The effect of arginine pretreatment on germination, growth and Physiological parameters in the increase of low temperature tolerance in *Pistacia vera* L. in vitro culture

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**ABSTRACT:** Cold stress is one of the important factors which will reduce crop yield and fruit production in world. Early spring cooling and freezing weather is mostly possible. This may considerable damage pistachio productions and farmer will lose most of their profit. Therefore study which show how pistachio plant cope freezing weather is valuable and important. The special role of amino acid in plant response stress full condition is quite clear. Therefore we investigated the unique physiological role of amino acid (Arginine) in inducing cold tolerance in pistachio seedlings under in vitro conditions. The results showed that when seedlings were exposed to freezing stress (-6°C) significant increasing in prolin, phenols, protein oxidation and reduction in soluble sugars content was observed. Arginine pretreatment slightly reduced in prolin, phenols, protein oxidation and increased in soluble sugars content. Using inhibitor of the NO production pathway (LNAM) showed that NO had important effect reduction of protein oxidation.

**Key words:** Nitric oxide, pheny alanine ammonia lyase, Prolin, Polyamines

**Abbreviations:** Arg- Arginine; LNAM- N-nitro-L-arginine-methyl ester hydrochloride; NO- Nitric oxide; PAL- Phenylalanine Ammonia-Lyase (EC 4.3.1.5); PPO- Polyphenol oxidase activity (EC 1. 14. 18. 1)

**INTRODUCTION**

Pistachio (*Pistasia vera* L.) tree is an economically important plant which is cultivated in vast areas of arid and semi arid environments of different elevations and precipitation rates in Iran. Hence, the plant is exposed to different environmental stresses biotic and abiotic factors. The plant is reproduced by grafting different cultivars on rootstocks and the assessment of the best cultivars with respect to their adaptation to the environmental stresses is likely to be necessary (Karimi et al., 2009). The economic value of pistachio exports to 66 countries is about one billion dollars/per year, ranking second among each nation’s sources of income after oil (Ayaz Tilkat et al., 2011).

Freezing temperatures (below 0°C) cause the movement of water from the protoplast to the extracellular space, resulting in the growth of extracellular ice crystals and ultimately, cell dehydration (Taiz and Zeiger, 2002). Plants have developed complex processes to survive and recover from unfavorable conditions. To tolerate cold stresses, plants develop multiple mechanisms, including the accumulation of cryoprotective molecules and proteins, alterations in membrane lipid composition, and primary and secondary metabolite composition, as well as changes in global gene and protein expression (Lissarre et al., 2010).

In addition, osmotic adjustment provides a potentially important mechanism for freezing tolerance and cold acclimation. This can be achieved via the accumulation of compatible solutes, such as soluble sugar and soluble protein, in protoplasm (Misra and Gupta, 2005). It has been suggested that they not only serve as osmo- protectants, but also, through their interaction with the lipid bilayer, play a role in protecting cellular membranes from damage caused by dehydration and freezing (Karimzadeh et al., 2006).

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 biosynthesis of polyamines (PAs), Agmatine and proline as well as the cell signaling molecules glutamine and nitric oxide (NO) (Chen et al., 2004; Liu et al., 2006). In higher plants,
it has also been proposed that both endogenous and exogenous arginine have roles in plant stress response, such as salinity, water and disease (Zeid, 2009; Nasibi et al., 2011; Zheng et al., 2011).

Alleviating effects of exogenous NO (Liu et al., 2011; Neill et al., 2003), PAs (He et al., 2002) and Prolin (Patton et al., 2007; Gothandam et al., 2010) in low temperature stress have been reported previously. However not any data are available on the effect of exogenous arginine as a precursor of these compounds in plants against freezing stress. The present study was conducted to evaluate the effect of arginine as NO, prolin precursor and or polyamines substrate on pistachio seedlings to elucidate the physiological mechanisms of exogenous Arg in increased tolerance of pistachio to freezing stress. Also In this research given to the importance of non-sexual reproduction of pistachio in tissue culture, the effect of arginine treatment on germination percentage and rate, shoot length and root length under in vitro conditions was investigated.

MATERIALS AND METHODS

The investigation was carried out with Ohadi cultivar. Pistachio seeds were collected from the orchards in Rafsanjan of Iran. First, Surface of seeds was sterilized with 70 % ethanol for 2min, seeds were washed with sterile water and then seeds were dipped in sodium hypochlorite 7% and tween-20 for 25min, ultimately seeds were washed with sterile water and placed for germination in vitro on basal MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.1% PVP, solidified with agar. Media adjusted to pH 5.8 prior to autoclaving at 120 °C for 25 min. Seeds was grown in concentrations of 0, 2, 4 and 8μM arginine. The cultures were placed in dark at 26-28°C. Ultimately seeds germination percentage and rate were investigated.

