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Molecular and Physiological Studies on Basil (*Ocimum basilicum* L.) Under Cadmium Stress

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Abstract: Cadmium toxicity is a serious problem in agriculture. Cadmium accumulation in soils may come from different sources, including air pollutants and soil applications of commercial fertilizers, sewage sludge, manure and lime. The cadmium exposure causes oxidative stress. Phenol and flavonoid are involved in this stress. The effect of different concentrations of cadmium chloride (0, 25, 50 and 75 µM) on the phenol, flavonoid and proline contents in Ardestan and Isfahan cultivars of basil (*Ocimum basilicum* L.) were studied. The samples were harvested after 18 days of treatment.With increasing concentration of cadmium chloride, the content of phenol, flavonoid and proline increased in both of the cultivars. The increase in Ardestan cultivar was more pronounced than Isfahan cultivar. According to the results, it appears that Ardestan and Isfahan cultivars are both tolerant to cadmium stress but Ardestan cultivar may do more compare to other one. Effective methods are needed to identify those genes that are differentially expressed under cadmium condition. The differential display PCR (DD-PCR) was conceived to allow the identification and molecular cloning of differentially expressed genes. This technique was devised to amplify messenger RNAs and display their 3' termini on polyacrylamide gels. It was concluded that the plant responded to the stress conditions by altering the gene expression of regulatory metabolic processes, especially the photosynthesis.

Key words: Ardestan Cultivar · Isfahan Cultivar · Heavy Metal Toxicity · Differential Display PCR

INTRODUCTION

Abiotic stress is the main factor negatively affecting crop growth and productivity worldwide. Plants are continuously confronted with the harsh environmental conditions (such as soil salinity, drought, heat, cold, flooding and heavy metal contamination). The heavy metal, Cd is commonly released into the arable soil from industrial processes and farming practices [1]. In spite of its high phytotoxicity, Cd is easily taken up by plant roots and transported to above-ground tissues and enters into the food chain where it may pose serious threats to human health [2, 3]. The International Agency for Research on Cancer in 1993 classified Cd as a human carcinogen and, interestingly, it has also been suggested that crops are the main source of Cd intake by humans [4, 5]. Being highly mobile in phloem, 16 Cd can be accumulated in all plant parts which causes stunted growth, chlorosis, leaf epinasty, alters the chloroplast ultrastructure, inhibits photosynthesis, inactivate enzymes in CO_2 fixation, induces lipid peroxidation, inhibits pollen germination and tube growth and also disturbs the nitrogen (N) and sulfur (S) metabolism and antioxidant machinery. However, Cd is a non-redox activemetal, but it induces the generation of reactive oxygen species (ROS) including superoxide radical (O_2^{0-}), hydrogen peroxide (H_2O_2) and hydroxyl radical which has to be kept under tight control because the presence of Cd lead to excessive production of ROS causing cell death due to oxidative stress such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acid. To repair the Cd-induced inhibitory effects of ROS, plants employ ROS detoxifying antioxidant defense machinery which includes nonenzymatic (proline, phenolic compounds; α-tocopherol and carotenoids) and enzymatic (superoxide dismutase, SOD; catalase, CAT and guaicol peroxidase, GOPX) antioxidants [6].

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Basil (*Ocimum basilicum* L.) is a member of the Lamiaceae familyand a very important medicinal plant. *Ocimum basilicum* contains phenolic compounds that act as powerful antioxidants, free radical-scavengers and metal chelators [7]. With its particular importance for human health, basil was used in this experiment to explore the cadmium stress effects on its genome.

Plant species have been studied for their responses (physiological/molecular) when subjected to different stresses [8, 9]. The differential screening has been helpful at molecular level in sorting out the novel endurance mechanisms, subsequently to find out the key responses (molecular) for model species [10, 11]. State-of-the-art methods for analyzing gene expression can be divided into two systems, those with a closed and those with an open architecture. While closed systems only allow analysis of predefined known genes, open systems allow analysis of the expression of genes that were previously unknown. The quality of closed systems therefore depends on the quality of the prior knowledge. A complete analysis of the transcriptome of a genome using closed systems is strictly dependent upon the completeness of knowledge of that transcriptome. In contrast to closed architecture systems, open systems require no *a priori* knowledge of the transcriptome. Therefore, open systems can identify novel transcripts in the absence of information on the modulation of expression of such a transcript [12]. The differential-display reverse transcription-PCR (DDRT-PCR) technique has been widely used in plants to isolate genes that are differentially expressed in response to various stresses. This technique is a suitable, low-cost technique to identify differentially displayed genes [13].

