INVESTIGATION OF RELATIONSHIP BETWEEN STRESS FACTORS AND CERTAIN METABOLITES INCLUDING CARDENOLIDES IN CALLUS CULTURES OF ENDEMIC TURKISH *DIGITALIS* L. SPECIES

by
GÜNCE ŞAHİN

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

MARCH 2012

Approval of the Graduate School of Natural and Applied Sciences.

Prof. Dr. Nihat ÇELEBİ
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. M. Tekin BABAÇ
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

Prof. Dr. Ekrem GÜREL
Supervisor

Examining Committee Members

1- Prof. Dr. Rukiye TIPIRDAMAZ ----------

2- Prof. Dr. M. Tekin BABAÇ ----------

3- Prof. Dr. Nusret ZENCİRCİ ----------

4- Prof. Dr. Ekrem GÜREL ----------

5- Assoc. Prof. Arzu TÜRKER ----------

ABSTRACT

INVESTIGATION OF RELATIONSHIP BETWEEN STRESS FACTORS AND CERTAIN METABOLITES INCLUDING CARDENOLIDES IN CALLUS CULTURES OF ENDEMIC TURKISH *DIGITALIS* L. SPECIES

Şahin, Günce
Ph.D., Department of Biology
Supervisor: Prof. Dr. Ekrem Gürel

March 2012, 126 pages

The genus *Digitalis* L., whose Latin name is "finger of a glove" referring to the shape of the flowers, is a member of the Plantaginaceae. Members of the genus *Digitalis* are medicinally and economically important plants which contain important cardioactive compounds used to treat heart problems. The plant is well-known in Turkey for its cardenolides (glycosides) content, which is used for myocardial infarction, edema, angina, cardiac dysfunction, hyertropy and arterial hypertension. Commercial production of bioactive secondary metabolites by traditional agriculture is an inefficient process. Strategies, based on in vitro culture methods, have been extensively studied to improve the production of specific plant derived chemicals. The aim of the present research is to obtain relationship between different stress treatments and content of cardiotonic glycosides (digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin) as secondary metabolites of commercial value

for the pharmaceutical industry and to determine the antioxidant metabolites against stress conditions. The effects of different stress treatments (media strength,macronutrient deficiency, oxidative, chemical, temperature and UV) on cardiotonic glycoside accumulation in *D. lamarckii* Ivanina, *D. trojana* Ivanina, *D. davisiana* Heywood and *D. cariensis* Boiss. ex Jaub. et Spach were investigated. HPLC analysis revealed that all stress conditions were significantly effective at 5% significance level according to their control groups. The predominant cardiac glycoside was lanatoside C (Lan C) followed by digitoxin, digoxigenin, gitoxigenin and digoxin. No digoxin was detected in all treatments as well as in control groups. Moreover, the effects of these stress treatments on the total phenolics, proline content and antioxidant enzymes activities were investigated. The results revealed that all stress conditions significantly increased the total phenolic and proline content. It was also concluded that CAT and SOD activities were markedly stimulated under all stress conditions in all *Digitalis* species, except for high temperature treatment. In contrast, high temperature stress resulted in lower CAT and SOD activities. All these results indicated that different stress treatments have induced changes in the redox state of callus cells and suggest that this alteration stimulates cardenolide formation in *Digitalis* callus cultures.

Keywords: Stress, *Digitalis* sp., cardiac glycosides, CAT, SOD, total phenolics, proline, HPLC.

ÖZET

TÜRKİYE'YE ENDEMİK *DIGITALIS* L. TÜRLERİNİN KALLUS KÜLTÜRLERİNDE STRESS FAKTÖRLERİ İLE KARDENOLİTLER DE DAHİL BELLİ METABOLİTLER ARASINDAKİ İLİŞKİNİN ARAŞTIRILMASI

Şahin, Günce
Doktora, Biyoloji Bölümü
Tez Danışmanı: Prof. Dr. Ekrem Gürel

Mart 2012, 126 sayfa

Digitalis L., çiçeklerinin şeklinden dolayı yüksük otu olarak bilinen ve Plantaginaceace familyasına ait iki veya çok yıllık bir bitkidir. Tıbbi ve ekonomik açıdan oldukça önemli olan bu türler, taşıdığı kalp üzerine etkili glikozitler nedeniyle miyokardiyal enfarktüs, ödem, anjin, kalp yetmezliği, arteryel hipertansiyon, kardiyak hipertrofi gibi kalp hastalıklarının tedavisinde kullanılmaktadırlar. Geleneksel tarım yöntemleri ile biyoaktif sekonder metabolitlerin ticari üretiminin yetersiz olmasından dolayı bu metabolitlerin üretimini arttırmak için in vitro kültür tekniklerine dayalı stratejiler kullanılmaktadır. Bu çalışmada, farklı stress uygulamaları ile ilaç endüstrisi için ticari değeri olan kalp glikozitleri (digoksigenin, gitoksigenin, lanatosit C, digoksin ve digitoksin) arasındaki ilişki ve strese karşı oluşan antioksidan metabolitleri belirlemek amaçlanmıştır. HPLC analizleri, stress

koşullarının, *D. lamarckii* Ivanina, *D. trojana* Ivanina, *D. davisiana* Heywood ve *D. cariensis* Boiss. ex Jaub. et Spach türlerine ait kardenolit üretiminin artışı üzerinde, kontrol gruplarına göre %5 anlamlılık düzeyinde önemli ölçüde etkili olduğunu ortaya koymuştur. Stres sonucu üretilen en etkili kardenolit Lan C'dir. Kontrol grupları da dahil olmak üzere, tüm uygulamalarda digoksin tespit edilememiştir. Ayrıca, bu çalışmada strese karşı oluşan toplam fenolik miktarı, prolin içeriği ve antioxidant enzim aktiviteleri tespit edilmiştir. Sonuçlar tüm stres uygulamalarının toplam fenolik miktarını ve prolin içeriğini önemli ölçüde arttırdığını ortaya koymuştur. Bunun yanısıra, sıcaklık stresi dışında tüm stres uygulamalarının, CAT ve SOD aktivitelerini arttırdığı gözlemlenmiştir. Sıcaklık stresi ise CAT ve SOD aktivitelerinde azalışa neden olmuştur. Tüm bu sonuçlar, stres uygulamalarının, kallus hücrelerindeki redoks durumunda değişikliğe neden olduğunu ve bu değişikliğin *Digitalis* türlerinde kardenolit oluşumunu tetiklediğini göstermiştir.

Anahtar Kelimeler: Stres, *Digitalis* sp., kalp glikozitleri, CAT, SOD, toplam fenolik, prolin, HPLC.

To My Family

ACKNOWLEDGEMENTS

I wish to express my gratitude to those who contributed to this work. Having completed this study, I am grateful to my supervisor, Prof. Dr. Ekrem Gürel, for his diligent support and professional guidance throughout the study. Without his encouragement and guidance, this thesis would not have been completed.

I am indebted to Prof. Dr. Rukiye Tıpırdamaz and Assoc. Prof. Dr. Arzu Türker for their great help and favour. Besides the scientific knowledge, they qualified me with social and cultural skills. I wish to express my sincere gratitude to Prof. Dr. M. Tekin Babaç and Prof. Dr. Hasan Akbayın for guidance on statistical analysis of the thesis.

I would like to thank Dr. Sandeep Kumar Verma who provided me a very friendly and encouraging environment to study, shared his knowledge and stood behind me whenever I needed. I would also like to thank Hatice Karadeniz for her useful advice on HPLC analysis. I greatly thank Assist. Prof. Dr. İsmail Eker for his help for the collection and characterization of plant materials. Special thanks to my colleague, Fatma Karakaş, for her close friendship.

I also thank to my mother, Binnaz Şahin, who nurtured and encouraged me throughout this long process; although he is now too far away from me, to my father, Güngör Şahin, for his strong encouragement; to my sister, Bilge Şahin, who provided useful advice on statistical analysis of the data; to the members of the rest of my family whose names are not mentioned here for their continous affection. Finally I would like to send my love to my fiance, Erol Cingöz, corresponding to his endless love and trust.

The financial support of the Scientific Research Projects (BAP) of Abant İzzet Baysal University for the 2010.03.01.341 is greatly appreciated.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURES

CHAPTER 1.

INTRODUCTION

Human beings are totally in need of plants as source of carbohydrates, proteins and lipids for food. In addition, plants are crucial source of a wide range of secondary metabolites used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. Over 80% of natural products coming from plant origin are approximately 30.000 (Phillipson 1990, Balandrin and Klocke 1988, Fowler and Scragg 1988). In 1985, approximately 3500 new chemical structures were identified, 2600 of which came from higher plants. All over the world, 121 clinically prescription drugs are derived from plants (Payne et al. 1991). In the coming centuries, because the chemistry of most plant species is to be designated, plants will continue to be used to provide both new products and chemical models for new drugs (Cox and Balick 1994). Since their complex structural features are difficult to synthesize, researchers still depend on biological sources for secondary metabolites which include pharmaceuticals despite improvements in synthetic chemistry (Pezzuto 1995). Some plant-derived pharmaceuticals are listed in Table 1 (Ramachandra and Ravishankar 2002).

Table 1. Plant-derived pharmaceuticals and their usage.

Product	**Plant species**	**Use**
Ajmalicine	*Catharanthus roseus* (L.) G. Don	Antihypertensive
Artemisinin	*Artemisia annua* L.	Antimalarial
Berberine	*Coptis japonica* (Thunb.) Makino	Intestinal ailment
Camptothecin	*Camptotheca acuminate* Decne.	Antitumour
Capsaicin	*Capsicum frutescens* L.	Counterirritan
Castanospermine	*Castanospermum austral* A.Cunn. & C.Fraser	Glycoside inhibitor
Codeine	*Papaver somniferum* L.	Sedative
Colchicine	*Colchicum autumnale* L.	Antitumour
Digoxin	*Digitalis lanata* Ehrh.	Heart stimulant
Diosgenin	*Dioscorea deltoidea* Wall. Ex Griseb.	Steroidal precursor
Ellipticine	*Ochrosia elliptica* Labill.	Antitumour
Forskolin	*Coleus forskohlii* (Willd.) Briq.	Bronchial asthma
Ginsenosides	*Panax ginseng* C.A. Mey.	Health tonic
Morphine	*Papaver somniferum*	Sedative
Podophyllotoxin	*Podophyllum peltatum* L.	Antitumour
Quinine	*Cinchona ledgeriana* (Howard) Bern.Moens ex Trimen	Antimalarial
Sanguinaria	*Sanguinaria canadensis* L.	Antiplaque
Shikonin	*Lithospermum erythrorhizon* Siebold & Zucc.	Antibacterial
Taxol	*Taxus brevifolia* Nutt.	Anticancer
Vincristine	*Catharanthus roseus*	Antileukemic
Vinblastine	*C. roseus*	Antileukemic

Plant cell cultures are useful alternative sources to produce high-value secondary metabolites (Ravishankar et al. 1999, Dornenburg and Knorr 1997, Scragg 1997, Alfermann and Petersen 1995, DiCosmo and Misawa 1995, Stockigt et al. 1995, Endress 1994, Ravishankar and Venkataraman 1990) (Table 2). Plant cells are biosynthetically totipotent; that is, each cell in culture contains whole genetic information and thus is able to produce the range of chemicals which is found in the parent plant. There are some outstanding advantages of plant cell cultures over the conventional agricultural production. One of them is the independence of geographical and seasonal variations and various environmental factors. Moreover, plant cell cultures offer a declared production system, which provides the perpetual

provision of products, unique quality and yield. Besides, producing novel compounds being not ordinarily found in parent plant is possible.

Table 2**.** Groups of natural products isolated from tissue and suspension cultures of higher plants.

Phenylpropanoids	**Alkaloids**	**Terpenoids**	**Quinones**	**Steroids**
Anthocyanins	Acridines	Carotenes	Anthroquinones	Cardiac glycosides
Coumarins	Betalaines	Monoterpenes	Benzoquinones	Pregnenolone derivatives
Flavonoids	Quinolizidines	Sesquiterpenes	Naphthoquinones derivatives	
Hydroxycinnamoyl	Furonoqui nones	Diterpenes		
Isoflavonoids	Harringtonines	Triterpenes		
Lignans	Isoquinolines			
Phenolenones	Indoles			
Proanthocyanidins	Purines			
Stilbenes	Pyridines			
Tanins	Tropane alkaloids			

However, despite these advantages, there are still many biological and biotechnological limitations for production of plant secondary metabolites by plant cell culture technology. One of the major drawbacks is the low yield of plant secondary metabolites in plant cell cultures. In order to improve the yield of such plant secondary metabolites, some strategies have been developed (Ramachandra and Ravishankar 2002).

A recent work for improving production of plant secondary metabolites was mainly focused on improving chemical processing and bioreactor performance or employing elicitors, abiotic and biotic stresses (Zhong 2001) and studying signal transduction pathways, transcription factors and their regulation mechanisms which include genetic manipulation of regulator genes (Memelink et al. 2001). Moreover, cloning

of secondary metabolite biosynthetic genes and genetic modification of key genes (Verpoorte and Memelink 2002) and studying metabolic flux and profiling metabolic intermediates (Sumner et al. 2003) and gene transcripts for plant secondary metabolism by profiling and analyzing global gene expression under distinct conditions (Goossens et al. 2003) are the main strategies which are effectively promote the production of a wide range of plant secondary metabolites, both in vivo and in vitro.

Table 3**.** Strategies to improve production of secondary metabolites in plant cell cultures.

1. Obtaining efficient cell lines for growth
2. Screening of high-growth cell line to produce metabolites of interest a. Mutation of cells b. Amenability to media alterations for higher yields
3. Immobilization of cells to enhance yields of extracellular metabolites and to facilitate biotransformations
4. Use of elicitors to enhance productivity in a short period of time
5. Permeation of metabolites to facilitate downstream processing
6. Adsorption of the metabolites to partition the products from the medium and to overcome feedback inhibition
7. Scale-up of cell cultures in suitable bioreactors

Next step is to describe stress which is one of these strategies mentioned above and used in this thesis for enhancing production of plant secondary metabolites.

1.1. Stress

Stress, in biological term, means deviation in the normal physiology, development and function of plants which can be injurious and can inflict irreversible damage to the plant system.

Environmental changes mainly originate from anthropogenic activities which have caused air and soil pollution, acid precipitation, soil degradation, salinity, increasing UV radiation, climate change etc. In addition, plants are exposed to natural climatic

or edaphic factors, for example, extreme temperatures, water deficit or abundance, high irradiation, heat, chilling, late frost, drought, flooding, pathogen and nutrient imbalance. All these stresses have decreased the biosynthetic capacity of plant organisms, altered their normal functions and caused damages which may lead to plant death (Lichtenthaler 1996, Levitt 1980a). Regarding all these information, environmental stresses can be divided into two groups; biotic stress, caused by other organisms and abiotic stress, caused by non-living effects of environment. List of the general stress elements are given in Table 4.

Table 4**.** General stress elements.

Biotic stress factors
Infection and competition by other organisms
Abiotic stress factors
Light (high, low intensity)
Temperature (high, low)
Water (deficit, excess)
Radiation (UV, visible, IR, ionizing)
Chemicals (salts, heavy metals)
Mechanical factors (wind, pressure)

1.1.1 Biotic Stress

Plants are under constant assault by biotic agents, including viral, bacterial and fungal pathogens, parasitic plants and insect herbivores, with enormous economic and ecological impact (Pimentel 1991, 2002).

When an attack is detected, plant metabolism must balance potentially competing demands for resources to support defense against the requirements for cellular maintenance, growth and reproduction (Herms and Mattson 1992, Zangerl and Berenbaum 1997, 2003, Berger et al. 2007).

Upon introduction of various elicitors, massive reprogramming of plant gene expression, hormonal and chemical defense responses are initiated (Tian et al. 2003, Zavala and Baldwin 2004). Because of the co-evolutionary relationships between specific plant species and the specific agents of damage, the defense responses to biotic attack are most variable. Faced with this diversity, it is remarkable that biotic attack triggers a uniform and apparently regulated reduction in transcription of nuclear genes coding for the major components of photosynthesis, regardless of the plant host or damage vector. A uniform down-regulation of photosynthetic gene transcription when faced with stress is not unprecedented, as environmental stresses, including drought, salinity and low temperatures, elicit a similar response (Saibo et al. 2009).

1.1.2 Abiotic Stress

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the retrogression of the environment. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer 1982, Bray et al. 2000). Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001). Drought, salinity, extreme temperatures and oxidative stress are often interconnected and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano et al. 1999, Zhu 2001a).

Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins (Smirnoff 1998). As a consequence, these diverse environmental stresses often activate similar cell signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2000, Knight and Knight 2001, Zhu 2001b, 2002) and cellular responses, such as the production of stress proteins, up-regulation of antioxidants and accumulation of compatible solutes (Vierling and Kimpel 1992, Zhu et al. 1997, Cushman and Bohnert 2000). The complex plant response to abiotic stress, which involves many genes and biochemical-molecular mechanisms, is schematically represented in Figure 1.

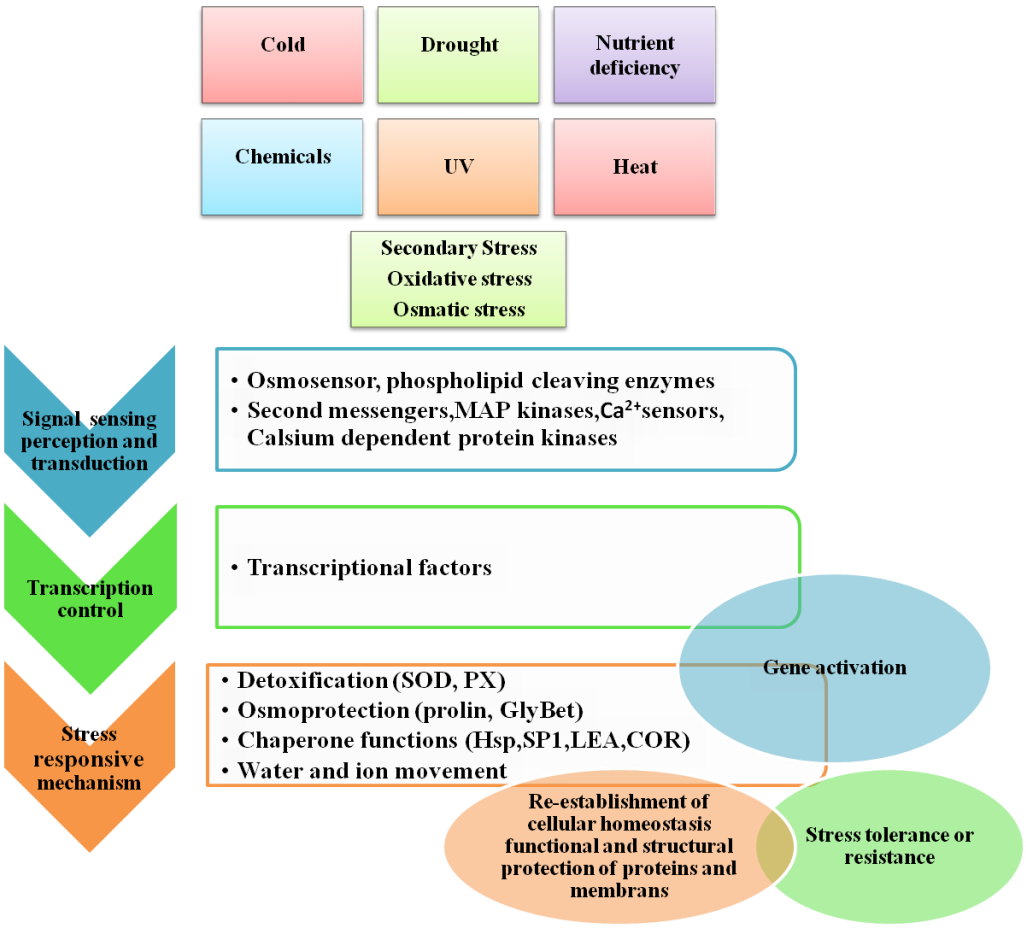

Figure 1. The complexity of the plant response to abiotic stress.

1.1.2.1 Nutrient Stress

Manipulation of the culture environment must be effective in increasing the product accumulation. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient strength, elimination of macro/micro nutrient and growth regulators.

1.1.2.1.1 MS Strength Stress

Most plant tissue culture media is still based on the nutrient composition described by Murashige and Skoog (MS) (1962) (Table 5), which is characterized by high levels of nitrogen in the forms of ammonium nitrate and potassium nitrate.

Table 5. MS media composition.

Macronutrients
Ammonium Nitrate
Ammonium Sulfate
Calcium Chloride Anhydrous
Potassium Nitrate
Potassium Phosphate Monobasic
Micronutrients
Boric Acid
Cobalt Chloride Anhydrous
Ferrous Sulfate-H_2O
Manganase Sulfate-H_2O
Molybdic Acid Sodium Salt $2H_2O$
Na_2-EDTA-$2H_2O$
Potassium Iodide
Zinc Sulfate-$7H_2O$

In an attempt to improve growth and production of secondary metabolite, one of the strategies of nutrient stress is changing strength of the culture medium. Medium salt strength induces to change in growth, physiology and secondary metabolite content in plants. Moreover, it is directly correlated with the total macro/micro nutrients level

in medium. That is an effective factor which contributes to the secondary metabolite production.

In *Hypericum perforatum* L. adventitious root cultures, 0.25-0.5 MS strengths were suitable for phenols and flavonoids (Cui et al. 2010). It was also found that lower concentration of MS major salts were significant for cardenolide content in *Digitalis obscur*a L. shoot-tip cultures (Gavidia and Perez-Bermudez 1997).

1.1.2.1.2 Macronutrient Stress

Nutrients have several structural, functional and regulatory roles in organisms. While some nutrients required at high levels are generally considered macronutrients, other needs much lower amounts and are considered micronutrients. Under this title, utilization of some macronutrients in plants and different strategies used to increase secondary metabolite concentration with changing exogenous nutrient conditions are to be discussed.

1.1. 2.1.2.1 Nitrate Levels

Nitrogen concentration was found to affect the level of protein or amino acid products in cell suspension cultures. The plant tissue culture medium such as MS, LS or B5 has both nitrate and ammonium as sources of nitrogen. However, the ratio of the ammonium/nitrate–nitrogen and overall levels of total nitrogen have been shown to markedly affect the production of secondary plant products. For example, reduced levels of NH_4^+ and increased levels of NO_3 promoted the production of shikonin and betacyanins, whereas higher ratios of NH_4^+ /NO_3 increased the production of berberine and ubiquinone (Bohm and Rink 1988, Nakagawa et al. 1984, Fujita et al. 1981, Ikeda et al. 1977). Reduced levels of total nitrogen improved the production of

capsaicin in *C. frutescens*, anthraquinones in *Morinda citrifolia* L. and anthocyanins in *Vitis* species (Yamakawa et al. 1983, Yeoman et al. 1980, Zenk et al. 1975). However, complete elimination of nitrate in cultures of *Chrysanthemum cinerariaefolium* (Trevir.) Vis. induced twofold increases in pyrethrin accumulation in the second phase of culture (Rajasekaran et al. 1991).

1.1.2.1.2.2 Phosphate Levels

The phosphate concentration in the medium can have a major effect on the production of secondary metabolites in plant cell cultures. Higher levels of phosphate were found to enhance the cell growth, where it had negative influence on secondary product accumulation. Sasse et al. (1982) gave a number of examples to show that a medium limited in phosphate either induces or stimulates both the product and the levels of key enzymes leading to the product. Thus, reduced phosphate levels induced the production of ajmalicine and phenolics in *Catharanthus roseus* (L.) G. Don., of caffeoyl putrescines in *Nicotiana tabacum* L. and of harman alkaloids in *Peganum harmala* L. Similar results were obtained in a separate study for the production of betacyanins in callus cultures of *Beta vulgaris* L. (Bohm and Rink 1988). In contrast, increased phosphate was shown to stimulate synthesis of digitoxin in *Digitalis purpurea* L. and of betacyanin in *Chenopodium rubrum* L. and *Phytolacca americana* L. (Bohm and Rink 1988, Hagimori et al. 1982a,b).

1.1.2.1.2.3 Ca Levels

Ca^{2+} plays a vital role in membrane stabilization and in the regulation of enzyme synthesis, e.g. proteinkinases or phosphatases. Disturbance of these processes causes cell and membrane degradation as a consequence of oxidative processes. On the other hand, it has been shown that exogenous application of Ca^{2+} can improve plant

growth under environmental stress. Besides, the function of calcium is clearly reflected by stabilization of the middle lamella as calcium pectate for strengthening the cell walls and plant tissue (Marschner 1993).

It has been demonstrated that extracellular calcium availability, calcium influx and/or intracellular calcium mobilization is necessary for accumulation of secondary metabolites (Merillon et al. 1991, Mahady and Beecher 1994, Preisig and Moreau 1994, Ishihara et al. 1996). Corchete et al. (1991) and Cacho et al. (1995) found that the elimination of calcium ions from undifferentiated cultures of *Digitalis thapsi* promoted the accumulation of cardenolides. However, contradictory reports have also appeared in the literature. Thus, calcium negatively affects the production of some alkaloids (Sierra et al. 1991, Indrayanto et al. 1993, Moreno-Valenzuela et al. 2003), cardenolides (Hagimori et al. 1983, Corchete et al. 1991, Cacho et al. 1995, 1999b, Paranhos et al. 1999) and, as mentioned above, flavonolignans (Cacho et al. 1999a).

1.1.2.1.2.4 Mg Levels

Mg also acts as activator or regulator of several kinases, ATPases, RUBP carboxylase/oxygenase and several other enzymes of carbohydrate metabolism (Marschner 1995). Thus, deficiency of Mg is likely to decrease the efficiency of Calvin cycle, reduce the utilization of reductive power (NADPH) and cause over-saturation of photosynthetic electron transport system. Under such highly reduced condition, electrons could pass-on to O_2 generating $O_2^{\cdot -}$ and other reactive oxygen species (ROS). Enhanced activities of antioxidative enzymes and concentrations of antioxidant molecules have been reported earlier in Mg deficient bean (Cakmak and Marschner 1992, Cakmak 1994), mentha (Candan and Tarhan 2003) and maize

(Tewari et al. 2004) plants. Moreover, Mg-deficient mentha (Candan and Tarhan 2003) and maize (Tewari et al. 2004) plants have also been shown to accumulate significantly higher amount of malondialdehyde (MDA, an index of lipid peroxidation). Putrescine (Put) accumulation was also reported in response to K and Mg deficiencies (Murthy et al. 1971, Smith 1973, Basso and Smith 1974).

1.1.2.2 Temperature Stress

Temperature stress is known to cause many physiological, biochemical and molecular changes in plant metabolism and alter the secondary metabolite production in plants possibly. A temperature range of 17–25 ^{o}C is normally used for the induction of callus tissues and growth of cultured cells. However, each plant species may favour a different temperature. Toivonen et al. (1992) found that lowering the cultivation temperature increased the total fatty acid content per cell in dry weight. When the temperature was maintained at 19 ^{o}C, biotransformation of digitoxin to digoxin is favoured, whereas 32 ^{o}C favours purpureaglycoside A, formation in *Digitalis lanata* Ehrh. cell cultures (Kreis and Reinhard 1992). Ikeda et al. (1977) observed a higher yield of ubiquinone in tobacco cell cultures at 32 ^{o}C when compared to either 24 or 28 ^{o}C. Courtois and Guren (1980) reported a 12-fold higher production of crude alkaloids in cell cultures of *C. roseus* at 16 ^{o}C as compared to the cultures grown at 27 ^{o}C.

1.1.2.3 UV Stress

Ultraviolet radiation is one important factor that in many cases stimulates the production of secondary metabolites. Secondary metabolites have often been obtained in callus and cell cultures when UV-B (radiation of wavelength 280–315 nm) or UV-C (wavelength <280 nm; usually mostly 254 nm) radiation has been

applied. Recent studies reported an increase of phenolic compounds and corresponding antioxidant activity in berry fruits, i.e. *Ribes nigrum* L., resulting from an UV-B mediated stress response (Huyskens-Keil et al. 2007). Duval et al. (2000) observed that exposure to UV-C effected the total phenolic content of *in Chlamydomonas nivalis* (Bauer) Wille cells.

1.1.2.4 Oxidative Stress

Some researchers have found that exogenous application of H_2O_2 is capable of inducing secondary metabolite formation. It was found that in cell suspensions and root cultures of *Uncaria tomentosa* (Willd. Ex Schult.) DC., the alkaloids production increased by 56% after addition of 200–1000 mM H_2O_2 compared to the control (Huerta-Heredia et al. 2009).

1.1.2.5 Chemical Stress

Heavy metals are defined as a group of elements that have specific weights higher than about 5g/cm^3. A number of them (Co, Fe, Mn, Mo, Ni, Zn, Cu) are essential micronutrients and are required for normal growth and take part in redox reactions, electron transfers and other important metabolic processes in plants. Metals which are considered nonessential (Pb, Cd, Cr, Hg etc.) are potentially highly toxic for plants (Sebastiani et al. 2004, Rai et al. 2004). Large areas of land are contaminated with heavy metals (the main group of inorganic contaminants) resulting from urban activities, agricultural practices and industry (Khan et al. 2000, Clemens 2001). Excessive concentrations of trace elements (Cd, Co, Cr, Hg, Mn, Ni, Pb and Zn) are toxic and lead to growth inhibition, decrease in biomass and death of the plant (Zenk 1996). Heavy metals inhibit physiological processes such as respiration, photosynthesis, cell elongation, plant-water relationship, N metabolism and mineral

nutrition. Secondary metabolism is also known to be affected by the presence or absence of heavy metals, as they may be responsible for activation of some of the biosynthetic pathways.

Copper (Cu) is an essential plant micronutrient that is important for the functioning of several proteins, including copper-zinc superoxide dismutase, ascorbate oxidase, polyphenol oxidase, cytochrome oxidase and plastocyanin. Cu is a redox active metal and catalyses the formation of reactive free oxygen radicals. Consequently, it increases the rate of lipid peroxidation and disintegration of plant cell membranes (Clijsters et al. 1999). Cu was shown to decrease photosynthetic yield through damage to the photosynthetic apparatus at the thylakoid level and consequently interfere with the photosystems (Clijsters et al. 1999) and enhance photoinhibition (Pätsikkä et al. 1998).

Also mercury (Hg) is a highly toxic, nonessential, persistent, immutable and non-biodegradable metal that undergoes many changes during transfer through different trophic levels of the food chain. Hg occurs naturally in the environment and exists in several forms such as metallic mercury, inorganic mercury and organic mercury. Toxic effects of mercury in plants include abscission of older leaves, growth reduction, and decreased vigor inhibition of root and leaf development, decreased chlorophyll content and nitrate reductase activity (Vyas and Puranik 1993). Other adverse effects caused by excessive mercury include membrane structure integrity disruption (Ma 1998), mineral nutrient uptake reduction (Cho and Park 2000, Patra and Sharma 2000) and photosynthesis and transpiration reduction (Krupa and Baszynski 1995).

Generation of reactive oxygen species (ROS) is the major response of plants to toxic concentrations of heavy metals including Cu and Hg (Schützendübel and Polle 2002, Maksymiec and Krupa 2002). One week old bean seedlings pigment analyses on the leaves revealed that the mercury, copper, cadmium and lead led to a decrease in chlorophyll a, chlorophyll b, total pigment I and total pigment II contents while increasing the carotenoid contents (Zengin and Munzuroglu 2005). Abou Zeid (2002) reported that the antifungal compounds produced in the leaves of *Corchorus olitorius* L. in response to treatment with $HgCl_2$ and $CuCl_2$.

1.2. Antioxidants

At some stage of stresses exposure, a common consequence of most biotic and abiotic stresses is that they result in an increased production of reactive oxygen species (Polle and Renenberg 1993) such as $O^{2\cdot-}$ and H_2O_2 in plant tissues. ROS are highly active molecules that can easily damage membrane and other cellular components. For a long time, ROS have been considered mainly as dangerous molecules, whose levels need to be kept as low as possible. Now, this opinion is changing rapidly. It has been realized that ROS play important roles in plant defense system and act as intermediate signaling molecules to regulate the expression of genes (May et al. 1998, Karpinski et al. 1999, Neill et al. 2002, Vranová et al. 2002). The control of oxidant levels is achieved by antioxidative systems. Antioxidation systems of the cell include the enzymatic ROS-scavenging system and non-enzymatic antioxidants. Scavenging mechanisms for ROS involve these enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and glutathione reductase (GR) (Payton et al. 2001).

Non-enzymatic antioxidants include pigments, vitamin E, phenols, flavanoids, tocopherol, ascorbic acid (AsA), and glutathione (GSH) (Wingsle and Hallgren 1993).

1.2.1 Enzymatic Antioxidants

The enzymatic defense against the reactive oxygen species is essential for plants under biotic or abiotic stress. The antioxidative enzymes, superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.1.1.11), glutathione reductase (GR; EC 1.6.4.2), and other ascorbate glutathione cycle enzymes (monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1)) catalyze the synthesis, degradation, and recycling of antioxidant molecules and can directly catalyze the removal of free radicals from the cells. ROS scavenging enzymatic antioxidants and reactions catalyzed by these enzymes are given in Table 6.

Table 6. Major ROS scavenging antioxidant enzymes.

