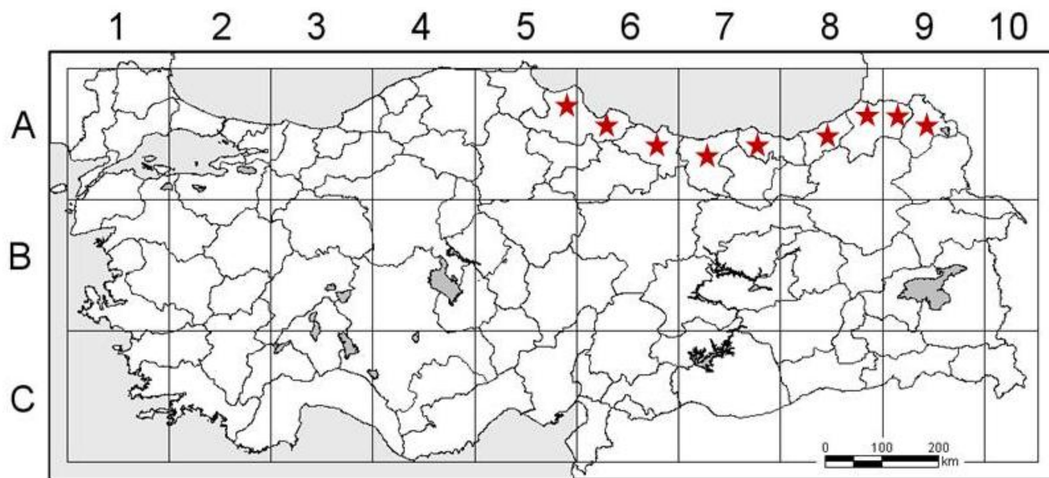


Figure 3. Distribution of *Digitalis ferruginea* subsp. *schischkinii* in Turkey (red stars represent natural range of the species).

1.3. *In vitro* culture of *Digitalis* spp.

Although there are some reports on the tissue culture of certain *Digitalis* spp, no studies were found on *D. ferrrigune schischkinii.*

Corduan and Spix (1975) obtained haploid callus and achieved regeneration of plants from anthers of *D. purpurea*. Li (1981) reported the plantlet regeneration from mesophyll protoplasts of *D. lanata*. Brisa and Segura (1987) investigated the isolation, culture and plant regeneration from mesophyll protoplasts of *D. obscura*. Arrillaga et al. (1987) studied somatic embryogenesis and plant regeneration from hypocotyl cultures of *D. obscura*.

Reinbothe et al. (1990) regenerated *D. lanata* from suspension cultures via somatic embryogenesis. Cacho et al. (1991) investigated the effects of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) either alone or in combination with kinetin or benzyladenine (BA) to assess the morphogenetic potential of leaf, root and hypocotyl explants of *D. thapsi*. Calli were obtained from all the three explant types in basal medium without the addition of growth regulators and in leaves, the calli formed roots. Application of 2,4-D, NAA or BA increased callus formation. The presence of NAA induced root formation and that of BA induced shoot formation via callus interphase. IAA alone only induced the generation of roots in the hypocotyl callus. Kinetin was ineffective in all the explants tested. Combinations of NAA with kinetin or BA were more effective in inducing organogenesis in leaf explants. Optimum responses were obtained in hypocotyl and root explants by using IAA in combination with BA, the highest rate of shoot regeneration being observed in hypocotyl explants.

Onisei et al. (1992) reported that stem tips of 3-month-old plants and 0.5-1.0 cm long explants of young spikes (15-months-old) of *D. lanata* and *D. purpurea*

were excised and cultured on MS medium supplemented with various growth regulators. Culture response by the 2 species was similar but *D. purpurea* explants exhibited a stronger and faster organogenetic capacity, and survival of regenerated plants exceeded 95%. *D. lanata* and *D. purpurea* regenerated, respectively, 10 and 15 shoots per explant on MS medium containing 2 mg/l BA. Rooting capacity of *D. lanata* explants was lower than that of *D. purpurea* and survival following soil acclimatization was only 65-70 %, compared with 95-98 % for *D. purpurea*.

Yürekli and Baba (1995) studied on *Digitalis cariensis,* an endemic species, and produced green callus from *in vitro* seedlings cultured on MS medium containing 3 mg/l BA and 0.4 mg/l NAA. However, regeneration was not achieved.

Bosila *et al*. (2003) investigated that explants (shoot-tip, leaf, hypocotyl and root) of *D. lanata*, which is a major source of digoxin and digitoxin, were cultured on aseptic MS basal solid medium containing 5.0 mg/l 2,4-D and 0.5 mg/l of BA. On the other hand, callus derived from different explants was recultured (in initial amounts of 49-63 mg) on the same new sterilized MS medium to increase the mass of callus. The cultures were then maintained in a growth chamber at 26 °C ± 2 °C and subjected to four different light photoperiods as follows (in hours): 10 light/14 dark, 14 light/10 dark, 16 light/8 dark, and 18 light/6 dark. The greatest callus production and the highest amount of glycosides occurred in callus derived from 2- or 20-week-old leaf explants among all the examined types and ages of explants. Repeating the subculturing three times decreased significantly the dry weight of callus, but it significantly favored digoxin and digitoxin content. The optimal callus growth was obtained at 16 h light per day but the best glycosidal content was achieved when callus was exposed to 18 h light per day.

A repeatable transformation system was established for *Digitalis minor* L. by using *Agrobacterium tumefaciens*. Leaf explants from 30-days old seedlings were incubated with either EHA105 (carrying the *nptII* and *gus A* genes) or AGL1 (with the *bar* and *gus A* genes) strains. Among tested factors influencing T-DNA transfer to plants, the EHA105 strain and the addition of acetosyringone to co-culture medium increased transformation. The highest transformation efficiency (8.4%) was obtained when freshly isolated explants, soaked in a bacterial suspesion with an OD_{550} of 0.9, were subcultured on selection medium after 4 days co-culture with bacteria. Evidence of stable transgene integration was obtained by PCR, growth on media selective for *nptII* or *bar* genes, and expression of the *gus A* gene. Southern hybridization performed in six plants provided information about the number of inserts more than 200 transgenic plants were recoverd from 65 independent explants. Thirty of these plants were sucessfully estabalished in soil (Sales et al., 2003).

Fatima et al. (2009) investigated the effect of plant growth regulators (PGRs) and carbohydrate sources on callus induction, callus growth, and plant regeneration in *Digitalis lanata* Ehrh. Explants were transfered onto MS medium with various levels of PGRs and carbohydrates to determine the optimum explant and effective combinations of PGR treatment. For callus induction, 6.0 mg/l of NAA and 3.0 mg/l of BA were highly responsive. Addition of cytokinins (BA and Kinetin) at 0.5-3.0 mg/l to media containing NAA enhanced callus growth. Shoot regeneration was best achieved in MS medium containing 6.0 mg/l of BA. Adenin sulphate (Ade) and casein hydrolysate (Ch) were added to the medium as a nitrogen source to improve plant growth and maximum growth was obtained on medium supplemented with 1.5 mg/l kinetin, 0.5 mg/l IAA and 500 mg Ch. Carbohydrates also influenced callus production and shoot regeneration potentially. Among all the tested carbohydrates

(sucrose, maltose, fructose and glucose and concentrations were between 3.0-6.0 g/l), the optimum carbohydrate concentration was 3.0 g/l and was applied to all carbohydrate cases.

Benli et al. (2009) studied on antimicrobial activity of the methanolic extracts of leaves and flowers of *D. lamarckii* Ivan, testing ten bacterial and four yeast strains. Effective antibacterial activity was observed in four bacterial strains. Minimum inhibitory concentration (MIC) was calculated by use of liquid culture tests and in all the four effective bacterial strains, the MIC was found to be ≥199.5 mg/ml. The minimum bactericidal concentrations (MBC) of *Bacillus subtilis, Staphylococcus aureus*, and *Listeria monocytogenes* were calculated to be ≥199.5 mg/ml, and MBC value for *Shigella* was calculated as ≥399 mg/ml.

Çörduk and Akı (2010) developed a protocol for direct shoot organogenesis from leaf explant of *D. trojana*. Leaf explants were cultured on MS medium supplemented with different concentrations of NAA (0.1, 0.5 and 1.0 mg/ml) and BAP (0.1, 0.5, 1.0 and 3.0 mg/ml) for shoot formation among explants cultured on MS medium with 0.1 mg/ml BAP.

Gurel et al. (2011) investigated that in vitro regenaration of *D. davisiana* and cardiotonic glycoside production from both *in vitro* produced materials as well as natural populations. Testing six different types of culture media revealed that Linsmaier and Skoog medium (1965) was the most effective for shoot production. Shoot regeneration efficiency was higher when flamigo-bill or hypocotyl explants were cultured on LS medium contaıng 0.5 mg/l TDZ and 0.25 mg/l IAA.

Verma et al. (2011) developed, for the first time, an efficient *in vitro* plant regeneration protocol for *Digitalis lamarckii* Ivan. (dwarf foxglove) via direct shoot organogenesis. Two sets of experiments were carried out; the first compared

different concentrations of 6-benzylaminopurine (BAP), kinetin, thidiazuron (TDZ), and zeatin alone using leaf explants excised from *in vitro* germinated seedlings, while the second set tested the combinations of indole-3-butyric acid (IBA) with BAP, kinetin, TDZ and zeatin for shoot multiplication from the leaf explants, which were already cultured and developed numerous shoots during the first set of experiments. For shoot regeneration (the first set of experiments), TDZ was the most effective at 1.0 mg/l concentration, producing a mean of 10.3 shoots per explant and was significantly more effective than BAP. For shoot multiplication (the second set of experiments), a combination of 0.2 mg/l IBA with 0.2 mg/l TDZ produced significantly more shoots per explant (16.5 shoots) than with BAP (11.0 shoots), zeatin (5.5 shoots), or kinetin (4.0 shoots).

The protocols described above provide important information about effective plant tissue culture methods and reveal biochemical properties, which would be useful for a large-scale production of cardenolides, germplasm conservation, genetic transformation studies in *Digitalis* species.

1.4. The objectives of study

The objectives of this study were to produce callus and regerenants from cotyledonary leaf and hypocotyl explants of *Digitalis ferruginea* subsp. *schischkinii* by using different PGR concentrations and combinations, and to determine total phenolic contents of plant samples regenerated on medium containing different concentrations and combinations of plant growth regulators by using UV-spectrophotometer.

CHAPTER II. MATERIALS AND METHODS

2.1. Plant material

Digitalis ferrigunea L. subsp. *schischkinii* (Ivan.) Werner were collected from wild plant populations growing at north part of the Rize province in Blacksea region of Turkey in September 2009. Identification of species was done according to Davis (1978), and voucher specimens (Eker-2536/b) were deposited at AIBU (Herbarium of Abant İzzet Baysal University, Bolu, Turkey).

