Effects of two arginine enantiomers (L and D) pre-treatment on some physiological parameters and the expression of arginase 1 gene in tomato plants under drought Stress

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Abstract

Arginine (Arg) is reported to contribute in reduction of destructive effects of biotic and abiotic stresses through three pathways and their end products (proline, polyamine and nitric oxide). Two isomers of arginine (L and D) have been applied as pre-treatment and then tomato plants were subjected to drought stress. The effects of these isomers on some physiological parameters and expression of arginase 1 (ARG I) (one of the enzymes in proline biosynthesis) pathway have been investigated. The results showed that leaf relative water content (RWC) of tomato plants under drought stress decreased and pre-treatment with L-Arg improved RWC, but D-Arg did not. Drought stress caused a significant decrease in photosynthetic pigments but arginine had no effect on these parameters. Sugar content and lipid peroxidation level increased under stress. However, arginine treatment caused decrease in lipid peroxidation while it did not change sugar content significantly. Proline also significantly increased under drought stress and L-Arg many time increased it content. Increment in expression of arginase1 may be the cause for this increment. On the other hand, the expression of arginase1 and proline content with D-Arg was less obvious. The results of the present study indicate that in tomato plant in addition of L-Arg, arginase can use D-Arg substrate to produce proline. However D-Arg has been reported as an analog of L-Arg which could block ADC pathway. Our molecular and biochemical results showed that this enantiomer could not inhibit arginase pathway.

Key words: arginase; D-Arg; drought stress; L-Arg; proline; tomato

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Introduction

Plants generally undergo a variety of biotic or abiotic stresses, including drought, salinity and coldness. These stresses influence their development, growth and productivity. Drought, being the most important environmental stress, severely impairs plant growth and development, limits plant production and the performance of crop plants, more than any other environmental factor (Shao et al., 2009). Drought impacts include growth, yield, membrane integrity, pigment content, osmotic

adjustment, water relations, and photosynthetic activity (Benjamin and Nielsen, 2006). Leaf water potential is considered to be a reliable parameter for quantifying plant water stress response (Jongdee et al., 2002). From a physiological perspective, leaf chlorophyll content is a parameter of significant interest and the accessory pigments like carotenoid also have very important role in photosynthesis. It is obvious that photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers.

It has been reported that both chlorophyll a and b are prone to soil drying; however, carotenoids have additional roles and partially help plants to withstand adversaries of drought (Farooq et al., 2009). Smirnoff (1993) reported that increase in water stress caused rapid damage to membrane and the leakiness of membranes is caused by an uncontrolled increase in free radicals, which cause lipid peroxidation. Adaptation is associated with maintaining osmotic homeostasis by metabolic adjustments that lead to the accumulation of metabolically compatible compounds such as soluble sugars and proline (Ge et al., 2006). Proline acts as a compatible osmolyte since it can accumulate to high concentrations without damaging cellular macromolecules. Proline can also serve as a nitrogen and carbon source in the cell (Galston and Sawhney, 1990). Moreover, proline has a protective action which prevents membrane damage and protein denaturation during severe drought stress (Shi et al., 2007). Concerning the effect of water stress on proline content, many researchers reported that proline is accumulated in all stressed organs of plants especially in leaves (Shi et al., 2007). The elevation in proline level could be due to increase in protein degradation and simultaneous decline in protein synthesis. In addition, some amino acids such as ornithine, arginine and glutamate may convert to proline (Liu et al., 2006). Several studies showed that some amino acids are accumulated during drought stress. For example proline, ornithine and glutamic acid are accumulated in rice (Yang et al., 2000), while asparagine, aspartic acid, serine, and glycine are accumulated in maize (Slukhai and Shvedova, 1972). These amino acids may be the cause for increase in proline content

under drought stress and induce tolerance (Singh et al., 1985).

It has been reported that arginine plays an important role in cell division, immune function in plants, and release of some endogenous hormones (Guak et al., 2003). Moreover, alleviation of destructive effects of environmental stress has been reported in wheat (Kakkar et al., 2000; Chen et al., 2004) and apple (Malaguti et al., 2001). It is also considered as a precursor for endogenous polyamines, putresine, nitric oxide, and proline (Guak et al., 2003). Arginine also can be hydrolyzed to ornithine and urea by arginase. Then ornithine which is synthesized by arginase can be used in either urea cycle or considered as a precursor for polyamine and proline synthesis.