MATERIALS AND METHODS

Physiological Assays: Seeds of basil (*Ocimum basilicum* L.) were selected uniformly and disinfected with 5% hypochlorite for 1min. After germination, uniform seedlings were transferred into plastic pots containing sand. Plants were treated with different concentrations of $CdCl_2$ (0, 25, 50 and 75 µM) under controlled environment. After 18 days of treatment period, plants were harvested for proline, phenolic contents and flavonoids contents assays.

Assay of Proline Contents: At first, 0.2 g of plant tissue was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate f iltered through Whatman # 2 filter paper. Then 2 ml of filtrate was boiled with 2 ml acid-ninhydrin and 2 ml of acetic acid in a test tube for 1 hour at 100°C. The samples promptly were transferred to ice bath in order to stop reaction (for 20 min). The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube and stirred for 15-20 sec, the absorbance of above phase has been read at 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight. For preparing of reagent, the acid-ninhydrin got ready by warming 1.25 g ninhydrin in 30 ml acetic acid and 20 ml phosphoric acid until dissolved [14].

Assay of Total Phenolic Contents: Initially, 0.3 g of plant tissue was weighed and pulverized in pounder containing 3 ml acidic methanol (99.5% methanol and 1% hydrochloric acid, 99 to 1 ratio). The extracts were spilled in falcon and placed in darkness at 4°C for 24 h. After 24 h, the extract was centrifuged in 4000g for 10 min. Then the supernatant was separated and 2ml of ether was added to it for removing of the remaining chlorophyll. Coarser solution was separated by decanter. The resulting solution was used for determining of phenol content. The absorbance of extracts was read at 280 nm by spectrophotometer. Gallic acid standard curve was applied to ascertain the concentration of phenol samples. Then absorbance curve was plotted according to various concentrations of gallic acid [15].

Assay of Flavonoids Contents: At the outset, 0.1g of plant leaves and 5 ml of absolute methanol was homogenized in the laboratory temperature. Then chlorophyll and carotenoid were removed by light ether oil with phase separation from the solution by decanter. In methanolic phase, the absorption spectrum in the region of 200-400nm and maximum absorptionpeak at 240nm was determined the final concentration of flavonoids was expressed based on mg g^{-1}fw [16].

Molecular Assays

RNA Extraction: Total RNA was isolated from leaf tissues of plant samples using QIAGEN RNeasy Plant Mini Kit according to the manufacturer's instructions. The quality of RNA was analyzed electrophorizing the samples on 1% agarose gel. After extraction of RNA, using from DNAase so that the amount of DNA with RNA was extracted, destroyed. This enzyme was used as follows: 2µl of RNA, 1µl of reaction buffer, ribonuclease inhibitor and DNAase 1RNAase-free was added to a tube and it arrived to 20 µl

volume with DEPC-treated water. After that, the samples were placed in thermoblock at 37°C for 30 min. Then 1 µl of RNA was added to it and placed at 65°C for 10 min.

cDNA Synthesis: cDNA synthesis was performed by using of Transcriptor First Strand cDNA synthesis Kit (Roche) in accordance with the manufacturer's instructions. The sequences of the primers (anchored arbitrary) used were including:

Anchored Primers: Sal1:AAGCTTTTTTTTTTA
Arbitrary Primers: Sal2:AAGCTTGATTGCC, Sal3: AAGCTTTGGTCAG, Sal4:AAGCTTTTACCGC

Polymerase Chain Reaction (PCR): PCR was performed by Fermentase Kit as indicated by the manufacturer's instructions. Then the samples were ready to load gel electrophoresis. Positive and negative control samples were placed for PCR. In positive control, of ubiquitine primer was used and for negative control distilled water was added to the compound PCR.

