The role of over expression of P5CS gene on proline, catalase, ascorbate peroxidase activity and lipid peroxidation of transgenic tobacco (*Nicotiana tabacum L.*) plant under *in vitro* drought stress

Somayyeh Zarei, Ali Akbar Ehsanpour[*] and Jalil Abbaspour

Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

Abstract

In this study proline content and activity of catalase (CAT), and ascorbate peroxidase (APX) and level of lipid peroxidation in terms of malondialdehyde (MDA) content were measured in transgenic tobacco (*Nicotiana tabacum* cv. Wisconsin), over expressing a Δ-1-pyrroline-5-carboxylate synthase (P5CS) gene, and non transgenic plants as control. Drought stress was applied using polyethylene glycol (PEG) 6000 at concentrations of 217, 264, 320, 637, 1292 mmol/kg equal to (0, 5, 10, 20, 30% respectively). Proline content, especially in transgenic plants, was increased in leaves and roots significantly. CAT and APX activities increased under drought stress and the highest activity was observed in 10 and 20% of the PEG treatment. MDA content was increased by increasing of PEG and the highest MDA content was revealed in transgenic and non transgenic plants at 20% and 30%, respectively. Our results suggest that P5CS is an inducible gene and over production of proline and induction of CAT and APX activities are involved in drought tolerance mechanism.

Keywords: Tobacco, drought stress, proline, catalase, ascorbate peroxidase, P5CS gene

Introduction

One of the most important abiotic stress is drought, which results in the disruption of water potential slopes, loss of turgor and decreasing of pressure potential. Abiotic stresses and osmotic adjustment contributes to pressure potential maintenance and stress tolerance of plants (Cherian et al., 2006). In response to water stress, plants accumulate osmolytes and protect themselves against drought stress. Proline is one of the most common compatible osmolytes and plays an overriding role in osmotic pressure adjustment (Yamch et al., 2005). Proline allows many plant species to survive under stress without interfering with the normal biochemical reactions (Stewart, 1981). The most important function of proline is turgor maintenance and scavenging the excess reactive oxygen species (ROS). It stabilizes protein, enzyme and other subcellular structures such as membranes. Proline also act as an antioxidant, and regulates cellular redox status under stress condition (Chinnusamy et al., 2005). The first response of plants to drought stress is closure of

stomata and a decrease in CO_2 concentration in leaf mesophyll tissue. Due to decrease in CO_2 concentration, accumulation of NADPH and loss of $NADP^+$ occur and oxygen accepts electrons and leads to ROS formation (Sairam et al., 1998; Asada, 1999). ROS results in injury to vital molecules such as nucleic acids, proteins, structural carbohydrates, and lipids (Mittler, 2002; Daveis, 1987). Lipid peroxidation of cellular membranes is the most important effect of ROS that finally leads to disruption of plant growth and development (Chen et al., 2000; Sreenivasulu et al., 1999).

Plants prevent or alleviate ROS damages by antioxidants protection system which provides protection against oxidative stress. Antioxidants are non enzymatic including glutathione, ascorbate and carotenoids, and enzymatic such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) (Apel and Hirt, 2004; Bhardwaj et al., 2007).

Ascorbate peroxidases have been found in higher plants, algae and some cyanobacteria (Sano et al., 2001; Sharma and Dubey, 2004). Ascorbate, as an electron donor, is utilized in reduction of hydrogen peroxide (Shigeoka et al., 2002). CAT and APX reduce H_2O_2 to water and O_2 (Gratao et al., 2005). Unlike APX, CAT acts without any electron donor

---
*Corresponding author E-mail:
 ehsanpou@sci.ui.ac.ir

or reducing agent (Mallick and Mohn, 2000).

In some plants such as *Vigna aconitifolia* and *Arabidopsis thaliana* reduction of glutamate to its semialdehyde intermediate is catalyzed by a single bifunctional enzyme, Δ-1-pyrroline-5-carboxylate synthase (P5CS), whose transcription is induced in plants subjected to salt and drought stress (Yoshiba et al., 1995). Furthermore, data indicated that, over expression of *V. aconitifolia* P5CS (VaP5CS) in transgenic tobacco plants increased proline level and rendered plants less sensitive to the osmotic stress (Kishor et al., 1995). However, there is a controversy discussion in balance activity of P5CS and P5CR enzyme (Verbruggen, 1995). It has been documented that transgenic plants, over expressing the P5CS gene, increases concentration of proline and resulted in more resistance to both drought and salt stress (Kishore et al., 1995). The objective of this work was to understand better the relationship between drought tolerance and proline content, the CAT and APX activity and lipid peroxidation in transgenic tobacco plants over expressing P5CS gene.