2.2. Methods

Sterilization: For surface sterilization, different types of procedures were employed. The seeds were washed in different concentrations of Domestos (commercial bleach containing 5% sodium hypochlorite) for varied periods as follows:

1) The seeds were washed with tap water for 5 minutes. Then, they were soaked in 20% Domestos for 15 minutes. Finally, seeds were rinsed with sterile distilled water (sd- H_2O) 4-5 times.
2) The seeds were treated with gibberellic acid (GA_3) for one day, then surface sterilized with 20% Domestos for 15 minutes. Lastly, they were rinsed with sterile distilled water 4-5 times.
3) The seeds were washed in 30% Domestos added with 0.1% Tween 20 for 20 minutes, rinsed throughly with sterile distilled water 5 times (Herrera et al., 1990).

Preparation of culture medium: MS (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose and 0.8% agar (Bacto agar) was used. The MS medium contains some inorganic and organic compounds (Table 1).

Table 1. The ingredients of MS basal medium (Sigma Aldrich).

Components	Mg/l
Ammonium nitrate	1650
Potassium nitrate	1900
Boric acid	6.2
Calcium chloride anhydrous	332.2
Cobalt chloride.6H_2o	0.025
Cupric sulfate.7H_2o	27.8
Na_2-EDTA	37.26
Ferrous sulfate.7H_2o	27.8
Magnesium sulfate	180.7
Manganese sulfate	16.9
Moliybdic acid(Sodium salt).2H_2o	0.25
Potasium iodide	0.83
Potasium phosphate monobasic	170
Zinc sulfate.7H_2o	8.6
Myo-Inositol	100
Thiamine.HCL	0.4

i) Germination medium: The seeds were germinated on medium containing 3% sucrose, 4.43 g/l MSMO (Murashige and Skoog's minimal organics) then pH of the media was adjusted to 5.7-5.8 and 0.8% agar was added, all suplemented materials autoclaved at 121 ^{0}C for 25 minutes at 105 kPa pressure.

1. The seeds were placed onto the germination medium (10-30 seeds per 25 ml petri dishes). Sterilized seeds in petri dishes were kept in stable growth chamber at 23^{0}C and 55-60% humidity under 16 h light/ 8 h dark condition. Germinated seeds were counted and recorded at 2 week intervals.

2. The seeds were placed onto the germination medium (10-20 seeds per 25 ml petri dishes). Sterilized seeds in petri dishes were covered by tin foil and kept in growth chamber to provide dark conditions for producing hypocotyls at high frequencies.

ii) Regeneration medium: The MS media were suplemented with several concentrations and combinations of plant growth regulators (Sigma Aldrich); thidiazuron (TDZ; 0.0; 0.5; 1.0 and 2.0 mg/l) combined with indole-3-acetic acid (IAA; 0.0; 0.1; 0.5 and 1.0 mg/l); benzyladenine (BA; 0.5; 1.0 and 2.0 mg/l) combined with IAA (0.0; 0.5 and 1.0 mg/l); kinetin (KIN; 0.5; 1.0 and 2.0 mg/l) combined with IAA (0.0; 0.5 and 1 mg/l). And also TDZ and IAA combinations were combined with 0.5 mg/l GA_3. All cultures were incubated at 22ºC under a 16-h photoperiod (cool-white flurecent lights, 22-28 µ mol m^{-2} s^{-1})

When the seedlings were 20-25 days old, 1-2 cm long hypocotyl and cotyledonary leaf explants were excised and cultured on regeneration media. Cotyledonary leaf explants were excised as shown in figure 4. After three weeks, regenerated explants, which were healthy and long, were transfered to magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MSMO medium with varying concentrations of different auxins; IAA (0.1; 0.5; 1.0 and 2.0 mg/l); NAA (0.1; 0.5; 1.0 and 2.0 mg/l); IBA (0.1; 0.5; 1.0 and 2.0 mg/l) for root formation in culture jars containing 40 ml medium. Rooted regenerants were transferred to plastic pots containing potting mixed soil and also added little tap water. The plants were covered with transparent polythene covers to maintain temperature and high humidity.

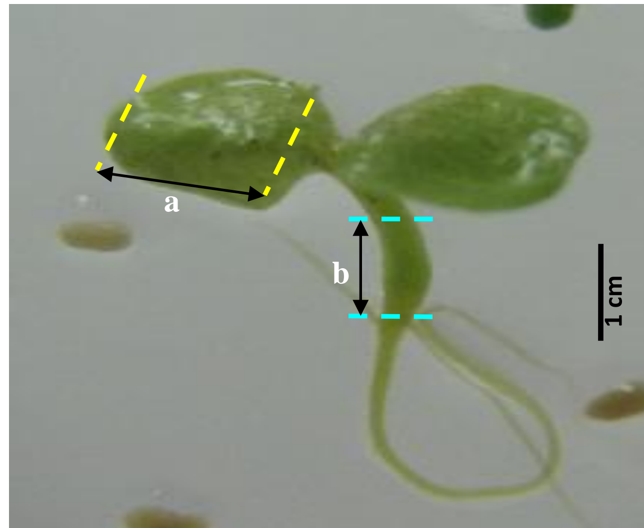


Figure 4. Explants excised from a seedling: a) cotyledonary leaf segment; b) hypocotyl segment

Data collection and statistical analysis: The shoot number and percentage of explants producing shoots were recorded after 4-5 weeks for all explants. Each treatment used 8 replications for each explant type and the experiments were repeated three times. In addition, after three weeks, the number of roots and percentge of explants producing roots were recorded. Results were expressed as means ± standart error (SE) of the mean. All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Test using SPSS version 15 (SPSS Inc, Chicago, IL, USA).

Analysis of phenolic contents: In the part of the study, all of the plant samples (regenerants that obtained from hypocotyl explants) were removed from media and dried seperately in an incubator (drying owen, Nüve EN 500) for 2-3 days at 37^0C. Then, all dried samples were grinded and used for the determination of total phenolic contents by UV-spectrophotometer regard to absorbance values.

To determine total phenolic contents, Folin-Ciocalteu reagent was applied (Ainsworth and Gillespie, 2007).

Reagents used are:

Methanol: 95% (vol/vol) methanol in water.

Sodium carbonte: 20% Na_2CO_3 in water.

Gallic acid: 50 uM-2.5 mM gallic acid (in 95% (vol/vol) methanol).

A gallic acid calibration graph was established for determination of the amount of total phenolics. To prepare stock solution, 0.05 g (50 mg) of gallic acid were weighed and solved in 5000 microliters of methanol. Based on increasing values in the form of stock solution of 50, 100, 200, 300, 400 and 500 ppm, UV-spectrophotometer absorbance values were determined according to the extraction method of phenolic compounds and the calibration graph was established for standards. R^2 was identified as ~ 0.9971 and the results evaluated depending upon the established formula. Gallic acid was used as a standard for determining the phenolic content of various analyses by the Folin-Ciocalteau assay (Marigo, 1973) and results were reported in gallic acid equivalents.

Extracts were prepared from crude samples and phenolic determination was done by using folin reagent. Regenerants were dried (in incubator at 37 ºC) and kept in room conditions for 15 days to analyse total phenolic contents. 0.05 mg dried and grinded samples were measured in 2.5 µl reaction test tubes. 2 µl MEOH was added and kept in dark room conditions for two days. Extracts were seperated by centrifugation at 14.000 rpm for 5 min Extracts were prepared using a modified Folin-Ciocalteau spectrophotometric method (Marigo, 1973). 20 ml extract was placed in a reaction test tube to which 1.58 ml of water and 100 ml of Folin-Ciocalteau reagent was added. The test tube allowed to stand for between 30 s and 8 min, and then 300 ml 20% Na_2CO_3 were added. After 20 min at 40 ^{0}C, absorbance was measured at 750 nm by UV-spectrophotometer (Hitachi U-1900). Total phenolic contents were expressed as mg gallic acid equivalents/g dry weight.

CHAPTER III. RESULTS

3.1. *In vitro* regeneration protocol

An efficient and reliable system for the *in vitro* propagation of *Digitalis ferruginea* L. subsp. *schischkinii* (Ivan.) Werner. was optimized. Different combinations and concentrations of plant growth regulators were tested for multiple shoot formation using cotyledonary leaf and hypocotyl explants.

3.1.1. Germination rate of seeds

At the begining of the study, seeds were germinated and germination rates of seeds were recorded weekly. The percentage of germination was 5.17% in the first week and this ratio increased in the following weeks, eventually reached to highest ratio (43%) at the end of the 4th week (Figure 5). In this process, length of seedlings reached to approximately 2 cm (Figure 6).

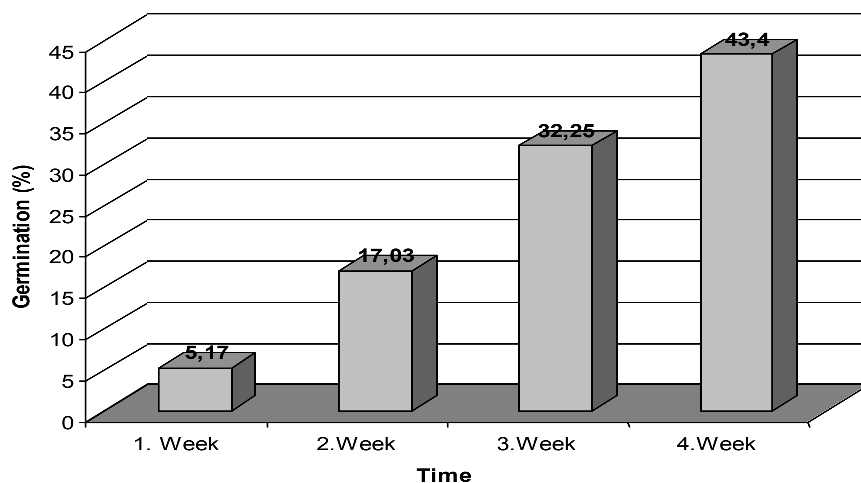


Figure 5. Germination rate of seeds with regard to time.

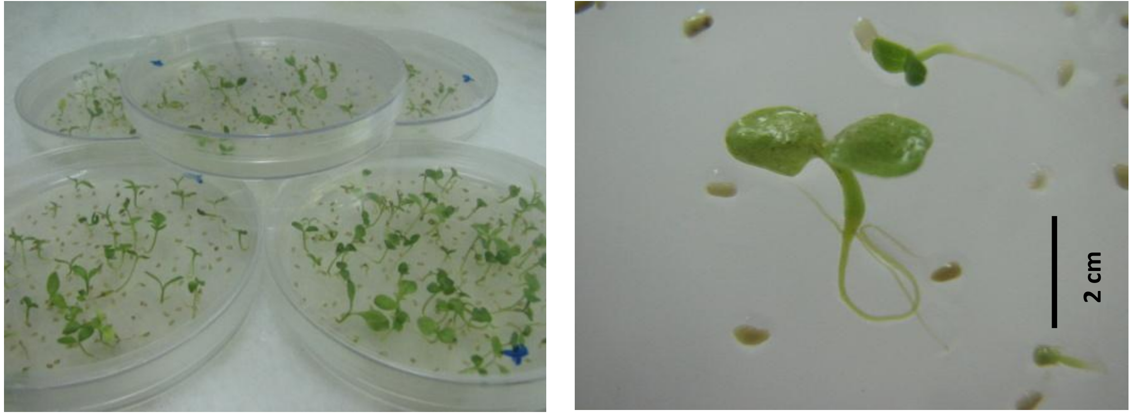


Figure 6. The four-week old seedlings.