It has been reported that arginase from leaves of jack bean, can also use D-Arg isomer as precursor in addition of L-Arg and L-canavanine (Kavanaugh et al., 1990). Some studies indicate that organisms like mouse, cow, pea, soybean and *Caragana spinosa* can use L-Arg as substrate but among them only *Caragana spinosa* can use D-Arg as substrate. There is no more information showing that other plants are also able to use D-Arg. Therefore the aim of this work was to study the effects of two isomers of arginine pre-treatment on alleviation of oxidative damages induced by drought stress. Comparing these responses can be useful in understanding the physiological and biochemical mechanisms of these compounds in plants which have to cope with drought stress.

Material and Methods

Tomato plants (*Lycopersicon esculentum*) were grown from seeds in trays of compost until the seeds were germinated. After germination, the seedlings were transferred to growth chamber with day/night temperature of 22 ^{o}C/18 ^{o}C and a 16 h photoperiod with a relative humidity of 50%. The seedlings were irrigated with water once a day and half-strength Hogland's nutrient solution once a week. After four weeks, the seedlings were transferred to bottles containing Hogland's nutrient solution aerated for 24h. The plants were then divided into 3 groups with 3 replicates. Two groups of

plant were sprayed either with (10 ml)1 mM L-Arg or (10 ml) 1 mM D-Arg solutions. The other group was sprayed with (10 ml) distilled water (as control) for one week (0.1% V/V Tween-20 was used as a surfactant and the pH of solution was 6.5). After one week, plants were subjected to *in vitro* water stress for 24 h. For this purpose 3 seedlings were placed in aerated bottle containing distilled water served as the control and polyethylene glycol (PEG-6000) of 11.2% strengths to achieve water (osmotic) stress level of -0.2 MPa. After 24 h of root osmotic stress the second leaves (counting from the bottom) were harvested and immediately frozen in liquid nitrogen and stored at -80 $^{\circ}$C for future analysis.

Chlorophyll and carotenoid content

Chlorophyll and carotenoid contents were estimated according to the method of (Lichtenthaler, 1987).

Leaf relative water content (RWC)

Leaf relative water content (RWC) was calculated as follows (wheatherley 1950):

RWC= [(fresh weight- dry weight) / (saturated weight – dry weight)] /100

Malondialdehyde (MDA) content

For measuring MDA, 0.1 g of the leaf tissue was homogenized in 10 ml of 0.1% TCA, then centrifuged at 10000× g for 15 min. MDA content was determined according to the method of Heath and Packer (1968) and an extinction coefficient (ε) of 1.55×10^5 M^{-1}Cm^{-1} was used for determination of MDA content.

Reducing sugars content

Reducing sugars were determined according to the Somogy-Nelson method (Somogy, 1952).

Proline determination

Determination of free proline content was performed according to Bates et al. (1973). Leaf samples (0.5 g) from each plant were homogenized in 3% (w/v) sulphosalycylic acid and the homogenate was filtered through filter paper No 1. After addition of ninhydrin acid and glacial acetic acid, the resulting mixture was heated at 100 °C for 1 h in water bath. Reaction was then stopped by using ice bath. The mixture was extracted with toluene and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was determined using calibration curve.

RNA extraction and RT-PCR

Total RNA was isolated from *Lycopersicon esculentum* leaves using the RNX ^{Plus} (*CinnaGen, Tehran, Iran*) according to manufacturer's instruction. To eliminate residual genomic DNA the RNA was treated with DNaseI (*Fermentase, Lituany, EN0521*) for 30 min at 37 °C, followed by inactivation of the enzyme at 70 °C for 10 min. One µg of total RNA was used as template in a 20 µl volume of cDNA synthesis reaction containing 0.2 µg random hexamer, 200 U RevertAid™ M-MuLV Reverse Transcriptase (*Fermentase*), 20U Ribonuclease Inhibitor (*Fermentase*) and 1 mM dNTPs, at 42 °C for 60 min. Two negative controls, without RNA and RT, also accompanied each reaction.

