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Effect of exogenous arginine pre-treatment on some physiological parameter and the expression of arginase I and II genes in tomato plant under drought stress

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ABSTRACT

In this research, we have used arginine as a precursor of nitric oxide synthase and N^{w}-Nitro- L- Arginine Methyl Ester (LNAM) as an inhibitor of NOS, and the effect of these compound on alleviation of oxidative damages under drought stress has been investigated. The result showed that relative water content and membrane stability index increased when tomato plants treated with Arg. These results correspond well with lipoxygenase activity, which the activity of this enzyme decreased in plants, which were pretreated with Arg. In this research drought stress increased the activity of PAL and total phenol content, but arginine pretreatment had no effect on PAL activity and total phenol content. Our findings showed that the effects of Arg and Arg+LNAM pretreatment had the same effects on many parameters, and it seems that in these situations other pathways of Arg metabolism rather than NOS may activate. Increment in proline content and the molecular analysis of this research confirm this idea because in the Arg pretreated plants, the expression of arginase ² and ²² genes were higher than the non -Arg pretreated plants. Therefore, it seems that under these situations, protective effect of Arg is related to polyamines and or proline biosynthesis.

KEYWORDS

Lycopersicun sculentum;
Nitric oxide;
Oxidative stress;
Polyamines;
Water stress.

INTRODUCTION

Drought stress is one of the major constraints affecting crop productivity in various regions of the world and understanding the cellular process that ameliorates the consequences of drought stress and conserves water are clearly important[1]. When plants are subjected to drought stress, a variety of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radicals, which cause oxidative damage in plants, are generated. Free radicals are toxic to living organisms unless removed rapidly, destroyed or inactivated by various cellular components. In the absence of effective mechanisms, which remove or scavenge free radicals, they can seriously damage plant by lipid peroxidation, protein degradation, breaking of DNA and cell death[2]. To control the level of reactive oxygen species, plants have evolved an antioxidant

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defense system comprising of enzymes such as the superoxide dismutase (SOD), Catalase (CAT) guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR) as well as non-enzymatic constituents such as ascorbate, glutathione, polyphenolic compounds and proline, which are responsible for scavenging excessively accumulated ROS in plants under stress conditions[3]. The regulation of these antioxidant constituents by an exogenous substance might mediate the plant tolerance to drought stress. L-arginine is one of the most functionally diverse amino acids in living cells. In addition to serving as a constituent of proteins, arginine is a precursor for the biosynthesis of polyamines, Agmatine and proline as well as the cell signaling molecules glutamine and nitric oxide[4,5]. Three most studied pathways of arginine metabolism are those catalyzed by nitric oxide synthase, arginase, and arginine decarboxylase (ADC). Nitric oxide synthase hydrolyzes arginine to nitric oxide and citrulline, while final production of arginase and ADC are mostly polyamines and proline[5]. Most studies of plant arginase have focused on its role in mobilizing arginine as a nitrogen source during post-germinative growth[6-8]. However, the molecular mechanism by which arginase expression in plants is regulated by developmental or stress-related cues remain to be determined. In plants, arginine-dependent NOS activity has been detected along with inhibition of NO production by NOS inhibitor[9-11]. However, no gene or protein with sequence similar to the large animal NOS proteins has been found even in the sequenced Arabidopsis genome[12]. Ninnemann and Maier (1996) showed, for the first time, the presence of NOS activity in higher plants. They used inhibition by the arginine analogues N-nitro-L-arginine (L-NNA) and N-nitro-L-arginine methyl ester (L-NAME) and the production of radiolabeled citrulline as evidence[13]. Nitric oxide synthase activity was detected also in roots and nodules of *Lupinus albus* and was inhibited by NOS inhibitor L-NAME[9]. Two mechanisms by which NO might abate stress has been postulated. First, NO might function as an antioxidant, by directly scavenging the reactive oxygen species (ROS), such as superoxide radicals, to form peroxy ($ONOO^-$), which is considerably less toxic than peroxides and thus limit cellular damage. Second, NO could function as a signaling molecule in the cascade of events leading to changes of gene expression[14]. Accumulating evidence suggests that NO is an important signal molecule involved in the plant response to biotic and abiotic stress, for example, NO production increased in tobacco plants in responses to salinity, hyper osmotic stress, and high temperature[15]. It is reported that NO production in plants at low concentration may rapidly eliminate lipid peroxyl radicals, and alter the species and component of reactive oxygen species, block the injury from ROS, induce the expression of antioxidant genes and the activity of antioxidant enzymes and protect plants from abiotic stress[14,16,17]. Tolerance to drought, salt and heat stress was enhanced in wheat (*Triticum aestiva*) and rice (*Oryza sativa*) seedlings when the plants were treated with the NO donor, sodium nitroprusside (SNP)[18,19]. In previous researches, SNP has been applied as a NO donor to counteract the effect of drought stresses on plants[18-20]. However, not any data is available on the effect of exogenous arginine as a precursor of NO in the plants to cope with stress. In this research, we have used arginine and N^w-Nitro- L- Arginine Methyl Ester (LNAM) as an inhibitor of NOS as pretreatment, and the effects of these compounds on alleviation of oxidative damages under drought stress were investigated. Comparing these responses can be useful in understanding the physiological and biochemical mechanisms of this compound in plants to cope with drought.