3.1.2. Effects of TDZ combined with IAA on shoot production from hypocotyl and cotyledonary leaf explants

Leaf lamina and hypocotyl explants were excised from three-week old seedlings and cultured on media containing various combinations and concentrations of TDZ and IAA (Table 2; Figure 7). The most effective medium for the formation of shoots was optimised.

For hypocotyl explants, the presence of high concentration of TDZ combined with IAA induced shoot formation while control group did not produce any shoots at all. TDZ, when used alone, also produced shoots. 0.5 mg/l TDZ alone was not very effective to form shoots (0.16 per hypocotyl explant) but, when combined with 0.5 mg/l IAA, the same concentration of TDZ resulted in a mean of 7.5 shoots per hypocotyl explant (Table 2; Figure 9). If TDZ concentration was doubled to 1.0 mg/l, shoot formation was significantly increased to 2.6 shoots per hypocotyl explant. When the highest TDZ (2.0 mg/l) was used alone, a mean of 4.1 shoots per hypocotyl explant was obtained and with the combination of 1.0 mg/l IAA, shoot number was highly reduced (0.8 shoots at 16% frequency). Considering all the combinations and concentrations of TDZ and IAA, the most effective results (11.0 shoots per hypocotyl explant at 100% frequency) were obtained when 2.0 mg/l TDZ

was combined with 0.5 mg/l IAA. It meant that higher concentrations of TDZ combined with lower concentrations of IAA were more effective to form shoots for hypocotyl explant.

Table 2. Comparison of cotyledonary leaf and hypocotyl explants in terms of shoot formation on medium containing different combinations and concentrations of TDZ and IAA after 3-4 weeks culture ($P<0.05$; Data represents mean values ±SE, experiments were repeated 3 times).

Plant Growth Regulators		Type of explants			
		Hypocotyl		Cotyledonary leaf	
TDZ (mg/l)	IAA (mg/l)	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explant forming shoots
0	0	0	0	0	0
0.5	0	0.16 ± 0.16^{g}	17%	0±0^{d}	0
1	0	2.66 ± 0.61^{e}	34%	0±0^{d}	0
2	0	4.16 ± 0.30^{cd}	34%	1.7±0.3^{cd}	16%
0.5	0.1	1.33 ± 0.61^{gf}	17%	0±0^{d}	0
1	0.1	1.16 ± 0.79^{gf}	33%	2.33±0.7^{bc}	33%
2	0.1	4.66 ± 0.21^{c}	66%	0±0^{d}	0
0.5	0.5	7.5 ± 0.22^{b}	67%	0±0^{d}	0
1	0.5	8.66 ± 0.42^{b}	83%	2.16±0.4^{bc}	33%
2	0.5	11 ± 0.51^{a}	100%	5.33±0.3^{a}	66%
0.5	1	3.16 ±0.16^{de}	25%	3.33±0.4^{b}	33%
1	1	2 ± 0.25^{fe}	16%	0±0^{d}	0
2	1	0.83 ± 0.30^{g}	16%	0±0^{d}	0
Means		3.94 ± 0.40	42%	1.22± 0.2	15%

For cotyledonary leaf explants, the combinations of TDZ and IAA showed different ranges of shoot formation (Table 2; Figure 7). While both 0.5 and 1.0 mg/l TDZ did not give any shoot response, the highest concentration of TDZ alone (2.0 mg/l) produced a mean of 1.7 shoots per cotyledonary leaf explant. For 1.0 mg/l TDZ combined with 0.5 mg/l IAA, mean number of shoots per cotyledonary leaf explant was 2.16. If TDZ concentration was increased to 2.0 mg/l in the same combination, the shoot formation was almost doubled; from 2.16 to 5.33 shoots per cotyledonary leaf explant, which was the most effective treatment for cotyledonary leaf explants.

Therefore, the combinations of TDZ and IAA were comparatively effective to produce shoots in same concentrations. In general, high concentrations of TDZ combined with lower concentrations of IAA were effective to induce shoot formation for both hypocotyl and cotyledonary leaf explants.

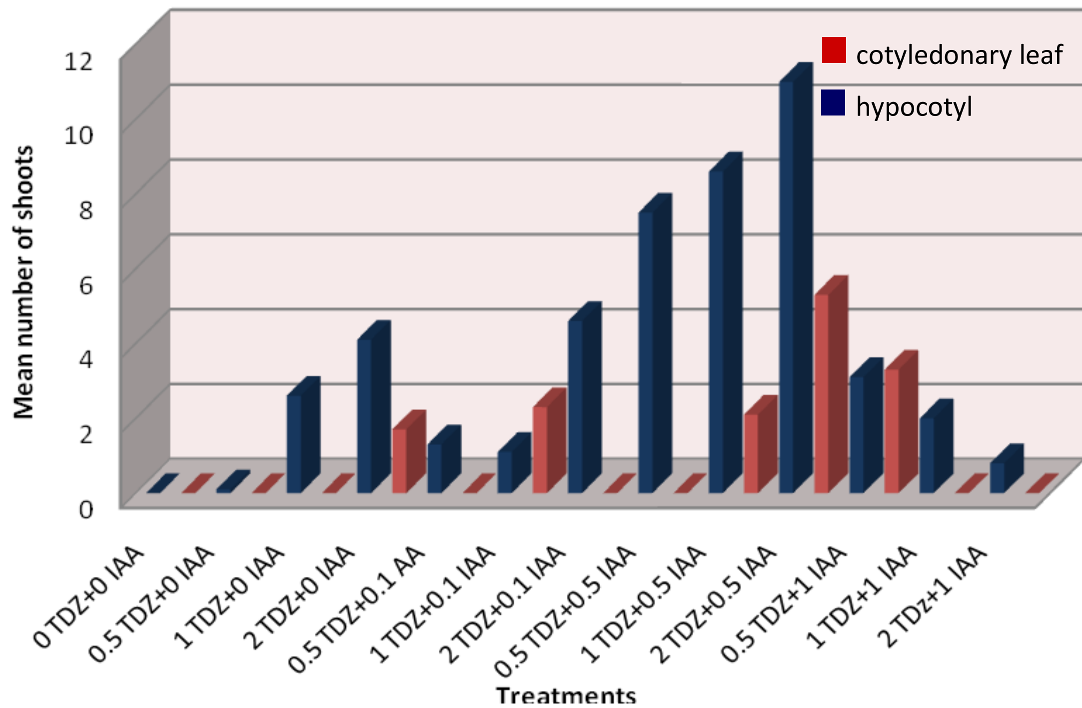
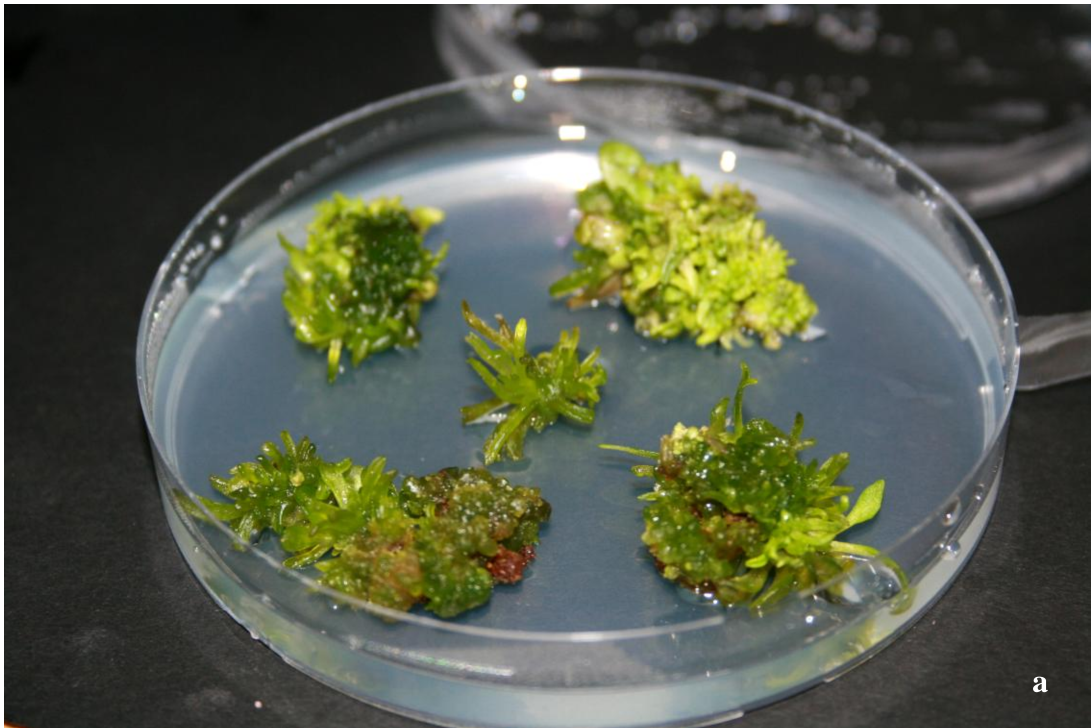


Figure 7. Comparison of cotyledonary leaf and hypocotyl explants in terms of shoot formation.

Consequently, all combinations of TDZ and IAA for hypocotyl explants were much more productive for shoot production than cotyledonary leaf explants (Figure 8). This is more prominent when TDZ was used alone, in which cotyledonary leaf explants produced no shoots at all.