The TBE buffer solution including the mixture of following material: 10.8 g l^{-1} of Tris-base, 5.5 g l^{-1} of boric acid, 0.7444 g l^{-1} of EDTA. Before adding EDTA, pH should regulate (pH 7). 0.5X buffer was used for electrophoresis tank.

Preparation of Polyacrylamide Gel: In the beginning, the glass plates and spacers were cleaned thoroughly and the plates were rinsed with deionized water and ethanol and set them aside to dry. The glass was assembled plates with spacers in gel caster. The 8% gel solution was prepared according to the following protocol: At first, 4.8 ml of 30% Acrylamide, 2.4 ml H_2O and 200 µl5x TBE were mixed. Then 10 µl TEMED and finally 4.8 ml 10% APS were added.

The glasses covered by agar were filled with this solution. The comb was been placed between two glasses that this action can be before adding the solution. The agar is typically used to cover is %0.6 that for preparation of it, 620 mg of agar was solved in 100 ml of water or 0.5X TBE. After sealing of gel, the comb was pulled out from gel. Then the samples and marker were loaded within the sink and run about for 5h and with 90 V.

The gel staining was carried out as follows: Firstly, it was placed in 15 ml 10% ethanol and 1.5 ml 1% acetic acid up and total volume was reached up to 150 ml, for 10 min. Then it was washed with water for 1min. It was placed in 2.25 ml 1.5% nitric acid that its volume arrived to 150 ml, for 3 min. The gel was rinsed for 1min. It was placed in 0.3g $AgNO_3$ and total volume was reached up to 150 ml, for 20 min. It was washed with water for 30 sec (two times). The gel placed in mixture of 7.5 g Na_2CO_3 and 135 µl formaldehyde that its volume arrived to 250 ml, for 4-7 min. At end, it was placed in 7.5 ml 5% nitric acid and total volume reached up to 150 ml volume, for 5 min [17].

Sequencing and Bioinformatics Analysis: The products were directly sequenced by Centre ofExcellence in Molecular biology (CEMB). The homology of the sequences was analyzed using BLASTN (Basic Local Alignment Search Tool) at NCBI website.

Statistical Analysis: Statistical analysis was done using SPSS (version 16) program. Differences were analyzed using one-way Duncan followed by post-hoc comparisons. Duncan was employed for statistical analysis of data. Statistical significance was defined as $P < 0.05$.

RESULTS

Physiological Assays

Proline Contents: Cd treatment caused a increase in the leaf proline contents in Ardestan and Isfahan cultivars of basil. This increase in both of cultivars, was highly significant in 75 µM $CdCl_2$ concentration. In general, the increase of proline in Ardestan cultivar was more than that of Isfahan cultivar (Figure 1).

Total Phenolic Contents: Increasing of cadmium chloride concentration caused an increase in phenol content in Ardestan and Isfahan cultivars of basil. This increase in both cultivars was highly significant under 50 and 75 µM $CdCl_2$ treatments compared to the control (Figure 2).

Flavonoid Contents: The leaf flavonoid levels increased as a result of rise in $CdCl_2$ treatment of cultivars (Figure 3).

Molecular Assay

Differential Display of Basil DNA Sequences: In this experiment, Sal2 and Sal4 primers did not have any informative bands. After cDNA synthesis, the PCR was performed. The PCR products were electrophoresed and stained. The length ofcDNA fragments were 200bp (Figure 4).

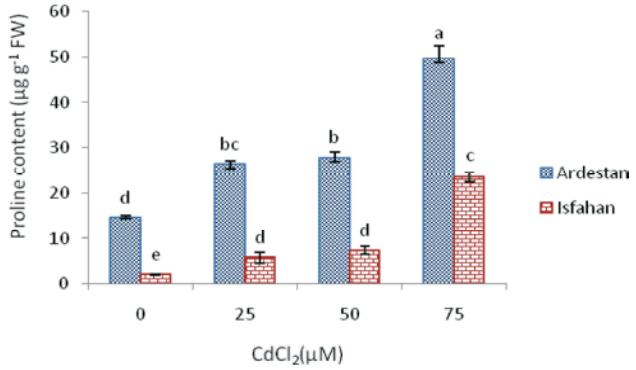

Fig. 1: The effect of $CdCl_2$ on proline contents in Ardestan and Isfahan cultivars