MATERIALS AND METHODS

Tomato plants (*Lycopersicon esculentum* Mill v Alicante) were grown from seeds (provided from Thomson and Morgan company.UK.) in trays of compost until the seeds were germinated. After germination, the seedlings were transferred to the growth chamber with day/night temperature of 22°C/18°C and a 16h photoperiod with a relative humidity of 50%. The seedlings were irrigated with water once a day and half-strength Long Aashton nutrient solution once a week. After four weeks, the seedlings were transferred to bottles containing nutrient solution aerated with air then the plants were divided into eight groups with 3 replicates. Four groups of plant were sprayed either with (10ml)1mM Arg or (10ml)1mMArg + 2mM LNAM (NOS inhibitor) solutions, other four groups were sprayed either with (10ml) distilled water or (10ml)2mM LNAM, for two days. (0.1% V/V Tween-20 was used

as a surfactant and the pH of solution was 6.5). In third day, after spraying the solutions, plants were subjected to in vitro water stress for 24 h. For this purpose, three seedlings were placed in aerated bottle containing distilled water served as a control and polyethylene glycol (PEG-6000) of 11.2% strengths to achieve water (osmotic) stress level of -0.2MPa. 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°C for future analysis.

Leaf relative water content (RWC)

Leaf relative water content (RWC) was calculated as follows: RWC= [(fresh weight- dry weight)/ (saturated weight – dry weight)] ×100[21].

Membrane stability index (MSI)

Membrane stability index (MSI) of leaves was determined by recording the conductivity of leakage in de-ionized water at 40 and 100°C[22]. Two similar leaf disks (0.1g) were cut to uniform size and placed in two separated test tubes containing 10 ml de-ionized water. One set of disks was kept at 40°C for 30min and its conductivity (C_1) was recorded using conductivity meter while second set was kept in a boiling water bath (100°C) for 10 min and its conductivity (C_2) was also recorded. The MSI was calculated using the formula: (MSI) = [1-(C1/C2)] × 100.

Enzyme extraction and activity determination

500 mg leaves were homogenized in 50mM potassium phosphate buffer (pH 7.0) containing 1% soluble PVP, 1mMEDTA and 1mM PMSF. The homogenate was centrifuged at 20000g for 20 min and the supernatant was used for assay of the activity of enzymes.

Lipoxygenase (LOX) activity

Lipoxygenase activity was estimated according to the method of Dodere et al. (1992)[23]. For measurement of LOX activity, the substrate solution was prepared by adding 35 µl linoleic acid to 5 ml distilled water containing 50µl Tween-20. The solution was kept at pH 9.0 by adding 0.2M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 6.5 by adding 0.2M HCl, 0.1M phosphate buffer (pH 6.5) was added to make a total volume of 100ml. LOX activity was determined spectrophotometrically by adding 50 µl of enzyme to 2.95 ml substrate. Solution absorbance was recorded at 234nm and the activity was expressed as a change in absorbance per minute per mg protein in the leaves.