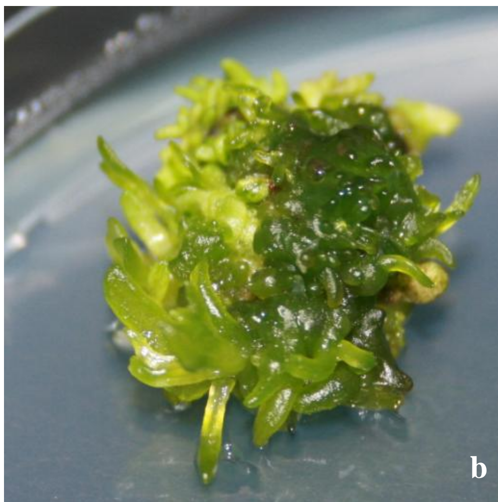
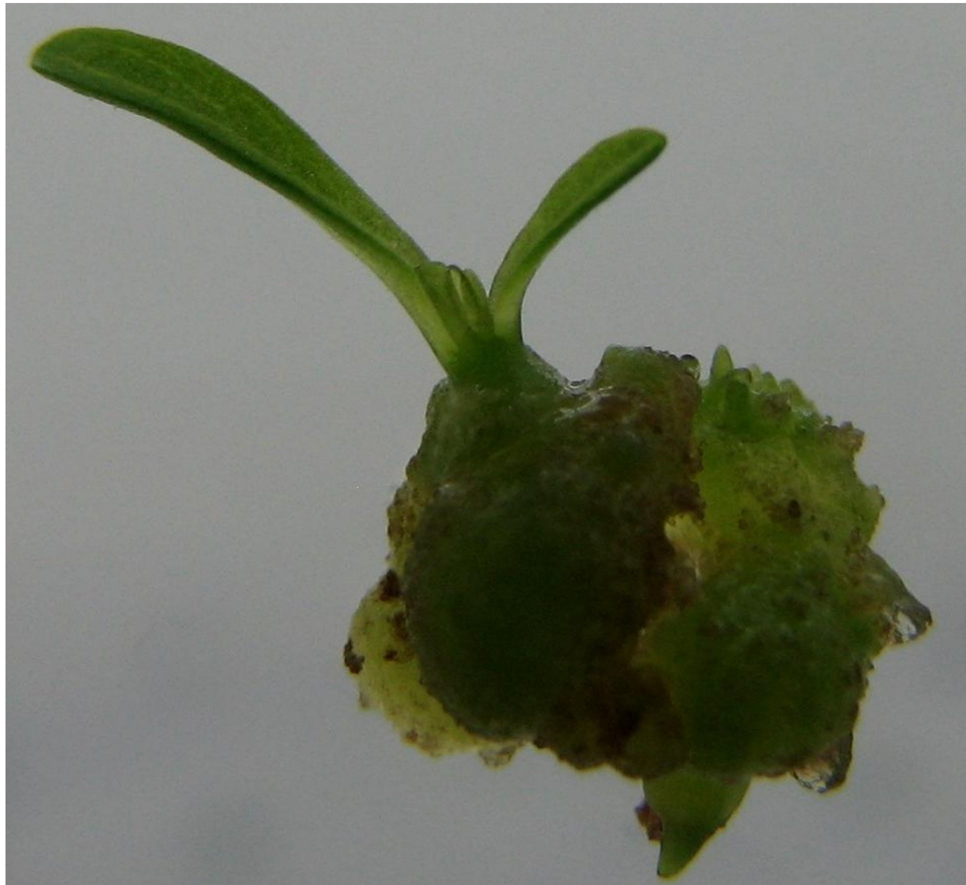


Figure 8. Shoot regeneration from hypocotyl explants cultured on medium containing different combinations and concentrations of TDZ and IAA: a-c) 1.0 mg/l TDZ + 0.5 mg/l IAA; d-e) 0.5 mg/l TDZ + 0.5 mg/l IAA.



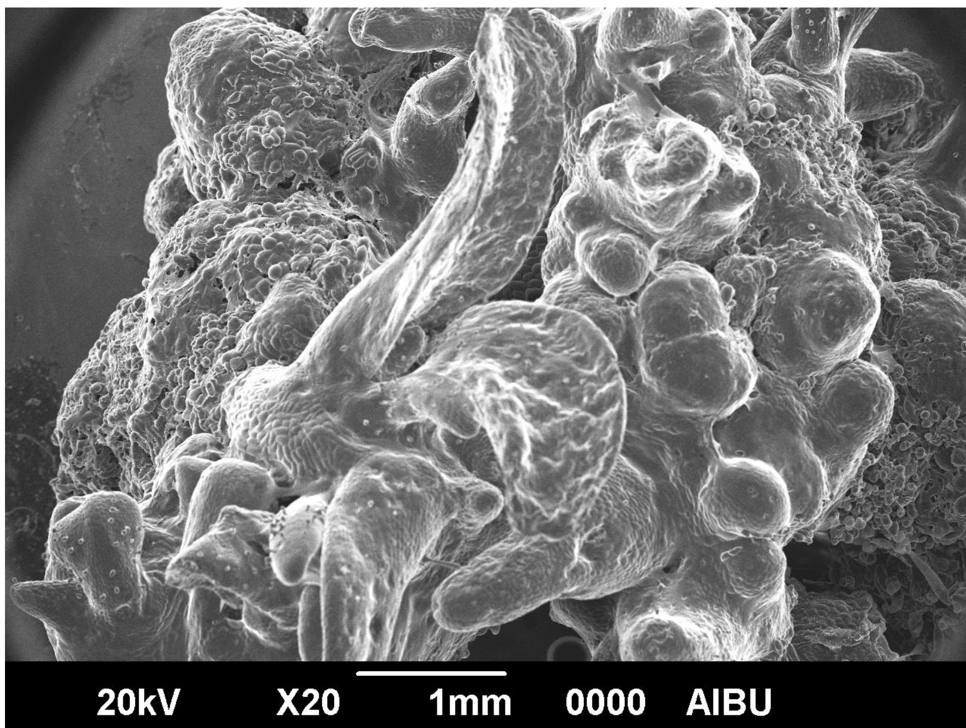


Figure 9. Normal (top) and SEM (bottom) picture of shoot regeneration from hypocotyl explant on medium containing 0.5 mg/l TDZ + 0.5 mg/l IAA.

3.1.3. Effects of BA combined with IAA on shoot production from hypocotyl and cotyledonary leaf explants

In the second part of the experiments, BA as a cytokinin and IAA as an auxin were combined for shoot formation. In this experiment, cotyledonary leaf and hypocotyl explants mostly produced different types of calli (Figure 10).

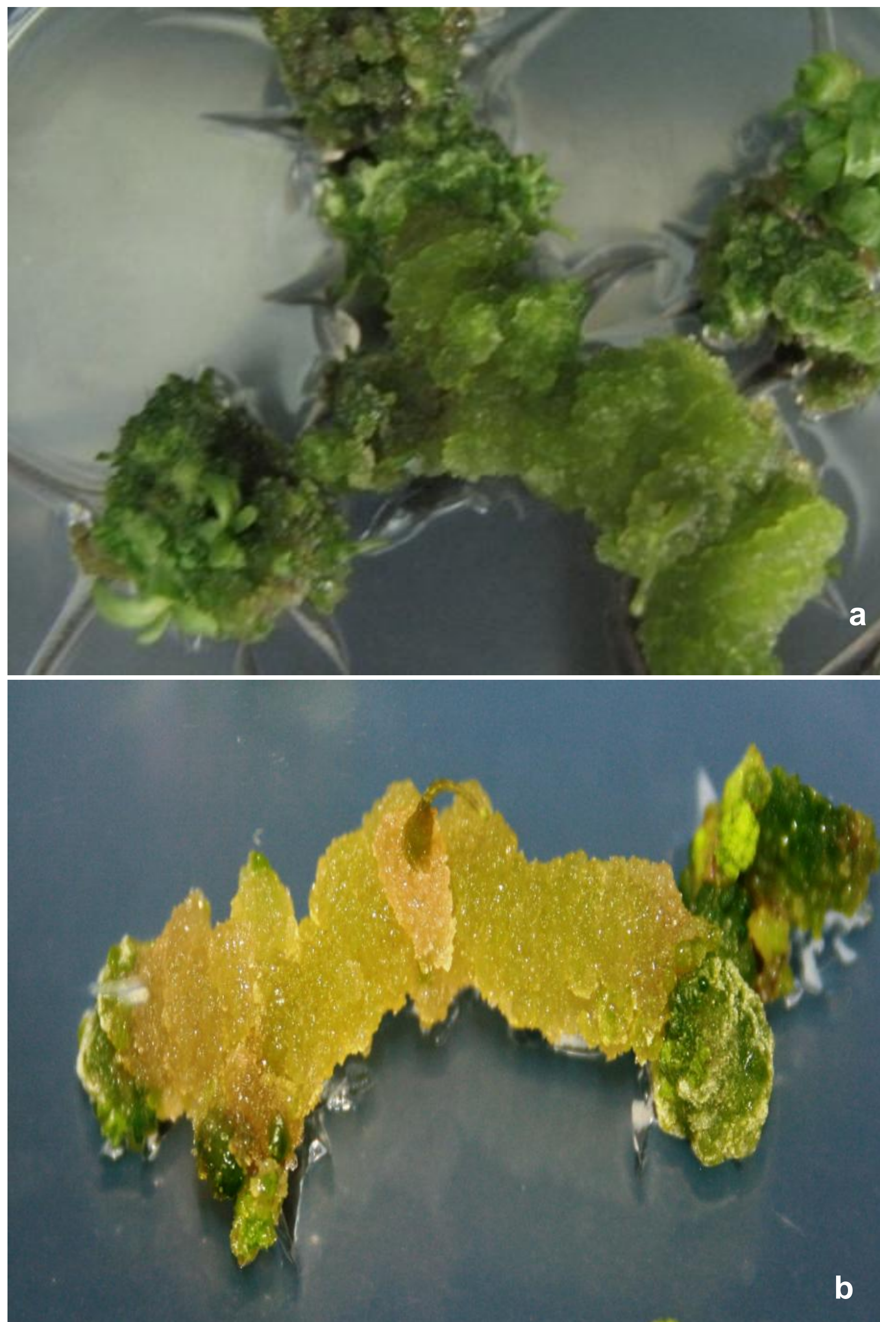


Figure 10. a) Green callus from cotyledonary leaf explant on medium containing 0.5 mg/l BA + 0.5 mg/l IAA; b) Yellowish callus from hypocotyl explant on medium containing 2.0 mg/l BA + 1.0 mg/l IAA.

All of concentrations (0.5, 1.0 and 2.0 mg/l) of BA did not give any shoot response for both hypocotyl and cotyledonary leaf explants. However, hypocotyl explants produced shoots (a mean of 1.5 shoots per hypocotyl explant) in the medium containing 0.5 mg/l BA combined with 0.5 mg/l IAA. If the concentration of BA was increased to 1.0 mg/l in the same combination, it produced the highest number of shoots, with a mean of 6.1 shoots per hypocotyl explant at 84% frequency and 1.66 shoots per cotyledonary leaf explant at 17% frequency. For cotyledonary leaf explant, only two treatments involving 0.5 mg/l IAA combined with 1.0 or 2.0 mg/l BA gave lower shoot formation (1.66 and 1.0 shoots per explant, respectively). However, almost all regenerated cotyledonary leaf explants showed callus formation while some explants showed necrosis, which then ended up with a complete death.

Table 3. Comparison of cotyledonary leaf and hypocotyl explants in terms of shoot formation on medium containing different combinations and concentrations of BA and IAA after 3-4 weeks culture ($P<0.05$; Data represents mean values ± SE, experiments were repeated 3 times).