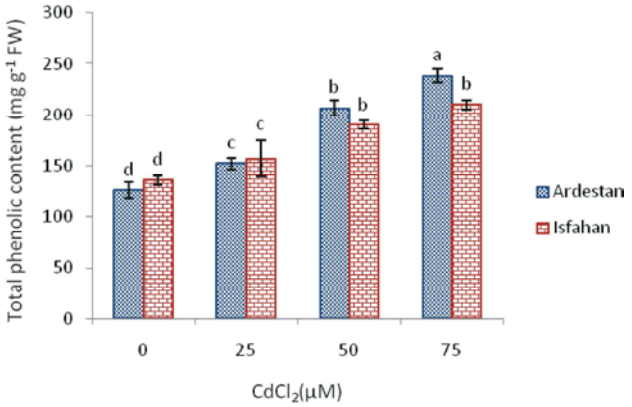

Fig. 2: The effect of $CdCl_2$ on total phenolic contents in Ardestan and Isfahan cultivars

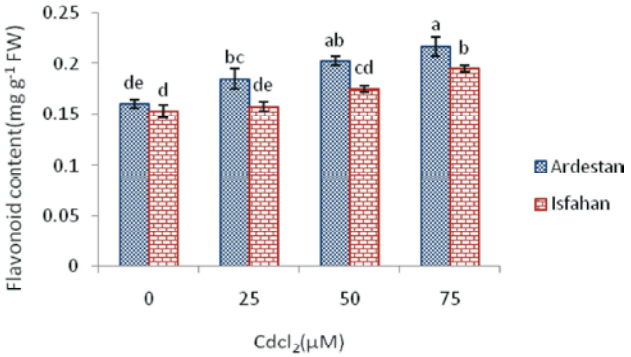

Fig. 3: The effect of $CdCl_2$ on Flavonoid contents in Ardestan and Isfahan cultivars

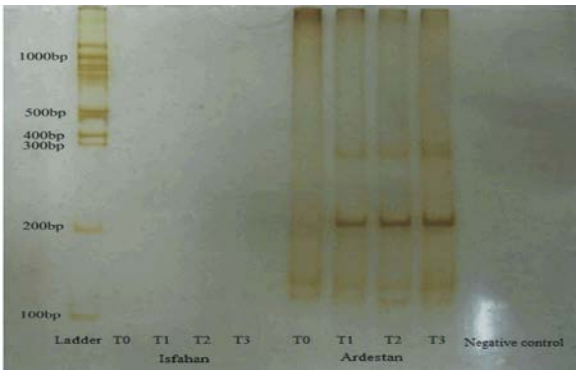

Fig. 4: PCR product in plants under different treatment

Fig. 5: Color key for alignment score

Table 1: The cDNA sequences producing significant alignment with other family members

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
JF412791.1	Cucumis melo subsp. melo chloroplast, complete genome	113	113	63%	7e-22	80%	
EU431223.1	Carica papaya chloroplast, complete genome	113	113	63%	7e-22	80%	
DQ865976.1	Cucumis sativus strain CHIPPER chloroplast, complete genome	113	113	63%	7e-22	80%	
DQ865975.1	Cucumis sativus cultivar GY14 chloroplast, complete genome	113	113	63%	7e-22	80%	
DQ119058.1	Cucumis sativus chloroplast, complete genome	113	113	63%	7e-22	80%	
JX664077.1	Averrhoa carambola PetA (petA) gene, partial cds; chloroplast	107	107	63%	3e-20	79%	
JQ228389.1	Theobroma cacao chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228388.1	Theobroma grandiflorum chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228387.1	Theobroma cacao genotype ICS-39 chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228386.1	Theobroma cacao genotype Pentagonum chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228385.1	Theobroma cacao genotype Stahel chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228379.1	Theobroma cacao genotype Criollo-22 chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228383.1	Theobroma cacao genotype ICS-06 chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228382.1	Theobroma cacao genotype Scavina-6 chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228381.1	Theobroma cacao genotype ICS-01 chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ228380.1	Theobroma cacao genotype Amelonado chloroplast, partial genome	107	107	63%	3e-20	79%	
JQ220304.1	Theobroma cacao genotype EET-64 chloroplast, partial genome	107	107	63%	3e-20	79%	
HQ244500.2	Theobroma cacao chloroplast, complete genome	107	107	63%	3e-20	79%	
HQ336404.2	Theobroma cacao chloroplast, complete genome	107	107	63%	3e-20	79%	
HQ207704.1	Corynocarpus laevigata chloroplast, complete genome	107	107	63%	3e-20	79%	
GQ861354.1	Brassica napus strain ZY036 chloroplast, complete genome	107	107	63%	3e-20	79%	
EU922543.1	Pelargonium cotyledonis cytochrome f (petA) gene, complete cds; cl	107	107	63%	3e-20	80%	
AP009367.1	Aethionema grandiflorum chloroplast DNA, complete sequence	107	107	63%	3e-20	80%	
AC189190.1	Brassica rapa subsp. pekinensis clone KBrB002G19, complete sequen	107	107	63%	3e-20	79%	
DQ231548.1	Brassica rapa subsp. pekinensis chloroplast sequence	107	107	63%	3e-20	79%	
AJ970307.1	Cucumis sativus chloroplast complete genome	107	107	57%	3e-20	81%	