Phenylalanine ammonia-lyase (PAL) activity assay

PAL activity was assayed according to the method of D×cünha (1996)[24]. The reaction mixture contained 100mM Tric-HCl buffer (pH 8.5), 1mM 2-mercaptoethanol, 50mM L-Phenylalanine and 100 µl of enzyme extract. The mixture was incubated at 30° for 15 min. The reaction was terminated by the addition of 6M HCl and absorbance of supernatant was measured at 290 nm. One unit of enzyme represents the conversion of 1µmol substrate to cinammic acid per min.

Total soluble proteins

Protein content was determined according to the method of Bradford (1976)[25] using Bovine serum albumin as standard.

Determination of total phenol contents

The total phenol content in leaves was determined by the method of Folin-Ciocalteu reduction, using gallic acid as standard. The phenol content was expressed as gallic acid equivalents in milligram on a dry weight[26].

Proline determination

Determination of free proline content performed according to Bates et al.(1973)[27]. Leaf samples (0.5 g) from each plant were homogenized in 3% (w/v) sulphosalycylic acid and the homogenate filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, 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 *Lycopersicun esculentum* leaves. RNA was isolated using the RNase Plant Mini Kit (*Qiagen*), according to the producer's instructions. Prior to the RT-PCR analyses, the RNA was treated with DNase I (*Fermentase, Lituany, EN0521*) for 30 min at 37°C, followed by inactivation

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of the enzyme at 7 °C for 10 min. One µg of total RNA was used as a template in a 20µl volume cDNA synthesis reaction containing 0.2µg random hexamer, 200U RevertAid™ M-MuLV Reverse Transcriptase (*Fermentase*), 20U Ribonuclease Inhibitor (*Fermentas*) and 1mM dNTPs, at 42 °C for 60 min. Two negative controls, without RNA and RT, also accompanied each reaction. PCR was performed in 25µl reaction volume containing 2µl of the cDNA, 1.25UTaq polymerase (*Cinnagen*), 1.5mM MgCl2, 200µM dNTPs, and 0.4µM 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 intensities 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, 1µl RNA and Random Hexamer (*Fermentase*) primers were used. The conditions were as follows: 25°C (10min), 42°C (60min) and 70°C (10min). Two µl of 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 the gel-documentation system. In all PCR reaction, GAPDH gene was used as control. Primers were designed as follow**:**

Arginase ^{2} (AY656837):
Arg^{2}-f: TCGGTGTGGAGCAATATGAA;
Arg^{2}-r: AACCACATCAGCACCAACAA
Arginase ^{22} (AY656838):
Arg2-f: CGTGGATGTTGACTGTTTGG;
Arg2-r: CAGTATCACGCTGTGGGTTG
GAPDH (U97257) GAPDH-f: GTGGTGCCAAGAAGGTTGTG;
GAPDH-r: CAGTTTTCTGGGTGGCAGTC

Statistical analysis

All the experiments were performed in triplicate. Values indicate mean values ± standard errors of the mean. Duncan's test was used to analyze the difference between treatments and one-way ANOVA taking $p< 0.05$ as significant.

RESULTS

Relative water content and membrane stability index

In the present investigation when plants were under water deficit, the mean relative leaf water content was reduced from 87% to 61% (Figure 1-A). However, the water deficit produced was not sufficient to cause significant wilting of the leaves and there was no significant difference between the total dry weights of leaves on the drought stressed and control plants (data not shown). The effect of drought stress on the membrane integrity was measured in terms of membrane stability index. As shown in Figure 1-B, membrane stability decreased from 80% in control to 55% in