Materials and Methods

Plant materials and treatment

Transgenic tobacco plants (*Nicotiana tabacum* cv. Wisconsin) (T1 seeds) carrying P5CS gene were surface sterilized in 70% ethanol and then grown on MS medium (Murashige and Skoog, 1962) and kept in the growth chamber (16/8 h light and dark respectively, with approximately 40 μm photon $m^{-2}s^{-1}$ light density) at 25°C. After 18-20 days, seedlings were transferred to MS medium supplemented with PEG (217, 264, 320, 637, 1292 mmol/kg). After 4 weeks proline content, CAT and APX activities and lipid peroxidation were measured in leaves and roots.

Proline measurement

Free proline accumulation was estimated using ninhydrin reaction based on method described by Bates (1973). A small portion (0.04 g) of leaves or roots was homogenized with 1.7 ml of 3% (w/v) sulphosalicylic acid (Merk). The homogenate was centrifuged at 13000 rpm for 20 min. Then ninhydrin reagent (1 ml) (Sigma) and glacial acetic acid (1 ml) were added to 1 ml of the centrifuged extract. The mixture was boiled for 1 h in a water bath and then cooled on ice. Then 2 ml toluene was added to each tube, and tubes were placed in the dark for 1 h. Absorption of chromophore was determined at 520 nm by spectrophotometer (Shimadzu UV-160, Japan). Toluene was used as

blank. Proline content was calculated using L-proline (Sigma) as a standard curve.

Enzyme assays

For enzyme extraction, fresh samples of leaves from control and stressed seedlings (0.1 g) were homogenized in an ice bath in 1 mL of phosphate buffer saline (PBS, pH:7.4) containing NaCl (8 g/l), KCl (0.2 g/l), Na_2HPO (41.44 g/l), KH_2PO_4 (24 g/l) and polyvinylpyrrolidone (PVP, 1%). The homogenate was centrifuged at 14000 rpm at 4 °C for 20 min. The supernatant was collected and used for enzyme (CAT and APX) activity analysis.

Catalase (EC 1.11.1.6)

The CAT activity was determined by measuring the decomposition of H_2O_2. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 10 mM H_2O_2, and 0.05 ml of the enzyme extract (Aebi, 1984). Then the absorbance at 240 nm was recorded every 10 sec. up to 1 min. The CAT activity was calculated and expressed as U/g FW^{-1} min $^{-1}$ (One unit of CAT activity is defined as the amount of enzyme required to consume 1 μmole H_2O_2 min–1). CAT activity was calculated using the coefficient of absorbance of 0.0394 mM^{-1} cm^{-1} by spectrophotometer (Shimadzu).

Ascorbate peroxidase (EC 1.11.1.11, APX)

APX was estimated by slightly modified procedure of Nakano and Asada (1981). APX activity was determined by measuring the consumption of ascorbate. Reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.1 mM H_2O_2, 50 μl of BSA, and 0.05 ml of the enzyme extract. The absorbance at 290 nm was recorded every 10 sec up to 1 min. One unit of APX activity was defined as the amount of enzyme required to consume 1 μmole ascorbate min^{-1}. Ascorbate peroxidase activity was calculated using the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}.

Lipid peroxidation

Fresh plant material (0.1 g) was homogenized in 2.5 ml 0.1% (w/v) trichloroacetic acid (TCA). The level of lipid peroxidation was measured using the malondialdehyde (MDA), thiobarbituric acid (TBA) reaction based on method of Heath and Packer (1968). Lipid hydroperoxides resulting from peroxidation of the cell membrane react with thiobarbituric acid (TBA) to form MDA, which is a crystalline pink pigment with absorption from 525 to 535 nm (Persky et al. 2000). The absorbance of extract was measured at 532 and 600 nm. The amount of MDA-TBA complex was calculated

from the coefficient of absorbance 155 mM^{-1}cm^{-1}.