Plant Growth Regulators		**Type of explants**			
		Hypocotyl		**Cotyledonary leaf**	
BA (mg/l)	**IAA (mg/l)**	**Mean number of shoots per explant(±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explant forming shoots**
0.5	0	$0±0^{d}$	0	$0±0^{c}$	0
1	0	$0±0^{d}$	0	$0±0^{c}$	0
2	0	$0±0^{d}$	0	$0±0^{c}$	0
0.5	0.5	$1.5±0.8^{c}$	67%	$0±0^{c}$	0
1	0.5	$6.16±0.1^{a}$	84%	$1.66±0.3^{a}$	17%
2	0.5	$5.83±0.1^{a}$	84%	$1±0^{b}$	17%
0.5	1	$3.66±0.2^{b}$	56%	$0±0^{c}$	0
1	1	$0±0^{d}$	0	$0±0^{c}$	0
2	1	$0±0^{d}$	0	$0±0^{c}$	0
Means		1.9±0.3	32%	0.29±0.08	3.7%

As a result, high concentrations of BA combined with lower concentrations of IAA induced shoot formation for both explants (Table 3; Figure 11). However, when the concentration of IAA was increased in the same media, the shooting response

was totally lost for both types of explants and only calli was observed. Also, the hypocotyl explants again had higher regeneration capacity than cotyledonary leaf explants as also observed in the previous experiment (see Table 2).

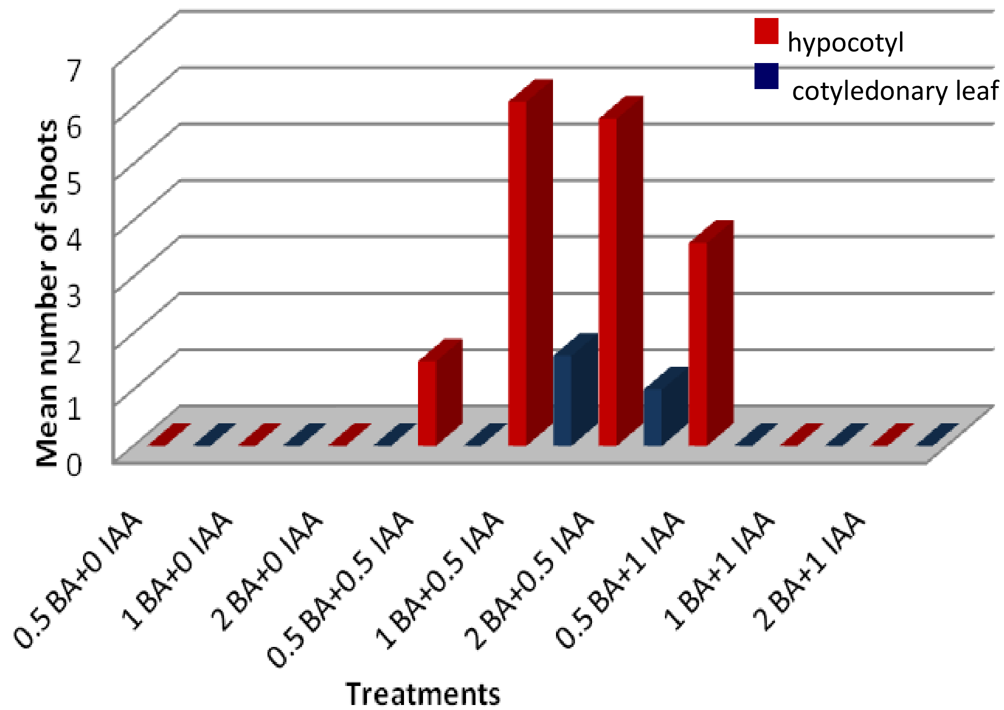


Figure 11. Comparison of cotyledonary leaf and hypocotyl explants in terms of shoot formation.

3.1.4. Effects of KIN combined with IAA on shoot production from hypocotyl and cotyledonary leaf explants

In the last step of *in vitro* regeneration studies, the different concentrations and combinations of KIN with IAA were tested for hypocotyl and cotyledonary leaf explants. While 0.5 mg/l KIN promoted shoot formation in only hypocotyl explants (3.6 shoots per explant), 1.0 and 2.0 mg/l of KIN did not give any shoot response for hypocotyl explants and gave a weak response for cotyledonary leaf explants. In the combination of 0.5 KIN and 0.5 IAA, shooting response was 3.6 shoots per hypocotyl explant and if IAA concentration was doubled to 1.0 mg/l, mean shoot

number increased to 6.3 shoots per hypocotyl explant. The most effective combination (2.0 mg/l KIN and 0.5 mg/l IAA) produced a mean of 7.0 shoots per hypocotyl explants at 88% frequency and 2.3 shoots per cotyledonary leaf explants at 33% frequency (Table 4; Figure 12). Also, root formation, callus formation and blackening were observed in some explants (Figure 13).

Table 4. Comparison of cotyledonary leaf and hypocotyl explants in terms of shoot formation on medium containing different combinations and concentrations of KIN and IAA after 3-4 weeks culture ($P<0.05$; Data represents mean values ± SE, experiments were repeated 3 times).

Plant Growth Regulators		Type of explants			
		Hypocotyl		Cotyledonary leaf	
KIN (mg/l)	IAA (mg/l)	Mean number of shoots per explant(±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explant forming shoots
0.5	0	$3.66±0.6^{b}$	33%	$0±0^{c}$	0
1	0	$0±0^{c}$	0	$0.16±0.1^{bc}$	8%
2	0	$0±0^{c}$	0	$0.33±0.2^{bc}$	17%
0.5	0.5	$3.66±0.33^{b}$	33%	$0.16±0.1^{bc}$	8%
1	0.5	$0.16±0.16^{c}$	10%	$0±0^{c}$	0
2	0.5	$7±0.25^{a}$	88%	$2.33±0.3^{a}$	33%
0.5	1	$6.33±0,91^{a}$	77%	$0.33±0.2^{bc}$	17%
1	1	$0.33±0.21^{c}$	9%	$0.66±0.2^{b}$	25%
2	1	$0±0^{c}$	0	$0±0^{c}$	0
Means		2.38±0.3	27%	0.44±0.1	12%

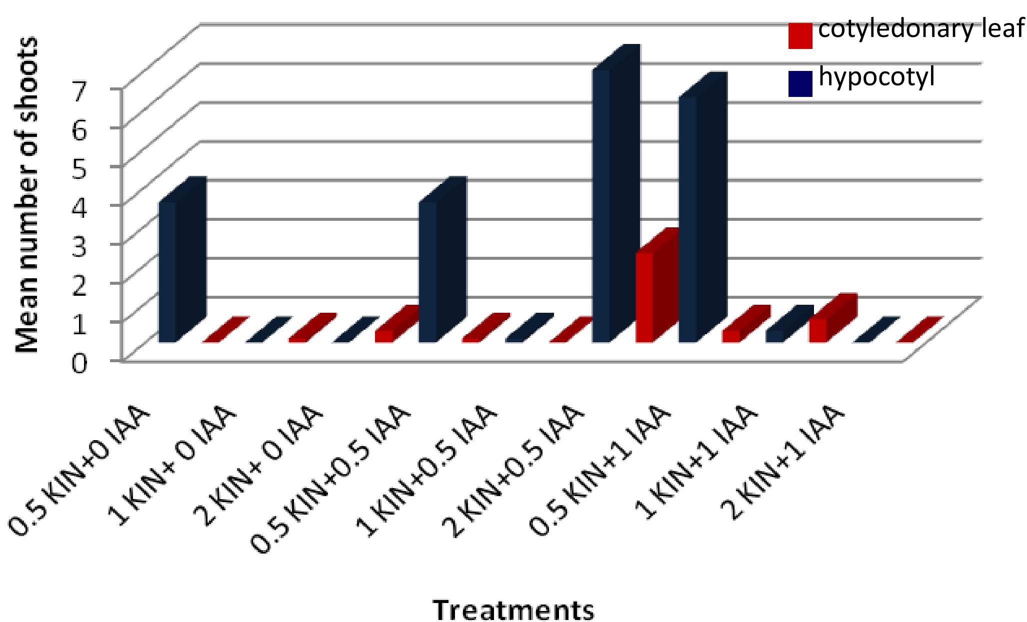


Figure 12. Comparison of cotyledonary leaf and hypocotyl explants in terms of shoot formation.

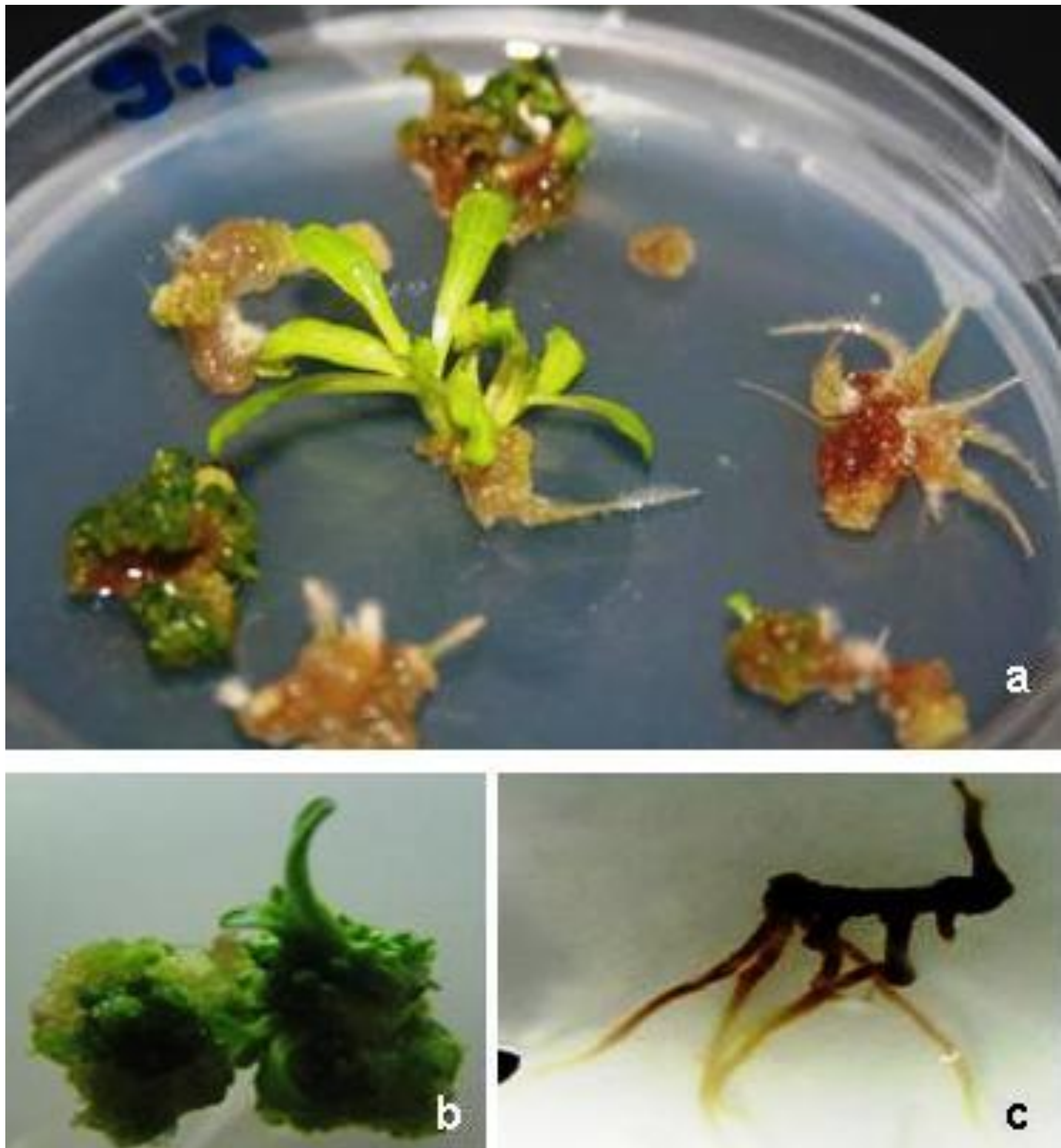


Figure 13. Shoot regeneration from hypocotyl explants cultured on medium containing different combinations and concentrations of KIN and IAA: a) 0.5 mg/l KIN + 0.5 mg/l IAA; b) 2.0 mg/l KIN + 0.5 mg/l IAA; c) 2.0 mg/l KIN + 1.0 mg/l IAA.