PCR was performed in 25 µl reaction volume containing 2 µl of the cDNA, 1.25U Taq polymerase (CinnaGen), 1.5 mM MgCl$_2$, 200 µM dNTPs, and 0.4 µM of each primer.

Amplification was done in Mastercycler (*Eppendorf*) machine under the following conditions: Initial denaturation at 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 40 s and a final extension of 72 °C for 5 min. The log phase of amplification was determined by running PCR products that were amplified in 25, 30 and 35 cycles and comparing the intensity of bands of different products including internal control gene. PCR products were separated on a 2% agarose gel, stained by EtBr and documented with G BOX HR (*Syngene*). The intensity of PCR product bands were measured in Gene Tools (*Syngene*) software. Each experiment was repeated at least three times to

obtain reproducible results. GAPDH gene expression was monitored as internal control in all experiments. For the reverse-transcription step, 1 µl RNA and Random Hexamer (*Fermentase*) primers were used. The conditions were as follows: 25 °C (10 min), 42 °C (60 min), and 70 °C (10 min). Two µl of the obtained 20 µl was then used in a PCR and then amplification for 35 cycles at 94 °C (5 min), 94 °C (30 s), 57 °C (30 s), 72 °C (40 s), and 72 °C (5 min). Gel images were generated by using gel-documentation system. In all PCR reactions GAPDH gene was used as control. Intensity of the band was compared semi quantitatively.

Primers were designed as follow:

Arginase I (AY656837)

ArgI-f: TCGGTGTGGAGCAATATGAA;

ArgI-r: AACCACATCAGCACCAACAA

GAPDH (U97257)

GAPDH-f: GTGGTGCCAAGAAGGTTGTG;

GAPDH-r: CAGTTTTCTGGGTGGCAGTC

Statistical analysis

The results were statistically analyzed using SPSS software. The mean comparisons among treatments were determined by Duncan multiple range test at 5% level of probability.

Result

Chlorophyll and carotenoids contents

In the present investigation, drought stress did not have any effect on chlorophyll a and carotenoids contents but pre-treatment of leaves with L-Arg and D-Arg caused a decrease in chlorophyll b and total chlorophyll (Fig. I).

Relative water content

When plants were under water deficit, the mean relative leaf water content was significantly reduced. Decrement in RWC of plants which were pre-treated with L-Arg was less than the other groups (Fig. II).
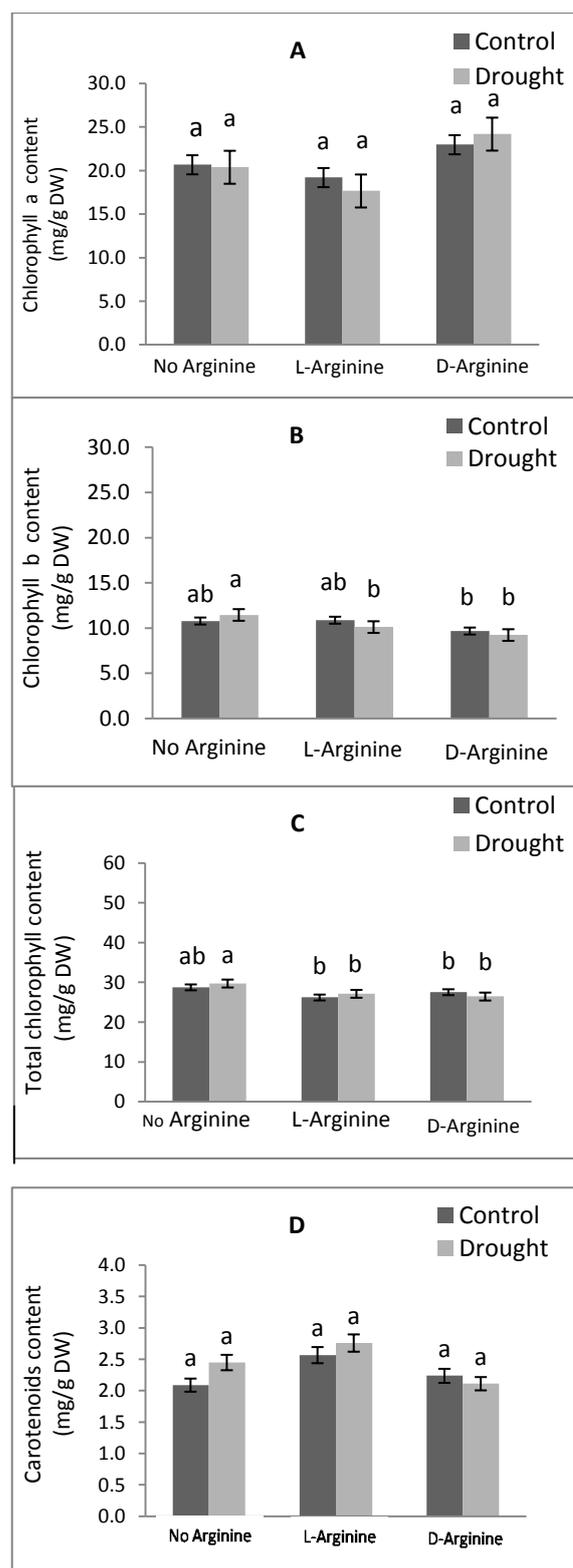