Firstly seeds were placed in non arginine medium that effects of arginine pretreatment on growth parameters in pistachio seedlings were investigated. Ultimately Seedlings were transferred to 4, 8 and 16μM arginine after seeds germination for 1 week. After 1 week, shoot length and root length under in vitro conditions was investigated.

In order to investigation of effect cold stress, After germination the seedlings were transferred to new mediums with 4μM arginine, 4μM arginine + 8μM LNAME (NOS inhibitor) and control medium for 10 days. Seedlings were kept at the temperature of 22±2°C, illumination of 50μmol m⁻² s⁻¹, photoperiod of 16/8 h (day/night). In order to inhibition of shoot necrosis, seedlings were transferred to fresh medium an interval of 3 days. After 10 days, seedlings were exposure to constant temperature of -6°C for 3h and then seedlings were placed at 22±2°C for 18h. Fresh sample were harvested for biochemical analysis.

Oxidation of proteins

The amount of protein oxidation was estimated by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine, as described by Levine and et al (1994). After the 2,4-dinitrophenylhydrazine reaction, the carbonyl content was calculated by absorbance at 370 nm, using an extinction coefficient for aliphatic hydrazones of 22mM⁻¹ cm⁻¹ and expressed as nano moles of carbonyl per milligram of protein.

Total soluble sugar

Total soluble sugar was determined using anthrone reagent and glucose as standard (Roe, 1955).

Total phenolic compounds

Total phenolic content was determined using the Folin-Ciocalteau method Gao (2000). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight.

Enzyme extraction and activity determination

Five hundred mg leaves were homogenized in an ice cold mortar using 50mM potassium phosphate buffer pH 7.0 containing 1mM EDTA, 1% (w/v) soluble PVP, and 1mM PMSF. After centrifugation (20 000 g, 20 min) the supernatant was used for determination of PAL and PPO activities.

PAL Activity (EC 4.3.1.5)

PAL activity was determined based on the rate cinnamic acid production. 1ml of the extraction buffer, 0.5ml of 10mM L-phenylalanine, 0.4ml of de ionized water and 0.1ml of enzyme extract were incubated at 37°C for 1h. The reaction was terminated by the addition of 0.5ml of 6M HCl and the cinnamic acid concentration was measured spectrophotometrically by the absorbance at 260nm. One unit of PAL activity is equal to 1μmol of cinnamic acid produced per min (D’cunha et al., 1996).
**PPO activity (EC 1.14.18.1)**

The PPO activity was assayed as described by Nicoli et al. (1991) with some modifications. The reaction mixture contained 200µL of 0.02M pyrogallol, freshly prepared in 50mM potassium phosphate buffer at pH 7, and a fixed quantity of enzyme. PPO activity was measured by the change in 420nm of the assay mixture based on the measurement of the disappearance of pyrogallol by enzymatic oxidation.

**Total soluble proteins**

Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

**Praline determination**

Determination of free proline content performed according to Bates and et al. (1973).

**Statistical analysis**

All determinations were carried out in three triplicate and data were subjected to analysis of variance. Analysis of variance was performed using the ANOVA procedure. Statistical analyses were performed according to the MSTATC software. Significant differences between means were determined by Duncan's multiple range tests. P values less than 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**Growth parameters**

As shown in table 1, Arginine 4 and 8µM had significant effects on germination percentage and rate. Also the concentrations of 4, 8 and 16µM arginine leading to increase of shoot and root length and growth quality under in vitro condition.

<table>
<thead>
<tr>
<th></th>
<th>Arg 0</th>
<th>2µM</th>
<th>4µM</th>
<th>8 µM</th>
<th>16 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third day of culture</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>80%</td>
<td>-</td>
</tr>
<tr>
<td>Sixth day of culture</td>
<td>20%</td>
<td>20%</td>
<td>60%</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>Eleventh day of culture</td>
<td>60%</td>
<td>60%</td>
<td>80%</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>Root length(Cm)</td>
<td>9.24c</td>
<td>-</td>
<td>11.3bc</td>
<td>13.3ab</td>
<td>15.59a</td>
</tr>
<tr>
<td>Shoot length(Cm)</td>
<td>6.40c</td>
<td>-</td>
<td>7.38bc</td>
<td>8.53b</td>
<td>10.60a</td>
</tr>
</tbody>
</table>

Data in Table 1 clear that arginine pretreatment increased significantly germination percentage and germination rate as compared with untreated samples. Since the germination rate of pistachio seeds is very low and germination time varied between 8-20 days after culturing, so that application of the substance can synchronize germination, it is important. Zied reported that Arginine and urea treatments stimulated germination of both unstressed and salinity-stressed seeds. the increased germination percentage in response to arginine and urea treatments was associated with increased content of polyamines (Zied, 2009). Many reports indicate the importance of exogenous NO in increment of germination rate and percentage in several plants such as Arabidopsis (Bethke et al., 2006) and wheat (Zheng et al., 2009).