3.1.5. Effects of combinations of TDZ, IAA and gibberellic acid for hypocotyl explants

After experimenting the successful combination of TDZ and IAA in terms of shoot induction for hypocotyl explants, the effects of including gibberellic acid as an additional PGRs were also tested. When the medium contained 2.0 mg/l TDZ and 0.5

mg/l IAA (see Table 2), a much higher mean number of shoots (11.0) per hypocotyl explant was obtained as compared to the addition of 0.5 mg/l gibberellic acid to the same combination, which produced a mean of 6.5 shoots per hypocotyl explant (Table 5). Likewise, 1.0 mg/l TDZ combined with 1.0 mg/l IAA gave 3.1 shoots per hypocotyl explant (see Table 2), and with the addition of 0.5 mg/l gibberellic acid to the same combination, shoot formation was not observed at all (Table 5, Figure 14). In general, it was clear that the presence of IAA was more critical for shoot induction from hypocotyl explants, and irrespective of the concentration of both TDZ and GA_3, 0.5 mg/l IAA was much more effective than 0.1 or 1.0 mg/l (Table 5).

Table 5. The shoot formation from hypocotyl explants cultured on medium containing different combinations and concentrations of TDZ, IAA and GA_3 after 3-4 weeks culture ($P<0.05$; Data represents mean values ± SE, experiments were repeated 3 times).

Plant Growth Regulators			**Type of explants**	
			Hypocotyl	
TDZ (mg/l)	**IAA (mg/l)**	**GA_3 (mg/l)**	**Mean number of shoots per explant(±SE)**	**% explants forming shoots**
0.5	0	0.5	$0±0^{f}$	0
1	0	0.5	$0.25±0.2^{f}$	25%
2	0	0.5	$2±0.4^{e}$	30%
0.5	0.1	0.5	$0±0^{f}$	0
1	0.1	0.5	$0±0^{f}$	0
2	0.1	0.5	$2.75±0.4^{d}$	25%
0.5	0.5	0.5	$4.25±0.2^{c}$	50%
1	0.5	0.5	$5.25±0.2^{b}$	50%
2	0.5	0.5	$6.5±0.3^{a}$	100%
0.5	1	0.5	$0.5±0.3^{f}$	20%
1	1	0.5	$0±0^{f}$	0
2	1	0.5	$0±0^{f}$	0
Means			1.79±0.3	25%

When the overall means of shoot formation induced by combinations of TDZ and IAA was taken into consideration, percentage of shoot response from hypocotyl explants was 42%. However, this ratio was reduced to 25% when gibberallic acid

was added. This results clearly indicates that gibberellic acid reduces shoot production capacity of hypocotyl explants dramatically.

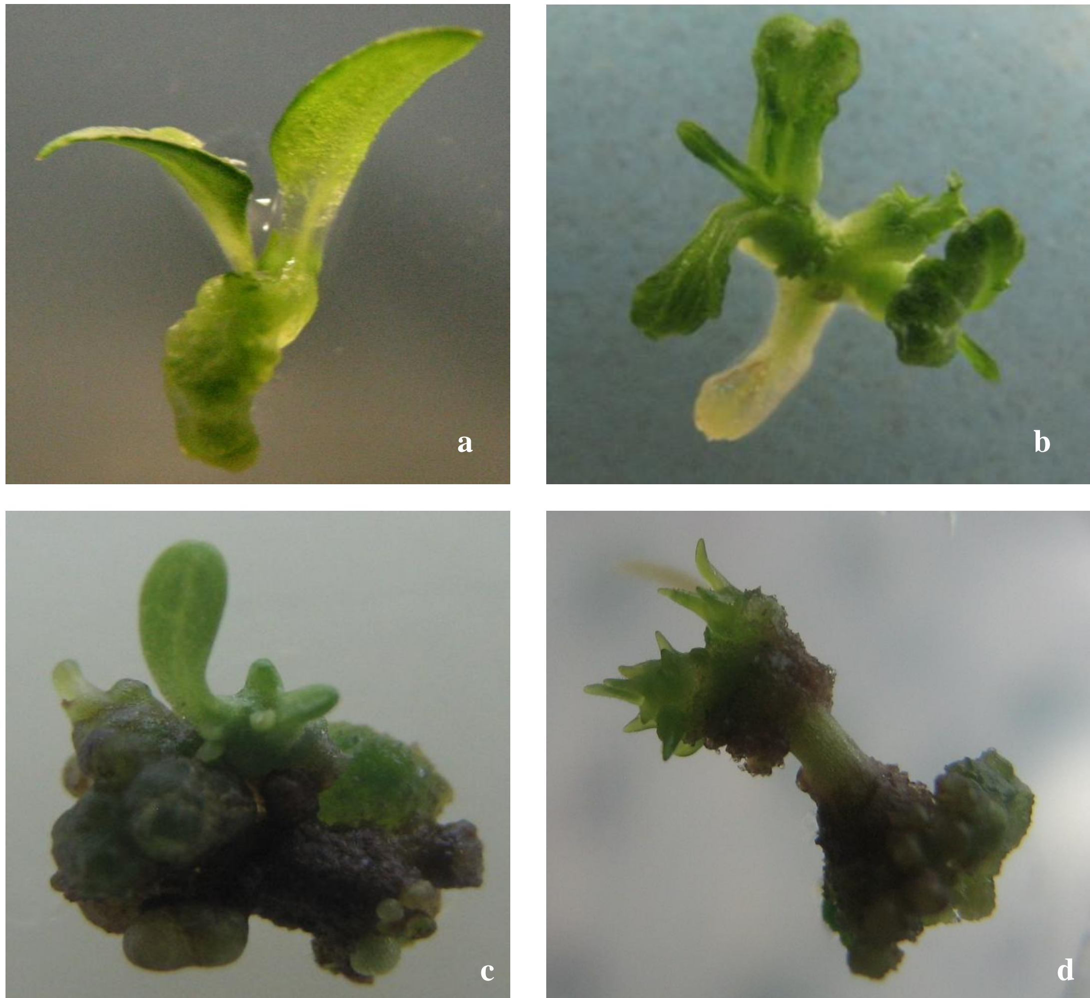


Figure 14. Shoot regeneration from hypocotyl explants cultured on medium containing different combinations and concentrations of TDZ, IAA and GA_3: a-b) 1 mg/l TDZ + 0.5 mg/l IAA + 0.5 mg/l GA_3; c-d) 2.0 mg/l TDZ + 0.5 mg/l IAA + 0.5 mg/l GA_3.

3.1.6. Root formation

After removing the callus, some healthy shoots were selected to transfer in the medium that contained different types of auxin (IAA, NAA and IBA) at different concentrations (Table 6; Figure 15). The root production was achieved from shoots succesfully in 4 to 5 weeks.

Table 6. Comparison of root formation on medium containing different concentrations of IAA, NAA and IBA after 3-4 weeks culture ($P<0.05$; Data represents mean values ± SE, experiments were repeated 3 times).

Plant growth regulators (mg/l)	Mean number of roots per explant	Percentage of explants forming roots
0.1 IAA	3,33±0.8	40%
0,5 IAA	5,66±1.2[bc]	50%
1 IAA	16,33±0.6[a]	100%
2 IAA	17,33±1.2[a]	100%
0.1 NAA	2,33±0.6[cd]	25%
0.5 NAA	3,33±0.8[cd]	40%
1 NAA	6±2.8[bc]	75%
2 NAA	7,33±0.6[b]	100%
0.1 IBA	1±0[d]	13%
0.5 IBA	4±0[bcd]	50%
1 IBA	5,33±0.3[bc]	60%
2 IBA	6±0.3[bc]	75%
Means	6,5±0.8	60%

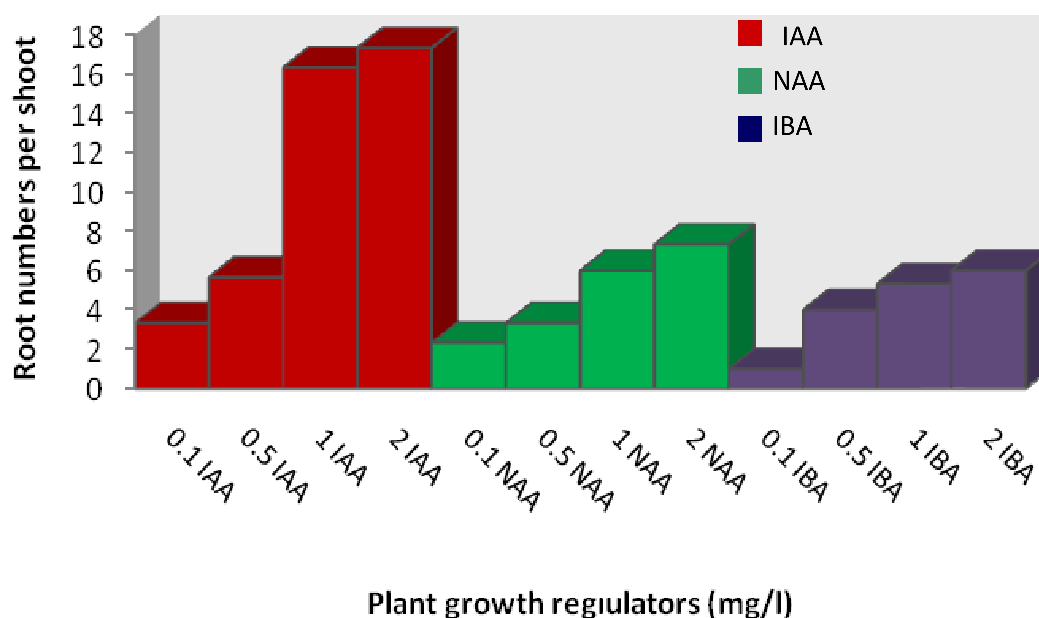


Figure 15. Comparison of root formation developed on medium containing different auxins.