Fig. I. Effect of L-Arg and D-Arg pretreatment on chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), and carotenoids (D) content of tomato leaves under drought stress condition; data are means ± SE of three replicates. The mean comparisons among treatments were determined by Duncan's multiple range test taking p<0.05 as significant.
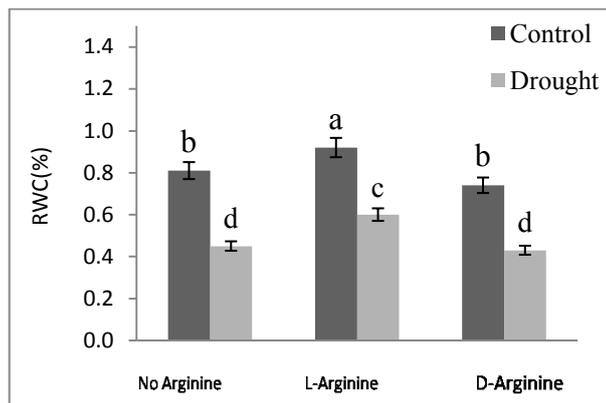
Fig. II. Effect of L-Arg and D-Arg pre-treatment on RWC percentage in tomato leaves under drought stress condition; data are means ± SE of three replicates. The mean comparisons among treatments were determined by Duncan's multiple range test taking p<0.05 as significant.
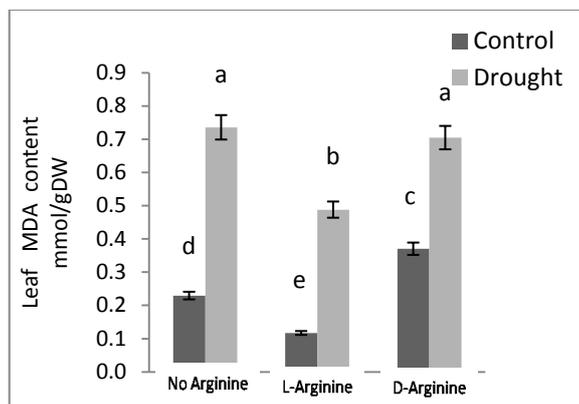

Fig. III. Effect of L-Arg and D-Arg pre-treatment on MDA content in tomato leaves under control and drought stress condition; data are means ± SE of three replicates. The mean comparisons among treatments were determined by Duncan's multiple range test taking p<0.05 as significant.

Malondialdehyde

The data showed that drought induced increment in the amount of MDA. L-Arg pre-treatment decreased lipid peroxidation in control and drought stressed plants. However, in drought condition, D-Arg had no significant effect on reducing MDA content (Fig. III).

Sugar content

Water deficit caused an increase in sugar content (Fig. IV). Arginine pre-treatment had no significant effect on increasing of sugar content in stress condition.