Enzymatic antioxidants	**Reactions catalyzed**
Superoxide dismutase (SOD)	$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow 2H_2O_2 + O_2$
Catalase (CAT)	$H_2O_2 \rightarrow H_2O + ½\ O_2$
Ascorbate peroxidase (APX)	$H_2O_2 + AA \rightarrow 2H_2O + DHA$
Guaicol peroxidase (GPOX)	$H_2O2 + GSH \rightarrow H_2O +$ GSSG
Monodehydroascorbate reductase (MDHAR)	$MDHA + NAD(P)H \rightarrow AA + NAD(P)^+$
Dehydroascorbate reductase (DHAR)	$DHA + 2GSH \rightarrow AA +$ GSSG
Glutathione reductase (GR)	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$

1.2.1.1 Superoxide Dismutase (SOD)

Metalloenzyme SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. It is well established that various environmental stresses often lead to the increased generation of ROS, where, SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. The SODs remove $O_2^{\cdot -}$ by catalyzing its dismutation, one $O_2^{\cdot -}$ being reduced to H_2O_2 and another oxidized to O_2 (Table 6.). It removes $O_2^{\cdot -}$ and hence decreases the risk of $OH^{\cdot}$ formation via the metal catalyzed HabereWeiss-type reaction. This reaction has a 10,000 fold faster rate than spontaneous dismutation. SODs are classified by their metal cofactors into three known types: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD), which is localized in different cellular compartments (Mittler 2002).

1.2.1.2 Catalases (CAT)

CATs are tetrameric heme containing enzymes with the potential to directly dismutate H_2O_2 into H_2O and O_2 (Table 6.) and is indispensable for ROS detoxification during stressed conditions (Garg and Manchanda 2009).

CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert ≅ 6 million molecules of H_2O_2 to H_2O and O_2 per minute. CAT is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration and purine catabolism.

1.2.2 Non enzymatic Antioxidants

The non-enzymatic defense mechanisms include glutathione, ascorbate (vitamin C), carotenoids, α-tocopherol (vitamin E), proline, and various phenylpropanoid derivatives (phenolic compounds) such as flavonoids, lignans, tannins, and lignins.

1.2.2.1. Proline (Pro)

Osmoprotectants can be defined as low molecular weight organic compounds that are used by cells to maintain turgor pressure and cell volume, especially under water stressed conditions. In addition, under stress conditions these compounds also play an important role in maintaining the structural integrity of enzymes, membranes, hormones and other cellular components. A major category of osmoprotectans consists of simple sugars (mainly glucose, fructose), sugar alcohols (glycerol, methylated inositols, mannitol, sorbitol) and complex sugars (trehalose, raffinose and fructans), others include quaternary aminoacid dervatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines (ectione) and sulfonium compounds (choline-o-sulfate). Proline accumulation has been shown in different abiotic stressed plants. It has been suggested that proline protects plants by functioning as a cellular osmotic regulator between cytoplasm and vacuole, and by detoxifying of ROS, thus protecting membrane integrity and stabilizing antioxidant enzymes (Sharp et al. 1990, Bandurska 1993, Bohnert and Jensen 1996).

1.2.2.2. Phenolics

Phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. They are mainly synthesized from cinnamic acid, which is formed from phenylalanine by the action of L-phenylalanine ammonia-lyase PAL (EC

4.3.1.5), the branch point enzyme between primary (shikimate pathway) and secondary (phenylopropanoid) metabolism (Dixon and Paiva 1995). Phenols are divided into several different groups, distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (simple phenols, benzoic acids, phenylopropanoids and flavonoids) (Chaudiere and Ferrari-Iliou 1999, Rice-Evans and Miller 1997, Harborne 1964).

Phenolics have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions (Diáz et al. 2001, Sakihama and Yamasaki 2002, Grace and Logan 2000, Lavola et al. 2000). The synthesis of isoflavones and some other flavonoids is induced when plants are infected or injured (Takahama and Oniki 2000, Ruiz et al. 2003), or under low temperatures and low nutrient conditions (Sakihama and Yamasaki 2002, Ruiz et al. 2003). Most of them have antimicrobial activity. Plants accumulate UV absorbing flavonoids and other phenolic compounds mainly in vacuoles of epidermal cells to prevent the penetration of UV-B into the deeper tissues of the plant (Kondo and Kawashima 2000). Flavonoids can be classified into flavonols, flavones, isoflavones, and anthocyanins based on their structure. Flavonoids are suggested to have many functions like flowers, fruits, and seed pigmentation, protection against UV light, defense against phytopathogens (pathogenic microorganisms, insects, animals), role in plant fertility and germination of pollen and acting as signal molecules in plant-microbe interactions (Michalak 2006).

1.3. *Digitalis* L.

The genus *Digitalis*, commonly known as the "foxglove," is a member of the Plantaginaceae. The name *Digitalis* is Latin for "finger of a glove," which refers to the shape of the flowers. All *Digitalis* species are biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are often crowded in basal rosettes. Flowers are zygomorphic and arranged in terminal, bracteate racemes, and vary in color with species, from purple to pink, white, and yellow. 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 (Bräuchler et al. 2004). Several *Digitalis* species are used therapeutically, as they are the main source of cardiac glycosides and most of them are of great ornamental value. The therapeutic action of cardiac glycosides depends on the structure of the aglycone and on the type of sugar (or sugar chain) attached to it. Two types of aglycones are known, namely the cardenolides, bearing an α,β-unsaturated five-membered lactone ring (butenolide) at C-17β, and the bufadienolides, with a sixmembered lactone ring (cumaline). The stereochemistry of these compounds is important for their biological activity. The typical *Digitalis* cardenolides (e.g., digoxin, digitoxin, methyldigoxin) are characterized by a steroid nucleus with its rings connected cis–trans–cis, possessing a 14β-hydroxyl group, at position 3β 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. More than 100 different cardenolides have been isolated from *Digitalis* species of which all have been analyzed for the occurrence of this important group of plant secondary metabolites (Clemente et al. 2001).

Cardenolides are formed by condensation of the C_{21} pregnane derivate with acetate. Progestrone is assumed to be a precurcor of cardenolides and it has been established that *Digitalis* plants are capable of transforming progesterone into cardenolides. Cholesterol is also transformed into cardenolides by *Digitalis* plants and assumed to be more distant precursor of cardenolides. The biosynthetic pathway leading from phytosterol precursors to the cardiac was basically deduced from studies using radiolabelled precursors. The more recent identification and characterization of several enzymes/genes involved in pregnane and cardenolide metabolism, such as 3β-hydroxysteroid dehydrogenase and progesterone 5β-reductase.

Reviews on the botany of foxglove date back to the 1950–1965 (Ivanina 1955, Werner 1960, 1965). Botanists, at that time, were supporting the well-known nomenclature and taxonomy of Werner (1965, 1966) who described 19 species of *Digitalis* and three species of *Isoplexis*. Currently, the genus *Digitalis* comprises of 23 species including the four species of the former genus *Isoplexis* (Bräuchler et al. 2004). In Turkey, the genus *Digitalis* is represented by 9 taxon belonging to 8 species as follows; (1) *D. cariensis*, (2) *D. davisiana*, (3) *D. ferruginea* L. subsp. *ferruginea* L., (4) *D. ferruginea* L. subsp. *schischkinii* (Ivanina) K. Werner, (5) *D. grandiflora* Mill., (6) *D. lamarckii*, (7) *D. lanata*, (8) *D. trojana* and (9) *D. viridiflora* Lindl. Besides, four species of which are currently present have been used in this thesis (*D. trojana*, *D. davisiana*, *D. cariensis* and *D. lamarckii*) and, in fact, are endemic to Turkey (Davis 1978). (Figure 2).

D. lamarckii

D. trojana

D. davisiana

D. cariensis

Figure 2. Endemic *Digitalis* species to Turkey.

CHAPTER 2.

MATERIAL AND METHODS

2.1. Plant Material

Seeds of four endemic *Digitalis* species (*D. lamarckii*, *D. trojana*, *D. davisiana*, *Digitalis cariensis*) were gathered in early September in 2010. Seeds of *D. trojona* were collected from National Park of Ida Mountains ($N39^0$ 38.885', $E026^0$ 57.402', 01.09.2010 Leg. Eker 2691 Det. İ.Eker 2010), seeds of *D. cariensis* ($N36^0$ 30.767', $E032^0$12.695', 03.09.2010 Leg. Eker 2692, Det. İ.Eker 2010) and *D. davisiana* were collected from Alanya-Mahmutlar ($N36^0$ 31.916', $E032^0$14.402', 03.09.2010, Leg. Eker 2693, Det. İ. Eker 2010) and seeds of *D. lamarckii* were collected from around Ankara-Kızılcahamam ($N40^0$37.709', $E032^0$26.265', 05.09.2010, Leg. Eker 2694, Det. İ. Eker 2010).Voucher specimen of the species were deposited in herbarium of AIBU.

2.2. Experimental Design

For germination, the seeds were cultured on MS medium including vitamins (Duchefa, Prod. No: M0222.0050) containing 3% sucrose and 0.8% agar (pH 5.7-5.8). In order to obtain callus, hypocotyl explants excised from germinated seedlings were cultured on MS medium including vitamins containing different auxin and cytokinin concentrations (0.5 ppm TDZ and 0.25 ppm IAA) (Gurel et al. 2011). After

30 days of culture, different sources of stresses were applied to the callus in order to expose the callus cultures to the stress conditions (media strength, macronutrient deficiency, oxidative, chemical, temperature and UV) (Figure 3).

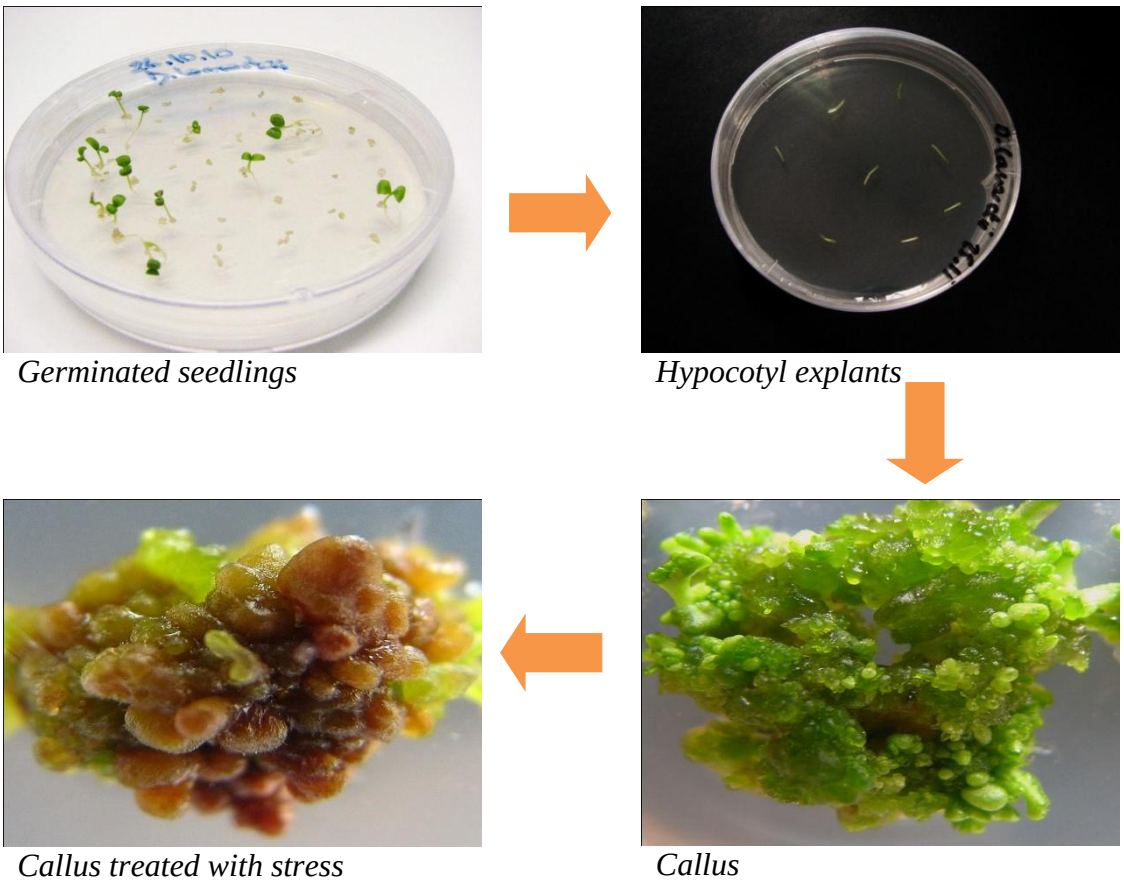

Figure 3. Experimental design.

2.3. Stress Conditions

Different strenghts of MS medium were used and some of macronutrients (Ca or Mg) in the medium were completely eliminated for creating *nutrient stress* and callus cultures were incubated for 15 days. For *oxidative stress* 10 mM H_2O_2 was used and developing callus cultures incubated for 6 hours. For control groups, subcultures which received no H_2O_2 were used. Callus cultures were held in 45 ^{0}C for 2 hours in order to expose the cells to *temperature stress*. Control groups grown at 25 ^{0}C. 50

µm $HgCl_2$ and 50 µm $CuCl_2$ were used in cultures for 10 days as *chemical stress*. To create *UV stress*, callus cultures were exposed to 254 nm UV ray for 10 min.

Following the incubation period of stress-treated calli, they were kept in –80 ^{0}C deep-freeze until they were used for cardenolide analysis by HPLC and total phenolics, proline, SOD and CAT by spectrophotometer.

2.4. Total Phenolic Assay

Total phenolic content of extracts was determined using a modified Folin-Ciocalteau spectrophotometric method (Marigo 1973). 20 µl extract was placed in a reaction test tube in which 1.58 ml of water and 100 µl of Folin-Ciocalteau reagent were added. The test tube was allowed to stand for 5 min, and then 300 µl 20% Na_2CO_3 was added into the tube. After 20 min at 40 ^{0}C, absorbance was measured at 750 nm. Total phenolic content was expressed as µg gallic acid (GA) equivalents/g dry weight.

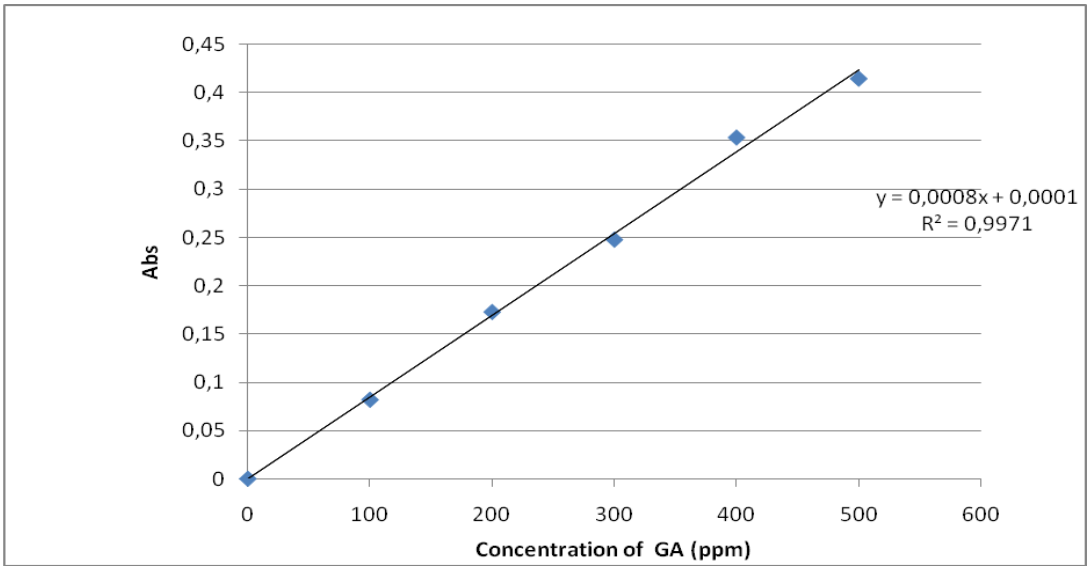

Figure 4. Standard curve of gallic acid (ppm).

2.5. Determination of Proline Content

Proline was determined according to the method of Bates et al. (1973). Plant callus tissues (0.5 g) were extracted with 5 ml 3% sulfosalicylic acid and centrifuged at 5000 rpm min^{-1} for 20 min. A 2 ml sample of the supernatant was reacted with 2 ml of ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100 ^{o}C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene and mixed vigorously with a test tube stirrer for 15–20 s. The chromophore containing toluene was extracted from the aqueous phase, then warmed to room temperature. Proline content was measured by a spectrophotometer at 520 nm and calculated as µmol g^{-1} dw against standart proline as below:

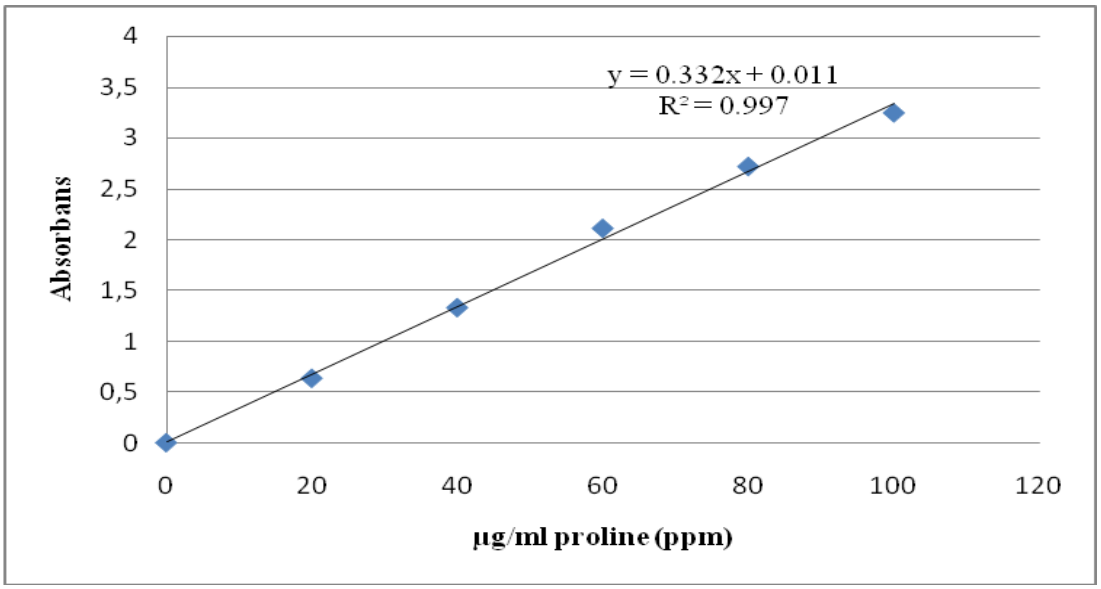

Figure 5. Standard curve of proline (ppm).

2.6. Extraction Method for Enzyme Assays

Tissue extraction of the samples was prepared for the analyses by homogenizing 0.1 g of callus material in 2 ml of ice cold 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na–EDTA and 1% (w/v) polyvinyl–polypirrolidone (PVP). The

homogenate was centrifuged at 12000 rpm min^{-1} and 4 ^{0}C for 10 min. Afterwards, tissue extract was stored at -80 ^{0}C, or immediately used for subsequent analyses of SOD and CAT.

2.7. Lowry Method

The soluble protein content was determined by the Lowry method (Lowry et al. 1951). From each sample 0.2 ml was taken and put into a test tube. 2 ml of alkaline copper sulphate reagent was added to the extract. After 10 min of incubation at room temperature, the absorbances were measured at 660 nm by spectrophotometer against blank. For the standard curve of protein, Bovine Serum Albumin (BSA) was used at concentrations of 0, 50, 100, 200, 600, 800 mg/l, respectively.

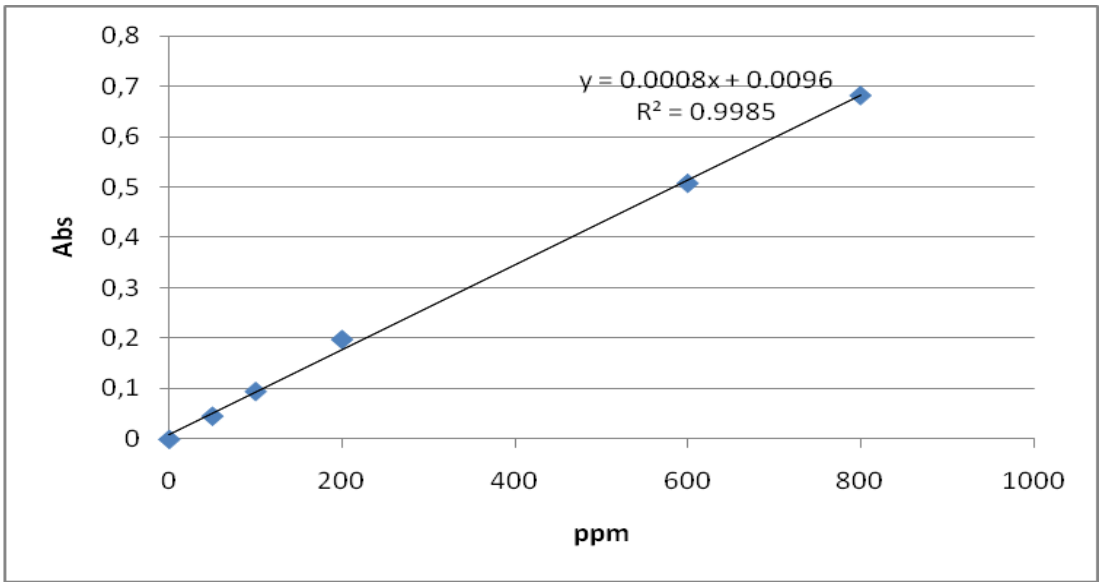

Figure 6. Standard curve of Bovine Serum Albumin (BSA).

2.8. Determination of CAT Activity

Catalase activity was determined according to the method of Lartillot et al. (1988). For CAT, the decomposition of H_2O_2 was followed by a decline in absorbance at 240 nm. 3 ml of reaction mixture was contained 50 mM phosphate buffer (pH 7.0) and 10

mM H_2O_2. Afterwards, 20 µl of enzyme extract was added into each tubes. The reaction was initiated by adding enzyme extract. CAT activity was determined by following consumption of H_2O_2 (extinction coefficient, 38.9 mM^{-1} cm^{-1}) at 240 nm over a 2 min interval. The reaction was terminated by adding 0.5 ml 1 M HCI solution (Ar). To detect initial absorbance of H_2O_2 (As); absorbance of solution containing 2.5 ml reaction mixture and 0.5 ml 1 M HCI was measured spectrophotometrically. In order to detect protein absorbance (At); absorbance of solution containing 20 µl enzyme extract 2.5 ml buffer and 0.5 ml 1 M HCI was measured spectrophotometrically. Absorbance change caused by enzymatic activity was calculated below:

$$A = (As + At) - Ar$$

Enzyme activity in terms of $Uml^{-1}/mg\ protein$ was calculated below:

$$Activity = (A \times Vt) / (\varepsilon \times t \times V_e) \times (1/(mg\ protein))$$

$V_t =$ $The\ total\ volume\ of\ reaction\ (ml)$

$V_e =$ $Volume\ of\ Enzyme\ extract\ (ml)$

$\varepsilon =$ $Extinction\ coefficient\ of\ H_2O_2$

$t =$ $Reaction\ time\ (min)$

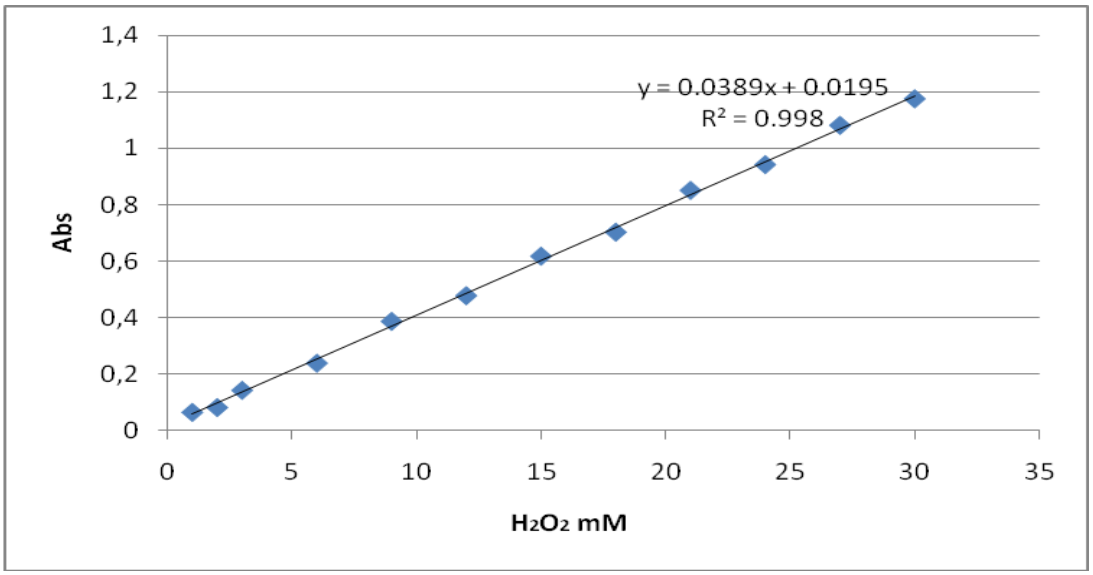

Figure 7. Standard Graphics of H_2O_2 extinction coefficient.

2.9. Determination of SOD Activity

SOD activity was determined according to the method of Sun et al. (1988). This assay for superoxide dismutase (SOD, EC 1.15.1.1) activity involved inhibition of nitroblue tetrazolium reduction, with xanthine-xanthine oxidase used as a superoxide generator. SOD assay reagent contained the following reagents in a 200 ml beaker, 40 ml of 0.3 mmol/l xanthine solution, 20 ml of 0.6 mmoI/l EDTA solution, 20 mL of 150 µmol/l nitroblue tetrazolium solution, 12 ml of 400 mmol/l Na_2CO_3 solution, and 6 ml of bovine serum albumin (1 g/l). 0.05 ml enzyme extract was added into the each test tubes containing 1.425 ml of the SOD assay reagent. Tubes were placed into a water bath adjusted to 25 ^oC. 0.025 µl of xanthine oxidase solution was put into the each tubes and incubated for 20 min, then the reaction was terminated by adding 0.05 ml of 0.8 mmol/l $CuCl_2$ solution to each tubes. The production of formazan was determined at 560 nm spectrophotometrically. Blank tubes containing 1.425 ml of the SOD assay reagent were placed into a water bath (25 ^oC) and then 0.025 µl of xanthine oxidase solution was addded into the each tubes and incubated

for 20 min. After, the reaction was terminated by adding 0.05 ml of 0.8 mmol/l $CuCl_2$ solution to each tubes. Finally, 0.05 ml enzyme extract was added into the each test tubes. In order not the results of this study to be affected by the absorbance which is caused by protein, these test tubes were read spectrophotometrically at 560 nm aganist distilled water. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The percent inhibition was calculated according to the formula below:

$$\%\ Inhibition = \frac{Absorbance\ blank - Absorbance\ sample \times 100}{Absorbance\ blank}$$

$$Activity\ (U/ml) = (\%\ Inhibition)/(50 \times 0.1)$$

$$Specific\ activity = (Activity\ (U/ml))\ /\ (mg/ml\ protein)$$

2.10. Extraction Method for Cardenolides

Extraction of cardenolides was determined according to the method of Wiegrebe and Witchtl (1993). Callus materials were ground in liquid nitrogen, then 50 mg of material was transferred to the centrifuge tube containing 1 ml 70 % methanol. After 15 min treatment in an ultrasonic bath at 70-75˚C, the extract was rapidly cooled on ice for 5 min and then centrifuged at 12000 rpm for 5 min. The supernatant was thoroughly mixed with 250 µl of lead acetate (15%) and centrifuged for 5 min at 12000 rpm. After elucidating the lead acetate residue, 500 µl of 4% NaH_2PO_4 was added and centrifuged again at 12000 rpm for 8 min. The supernatant was transferred into the 2 ml centrifuge tube, diluted to a final volume of 2 ml with water and mixed by vortex for 1 min. Then, the extract was divided into two 1.5 ml new tubes. Next, 500µl of chloroform:isopropanol (3:2) was added each tubes and were centrifuged

for 5 min at 12000 rpm. The lower phase were transferred into 2 ml centrifuge tubes as the first extraction. The remaining methanolic solutions were used for the second extraction by adding 500µl of chloroform. Then, the lower phases were taken to the tubes. It was called as second extraction. Afterwards, Na_2SO_4 was added to the tubes and mixed by hand and centrifuged at 12000 rpm for 1 min in order to remove water. Extractions were evaporated to dryness under gentle stream of nitrogen and finally the volume was completed to 0.5 ml with HPLC grade methanol.

2.11. HPLC Determination

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 HP model integrator (Agilent 1100 series, Germany). For columns, GL Sciences Inc. Inertsil ODS-3 (4.6 x 150 mm) for RP-HPLC was used throughout. A 20 µl aliquot of pretreated extract described above was analyzed with HPLC. For the calibration curves, concentrations of 5, 10, 20, 30 and 40 mg/l digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin were used (*R* values were 0.99 for digoxigenin, gitoxigenin, lanatoside C, digoxin and digitoxin) (Fig. 4). Cardenolides were eluted with acetonitrile (A) and water (B) gradients as follows: 0 to 20 min 20% (A), 80% (B); 20 to 23.40 min 30% (A), 70% (B); 23.40 to 30 min 25% (A), 75% (B) and 30 to 40 min 40% (A), 60%(B). Average peak area of the glycoside in samples was automatically calculated and monitored by ChemStation LC/MS software against that of standards. HPLC runs were repeated three times for every sample group.

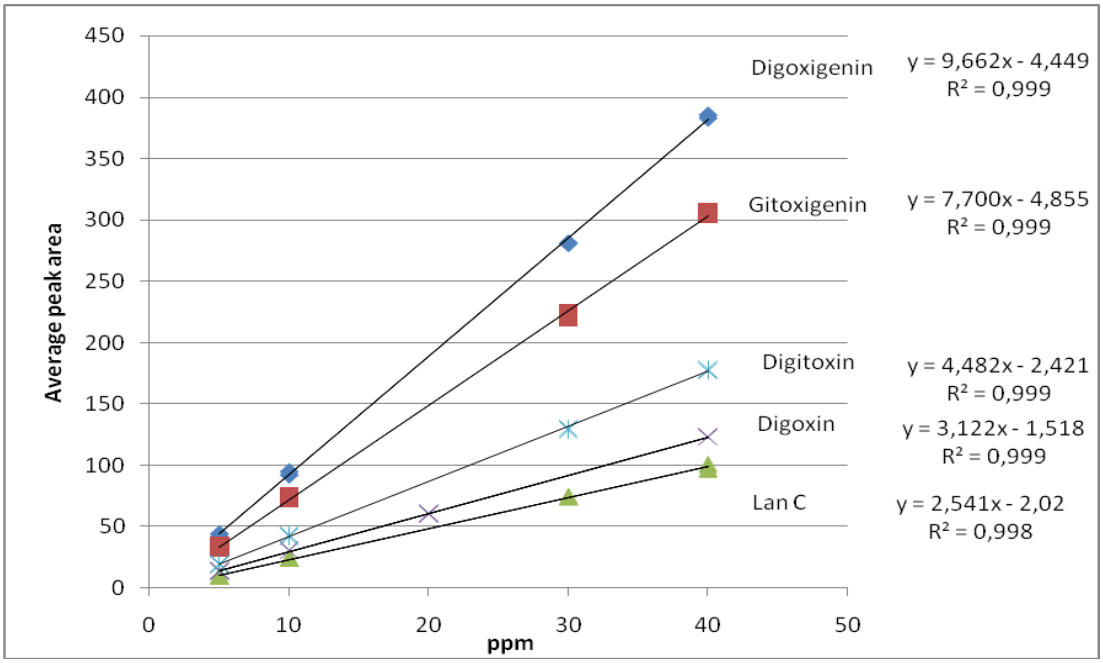

Figure 8**.** Calibration curves and R^2 values for each standard samples.

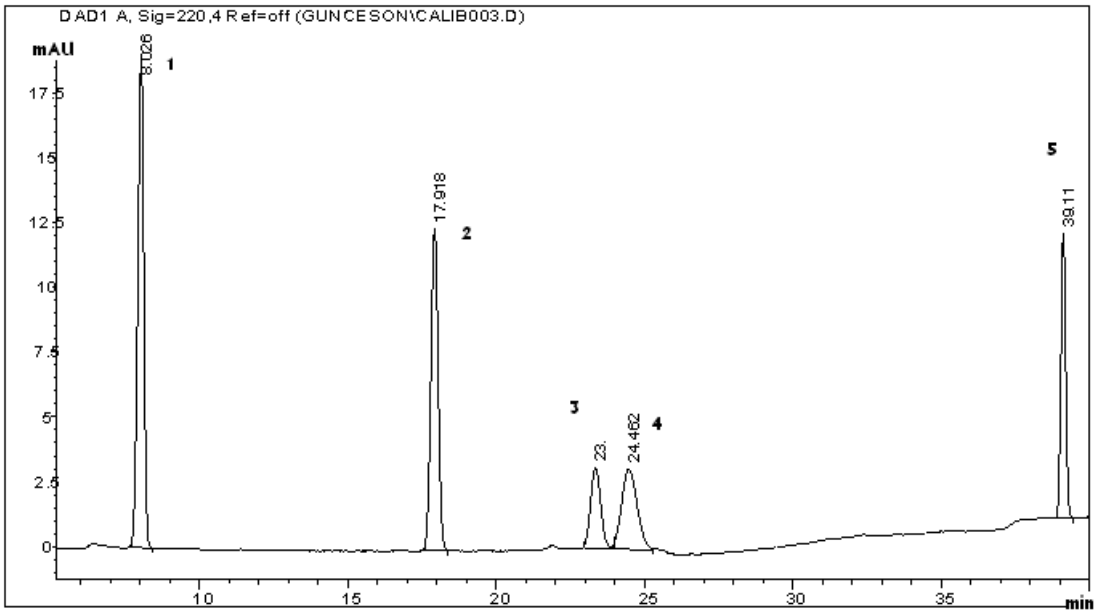

Figure 9**.** Typical HPLC pattern of reference samples (1: digoxigenin, 2: gitoxigenin, 3: lanatoside C, 4: digoxin, 5: digitoxin).