Effects of IAA on root formation: The healty shoots developed on media containing different types of PGRs were transferred to auxin-containing for root formation. In this experiment, different concentrations of IAA (0.1, 0.5, 1.0 and 2.0 mg/l) were used. While 0.1 mg/l IAA produced 3.3 roots per shoot, 0.5 mg/l IAA produced 5.6 roots per shoot. When the concentration of IAA was increased from 0.5 to 1.0 mg/l, root number significantly increased from 5.6 to 16.33 roots per shoot. Among all of the treatments, medium containing 2.0 mg/l IAA produced the highest number of roots (17.33 roots per shoot at 100% frequency; Figure 16). IAA was more effective to produce roots than the other auxins used (NAA and IBA). The average frequency of roots per shoot was 72.5% in different ranges of IAA.

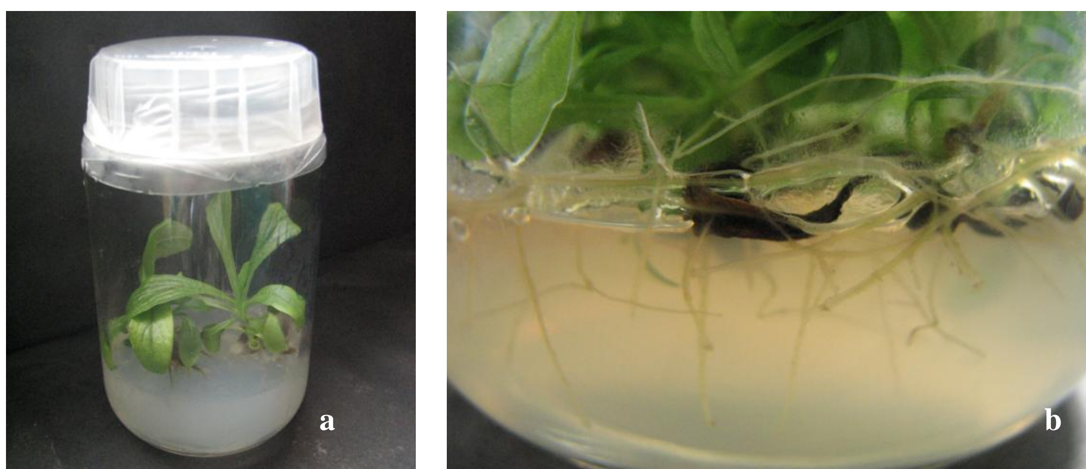


Figure 16. The root formation on medium containing different concentrations of IAA: a) 1.0 mg/l IAA; b) 2.0 mg/l IAA.

Effects of NAA on root formation: The range of 0.1, 0.5, 1.0 and 2.0 mg/ l NAA were used to produce roots from healthy shoots and the number of roots were 2.3, 3.3, 6.0 and 7.3 roots per shoot, respectively (Figure 17). For NAA, the highest number of roots (7.3) was obtained at the concentration of 2.0 mg/l, while the highest number for IAA was 17.33 roots per shoot at 2.0 mg/l. Thus, NAA was less effective than IAA for root induction.

Figure 17. The root formation on medium containing 1.0 mg/l NAA.

<u>Effects of IBA on root formation:</u> The effects of IBA at different concentrations (0.1, 0.5, 1.0 and 2.0 mg/ l) was also examined for rooting. For 0.1 mg/l IBA, the rooting response was 1.0 root per shoot. If the concentration was icreased to 0.5, 1.0 or 2.0 mg/l, root formation increased to 4.0, 5.33 and 6.0 roots per shoot, respectively (Figure 18). Among all of the auxins, IBA was the least effective for root induction and development.

Figure 18. The root formation on medium containing different concentrations of IBA: a) 0.5 mg/l IBA; b) 2.0 mg/l IBA.

3.1.7. Transferring plantlets to pots

The rooted plants were transfered to Magenta containers or pots including sterile potting soil and kept under growth room conditions. Approximately 70% of regenerated plants survived through the hardening off process (Figure 19).

Figure 19. Rooted shoots were transferred to plastic pots containing sterile potting soil (acclimatization).

3.2. Total phenolic contents

In the last part of the study, regenerated shoots which obtained from hypocotyl explants in different PGRs were dried and powdered. Then, samples were analyzed for total phenolic contents (Figure 20) and the results were compared.

Figure 20. Some pre-stages for analysis of total phenolic contents.

Total phenolic content was expressed as mg gallic acid equivalents/g dry weight. The different concentrations of gallic acid standarts were measured by UV-spectrophotometer and according to absorbance values the gallic acid curve was established (Figure 21).

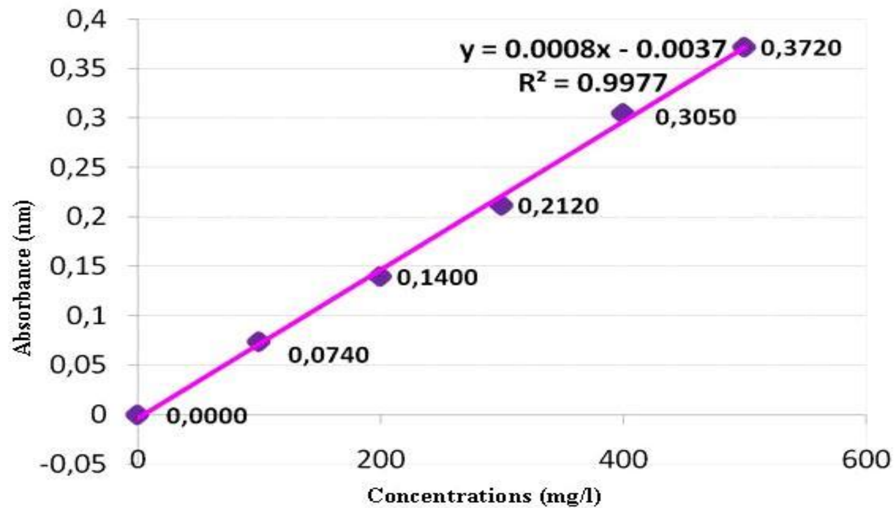


Figure 21. Gallic acid standart curve for total phenolic analysis.

The values of total phenolic contents were similar for the shoots obtained from hypocotyl explant in different concentrations and combinations of PGRs (Table 7). Total phenolic contents of 0.2 mg/l TDZ combined with IAA ranging from 0.5, 1.0 to 2.0 mg/l were 915.33, 1149.7 and 991.33 µg/g dw, respectively. This value was 935.03 µg/g dw for the shoots obtained from the medium containing 0.1 mg/l TDZ only. While the total phenolic value of 2.0 mg/l TDZ combined with 0.1 mg/l IAA was 967.62 µg/g dw, this value was 1005.23 µg/g dw for 2.0 mg/l TDZ combined with 0.5 mg/l IAA. The amount of total phenolic for both low and high doses of TDZ did not show a significant change.

When KIN was used with IAA, total phenolic content of the combination of 0.5 mg/l KIN and 0.1 IAA mg/l was 1039.24 µg/g dw, and with the ten-fold increase of IAA (1.0 mg/l) in the same combination, this value increased to 1205.8 µg/g dw.

Consequently, the highest amount of total phenolics was 1205.08 µg/g dw at 0.5 mg/l KIN combined with 1.0 mg/l IAA while the lowest amount of total phenolic content was 915.33 µg/g dw at 0.2 mg/l TDZ combined with 0.5 mg/l IAA (Table 7; Figure 22).

Table 7. Comparison of total phenolic contents for shoots that obtained from different types of plant growth regulators.

Plant growth regulators(mg/l)	Total phenolic contents (µg/g dw)
0.2 TDZ + 1 IAA	1149.7±0.2
0.2 TDZ + 0.5 IAA	915.33±0.5
0.2 TDZ + 2 IAA	991.33±0.5
0.1 TDZ + 0 IAA	935.03±0.05
0.5 TDZ + 1 IAA	1123.19±0.7
2 TDZ + 0.1 IAA	967.62±0.06
2 TDZ + 0.5 IAA	1005.23±0.3
0.5 KIN + 0.1 IAA	1039.24±0.3
0.5 KIN + 1 IAA	1205.08±0.1
2 KIN + 0.1 IAA	1002.39±0.3
2 KIN + 1 IAA	999.27±0.02

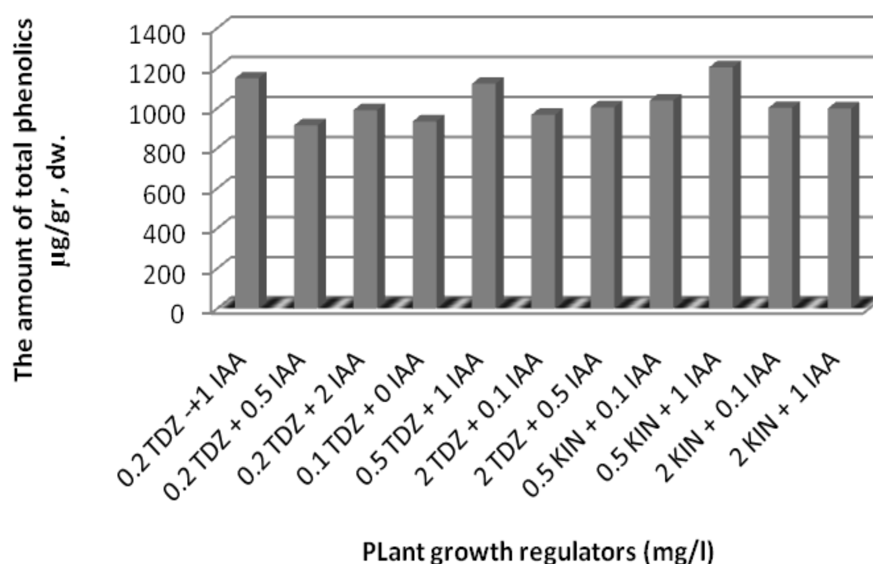


Figure 22. Comparison of total phenolic contents for different shoots that obtained from hypocotyl explants in media containing different plant growth regulators at varying concentrations.

CHAPTER IV. DISCUSSION

This study aimed at producing shoots from hypocotyl and cotyledonary leaf explants of *D. ferruginea* subsp. *schischkinii* by using different plant growth regulators. Among the all concentrations of TDZ and IAA combinations, the highest value of shoot production (11.0 shoots per explant) was observed in the combination of 2.0 mg/l TDZ and 0.5 mg/l IAA for hypocotyl explant. This result is compatible with the results established by Gürel et al. (2011) where high concentration of TDZ (0.5 mg/l) combined with lower IAA levels (especially 0.25 mg/l) was most effective for shoot regeneration, particularly from hypocotyl and filamingo bill type explants of *Digitalis davisiana* Heywood, producing a mean of 6.3 and 5.9 shoots per explant, respectively. Our experiments showed that all combinations of TDZ and IAA for hypocotyl explants are more productive than leaf explants for shoot production and also high levels of TDZ in the combination is significantly effective to induce shoot formation, especially for hypocotyl explants. In another study with *Digitalis lamarckii* Ivan., different concentrations ranging from 0.1, to 0.5 and 1.0 mg/l of TDZ were used and highly effective results were observed for leaf explants (Verma et al. 2011). This study revealed that 1.0 mg/l of TDZ was most effective for shoot production, giving 10.3 shoots per explant. Increasing TDZ to 3.0 mg/l induced no shoot regeneration, but formation of some calli only (Verma et al. 2011). In our study, the values of 0.5, 1.0 and 2.0 mg/l TDZ were compared for hypocotyl explants. While a significant difference was not observed for shoot formation at

concentrations of 0.5 and 1.0 mg/l TDZ, 2.0 mg/l TDZ produced significantly more shoots, with an average of 3 shoots per explant. The shoot formation has considerably increased in the same doses of TDZ with low doses of IAA. Consequently, the increasing doses of TDZ alone or in combination with the IAA were effective for shoot development from cotyledonary leaf and hypocotyl explants. TDZ has been widely used to induce shoot regeneration in different explants of various plant species (Ernst, 1994; Chen and Piluek, 1995; Chang and Chang, 1998; Bacchetta et al., 2003; Malabadi et al., 2004; Yucesan et al., 2007; Ling Fei et al., 2009; Turker et al., 2009a, 2009b).