Proline

Proline significantly increased under drought stress and L-Arg and D-Arg many time increased it content. However L-Arg was more effective than D-Arg (Fig. V).

Gene expression

Expression of arginase1 in every replicates of all control groups (in control without arginine, control with L-arginine, and control with D-arginine) was low and random but it was high in plants which were under drought stress, specially the group pre-treated with L-arginine. In this study, the length of bands was 230 bp, but an unknown band in 630 bp which was not reported in literature was seen. Therefore it was sent for sequencing and analyzing and then blasted in NCBI, and was found that it is part of intron then it was sent to the Gene Bank which is available to the interested researchers by the accession number of HQ84U971.1.

Semi-quantitative result of gene expression showed that in plants which were under drought stress, expression of arginase1 increased and pre-treatment with arginine improved it and L-Arg was more effective than D-Arg (Fig. VI).
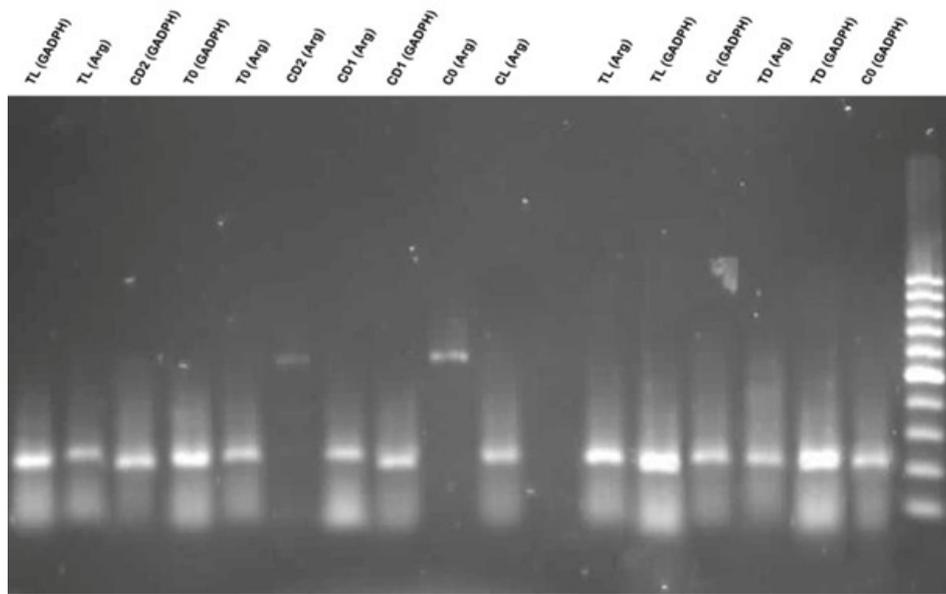
Discussion

Fig. VI. Expression of ARGI in leaves of tomato plant; (C0) control without arginine, (CD) control with D-Arg, (CL) control with L-Arg, (T0) drought without arginine, (TD)drought with D-Arg, (TL) drought with L-Arg.
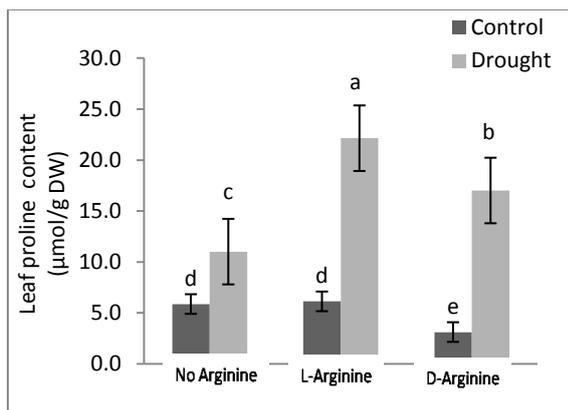