2.12. Limit of Detection (LOD)

To examine the capability of the systems for low concentrations, the detection limits were calculated for HPLC by ten replicates of 5 ppm standard solutions. Table 7. shows the limit of detection values (LOD), retention times (RT) and peak areas of standards.

Table 7**.** Limit of detection values of standards.

Cardenolides	RT±SD	Area±SD	LOD (ppm)
Digoxigenin	8.325±0.0343	27.294±0.902	0.29
Gitoxigenin	18.554±0.323	27.504±0.869	0.35
Lanatoside C	22.7316±0.860	4.778±0.783	0.98
Digoxin	25.308±0.937	5.747±0.927	0.95
Digitoxin	39.340±0.070	15.256±0.832	0.59

2.13. Statistical Analysis

The measurements were made in triplicate and they were statistically evaluated using SPSS and Duncan's multiple range test ($P \leq 0 \pm 05$) to determine whether statistically significant differences existed among treatments and control at a given time.

CHAPTER 3.

RESULTS

3.1. Cardenolide Production

The effects of different strength of MS media (Murashige and Skoog 1962), UV stress (254 nm, 10 min), chemical stress (50 μm $HgCl_2$, 50μm $CuSO_4$), macronutrient stress (Ca and Mg deficiency), temperature stress (45 ^{0}C, 2h), oxidative stress (10 mM H_2O_2, 6h) on cardiotonic glycoside accumulation in *D. lamarckii*, *D. trojana, D. davisiana* and *D. cariensis* were investigated. All stress conditions were significantly effective at 5% significance level as compared to the related control group (see Tables 8 through 11).

The predominant cardiac glycoside was lanatoside C (Lan C) followed by digitoxin, digoxigenin, gitoxigenin and digoxin. The assay results obtained for 4 *Digitalis* species indicated that the average content of lanatoside C of control groups per 1 g of the callus powder was 276.26 μg/g dw for *D. lamarckii*, 231.17 μg/g dw for *D. trojana*, 256.65 μg/g dw for *D. davisiana* and 274.1 μg/g dw for *D.cariensis*, with excellent reproducibility.

On the other hand, in all treatments as well as in control groups, there was no digoxin detected.

3.1.1. Cardenolide Production for *D. lamarckii*

The observed measurements for cardenolides of the *Digitalis lamarckii* are represented in Table 8. The predominant cardiac glycoside was Lan C followed by digitoxin, digoxigenin, gitoxigenin and digoxin. There was not any digoxin detected in all treatments as well as in control groups.

It should be pointed out that, although Ca and Mg elimination stimulated the cardenolide accumulation, the cardenolide yield was very low in control group. Deficiency of both Ca and Mg from the medium was followed by a significant increase of Lan C (1962.4±7.84 µg/g dry weight, dw). The control (non-treated) callus produced 251.2±4.62 µg/g dw Lan C while those cultured on medium lacking either Ca or Mg producing 883.66±9.71 µg/g dw or 561.53±4.55 µg/g dw Lan C, respectively. The both deficiencies of these macronutrients resulted in the highest level of digitoxin (38.55±2.03µg/g dw), digoxigenin (9.74±0.26 µg/g dw) and gitoxigenin (7.68±0.40 µg/g dw).

A remarkable increase of Lan C (752.50±7.50 µg/g dw), digoxigenin (12.13±0.41 µg/g dw) and digitoxin (13.32±1.43 µg/g dw) content was prominent in high temperature applied callus when compared with the concentrations of Lan C (237.19±3.35 µg/g dw), digoxigenin (9.81±0.15 µg/g dw) and digitoxin (9.42±2.28 µg/g dw) under the condition of no stress conditions. There was not a significant change in gitoxigenin level at 5% significance level.

The application of UV stress was noted, with correlated increase of the amounts of Lan C (280.59±6.42 µg/g dw), digoxigenin (5.82±0.24 µg/g dw) and digitoxin (5.90±0.22 µg/g dw) to the levels of 453.42±6.30 µg/g dw, 13.73±2.68 and

9.76±1.17 µg/g dw, respectively. There was not any significant change in gitoxigenin level observed at 95% confidence level.

In callus that was subjected to oxidative stress, all cardenolides were greatly promoted. The control (non-treated) callus produced 273.62±6.45 µg/g dw Lan C while that cultured on under oxidative stress by H_2O_2 producing 551.80±2.39 Lan C. In addition to this, the concentration of digoxigenin was 5.82±0.58 µg/g dw, gitoxigenin was 6.33±1.41 µg/g dw and digitoxin was 8.51±0.05 µg/g dw under the condition of no stress. When 6 h of oxidative stress by H_2O_2 was applied, these concentrations revealed a remarkable increase, with the following results obtained: 10.03±0.66 µg/g dw, 8.53±0.10 µg/g dw and 16.29±5.17 µg/g dw for digoxigenin, gitoxigenin and digitoxin, respectively.

The use of $CuSO_4$ and $HgCl_2$ led to a drastic increase in the accumulation of Lan C in the chemical stress experiments. The control (non-treated) callus produced 302.04±7.49 µg/g dw Lan C while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 721.80±8.78 µg/g dw and 377.23±5.20 µg/g dw Lan C respectively. It is apparent that, at 5% significance level, there was not any significant change observed between control groups and digoxigenin, gitoxigenin and digitoxin content under chemical stress.

The level of Lan C in callus tissues cultivated on ½ strength MS was found to be 721.9±7.39 µg/g dw and to be 413.14±5.66 µg/g dw cultured onto ¼ strength MS medium compared to non-treated callus grown on MS (312.99±1.48 µg/g dw). The control (non-treated) callus produced 16.51±0.29 µg/g dw digitoxin while those cultured on ½ strength MS and on ¼ strength MS producing 31.46±0.63 µg/g dw and 29.80±0.88 µg/g dw digitoxin, respectively. The amount of digoxigenin 8.44±1.87

µg/g dw and gitoxigenin 9.97±1.31 µg/g dw exhibited a drastic increase when they were cultured on ½ strength MS medium. However, no significant change was observed when they were cultivated on ¼ strength MS medium.

Table 8**.** Analysis of five different cardenolides content of control (non-treated) and stress-treated callus tissues of *D. lamarckii*.

D. lamarckii						
Types of stress	**Stress factor**	**Amount (µg/g dw)**				
		Digoxigenin	**Gitoxigenin**	**Lan C**	**Digoxin**	**Digitoxin**
Media strength stress	**Control**	**5.66^{f}±0.54**	**5.69^{d}±0.16**	**312.99^{i}±1.48**	<LOD	**16.51^{d}±0.29**
	½ MS	**8.44^{de}±1.87**	**9.97^{a}±1.31**	**721.9^{d}±7.39**	<LOD	**31.46^{b}±0.63**
	¼ MS	**6.06^{f}±1.20**	**6.20^{d}±1.35**	**413.14^{g}±5.66**	<LOD	**29.80^{b}±0.88**
Macronutrients stress	**Control**	**7.37^{ef}±0.34**	**5.82^{d}±0.24**	**251.20^{l}±4.62**	<LOD	**11.24^{ef}±0.78**
	-Ca	**9.5^{cd}±0.45**	**6.38^{d}±0.35**	**883.66^{b}±9.71**	<LOD	**32.10^{b}±0.53**
	-Mg	**9.07^{de}±0.20**	**6.63^{cd}±0.26**	**561.53^{e}±4.55**	<LOD	**23.47^{c}±3.92**
	-Ca -Mg	**9.74^{cd}±0.26**	**7.68^{bc}±0.40**	**1962.4^{a}±7.84**	<LOD	**38.55^{a}±2.03**
Oxidative stress	**Control**	**5.82^{f}±0.58**	**6.33^{d}±1.41**	**273.62^{k}±6.45**	<LOD	**8.51^{fg}±0.05**
	H_2O_2	**10.03^{cd}±0.66**	**8.53^{b}±0.10**	**551.80^{e}±2.39**	<LOD	**16.29^{d}±5.17**
Chemical stress	**Control**	**9.04^{de}±0.62**	**6.46^{d}±0.36**	**302.04^{j}±7.49**	<LOD	**8.67^{fg}±0.49**
	$CuSO_4$	**11.41^{bc}±1.96**	**6.41^{d}±0.51**	**721.80^{d}±8.78**	<LOD	**11.83^{ef}±3.78**
	$HgCl_2$	**10.37^{bcd}±0.41**	**6.49^{d}±0.57**	**377.23^{h}±5.20**	<LOD	**9.27^{efg}±2.08**
UV stress	**Control**	**5.82^{f}±0.24**	**5.73^{d}±0.17**	**280.59^{k}±6.42**	<LOD	**5.90^{g}±0.22**
	UV	**13.73^{a}±2.68**	**5.83^{d}±0.02**	**453.42^{f}±6.30**	<LOD	**9.76^{efg}±1.17**
Temperature stress	**Control**	**9.81^{cd}±0.15**	**6.16^{d}±0.02**	**237.19^{m}±3.35**	<LOD	**9.42^{efg}±2.28**
	Temp.	**12.13^{ab}±0.41**	**6.05^{d}±0.33**	**752.50^{c}±7.50**	<LOD	**13.32^{de}±1.43**

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different ($P<0.05$)

3.1.2. Cardenolide Production for *D. trojana*

The results comprised of the cardenolides of *D. trojana* investigated are in Table 9. The predominant cardiac glycoside was Lan C followed by digitoxin, digoxigenin, gitoxigenin and digoxin. None of digoxin was detected in the treatments as well as control groups.

Similar to *D. lamarckii*, although the cardenolide yield was very low in control group, Ca and Mg elimination enhanced the cardenolide accumulation. When both Ca and Mg were eliminated from the medium, the level of Lan C increased significantly (1344±6.97 µg/g dw). 225.63±7.58 µg/g dw Lan C was produced in the control (non-treated) group compared to those cultured on medium lacking either Ca or Mg producing 890.83±9.6 µg/g dw or 562.56±7.00 µg/g dw Lan C, respectively. Moreover, the highest digitoxin (26.65±4.43 µg/g dw) and digoxigenin (8.64±0.38 µg/g dw) levels were detected when both Ca and Mg were eliminated from the medium. On the basis of data given below, at 95% confidence level, there was not a significant change in gitoxigenin level between control group and treated-group under macronutrient stress.

A great increase of Lan C, gitoxigenin and digitoxin content due to high temperature was measured as 979.16±8.09 µg/g dw, 15.48±0.73µg/g dw and 11.35±1.39 µg/g dw from the beginning levels of 287.52±4.50 µg/g dw, 8.52±2.09 µg/g dw and 8.39±0.83 µg/g dw, respectively. In the analysis between control groups and digoxigenin content under temperature stress, no significant difference was recorded.

The amount of Lan C (245.18±5.31 µg/g dw), digoxigenin (6.92±1.58 µg/g dw), digitoxin (12.63±1.11 µg/g dw**)** and gitoxigenin (6.21±0.25 µg/g dw) revealed a

remarkable increment and the following results were obtained after a UV treatment: 293.13±7.76 µg/g dw, 7.18±1.99, 22.50±2.08 and 8.58±0.39 µg/g dw, respectively.

There was a noticeable increase in all cardenolides of callus that were exposed to the oxidative stress. The control (non-treated) callus produced Lan C 159.51±2.34 µg/g dw while callus cultured under oxidative stress by H_2O_2 producing 229.80±4.48 µg/g dw Lan C. In addition, 6 h of oxidative stress by H_2O_2 was followed by a significant increase in the concentrations of digoxigenin, gitoxigenin and digitoxin from 13.09±0.41 µg/g dw, 5.81±0.26 µg/g dw and 18.51±1.88 µg/g dw to the levels of 16.58±0.10 µg/g dw, 6.92±0.24 µg/g dw and 29.09±1.47 µg/g dw, respectively.

The positive correlation between the accumulation of Lan C and $CuSO_4$ - $HgCl_2$ application was detected in chemical stress experiments. 285.63±2.57 µg/g dw Lan C was found in the control (non-treated) callus while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 619.83±7.23 µg/g dw and 346.37±9.47 µg/g dw Lan C, respectively. The amount of digoxigenin was recorded as 12.96±0.56 µg/g dw under the condition of no stress, whereas the level of digoxigenin significantly increased to 17.13±1.31 µg/g dw when $CuSO_4$ was applied on callus. On the other hand, no significant change was observed in digoxigenin content when $HgCl_2$ was exposed on callus. Furthermore, digitoxin content was examined and the following results were obtained: 7.48±0.50 µg/g dw for the control callus and 9.66±0.77 µg/g dw and 8.68±0.77 µg/g dw for the callus treated with chemical stress by $CuSO_4$ and $HgCl_2$, respectively. Although a slight increase was observed in gitoxigenin content under the exposure of $CuSO_4$ (7.62±1.13 µg/g dw), there was not any significant increase under the exposure of $HgCl_2$ (6.61±0.83 µg/g dw) compared to non-treated callus (6.12±0.13 µg/g dw).

Lower media strength had beneficial effects on cardenolide production. The increment of Lan C level was associated with the cultivation of callus on ½ strength MS medium (293.86±3.18 µg/g dw) and on ¼ strength MS medium (235.11±3.03 µg/g dw) in comparison with non-treated callus grown on MS medium (183.54±6.21 µg/g dw **)**. The control (non-treated) callus produced 20.92±1.33 µg/g dw digitoxin while those cultured on ½ strength MS and on ¼ strength MS producing 38.89±1.75 µg/g dw and 30.47±1.75 µg/g dw digitoxin, respectively. The amount of digoxigenin was 11.33±0.30 µg/g under the condition of no stress, but the level of digoxigenin enhanced to 14.25±0.31 µg/g and 12.30±0.54 µg/g when media strength stress applied by ½ MS and ¼ MS, respectively. The amount of gitoxigenin in a drastic increase when the callus was cultured on ½ strength MS medium (7.66±2.35 µg/g dw). On the contrary, no significant change was recorded when it was cultivated on ¼ strength MS medium (6.68±0.44 µg/g dw) compared to non-treated callus (6.15±0.12 µg/g dw).

Table 9**.** Analysis of five different cardenolides content of control (non-treated) and stress-treated callus tissues of *D. trojana*.

D. trojana						
Types of stress	**Stress factor**	**Amount (µg/g dw)**				
		Digoxigenin	**Gitoxigenin**	**Lan C**	**Digoxin**	**Digitoxin**
Media strength stress	**Control**	**11.33^{d}±0.30**	**6.15^{cd}±0.12**	**183.54^{j}±6.21**	<LOD	**20.92^{de}±1.33**
	½ MS	**14.25^{b}±0.31**	**7.66^{bc}±2.35**	**293.86^{g}±3.18**	<LOD	**38.89^{a}±1.75**
	¼ MS	**12.30^{cd}±0.54**	**6.68^{cd}±0.44**	**235.11^{hi}±3.03**	<LOD	**30.47^{b}±1.75**
Macronutrients stress	**Control**	**7.07^{efg}±0.09**	**5.94^{cd}±0.21**	**225.63^{i}±7.58**	<LOD	**11.69^{fgh}±1.40**
	-Ca	**8.42^{ef}±0.14**	**6.45^{cd}±0.42**	**890.83^{c}±9.60**	<LOD	**19.33^{de}±1.43**
	-Mg	**8.36^{ef}±0.22**	**6.45^{cd}±0.20**	**562.56^{e}±7.00**	<LOD	**14.47^{f}±3.78**
	-Ca -Mg	**8.64^{e}±0.38**	**6.46^{cd}±0.50**	**1344^{a}±6.97**	<LOD	**26.65^{c}±4.43**
Oxidative stress	**Control**	**13.09^{cb}±0.41**	**5.81^{d}±0.26**	**159.51^{k}±2.34**	<LOD	**18.51^{e}±1.88**
	H_2O_2	**16.58^{a}±0.10**	**6.92^{bcd}±0.24**	**229.80^{i}±4.48**	<LOD	**29.09^{bc}±1.47**
Chemical stress	**Control**	**12.96^{bcd}±0.56**	**6.12^{cd}±0.13**	**285.63^{g}±2.57**	<LOD	**7.48^{i}±0.50**
	$CuSO_4$	**17.13^{a}±1.31**	**7.62^{bc}±1.13**	**619.83^{d}±7.23**	<LOD	**9.66^{gh}i±0.77**
	$HgCl_2$	**12.49^{cd}±1.10**	**6.61^{cd}±0.83**	**346.37^{f}±9.47**	<LOD	**8.68^{hi}±0.77**
UV stress	**Control**	**6.92^{fg}±1.58**	**6.21^{cd}±0.25**	**245.18^{h}±5.31**	<LOD	**12.63^{fg}±1.11**
	UV	**7.18^{efg}±1.99**	**8.58^{b}±0.39**	**293.13^{g}±7.76**	<LOD	**22.50^{d}±2.08**
Temperature stress	**Control**	**5.22^{h}±1.16**	**8.52^{b}±2.09**	**287.52^{g}±4.50**	<LOD	**8.39^{hi}±0.83**
	Temp.	**6.22^{gh}±1.14**	**15.48^{a}±0.73**	**979.16^{b}±8.09**	<LOD	**11.35^{fgh}±1.39**

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.1.3. Cardenolide Production for *D. davisiana*

The observed measurements for five cardenolides of *D. davisiana* investigated are given in Table 10. The predominant cardiac glycoside was Lan C followed by digitoxin, digoxigenin, gitoxigenin and digoxin. Any digoxin was not detected in the treatments and control groups.

With the elimination of both Ca and Mg from the medium, the amount of Lan C was increased to 843.95±3.37 μg/g dw. The control (non-treated) callus produced 274.417±2.74 μg/g dw Lan C while those cultured on medium lacking either Ca or Mg producing 463.28±5.48 or 317.08±2.55 μg/g dw Lan C, respectively. On the basis of the analysis, the highest digitoxin (28.80±0.20 μg/g dw), digoxigenin (11.16±0.39 μg/g dw) and gitoxigenin (15.95±1.06 μg/g dw) levels were estimated when both Ca and Mg discarded from the medium.

It is apparent that the callus exposed to the temperature stress was found to increase in the Lan C (447.70±3.14 μg/g dw), digoxigenin (9.14±0.55 μg/g dw) and digitoxin (16.25±0.49 μg/g dw) content from the levels of 266.27±1.11 μg/g, 7.69±0.51 μg/g dw and 9.65±1.69 μg/g dw , respectively, under the condition of no stress. There was not any significant difference observed in gitoxigenin content between non-treated and treated groups.

With the application of 10 min 254 nm UV on callus compared to non-treated group (262.10±7.90 μg/g dw), the amount of Lan C was significantly increased (397.54±3.85 μg/g dw). The amount of digoxigenin (8.68±0.27 μg/g dw), digitoxin (15.23±2.06 μg/g dw) and gitoxigenin (5.62±0.22 μg/g dw) sharply increased and the following results were found after UV treatment: 9.1±0.48 μg/g dw digoxigenin, 15.90±0.69 μg/g dw digitoxin and 6.24±0.24 μg/g dw gitoxigenin.

The exposure of oxidative stress gave rise to a marked increase in concentrations of cardenolides tested. The control (non-treated) callus produced Lan C 245.74±5.72 μg/g dw whereas callus cultured on under oxidative stress by H_2O_2 produced 469.09±9.49 μg/g dw Lan C. Besides, the concentration of digoxigenin was 6.97±0.13 μg/g dw, gitoxigenin was 6.89±0.17 μg/g dw and digitoxin was 9.79±0.15

μg/g dw under the condition of no stress. Moreover, it was also found that these values increased to 11.71±0.65 μg/g dw, 8.39±0.72 μg/g dw and 17.60±0.84 μg/g dw, respectively, when 6 h of oxidative stress by H_2O_2 was applied.

In chemical stress experiments, the accumulation of Lan C was significantly induced as a concequence of $CuSO_4$ and $HgCl_2$. Lan C of control was 259.85±8.17 μg/g dw while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 844.097±7.89 μg/g dw and 461.13±6.08 μg/g dw Lan C, respectively. Under the condition of no stress, the amount of digoxigenin was 9.56±0.10 μg/g dw and this value markedly increased to 18.99±0.17 μg/g dw in callus submitted to $CuSO_4$ and to 15.44±0.77 μg/g dw in callus submitted to $HgCl_2$. Moreover, in present study, the control (non-treated) callus produced 7.55±0.50 μg/g dw digitoxin whereas those cultured on under chemical stress by $CuSO_4$ producing 13.52±0.93 μg/g dw, and by $HgCl_2$ producing 9.33±0.31 μg/g dw. A slight increment was observed in gitoxigenin content while callus was treated with $CuSO_4$ (7.75±0.21 μg/g dw) and $HgCl_2$ (7.34±0.33 μg/g dw) compared to non-treated callus (5.16±0.2 μg/g dw).

According to our results, the level of Lan C was significantly increased with the cultivation of callus on ½ strength MS to 549.30±7.76 μg/g dw and on ¼ strength MS to 330.01±6.18 μg/g dw in comparison with non-treated callus grown on MS (231.50±7.50 μg/g dw). 16.40±1.48 μg/g dw digitoxin was produced in control group (non-treated) while those cultured on ½ strength MS and on ¼ strength MS produced 39.56±2.33 μg/g dw and 21.38±1.04 μg/g dw digitoxin, respectively. The amount of digoxigenin was 8.83±0.17 μg/g dw under the condition of no stress, but the increased amount of digoxigenin was 10.73±0.66 μg/g dw and 9.36±0.52 μg/g dw when media strength stress applied by ½ MS and ¼ MS, respectively. Besides, the

amount of gitoxigenin was rised when callus was cultured on ½ strength MS medium (9.59±0.53 µg/g dw) and on ¼ strength MS medium (7.80±0.16 µg/g dw) compared to non- treated callus (6.29±0.27 µg/g dw).

Table 10**.** Analysis of five different cardenolides content of control (non-treated) and stress-treated callus tissues of *D. davisiana.*

D. davisiana						
Types of stress	**Stress factor**	**Amount (µg/g dw)**				
		Digoxigenin	**Gitoxigenin**	**Lan C**	**Digoxin**	**Digitoxin**
Media strength stress	**Control**	**8.83^{efg}±0.17**	**6.29^{efgh}±0.27**	**231.50^{k}±7.50**	<LOD	**16.40^{de}±1.48**
	½ MS	**10.73^{cd}±0.66**	**9.59^{b}±0.53**	**549.30^{b}±7.76**	<LOD	**39.56^{a}±2.33**
	¼ MS	**9.36^{e}±0.52**	**7.80^{cde}±0.16**	**330.01^{f}±6.18**	<LOD	**21.38^{c}±1.04**
Macronutrients stress	**Control**	**6.23^{i}±2.17**	**7.63^{cdef}±2.10**	**274.417^{h}±2.74**	<LOD	**11.51^{ij}±0.58**
	-Ca	**9.11^{ef}±0.99**	**15.20^{a}±1.13**	**463.28^{d}±5.48**	<LOD	**13.77^{fgh}±1.26**
	-Mg	**7.82^{fgh}±0.50**	**8.91^{bc}±1.83**	**317.08^{g}±2.55**	<LOD	**12.91^{hi}±1.86**
	-Ca Mg	**11.16^{c}±0.39**	**15.95^{a}±1.06a**	**843.95^{a}±3.37**	<LOD	**28.80^{b}±0.20**
Oxidative stress	**Control**	**6.97^{hi}±0.13**	**6.89^{defg}±0.17**	**245.74^{j}±5.72**	<LOD	**9.79^{j}±0.15**
	H_2O_2	**11.71^{c}±0.65**	**8.39^{bcd}±0.72**	**469.09^{cd}±9.49**	<LOD	**17.60^{d}±0.84**
Chemical stress	**Control**	**9.56^{de}±0.10**	**5.16^{h}±0.20**	**259.85^{i}±8.17**	<LOD	**7.55^{k}±0.50**
	$CuSO_4$	**18.99^{a}±0.17**	**7.75^{cde}±0.21**	**844.097^{a}±7.89**	<LOD	**13.52^{ghi}±0.93**
	$HgCl_2$	**15.44^{b}±0.77**	**7.34^{cdef}±0.33**	**461.13^{d}±6.08**	<LOD	**9.33^{jk}±0.31**
UV stress	**Control**	**8.68^{efg}±0.27**	**5.62^{gh}±0.22**	**262.10^{i}±7.90**	<LOD	**15.23^{efg}±2.06**
	UV	**9.1^{ef}±0.48**	**6.24^{efgh}±0.24**	**397.54^{e}±3.85**	<LOD	**15.90^{def}±0.69**
Temperature stress	**Control**	**7.69^{gh}±0.51**	**6.09^{fgh}±0.03**	**266.27^{hi}±1.11**	<LOD	**9.65^{jk}±1.69**
	Temp.	**9.14^{ef}±0.55**	**6.14^{fgh}±0.19**	**447.70^{c}±3.14**	<LOD	**16.25^{de}±0.49**

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.1.4. Cardenolide Production for *D. cariensis*

The observed values of five cardenolides of *D. cariensis* investigated are presented in Table 11. The predominant cardiac glycoside was Lan C followed by digitoxin, digoxigenin, gitoxigenin and digoxin. In all treatments similar to control groups, no digoxin was detected.

It should be pointed out that, although Ca and Mg elimination gave rise to an enhancement in the cardenolide accumulation, the cardenolide yield was very low in comparison to control group. Lan C was significantly increased to 968.18±9.49 µg/g dw when both Ca and Mg were eliminated from the medium. The control (non-treated) callus produced 251.35±5.50 µg/g dw Lan C while those cultured on medium lacking either Ca or Mg producing 684.18±9.76 µg/g or 462.23±5.56 µg/g dw Lan C, respectively. The highest digitoxin (39.22±2.55 µg/g dw), digoxigenin (19.35±0.33 µg/g dw) and gitoxigenin (11.90±0.33 µg/g dw) levels were recorded with the deficiency of both macronutrients.

Callus subjected to high temperature showed a great increase in the formation of Lan C (575.06±5.84 µg/g dw), digoxigenin (11.57±0.90 µg/g dw), digitoxin (14.77±0.59 µg/g dw) and gitoxigenin (11.86±0.99 µg/g dw) while the beginning concentrations of Lan C, digoxigenin, digitoxin and gitoxigenin were 280.10±5.89 µg/g, 10.85±0.40 µg/g dw, 8.14±0.25 µg/g dw and 8.68±0.50 µg/g dw, respectively, under the condition of no stress.

A drastic increase in Lan C (401.58±7.40 µg/g dw) for callus submitted to 10 min 254 nm UV was observed where the level was 286.04±6.41 µg/g dw in non-treated group. After UV treatment, the amount of digoxigenin (10.82±0.24 µg/g dw), digitoxin (6.76±0.39 µg/g dw) and gitoxigenin (8.88±0.19 µg/g dw) sharply increased to 13.54±0.60 µg/g dw, 13.53±0.24 µg/g dw and 9.12±0.65 µg/g dw, respectively.

Exogenous H_2O_2 stimulated cardenolide production significantly. In this condition, a noticeable increase in the formation of Lan C (341.20±7.66 µg/g dw) was observed compared to the control group (261.11±5.12 µg/g dw). Moreover, H_2O_2 stimulated

the concentrations of digoxigenin (10.87±0.29 µg/g dw), gitoxigenin (7.65±0.59 µg/g dw) and digitoxin (12.11±0.67 µg/g dw) to the levels of 11.86±0.41 µg/g dw, 8.46±0.80 µg/g dw and 15.08±0.20 µg/g dw respectively.

$CuSO_4$ as well as $HgCl_2$ stress was followed by a significantly enhanced accumulation of Lan C in chemical stress experiments. The control (non-treated) callus produced 280.71±7.14 µg/g dw Lan C while those cultured on under chemical stress by $CuSO_4$ and $HgCl_2$ producing 939.21±9.09 µg/g dw and 673.23±6.58 µg/g dw Lan C respectively. The amount of digoxigenin under the condition of no stress (10.73±0.17 µg/g dw) rised to 14.09±0.59 µg/g dw and 13.07±0.43 µg/g dw under the exposure of $CuSO_4$ and $HgCl_2$, respectively. Digitoxin concentration under chemical stress by $CuSO_4$ increased to 16.13±0.54 µg/g dw and by $HgCl_2$ rised to 13.92±0.16 µg/g dw as compared with the control group (9.06±0.27 µg/g dw). A noticeable increase in the formation of gitoxigenin content was recorded while $CuSO_4$ (12.16±0.63 µg/g dw) and $HgCl_2$ **(**10.01±0.67 µg/g dw**)** were applied on callus, seperately, compared to non-treated callus (8.33±0.47 µg/g dw**)**.

Media strength significantly influenced the cardenolide production in callus cultures. In our studies, ½ MS strength (394.16±3.17 µg/g dw) and ¼ MS strength (342.71±7.83 µg/g dw) treated cultures obtained higher Lan C content than non-treated callus (285.29±6.46 µg/g dw). In addition, lower media strength had beneficial effects on digitoxin production. The control (non-treated) callus produced 12.35±0.87 µg/g dw while those cultured on ½ strength MS and on ¼ strength MS µg/g producing 38.89±1.75 µg/g dw and 31.37±1.82 µg/g dw, respectively. Moreover, in our experiment, it was also found that the amount of digoxigenin was 11.23±0.25 µg/g dw under the condition of no stress, but this level increased to

14.25±0.31 µg/g and to 13.86±0.48 µg/g dw when media strength stress applied by ½ MS and ¼ MS, respectively. Besides, the amount of gitoxigenin was increased when callus was cultured on ½ strength MS medium (11.03±0.59 µg/g dw) but no significant change was recorded when cultured on ¼ strength MS medium (6.68±0.44 µg/g dw) compared to non- treated callus (7.26±0.06 µg/g dw).

Table 11**.** Analysis of five different cardenolides content of control (non-treated) and stress-treated callus tissues of *D. cariensis*.

D. cariensis						
Types of stress	**Stress factor**	**Amount (µg/g dw)**				
		Digoxigenin	**Gitoxigenin**	**Lan C**	**Digoxin**	**Digitoxin**
Media strength stress	**Control**	**11.23^{def}±0.25**	**7.26^{f}±0.06**	**285.29^{h}±6.46**	<LOD	**12.35^{fg}±0.87**
	½ MS	**14.25^{b} ±0.31**	**11.03^{b}±0.59**	**394.16^{f}±3.17**	<LOD	**38.89^{a}±1.75**
	¼ MS	**13.86^{bc} ±0.48**	**6.68^{fg}±0.44**	**342.71^{g}±7.83**	<LOD	**31.37^{c}±1.82**
Macronutrients stress	**Control**	**10.37^{f} ±0.65**	**6.17^{g}±0.31**	**251.35^{i}±5.50**	<LOD	**11.64^{g}±0.69**
	-Ca	**13.16^{c} ±0.34**	**8.91^{d}±0.31**	**684.18^{c}±9.76**	<LOD	**34.10^{b}±0.47**
	-Mg	**11.74^{d} ±0.55**	**8.63^{de}±0.88**	**462.23^{e}±5.56**	<LOD	**30.85^{c}±0.68**
	-Ca -Mg	**19.35^{a} ±0.33**	**11.90^{ab}±0.33**	**968.18^{a}±9.49**	<LOD	**39.22^{a}±2.55**
Oxidative stress	**Control**	**10.87^{ef} ±0.29**	**7.65^{ef}±0.59**	**261.11^{i}±5.12**	<LOD	**12.11^{fg}±0.67**
	H_2O_2	**11.86^{d} ±0.41**	**8.46^{de}±0.80**	**341.20^{g}±7.66**	<LOD	**15.08^{de}±0.20**
Chemical stress	**Control**	**10.73^{ef}±0.17**	**8.33^{de}±0.47**	**280.71^{h}±7.14**	<LOD	**9.06^{h}±0.27**
	$CuSO_4$	**14.09^{b}±0.59**	**12.16^{a}±0.63**	**939.21^{b}±9.09**	<LOD	**16.13^{d}±0.54**
	$HgCl_2$	**13.07^{c}±0.43**	**10.01^{c}±0.67**	**673.23^{c}±6.58**	<LOD	**13.92^{ef}±0.16**
UV stress	**Control**	**10.82^{ef}±0.24**	**8.88^{d}±0.19**	**286.04^{h}±6.41**	<LOD	**6.76^{i}±0.39**
	UV	**13.54^{bc}±0.60**	**9.12^{cd}±0.65**	**401.58^{f}±7.40**	<LOD	**13.53^{ef}±0.24**
Temperature stress	**Control**	**10.85^{ef}±0.40**	**8.68^{de}±0.50**	**280.10^{h}±5.89**	<LOD	**8.14^{hi}±0.25**
	Temp.	**11.57^{de}±0.90**	**11.86^{ab}±0.99**	**575.06^{d}±5.84**	<LOD	**14.77^{de}±0.59**

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.2 CAT Activity

The activity of the catalase enzyme in callus of *Digitalis* species treated with different stress factors was determined (Table 12).

Table 12**.** CAT activity of *Digitalis* species.