Results

Statistical analysis

All experiments were carried out in a completely randomized design. The mean values of proline, CAT and APX activity and lipid peroxidation level were taken from the measurements of four replicates and the "Standard Error" of the means was calculated. Two-way ANOVA was applied to determine the mean between different treatments and then Tukey test was performed and significance was determined at $P < 0.05$. All statistical analyses were carried out using SPSS Software program version 10.

Proline content

As the PEG concentration increased, proline level of shoot in transgenic and non transgenic plants, was increased significantly. In transgenic plants, either in leaf or root the proline content was significantly higher than non transgenic (figures 1A and 1B). In both plant types, 20 and 30% PEG showed the highest level of proline content. In the root, proline content was increased (1.3 and 1.2 fold) by increasing of PEG particularly in the highest level at 20 and 30% PEG compared to non transgenic plants.
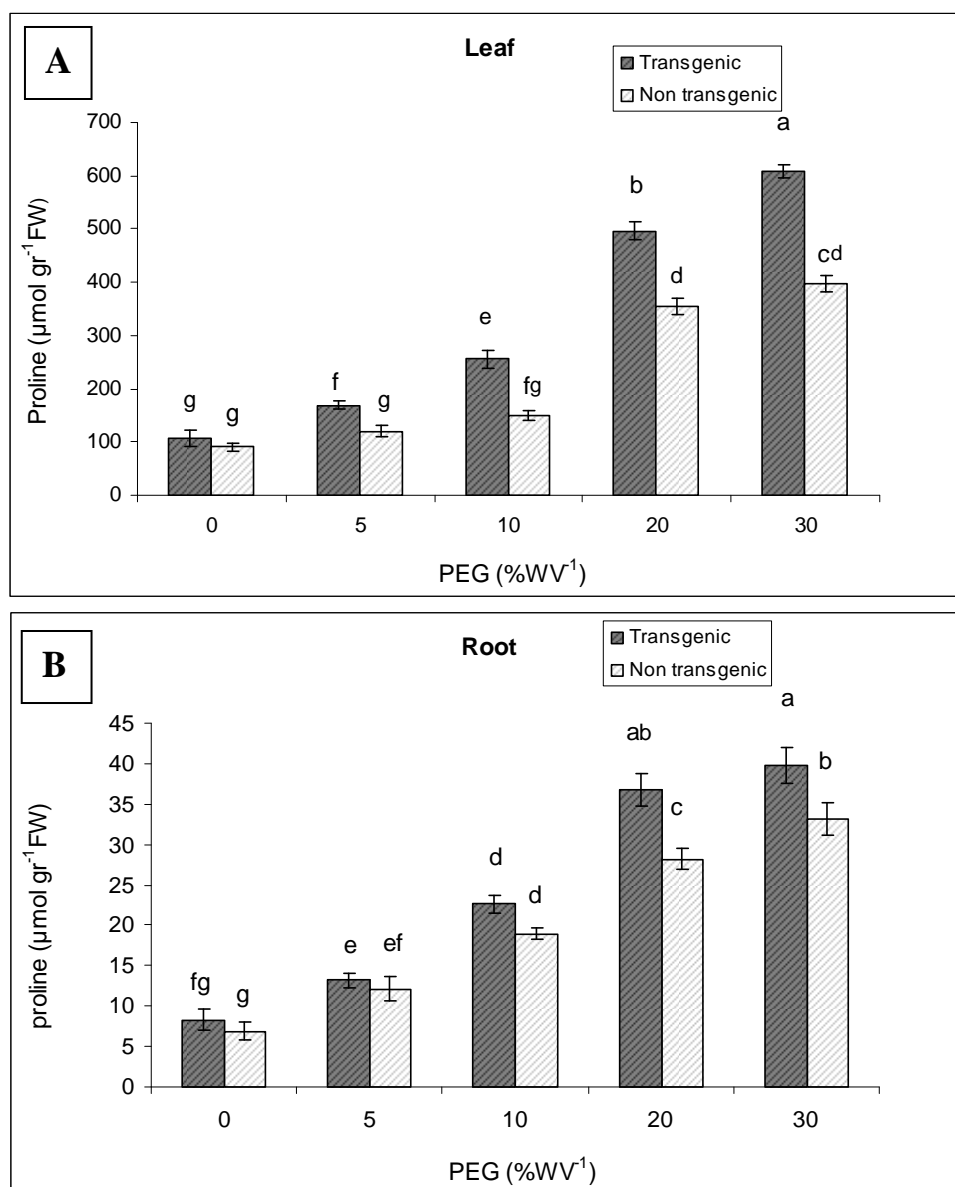