Fig. V. Effect of L-Arg and D-Arg pre-treatment on leaf proline content in tomato leaves under drought stress condition; the mean comparisons among treatments were determined by Duncan,s multiple range test at 5% level of probability.
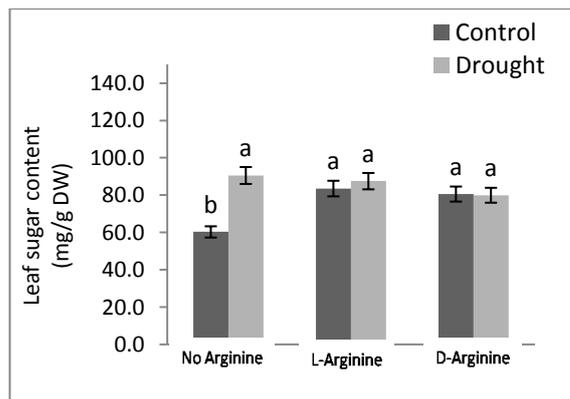

Fig. IV. Effect of L-Arg and D-Arg pre-treatment on sugar content in tomato leaves under drought stress condition; data are means ± SE of three replicates. The mean comparisons among treatments were determined by Duncan's multiple range test taking p<0.05 as significant.

It has been reported that drought stress causees changes in the ratio of chlorophyll 'a' and 'b' and carotenoids (Anjum et al., 2003b; Farooq et al., 2009). A reduction in chlorophyll content was reported in drought stressed cotton (Massacci et al., 2008). Carotenoids are a large class of isoprenoid molecules which are de novo synthesized by all photosynthetic and many non-photosynthetic organisms (Andrew et al., 2008). In this investigation, chlorophyll and carotenoid content did not change under drought stress but pre-treatment with L-Arg, D-Arg decreased

chlorophyll b and total chlorophyll content. It has been shown that with growth of plant and increment of leaf surface, content of chlorophyll per unit surface will decrease (Harn et al., 2001) which is in agreement with our findings.

In this study, carotenoids content did not change and it can be concluded that pre-treatment of plants with arginine and exposing plants to drought stress could not affect carotenoids content; therefore, it seems that carotenoids do not have effective role as antioxidant in non-enzymatic defense

mechanisms and enzymatic defense mechanism would be more effective.

Relative water content (RWC) is considered as a measure of plant water status, reflecting the metabolic activity in tissues and is used as the most meaningful index for dehydration tolerance. RWC of leaves is higher in the initial stages of leaf development and declines as the dry matter accumulates and the leaf matures.

A decrease in relative water content (RWC) in response to drought stress has been noted in a wide variety of plants as reported by Nayyar and Gupta (2006). Results showed that those plants which were under drought stress had lower RWC when compared with control (Fig.II) while treatment of plants with Arg increased RWC in control and water stressed plants. In previous studies it has been reported that stomata closed as leaves sense water deficit (Mata and Lamatina, 2001). Therefore, plants which had the ability to close stomata could maintain higher RWC. However, in this experiment stomata conductance was not measured but it could be considered as the case. MDA levels have been utilized as a suitable marker for membrane lipid peroxidation. Lipid peroxidation has been reported to be one of the most important causes of cell deterioration during drought stress (Smirnoff, 1993). It has been reported that L-Arg through its three by-products has an effect on decreasing lipid peroxidation under drought stress e.g. NO inhibits lipid peroxidation (Beligni and Lamatina, 1999). Polyamines have an effect on decrement of lipid peroxidation under stress (Verma and Mishra, 2005) and proline has antioxidant effect and can react with oxygen and hydroxyl radicals inhibiting their destructive effects (Matysil et al., 2002). Therefore, decrease in MDA content in this study could be related to the effects of by-products of L-Arg. In an earlier study it has been reported that D-Arg blocked ADC pathway (polyamines pathway) (Liu et al., 2006) so these isomers could improve drought tolerance by NOS or arginase pathways.

Under drought, the maintenance of leaf turgor may also be achieved by osmotic adjustment in response to the accumulation of proline, sucrose, soluble carbohydrates, glycine-betaine, and other solutes in cytoplasm improving water uptake from drying soil. It has been reported that in wheat accumulation and mobilization of proline is responsible for enhancing tolerance to water stress (Nayyar and Walia, 2003). Our results showed sugar content was increased under drought stress. This is in agreement with the results of Jones and Turner (1980). It has been reported that application of 2.5 mM arginine caused increment in sugar content in wheat under drought stress (Anjum et al., 2011). However, in this study pretreatment with arginine had no effect on this parameter and it seems that other osmolytes like proline caused osmotic adjustment. Proline accumulation is the first response of plants exposed to water-deficit in order to reduce injury to cells. It has been shown that progressive drought stress induced a considerable accumulation of proline in water stressed maize plants (Anjum et al., 2011).