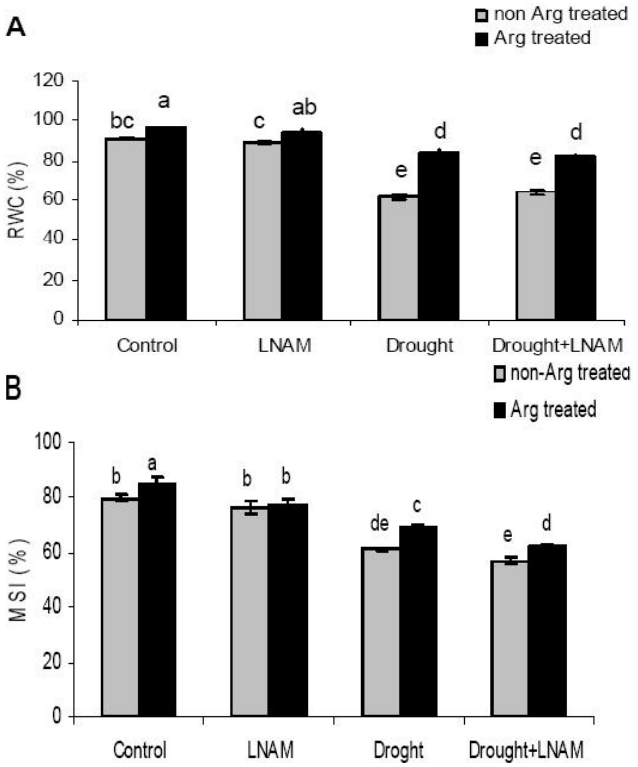

Figure 1: **Effect of Arg and Arg+LNAM pretretment on RWC(A) and MSI(B) in tomato plant leaves under control and drought stress condition. Data are means± SE of three replicates.The significant of different between treatments was determined by one-way ANOVA taking $p<0.05$ as significant.**

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drought stress. Pretreatment of leaves with Arg showed a slight decreased of ion leakage and increased MSI in control and drought stressed plants. In drought stressed plants when the plant was pretreated with Arg+LNAM the effect of Arg on maintaining of membrane integrity declined significantly (Figure 1-B).

Lipoxygenase activity

Lipoxygenase is an oxidative enzyme that contributes in oxidation of polyunsaturated fatty acids. The activity of this enzyme showed that under water deficit, activity increased about 8 fold when compared with control (Figure 2). About 60% decrease in activity of this enzyme was observed in Arg pretreatment of drought stressed plants. However application of Arg+LNAM pretreatment decreased the activity of the enzyme compared to the application of Arg alone but the decrease was not statistically significant.

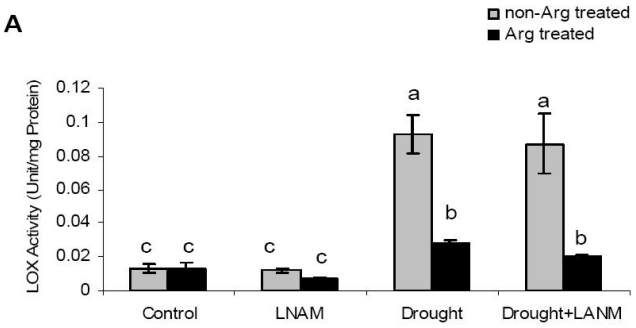

Figure 2: **Effect of Arg and Arg+LNAM pretretment on LOX activity in tomato plant leaves under control and drought stress condition. Data are means± SE of three replicates.The significant of different between treatments was determined by one-way ANOVA taking p<0.05 as significant.**

PAL activity and total phenol contents

As shown in figure 3 the activity of PAL and total phenol content increased in tomato plants which were under drought stress. Arg pretreatment did not have any effect on PAL activity and total phenol content (Figure 3-A and B).

Proline content

The amounts of proline increased significantly under drought stress. Treatment of plants with Arg and Arg+LNAM in the same manner increased the proline content under drought stress.

Expression of arginase I and II genes

Results of molecular studies were showed in Figure 5. In Figure 5, expression of ARG² and ARG²² were compared between the Arg and non-Arg pretreated plants. As it shown in this Figure, Drought stress caused an increment in ARG² and ARG²² transcription level, indicating the responses of ARG² and ARG²² to drought stress. However, pretreatment of plants with Arg led to an increased expression level of ARG² and ARG²² in stress condition. In this research, the expression of TGAPDH studied as control and the expression pattern of this gene confirm the quality of extracted RNA from leave of tomato.