Figure 1. Proline content in shoot (A) and roots (B) of transgenic and non transgenic tobacco seedlings. Values are means ± Sd. Uncommon letters are significant based on Tukey test ($P < 0.05$). 0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively.

APX activity

The APX, showed a high significant activity by increasing of PEG up to 20% either in transgenic or non transgenic plants. Transgenic plants showed higher APX activity than non transgenic plants in all PEG concentrations. In transgenic plants, both plant types, the APX activity decreased at 30% PEG. (figure 2).

CAT activity

Results indicated that the CAT activity was increased by increasing of PEG concentration. In transgenic plants, CAT activity at 5, 10 and 20%

PEG while in non transgenic plants at 10 and 20% PEG increased significantly however, at 30% the activity of CAT decreased compared to the control plants (figure 3).

Lipid peroxidation

Lipid peroxidation data (measured as MDA content), showed a significant increase in both plant types in 10, 20 and 30% of PEG. MDA was increased significantly in comparison to control plants. In non transgenic plants at 10 and 20% PEG it was significantly higher than transgenic ones (figure 4).
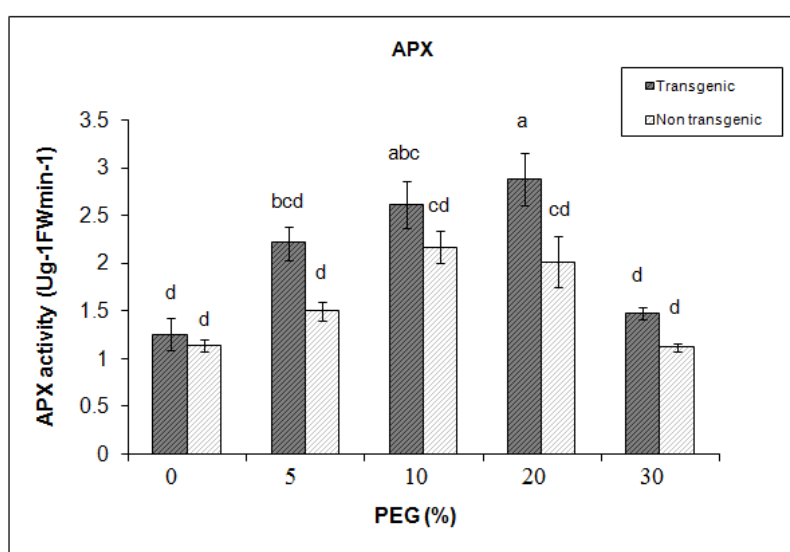

Figure 2. Ascorbat peroxidase (APX) activity of leaves of tobacco seedlings in response to drought stress. Values are means ± SE. Uncommon letters are significant based on Tukey test (P < 0.05). 0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively.
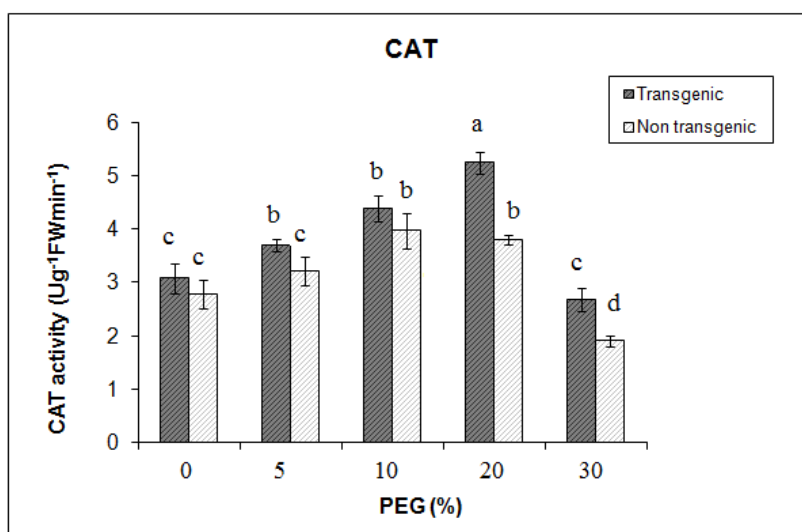