In this study, pre-treatment of plants with L-Arg and D-Arg increased proline content under drought stress, while L-Arg was more effective than D-Arg. This is not surprising because L-Arg is a precursor of arginase for production of proline while it was not reported that D-Arg can be used as a precursor of arginase in plants. Our results showed that D-Arg also can be used by arginase1 as a substrate to produce proline and the molecular results confirm that (Fig. V and VI).

Most studies used L-Arginine as substrate for arginase1 but there is no report on D-Arg (analog of L-Arg) as substrate for arginase1 enzyme. However, D-Arg has been reported as an analog of L-Arg which could block ADC pathway (Liu et al., 2006). The molecular and biochemical results of the present study showed that this enantiomer could not inhibit arginase pathway.

References

Andrew, J. S., H. Moreau, M. Kuntz, , G. Pagny, , C. Lin, , S. Tanksley, and J. McCarthy . 2008. 'An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea Arabica*', *J. Plant Physiol*, 165: 1087-1106.

Anjum, S. A., L.C. Wang, M. Farooq, I. Khan and L. L. Xue. 2011. 'Methyl jasmonate-induced alteration in lipid peroxidation,

antioxidative defence system and yield in soybean under drought, J. Agro. Crop. Sci, 197: 296-301.

Anjum, F., M. Yaseen, E. Rasul, A. Wahid and **S. Anjum.** 2003a. 'Water stress in barley (*Hordeum vulgare* L.). I. Effect on morphological characters'. *Pakistan J Agri Sci*, 40: 43–44.

Anjum, F., M. Yaseen, E. Rasul, A. Wahid and **S. Anjum.** 2003b. 'Water stress in barley (*Hordeum vulgare* L.). II. Effect on chemical composition and chlorophyll contents'. *Pakistan J Agric Sci*, 40: 45–49.

Benjamin, J.G. and **D. C. Nielsen**.2006. 'Water deficit effects on root distribution of soybean, field pea and chickpea'. *Field Crops Res*., 97(2): 248-253.

Chen, H., B. C. McCaig, M. Melotto, H. Yang, S. He and **G. A. Howo**. 2004.' Regulation of plant arginase by wounding, jasmonate and the phytotoxin coronation'. *The J Biol. Chem*., 279: 45998-46007.

Farooq, M., A. Wahid, N. Kobayashi, D. Fujita and **M. Basra**. 2009. 'Plant drought stress: effects, mechanisms and management'. *Agro Sustain Develop*, 29: 185–212.

Galston, A.W. and **R. K. Sawhney**. 1990. 'Polyamines in plant physiology'. *Plant Physiol*, 94(2): 404-410.

Ge, T. D., F. G. Sui, L.P. Bai, Y. Y. Lu and **G. S. Zhou**.2006. 'Effects of water stress on the protective enzyme activities and lipid peroxidation in roots and leaves of summer maize'. *China Agri Sci*, 5: 291-298.

Guak, S., D. Neilsen, P. Millard, R. Wendler and **G. H. Neilsen**. 2003. 'Determining the role of N remobilization for growth of apple (*Malus domestica* Borkh.) trees by measuring xylem-sap N flux'. *J. Exp. Bot*, 54:2121-2131.

Heath, R. L. and **L. Packer**. 1986. 'Photoperoxidation in isolated chloroplast. I. kinetics and stoichiometry of fatty acids peroxidation'. *Arch Biochem Biophy*, 125(1):189-198.

Jones, M. M. and **N. C. Turner**. 1980. 'Osmotic adjustment in expanding and fully expanded leaves of sunflower in response to water deficits'. *Aust J Plant Physiol*, 7: 181-192.

Jongdee, B., S. Fukai and **M. Cooper**. 2002. 'Leaf water potential and osmotic adjustment as physiological traits to improve drought tolerance in rice'. *Field Crops Res*, 76: 153-163.