Using BLASTN program, cDNA fragment showed sequence similarity to some plant families. The closet members of Cucurbitaceae and Oxalidaceae family that *Cucumismelo* and *Cucumissativus* from Cucurbitaceae family and *Averrhoacarambola* from Oxalidaceae family can be named. This fragment has80% homology to the *C.melo* and *C.sativus* chloroplast DNA. Of chloroplast genes that are effective in stress include *psbA*and *psbD* that products of these genes are D_1 and D_2 proteins respectively which are in photosystem II. This fragment has 79% homology to *pet A*gene in *A. carambola*that the product of *pet A* gene is cytochrome *f* (Figure 5 and Table 1).

DISCUSSION

Proline accumulation,could be accepted as an indicator of environmental stress especially salt stress,

It is also considered as an important protective roles. Heavy metal stress leads to prolineaccumulation. It results from (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization and (d) hydrolysis of proteins [18]. For a long time, proline was considered as an inert compatible osmolyte that protects subcellular structures and macromolecules under osmotic stress. However, proline accumulation can influence stress tolerance in multiple ways. Proline has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes. Examples of such roles include the prevention of protein aggregation and protection of nitrate reductase during heavy metal and osmotic stress [19].

Phenolic compounds such as flavonoids, phenolic acids and tannins are widely distributed in plants [20], which have gained much attention, due to their antioxidant activities and free radical scavenging abilities, which potentially have beneficial implications for human health [21]. The antioxidant activity of the phenolic compounds were attributed to its redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and have also metal chelating properties [22]. Phenolic compounds are shown to have strong antioxidant activity in plants growing under heavy metal stress. It has been suggested that their antioxidant act resides chiefly in their chemical structure. Phenols are oxidized by peroxidase and contribute inscavenging H_2O_2 [23]. The phenolic compounds play an important role in the cadmium defense of *E. andevalensis*. Phenolic compounds could be used as indicators of metal presence in the leaves of plant species that this results are agree with our results [24].

Flavonoids are a large group with low molecular weight, ubiquitously distributed, polyphenolic secondary metabolites. These compounds play a significant role in various stages of plant growth and their existence in the environmental stresses. Flavonoids are remarkable reactive oxygen species scavengers and fight continuously against polluted atmosphere. Flavonoids play a variety of significant roles in plants. They act as signal molecules, phytoalexins, detoxifying agents, stimulants for germination of spores, play significant activities in seeds germination, act as UVfilters, flavonoids in temperature acclimation, in drought resistance, pollinator attractants and allelochemical agents [25].

The studies have provided evidence that flavonoids could be a factor in heavy metal tolerance in *Arabidopsis thaliana* that are consistent to our results [26]. Flavonoids serve as ROS scavengers by locating and neutralizing radicals before they damage the cell thus important for plants under adverse environmental conditions [27].

Plants have the potential to survive under stress conditions through alteration in expression of specific genes (stress-responsive genes). The objective of the current study was to appraise the expression of gene/ DNA sequences controlling the key metabolic processes of cadmium tolerance in basil (*Ocimum basilicum*). The Differential display was used to detect the fragments of gene/DNA sequences, expressed differentially under stress conditions.