Types of stress	Stress factor	CAT activity (µmol H_2O_2/min/mg protein)			
		D. lamarckii	***D. trojana***	***D. davisiana***	***D. cariensis***
Media strength stress	**Control**	7.39^{i}±0.66	10.21^{j}±0.99	15.70^{f}±0.56	11.39^{hi}±0.67
	½ MS	13.45^{f}±1.03	11.56^{ij}±0.78	22.78^{c}±1.00	16.33^{d}±0.50
	¼ MS	11.69^{fg}±1.09	11.54^{ij}±0.68	21.44^{d}±0.64	14.33^{ef}±0.76
Macronutrients stress	**Control**	6.83^{i}±0.80	12.51^{i}±0.62	9.41^{i}±0.39	12.47^{gh}±1.19
	-Ca	10.52^{gh}±1.12	16.59^{gh}±0.80	20.29^{d}±0.21	18.49^{c}±0.69
	-Mg	9.57^{h}±1.48	15.65^{h}±0.96	18.08^{e}±0.39	15.54^{de}±0.51
	-Ca -Mg	13.40^{f}±1.01	24.03^{bc}±1.38	25.20^{b}±0.38	26.66^{a}±0.70
Oxidative stress	**Control**	11.51^{g}±0.86	17.38^{fgh}±0.94	17.42^{e}±1.10	9.76^{j}±0.54
	H_2O_2	26.47^{b}±1.08	34.53^{a}±0.84	34.04^{a}±1.30	21.31^{b}±0.76
Chemical stress	**Control**	19.30^{d}±0.70	15.55^{h}±0.98	11.45^{h}±0.59	8.24^{k}±0.93
	$CuSO_4$	28.61^{a}±1.20	23.50^{cd}±1.31	17.68^{e}±1.05	12.66^{gh}±0.63
	$HgCl_2$	21.60^{c}±0.94	21.85^{de}±1.41	14.82^{fg}±0.44	10.69^{ij}±0.63
UV stress	**Control**	11.24^{gh}±0.93	21.40^{e}±1.12	13.57^{g}±0.50	13.52^{fg}±0.93
	UV	18.36^{de}±1.04	25.47^{b}±1.24	15.34^{f}±1.01	15.79^{de}±1.25
Temperature stress	**Control**	21.04^{c}±1.36	19.12^{f}±1.12	12.21^{h}±0.61	13.66^{fg}±1.30
	Temp.	16.70^{e}±0.99	18.20^{fg}±0.72	11.20^{h}±0.88	11.51^{hi}±1.11

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.2.1. CAT Activity for *D. lamarckii*

According to our results, CAT activity was significantly increased when callus was cultivated on ½ strength MS (13.45±1.03 µmol H_2O_2/min/mg protein) and on ¼ strength MS (11.69±1.09 µmol H_2O_2/min/mg protein) compared to non-treated callus grown on MS (7.39±0.66 µmol H_2O_2/min/mg protein).

CAT activity was greatly enhanced (13.40±1.01 µmol H_2O_2/min/mg protein) when both Ca and Mg were eliminated from the medium. Besides, CAT activity of callus cultured on medium lacking either Ca (10.52±1.12 µmol H_2O_2/min/mg protein) or

Mg (9.57±1.48 µmol H_2O_2/min/mg protein) was significantly rised compared to the control group (6.83±0.80 µmol H_2O_2/min/mg protein).

Oxidative stress gave rise to a noticeable increase in CAT activities in callus cultures. The CAT activity of control (non-treated) callus produced 11.51±0.86 µmol H_2O_2/min/mg protein while that cultured on under oxidative stress by H_2O_2 producing 26.47±1.08 µmol H_2O_2/min/mg protein.

As a concequence of $CuSO_4$ and $HgCl_2$, the CAT activity was significantly increased in chemical stress experiments. The CAT activity of control (non-treated) was 19.30±0.70 µmol H_2O_2/min/mg protein while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 28.61±1.20 µmol H_2O_2/min/mg protein and 21.60±0.94 µmol H_2O_2/min/mg protein, respectively.

The decrease of CAT activity was prominent in high temperature applied callus (16.70±0.99 µmol H_2O_2/min/mg protein) as compared with non-treated callus (21.04±1.36 µmol H_2O_2/min/mg protein).

UV stress was imposed on callus to study the expression of CAT activity which increased to 18.36±1.04 µmol H_2O_2/min/mg protein as compared with control group (11.24±0.93 µmol H_2O_2/min/mg protein).

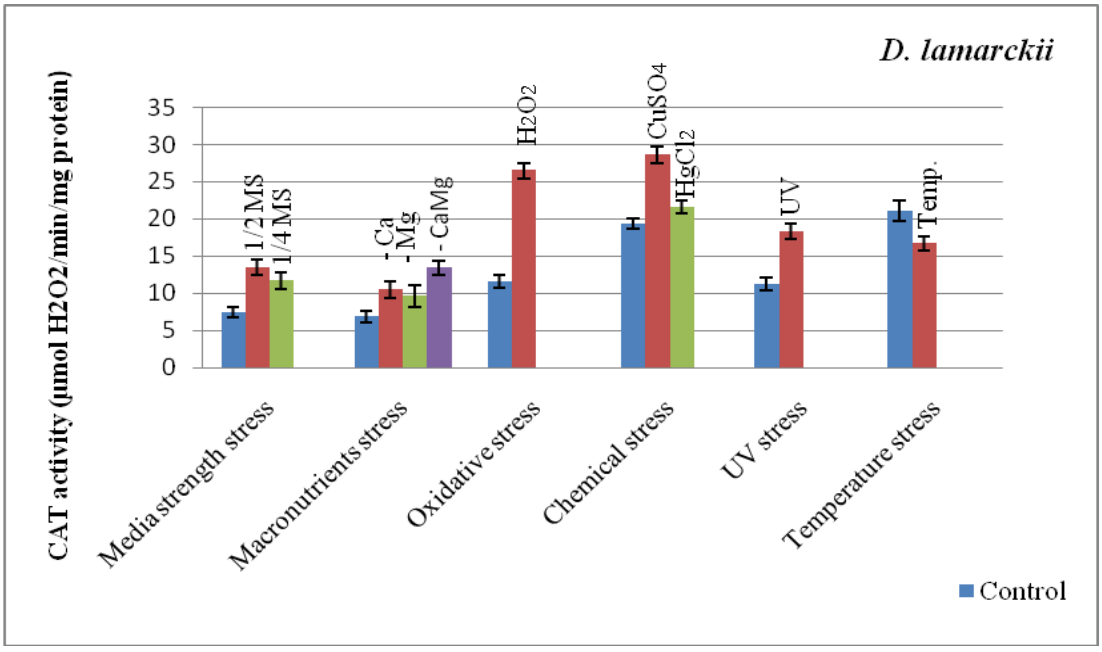

Figure 10**.** CAT activity in callus of control and treated plants of *D. lamarckii*.

3.2.2 CAT Activity for *D. trojana*

Similar to *D. lamarckii*, CAT activity showed an increase when callus was cultivated on ½ strength MS (11.56±0.78 µmol H_2O_2/min/mg protein) and on ¼ strength MS (11.54±0.68 µmol H_2O_2/min/mg protein) compared to non-treated callus grown on MS (10.21±0.99 µmol H_2O_2/min/mg protein) in *D.trojana*.

Lacking either Ca (16.59±0.80 µmol H_2O_2/min/mg protein) or Mg (15.65±0.96 µmol H_2O_2/min/mg protein) from growth medium caused an enhancement of CAT activity. The both deficiencies of these macronutrients resulted in the highest level of CAT activity (24.03±1.38 µmol H_2O_2/min/mg protein) compared to the control (12.51±0.62 µmol H_2O_2/min/mg protein).

Compared to control group (17.38±0.94 µmol H_2O_2/min/mg protein), a considerable increment of CAT activity (34.53±0.84 µmol H_2O_2/min/mg protein) was resulted from H_2O_2 treatment.

Concentrations of CAT increased with $CuSO_4$ (23.50±1.31 µmol H_2O_2/min/mg protein) in a similar way with $HgCl_2$ (21.85±1.41 µmol H_2O_2/min/mg protein) compared to the control group (15.55±0.98 µmol H_2O_2/min/mg protein).

High temperature stress resulted in lower CAT activity (18.20±0.72 µmol H_2O_2/min/mg protein) than that of control group (19.12±1.12 µmol H_2O_2/min/mg protein).

UV treatment led to a remarkable increase in CAT activity (25.47±1.24 µmol H_2O_2/min/mg protein) with regards to comparison group (21.40±1.12 µmol H_2O_2/min/mg protein).

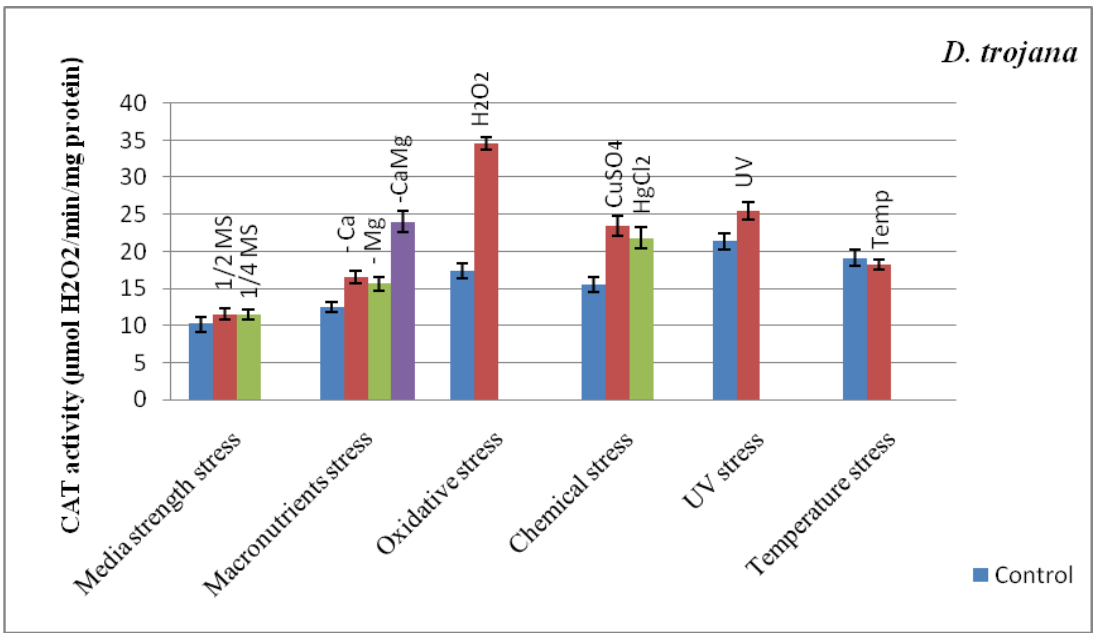

Figure 11**.** CAT activity in callus of control and treated plants of *D. trojana.*

3.2.3 CAT Activity for *D. davisiana*

The level of CAT activity in callus tissues submitted to ½ strength MS was found to be 22.78±1.00 μmol H_2O_2/min/mg protein and to be 21.44±0.64 μmol H_2O_2/min/mg protein cultured onto ¼ strength MS medium compared to non-treated callus grown on MS (15.70±0.56 μmol H_2O_2/min/mg protein).

Elimination of either Ca (20.29±0.21 μmol H_2O_2/min/mg protein) or Mg (18.08±0.39 μmol H_2O_2/min/mg protein) from growth medium caused to improve in CAT activity. The both deficiencies of these macronutrients resulted in the highest level of CAT activity (25.20±0.38 μmol H_2O_2/min/mg protein) compared to the control group (9.41±0.39 μmol H_2O_2/min/mg protein).

In comparison to control cultures (17.42±1.10 μmol H_2O_2/min/mg protein), under oxidative stress, CAT activity was drastically enhanced to 34.04±1.30 μmol H_2O_2/min/mg protein.

$CuSO_4$ as well as $HgCl_2$ stress was followed by a significant increase of the CAT activity. The CAT activity of control group (non-treated) was 11.45±0.59 μmol H_2O_2/min/mg protein while those cultured on under chemical stress by $CuSO_4$ and $HgCl_2$ producing 17.68±1.05 μmol H_2O_2/min/mg protein and 14.82±0.44 μmol H_2O_2/min/mg protein, respectively.

High temperature treatment decreased the CAT activity (11.20±0.88 μmol H_2O_2/min/mg protein) as compared with non-treated callus (12.21±0.61 μmol H_2O_2/min/mg protein). However, these changes were not statistically significant at 5% significance level.

UV stress was imposed on callus to examine the expression of CAT activity which increased to 15.34±1.01 µmol H_2O_2/min/mg protein as compared with control group (13.57g±0.50 µmol H_2O_2/min/mg protein).

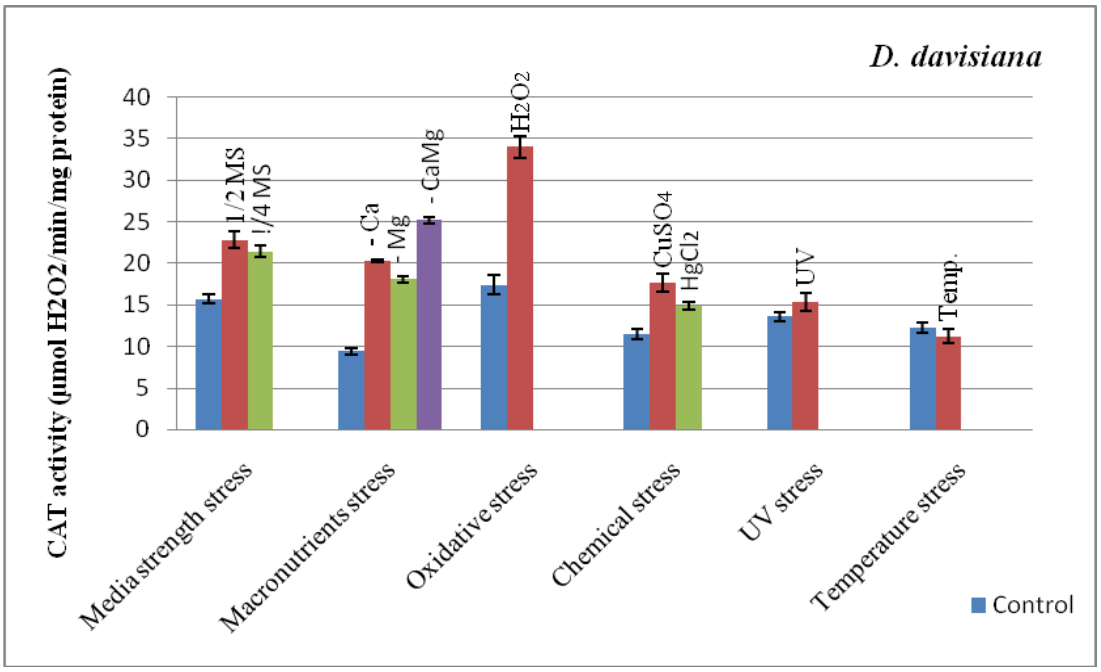

Figure 12**.** CAT activity in callus of control and treated plants of *D. davisiana.*

3.2.4. CAT Activity for *D. cariensis*

With the cultivation of callus on ½ strength MS (16.33±0.50 µmol H_2O_2/min/mg protein) and on ¼ strength MS (14.33±0.76 µmol H_2O_2/min/mg protein), CAT activity showed a significant increase compared to non-treated callus grown on MS (11.39±0.67 µmol H_2O_2/min/mg protein).

Exclusion of either Ca (18.49±0.69 µmol H_2O_2/min/mg protein) or Mg (15.54±0.51 µmol H_2O_2/min/mg protein) from culture medium gave rise to an enhancement of CAT activity. The both deficiencies of these macronutrients resulted in the highest level of CAT activity (26.66±0.70 µmol H_2O_2/min/mg protein) with regards to comparison group (12.47±1.19 µmol H_2O_2/min/mg protein).

CAT activity was significantly increased under H_2O_2 treatment. The CAT activity of control (non-treated) callus produced 9.76±0.54 μmol H_2O_2/min/mg protein while that cultured on under oxidative stress by H_2O_2 producing 21.31±0.76 μmol H_2O_2/min/mg protein.

In chemical stress experiments, CAT activity was greatly increased with $CuSO_4$ and $HgCl_2$ stress. The CAT activity of control (non-treated) was 8.24±0.93 μmol H_2O_2/min/mg protein while those treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 12.66±0.63 μmol H_2O_2/min/mg protein and 10.69±0.63 μmol H_2O_2/min/mg protein, respectively.

A decline in CAT activity was observed at high temperature (11.51±1.11 μmol H_2O_2/min/mg protein) as compared with control group (13.66±1.30 μmol H_2O_2/min/mg protein).

UV treatment notably enhanced the CAT activity (15.79±1.25 μmol H_2O_2/min/mg protein) as compared with control group (13.52±0.93 μmol H_2O_2/min/mg protein).

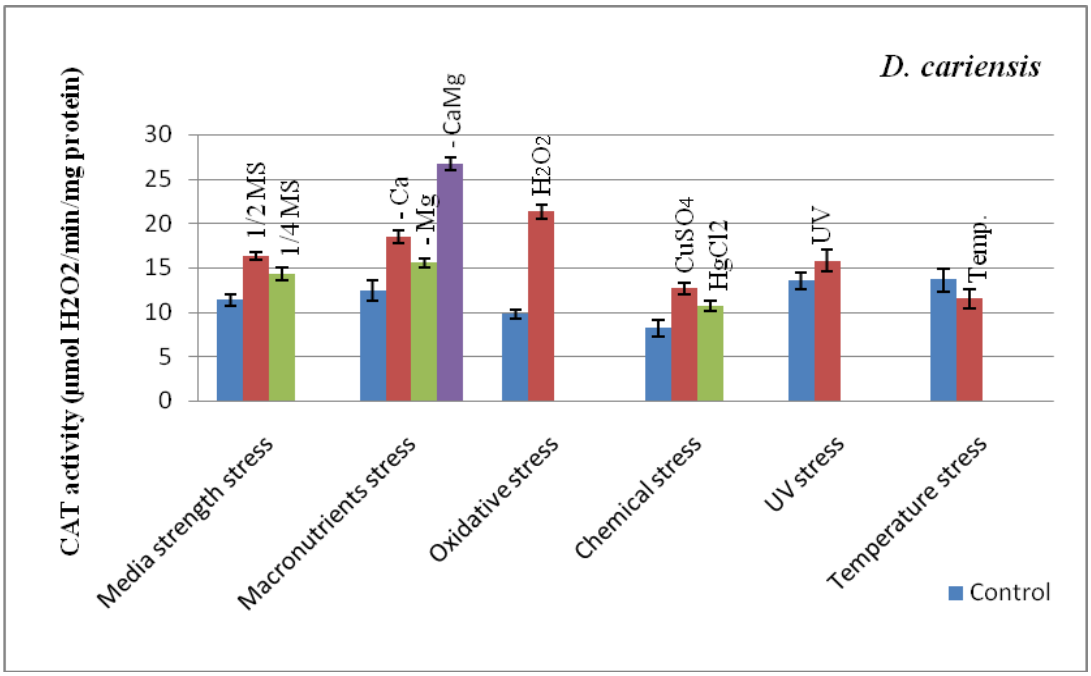

Figure 13**.** CAT activity in callus of control and treated plants of *D. cariensis.*

3.3. SOD Activity

The activity of the SOD enzyme in callus of *Digitalis* species treated with different stress factors was determined (Table 13).

Table 13**.** SOD activity of *Digitalis* species.

Types of stress	**Stress factor**	**SOD activity (U/mg protein)**			
		D. lamarckii	***D. trojana***	***D. davisiana***	***D. cariensis***
Media strength stress	**Control**	0.40^{fg}±0.02	0.20^{h}±0.06	0.16^{j}±0.03	0.37^{i}±0.04
	½ MS	1.30^{b}±0.009	1.17^{c}±0.10	1.13^{b}±0.06	1.47^{b}±0.01
	¼ MS	0.74^{d}±0.04	0.91^{d}±0.03	0.55^{f}±0.01	0.91^{e}±0.02
Macronutrient stress	**Control**	0.51^{ef}±0.04	0.44^{f}±0.13	0.49^{fg}±0.05	0.53^{h}±0.04
	-Ca	1.09^{c}±0.17	1.60^{b}±0.11	1.01^{c}±0.04	1.22^{d}±0.1
	-Mg	0.75^{d}±0.05	1.08^{c}±0.14	0.90^{d}±0.02	0.89^{ef}±0.04
	-Ca -Mg	2.38^{a}±0.16	1.91^{a}±0.05	1.50^{a}±0.04	2.21^{a}±0.04
Oxidative stress	**Control**	0.27^{h}i±0.02	0.23^{gh}±0.04	0.13^{j}±0.02	0.5^{h}±0.02
	H_2O_2	1.03^{c}±0.02	0.44^{f}±0.04	0.33^{i}±0.01	1.3^{c}±0.03
Chemical stress	**Control**	0.14^{j}±0.04	0.16^{h}±0.05	0.48^{fg}±0.05	0.46^{h}±0.03
	$CuSO_4$	0.55^{e}±0.07	0.46^{f}±0.08	0.88^{d}±0.04	1.14^{d}±0.01
	H_gCl_2	0.31^{ghi}±0.03	0.36^{fg}±0.09	0.78^{e}±0.02	0.64^{g}±0.05
UV stress	**Control**	0.21^{ij}±0.01	0.24^{gh}±0.01	0.45^{g}h±0.08	0.21^{j}±0.05
	UV	1.00^{c}±0.01	0.69^{e}±0.02	0.72^{e}±0.03	0.82^{f}±0.06
Temperature stress	**Control**	0.39^{fgh}±0.03	0.83^{d}±0.01	0.42^{gh}±0.06	0.30^{i}±0.03
	Temperature	0.27^{hi}±0.02	0.49^{f}±0.03	0.39^{hi}±0.06	0.15^{j}±0.02

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.3.1. SOD Activity for *D. lamarckii*

A significant increase in SOD activity was observed when callus was cultivated on ½ strength MS (1.30±0.009 U/mg protein) and on ¼ strength MS medium (0.74±0.04 U/mg protein) compared to non-treated callus grown on MS (0.40±0.02 U/mg protein).

Lacking either Ca (1.09±0.17 U/mg protein) or Mg (0.75±0.05 U/mg protein) from growth medium caused to enhance of SOD Activity. Moreover, the both deficiencies of these macronutrients gave rise to the highest level of SOD Activity (2.38±0.16 U/mg protein) as regards with the comparison group (0.51±0.04 U/mg protein).

Exposure of H_2O_2 was followed by a remarkable increase in SOD activity (1.03±0.02 U/mg protein) compared to control group (0.27±0.02 U/mg protein).

UV stress induced the increase of SOD activity (1.00±0.01 U/mg protein) while the SOD activity of control (non-treated) callus was 0.21±0.01 U/mg protein.

In chemical stress experiments the SOD activity was significantly increased as a concequence of $CuSO_4$ and $HgCl_2$. The SOD activity of control (non-treated) was 0.14±0.04 U/mg protein while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 0.55±0.07 U/mg protein and 0.31±0.03 U/mg protein, respectively.

SOD activity responded to high temperature as showing a decrease in applied callus (0.27±0.02 U/mg protein) as compared with non-treated callus (0.39±0.03 U/mg protein).

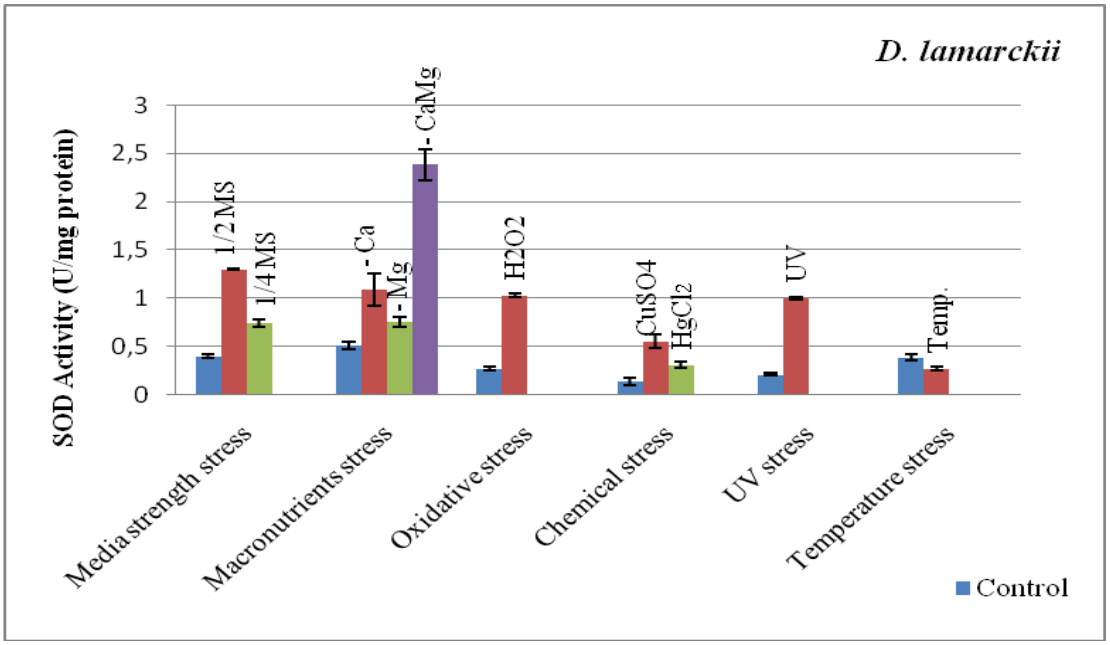

Figure 14**.** SOD activity in callus of control and treated plants of *D. lamarckii*.

3.3.2. SOD Activity for *D. trojana*

In comparison with control cultures (0.20±0.06 U/mg protein), ½ strength MS medium (1.17±0.10 U/mg protein) and ¼ strength MS medium (0.91±0.03 U/mg protein) enhanced total SOD activity.

SOD activity dramatically increased when both Ca and Mg were removed from the medium. The control (non-treated) callus produced 0.44±0.13 U/mg protein while those cultured on medium lacking either Ca or Mg producing 1.60±0.11 U/mg protein or 1.08±0.14 U/mg protein, respectively. With the elimination of both Ca and Mg from the medium, the highest SOD activity (1.91±0.05 U/mg protein) was estimated.

Compared to control cultures (0.23±0.04 U/mg protein), in callus, which was subjected to oxidative stress, SOD activity was greatly promoted (0.44±0.04 U/mg protein).

SOD activity (0.16±0.05 U/mg protein) under the condition of no stress increased to 0.46±0.08 U/mg protein and 0.36±0.09 U/mg protein under the application of $CuSO_4$ and $HgCl_2$, respectively.

SOD activity decreased to 0.49±0.03 U/mg protein under high temperature treatment whereas the level was 0.83±0.01 U/mg protein in non-treated callus.

In our studies, higher level of SOD activity (0.69±0.02 U/mg protein) treated cultures obtained under UV treatment than that of comparison group (0.24±0.01 U/mg protein).

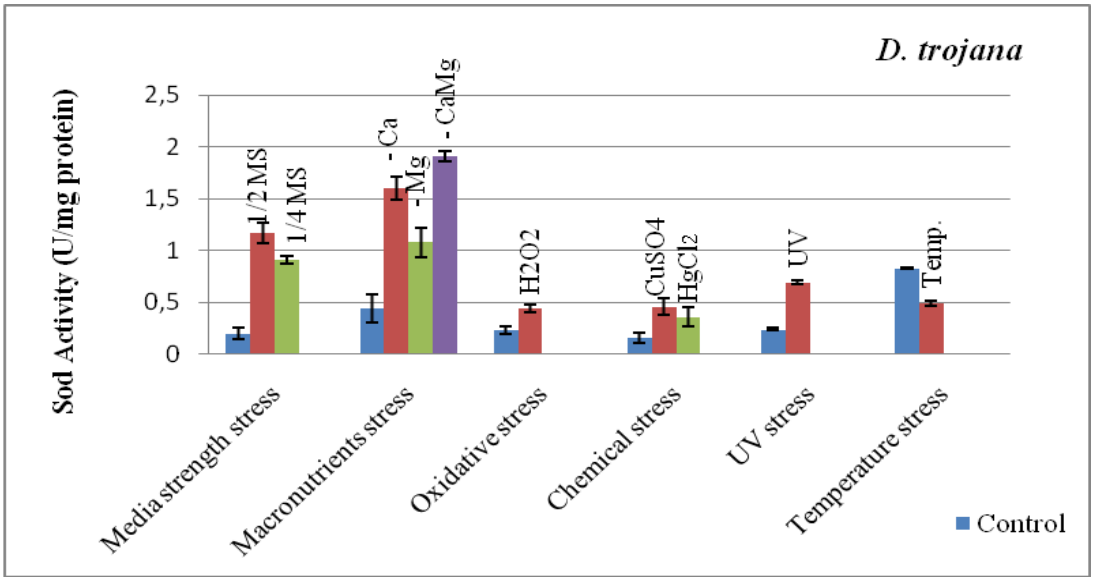

Figure 15**.** SOD activity in callus of control and treated plants of *D. trojana.*

3.3.3. SOD Activity for *D. davisiana*

A noticeable effect of ½ strength MS medium (1.13±0.06 U/mg protein) and ¼ strength MS (0.55±0.01 U/mg protein) medium on SOD activity was observed as compared with control group (0.16±0.03 U/mg protein). The highest SOD activity was observed on half-strength medium.

SOD activity significantly increased when both Ca and Mg were eliminated from the medium. While callus cultured on medium lacking either Ca (1.01±0.04 U/mg protein) or Mg (0.90±0.02 U/mg protein) resulted in the increment of SOD activity, the control (non-treated) callus producing 0.49±0.05 U/mg protein. The highest SOD activity (1.50±0.04 U/mg protein) was estimated when both Ca and Mg were discarded from the medium.

H_2O_2 treatment led to a significant increase in SOD activity (0.33±0.01 U/mg protein) as compared with non-treated callus (0.13±0.02 U/mg protein).

SOD activity was drastically increased due to $CuSO_4$ and $HgCl_2$ in chemical stress experiments. The SOD activity of control (non-treated) was 0.48±0.05 U/mg protein while callus under chemical stress by $CuSO_4$ and $HgCl_2$ producing 0.88±0.04 U/mg protein and 0.78±0.02 U/mg protein, respectively.

The inverse relationship between high temperature treatment and SOD activity (0.39±0.06 U/mg protein) was recorded according to comparison group 0.42±0.06 U/mg protein).

UV treatment enhanced the SOD activity (0.72±0.03 U/mg protein) in treated callus tissues compared to control group (0.45±0.08 U/mg protein).

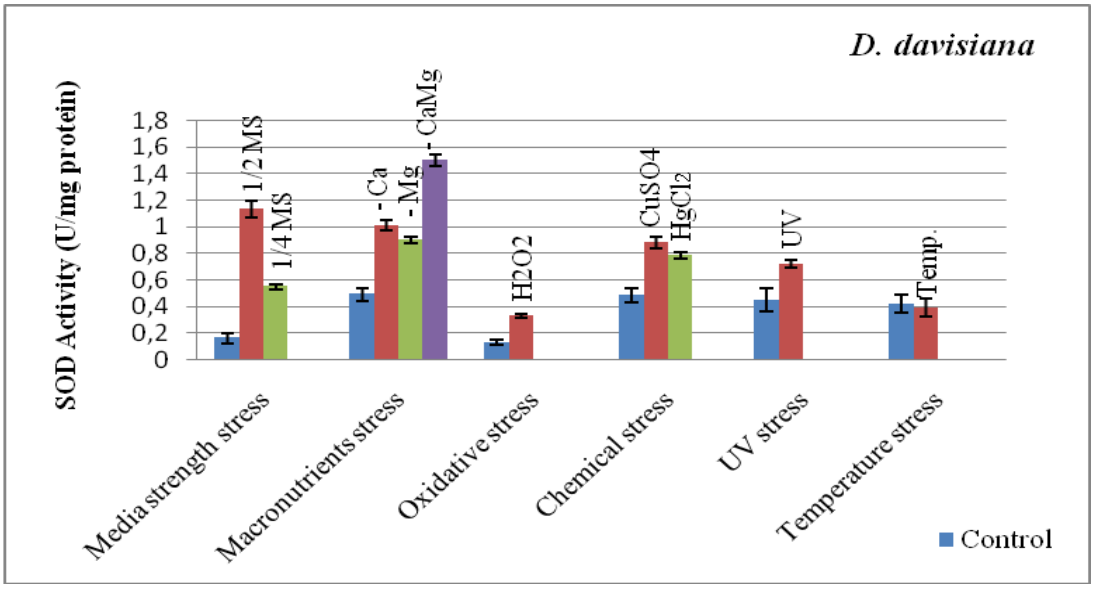

Figure 16**.** SOD activity in callus of control and treated plants of *D. davisiana.*

3.3.4. SOD Activity for *D. cariensis*

A significant increase in SOD activity was associated with the cultivation of callus on ½ strength MS (1.47±0.01 U/mg protein) and on ¼ strength MS medium (0.91±0.02 U/mg protein) compared to non-treated callus grown on MS (0.37±0.04 U/mg protein).

SOD activity was noticeably increased (2.21±0.04 U/mg protein) when both Ca and Mg were removed from the medium. In addition, SOD activity of callus cultured on medium lacking either Ca (1.22±0.1 U/mg protein) or Mg (0.89±0.04 U/mg protein) was significantly enhanced with regards to the comparison group (0.53±0.04 U/mg protein).

As compared with control group (0.50±0.02 U/mg protein) a considerable increase of SOD activity (1.30±0.03 U/mg protein) was resulted from exogenous H_2O_2 application.

In chemical stress experiments, the SOD activity was remarkably influenced with $CuSO_4$ and $HgCl_2$ stress. The SOD activity of control was 0.46±0.03 U/mg protein while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 1.14±0.01 U/mg protein and 0.64±0.05 U/mg protein, respectively.

A reduction of SOD activity (0.15±0.02 U/mg protein) resulted from the high temperature in comparison to non-treated callus (0.30±0.03 U/mg protein).

The application of UV stress was noted, with correlated increase of SOD activity (0.82±0.06 U/mg protein) with regards to control group (0.21±0.05 U/mg protein).