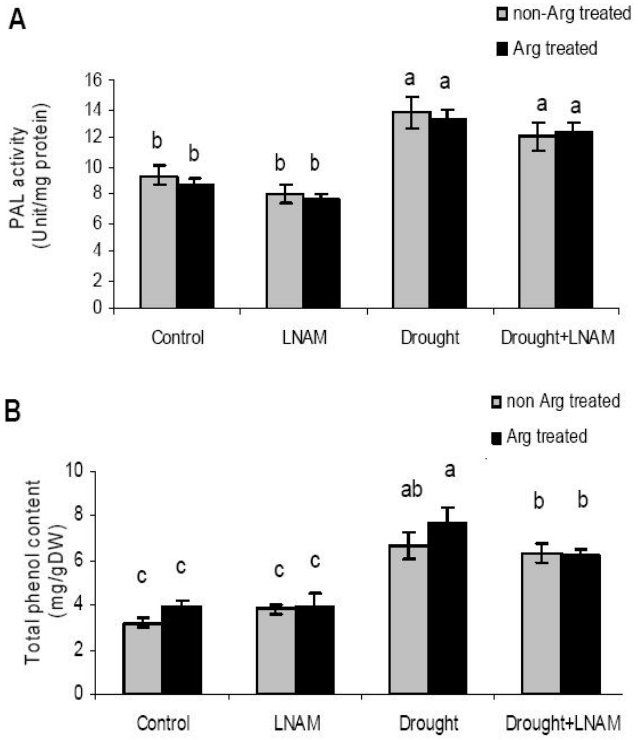

Figure 3 : **Effect of Arg and Arg+LNAM pretretment on PAL activity(A) and total phenol content(B) in tomato plant leaves under control and drought stress condition.The significant of different between treatments was determined by one-way ANOVA taking p<0.05 as significant.**

DISCUSSION

Two pathways for metabolism of Arg have been reported, which were catalyzed by either arginase or nitric oxide synthase so that the end product will be ornithine or nitric oxide respectively. Ornithine is a precursor for the polyamines (PAs) biosynthesis. It has been reported that arginine in tomato plants could produce NO and Pas[5]. In this study, LNAM was used as an inhibitor of NOS, to study the role of NO in some physiological parameter under drought stress. The result showed that those plants which were under drought

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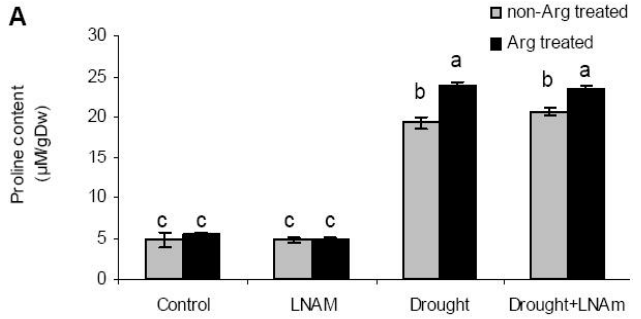
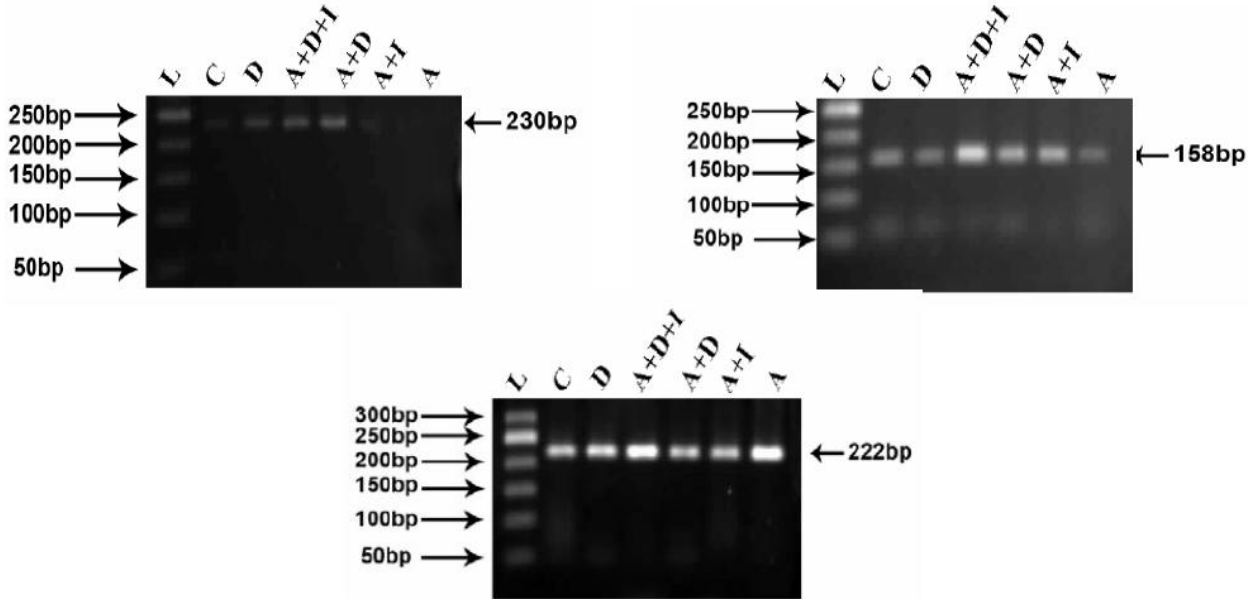
stress had lower RWC when compared with control (Figure 1-A) while treatment of plants with Arg increased RWC in control, and water stressed plants. In previous studies, it has been reported that stomata closed as leaves sense water deficit[18]. Therefore, plants, which had an ability to close stomata, could maintain higher RWC. Based on the report by Mata and Lamatina (2001)[18], nitric oxide could induce stomata closure and enhance the adaptive of wheat seedling responses to drought stress. They showed that NO can increase intracellular Ca^{+2} concentration via cGMP dependent signaling pathway. This is because when they used Ca^{+2} chelator (EGTA), the effect of NO was completely reversed, suggesting that NO could be acting upstream of Ca^{+2}. Neill et al (2003b) confirmed these findings in Arabidopsis plants. They observed that when cPTIO (NO scavenger) was applied, the percentage of open stomata increased, which is an indication of the role of NO in stomata closure[28]. In previous in our research, the application of Arg or Arg+LNAM had the same effect on RWC. Our findings showed that the protective effect of Arg on RWC could relate to another pathway metabolism of Arg except NOS pathway. One of the described damages provoked by water deficit stress is the membrane injury and liberation of ions from the cell to extra cellular space[29]. This is a consequence of an oxidative burst leading to lipid peroxidation, membrane permeability and cell injury[30]. As shown in Figure1-B, MSI declined in plants, which were subjected to drought. These results are corresponded well with the results of MDA, other aldehydes and H_2O_2 content[31]. Lipoxygenase is another enzyme that was studied in this research. This enzyme is an oxidative enzyme, which can contribute to lipid peroxidation. It is a non-heme enzyme that contains a single iron atom which is thought to oscillate between ferrous (inactive) and ferric (active) forms during each cycle of catalysis. Nitric oxide was thought to inhibit enzyme activity by reducing the iron of the active site from an active Fe^{+3} to an inactive Fe^{+2} form and trapping the iron in a reduced inactive form[32]. Our results show that activity of this enzyme increased in drought conditions and when plants were pretreated