The Role of Cytochrome *f* in Plant Stress: Cytochrome *f* is one of the main subunits of a membrane protein complex, the cytochrome *b6 f*, which is one of the major redox complexes of the thylakoid membrane and an electron transfer and proton-translocating enzyme in photosynthesis. Cytochrome *f* is encoded by the single-copy chloroplast *petA* gene and thus the protein is not imported from the cytosol [28]. In plants, most algae and some cyanobacteria are plastocyanin (Pc), a water soluble metallo-protein containing copper, which accepts electrons from the cyt*f* and transfers to the chlorophyll reaction centre of photosystem I. The interaction of the cytochrome and two photosystems is essential in proper plant functioning and growth. It has been reported that heavy metals such as zinc, cadmium or copper influence many features of plant life. They can cause the inhibition of plant enzymes due to making catalytically active groups react with other ions or compete for the metal sites of metal-binding proteins involved in many processes [29]. It has been found that the metalions soluble in water can either adhere to the protein in some in soluble forms or compete for the metal sites of metal-binding proteins [30].

In *C. saccharophila* following the HS was released cytochrome *f*from thylakoid membranes into the cytosol. Moreover, a partial *petA*cDNA from *C. saccharophila* (*ChspetA*) was isolated and identified and its expression analysed during HS-induced PCD.

By a search of the cytochrome *f* sequences from the GenBank database and multiple sequence alignments deduced *C. saccharophila* cytochrome *f* amino acid sequence has the highest percentage of similarity with plants (about 90%) and *Chara vulgaris* (77%), whereas the similarity reduces to 67–69% if the *C. saccharophila* cytochrome *f* amino acid sequence is compared with green algae. Multiple alignments of the cytochrome *f* amino acid sequences revealed that the C-terminal domain is highly

conserved in *C. saccharophila*. The conservation of the C-domain amongst plants and algae cytochrome *f* is high [28].

The Role of D_1 and D_2 Proteinsin Plants Stress: PSII is a multisubunit pigment-protein complex with the enzymatic activity of light-dependent plastoquinonereductase, leading to the release of electrons, protons and molecular oxygen. Most of the heavy metals inhibit PSII activity. They affect the oxygen-evolving complex (OEC) with the loss of all or part of the Mn^{2+} cluster together with some of the extrinsic polypeptides associated with the water oxidation mechanism. Cd inhibits PSI and PSII activity. PSII is more sensitive to Cd than PSI and it is the primary site of action of Cd in photosynthetic electron flow. Moreover, biosynthesis of chlorophyll is impeded by Cd. Heavy metals such as Cd^{2+}, $Pb^{2+}$and Ni^{2+} replace the central Mg^{2+} of chlorophyll in plants. Such substitution is expected to prevent light harvesting and cause impairment of photosynthesis. Mycorrhiza, by improving uptake of Mg can support a higher chlorophyll concentration and subsequently lead to a higher production of photosynthateand biomass.

In PSI due to blockage of electron flow, free radicals can be produced that lead to generation of free radicals and initiate peroxidation reactions. Enhanced peroxidation activity contributes significantly to the decreased level of chlorophyll and decreased photosynthetic rate observed under Cd treatment [6].

The influence of Cd^{2+} on the function, structure and composition of PSII domains in the thylakoid membranes has been emphasised in several reports. Cd^{2+} has been demonstrated to cause an initial stimulation of D1 protein turnover and PSII activity [31]. D_1 protein of thylakoid membranes was shown as a sensitive protein to environmental stress conditions: under various unfavorable conditions like drought, nutrition deficiency, heat, chemical stress, ozone fumigation as well as UV-B and visible light stresses can influence the turnover of D_1 protein. In *Spirulinaplatensis*, it has been proposed that salt stress in combination with moderate light intensities could damage the D_1 protein [32]. The reaction centre D_1 protein of PSII is vulnerable not only to light, but also to high temperature. The almost complete D_1 dephosphorylation occurs in the leaves exposed to 42°C. The heat-shock-induced dephosphorylation of N-terminal threonine residue in D_1, as well as in D_2 is catalyzed by a cyclophilin-regulated PP2A-like protein phosphatase intrinsic to thylakoid membranes. This protein phosphatase is specific in dephosphorylationof N-terminal threonine residues of PSII subunits [33]. PSII is believed to play an important role in the response of leaf photosynthesis to environmental stresses. In particular, the susceptibility of PSII to several stress factors such as heat, high light, air pollution as well as nutrient deficiency has been affirmed [34]. In *Synechocystis*, it has been reported that the decreased PSII activity by salt stress can be explained by the fact that salt stress inhibits the repair of photodamaged PSII by suppressing the transcription and translation of *psbA* genes [35]. Application of biochemical techniques detecting in vivo phosphorylated proteins has also revealed significant and differential environment- dependent changes in phosphorylation of the photosynthetic proteins, particularly in stressful conditions [33].