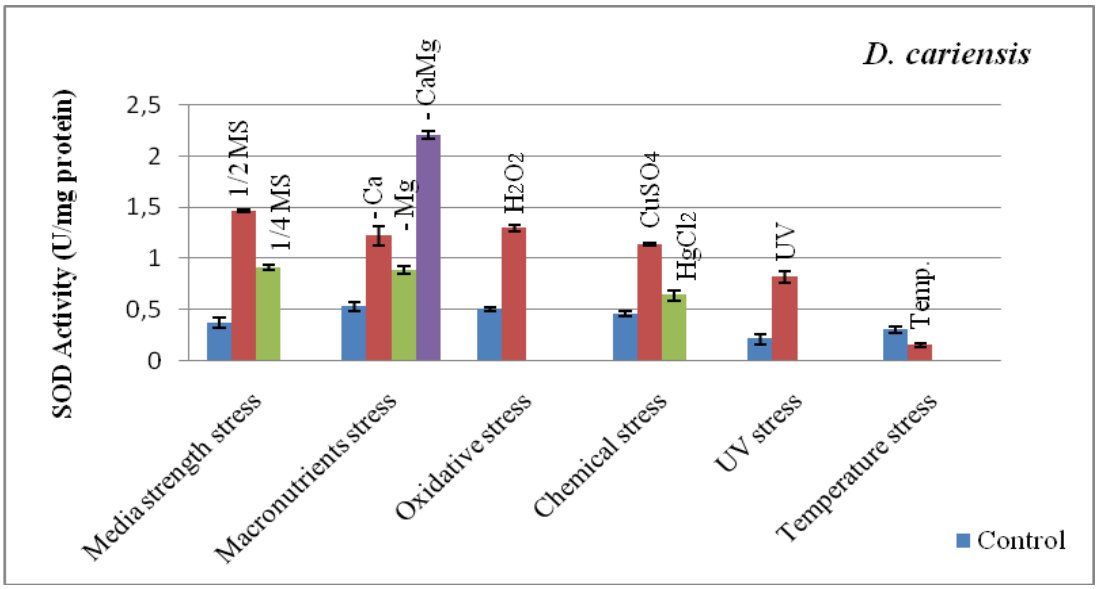

Figure 17**.** SOD activity in callus of control and treated plants of *D. cariensis.*

3.4. Total Phenolics Analysis

The content of total phenolic in callus of *Digitalis* species treated different stress factors was determined (Table 14).

Table 14**.** Total phenolic content of *Digitalis* species

Types of stress	**Stress factor**	**Total Phenolic (µg GA/mg, dw)**			
		D. lamarckii	***D. trojana***	***D. davisiana***	***D. cariensis***
Media strength stress	**Control**	172.37^{n}±1.25	229.87^{n}±1.25	294.87^{g}±0.88	236.95^{o}±0.72
	½ MS	399.45^{a}±0.72	515.70^{c}±0.72	421.54^{c}±1.76	458.20^{e}±0.72
	¼ MS	383.20^{b}±0.72	481.54^{b}±1.44	373.20^{e}±0.88	371.54^{h}±0.72
Macronutrient stress	**Control**	232.79^{h}±1.44	251.12^{l}±1.25	277.37^{h}±1.25	264.87^{k}±1.25
	-Ca	315.70^{d}±0.72	475.70^{d}±0.72	525.29^{b}±0.63	496.95^{b}±0.72
	-Mg	268.62^{e}±1.25	372.79^{g}±0.72	374.04^{e}±1.27	334.87^{i}±1.25
	-Ca -Mg	334.45^{c}±1.44	581.54^{a}±0.72	626.95^{a}±0.63	633.20^{a}±0.72
Oxidative stress	**Control**	186.95^{l}±0.72	231.54^{n}±0.72	187.37^{k}±0.88	248.20^{n}±0.72
	H_2O_2	235.70^{g}±1.90	399.45^{f}±2.60	388.63^{d}±0.88	412.37^{f}±1.25
Chemical stress	**Control**	154.45^{p}±0.72	219.46^{o}±0.72	198.62^{j}±1.76	252.38^{m}±1.25
	$CuSO_4$	196.12^{j}±1.25	276.54^{j}±1.90	371.95^{e}±0.88	476.95^{c}±0.72
	H_gCl_2	174.87^{m}±0.72	238.20^{m}±1.44	277.37^{h}±1.76	330.29^{j}±1.90
UV stress	**Control**	213.20^{i}±1.44	279.45^{i}±1.44	165.29^{l}±0.63	264.87^{k}±1.25
	UV	237.79^{f}±0.72	414.87^{e}±1.25	329.86^{f}±0.72	461.54^{d}±0.72
Temperature stress	**Control**	164.45^{o}±0.72	258.20^{k}±1.25	159.45^{m}±0.63	256.95^{l}±0.72
	Temp.	191.54^{k}±1.90	288.62^{h}±0.72	240.70^{i}±0.63	378.20^{g}±0.72

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.4.1. Total Phenolic Content for *D. lamarckii*

Total phenolic content was significantly increased with the cultivation of the callus on ½ strength MS (399.45±0.72 µg/g GA, dw) and ¼ strength MS medium (383.20±0.72 µg/g GA, dw) compared to non-treated callus grown on MS (172.37±1.25 µg/g GA, dw).

Elimination of both Ca and Mg from the medium was followed by a significant increase of total phenolic content. The control (non-treated) callus produced 232.79±1.44 µg/g GA, dw while callus cultured on medium lacking Ca producing 315.70±0.72 µg/g GA, dw and lacking Mg producing or 268.62±1.25 µg/g GA, dw.

Moreover, the highest total phenolic content (334.45±1.44 µg/g GA, dw) was recorded when both Ca and Mg were eliminated from the medium.

With regards to the control cultures (186.95±0.72 µg/g GA, dw), under oxidative stress, total phenolic content was markedly enhanced (235.70±1.90 µg/g GA, dw).

The use of $CuSO_4$ and $HgCl_2$ led to a drastic increment in total phenolic content in chemical stress experiments. Total phenolic content of control was 154.45±0.72 µg/g GA, dw while callus which was treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 196.12±1.25 µg/g GA, dw and 174.87±0.72 µg/g GA, dw, respectively.

The greatly increased total phenolic content due to high temperature was measured as 191.54±1.90 µg/g GA, dw in comparison to non-treated callus (164.45±0.72 µg/g GA, dw).

An increase in total phenolic content in callus submitted to UV treatment was observed in treated group and in non-treated group with the levels of 237.79±0.72 µg/g GA, dw and 213.20±1.44 µg/g GA, dw, respectively.

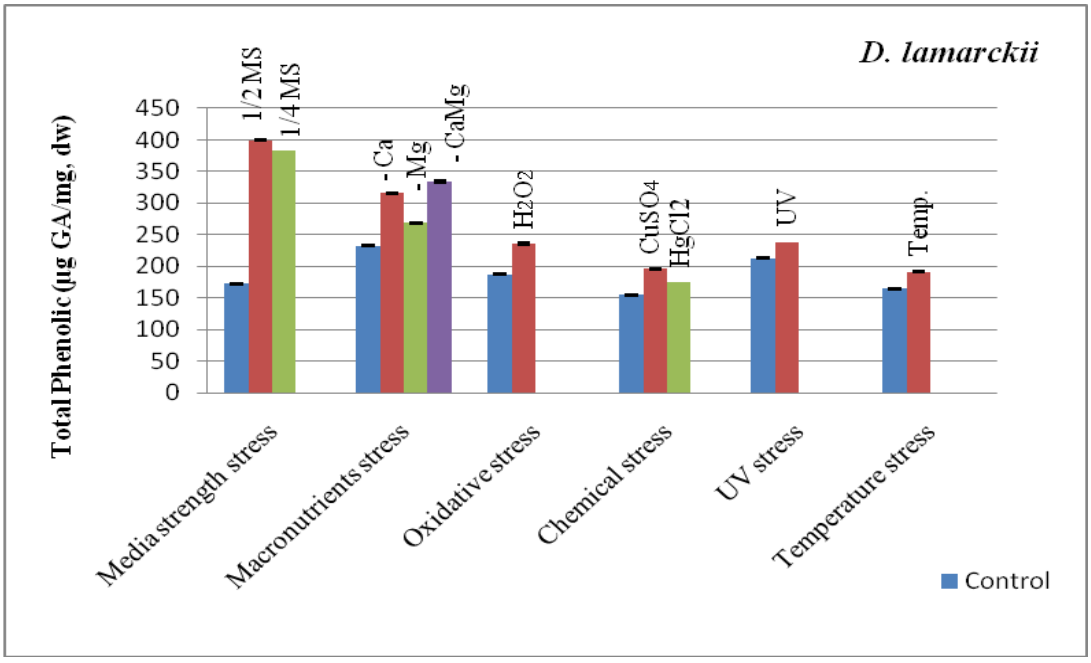

Figure 18**.** Content of total phenolics in *D. lamarckii* (µg/mg).

3.4.2. Total Phenolic Content for *D. trojana*

Total phenolic content was significantly improved as a result of cultivation of callus on ½ strength MS (515.70±0.72µg/g GA, dw) and on ¼ strength MS medium (481.54±1.44 µg/g GA, dw) compared to non-treated callus grown on MS (229.87±1.25 µg/g GA, dw).

Similarly, deficiency of both Ca and Mg gave rise to a remarkable increment in total phenolic content. The control callus produced 251.12±1.25 µg/g GA, dw while those cultured on medium lacking either Ca or Mg producing 475.70±0.72 µg/g GA, dw or 372.79±0.72 µg/g GA, dw, respectively. When both Ca and Mg were discarded from the medium, the highest total phenolic content was noted (581.54±0.72 µg/g GA, dw).

In comparison to control cultures (231.54±0.72 µg/g GA, dw), callus submitted to oxidative stress showed a markedly enhanced level of phenolic content as 399.45±2.60 µg/g GA, dw.

Exposure of $CuSO_4$ and $HgCl_2$ on callus in chemical stress experiments was followed by a significant increment in total phenolic content. Total phenolic content of control was 219.46±0.72 µg/g GA, dw while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 276.54±1.90 µg/g GA, dw and 238.20±1.44 µg/g GA, dw, respectively.

Callus subjected to high temperature showed a great increase in total phenolic content (288.62±0.72 µg/g GA, dw) with regards to non-treated callus (258.20±1.25 µg/g GA, dw).

The application of UV stress was noted, with correlated increase of total phenolic content (414.87±1.25 µg/g GA, dw) compared to the control group (279.45±1.44 µg/g GA, dw).

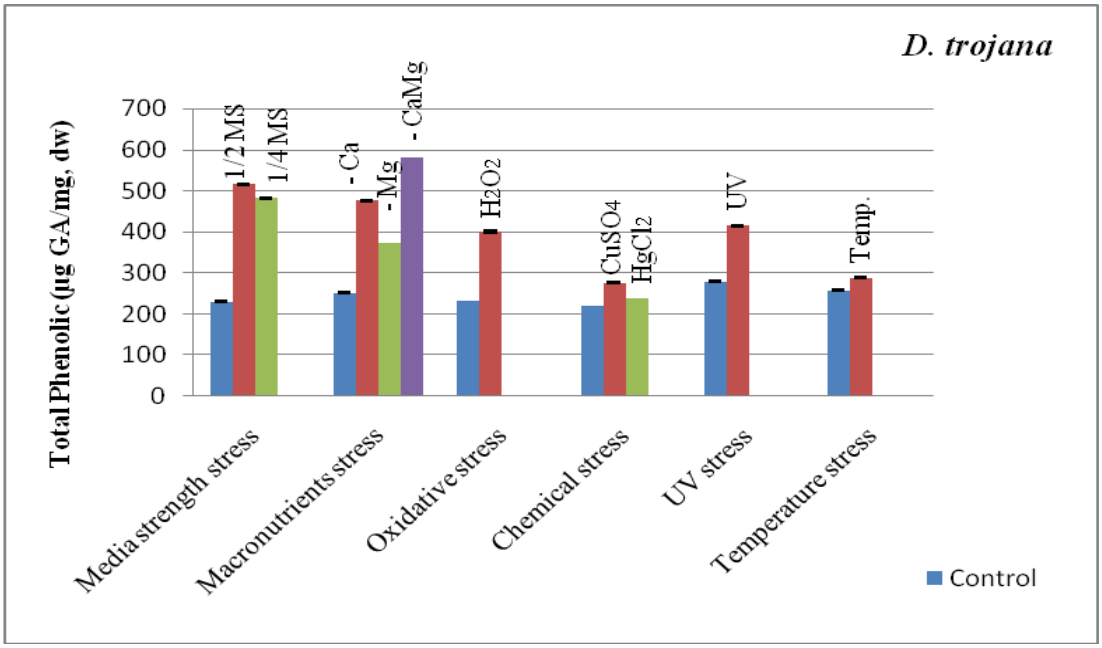

Figure 19**.** Content of total phenolics in *D. trojana* (µg/mg).

3.4.3. Total Phenolic Content for *D. davisiana*

When callus was cultivated on ½ strength MS and on ¼ strength MS medium, total phenolic content was significantly stimulated and the following results were obtained: 421.54±1.76 µg/g GA, dw for ½ strength MS and 373.20±0.88 µg/g GA, dw for ¼ strength MS compared to non-treated callus grown on MS (294.87±0.88 µg/g GA, dw).

Total phenolic content was markedly enhanced when both Ca and Mg were excluded from the medium. While callus cultured on medium lacking either Ca or Mg producing 525.29±0.63 µg/g GA, dw or 374.04±1.27 µg/g GA, dw, respectively, the control (non-treated) callus producing 277.37±1.25 µg/g GA, dw. The highest level of total phenolic content (626.95±0.63 µg/g GA, dw) was recorded when both Ca and Mg were discarded from the medium.

In comparison to control cultures (187.37±0.88 µg/g GA, dw), total phenolic content markedly enhanced in callus which was under oxidative stress (388.63d±0.88 µg/g GA, dw).

Similarly, total phenolic content significantly improved in callus as a result of exposure of $CuSO_4$ and $HgCl_2$ in chemical stress experiments. Total phenolic content of control was 198.62±1.76 µg/g GA, dw while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 371.95±0.88 µg/g GA, dw and 277.37±1.76 µg/g GA, dw, respectively.

Callus which was treated high temperature showed an increase of total phenolic content (240.70±0.63 µg/g GA, dw) as compared with non-treated callus (159.45±0.63 µg/g GA, dw).

UV stress induced the increase of total phenolic content to 329.86±0.72 µg/g GA, dw while total phenolic content of control (non-treated) callus was 165.29±0.63 µg/g GA, dw.

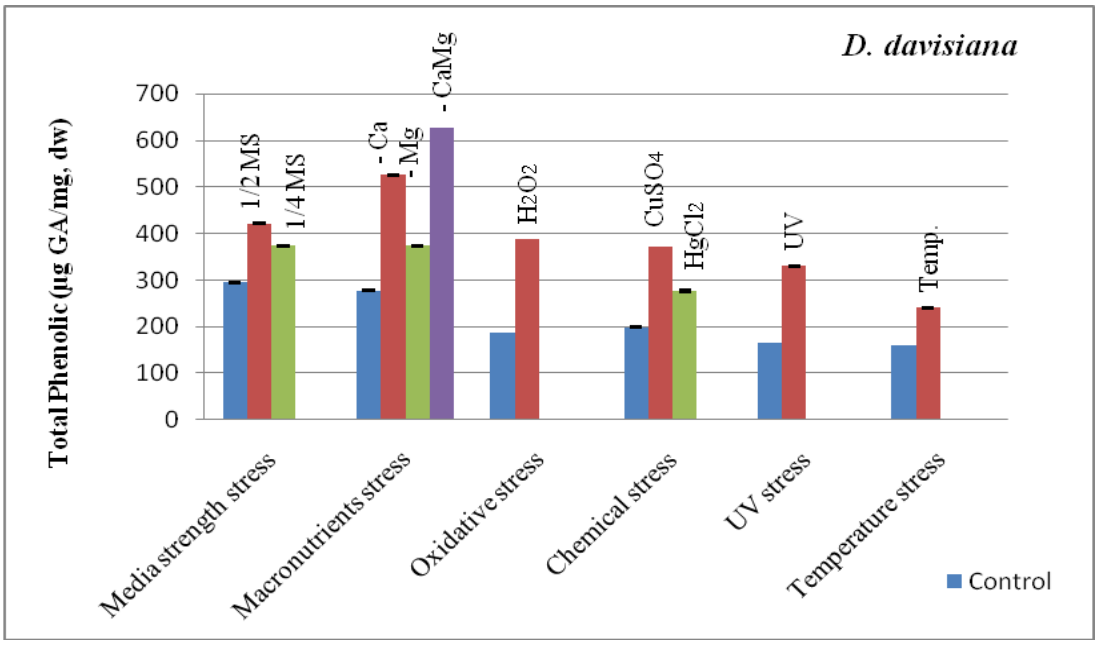

Figure 20**.** Content of total phenolics in *D. davisiana* (µg/mg).

3.4.4. Total Phenolic Content for *D. cariensis*

According to our results, total phenolic content was significantly increased when callus was cultivated on ½ strength MS (458.20±0.72 µg/g GA, dw) and on ¼ strength MS (371.54±0.72 µg/g GA, dw) compared to non-treated callus grown on MS (236.95±0.72 µg/g GA, dw).

Lacking either Ca (496.95±0.72 µg/g GA, dw) or Mg (334.87±1.25 µg/g GA, dw) from growth medium caused to enhance total phenolic content. The both deficiencies of these macronutrients resulted in the highest level of CAT Activity (633.20±0.72 µg/g GA, dw) compared to the control group (264.87±1.25 µg/g GA, dw).

Total phenolic content markedly rised to 412.37±1.25 µg/g GA, dw under oxidative stress whereas the level was 248.20±0.72 µg/g GA, dw in control cultures.

$CuSO_4$ as well as $HgCl_2$ stress was followed by a significantly enhanced total phenolic content in chemical stress experiments. The total phenolic content of control group was 252.38±1.25 µg/g GA, dw while callus under chemical stress by $CuSO_4$ and $HgCl_2$ producing 476.95±0.72 µg/g GA, dw and 330.29±1.90 µg/g GA, dw, respectively.

A remarkable increase of total phenolic content was prominent in high temperature applied callus (378.20±0.72 µg/g GA, dw) with regards to comparison group (256.95±0.72 µg/g GA, dw).

In our studies, higher level of total phenolic content (461.54±0.72 µg/g GA, dw) treated cultures obtained under UV treatment than that of comparison group (264.87±1.25 µg/g GA, dw).

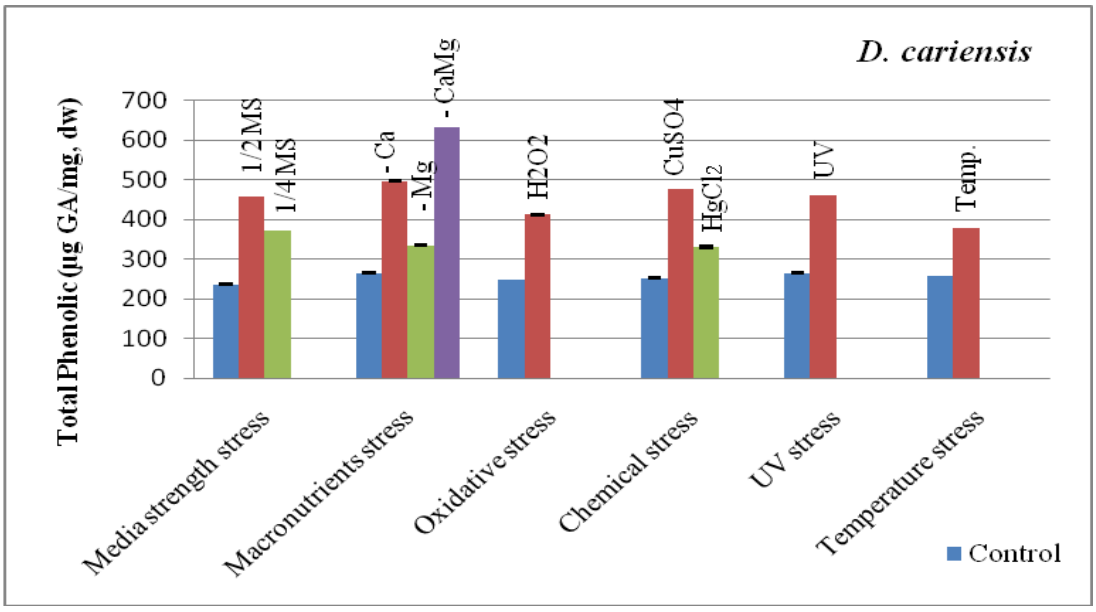

Figure 21**.** Content of total phenolics in *D. cariensis* (µg/mg).

3.5. Proline Content

Proline accumulation in callus of *Digitalis* species treated with different stress factors was determined.

Table 15**.** Proline content of *Digitalis* species.

Types of stress	**Stress factor**	**Proline content (µmol/g, dw)**			
		D. lamarckii	***D. trojana***	***D. davisiana***	***D. cariensis***
Media strength stress	**Control**	0.85^{l}±0.006	0.60^{o}±0.010	0.77^{k}±0.006	0.84^{l}±0.006
	½ MS	1.86^{d}±0.006	1.40^{g}±0.010	1.67^{g}±0.010	1.99^{g}±0.006
	¼ MS	1.56^{f}±0.010	1.23^{i}±0.006	1.55^{h}±0.006	1.67^{i}±0.010
Macronutrient stress	**Control**	0.87^{k}±0.006	0.68^{m}±0.010	0.74^{l}±0.010	0.91^{k}±0.010
	-Ca	2.46^{b}±0.010	2.32^{d}±0.010	2.38^{c}±0.006	2.94^{d}±0.006
	-Mg	2.31^{c}±0.006	2.21^{e}±0.010	2.15^{e}±0.010	2.65^{e}±0.006
	-Ca -Mg	3.53^{a}±0.015	4.10^{a}±0.020	3.45^{b}±0.010	5.45^{a}±0.010
Oxidative stress	**Control**	0.88^{j}±0.006	0.73^{l}±0.006	0.85^{j}±0.006	0.93^{j}±0.015
	H_2O_2	2.46^{b}±0.010	2.68^{b}±0.010	4.13^{a}±0.006	5.42^{b}±0.010
Chemical stress	**Control**	0.76^{n}±0.006	0.72^{l}±0.010	0.76^{k} ±0.006	0.81^{m}±0.006
	$CuSO_4$	1.33^{g}±0.006	1.29^{h}±0.006	1.68^{g}±0.012	1.82^{h}±0.006
	H_gCl_2	1.23^{i}±0.012	1.13^{j}±0.006	1.46^{i}±0.010	1.66^{i}±0.010
UV stress	**Control**	0.80^{m}±0.010	0.78^{k}±0.010	0.73^{l}±0.006	0.84^{l}±0.006
	UV	1.76^{e}±0.010	2.36^{c}±0.006	2.32^{d}±0.012	3.98^{c}±0.010
Temp. stress	**Control**	0.79^{m}±0.006	0.66^{n}±0.006	0.70^{m}±0.010	0.75^{n}±0.012
	Temp.	1.26^{h}±0.015	1.98^{f}±0.012	1.99^{f}±0.006	2.14^{f}±0.010

Data are means ± SD from three replicates
Values followed by different letters in the same column are significantly different (P<0.05)

3.5.1. Proline Content for *D. lamarckii*

Media strength significantly influenced the proline content in callus cultures. In our studies, ½ MS strength (1.86±0.006 µmol/g, dw) and ¼ MS strength (1.56±0.010 µmol/g, dw) treated cultures obtained higher proline content than non-treated callus grown on MS (0.85±0.006 µmol/g, dw).

The proline content was greatly enhanced (3.53±0.015 µmol/g, dw) when both Ca and Mg were eliminated from the medium. Besides, the proline content of callus cultured on medium lacking either Ca (2.46±0.010 µmol/g, dw) or Mg (2.31±0.006 µmol/g, dw) was significantly rised compared to the control group (0.87±0.006 µmol/g, dw).

The exposure of oxidative stress gave rise to a marked increase of the proline content in treated cultures (2.46±0.010 µmol/g, dw) compared to the control cultures (0.88±0.006 µmol/g, dw).

In chemical stress experiments the proline content was significantly enhanced as a concequence of $CuSO_4$ and $HgCl_2$. The proline content of control was 0.76±0.006 µmol/g, dw while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 1.33±0.006 µmol/g, dw and 1.23±0.012 µmol/g, dw, respectively.

A considerable increment of the proline content (1.26±0.015 µmol/g, dw) was resulted from high temperature in comparison to non-treated callus (0.79±0.006 µmol/g, dw).

UV stress induced the increase of total proline content (1.76±0.010 µmol/g, dw) while total proline content of control (non-treated) callus was 0.80±0.010 µmol/g, dw.

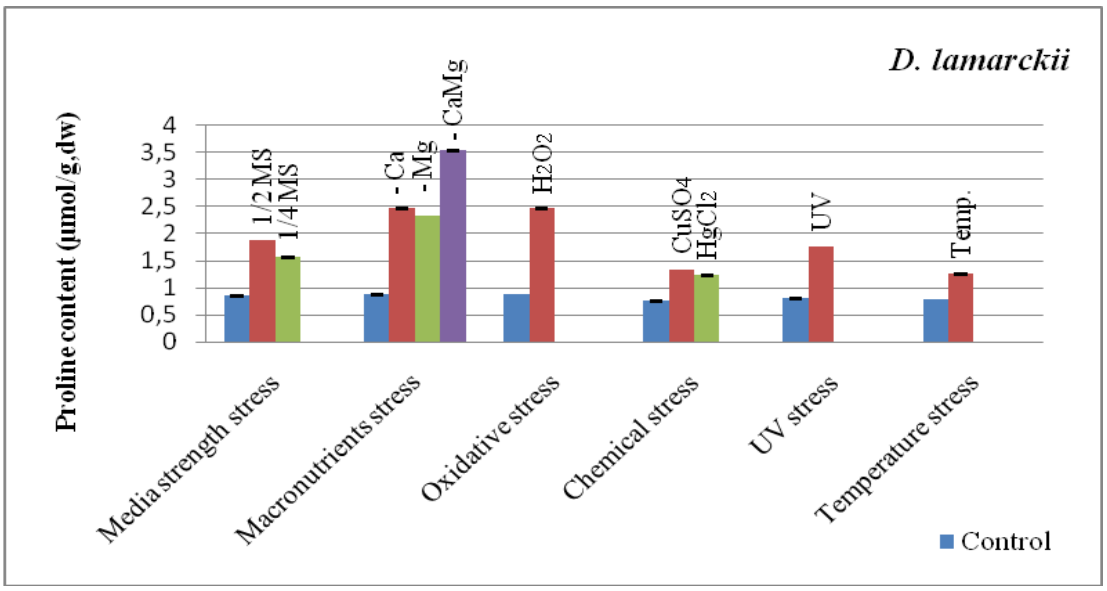

Figure 22**.** Changes in proline content in callus tissues of *D. lamarckii* after stress treatment.

3.5.2. Proline Content for *D. trojana*

Proline content was significantly increased when callus was submitted to ½ strength MS (1.40±0.010 µmol/g, dw) and to ¼ strength MS (1.23±0.006 µmol/g, dw) compared to non-treated callus grown on MS (0.60±0.010 µmol/g, dw).

Exclusion of either Ca (2.32±0.010 µmol/g, dw) or Mg (2.21±0.010 µmol/g, dw) from culture medium gave rise to an enhancement of proline content. The both deficiencies of these macronutrients resulted in the highest level of proline content (4.10±0.020 µmol/g, dw) compared to the control group (0.68±0.010 µmol/g, dw).

Oxidative stress caused a noticeable increase in the proline content in callus cultures. The proline content of control (non-treated) callus produced 0.73±0.006 µmol/g, dw while that cultured on under oxidative stress by H_2O_2 producing 2.68±0.010 µmol/g, dw.

Concentrations of the proline content increased with $CuSO_4$ (1.29±0.006 µmol/g, dw) in a similar way with $HgCl_2$ (1.13±0.006 µmol/g, dw) compared to the control group (0.72±0.010 µmol/g, dw).

A considerable increase in the proline content was prominent in high temperature applied callus (1.98±0.012 µmol/g, dw) as compared with non-treated callus (0.66±0.006 µmol/g, dw).

UV stress was imposed on callus to study the expression of proline content which increased to 2.36±0.006 µmol/g, dw as compared with control group (0.78±0.010 µmol/g, dw).

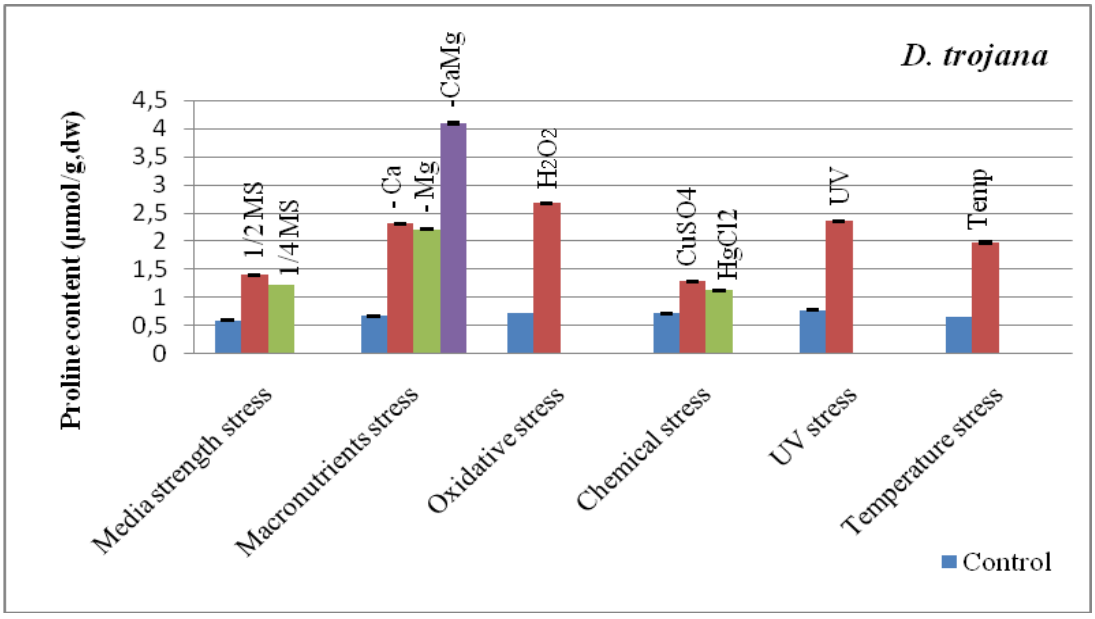

Figure 23**.** Changes in proline content in callus tissues of *D. trojana* after stress treatment

3.5.3. Proline Content for *D. davisiana*

According to our results, proline content was eminently increased when callus was cultivated on ½ strength MS (1.67±0.010 µmol/g, dw) and on ¼ strength MS (1.55±0.006 µmol/g, dw) with regards to non-treated callus grown on MS (0.77±0.006 µmol/g, dw).

The proline content was noticeably increased when both Ca and Mg were discarded from the medium. The control (non-treated) callus produced 0.74±0.010 µmol/g, dw while those cultured on medium lacking either Ca or Mg producing 2.38±0.006 µmol/g or 2.15±0.010 µmol/g, dw respectively. The highest proline content (3.45±0.010 µmol/g, dw) was estimated when both Ca and Mg were eliminated from the medium.

With regards to the control cultures (0.85±0.006 µmol/g, dw), a notable increase in proline content (4.13±0.006 µmol/g, dw) was resulted from oxidative stress.

The proline content was significantly enhanced with $CuSO_4$ and $HgCl_2$ stress in chemical stress experiments. Proline content of control was 0.76±0.006 µmol/g, dw while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 1.68±0.012 µmol/g, dw and 1.46±0.010 µmol/g, dw, respectively.

Temperature treatment greatly gave rise to an increase of the proline content (1.99±0.006 µmol/g, dw) as compared with non-treated callus (0.70±0.010 µmol/g, dw).

UV treatment led to an increase of the proline content (2.32±0.012 µmol/g, dw) compared to control group (0.73±0.006 µmol/g, dw).

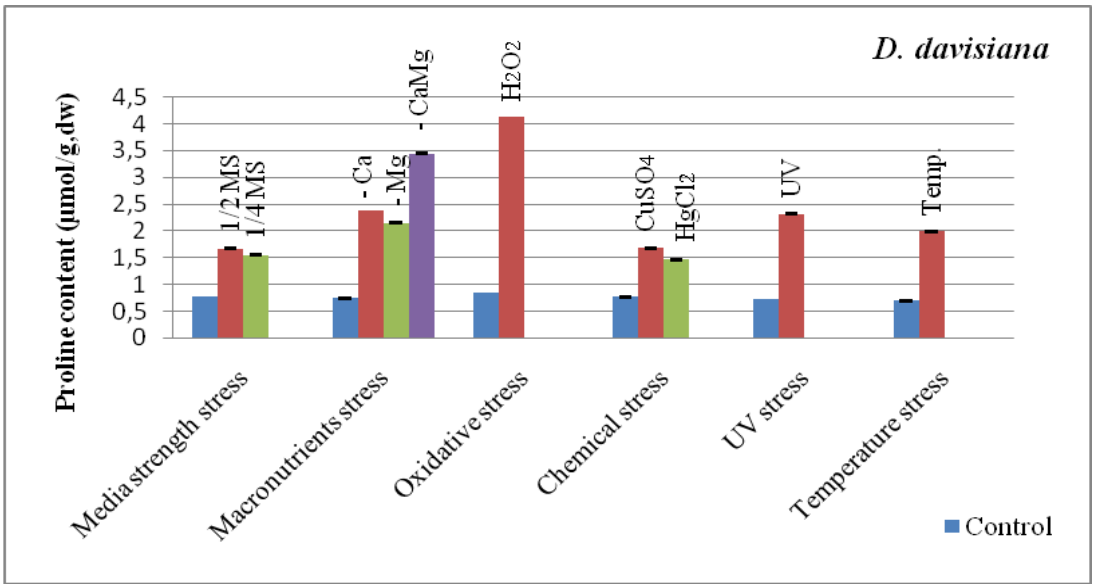

Figure 24**.** Changes in proline content in callus tissues of *D. davisiana* after stress treatment

3.5.4. Proline Content for *D. cariensis*

The level of the proline content in callus tissues cultivated on ½ strength MS was found to be 1.99±0.006 µmol/g, dw and to be 1.67±0.010 µmol/g, dw cultured onto ¼ strength MS medium compared to non-treated callus grown on MS (0.84±0.006 µmol/g, dw).