Figgure 4 : Effect of Arg and Arg+LNAM pretretment proline content in tomato plant leaves under control and drought stress condition. The significant of different between treatments was determined by one-way ANOVA taking p<0.05 as significant.



Figgure 5 : Expression of ARG² (230bp), ARG²² (158bp) and TGAPDH (222bp) genes in Arg pretreated plants. L(DNA Ladder), C(control plant without Arg pretreatment), D(drought stressed plants without Arg pretreatment), A+D+I(drought stressed plants which is pretreated with Arg+LNAM), A+D(Drought stressed plants with Arg pretreatment), A+I(control plants which pretreated with Arg+LNAM), A(control plants which pretreated with Arg).

with Arg, MSI increased while the activity of LOX decreased and this effect is very important for drought stress tolerance (Figure 3). Increment of MSI and the reduction in LOX activity is expected because of the role of Arg in releasing NO, either directly or indirectly. In this research, the amounts of proline increased significantly in drought stressed plants. Treatment of plants with Arg and Arg+LNAM in the same manner increased the proline content under drought stress condition (Figure 4). When Arg was used as a precursor of NO, the amelioration of the drought effect on tomato plants was observed, which is could be indication that these effects may be related to NOS activity and NO production. To prove that, we applied Arg+LNAM and in almost all parameters, which we measured in this study, Arg and Arg+LNAM pretreatment had the same effects, and it seems that in these situation's other pathways of Arg metabolism rather than NOS may activate. The molecular analysis of this research confirmed this idea because in the Arg pretreated plants, the expression of arginase I and [22] Genes were higher than the non- Arg pretreated plants. Therefore, it seems that under these situations, protective effect of Arg is related to polyamines or indirect synthesis of NO from polyamines, which has been reported more recently[33] and or proline biosynthesis.

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