REFERENCES

1. Salt, D.E. and W.E. Rauser, 1995. Mg-ATP dependent transport of phytochelatins across the tonoplast of oat roots. PlantPhysiology, 107: 1293-1301.
2. Bertrand, M. and J.C. Guary, 2002. How plants adopt their physiology to an excess of metals. In: Pessarakli M., ed. Handbook of Plant and Crop Physiology. 2nded. New York: Marcel Dekker, pp: 751-761.
3. Ouzounidou, G., 1993. Changes in variable chlorophyll fluorescence as a result of Cu-treatment: Dose-response relations in Sileneand Thlaspi. Photosynthetica, 29: 445-462.
4. Hirschi, K.D., V.D. Korenkov, N.L. Wilganowski and G.J. Wagner, 2000. Expression of Arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiology, 124: 125-133.
5. Molas, J., 2002. Changes of chloroplast ultrastructure and total chlorophyll concentration in cabbage leaves caused by excess of organic Ni(II) complexes. Environmental.Experimental. Botany, 47: 115-126.
6. Mishra, S. and R.S. Dubey, 2005. 44 Heavy Metal Toxicity InducedAlterations in Photosynthetic Metabolism in Plants.
7. Sgherri, C., S. Cecconami, C. Pinzino, F. Navari-Izzo and R. Izzo, 2010. Levels of antioxidants and nutraceuticals in basil grown in hydroponics and soil. Food Chemistry, 123: 416-422.
8. Kang, J., W. Xie, Y. Sun, Q. Yangand M. Wu, 2010. Identification of genes induced by salt stress from *Medicagotruncatula* L. seedlings. Biotechnology, 9: 7589-7594.