In the precence of 0.5 mg/l GA_3 in addition to different combinations of TDZ and IAA, shoot regeneration from hypocotyl explant was also investigated. The combination of 2.0 mg/l TDZ, 0.5 mg/l IAA and 0.5 mg/l GA_3 exhibited the highest number of shoots; a mean of 6.5 shoots per explant. Gibberellic acid has been used most succesfully in shoot-tip cultures in order to preserve the integrity of apical buds and to obtain rapid shoot proliferation and elongation (Hu and Wang, 1983; George and Sherrington, 1984). However, the same combinations applied without 0.5 mg/l GA_3 increased shoot formation up to 11.0 shoots per explant in our experiments, with few exceptions, e.g. in spinach, where GA_3 was required for adventitious shoot or root differentiation (Al-Khayri et al. 1992). One of the marked effects of giberellins is the inhibition of organ formation (George and Sherrington, 1984). It also the case for *Digitalis obscura* L. cultures since GA_3, especially at high dosages, inhibited caulogenesis and rhizogenesis. Similarly, high GA_3 concentrations repressed both organogenic responses in *Digitalis lanata* Ehrh. (Lui and Staba, 1981) and *Digitalis purpurea* L. (Hagimori et al. 1982, Rücker, 1982). Thus, even giberellins play

important roles in plant growth and development, they sometimes have dramatic effects on *in vitro* morphogenesis (Meins 1986).

The concentrations of BA ranging from 0.5 to 1.0 and 2.0 mg/l did not give any shoot response for both hypocotyl and cotyledonary leaf explants. Also, the high concentrations of BA produced different types of calli from both leaf and some of the hypocotyl explants. In a previous study related to *Digitalis purpurea*, Hagimori et al. (1982) used different concentrations of BA (from 0.01 to 1.0 mg/l) alone or its combinations with auxins and they exhibited that BA did not affect the growth significantly. The presence of high concentrations of BA (3.0, 4.0, 5.0 mg/l) in combination with IAA promoted callus formation and shoot organogenesis from leaf explant of *Digitalis thapsi* L. (Cacho et al., 1991). However, when the combination of 1.0 mg/l BA and 0.5 mg/l IAA were used for hypocotyl and cotyledonary leaf explants, the highest value was 6.16 and 1.66 shoots per explant, respectively. Our results related to the effects of plant growth regulators are similar with the report on *Digitalis obscura* in the combinations of 1.0 or 2.0 mg/l BA and 0.5 mg/l IAA, in which case the shoot formation was the highest (9.9 and 8.7 per explant, respectively) (Pérez-Bermúdez et al. 1987). Sales et al. (2002) reported that BA promoted adventitious bud differentiation alone, but addition of auxin significantly increased the bud forming capacity of leaf explants of *Digitalis minor* L. In a recent study on *Digitalis trojana* Ivan. (Çördük and Akı, 2010), different concentrations of BA were combined with NAA, and a high concentration of BA (3.0 mg/l) with the low concentration of NAA (0.1 mg/l) gave the highest value (28.0 shoots per explant) of shoot formation in leaf explants. However, shoot formation was not observed at low concentrations of BA (0.1, 0.5 or 1.0 mg/l) in combination with 0.5 mg/l NAA. In our study, different concentrations of BA (0.5, 1.0 and 2.0 mg/l) in

combination with 1.0 mg/l IAA did not produce shoot formation. In this respect, it can be said that the high doses of IAA adversely affected the promoting influence of BA. Despite these, in another study on *Digitalis lamarckii* (Verma et al. 2011), 0.5 and 1.0 mg/l BA formed shoots. But, if the lowest and the highest values of BA (0.1 and 3.0 mg/l) were used, shoot formation was not observed. Garve et al. (1980) showed that the combination of high level of BA (2.0 mg/l) with low level of IAA (0.1 mg/l) was the most effective combination for shoot formation for *Digitalis lanata*.

In our experiments, in addition to BA and TDZ as a cytokinin, KIN was also tested for cotyledonary leaf and hypocotyl explants. 2.0 mg/l KIN and 0.5 mg/l IAA was the best combination that produced a mean of 7.0 shoots per explant. Pérez-Bermúdez et al. (1987) introduced that direct shoot formation was occasionally observed in a few explants of *Digitalis obscura* and the highest number of shoots was obtained from the combination including 0.1 mg/l IAA and 1.0 mg/l KIN. In our study, the other concentrations of KIN (0.5, 1.0 and 2.0 mg/l) did not give a significant level of shoots (0, 0.16, 0.3 shoots per explant, respectively); some calli and root formation with necrosis were observed instead. Similar results are observed in Verma et al.'s (2011) study regarding *Digitalis lamarkii* where several concentrations of KIN were used and there was no shoots produced as leaf explants became necrotic. In a smiliar study for *Digitalis thapsi*, an *in vitro* study was conducted using hypocotyl and root explants and it was found that KIN alone did not cause any shoot reponse. Only 0.5 mg/l KIN and 1.0 mg/l NAA produced low numbers of shoots (Cacho et al., 1991). This observation agrees with the fact that KIN has relatively low biological activity in certain bio-assays (Bogaert et al., 2006).

After shoot proliferation, shoots were transfered to auxin-containing media for rooting. For this, IAA, NAA and IBA were used in different concentrations and nearly all shoots formed roots. In a previous study about root formation, different concentrations of IAA, NAA and IBA were used and root formation of *Digitalis lanata* was achieved (Fatima et al., 2009). Gürel et al. (2011) compared IAA and IBA for root formation in *Digitalis davisiana* Heywood and IAA was found more effective than IBA for promoting roots, in which the highest root number (17.3 roots per shoot) was obtained at 2.0 mg/l IAA.

In the last stage of tissue culture studies, the rooted shoots were succesfully transfered to pots, and the range of root forming frequency reached 100%, especially for IAA and NAA. Approximately 70% of regenerated plants survived through the hardening off process. Our results was similar to that of Cacho et al. (1991)'s study in which all rooted plantlets of *Digitalis thapsi* transferred to the pots and 70% of them were survived. In another study on *Digitalis trojana,* an *in vitro* study was established and rooted plantlets were adapted to *ex vitro* conditions and then transplanted to vials containing soil, all of *in vitro* regenerated plantles growing healthy (Çördük and Akı, 2010).

Although many *in vitro* studies have been performed about the genus *Digitalis* which contains economically important cardenolides, no *in vitro* study has been found on *D. ferruginea* subsp. *schischkinii* in literature. Consequently, an *in vitro* protocol system was successfuly developed from cotyledonary leaf and hypocotyl explants of *D. ferruginea* subsp. *schischkinii* for the first time. The various concentrations and combinations of plant growth regulators were compared and TDZ was found to be more effective than both BA and IAA in terms of shoot induction. On the other hand, among the all treatments, the most effective combination was

TDZ and IAA. To observe the effects of gibberellic acid in the same combination, different treatments were also tested and it was found that gibberellic acid prevented shoot production for hypocotyl explants dramatically.

BA and KIN were not very effective for shoot production and in some media callus formation, root formation and blackening was observed. However, the combinations of higher levels of KIN and BA, when combined with IAA, also induced shoot formation. Although not a significant difference, combination of KIN and IAA was found more effective than combination of BA and IAA. In addition, the effects of explants were found in different plant growth regulators. Generally, hypocotyl explants were more productive for shoot production than cotyledonary leaf explants.

Few studies have been performed on the cardenolides of natural populations of *D. ferruginea* subsp. *schischkinii*. In these studies, lanatosides A, B, C, D and E as well as the secondary glycosides acetyldigoxin and acetyldigitoxin were isolated as the major glycosides from the leaves of *D. ferruginea* subsp. *schischkinii* from Turkey (İmre and Ersoy, 1976). And, also cardenolide glycosides of stem leaves of *D. ferruginea* subsp. *schischkinii* of the same physiological age and location were analysed quantitatively over several vegetations (İmre et al., 1982). Our study presents an analysis of *in vitro* total phenolic content for *D. ferruginea* subsp. *schischkinii* for the first time and the highest level of total phenolic content was 1205.08 µg/g in the samples regenerated on medium containing the combination of 0.5 mg/l KIN and 1.0 mg/l IAA. In general, the range of total phenolic contents were not significantly different in shoots developed on medium containing different concentrations and combinations of various plant growth regulators. Although the

combinations of TDZ and IAA resulted in much better shoot formation, the highest values for total phenolic amount were obtained when KIN and IAA were combined.

CHAPTER V. CONCLUSIONS

An *in vitro* protocol system was successfuly developed from cotyledonary leaf and hypocotyl explants of *D. ferruginea* subsp. *schischkinii* for the first time. And also, Our study presents an analysis of *in vitro* total phenolic content for this plant for the first time.

The various concentrations and combinations of PGRs were compared and TDZ was found to be more effective than both BA and KIN in terms of shoot induction. Generally, hypocotyl explants were more productive for shoot production than leaf explants.

To observe the effects of gibberellic acid in the same combination, different treatments were also tested and it was found that gibberellic acid prevented shoot production for hypocotyl explants dramatically.

BA and KIN were not very effective for shoot production and in some media callus formation, root formation and darkening was observed. However, the combinations of higher levels of KIN and BA, when combined with IAA, also induced shoot formation.

For root formation different types of auxins were used. Among them, IAA was the most effective PGRs to produce roots from shoots.

All healthy rooted shoots were transferred to the pots for acclimatization and kept in plant growth chamber, % 70 plantlets were survived.

The values of total phenolic contents were measured for the shoots obtained from hypocotyl explant in different concentrations and combinations of PGRs first time. In general the total phenolic values were similar but the highest value was observed from KIN and IAA combination.

The next target is that to determine the cardiac glycosides contents of *D. ferruginea* subsp. *schischkinii* in *in vitro* samples by HPLC. *In vitro* production of *D. ferruginea* subsp. *schischkinii* may be enhanced and cardiac glicosides which has economical and medicinal imporatance can be produced rapidly. Besides, in terms of anti-cancer properties of *Digitalis* species, detailed transformation studies may be performed.

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