Similarly, deficiency of both Ca and Mg caused to a remarkable increment of the proline content. The control callus produced 0.91±0.010 µmol/g, dw while those cultured on medium lacking either Ca or Mg producing 2.94±0.006 µmol/g or 2.65±0.006 µmol/g, dw, respectively. When both Ca and Mg were discarded from the medium, the highest total phenolic content was noted (5.45±0.010 µmol/g, dw).

Oxidative stress induced a noticeable increase in the proline content in callus cultures. The proline content of control (non-treated) callus produced 0.93±0.015 µmol/g, dw while that cultured on under oxidative stress by H_2O_2 producing 5.42±0.010 µmol/g, dw.

In chemical stress experiments the proline content was noticeably improved with $CuSO_4$ and $HgCl_2$ stress. The proline content of control was 0.81±0.006 µmol/g, dw while callus treated with chemical stress by $CuSO_4$ and $HgCl_2$ producing 1.82±0.006 µmol/g, dw and 1.66±0.010 µmol/g, dw, respectively.

Temperature treatment was followed by a great increase of the proline content (2.14±0.010 µmol/g, dw) with regards to non-treated callus (0.75±0.012 µmol/g, dw).

Higher level of total proline content (3.98±0.010 µmol/g, dw) treated cultures was received under UV treatment than that of comparison group (0.84±0.006 µmol/g, dw).

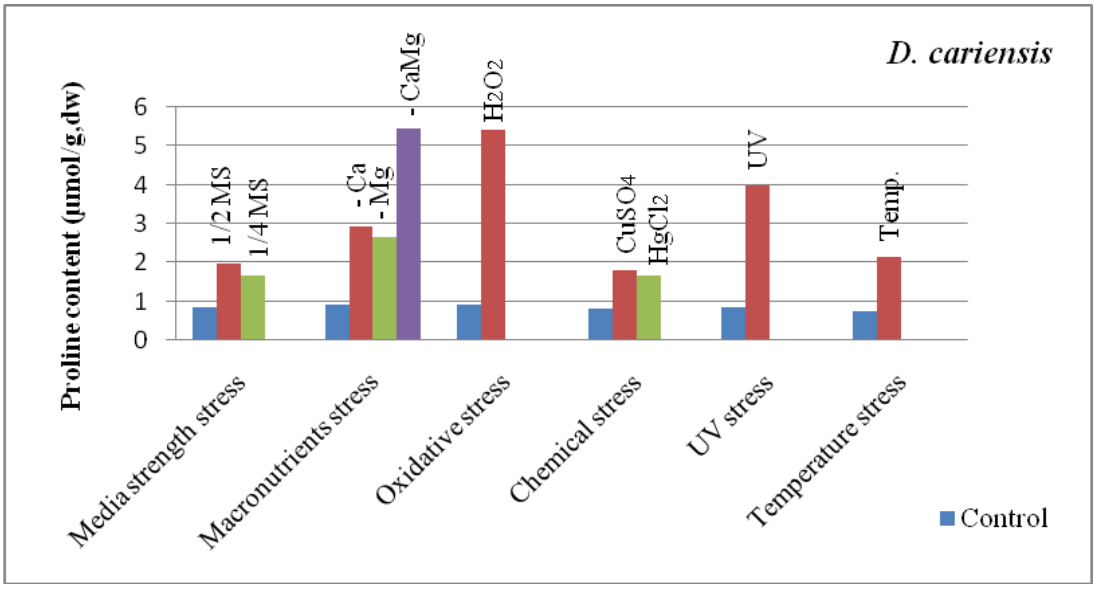

Figure 25**.** Changes in proline content in callus tissues of *D. cariensis* after stress treatment.

CHAPTER 4.

DISCUSSION

4.1. Cardenolide Production

Earlier studies showed the use of cell and tissue cultures of *Digitalis* genus for production of cardenolides, such as suspension cultures (Kreis et al. 1986), embryogenic cell cultures (Lindemann and Luckner 1997), as well as root (Shimomura et al. 1992) or shoot cultures (Hagimori et al. 1983, Seitz and Gartner 1994). Most scientists have reported that undifferentiated cultured cells either did not produce cardenolides or contained only trace amounts of cardenolides. Results indicated that cardenolide biosynthesis is basically dependent on morphological differentiation (Eisenbeiβ et al. 1999).

Strategies, based on in vitro culture methods, have been extensively used to improve the production of specific plant chemicals by changing nutrients, growth regulators, elicitors (Hagimori et al. 1983, Gavidia and Perez-Bermudez 1997, Debjani and Brattati 2005, Palacio et al. 2008), biosynthetic precursor feeding (Zhao et al. 2001, Silvestrini et al. 2002) and culturing cells or organs in liquid medium systems such as bioreactors (Paek et al. 2005, Jeong et al. 2006, Russowski et al. 2006, Tisserat and Vaughn 2008). There is only scant literature available on *Digitalis* tissue cultures in

which improvements in cardenolide production have been achieved with a specific elicitor, in contrast to what occurs in other plant species in which the addition of many biotic and abiotic agents promotes an increased accumulation of secondary metabolites (Walters and Eilert 1993). In most conditions, this enhancement in production was associated with the generation of ROS, such as H_2O_2 and superoxide radicals. However, there is currently no evidence that relates oxidative stress to secondary metabolite production in *Digitalis* cells.

In our studies, all stress conditions were significantly effective at 5% significance level as compared to the related control group. The predominant cardiac glycoside was Lan C followed by digitoxin, digoxigenin, gitoxigenin and digoxin. On the other hand, in all treatments as well as in control groups, there was no digoxin detected. It is well known that Lan C is transformed into digoxin by deglucosylation using digilanidase in the leaves and subsequent deacetylation (Ikeda et al. 1992). This was probably due to the reason that the amount of digoxin in the callus tissues examined was found to be below the detection limit of the determination used.

There is a lot of evidence that medium salt strength induces to change in growth, physiology and secondary metabolite content in plants. Gavidia and Perez-Bermudez (1997) found that lower salt strength increased cardenolide accumulation in *D. obscura* shoot-tip cultures. A similar observation was also in *H. perforatum* adventitious root cultures, 0.25 and 0.5 MS strengths were suitable for phenols and flavonoids (Cui et al. 2010). Wu et al. (2006) also found that 0.25 and 0.50 MS strengths showed better performance to enhance dry biomass and secondary metabolite accumulation in adventitious root cultures of *Echinacea angustifolia* DC. In our studies, lower salt strength (0.25-0.5 MS) treated callus cultures obtained

higher cardenolide content. Moreover, it was also observed in our experiment that when MS strength was reduced from 0.5 to 0.25, the cardenolide content began to decrease. A similar observation was also made by Gavidia and Perez-Bermudez (1997) who reported that lower concentration of MS major salts were significant for cardenolide content in *D. obscura* shoot-tip cultures. It was also found that when the salt strength was reduced from 0.5 to 0.25, the trend of cardenolide content began to go down. Zhang et al. (2011) reported that 0.5 MS media was more beneficial for ginsenoside production than 0.25 MS media in *Periploca sepium* Bunge adventitious root cultures. This was probably due to the reason that too low macro/micro nutrients content caused by too low medium strength might hinder the synthesis of enzyme related to secondary metabolism, which might restrain the cardenolide synthesis to a certain extent. In other words, the inverse relationship between medium strength and secondary metabolite accumulation could be possibly explained by the fact that full strength media predominantly promote primary metabolism and cellular growth, in some cases hampering morphological and biochemical tissue differentiation.

The effect of different macro elements and their varying concentrations plays an important role in the secondary metabolite production. The elimination of calcium ions from undifferentiated cultures of *D. thapsi* promoted the accumulation of cardenolides (Corchete et al. 1991, Cacho et al. 1995). In our studies, elimination of calcium ions significantly induced cardenolide production in all *Digitalis* species tested. The enhanced production of H_2O_2 and cardenolide accumulation in the absence of calcium, supported by experiments with the chelator and the channel blocker, are striking in the sense that extracellular calcium is necessary for the elicitor-induced formation of active oxygen and phytoalexin biosynthesis in many plant systems (Atkinson et al. 1990, Schwacke and Hager 1992). Although any

analogous study has not been performed yet, our results are in line with several observations reporting an elicitor-induced down-regulation of steroid biosynthesis. Vögeli and Chappell (1988) demonstrated that isoprenoid metabolism in plants is subject to both positive and negative regulation. This conclusion was supported by the fact that when tobacco cultures were challenged with pathogens, a coordinated induction of enzyme activities leading to sesquiterpenoid biosynthesis was accompanied by a coordinated suppression of squalene synthetase, the enzyme that catalyses the first committed step in sterol synthesis. In our system, assuming the existence of a common pool of isoprenoid intermediates, the differential regulation of enzyme activities by the alterations induced in the absence of calcium could thus have contributed to cardenolide production. Mg also acts as activator or regulator of several kinases, ATPases, RuBP carboxylase/oxygenase and several other enzymes of carbohydrate metabolism (Marschner 1995). Thus, deficiency of Mg is likely to decrease the efficiency of Calvin cycle, reduce the utilization of reductive power (NADPH) and cause over-saturation of photosynthetic electron transport system. Under such highly reduced condition, electrons could pass-on to O_2 generating $O_2^{\cdot -}$ and other reactive oxygen species (ROS). There are not any reports of *Digitalis* tissue cultures in which improvements in cardenolide production have been achieved by Mg depletion. Potassium, sulphur and magnesium deficiency are also reported to increase phenolic concentrations (Gerschenzon 1983). Putrescine (Put) accumulation was reported in response to K and Mg deficiencies (Murthy et al. 1971, Smith 1973, Basso and Smith, 1974). Magnesium deficiency is also known to increase Put levels in barley leaves (Smith 1973). In our studies, magnesium deficiency significantly increased cardenolide production in all *Digitalis* species tested, but more information concerning the possible role of Mg in the biosynthesis of cardiac glycosides is

necessary to determine whether this element is directly linked to the metabolic machinery, as happens in the synthesis of terpenoids.

Some researchers have found that exogenous application of H_2O_2 is capable of inducing secondary metabolite formation. Some evidence suggests that H_2O_2 is also functioning as a plant signal molecule (Lamb and Dixon 1997, Pitzschke and Hirt 2006). Expression of defense-related genes such as GST encoding glutathione-S transferase and PAL encoding phenylalanine ammonia lyase, both are induced by H_2O_2 (Levine et al. 1994, Grant et al. 2000). Furthermore, several studies have indicated that H_2O_2 is a key factor mediating programmed cell death (PCD) in response to pathogens, elicitors, and hormones. Araceli et al. (2007) showed that H_2O_2 induced capsidiol accumulation in pepper fruits (*Capsicum annuum* L.). Huerta-Heredia et al. (2009) found that in *U. tomentosa* cell suspension cultures, H_2O_2 stimulated the production of monoterpenoid oxindole alkaloids. A similar observation was also found in our experiment that exogenous application of H_2O_2 induced cardenolide production in all *Digitalis* species. It is assumed that the different forms of ROS interact with molecular targets, which are sensitive to changes in the ROS concentration within and outside the cells and thus transform this information into alterations in gene expression (Laloi et al. 2004). Among all ROS, hydrogen peroxide is the most appropriate molecule for this function because of its longer half-life and higher stability. In our studies, enhancement in cardenolide production was in most cases associated with H_2O_2 pretreatment caused alterations in the activity of gene related to cardenolides.

Copper sulfate and mercuric chloride are usually used to induce secondary metabolite production in plants (Bhuiyan and Adachi 2003, Engelmann et al. 2009,

Hakamatsuka et al. 1991, Liu and Dixon 2001, Keen and Bruegger 1977, Moesta and Grisebach 1980). Korsangruang et al. (2010) found that the effects of $CuSO_4$ in *P Pueraria candollei* cell suspension culture were useful in enhancing the accumulation of isoflavonoid compounds in such cells. Ali et al. (2006) reported that the concentration and the time of Cu exposure affected the growth of *P. ginseng* root cultures, including the ginsenoside content and other enzyme activities. Similar data for increased content of some phenolic compounds after Cu^{2+} treatment were reported also for *Raphanus sativus* L. (Sgherri and Navari-Izzo 2003). Mercuric chloride has been used to stimulate the accumulation of many of the reported phytoalexins and can be considered a universal abiotic elicitor. Phytoalexins produced in plants exposed to $HgCl_2$ include ipomeamarone in sweet potatoes (Uritani et al. 1960), pisatin in peas (Cruickshank and Perrin 1963, Hargreaves 1979), glyceolin in soybeans (Yoshikawa et al. 1978), and medicarpin in jackbean callus (Gustina 1978) and alfalfa (Higgins and Miller 1968); four phytoalexins of the phaseollin family were also produced in bean (Cruickshank et al. 1974, Fraile et al. 1980). Smith et al. (2001) showed that production of umbelliferone, which is a phytoalexin produced in response to stress or infection in whole plants (Modafar et al. 1993), was stimulated in suspension cultures of *Ipomoea batatas* (L.) Poir. using $HgCl_2$. A similar observation was also made by Gustine (1981) who reported that $HgCl_2$ was effective in stimulating the phytoalexin medicarpin in callus cultures of *Trifolium repens* L. Abou Zeid (2002) reported that the antifungal compounds produced in the leaves of *C. olitorius* in response to treatment with heavy metal salts ($HgCl_2$ and $CuCl_2$). It was also found that $CuCl_2$ solution was the most suitable stress agent for the production of phytoalexins compared with $HgCl_2$ stress. A similar observation was also recorded in our experiment that heavy metal stress induced

cardenolide production in callus cultures of *Digitalis* species and $CuSO_4$ treated callus cultures contained higher cardenolide content than $HgCl_2$ applied callus cultures. Generation of reactive oxygen species (ROS) is the major response of plants to toxic concentrations of heavy metals including Cu and Hg (Schützendübel and Polle 2002). ROS levels are controlled by the antioxidant system which includes enzymes and secondary metabolites (Schützendübel and Polle 2002, Inmaculada 2005). This enhancement in production was in most cases associated with the generation of ROS.

UV light acts as an abiotic factor which stimulates the biosynthesis of secondary metabolites (Broeckling et al. 2005). Accumulation of UV-absorbing compounds, mainly those of phenolic nature, is a typical defense mechanism of plants to increased UV radiation, and is the most common response produced by vascular plants (Searles et al. 2001). Thus, it has been shown that UV-B light induces both the formation of dimeric terpenoid indole alkaloids and tryptophan decarboxylase and strictosidine synthase mRNA accumulation in *C. roseus* (Ouwerkerk et al., 1999). Ramani and Jayabaskaran (2008) also observed the enhanced production of catharanthine and vindoline from *C. roseus* cell cultures, when cells were irradiated with UV-B for 5 min. In a similar way, Gläβgen et al. (1998) studied the effect of continuous irradiation with UV-containing white light (315-420 nm) on the anthocyanin content of *Daucus carota* L. cell cultures after 7 days of culture, and observed that the total anthocyanin concentration was strongly enhanced by the UV treatment. Ku et al. (2005) found that synthesis of piceatannol and resveratrol were promoted by UV radiation in callus cultures of groundnut. Schmidlin et al. (2008) recorded that a whole range of stilbene derivatives, including resveratrol, were induced in leaves of grapevine by UV stress. Likewise, Zagoskina et al. (2003, 2005)

found phenolics in callus cultures of the tea plant to be increased by UV-B radiation. It has been concluded that the content of carthamin in *Carthamus* flowers can be increased by ultraviolet radiation. Hajnos et al. (2001) studied the effect of ultraviolet radiation on the accumulation of two terpenoids, 10-DAB III and paclitaxel, in *Taxus bacchata* and found that the amounts of both were increased during 48 h of UV irradiation of excised twigs. According to Lamikanra et al. (2002), UV treatment induced the production of the following terpenoids: β-cyclocitral, cis- and trans- β-ionone, terpinyl acetate, geranylacetone and dihydroactinidiolide in cantaloupe melon (*Cucumis melo* L. var. *reticulatus*). It was also observed in our experiment that cardenolide production was promoted by UV radiation in callus cultures of *Digitalis* species. The induction of secondary metabolite production by higher plant tissues is widely recognized in response to UV treatment and other plant stressors. Depending on the plant, the specific metabolite and the stressor, variation in metabolite production over time is frequently observed. It is hypothesized that these metabolites are often unstable and are typically degraded and recycled in the plant. In other studies evaluating UV-induced responses in secondary product formation by higher plants, similar trends have been observed.

Temperature stress can cause many physiological, biochemical and molecular changes in plant metabolism such as protein denaturation, lipid liquefaction or perturbation of membrane integrity (Levitt 1980b). Many of these changes can alter the secondary metabolites concentrations in the plant tissues. Zobayed et al. (2005) found that high temperature stress in *St. John's wort* significantly increased the secondary metabolite concentrations in the shoot tissues. Nacif de Abreu and Mazafera (2005) also found that environmental factors, particularly temperature, have a marked influence on the content of pharmacologically active secondary

metabolites in *Hypericum brasiliense* Choisy. By contrast, in *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. somatic embryos, high (30 ^{0}C) temperature caused significant decrease in total phenolics, flavonoids and total eleutheroside accumulation (Shohael et al. 2006). The results presented in this thesis showed that high temperature significantly increased cardenolide production in *Digitalis* callus cultures. The increase in secondary metabolite concentrations in the callus cultures under high temperature stress may also be associated with the partitioning and translocation of carbon to incorporate into carbon based secondary compounds. Under stress condition, carbon may be available for the biosynthesis of these compounds as compared to the carbon demand associated with plant growth. Transpiration rate is known to be significantly influenced by temperature and thus transpiration rate may be also responsible to modify concentrations of the active constituents in *Digitalis* plant. It is also known that drought stress induces the biosynthesis of isopentenyl pyrophosphate, the primary precursor of terpenes in plants, with a consequent increase in abscisic acid, carotenoids and xanthophylls (Milborrow 2001, Turtola et al. 2003).

4.2 CAT and SOD Activities

It is well known that exposure of plants to abiotic factors causes oxidative stress in which increased production of reactive oxygen species (ROS) is evident (Aroca et al, 2001). Plants protect cells and subcellular systems from the effects of ROS by enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase, glutathione reductase, polyphenol oxidase and non-enzymic antioxidants such as ascorbate, glutathione, prolin and phenolics.

It is also known that exposure of plants to abiotic factors including low and high temperature causes oxidative stress in which increased production of reactive oxygen species (ROS) is evident (Aroca et al. 2001). An increase in the activities of CAT and SOD was observed in three mulberry genotypes (*Morus alba* L.) under high temperature (Chaitanya et al. 2001). Lu et al. (2008) found that *Eupatorium adenophorum* Spreng. and *E. odoratum* L. exhibited an increase in SOD activity in both heat and cold stresses. It was also recorded that CAT activity was depressed by high temperature in leaves of *E. adenophorum* while CAT activity increased in leaves of *E. odoratum*. An increase in SOD activity with a decrease in CAT activity has been reported when the plants were subjected to abiotic stress (Saruyama and Tanida 1995, Fu and Huang 2001, Jung 2003). Others have found that CAT activity is photoinactivated under low and high temperature stresses and CAT activity varies with plant species (Feierabend et al. 1992). Shohael et al. (2006) observed that high temperature significantly decreased antioxidant enzymes activities in somatic embryos of *E. senticosus*. A similar observation was also concluded in our experiment that CAT and SOD activities decreased in callus cultures of all *Digitalis* species treated with high temperature. The decrement in SOD and CAT activity at high temperature stress might be due to the inactivation of these enzymes. Although several environmental stresses in plants are known to induce the expression and/or increasing levels of antioxidative enzymes and their mRNAs (Pan and Yau 1992, Kliebenstein et al. 1998, Morita et al. 1999), the mechanism by which these activated oxygen-scavenging enzymes can work co-operatively, in response to temperature stress is not yet known. Further analysis of the regulation of gene expression in these enzymes should elucidate the mechanism of different temperature tolerances.

Activation of antioxidant enzymes by UV has earlier been observed in several plant species with respect to SOD, CAT, APX, POD and GR (Rao et al. 1996). Our results clearly demonstrated that in *Digitalis* species subjected to UV radiation, CAT and SOD increased markedly. Changes in SOD activity and overproduction of H_2O_2 as a result of exposure to UV-B radiation in sunflower cotyledons (Yannarelli et al. 2006) and *Picea asperata* Mast. seedlings (Han et al. 2009) were observed. This suggests that under UV stress H_2O_2 was formed intensively as a result of enzymatic superoxide anion dismutation. A similar study carried out in rice (*Oryza sativa* L.) leaves demonstrated that after supplemental UV-B radiation, SOD and CAT activities were enhanced (Dai et al. 1997). Mahdavian et al. (2008b) also found that UV-B and UV-C treatments also increased CAT and SOD activities in both leaf and root of *C. annuum*. Activation of antioxidant enzymes by UV-B was observed in *Arabidopsis thaliana* (L.) Heynh. (Rao et al., 1996), wheat (Sharma et al. 1998), and cucumber (Takeuchi et al. 1996). Rybus-Zając and Kubiś (2010) also reported that UV-B irradiation stress induced increased CAT and SOD activity in cucumber. Similar changes were reported by Baumbusch et al. (1998), Kubo et al. (1999) and Kubiś and Rybus-Zając (2008). The results of the present work illustrated that in *Digitalis* species, UV radiation generates antioxidant substances that provide protection against UV radiation.

It has been proved that pretreatment with H_2O_2 causes alteration in the activity of several antioxidant enzymes and/or antioxidants. Increased data evidence the biological activity of H_2O_2 as a stress signal molecule in plants (Dat et al. 2000, Overmyer et al. 2003, Hung et al. 2005). Available information suggests that H_2O_2 directly regulates the expression of numerous genes involved in plant defense and the related pathways such as antioxidant enzymes, defense proteins and transcription

factors (Kovtun et al. 2000, Robert and David 2004, Hung et al. 2005). A minor increase in the activity of SOD was detected in the H_2O_2-treated pea seedlings while treatment with H_2O_2 did not influence activity of catalase (Moscova et al. 2009). In contrast, SOD and CAT activity increased in wheat seedlings treated with exogenous H_2O_2 (Li et al. 2011). He et al. (2009) showed that H_2O_2 pretreatment enhanced higher expression of CAT in (*Triticum aestivum* L.) seedlings. Liu et al. (2010) proposed that the combined effect of osmotic stress and exogenous H_2O_2 resulted in the highest antioxidant activities in both cucumber ecotypes. A similar observation was also found in our experiment that SOD and CAT activity significantly increased in *Digitalis* species callus cultures treated with H_2O_2. The present data suggested that H_2O_2, a stress signal, could trigger the activation of antioxidants.

The activities of antioxidative enzymes were generally enhanced by the deficiencies of different macronutrients and associated with an increased production of ROS. ROS were hypothesized to serve as subcellular messenger in gene regulatory and signal transduction pathways (Bowler et al. 1992, Foyer and Noctor 2000, Allen and Tresini 2000) and have been implicated in the modulation of gene expression by redox mediated transcription factors (Dinesh-Kumar et al. 1995). Tewari et al. (2004) showed that deficiencies of P, K and Ca increased the activity of CAT significantly, while the effect of N, Mg or S deficiency on CAT activity was insignificant and SOD activity was significantly enhanced due to deficient supplies of all macronutrients except Ca, where the increase in SOD activity was insignificant in *Zea mays* L. They also found that deficiencies of each N, K and Ca induced a new isoform of SOD while P and Mg caused induction of three new SOD isoforms. It was suggested that while SOD activity increased due to the deficiency of each of the macronutrients, induction of new SOD isoforms appeared. Though, Schmitz-Eiberger et al. (2002)

reported that decrease in SOD activity in Ca deficient tomato plants, enhancements in SOD activity in plants deficient in Ca (Paranhos et al. 1999), Mg (Cakmak and Marschner 1992, Candan and Tarhan 2003) and N (Logan et al. 1999) were also reported earlier. Paranhos et al. (1999) showed that while SOD activity was significantly increased in Ca deficient undifferentiated cell cultures of *D. thapsi*, CAT activity was decreased. CAT and SOD activity were significantly increased in the leaves of Mg-deficient in mulberry plants (Tewari et al. 2006).

In our studies, elimination of calcium and magnesium ions significantly induced CAT and SOD activity in all *Digitalis* species. Moreover, it was also found in our experiment that CAT and SOD activity were highest when both Ca and Mg were eliminated from the medium. This was probably due to the reason that enhanced ROS generation due to macronutrient deficiencies was responsible for transcriptional activation of genes responsible for antioxidative enzymes.

There are many reports concerning the response of the antioxidant systems in plants to metal-induced oxidative stress. The strong effect of heavy metals on oxidative processes (Gratao et al. 2008) is the base for other connections with signaling response. One of the major consequences of heavy metals action is enhanced production of ROS. In the light of earlier studies, it was concluded that H_2O_2 increase occurred after Cu, Cd (Drazkiewicz et al. 2004, Romero-Puertas et al. 2004, Maksymiec and Krupa 2006) and Hg (Cho and Park 2000) treatment of *A. thaliana* and tomato plants, respectively. The increase in SOD and decrease in CAT activities response to an excess supply of heavy metals were reported (Chaoui et al. 1997, Pandey and Sharma 2002, Cho and Seo 2005, Lombardi and Sebastiani 2005, Pandey et al. 2009). Ali et al. (2006) showed that while the activity of SOD was enhanced

with Cu, CAT activity remained unchanged in the suspension culture of *P. ginseng*. In a different direction, Yürekli and Porgalı (2006) reported that Cu treatment increased the activity of superoxide dismutase and catalase in leaf tissue of bean seedlings (*Phaseolus vulgaris* L. cv. Akman). Lotmani and Mesnoua (2011) found one new SOD isoforme and two new CAT isoformes in *Atriplex halimus* L. leaves as response to high concentration of Cu and the new bands found suggested that stress of copper, stimulate the expression of a novel genes of SOD involved in plant defense. Substantial increases of CAT and SOD were observed in Hg-stressed plants in *Lycopersicon esculentum* Mill. (Cho and Park 2000). A similar observation was also found in our studies that CAT and SOD activity were significantly increased with the $CuSO_4$ and $HgCl_2$. According to Romero-Puertas et al. (2004), many heavy metals could result in increased NADPH oxidase activity partially connected with increased of O_2^- formation. Correspondingly, heavy metal-induced O_2^- formation could cause to transcriptional activation of genes responsible for antioxidative enzymes. The present study clearly indicated that Cu and Hg exposure resulted in an increase in H_2O_2 content in plants. H_2O_2 formation could be associated with an increased activity of SOD for O_2^- conversion and parallel increased activity of CAT.

Numerous reports proposed the significant role of medium salt strength to improve biomass production and secondary metabolite accumulation in cell cultures of several plant species. But no efforts have been paid to investigate the impact of medium salt strength on the antioxidant system. CAT and SOD activity was found to be significantly higher at lower salt strength (0.25) in adventitious roots of *Morinda citrifolia* L. (Baque et al. 2010). In our study, SOD activity was significantly enhanced in callus cultures treated with 0.5-0.25 MS. This was accompanied by increase in CAT. Besides, CAT activity was also found to be significantly higher at

lower salt strength. These data suggest that, possibly, CAT mitigated the toxic effects of H_2O_2 at low salt strength treated callus cultures.

4.3. Total Phenolic Content

Phenolics, including flavonoids, anthocyanins, lignins, etc., are the most important class of secondary metabolites in plants and play a variety of roles including tolerance to abiotic stresses (Chalker-Scott 2002, Wahid and Ghazanfar 2006, Wahid 2007).

High-temperature stress induces production of phenolic compounds such as flavonoids and phenylpropanoids. Phenylalanine ammonia-lyase (PAL) is considered to be the principal enzyme of the phenylpropanoid pathway. Increased activity of PAL in response to thermal stress is considered as the main acclimatory response of cells to heat stress. Thermal stress induces the biosynthesis of phenolics and suppresses their oxidation, which is considered to trigger the acclimation to heat stress for example as in watermelon, *Citrullus vulgaris* Schrad. ex Eckl. & Zeyh. (Rivero et al. 2001). A similar observation was also found in our studies that temperature stress induced total phenolic content in *Digitalis* species. Our studies suggested that accumulation of soluble phenolics under heat stress was accompanied with increased phenyl ammonia lyase (PAL).

Levizou and Manetas (2002) showed significant correlations between total phenolic levels and UV absorbing capacity. Studies of vascular terrestrial plants showed that UV-B (280–320 nm) stimulates the production of shikimate pathway metabolites including phenylpropanoids and flavonols (Tevini 1993), that may serve as UV-screening compounds (Reuber et al. 1996). The increase in phenolic compounds after UV exposure was reported in *Chlamydomonas nivalis* (Bauer) Wille (Duval et al.

2000). A similar observation was also found by Liu et al. (1995), who report slight increases in UV-A induced flavonoid accumulation in barley, but greater secondary phenolic induction after exposure to shorter wave-length UV. Ballare et al. (1995) also reported 15–20% increases in flavonoid accumulations in ethanol extracts of tomatoes exposed to continuous UV-B light. Recent studies reported an increase of phenolic compounds and corresponding antioxidant activity in berry fruits, resulting from an UV-B mediated stress response (Huyskens-Keil et al. 2007). Eichholz et al. (2011) also reported that UV-B exposure resulted in an increase in phenolic compounds of *Vaccinium corymbosum* L. Likewise Zagoskina et al. (2005) found phenolics in callus cultures of the tea plant to be increased by UV-B radiation. It was assumed that UV stress mediated the increase of phenylalanine ammonia–lyase activity (PAL), the key enzyme of the phenolpropanoid metabolism, leading to an acceleration of the biosynthesis of phenolic compounds (Pluskota et al. 2005). In our studies, UV stress induced the marked increase of total phenolic content in *Digitalis* species. This was probably due to the reason that UV radiation stimulated gene transcription and expression of key enzymes lead to the induction of phenolic UV-absorbing compounds.

Stress factors often induce oxidative stress in plant cells generally leading to the synthesis of signal molecules that activate a range of signal transduction pathways and antioxidants. One of the signal molecules produced under stress is H_2O_2, which has been associated to production in vitro of secondary metabolites and to antioxidant activity. H_2O_2 contents increased under both biotic and abiotic stresses and induced expression of PAL (Levine et al. 1994, Grant et al. 2000) and studies suggested that accumulation of soluble phenolics under biotic and abiotic stress was accompanied with increased phenyl ammonia lyase (PAL). Although, numerous

reports proposed the effects of exogenous application of H_2O_2 to improve antioxidant enzymes and secondary metabolite production, no study has been performed to clear that relationship between total phenolic content and exogenous application of H_2O_2. In present study, total phenolic content of *Digitalis* species was enhanced by exogenous addition of H_2O_2. According to information given above, accumulation of soluble phenolics under H_2O_2 stress was accompanied with increased PAL.

Nutrient stress also has a marked effect on phenolic levels in plant tissues (Chalker-Scott and Fuchigami 1989). Potassium, sulphur and magnesium deficiency was also reported to increase phenolic concentrations (Gerschenzon 1983). Removing calcium ions in callus and cell suspension cultures of *Silybum marianum* L. Gaertn. promoted flavonolignan accumulation (Cacho et al. 1999a). Ca^{2+} deficiency induced anthocyanin accumulation in cabbage (Bassim and Pecet 1975). The metabolism of *Prunus* callus tissues in vivo was affected by Ca^{2+} deficiency and responded with an accumulation of phenolic stress metabolites (Yuri et al. 1990). In our studies, Ca and Mg deficiency led to increased total phenolic content in *Digitalis* species. It could be considered that the accumulation of phenolic compounds was the consequence of an increased PAL activity.

Numerous reports proposed the significant role of medium salt strength to improve secondary metabolite accumulation in cell cultures of several plant species. Secondary metabolites, phenolic compounds in particular, are involved in plant response to biotic and abiotic stresses and provide a significant contribution to the antioxidant activity of plant tissues (Ferrat et al. 2003). The highest average phenolic content was observed on *Mentha spicata* L. shoot and root extracts cultured onto quarter and half strength medium (Fadel et al. 2010). A similar observation was also

made by Baque et al. (2010) who reported that 0.25 MS treated culture was significant for total phenolics and flavonoids in *M. citrifolia* adventitious roots cultures. It was also found that half-strength MS medium was effective to enhance phenol synthesis in adventitious roots of *Echinacea angustifolia* DC. (Wu et al. 2006). In our studies, higher total phenolic content was obtained in lower salt strength (0.25-0.5 MS) treated callus cultures. Most natural phenolic compounds in plants are derived from trans-cinnamic acid formed by deamination of L-phenylalanine by L-phenylalanine ammonia-lyase (PAL) (Boudet 2007). We observed stimulatory effects of medium salt strength on PAL activity in relation to accumulation of phenolic compound.

An increase of phenolics correlated to the increase in activity of enzymes involved in phenolic compounds metabolism was reported, suggesting de novo synthesis of phenolics under heavy metal stress. Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (Jung et al. 2003). The roots of many plants exposed to heavy metals exude high levels of phenolics (Winkel-Shirley 2002). The content of free phenols was found to increase in two lines of wheat (*T. aestivum*) with increasing Cu^{2+} concentration in the medium. (Ganeva and Zozikova 2007). Similar data for increased content of some phenolic compounds after Cu^{2+} treatment were reported also for *R. sativus* (Sgherri et al. 2003). Cu was observed to induce leaf accumulation of total soluble phenolics (Santiago et al. 2000). A strong increase of total thiols and phenolics was found in Hg treated roots of *Lupinus albus* L. (Esteban et al. 2008). In our studies, the increase in phenolic levels observed in the callus cultures of *Digitalis* species under Cu and Hg exposure could be a combined strategy to reduce the oxidative stress caused by Cu and Hg.