Figure 3. Catalase (CAT) activity of leaves of tobacco seedlings. Values are means ± SE. Uncommon letters are significant based on Tukey test (P < 0.05). 0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively.
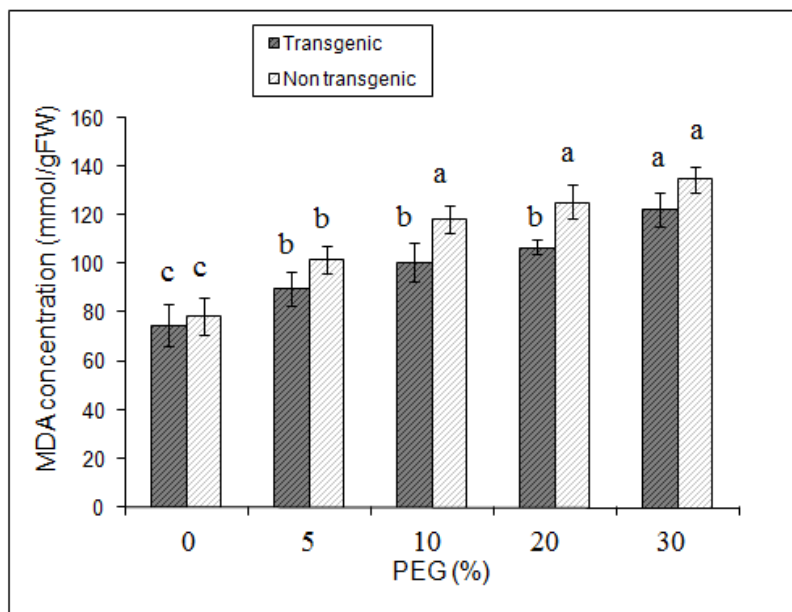
Figure 4. Effect of PEG on MDA content in transgenic and non transgenic tobacco plants. Values are means ± SE. Similar letters indicate not significant based on Tukey test ($P < 0.05$). 0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively.

Discussion

Results of this study showed that proline was accumulated in shoot and roots of transgenic and non transgenic plants when subjected to drought stress. Accumulation of proline in shoots and roots of the transgenic plants was much higher than that in non transgenic plants. It seems that over expression of P5CS gene in transgenic plants, have a remarkable role in proline synthesis and accumulation. As P5CS gene is a rate-limiting enzyme in the proline biosynthetic pathway in plants and has an important role in transgenic plants over expressing P5CS gene, it is expected to be responsible for more proline accumulation in the transgenic plants. As proline plays an important role in osmotic adjustment as well as membrane protection, free radical scavenging, and redox buffering (Verbruggen and Hermans, 2008; Kishor et al., 2005), high level of proline, accumulation by over expression of P5CS, can tolerate plants against osmotic stress (Hank and Hwang, 2003). Similar data was obtained when our transgenic and non transgenic tobacco plants exposed to osmotic (PEG) stress. These results are in agreement with the report from Kishore et al. (2005). Transgenic tobacco in the present study, plants of the over expressing P5CS gene, produced a high level of the antioxidant enzymes and synthesized more proline than the controls.

Our results showed that drought stress changed antioxidant enzymes, activity in leaves of both plant types. APX and CAT activities were induced in leaves of the transgenic plants compared to the control ones. These finding are in accordance to the results obtained by Khedr et al. (2003) and Ghorbanli et al. (2004). On the other hand, it has been known that abiotic stresses, including drought stress, induce ROS accumulation, resulting oxidative damage to the membrane lipids, proteins, and nucleic acids (Smirnoff, 1993). Plants increase activity of detoxifying enzymes such as superoxide dismutase, catalase, and ascorbat peroxidase to combat oxidative stress. Proline has a role in scavenging ROS and protect proteins against denaturation (Alia et al., 1991), increasing of CAT and APX activities may cooperates with the other antioxidant enzymes for giving more tolerance to drought stress in our tobacco plants.