Kavanaugh, D., A. Milan and **A. Gerald**. 1990. 'A higher plant enzyme exhibiting broad acceptance of stereoisomers'. *Plant Physiol.*, 94: 67-70.

Lichenthaler, H. K. 1987. 'Chlorophyll and carotenoids: pigments of photosynthetic biomembranes'. *Method in Enzymol*, 148: 350-382.

Liu, J.H., K. Nada, C. Honda, H. Kitashiba and **X. P. Wen.** 2006. 'Polyamine biosynthesis of apple callus under salt stress. Importance of the arginine decarboxylase pathway in stress response'. *J Exp Bot*, 57: 2589-2599.

Massacci, A., S. M. Nabiev, L. Pietrosanti, S. K. Nematov, T. N. Chernikova, K. Thor and **J. Leipner.** 2008. 'Response of the photosynthetic apparatus of cotton (*Gossypium hirsutum*) to the onset of drought stress under field conditions studied by gas-exchange analysis and chlorophyll fluorescence imaging'. *Plant Physiol. Biochem.* 46(2): 189–195.

Nayyar, H. and **D. Gupta**. 2006. 'Differential sensitivity of C_3 and C_4 plants to water deficit stress: association with oxidative stress and antioxidants'. *Environ Exp. Bot.*, 58: 106-113.

Nayyar, H. and **D. Walia**. 2003. 'Water stress induced proline accumulation in contrasting wheat genotypes as affected by calcium and ABA'. *Biol Plant*, 46(2): 275-279.

Neill, S. J. and **E. C. Burnett**. 2003. 'Regulation of gene expression during water deficit stress'. *Plant Growth Regul.*, 29: 23-33.

Shao, H.B., L. Y. Chu, C. A. Jaleel, P. Manivannan, R. Panneerselvam and **M. A. Shao**. 2009. 'Understanding water deficit stress-induced changes in the basic metabolism of higher plants-biotechnologically and sustainably improving agriculture and the eco environment in arid regions of the globe'. *Critical Rev Biotec*, 29: 131-151.

Shi, Q., F. Ding, X. Wang and **M. Wei.** 2007. 'Exogenous nitric oxide protects cucumber roots against oxidative stress induced by salt stress'. *Plant Physiol Biochem*, 45: 458-461.

Singh, T.N., D. Aspinall and **L. G. Paleg**. 1972. 'Proline accumulation and varietal adaptation

to drought in barley, a potential metabolic measure of drought resistance'. *Nature New Biol*, 236: 188-190.

Slukhai, S. I. and **O. E. Shvedova**. 1972.' Dynamics of free amino acid content in maize plants in connection with soil water regimes'. *Russ J Plant Physiol*, 45(4):151-156.

Smirnoff, N. 1993. 'The role of active oxygen in the response of plants to water deficit and desiccation'. *New Phytol*, 125(1): 27 – 58.

Somogy, M. 1952. 'Notes on sugar determination'. *J Biol Chem*, 195: 19-29.

Souza, R.P., E. C. Machado and J. A. Silveria. 2006. 'Photosynthetic gas exchange and chlorophyll fluorescence and associated metabolic change in cow pea (*Vigna unguiculata*) during water stress'. *Environ. Exp. Bot*. 51: 45-56.

Verma, S. and **S. N. Mishra.** 2005. 'Putrescine alleviation of growth in salt stressed *Brassica Juncea* by inducing antioxidative defense system' *J Plant Physiol*, 162(6): 669-677.

Wheutherley, P. E. 1950. 'Studies in water relations of cotton plants, the field measurement of water deficit in leaves'. *New Phytol*. 49: 81-87.

Yang, J., J. Zhang, Z.Q. Wang and **W. Wang**. 2000. 'Hormonal changes in the grains of rice subjected to water stress during grain fillings'. *Plant Physiol*, 127: 315-323.

Zhang, X., L. Shen, F. Li, D. Meng and J. Sheng.2013. 'Amelio ration of chilling stress by arginine in tomato fruit: Changes in endogenous arginine catabolism'. *Post Biol Technol*, 76: 106-111.