4.4. Proline Content

Proline is such an antioxidant which accumulates in response to biotic and abiotic stresses, including water stress (Zhang et al. 1995), salt stress (Fedina et al. 2006), extreme temperatures (Ruiz et al. 2002) and heavy metal toxicity (Chen et al. 2001). Stress induced proline accumulation in plants can arrest photoinhibitory damage by reducing photochemical activity losses and the production of free radicals in the thylakoid membrane of the chloroplast (Alia et al. 1991).

Duval et al. (2000) showed that free proline was not affected by UV-A, but increased markedly after UV-C exposure. Increments in proline and phenol contents in *Acorus calamus* L. (Sweet flag) were observed due to UV-B (Kumari et al. 2010). UV-B and UV-C increased proline concentrations in leaves of *C. annuum* (Mahdavian et al. 2008a). The proline accumulated in the seedling shoots of rice (*Oryza sativa*), mustard (*Brassica juncea*) and mung bean (*Vigna radiate*) exposed to UV-B radiation was studied by Saradhi et al. (1995). They concluded that the level of proline in the seedling increased significantly with increase in UV-B exposure time. Salama et al. (2011) showed that the different levels of UV radiation induced increases in shoot and root proline contents in *Malva parviflora* L., *Plantago major* L., *Rumex vesicarius* L. and *Sisymbrium erysimoides* Desf. UV induced proline accumulation may be critical for stimulating the pentose phosphate pathway in order to provide key precursors for the phenylpropanoid pathway (Shetty 1997, Kwok and Shetty 1997). The removal of excess H^+ occurring as a result of proline synthesis may have a positive effect on reduction of the UV induced damage. This lead us to believe that UV radiation induced proline accumulation protects plants against UV

radiation promoted peroxidation processes. In the light of these information, UV radiation induced the level of proline content in callus cultures of *Digitalis* species.

Proline accumulation in high temperature was reported in barley and radish leaves (Chu et al. 1974), in tomato floral buds and leaves (Kou et al. 1986), 1.5-fold high in mulberry leaves (Chaitanya 2001), in cotton leaves (Ronde et al. 2001), in cabbage and Chinese cabbage (Hossain et al. 1995), in apple (Park et al. 2001), in flag leaves of wheat (Hasan et al. 2007) and in *Eucalyptus grandis* W. Hill shoots (Souza et al. 2004). A similar observation was also found in our experiments that high temperature induced proline content in all *Digitalis* species. The relationship between proline accumulation and environmental stress suggested that proline could have some protective function. The accumulation of free proline might also contribute to the scavenging of heat stress induced active oxygen species by enhancing photochemical electron transport activities (Alia et al. 1991).

The slight increase in proline concentration was observed in H_2O_2-treated leaf samples of *O. sativa* (Upadhyaya et al. 2007). Ozden et al (2009) showed that treatment of samples with H_2O_2 caused to an increase in endogenous proline accumulation in leaves of grapevine. He et al. (2009) found that H_2O_2 pretreatment significantly enhanced free proline content in *Triticum aestivum* L. seedlings. A similar observation was also made by Yang et al. (2009) who reported that exogenous H_2O_2 treatment led to a significant accumulation of proline in maize seedlings. Verslues et al. (2007) found that increased H_2O_2 levels stimulated ABA-induced proline accumulation and gene expression in *Arabidopsis*, implying that H_2O_2 signaling may participate in proline biosynthesis. Our present results clearly showed that H_2O_2 treatment induced a rapid accumulation of proline in *Digitalis*

species. The present data suggested that H_2O_2 could regulate enzyme activities of proline metabolism.

Under several conditions of stress such as macronutrient deficiency, the plant is forced to accumulate a large quantity of free proline. Deficiencies in N, P, Mg and Ca resulted in an increase in proline content of leaves of cowpea (*Vigna unguiculata* (L.) Walp.) compared to full nutrition (Somal and Yapa 1998). Deficiency of B, Ca, P, K, Mg, and S caused an increase in proline content in different parts (root, stem, and leaf) of the tobacco plants (*N. tabacum*) (Tso and McMurtrey 1960). In our studies, Ca and Mg deficiency led to increased in proline content in *Digitalis* species. The higher proline levels in plants under stress have been linked to enhanced activities of pyrroline 5-carboxylate reductase (P5C reductase), the enzyme involved in proline biosynthesis (Charest and Pan 1990, Kohl et al. 1990). The present data suggested that Ca and Mg deficiency could regulate enzyme activities of proline metabolism.

Many plants accumulate high concentrations of proline when treated with toxic concentrations of heavy metals (Bassi and Sharma 1993ab, Costa and Morel 1994, Schat et al. 1997). Some researchers assume that proline accumulation is a symptom of injury which does not confer protection against metal stress (Lutts et al. 1996). On the contrary, it has been suggested that proline might protect plants from heavy metal toxicity (Mehta and Gaur 1999, Siripornadulsil et al. 2002). Mahta and Gaur (1999) reported that exposure of *Chlorella vulgaris* Buitenzorg to copper, chromium, nickel and zinc led to accumulation of free proline. Wang et al. (2009) also showed that 12 h of the Hg treatment increased the content of proline in rice. It was also found that proline content in sunflower (*Helianthus annuus* L.) leaves increased with the

increase of copper concentration (Zengin and Kirbag 2007). Zengin and Munzuroglu (2005) showed that copper and mercury toxicity increased the proline content of the leaves of bean (*Phaseolus vulgaris* L.) seedlings. A similar observation was also recorded in our experiment that heavy metal stress induced proline content in callus cultures of *Digitalis* species. The present observations showing a positive relationship between metal toxicity and proline accumulation suggest a protective role of this amino acid against heavy metal toxicity.

In many plants, free proline also accumulates in response to a wide range of biotic and abiotic stresses, including nutrient deficiency. In general, although only scant literature is available on this subject, it appears that there was a positive relationship between MS strength and proline accumulation. In our studies, lower salt strength (0.25-0.5 MS) treated callus cultures obtained higher proline content. Lattanzio et al. (2009) reported that nutritional deficiency (0.5 MS) caused to increase of free proline in shoot cultures of oregano (*Origanum vulgare* L.). As a consequence of the imposed nutritional stress, plant tissues exhibit an increase of proline that enhances the tolerance of cellular components to reactive oxygen species (ROS), synthesized by plants experiencing stress conditions (Smirnoff 1993).

CHAPTER 5.

CONCLUSIONS

Commercial production of bioactive secondary metabolites by traditional agriculture is an inefficient process. Strategies, based on in vitro culture methods, have been extensively studied to improve the production of specific plant derived chemicals. In this work, we reported the effect of stress factors as an abiotic stimulus used to promote secondary metabolite accumulation on the endemic Turkish *Digitalis* species (*D. lamarckii*, *D. trojana*, *D. davisiana* and *D. cariensis*). The results revealed that *Digitalis* cardenolides were induced rapidly in callus cultures under media strength stress, macronutrient deficiency stress, oxidative stress, chemical stress, temperature stress and UV stress. The protocol described here can have an important contribution to the future efforts for a large scale production of cardenolides of these endemic medicinal species.

Besides, there was an increased accumulation of total phenolic and proline content stimulated by stress factors in our study. It was concluded that the higher increase in total phenolic and proline content might be the main determining factor in protective mechanism against stress factors. It was also found that CAT and SOD activities were markedly induced under all stress conditions in all *Digitalis* species, except for high temperature treatment which resulted in lower CAT and SOD activities. The different responses of antioxidant enzymes to high temperature stress in contrast to

other stress treatments could be possibly explained by the temperature sensitivities of *Digitalis* species.

We expect that further analysis of cardenolides and antioxidant molecules will provide insights into the regulatory relationships among these molecules and the role of these molecules in the establishment of mechanism for cardenolide production. The results presented in the thesis regarding the analysis of cardenolides under stress conditions are believed to create a useful base for the future studies in understanding the antioxidant mechanism which can be employed for the improvement of a large scale production of cardenolides.

REFERENCES

Abou-Zeid AHS (2002). Stress metabolites from *Corchorus olitorius* L. leaves in response to certain stress agents. Food Chemistry 76:187-195.

Alfermann AW, Petersen M (1995). Natural products formation by plant cell biotechnology. Plant Cell Tissue Org Cult 43:199-205.

Ali MB, Hahn E-J, Paek K-Y (2006). Copper-induced changes in the growth, oxidative metabolism, and saponin production in suspension culture roots of *Panax ginseng* in bioreactors Plant Cell Rep 25: 1122–1132.

Alia A, Saradhi PP, Mohanty P (1991). Proline enhances primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photo inhibitory damage. Biochem Biophysic Res Commun 181:1238-1244.

Allen RG, Tresini M (2000). Oxidative stress and gene regulation, Free Radic Biol Med 28:463-499.

Aracelia A-C, Eldaa C-M, Edmundob L-G, Ernesto G-P (2007). Capsidiol production in pepper fruits (*Capsicum annuum* L.) induced by arachidonic acid is dependent of an oxidative burst. Physiological and Molecular Plant Pathology 70:69-76.

Aroca R, Irigoyen JJ, Sanchez-Diaz M (2001). Photosynthetic characteristics and protective mechanisms against oxidative stress during chilling and subsequent recovery in two maize varieties differing in chilling sensitivity. Plant Sci 161: 719-726.

Atkinson MM, Keppler LD, Orlandi WE, Baker CJ, Mischke CF (1990). Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive responses in tobacco. Plant Physiology 92:215-221.

Balandrin MJ, Klocke JA (1988). Medicinal, aromatic and industrial materials from plants. In: Bajaj YPJ (ed). Biotechnology in agriculture and forestry. Medicinal and aromatic plant, Berlin, Springer-Verlag, pp 4:1-36.

Ballare CL, Barnes PW, Flint SD, Price S (1995). Inhibition of hypocotyl elongation by ultraviolet-B radiation in de-etiolating tomato seedlings. II. Time-course, comparison, with flavonoid responses and adaptive significance. Physiol Plant 93: 593–601.

Bandurska H (1993). In vitro and in vivo effect of proline on nitrate reductase activity under osmotic stress in barley. Acta Physiol Plant 15:83-88.

Baque MA, Lee E-J, Paek K-Y (2010). Medium salt strength induced changes in growth, physiology and secondary metabolite content in adventitious roots of *Morinda citrifolia*: the role of antioxidant enzymes and phenylalanine ammonia lyase. Plant Cell Rep 29:685–694.

Bassi R, Sharma SS (1993a). Changes in proline content accompanying the uptake of zinc and copper by *Lemna minor*. Annals of Botany 72:151-154.

Bassi R, Sharma SS (1993b). Proline accumulation in wheat seedlings exposed to zinc and copper. Phytochemistry 33:1339-1342.

Bassim TAH, Pecket RC (1975). The effect of membrane stabilizers on phytochrome-controlled anthocyanin biosynthesis in *Brassica oleraceae*. Phytochemistry 14:731–733.

Basso LC, Smith TA (1974). Effect of mineral deficiency on amine formation in higher plants. Phytochemistry 13:875-883.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water-stress studies. Plant Soil 39:205-207.

Baumbusch LO, Eiblmeier M, Schnitzler JP, Heller W, Sandermann H, and Polle JA (1998). Interactive effects of ozone low UV-B radiation on antioxidants in spruce (*Picea abies*) and pine (*Pinus silvestris*) needles. Physiologia Plantarum 104:248-254.

Berger S, Sinha AK, Roitsch T (2007a). Plant physiology meets phytopathology: plant primary metabolism and plant-pathogen interactions. Journal of Experimental Botany 58:4019-4026.

Bhuiyan MNH, Adachi T (2003). Stimulation of betacyanin synthesis through exogenous methyl jasmonate and other elicitors in suspension-cultured cells of *Portulaca*. J Plant Physiol 160:1117-1124.

Bohm H, Rink E. Betalaines (1988). In: Constabel F, Vasil I (eds). Cell culture and somatic cell genetics of plants, Academic Press,New York, pp 5:449-63.

Bohnert HJ, Jensen RG (1996). Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14:89-97.

Boudet AM (2007). Evolution and current status of research in phenolic compounds. Phytochem 68:2722-2735.

Bowler C, Van Montagu M, Inzé D (1992). Superoxide dismutase and stress tolerance. Ann Rev Plant Physiol Plant Mol Biol. 43:83-116.

Boyer JS (1982). Plant productivity and environment. Science 218:443-448.

Bräuchler C, Meimberg H, Heubl G (2004). Molecular phylogeny of the genera *Digitalis* L. and *Isoplexis* (Lindley) *Loudon* (Veronicaceae) based on ITS and *trn*L-F sequences. Plant Syst Evol 248:111-128.

Bray EA, Bailey-Serres J, Weretilnyk E (2000). Responses to abiotic stresses. In: Gruissem W, Buchannan B, Jones R (eds). Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, MD, pp 1158-1249.

Broeckling CD, Huhman DV, Farag MA, Smith JT, May GD,Mendes P, Dixon RA, Sumner LW (2005). Metabolic profiling of *Medicago trunculata* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. Journal of Experimental Botany 53:323-326.

Cacho M, Morán M, Corchete P, Fernández-Tárrago J (1999a). Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L.) Gaertn. Plant Sci 144:63-68.

Cacho M, Morán M, Corchete P, Fernández-Tárrago J (1999b). Effect of calcium restriction on cardenolide accumulation in two cell lines of *Digitalis thapsi* grown under different light regimes. Acta Physiol Plant 21:335-340.

Cacho M, Morán M, Fernández-Tárrago J, Corchete P (1995). Calcium restriction induces cardenolide accumulation in cell suspension cultures of *Digitalis thapsi* L. Plant Cell Rep 14:786-789.

Cakmak I, Marschner H (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. Plant Physiol 98: 1222-1227.

Cakmak, I (1994). Activity of ascorbate-dependent H_2O_2-scavenging enzymes and leaf chlorosis are enhanced magnesium and potassium-deficient leaves, but not in phosphorus-deficient leaves. J Exp Bot 45:1259-1266.

Candan N, Tarhan L (2003). Relationship among chlorophyll-carotenoid content, antioxidant enzyme activities and lipid peroxidation levels by Mg^{2+} deficiency in the *Mentha pulegium* leaves. Plant Physiol Biochem 41:35-40.

Chaitanya KV, Sundar D , Reddy AR (2001). Mulberry leaf metabolism under high temperature stress. Biol Plant 44:379-384.

Chaitanya KV, Sundar D, Masilamani S, Ramachandra-Reddy A (2001). Variation in heat stress-induced antioxidant enzyme activities among three mulberry cultivars. Plant Growth Regulation 00: 1-6.

Chalker-Scott L (2002). Do anthocyanins function as osmoregulators in leaf tissues? Adv Bot Res 37:103–106.

Chalker-Scott L, Fuchigami LH (1989). The role of phenolic compounds in plant stress responses. In Paul HL (ed). Low Temperature Stress Physiology in Crops. CRC Press Inc, Boca Raton, Florida, 27-40.

Chaoui A, Mazhoudi MH, Ghorbal S, Ferjani EE (1997).Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). Plant Sci 127:139-147.

Charest C, Pan CT (1990). Cold acclimation of wheat (*Triticum astivum*): Properties of enzymes involved in proline metabolism. Physiol Plant 80:159-168.

Chaudiere J, Ferrari-Iliou R (1999). Intracellular antioxidants: from chemical to biochemical mechanisms. Food Chem Tox 37:949-962.

Chen CT, Chen LM, Lin CC, Kao CH (2001). Regulation of Proline Accumulation in Detached Rice Leaves Exposed to Excess Copper. Plant Sci 160:283-290.

Cho U, Park J (2000). Mercury induced oxidative stress in tomato seedlings. Plant Science 156:1-9.

Cho U-H, Seo N-H. (2005). Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Sci 168: 113-120.

Chu TM, Aspinall D, Paleg LG (1974). Stress metabolism. VI. Temperature stress and the accumulation of proline in barley and radish. Aust J Plant Physiol 1:87-89.

Clemens S (2001). Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212:475-486.

Clemente ES, Müller-Uri F, Nebauer SG, Segura J, Kreis W, Arrillaga I (2011). Wild Crop Relatives: Genomic and Breeding ResourcesPlantation and Ornamental Crops. In: Kole J (ed). *Digitalis.* Springer, Berlin, Heidelberg, pp 73-112.

Clijsters H, Cuypers A, Vangronsveld J (1999). Physiological responses to heavy metals in higher plants; defence against oxidative stress. Zeitschrift fur Naturforschung 54:730-734.

Corchete P, Jimenez MA, Morán M, Cacho M, Fernández-Tárrago J (1991). Effect of calcium, manganese and lithium on growth and cardenolide content in cell suspension cultures of *Digitalis thapsi* L. Plant Cell Rep 10:394-396.

Corchete P, Sanchez JM, Cacho M, Moran M, Fernández-Tárrago J (1990). Cardenolide content in suspension cell cultures derived from leaf and root callus of *Digitalis thapsi* L. Journal of Plant Physiology 137: 196-200.

Costa G, Morel J-L (1994). Water relations, gas exchange and amino acid content in cadmium-treated lettuce. Plant Physiology and Biochemistry 32:561-570.

Courtois D, Guren J (1980). Temperature response of *Catharanthus roseus* cells cultivated in liquid medium. Plant Sci Lett 17:473-482.

Cox PA, Balick MJ (1994).The ethanobotanical approach to drug discovery. Sci Am 270:82-87.

Cruickshank I, Biggs DR, Perrin DR, Whitle CP (1974). Phaseollin and phaseollidin relationships in infection droplets on endocarp of *Phaseolus vulgaris*. Physiol Plant Pathol 4: 261-276

Cruickshank IAM, Perrin DR (1963). Studies on phytoalexins. VI. Pisatin: the effect of some factors on its formation in *Pisum sativum* L. and the significance of pisatin in disease resistance. Aust J Biol Sci 16: 111-128.

Cui XH, Charkrabarty D, Lee EJ, Paek KY (2010). Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. Bioresour Technol 101:4708-4716.

Cushman JC, Bohnert HJ (2000). Genomic approaches to plant stress tolerance. Curr Opin Plant Biol 3:117-124.

Dai Q, Yan B, Huang S, Liu X, Peng S, Miranda ML, Chavez AQ, Vergara BS, and Olszyk DM (1997). Response of oxidative stress defense systems in rice (*Oryza sativa* L.) leaves with supplemental UV-B radiation. Physiologia Plantarum 101: 301-308.

Dat JF, Vandendede F, Vranova E, Montagu MV, Inze D, Breusegem FV (2000). Dual action of the active oxygen species during plant stress response. Cells Mol Life 57: 779-795.

Davis PH (1978). Flora of Turkey and the East Aegean Islands, Edinburg University Press, Edinburgh, pp 6:680–687.

Debjani D, Brattati D (2005). Elicitation of diosgenin production in Dioscorea floribunda by ethylene-generating agent. Fitoterapia 76:153-156.

Diáz J, Bernal A, Pomar F, Merino F (2001). Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annum* L.) seedlings in response to copper stress and its relation to lignification. Plant Sci 161:179-188.

DiCosmo F, Misawa M (1995). Plant cell and tissue culture: alternatives for metabolite production. Biotechnol Adv 13:425-35.

Dinesh-Kumar SP, Witham S, Choi D, Hehel R, Corr C, Baker B (1995). Transposon tagging of tobacco mosaic virus resistance gene N: Its possible role in the TMV-N-mediated signal transduction pathway. Proc Natl Acad Sci ,USA, 192:4175-4180.

Dixon R, Paiva NL (1995). Stress-induced phenylopropanoid metabolism. Plant Cell 7:1085-1097.

Dornenburg H, Knorr D (1997). Challenges and opportunities for metabolite production from plant cell and tissue cultures. Food Technol 51:47-54.

Drazkiewicz M, Skorzynsla-Polit E, Krupa Z (2004). Copperinduced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. Biometals 17:379-387.

Duval B, Shetty K, Thomas WH (2000). Phenolic compounds and antioxidant properties in the snow alga *Chlamydomonas nivalis* after exposure to UV light. Journal of Applied Phycology 11:559-566.

Eichholz I, Huyskens-Keil S, Keller A, Ulrich D, Kroh LW, Rohn S (2011). UV-B-induced changes of volatile metabolites and phenolic compounds in blueberries (*Vaccinium corymbosum* L.). Food Chemistry 126:60–64.

Eisenbeiβ M, Kreis W, Reinhard E (1999). Cardenolide biosynthesis in light- and dark-grown *Digitalis lanata* shoot cultures. Plant Physiol Biochem 37:13-23.

Endress R (1994).Culturing of plant cells. Freytag GF (ed). Plant cell biotechnology. Berlin, Springer-Verlag, pp 46-84.

Engelmann NJ, Reppert A, Yousef G, Rogers RB, Lila MA (2009). In vitro production of radiolabeled red clover (*Trifolium pretense*) isoflavones. Plant Cell Tiss Org Cult 98:147-156.

Esteban E, Morenoa E, Penalosa J, Cabrero JI, Millan R, Zornoza P (2008). Short and long-term uptake of Hg in white lupin plants: Kinetics and stress indicators. Environmental and Experimental Botany 62:316-322.

Fadel D, Kintzios S, Economou1AS, Moschopoulou G, Constantinidou H-IA (2010). Effect of Different Strength of Medium on Organogenesis, Phenolic Accumulation and Antioxidant Activity of Spearmint (*Mentha spicata* l.) The Open Horticulture Journal 3:31-35.

Fedina I, Georgieva K, Velitchkova M, Grigorova I (2006). Effect of pretreatment of barley seedlings with different salts on the level of UV-B induced and UV-B absorbing compounds. Environ Exp Bot 56:225-230.

Feierabend J, Schaan C, Hertwig B (1992). Photoinactivation of catalase occurs under both high- and low-temperature stress conditions and accompanies photoinhibition of photosystem II. Plant Physiol 100:1554-1561.

Ferrat L, Pergent-Martini C, Romeo M (2003). Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. Aquat Toxicol 65:187–204.

Fowler MW, Scragg AH (1988). Natural products from higher plants and plant cell culture. In: Pais MSS, Mavituna F, Novais JM (eds). Plant cell biotechnology. NATO ASI Series, Berlin, Springer-Verlag, pp 18:165-77.

Foyer CH, Noctor G (2000). Oxygen processing in photosynthesis: regulation and signalling. New Phytol 146:359-388.

Fraile A, Garcia-Arenal F, Sagasta EM (1980). Phytoalexin accumulation in bean (*Phaseolus vulgaris*) after infection with *Botrytis cinerea* and treatment with merucric chloride. Physiol Plant Pathol 16:9-18.

Fu JM, Huang BR (2001). Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ Exp Bot 45:105-114.

Fujita Y, Hara Y, Ogino T, Suga C (1981). Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*: I. Effects of nitrogen sources on the production of shikonin derivatives. Plant Cell Rep 1:59-60.

Ganeva G, Zozikova E, (2007). Effect of increasing Cu^{2+} concentrations on growth and content of free phenols in two lines of wheat (*Triticum aestivum*) with different tolerance. Gen Appl Plant Physiology 33:75-82.

Garg N, Manchanda G (2009). ROS generation in plants: boon or bane? Plant Biosys 143:8-96.

Gavidia I, Perez-Bermudez P (1997). Cardenolides of *Digitalis obscura*: the effect of phosphate and manganese on growth and productivity of shoot-tip cultures. Phytochemistry 45:81-85.

Gavidia I, Perez-Bermudez P (1997). *Digitalis obscura* cardenolides effect of macronutrient concentration and N source on growth and productivity of shoot –tip cultures. Phytochemistry Vol: 46:273-238.

Gerschenzon J (1983). Changes in the levels of plant secondary metabolites under water and nutrient stress. In: Timmermann BN, Steelink C, Loewus FA (eds). Phytochemical Adaptations to Stress. Plenum Press, New York, 273-299.

Gläβgen WE, Rose A, Madlung J, Koch W, Gleitz J, Seitz HU (1998). Regulation of enzymes involved in anthocyanin biosynthesis in carrot cell cultures in response to treatment with ultraviolet light and fungal elicitors. Planta 204:490-498.

Goossens A, Hakkinen ST, Laakso I, Seppanen-Laakso T, Biondi S, De Sutter V (2003). A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci 100:8595-8600.

Grace SC, Logan BA (2000). Energy dissipation and radical scavenging by the plant phenylopropanoid pathway. Phil Trans R Soc Lond 355:1499-1510.

Grant JJ, Yun B-W, Loake GJ (2000). Oxidative burst and cognate redox signaling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. Plant J 24: 569-582.

Gratao PL, Monteiro CC, Antunes AM, Peres LEP, Azevedo RA (2008). Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. Ann Appl Biol 153: 321-333.

Gurel E,Yucesan B, Aglic E, Gurel S, Verma SK, Sokmen M, Sokmen A (2011). Regeneration and cardiotonic glycoside production in *Digitalis davisiana* Heywood (Alanya Foxglove). Plant Cell Tiss Organ Cult 104:217–225.

Gustina DL, Sherwood RT, Vance CP (1978). Regulation of phytoalexin synthesis in jackbean callus cultures: stimulation of phenylalanine ammonia-lyase and o-methyltransferase. Plant Physiol 61:226-230.

Gustine DL (1981). Evidence for sulfhydryl involvement in regulation of phytoalexin accumulation in *Trifolium repens* callus cultures. Plant Physiol 68: 1323-1326.

Hagimori M, Matsumoto T, Obi Y (1982a). Studies on the production of *Digitalis* cardenolides by plant tissue cultures. Plant Cell Physiol 23:1205-1211.

Hagimori M, Matsumoto T, Obi Y (1982b). Studies on the production of *Digitalis purpurea* cardenolides by plant tissue culture. Plant Physiol 69:653-656.

Hagimori M, Matsumoto T, Obi Y (1983). Effects of mineral salts, initial pH and precursors on digitoxin formation by shoot forming cultures of *Digitalis purpurea* L. grown in liquid media. Agric Biol Chem 47:565-571.

Hajnos ML, Zobel AM, Glowniak KL (2001). The influence of ultraviolet radiation on the content of pharmacologically active taxoids in yew tissues. Phytomedicine 8:139-143.

Hakamatsuka T, Ebizuka Y, Sankawa U (1991). Induced isoflavonoids from copper chloride-treated stems of *Pueraria lobata*. Phytochemistry 30:1481-1482.

Han C, Liu Q, Yang Y (2009). Short-term effects of experimental warming and enhanced ultraviolet-B radiation on photosynthesis and antioxidant defense of *Picea asperata* seedlings. Plant Growth Regulation 58:153-162.

Harborne JB (1964). Biochemistry of phenolic compounds. Academic, London, pp 511-543.

Hargreaves JA (1979). Investigations into the mechanism of mercuric chloride stimulated phytoalexin accumulation in *Phaseolus vulgaris* and *Pisum sativum*. Physiol Plant Pathol 15: 279-287.

Hasan MA, Ahmed JU, Bahadur MM, Haque MM, Sikder S (2007). Effect of late planting heat stress on membrane thermostability, proline content and heat susceptibility index of different wheat cultivars. J Natn Sci Foundation Sri Lanka 35:109-117.

He L, Gao Z, Li R (2009). Pretreatment of seed with H_2O_2 enhances drought tolerance of wheat (*Triticum aestivum* L.) seedlings. African Journal of Biotechnology 8:6151-6157.

Herms DA, Mattson WJ (1992). The dilemma of plants: to grow or defend. Quarterly Review of Biology 67:283-335.

Higgins VJ, Millar RL (1968). Phytoalexin production by alfalfa in response to infection by *Colletotrichum phomoides*, *Helminthosporium turcicum, Stemphylium loti*, and *S. botyrosum*. Phytopathology 58: 1377-1383.

Hossain MM, Takeda H, Senboku T (1995). Proline content in *Brassica* under high temperature stress. JIRCAS J 2:87-93.

Huerta-Heredia AA, Marın-Lopez R, Ponce-Noyola T, Cerda-Garcıa-Rojas CM, Trejo-Tapia G, Ramos-Valdivia AC (2009). Oxidative stress induces alkaloid production in *Uncaria tomentosa* root and cell cultures in bioreactors. Eng Life Sci 3: 211-218.

Hung SH, Yu CW, Lin CH (2005). Hydrogen peroxide functions as a stress signal in plants. Bot Bull Acad Sin 46: 1-10.

Huyskens-Keil S, Eichholz I, Kroh LW, Rohn S (2007). UV-B induced changes of phenol composition and antioxidant activity in black currant fruit (*Ribes nigrum* L.). Journal of Applied Botany and Food Quality 81:140-144.

Ikeda T, Matsumoto T, Noguchi M (1977). Effects of inorganic nitrogen source and physical factors on the formation of ubiquinone by tobacco plant cells in suspension culture. Agric Biol Chem 41:1197-201.

Ikeda Y, Fujii Y, Yamazaki M (1992). Determination of Lanatoside C and Digoxin in *Digitalis lanata* by HPLC and its Application to Analysis of the Fermented Leaf Powder. Journal of Natural Products 55:748-752.

Indrayanto G, Rahayu L, Rahman A, Noeraeni PE (1993). Effect of calcium, strontium and magnesium ions on the formation of phytoesteroids in callus cultures of *Agave amaniensis*. Planta Med 59:97-98.

Inmaculada Y (2005). Copper in plants. Braz J Plant Physiol 17:145- 156.

Ishihara A, Miyagawa H, Kuwahara Y, Ueno T, Mayama S (1996). Involvement of Ca^{2+} ion in phytoalexin induction in oats. Plant Sci 115:9-16.

Ivanina LI (1955). Die Gattung *Digitalis* L. (Fingerhut) und ihre praktische Verwendung. In: Schischkina BK (ed) Flora und Systematik der ho¨heren Pflanzen. Acta Inst Bot Acad Sci URSS, Akademie der Wissenschaften der UDSSR, Moskau, pp 71-88.

Jeong CH-S, Chakrabarty D, Hahn EJ, Lee H, Paek KY (2006). Effects of oxygen, carbon dioxide and ethylene on growth and bioactive compound production in bioreactor culture of ginseng adventitious roots. Biochem Eng J 27:252-263.

Jung CH, Maeder V, Funk F, Frey B, Sticher H, Frosserd E (2003). Release of phenols from *Lupinus albus* L. roots exposed to Cu and their possible role in Cu detoxification. Plant soil 252:301-312.

Jung S (2003). The combined action of catalase (CAT) and superoxide dismutase (SOD) is critical in mitigating the effects of oxidative stress. Pesticide Biochem Physiol 75:9-17.

Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G. Mullineaux P (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science 284:654-657.

Keen NT, Bruegger B (1977). Phytoalexins and chemicals that elicit their production in plants. In: Hedin PA (ed) Host Plant Resistance to Pests. American Chemical Society, Washington, DC, 1-26.

Khan AG, Kuek TM, Chaudhury TM, Khoo CS, Hayes WJ (2000). Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere 41:197-207.

Kliebenstein DJ, Monde RA, Last RL (1998). Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118:637-650.

Knight H, Knight MR (2001). Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci 6:262-267.

Kohl DH, Lin JJ , Shearer G, Scubert KR (1990). Activities of the pentose pathway and enzymes of proline metabolism in legume root nodules. Plant Physiol 94:1258-1264.

Kondo N, Kawashima M (2000). Enhancement of tolerance to oxidative stress in cucumber (Cucumis sativus L.) seedlings by UV B irradiation: possible involvement of phenolic compounds and antioxidant enzymes. J Plant Res 113:311-317.

Korsangruang S, Soonthornchareonnon N, Chintapakorn Y, Saralamp P, Prathanturarug S (2010). Effects of abiotic and biotic elicitors on growth and isoflavonoid accumulation in *Pueraria candollei* var. *candollei* and *P. Candollei* var. *mirifica* cell suspension cultures. Plant Cell Tiss Organ Cult 103:333-342.

Kou CG, Chen HM, Ma LH (1986). Effect of high temperature on proline content in tomato floral buds and leaves. J Am Soc Hort Sci 11:734-750.

Kovtun Y, Chiu WL, Tena G, Sheen J (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci ,USA, 97: 2940-2945.

Kreis W, May U, Reinhard E (1986). UDP-Glucose: digitoxin 160 O-glucosyltransferase from suspension cultured *Digitalis lanata* cells. Plant Cell Rep 5:442-445.

Kreis W, Reinhard E (1992). 12β-hydroxylation of digitoxin by suspension cultured *Digitalis lanata* cells: production of digoxin in 20 L and 300 L airlift reactors. J Biotechnol 26:257-73.

Krupa Z, Baszynski T (1995). Some aspects of heavy metal toxicity towards photosynthetic apparatus: direct and indirect effects on light and dark reaction. Acta Physiologiae Plantarum 17:177-190.

Ku K-L, Chang P-S, Cheng Y-C, Lien C-Y (2005). Production of stilbenoids from the callus of *Arachis hypogaea*: A novel source of the anticancer compound piceatannol. Agric Food Chem 53:3877-3881.

Kubiś J, Rybus-Zając M (2008). Drought and excess UV-B irradiation differentially alter the antioxidant system in cucumber leaves. Acta Biologica Cracoviensia Series Botanica 50: 35-41.

Kubo A, Aono M, Nakaliman N, Saji H, Tanaka K, Kondo N (1999). Differential responses in activity of antioxidant enzymes to different environmental stresses in *Arabidopsis thaliana*. Journal of Plant Research 112: 279-290.

Kumari R, Singh S, Agrawal SB (2010). Response of ultraviolet-B induced antioxidant defense system in a medicinal plant, *Acorus calamus*. Journal of Environmental Biology 31:907-911.

Kwok D, Shetty K (1997). Effects of proline and proline analogs on total phenolic and rosmarinic acid levels in shoot clones of thyme (*Thymus vulgaris* L). J Food Biochem 22:37-51.

Laloi C, Apel K, Danon A (2004). Reactive oxygen signaling: the latest news. Plant Biol 7:323-328.

Lamb C, Dixon RA (1997). The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251-275.