9. Selvam, J.N., N. Kumaravadivel, A. Gopikrishnan, B.K. Kumar, R. Ravikesavan and M.N. Boopathi, 2009. Identification of a novel drought tolerance gene in *Gossypiumhirsutum* L. cv KC3. Communications in Biometry and Crop Science, 4: 9-13.
10. Bohnert, H.J., Q. Gong, P. Li and S. Ma, 2006. Unraveling abiotic stress tolerance mechanisms-getting genomics going. Current Opinion in Plant Biology, 9: 180-188.
11. Valliyodan, B. and H.T. Nguyen, 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Current Opinion in Plant Biology, 9: 189-195.
12. Green, C.D., J.F. Simons, B.E. Taillon and D.A. Lewin, 2001. Open systems: panoramic views of gene expression. Immunology. Methods, 250: 67-79.
13. Medini, M., M. Baum and S. Hamza, 2009. Transcript accumulation of putative drought responsivegenes in drought-stressed chickpea seedlings. Biotechnology, 8(18): 4441-4449.
14. Bates, L.S., R.P. Waldreman and I.D. Tear, 1973. Rapid determination of free proline for water stress studies. Plant Soil, 39: 205-207.
15. Dai, P., Z.T. Xiong, Y. Hung and M.J. Li, 2006. Cadmium induced changes in pigments total phenolic and phenylalanine ammonialiase activity in fronds in Azolla imbricate. Environmental Toxicology, 21(5): 505-513.
16. Swain, T., 1976. Flavonoids. In: T.W. Goodwin, (eds), chemistry and biochemistry of plant pigments. Academic. Press, London, pp: 166-206.
17. Creste, S., A.T. Neto and A. Figueira, 2001. Detection of singlesequencerepeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter, 19: 299-306.
18. Pant, P.P., A.K. Tripathi and V. Dwivedi, 2011. Effect of Heavy Metals on Some Biochemical Parameters of Sal (*Shorearobusta*) Seedling at Nursery Level, Doon Valley, India. Agriculture Science, 2(1): 45-51.
19. Szabados, L. and A. Savoure, 2009. Proline: a multifunctional amino acid. Trends in Plant Science, 15(2): 89-97.
20. Li, B.B., B. Smith and M.M. Hossain, 2006. Extraction of phenolics from citrus peels: I. Solvent extraction method. Separation. Purification. Technology, 48: 182-188.
21. Govindarajan, R., D.P. Singh and A.K.S. Rawat, 2007. High-performance liquidchromatographic method for the quantification of phenolics in 'Chyavanprash' a potent Ayurvedic drug. Pharmacy. Biomedicine. Analytical, 43: 527-532.
22. Rice-Evans, C.A., N.J. Miller, P.G. Bolwell, P.M. Bramley and J.B. Pridham, 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radical. Resoure, 22: 375-383.
23. Singh, Y. and C.P. Malik, 2011. Phenols and their antioxidant activity in *Brassica juncea* seedlings growing under $HgCl_2$ stress. Microbiological. Biotechnological. Response, 1(4): 124-130.
24. Marquez-Garcia, B., A.M. Fernandez-Recamales and F. Cordoba, 2011. Effects of Cadmium on Phenolic Composition and Antioxidant Activities of *Erica and evalensis*. Botany, doi: 10.1155/ 2012/ 936950.
25. Samanta, A., G. Das and S.K. Das, 2011. Roles of flavonoids in plants. Pharmacy Science Technology, 6(1): 12-35
26. Keilig, K. and J. Ludwig-Müller, 2009. Effect of flavonoids on heavy metal tolerance in *Arabidopsis thaliana* seedlings. Botanical Studies, 50: 311-318.
27. Lovdal, T., K.M. Olsen, R.V. Slimestad, M. Erheul and C. Lillo, 2010. Synergetic effects of nitrogen depletion, temperature and light on the content of phenolic compounds and gene expression in leaves of tomato. Phytochemistry, 71: 605-613.
28. Zuppini, A., C. Gerotto, R. Moscatiello, E. Bergantino and B. Baldan, 2009. *Chlorella saccharophila* cytochrome f and its involvement in the heat shock response. Experimental Botany, 60(14): 4189-4200.
29. Sujak, A., 2005. Interaction between cadmium, zinc and silver-substituted plastocyanin and cytochrome b6f complex-heavy metals toxicity towards photosynthetic apparatus. Physiologiae Plantarum, 27(1): 61-69.
30. Roberts, A.G., M.K. Bowman and D.M. Kramer, 2002. Certain metal ions are inhibitors of cytochrome b6fcomplex 'Rieske' iron-sulfur protein domain movements. Biochemistry, 41: 4070-4079.
31. Giardi, M.T., M. Koblizek and J. Masojidek, 2001. Photosystem II-based biosensors for the detection of pollutants. Biosensors and Bioelectronics, 16: 1027-1033.

32. Sudhir, P.R., D. Pogoryelov, L. Kovacs, G. Garab and S.D.S. Murthy, 2005. The Effects of Salt Stress on Photosynthetic Electron Transport and Thylakoid Membrane Proteins in the Cyanobacterium *Spirulinaplatensis*. Biochemistry and Molecular Biology, 38(4): 481-485.
33. Vener, A.V., 2006. Environmentally modulated phosphorylation and dynamics of proteins in photosynthetic membranes. Biochim. Biophys Acta., 1767(6): 449-57.
34. He, J.X., J. Wang and H.G. Liang, 1995. Effects of water stress on photochemical function and protein metabolism of photosystemII in wheat leaves. Physiologia Plantarum, 93: 771-777.
35. Gong, H., Y. Tang, J. Wang, X. Wen, L. Zhangand C. Lu, 2008. Characterization of photosystem II in salt-stressed cyanobacterial *Spirulinaplatensis* cells. Biochimicaet Biophysica Acta, 1777: 488-495.