It is well known that ROS induce lipid peroxidation of the membranes. The change in MDA content is often used as an indicator of oxidative damage (Sung, 1996; Goel and Sheoran, 2003). Our data showed that MDA content enhanced by increasing of drought stress intensity. It has been reported that MDA content, as lipid peroxidation criteria, in the transgenic plants was lower than non transgenic plants (Smirnoff and Cumbes, 1989; Matysik et al., 2002). It has been shown that proline can reduce lipid peroxidation in alga cells, exposed to heavy metals (Mehta and Gaur, 1999). Therefore it can be speculating that similar role might be responssible for proline accumulation due to P5CS over expression in tobacco plants too. The lower level of

lipid peroxidation in leaves of the transgenic plants suggests that, these plants are better protected from the oxidative damage under drought stress than non transgenic ones. It can be concluded that over expression of the P5CS gene in tobacco plants and consequent proline accumulation along with alleviation of with CAT and APX activities increase drought tolerance in tobacco plants.

Acknowledgment

References

1- Aebi H. (1984) Catalase in vitro. Methods of Enzymology 105: 121-126.
2- Alia P. S. P., Pardha S. P. and Mohanty P. (1991) Proline enhances primary photochemical activities in isolated thylakoid membranes of Brassica juncea by arresting photoinhibitory damage. Biochemical and Biophysical Research Communications 181: 1238–1244.
3- Apel K. and Hirt H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55: 373-399.
4- Asada K. (1999) The water- water cycle in chloroplast; scavenging of active oxygen and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology 50: 601- 639.
5- Bates L. S. (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39: 205-207.
6- Bhardwaj R. N., Arora P., Sharma H. and Arora K. (2007) Effects of 28-homobrassinolide on seedling growth, lipid peroxidation and antioxidative enzyme activities under nickel stress in seedlings of *Zea mays* L. Asian Journal of plant Science 6: 756-772.
7- Chen W. P., Li P. H. and Chen T. H. H. (2000) Glycinebetaine increases chilling tolerance and reduces chilling-induced lipid peroxidation in *Zea mays* L. Plant Cell Environment 23: 609-618.
8- Cherian S., Reddy M. P. and Ferreira R. B. (2006) Transgenic plants with improved dehydration- stress tolerance: Progress and future Prospects. Biologia Plantarum 50: 481- 495.
9- Chinnusamy V., Jagendorf A. and Zhu J. K. (2005) Understanding and improving salt tolerance in plants. Crop Science 45: 437–448.
10- Davies K. J. (1987) Protein damage and degradation by oxygen radicals. I. General aspects. Journal of Biological Chemistry 262: 9895-9901.
11- Goel A. and Sheoran L. S. (2003) Lipid peroxidation and peroxide scavenging enzymes in cotton seeds under natural ageing. Biologia Plantarum 46: 429–434.
12- Ghorbanli M., Ebrahimzadeh H. and Sharifi M. (2004) Effects of NaCl and mycorrhizal fungi on