Lamikanra O, Richard OA, Parker A (2002). Ultraviolet induced stress response in fresh cut cantaloupe. Phytochem 60:27-32.

Lartillot S, Kedziora P, Athias A (1988). Purification and characterization of a new fungal catalase. Prep Biochem 18:241-246.

Lattanzioa V, Cardinali A, Ruta C, Fortunato IM, Lattanzio VMT, Linsalata V, Cicco N (2009). Relationship of secondary metabolism to growth in oregano (*Origanum vulgare* L.) shoot cultures under nutritional stress. Environmental and Experimental Botany 65:54-62.

Lavola A, Julkunen-Tiitto R, De La Rosa TM, Lehto T, Aphalo PJ (2000). Allocation of carbon to growth and secondary metabolites in birch seedlings under UV-B radiation and CO2 exposure. Physiol Plant 109:260-267.

Levine A, Tenhaken R, Dixon R, Lamb C (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583-593.

Levitt J. (1980a) Responses of plants to environmental stresses: chilling, freezing and high temperatures stresses. In: Kozlowsky TT (ed). Physiological Ecology: A Series of Monographs, Texts and Trea- tises. Ed 2, Academic Press, New York, pp 1:23-64

Levitt J. (1980b). Drought tolerance. In: Larcher W (ed). Responses of Plants to Environmental Stresses. Water, Radiation, Salt, and Other Stresses. Academic Press II, New York, pp 129-280.

Levizou E, Manetas Y (2002). Spectrophotometric assessment of leaf UV-B absorbing compounds and chemically determined total phenolic levels are strongly correlated. Can J Bot 80:690–694.

Li JT ,Qiu ZB, Zhang XW, Wang LS (2011) . Exogenous hydrogen peroxide can enhance tolerance of wheat seedlings to salt stress. Acta Physiol Plant 33:835-842.

Lichtenthaler HK (1996). Vegetation stress: an introduction to the stress concept in plants. J Plant Physiology 148:4-14.

Lindemann P, Luckner M (1997). Biosynthesis of pregnane derivatives in somatic embryos of *Digitalis lanata*. Phytochemistry 46:507-513.

Liu C-J, Dixon RA (2001). Elicitor-induced association of isoflavone O-methyltransferase with endomembranes prevents the formation and 7-O-methylaiton of daidzein during isoflavonoid phytoalexin biosynthesis. Plant Cell 13:2643-2658.

Liu L, Gitz DC, McClure JW (1995). Effects of UV-B on flavonoids, ferulic acid, growth and photosynthesis in barley primary leaves. Physiol Plant 93:725–733.

Liu Z-J, Guo Y-K, Bai J-G (2010). Exogenous Hydrogen Peroxide Changes Antioxidant Enzyme Activity and Protects Ultrastructure in Leaves of Two Cucumber Ecotypes Under Osmotic Stress. J Plant Growth Regul 29:171-183.

Logan BN, Demming-Adams B, Rosenstiel TN, Adams WW III (1999). Effect of nitrogen limitation on foliar antioxidants in relationship to other metabolic characteristics. Planta 209:213-220.

Lombardi L, Sebastiani L (2005). Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzyme responses of in vitro grown plants. Plant Sci 168:797-802.

Lotmani B, Mesnoua M (2011). Effects of copper stress on antioxidative enzymes, chlorophyll and protein content in *Atriplex halimus.* African Journal of Biotechnology 10:10143-10148.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin Phenol Reagent. J Biol Chem 193:265-75.

Lu P, Sang WG, Ma KP (2008). Differential responses of the activities of antioxidant enzymes to thermal stresses between two invasive *Eupatorium* species in China. J Integr Plant Biol 50:393-401.

Lutts S, Kinet JM, Bouharmont J (1996). Effects of various salts and of mannitol on ion and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) callus cultures. Journal of Plant Physiology 149:186-195.

Ma C (1998). Mercury harm on cell membrane of rape leaf and cell endogenous protection effect. YingYong Shengtai Xuebao 9:23-26.

Mahady GB, Beecher CWW (1994). Elicitor-stimulated benzophenanthridine alkaloid biosynthesis in bloodroot suspension cultures is mediated by calcium. Phytochemistry 37:415-419.

Mahdavian K, Ghorbanli M, Kalantari KM (2008a).The Effects of Ultraviolet Radiation on the Contents of Chlorophyll, Flavonoid, Anthocyanin and Proline in *Capsicum annuum* L. Turk J Bot 32:25-33.

Mahdavian K, Ghorbanli M, Kalantari KM (2008b).The effects of ultraviolet radiation on some antioxidant compounds and enzymes in *Capsicum annuum* L. Turk J Bot 32:129-134.

Maksymiec W, Krupa Z (2006). The effects of short term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. Environ Exp Bot 57:187-194.

Maksymiec W, Krupa Z (2002). The in vivo and in vitro influence of methyl jasmonate on oxidative processes in Arabidopsis thaliana leaves. Acta Physiol Plant 24:351-357

Marigo G (1973). Sur une méthode de'fractionnement et d' estimation des composés phénoliques chez les vegétaux. Analusis 2:106–110.

Marschner H (1995). Mineral Nutrition of Higher Plants. Ed II, Academic Press Inc, London, pp 889-900.

Marschner H (1993). Functions of mineral nutrients: Macronutrients. In: Marschner H (ed). Mineral Nutrition of Higher Plants. 2nd ed, pp 229-299.

May M, Vernoux T, Leaver C, Van Montagu M, Inzé D (1998). Glutathione homeostasis in plants: implications for environmental sensing and plant development. Journal of Experimental Botany 49:649-667.

Mehta SK, Gaur JP (1999). Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chiorella vulgaris*. New Phytol 143:253-259.

Memelink J, Kijne JW, van der Heijden R, Verpoorte R (2001). Genetic modification of plant secondary metabolite pathways using transcriptional regulators. Adv Biochem Eng Biotechnol 72:103-125.

Merillon JM, Liu D, Hughet F, Chenieux JC, Rideau M (1991). Effects of calcium entry blockers and calmodulin inhibitors on cytokinin-enhanced alkaloid accumulation in *Catharanthus roseus* cell cultures. Plant Physiol Biochem 29:289-296.

Michalak A (2006). Phenolic Compounds and Their Antioxidant Activity in Plants Growing under Heavy Metal Stress. Polish J Of Environ Stud 15:523-530.

Milborrow BV (2001). The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. J Exp Bot 52:1145-1164.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405-410.

Modafar CE, Clerivet A, Fleuriet A, Macheix JJ (1993). Inoculation of *Platanus acerifolia* with *Ceratocystis fimbriata* F. sp. *platani* induces scopoletin and umbelliferone accumulation. Phytochemistry 34:1271-1276.

Moesta P, Grisebach H (1980). Effects of biotic and abiotic elicitors on phytoalexin metabolism in soybean. Nature 286:710-711.

Moreno-Valenzuela OA, Minero-Garcia Y, Chan W, Mayer- Geraldo E, Carbajal E, Loyola-Vargas V (2003). Increase in the indole alkaloid production and its excretion into the culture medium by calcium antagonists in *Catharanthus roseus* hairy roots. Biotechonol Lett 25:1345-1349.

Morita S, Kaminaka H, Masumura T, Tanaka K (1999). Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress; the involvement of hydrogen peroxide in oxidative stress signalling. Plant Cell Physiol. 40:417-422.

Moskova I, Todorova D, Alexieva V, Ivanov S, Sergiev I (2009). Effect of exogenous hydrogen peroxide on enzymatic and nonenzymatic antioxidants in leaves of young pea plants treated with paraquat Plant Growth Regul 57:193-202.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.

Murthy KS, Smith TA, Bould C (1971). The relation between the putrescine content and potassium status of black currant leaves. Ann Bot 35:687-695.

Nacif de Abreu I, Mazzafera P (2005). Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. Plant Physiology and Biochemistry 43:241-248.

Nakagawa K, Konagai A, Fukui H, Tabata H (1984). Release and crystallization of berberine in the liquid medium of *Thalictrum minus* cell suspension cultures. Plant Cell Rep 3:254-257.

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. Journal of Experimental Botany 53:1237-1247.

Ouwerkerk PBF, Trimborn TO, Hilliou F, Memelink J (1999). Nuclear factors GT-1 and 3AF1 interact with multiple sequences within the promoter of the Tdc gene from Madagascar periwinkle: GT-1 is involved in UV light-induced expression. Molecular General Genetics 261:610-622.

Overmyer K, Brosche H, Kangasjarvi J (2003). Reactive oxygen species and hormonal control of cell death. Trends Plant Sci 8:335–342.

Ozden M, Demirel U, Kahraman A (2009). Effects of proline on antioxidant system in leaves of grapevine (*Vitis vinifera* L.) exposed to oxidative stress by H_2O_2. Scientia Horticulturae 119:163-168.

Paek KY, Chakrabarty D, Hahn EJ (2005). Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tiss Org Cult 81:287-300.

Palacio L, Baeza C, Cantero JJ, Cusido´ R, Goleniowsi ME (2008) In Vitro propagation of ‘‘Jarilla’’ (Larrea divaricata CAV.) and secondary metabolite production. Biol Pharm Bull 31:2321-2325.

Pan SM, Yau YY (1992). Characterisation of superoxide dismutase in *Arabidopsis*. Taiwania 37:58-66.

Pandey N, Pathak GC, Pandey DK, Pandey R (2009). Heavy metals Co,Ni, Cu, Zn and Cd, produce oxidative damage and evoke differantial antioxidant responses in spinach.

Pandey N, Sharma CP (2002). Effect of heavy metals Co^{2+}, Ni^{2+} and Cd^{2+} on growth and metabolism of cabbage. Plant Sci 163:753-758.

Paranhos A, Fernández-Tarrago J, Corchete P (1999). Relationship between active oxygen species and cardenolide production in cell culture of *Digitalis thapsi*: effect of calcium restriction. New Phytol 141:51-60.

Paranhos A, Fernández-Tarrago J, Corchete P (1999). Relationship between active oxygen species and cardenolide production in cell cultures of *Digitalis thapsi*: effect of calcium restriction. New Phytol 141:51-60.

Park J, Ro H, Hwang K, Yiem MS (2001). Effect of water stress induced by polyethylene glycol and root zone temperature on growth and mineral contents of Fuji/M. 26 apple. J Korean Soc Hort Sci 42:435-438.

Patra M, Sharma A (2000). Mercury toxicity in plants. The Botanical Review 66:379-422.

Pätsikkä E, Aro E-M, Tyystjärvi E (1998). Increase in the quantum yield of photoinhibition contributes to copper toxicity in vivo. Plant Physiology 117:619-627.

Payne GF, Bringi V, Prince C, Shuler ML (1991). Plant cell and tissue culture in liquid systems. Munich, Hanser Publ 1-10.

Payton P, Webb R, Kornyeyev D, Allen R, Holaday AS (2001). Protecting cotton photosynthesis during moderate chilling at high light intensity by increasing chloroplastic antioxidant enzyme activity. J Exp Bot 52:2345-2354.

Pezzuto JM (1995). Natural product cancer chemoprotective agents. In: Arnason JT, Mata R, Romeo JT (eds). Recent advances in phytochemistry. Phytochemistry of medicinal plants, New York, Plenum, pp 29:19-45.

Phillipson JD (1990). Plants as source of valuable products. In: Charlwood BV, Rhodes MJC (eds). Secondary products from plant tissue culture. Clarendon Press, Oxford, pp 1-21.

Pimentel D (1991). Diversification of biological control strategies in agriculture. Crop Protection 10:243-253.

Pimentel D (2002). Biological Invasions: Economic and Environmental Costs of Alien Plant, Animal and Microbe Species. CRC Press, Boca Raton, FL, USA, pp 384.

Pitzschke A, Hirt H (2006). Mitogen-activated protein kinases and reactive oxygen species signaling in plants. Plant Physiol 141:351-356.

Pluskota WE, Michalczyk DJ, Górecki RJ (2005). Control of phenylalanine ammonia–lyase gene promoters from pea by UV radiation. Acta Physiologiae Plantarum 27:229–236.

Polle A, Renenberg H (1993). Signifiance of antioxidants in plant adaptation to environmental stress. In : Mansfield T, Fowden L, Stoddard F (eds). Plant adaptation to environmental stress. London, Chapman & Hall pp 263-273.

Preisig CL, Moreau RA (1994). Effects of potential signal transduction antagonists on phytoalexin accumulation in tobacco. Phytochemistry 36:857-863.

Rai V, Vaypayee P, Singh SN, Mehrotra S (2004). Effect of chromium accumulation on photosynthetic pigments, oxidative stress defence system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. Plant Sci 167:1159-1169.

Rajasekaran T, Ravishankar GA, Venkataraman LV (1991). Influence of nutrient stress on pyrethrin production by cultured cells of pyrethrum (*Chrysanthemum cinerariaefolium*). Curr Sci 60:705-707.

Ramachandra RS, Ravishankar GA (2002). Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances 20:101-153.

Ramani S, Jayabaskaran C (2008). Enhanced catharathine and vindoline production in suspension cultures of *Catharanthus roseus* by ultraviolet-B light. Journal of Molecular Signaling 3:1-6.

Rao MV, Paliyath G, Ormrod DP (1996). Ultraviolet-B and ozoneinduced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol 110:125-136.

Ravishankar GA, Bhyalakshmi N, Ramachandra RS (1999). Production of food additives. In: Ramawat KG, Merillon JM (eds). Biotechnology: secondary metabolites. New Delhi, Oxford IBH, pp 89-110.

Ravishankar GA, Venkataraman LV (1990). Food applications of plant cell cultures. Curr Sci 59:914-920.

Reuber S, Bornman JF, Weissenbock G (1996). A flavonoid mutant of barley (*Hordeum vulgare* L.) exhibits increased sensitivity of UV-B radiation in the primary leaf. Plant Cell Environ. 19:593– 601.

Rice-Evans CA, Miller NJ, Paganga G (1997). Antioxidant properties of phenolic compounds. Trends Plant Sci 2:152-159.

Rivero RM, Ruiz JM, Garcia PC, Lopez-Lefebre LR, Sanchez E, Romero L (2001). Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. Plant Sci 160:315-321.

Robert B, David O (2004). Arabidopsis OXS2 is a transcription factor in the oxidative stress response. Abstract of annual meeting of the American Society of Plant Biologists, Orlando, FL. July 24-28.

Romero-Puertas MC, Perazzolli M, Zago ED, Delledonne M (2004). Nitric oxide signalling functions in plant-pathogen interactions. Cellu Microbiol 6:795-803.

Romero-Puertas MC, Rodriquez-Serrano M, Corpas FJ, Gomez M, Del Rio LA, Sandalio LM (2004) Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. Plant Cell Environ 27:1122-1134.

Ronde J, Mescht A, Steyn HSF (2001). Proline accumulation in response to drought and heat stress in cotton. African Crop Sci J 8:85-91.

Ruiz JM, Rivero RM, Lopez-Cantarero I, Romero L (2003). Role of Ca^{2+} in metabolism of phenolic compounds in tabacco leaves (*Nicotiana tabacum* L.). Plant Growth Reg 41:173-177.

Ruiz JM, Sanchez E, Garcia P, Lopez-Lefebre LR, Rivero RM, Romero L (2002). Proline Metabolism and NAD Kinase Activity in Green Bean Plants Subjected to Cold-Shock. Phytochemistry 59:473-478.

Russowski D, Maurmann N, Rech SB, Fett-Neto A (2006). Role of light and medium composition on growth and valepotriate contents in *Valeriana glechomifolia* whole plant liquid cultures. Plant Cell Tiss Org Cult 86:211-218.

Rybus-Zając M, Kubiś J (2010). Effect of UV-B radiation on antioxidative enzyme activity in cucumber cotyledons. Acta Biologica Cracoviensia Series Botanica 52: 97-102.

Saibo NJ, Lourenco T, Oliveira MM (2009). Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Annals of Botany 103:609-623.

Sakihama Y, Yamasaki H (2002). Lipid peroxidation induces by phenolics in cinjunction with aluminium ions. Biol Plantarum 45:249-254.

Salama HMH, Al-Watban AA, Al-Fughom AT (2011). Effect of ultraviolet radiation on chlorophyll, carotenoid, protein and proline contents of some annual desert plants. Saudi Journal of Biological Sciences 18:79-86.

Santiago LJM, Louro RP, De Oliveira DE (2000). Compartmentation of phenolic compounds and phenylalanine ammonia-lyase in leaves of *Phyllanthus tenellus* Roxb. & their induction by copper sulphate. Ann Bot 86:1023-1032.

Saradhi PP, Sandeep A, Prasad K, Arora S (1995). Proline accumulates in plants exposed to UV radiation and protects them against UV induced peroxidation. Biochem Biophys Res Commun 209:1-5.

Saruyama H, Tanida M (1995). Effect of chilling on activated oxygen scavenging enzymes in low temperature-sensitive and tolerant cultivars of rice (*Oryza sativa* L.). Plant Sci 109:105-113.

Sasse F, Knobloch K, Berlin J (1982). Induction of secondary metabolism in cell suspension cultures of *Catharanthus roseus, Nicotiana tabacum* and *Peganum harmala*. In: Fujiwara A (ed). Proceedings of the 5th International Congress of Plant Tissue and Cell Culture. Tokyo, Abe Photo Printing, pp 343-344.

Schat H, Sharma SS, Vooijs R (1997). Heavy metal-induced accumulation of free proline in metal-tolerant and a nontolerant ecotype of *Silene vulgaris*. Physiologia Plantarum 101:477-482.

Schmidlin L, Poutaraud A, Claudel P, Mestre P, Prado E, Santos-Rosa M (2008). A Stress-Inducible Resveratrol O-Methyltransferase Involved in the Biosynthesis of Pterostilbene in Grapevine. Plant Physiol 148:1630-1639.

Schmitz-Eiberger M, Haefs R, Noga G (2002). Calcium deficiency- influence on the antioxidative defense system in tomato plants. J Plant Physiol 159:733-742.

Schützendübel A, Polle A (2002). Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351-1365.

Schwacke R, Hager A (1992). Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca++ and protein-kinase activity. Planta 187: 136-141.

Scragg AH (1997). The production of aromas by plant cell cultures. In: Schepier T, editor. Adv Biochem Eng Biotechnol. Berlin, Springer-Verlag, pp 55:239-263.

Searles PS, Kropp BR, Flint SD, Caldwell MM (2001). Influence of solar UV-B radiation on peatland microbial communities of southern Argentinia. New Phytologist 152:213-221.

Sebastiani L, Scebba F, Tongetti R (2004). Heavy metal accumulationand growth responses in poplar clones Eridano (*Populus deltoides* × *maximowiczii*) and I-214 (*P.* × *euramericana*) exposed to industrial waste. Env Exp Bot 52:79-88.

Seitz HU, Gartner D (1994). Enzymes in cardenolide-accumulating shoot cultures of *Digitalis purpurea* L. Plant Cell Tiss Org Cult 38:337-344.

Serrano R, Mulet JM, Rios G, Marquez JA, De Larrinoa IF, Leube MP, Mendizabal I, Pascual-Ahuir A, Proft M, Ros R, Montesinos C (1999). A glimpse of the mechanisms of ion homeostasis during salt stress. J Exp Bot 50:1023-1036.

Sgherri C, Cosi E, Navari-Izzo F (2003). Phenols and antioxidative status of *Raphanus sativus* grown in copper excess. Physiologia Plantarum 118:21-28.

Sharma PK, Anand P, Sankhalkar S (1998).Oxidative damage and changes in activities of Antioxidant enzymes in wheat seedlings exposed to ultraviolet-B radiation. Curr Sci 75:359-366.

Sharp RE, Hsiao TC, Silk WK (1990). Growth of maize primary root at low water potential's. Plant Physiol 93:1337-1346.

Shetty K (1997). Biotechnology to harness the benefits of dietary phenolics; focus on Lamiaceae. Asia Pacific J Clin Nutr 6:162-171.

Shimomura K, Yoshimatsu K, Sauerwein M, Christen P, Toda Y, Aoki T (1992). Production of biologically active compounds by transformed cultures. In: Oono R, Hirabayashi T, Kiruchi S, Handa H, Kahwara S (eds). Plant tissue culture and gene manipulation for breeding and formation of phytochemicals. National Institute of Agrobiological Resources, Tsukuba, Japan, pp 293-296.

Shinozaki K, Yamaguchi-Shinozaki K (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signalling pathways. Curr Opin Plant Biol 3:217-223.

Shohael AM, Ali MB, Yu KW, Hahn EJ, Paek KY(2006). Effect of temperature on secondary metabolites production and antioxidant enzyme activities in *Eleutherococcus senticosus* somatic embryos. Plant Cell Tissue and Organ Culture 85: 219-228.

Sierra MI, Dagnino D, Van der Heijden R, Verpoorte R (1991). Influence of calcium on peroxidase activity and alkaloid formation in Tabernaemontana divaricata cell suspension cultures. In: Lobarzewski J, Grepin H, Penel C, Gaspar TH (eds). Biochemical, molecular and physiological aspects of plant peroxidases. Geneva, Switzerland, University of Geneva pp 295-304.

Silvestrini A, Pasqua G, Botta B, Monacelli B, Van Der Heijden R, Verpoorte R (2002). Effect of alkaloid precursor feeding on a *Camptotheca acuminate* cell line. Plant Physiol Biochem 40:749-753.

Siripornadulsil S, Train S, Verma DPS, Sayre RT (2002). Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 14:2837-2847.

Smirnoff N, (1993). The role of active oxygen in the response of plants towater deficit and desiccation. New Phytol 125:27-58.

Smirnoff N (1998). Plant resistance to environmental stress. Curr Opin Biotech 9:214-219.

Smith RA, Drummond S, Haines A, Porter JR, Hock RS (2001). Induction of umbelliferone in sweet potato cell suspension culture using mercuric chloride. Biotechnology Letters 23: 1397-1400.

Smith TA (1973). Amine levels in mineral-deficient *Hordeum vulgare* leaves. Phytochemistry 12:2093-2100.

Somal TLC, Yapa PAJ (1998). Accumulation of Proline in Cowpea Under Nutrient, Drought, and Saline Stresses. Journal of Plant Nutrition 21: 2465-2473.

Souza GM, Cardoso VJM, Gonçalves AN (2004). Proline Content and Protein Patterns in Eucalyptus grandis Shoots Submitted to High and Low Temperature Shocks. Brazilian Archives of Biology and Technology 47:355-362.

Stockigt J, Obitz P, Flakenhagen H, Lutterbach R, Endress R (1995). Natural products and enzymes from plant cell cultures. Plant Cell Tissue Org Cult 43:914-20.

Sumner LW, Mendes P, Dixon RA (2003). Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochem 62:817-36.

Sun Y, Oberley LW, Li Y (1988). A simple method for clinical assay of superoxide dismutase. Clin Chem 34:497-500.

Takahama U, Oniki T (2000). Flavonoid and some other phenolics as substrates of peroxidase: physiological significance of the redox reactions. J Plant Res 113:301-309.

Takeuchi Y, KuboH , KasaharaH ,Sakaki T (1996).Adaptive alteration in the activities of Scavengers of active oxygen in cucumber cotyledons irradiated with UV-B. Plant Physiol 147:589-592.

Tevini M (1993). Effects of enhanced UV-B radiation on terrestrial plants. In: Tevini M (ed). UV-B Radiation and Ozone Depletion. Lewis Publ, Boca Raton, FL, pp 125-153.

Tewari RK, Kumar P, Sharma PN (2006). Magnesium deficiency induced oxidative stress and antioxidant responses in mulberry plants. Scientia Horticulturae 108:7-14.

Tewari RK, Kumar P, Tewari N, Srivastava S, Sharma PN (2004). Macronutrient deficiencies and differential antioxidant responses-influence on the activity and expression of superoxide dismutase in maize. Plant Sci 166:687-694.

Tian D, Traw MB, Chen JQ, Kreitman M, Bergelson J (2003). Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana*. Nature 423:74-77.

Tisserat B, Vaughn S (2008). Growth, morphogenesis, and essential oil production in *Mentha spicata* L. plantlets in vitro. In Vitro Cell Dev Biol-Plant 44:40-50.

Toivonen L, Laakso S, Rosenqvist H (1992). The effect of temperature on hairy root cultures of *Catharanthus roseus*: growth, indole alkaloid accumulation and membrane lipid composition. Plant Cell Rep 11:395-399.

Tso TC, McMurtrey JE (1960).Mineral Deficiency and Organic Constituents in Tobacco plants. II. Amino Acids. Plant Physiol 35:865-870.

Turtola S, Manninen AM, Rikala R, Kainulainen P (2003). Drought stress alters the concentration of wood terpenoids in Scots pine and Norway spruce seedlings. J Chem Ecol 29:1981-1995.

Upadhyaya H, Khan MH, Panda SK (2007). Hydrogen peroxide induces oxidative stress in detached leaves of *Oryza sativa* L. Gen Appl Plant Physiology 33:83-95.

Uritani I, Uritani M, Yamada H (1960). Similar metabolic alterations induced in sweet potato by poisonous chemicals and by *Ceratostomella fimbriata*. Phytopathology 50:30-34.

Verpoorte R, Memelink J (2002). Engineering secondary metabolite production in plants. Curr Opin Biotechnol 13:181-187.

Verslues PE, Kim YS, Zhu JK (2007). Altered ABA, proline and hydrogen peroxide in an *Arabidopsis* glutamate:glyoxylate aminotransferase mutant. Plant Mol Biol 64:205–17.

Vierling E, Kimpel JA (1992). Plant responses to environmental stress. Curr Opin Biotech 3:164-170.

Vögeli U, Chappell J (1988). Induction of sesquiterpene-cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitors. Plant Physiology 88:1291-1296.

Vranová E, Inzé D, Van Breusegem F (2002). Signal transduction during oxidative stress. Journal of Experimental Botany 53:1227-1236.

Vyas J, Puranik RM, (1993). Inhibition of nitrate reductase activity by mercury in bean leaf segments. Indian Journal of Plant Physiology 36:57-60.

Wahid A (2007). Physiological implications of metabolites biosynthesis in net assimilation and heat stress tolerance of sugarcane sprouts. J Plant Res 120:219-228.

Wahid A, Ghazanfar A (2006). Possible involvement of some secondary metabolites in salt tolerance of sugarcane. J Plant Physiol 163:723-730.

Walters B, Eilert U (1983). Elicitoren Ausloöser der Akkumulation von Pflanzenstoffen. Deutsche Apotheker Zeitung 123:659-667.

Wang F, Zeng B, Sun Z, Zhu C (2009). Relationship Between Proline and Hg^{2+}-Induced Oxidative Stress in a Tolerant Rice Mutant. Arch Environ Contam Toxicol 56:723-731.

Wang WX, Vinocur B, Shoseyov O, Altman A (2001). Biotechnology of plant osmotic stress tolerance: physiological and molecular considerations. Acta Hort 560:285-292.

Werner K (1960). Zur Nomenklatur und Taxonomie von *Digitalis* L. Bot Jahrb 79:218-254.

Werner K (1965). Taxonomie und Phylogenie der Gattungen *Isoplexis* (Lindl.) Benth. und *Digitalis* L. Feddes Rep 70:109-135.

Werner K (1966). Die Wuchsformen der Gattungen *Isoplexis* (Lindl.) Benth. und *Digitalis* L. Bot Jahrb 85:88-149.

Wiegrebe H, Wichtl M (1993). High-performance liquid chromotographic determination of cardenolides in Digitalis leaves after solid-phase extraction. J Chromatog A 630:402-407.

Wingsle G, Hallgren JE (1993). Influence of SO_2 and NO_2 exposure on glutathione, superoxide dismutase and glutathione reductase activities in Scots pine needles. J Exp Bot 44:463-470.

Winkel-Shirley B (2002). Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218-223.

Wu CH, Dewir YH, Hahn EJ, Paek KY (2006). Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. J Plant Biol 49:193-199.

Yamakawa T, Kato S, Ishida K, Kodama T, Minoda Y (1983). Production of anthocyanins by Vitis cells in suspension culture. Agric Biol Chem 47:2185-91.

Yang S-L, Lan S-S, Gong M (2009). Hydrogenperoxide-induced proline and metabolic pathway of its accumulationin maize seedlings. Journal of PlantPhysiology 166:1694-1699.

Yannarelli GG, Gallego SM, and Tomaro ML (2006). Effect of UV-B radiation on the activity and isoforms of enzymes with peroxidase activity in sunflower cotyledons. Environmental and Experimental Botany 56:174-181.

Yeoman MM, Meidzybrodzka MB, Lindsey K, McLauchlan WR (1980). The synthetic potential of cultured plant cells. In: Sala F, Parisi B, Cella R, Cifferi O (eds). Plant cell cultures: results and perspectives. Amsterdam, Elsevier, pp 327-343.

Yoshikawa M (1978). Diverse modes of action of biotic and abiotic phytoalexin elicitors. Nature 275: 546-547.

Yuri A, Schmitt E, Feucht W, Treutter D (1990). Metabolism of *Prunus* tissues affected by Ca^{2+} - deficiency and addition of prunin. J Plant Physiol 135:692–697.

Yürekli F, Porgalı ZB (2006). The effects of excessive exposure to copper in bean plants. Acta Biologica Cracoviensia Series Botanica 48: 7-13.

Zagoskina NV, Alyavina AK, Gladyshko TO, Lapshin PV, Egorova EA, Bukhov NG (2005). Ultraviolet rays promote development of photosystem II photochemical activity and accumulation of phenolic compounds in the tea callus culture (*Camellia sinensis*). Russ J Plant Physiol 52:731–739.

Zagoskina NV, Dubravina GA, Alyavina AK, Goncharuk EA (2003). Effect of ultraviolet (UV-B) radiation on the formation and localization of phenolic compounds in tea plant callus cultures. Russ J Plant Physiol 50:270-275.

Zangerl AR, Berenbaum MR (1997). Cost of chemically defending seeds: furanocoumarins and Pastinaca sativa. The American Naturalist 150:491–504.

Zangerl AR, Berenbaum MR (2003). Phenotype matching in wild parsnip and parsnip webworms: causes and consequences.Evolution 57:806-815.

Zavala JA, Baldwin IT (2004). Fitness benefits of trypsin proteinase inhibitor expression in Nicotiana attenuata are greater than their costs when plants are attacked. BMC Ecology 4, 11.

Zengin FK, Kirbag S (2007). Effects of copper on chlorophyll, proline, protein and abscisic acid level of sunflower (*Helianthus annuus* L.) seedlings. Journal of Environmental Biology 28: 561-566.

Zengin FK, Munzuroğlu Ö (2005). Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (*Phaseolus vulgaris* L.) seedlings. Acta Biologica Cracoviensia Series Botanica 47:157-164.

Zenk MH (1996). Heavy metal detoxification in higher plants–a review. Gene 179:21-30.

Zenk MH, EI-Shagi H, Schulte U (1975). Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. Planta Med Suppl 79-101.

Zhang CS, Lu Q, Verma DPS (1995). Removal of Feedback Inhibition of P5CS, a Bifunctional Enzyme Catalyzing the First Two Steps of Proline Biosynthesis in Plants. J Biol Chem 270:20491-20496.

Zhang J ,Gao W-Y ,Wang J, Li X-L (2011). Effects of explant types and media salt strength on growth and secondary metabolite accumulation in adventitious roots of *Periploca sepium* Bunge. Acta Physiol Plant 33:2447-2452.

Zhao J, Zhu W, Hu Q (2001). Effects of stress factors, bioregulators and synthetic precursor on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. Appl Microbiol Biotechnol 55:693-698.

Zhong JJ (2001). Biochemical engineering of the production of plant-specific secondary metabolites by cell cultures. Adv Biochem Eng Biotechnol 72:1-26.

Zhu JK (2001a). Plant salt tolerance. Trends Plant Sci 6:66-71.

Zhu JK (2001b). Cell signaling under salt, water and cold stresses. Curr Opin Plant Biol 4:401-406.

Zhu JK (2002). Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247-73.

Zhu JK, Hasegawa PM, Bressan RA (1997). Molecular aspects of osmotic stress in plants. Crit Rev Plant Sci 16:253-277.

Zobayed SMA, Afreen F, Kozai T (2005). Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort. Plant Physiology and Biochemistry 43:977-984.

CURRICULUM VITAE

Name : Günce ŞAHİN

Permanent Address : Abant Izzet Baysal University, Faculty of Arts and Sciences, Department of Biology, 14280 BOLU/TURKEY

Degree and date to be conferred : Ph.D., 2012

Place and Date of Birth : Ankara, 29 August 1980

Secondory education : Mimar Kemal Lisesi, Ankara, 2000

Collegiate institutions attended	Date	Degree	Date of degree
Ankara University, Dept of Biology	2000-2004	B.S	2004
Ankara University, Dept of Biology	2004-2007	M.S	2007
Abant Izzet Baysal University, Dept of Biology	2007-2012	Ph.D.	2012

Publications :

Şahin G, De Tullio MC (2010) A winning two-pair: role of the AsA/DHA and GSH/GSSG redox couples in signal transduction. In : Anjum NA, Chan MT, Umar S (eds). Ascorbate-glutathione pathway and stress tolerance in plants. Springer, Dordrecht, The Netherlands, pp 251-263.

Verma SK, Yucesan B, **Sahin G**, Gurel E. (2010) High frequency plant regeneration via direct and indirect somatic embryogenesis and cardiotonic glycoside production in *Digitalis ferruginea*. In Vitro Cell Dev Biol Animal 46 (Supl 1): S93-S211.

Verma SK, Yucesan B, **Sahin G**, Gurel S, Gurel E. (2011). Direct shoot regeneration from leaf explants of *Digitalis lamarckii* Ivan, an endemic medicinal species. Turkish J Bot 35:689-695.

Verma SK, **Sahin G**, Yucesan B , Eker I, Sahbaz N, Gurel S, Gurel E. (2012). Direct somatic embryogenesis from hypocotyl segments of *Digitalis trojana* Ivan and subsequent plant regeneration. Industrial Crops and Products 40:76-80.

Positions held : Research assistant in the Department of Biology, AIBU since 2006.