It is clear from the present results that increasing concentrations of Arg increase the growth parameters (table 1). Reported that the application of arginine significantly promoted the growth and increased the fresh and dry weights, certain endogenous plant growth regulators, chlorophylls a and b and carotenoids in bean (Nassar et al., 2003) in wheat (El-Bassiouny et al., 2008). Also, arginine treatment resulted in increasing growth criteria of the seedlings, which was associated with increased content of Spd and Spm (Zied, 2009).

**Total soluble sugar**

Results showed that total soluble sugar decreased in plants which were under freezing stress in comparison with control plants (Fig1). Both Arg and Arg+LNAME pretreatment increased the soluble sugar in
stressed plants in comparison with non-pretreated plants. Also, Application of Arg had significant effects on control plants.

Low temperatures retard metabolism, delay energy dissipation, and induce the formation of free radicals, resulting in oxidative damage (Beck et al., 2007). In this study soluble sugar decreased in plants which were under freezing stress that this reduction may be related to metabolism retarding. As low temperatures reduce enzymatic activity, alter metabolism and decrease the photosynthetic capacity of plant tissues (Dubey, 1997).

When plants were pretreated with Arg or Arg+LNAM, the amounts of soluble sugar increased (Fig1). That this treatments inhibit metabolism retarding in plants under freezing stress. Sugars might protect plant cell membranes during cold-induced dehydration, replacing water molecules in establishing hydrogen bonds with lipid molecules (Uemura et al., 2003; Ruelland et al., 2009). arginine and arginine+LNAM pretreatment had the same effects and it seems that in these situations other pathways of arginine metabolism rather than NOS may be activated.

**Total phenolic compounds**

Freezing stress induced an increase in phenolic compounds (Figure 2). Both Arg and Arg+LNAME pretreatment decreased the amounts of phenolic compounds in plants under stress and control condition.

**PAL and PPO activity**
The effect of freezing stress on PAL and PPO in pistachio plants, either with or without Arg pretreatment was investigated. Results showed that the activity of PAL and PPO was lower in stressed plants than those of the control groups (Fig 3). Arg or Arg +LNAM pretreatment increased the activity of enzymes in plants under stress situation. However Arg was more effective than Arg+LNAME pretreatment. Application of Arg or Arg +LNAM pretreatment decreased the activity enzymes in control plants (Figure 3).

![Figure 3. Effect of Arg and Arg+LNAME pretreatments on PAL (A) and PPO activity (B) in Pistachio plant under control and freezing stress conditions. The mean comparisons of treatments were done using Duncan method at P < 0.05 significant level.](image)

It is well established fact that browning of the tissue is correlated with excessive accumulation of phenolics (Dubravina et al., 2005). Tissue browning and blackening are major impediments of In vitro culture of plants (Naz, 2008). In this study results showed that Pretreatment of plants with Arg or Arg+LNAM decreased the amounts of phenolic compounds in control plants (Fig 2). This reduction harmonies with reduction of PAL activity (Fig 3-A). PAL plays a pivotal role in the production of phenolic compounds. PPO activity also decreased in these treatments (Fig 3-B). The oxidation of phenolic compounds into quinones is mainly catalyzed by polyphenol oxidase. The brown colour that develops in callus cultures of diverse plant cultures is due to the formation of quinones which are inhibitory to plant cellular growth. Accumulation of quinones to a level which is detrimental for In vitro growth is common in some very important plants of economic importance e.g, coffee, mango, chickpea, (Iqbal et al., 1991), sugarcane (Chen et al., 1990), guava, date palm, (Daayf et al., 2003), cotton (Ozyigit et al., 2007) and pistachio. It is very important to reduce quinones in culture medium.

Plants exposed to low temperatures usually experience oxidative stress caused by reactive oxygen species (ROS) which can damage cells by oxidizing lipids, proteins and nucleic acids (Ibrahim and Bafeel, 2008). Phenolic compounds are known to have antioxidant activity (Tepe et al., 2006). But it must be emphasized that most plant-derived polyphenolic anti-oxidants also act as pro-oxidants under high concentrations condition (Khan et al., 2000).

Plants could indicate an accumulation of phenolics compounds in the plants in response to heat and cold stress respectively, caused by activation of enzyme PAL, as proposed by other authors (Nozolillo et al., 1990; Bharti et al., 1997). PAL is an extremely sensitive indicator of stress conditions and it is commonly considered as a biochemical marker indicating the synthesis of both structural and protective compounds (Vogt, 2010).