antioxidative enzymes in soybean. Biologia Plantarum 48: 575-581.
13- Gratao P. L., Polle A., Lea P. J. and Azevedo R. A. (2005) Making the life of heavy metal-stressed plants a little easier. Functional Plant Biology 32: 481–494.
14- Hank H. and Hwang C. H. (2003) Salt tolerance enhanced by transformation of a P5CS gene in carrot. Journal of Plant Biotechnology 5: 149–153.
15- Heath R. L. and Packer L. (1969) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125: 189-198.
16- Kavi Kishor P. B., Sangam S., Amrutha R. N., Laxmi P. S., Naidu K. R., Rao K. R. S. S., Rao S., Reddy K. J., Theriappan P. and Sreenivasulu N. (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Current Science 88: 424-38.
17- Khedr A. H. A., Abbas M. A., Wahid A. A. A., Quick W. P. and Abogadallah G. M. (2003) Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of Pancratium maritmum L. to salt stress. Journal of Experimental Botany 54: 2553-2562.
18- Kishore B., Hong Z., Miao G., Hu C. and Verma D. (1995) Over expression of Δ- Pyrroline-5-carboxylate synthetase increase proline production and confers osmotolerance in transgenic plants. Plant Phsiology 108: 1387-1394.
19- Kishore P. B. K., Sangam S., Amrutha R. N., Laximi P., Naidu K., Rao K. R., Rao S., Reddy K. J., Theriappon P. and Sreenivasula N. (2005) Regulation of proline biosynthesis, degredation, uptake and transport in higher plants: its implication in plant growth and abiotic stress tolerance. Current Science 88: 427-438.
20- Mallick N. and Mohn F. H. (2000) Reactive oxygen species: Response to algal cells. Journal of Plant Physiology 157: 183-193.
21- Matysik J., Alia B. B. and Mohanty P. (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science 82: 525-532.
22- Mehta S. K. and Gaur J. P. (1999) Heavy metal-induced proline accumulation and its role in ameliorating metal toxicity in Chlorella vulgaris. New Phytologist 143: 253-259.
23- Mittler R. (2002) Oxidative stress, antioxidntas and stress tolerance. Trends in Plant Science 7: 405-410.
24- Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.
25- Nakano Y. and Asada K. (1987) Purification of ascorbate peroxidase in spinach chloroplasts: its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. Plant and Cell Physiology 28: 131-140.
26- Sairam R. K., Deshmukh P. S. and Saxna D. C. (1998) Role of antioxidant systemes in wheat genotype tolerance to water stress. Biologia Plantarum

41: 387-394.

27- Savouré A., Jaoua S., Hua X. J., Ardiles W., Van Montagu M. and Verbruggen N. (1995) Isolation, characterization, and chromosomal location of a gene encoding the 11-pyrroline-5-carboxylate synthase in *Arabidopsis thaliana*. Federation of European Biochemical Societies (FEBS) Letter 372: 13–19

28- Sano S., Ueda M., Kitajima S., Takeda T., Shigeoka S., Kurano N., Miyachi S., Miyake C. and Yokota A. (2001) Characterization of ascorbate peroxidases from unicellular red alga Galdieria partita. Plant and Cell Physiology 42: 433–440.

29- Sharma P. and Dubey R. S. (2004) Ascorbate peroxidase from rice seedlings: properties of enzyme isoforms, effects of stresses and protective roles of osmolytes. Plant Science 167: 541–550.

30- Shigeoka S., Ishikawa T., Tamoi M., Miyagawa Y., Takeda T., Yabuta Y. and Yoshimura K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany 53: 1305–1319.

31- Smirnoff N. and Cumbes Q. J. (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057-1060.

32- Smirnoff N. (1993) The role of active oxygen in the response to plants to water deficit and desiccation. New Phytologist 125: 27-58.

33- Sreenivasulu N., Ramanjulu S., Rmachandra-Kini K., Prakash H. S., Shekar-Shetty H., Savithri H. S., Sudhakar C. (1999) Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. Plant Science 141: 1-9.

34- Stewart C. R. (1981) Proline accumulation, Biochemical aspects. In: Paleg L. G., Aspinall D. (Eds), Physiology and Biochemistry of drought resistance in plants 243-251 pp.

35- Sung J. M. (1996) Lipid peroxidation and peroxide-scavenging in soybean seeds during aging. Physiologia Plantarum 97: 85–89.

36- Verbruggen N. and Hermans C. (2008) Proline accumulation in plants: a review. Amino Acids 35: 753-759.

37- Yamchi A., Rastgar Jazii F., Ghobadi C., Mousavi A. and Karkhanehee A. A. (2005) Increasing of tolerance to osmotic stresses in tobacco *Nicotiana tabacum* cv. xanthi through overexpression of p5cs gene. Journal of Science and Technology of Agriculture and Natural Resources 8: 40- 49.

38- Yoshiba Y., Kiyosue T., Katagiri T., Ueda H., Mizoguchi T., Yamaguchi-Shinozaki K., Harada Y., Shinozaki K. (1995) Correla- tion between the induction of a gene for 11-pyrroline-5- carboxylate synthase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant Journal 7: 751–760**.**