The data from this study showing that the amounts of phenolic compounds increased in plants under stress. PAL activity was decreased significantly but despite increasing of phenolic compounds in stress condition. It seems that PAL enzyme deactivation due to stress intensity and the passage of time. Application of Arg and Arg + LNAM increased PAL activity in stressed plants. The effect of temperature (0-4ºC) on watermelons, tomatoes and canola plants have shown that the enzyme polyphenol oxidase activity is reduced in the cold. So leads to the accumulation of phenolic compounds in plants (Sonald and Laima, 1999; Sosa et al., 2002). As shown in Fig. 3-b, PPO activity declined in plants which were subjected to cold and this reduction associated with accumulation of phenolic compounds in plants. When plants were pretreated with Arg and Arg + LNAM increased PPO activity in freezing stress. Results showed that those plants which were under cold stress had lower phenolic compounds when compared with control (Fig.2). Our findings showed that in cold stressed plants arginine treatment is more effective than the Arg + LNAM treatment in increment of the PPO and PAL enzymes activity, thus the effects of Arg on Pal enzymes may be related to the NO production in this situation.
Proline content
The amounts of proline increased significantly in plants which were under stress. Treatment of plants with Arg decreased the amounts of proline while when Arg was used with LNAME, proline content increased significantly in comparison with Arg pretreated and non-pretreated plants under stress condition.

In higher plants, proline accumulation in response to chilling stress plays a major role in antioxidative stress as a hydroxyl radical scavenger, in regulation of the NAD+/NADH ratio and as a protein-compatible hydrotrope (Yadegari et al., 2007). In this research proline content increased in plants which were under cold stress (Fig.4). Application of Arg in plants which were under cold stress decreased the proline content while Arg+LNAME increased the proline content. In this study it seems that Arg could increase the cold tolerance of pistachio plants preferably through the NO production so proline synthesis decreased in Arg pretreated plants and when LNAME was used as NOS inhibitor proline content increased significantly. In this study proline accumulation under stress conditions may be related to activated the glutamate pathway (Delauney and Verma, 1993). It has been reported that prolin to reduce under the influence of nitric oxide. For example application of exogenous nitric oxide decreased root prolin in cucumber plants under salt stress (Arasimowicz-Jelonek et al., 2009).

Protein oxidation is defined as covalent modification of a protein induced by ROS or byproducts of oxidative stress. Most types of protein oxidations are essentially irreversible. Protein carbonylation is widely used as marker of protein oxidation (Moller et al., 2007). It has been found that various stresses lead to the carbonylation
of proteins in tissues (Job et al, 2005). In this study results showed that cold stress increased the amounts of carbonyl groups (Fig 5). Pretreatment of plants with Arg decreased the oxidation of proteins under cold stress. However the oxidation of protein increased in those plants which were pretreated with Arg+LNAM in comparison with Arg pretreated plants. The effect of exogenous arginine treatment on alleviating protein oxidation could related to the ability of NO in the reduction of protein oxidation. The exogenous supply of NO protects plants from oxidation damage by eliminating the superoxide anion O_2^- and lipid radical R' and activating the antioxidant enzyme activities specially superoxide dismutase (Kopyra and Gwozdz 2003). NO has been proven to combine with superoxide anion and produce proxynitrite (ONOO'). Peroxynitrite has been shown to react with H2O2 to yield nitrite ion and oxygen (Beligni and Lamatina 1999; Hsu and Kao 2004).

Protective effect of Arg pretreatment in reduction of protein oxidation could related to synthesis of polyamines. Overall, cold acclimation results in protection and stabilization of the integrity of cellular membranes, enhancement antioxidative mechanisms, increased intercellular sugar levels as well as accumulation of other cryoprotectants including polyamines that protect the intracellular proteins by inducing the genes encoding molecular chaperones (Guy and Li 1998). All these modifications help the plant to withstand and surpass the severe dehydration associated with freezing stress.

In this research in some parameters, Arg and Arg+LNAM pretreatment had the same effects and it seems that in these situations other pathways of Arg metabolism rather than NOS may be activated. However in the case of, protein oxidation the role of NO were more effective than other products because in the present of LNAM the effects of Arg on these parameters decreased significantly.

**Protein oxidation and carbonyl groups**

As it is shown in Fig 5, freezing stress caused an increase in carbonyl groups which is shown that protein oxidation increased under this condition. Arginine or Arg+LNAM pretreatment decreased the amounts of carbonyl groups. However Arg was more effective than Arg+LNAM pretreatment. Pretreatment of plants with Arg or Arg+LNAM had no significant effects on control plants.

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