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Arginine Induced Photosynthetic Adaptability of Ajwain (*Trachyspermum ammi*) under Osmotic Stress

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Abstract

Compounds which are able to reduce damaging effects of various stresses such as drought could be of great importance. In this research, arginine was used as a precursor of nitric oxide or polyamines and the effect of this compound on alleviation of oxidative damages under drought stress has been investigated. Experimental treatments included arginine at three levels (0, 10 and 20 µmol) and, drought stress (induced by polyethylene glycol 6000) at the levels of 0, 13.5% and 17% (W/V). This experiment was conducted as a completely randomized design in a factorial arrangement with three replications. The application of arginine through the root medium, increased drought tolerance of ajwain (*Trachyspermum ammi*) seedlings. Arginine profoundly induced the activities of phenylalanine ammonia-lyase and, polyphenol oxidase in plants which led to reduction in electrolyte leakage and increasing in relative water content, photosynthetic pigments (chlorophyll a, b, total chlorophyll and, carotenoids), polyphenol compounds, flavonoids, anthocyanin content and, soluble sugar content. It is concluded that the application of arginine appeared to induce pre-adaptive responses to drought stress, leading to promote protective reactions.

1. Introduction

Plants have been used as medicine throughout history. Medicinal plants have a wide range of uses so that they can be used as food supplements, applied to the skin care in the form of soaps and creams and, one third of drugs have been derived from natural sources. Due to the high value of these medicinal plant resources both for local communities use and also as an income source, the cultivation of herbs has been increased in recent years (Rout *et al*., 2000). Ajwain is one of the most important plants in this regard. Ajwain (*Trachyspermum ammi*) belongs to the family of umbellifera (Apiaceae). It is used in folk medicine as a carminative, anti-spasmodic, anti-bacterial, antimicrobial, anti-nausea and anti-inflammatory; it is also a plant with the highest amount of thymol in the world. This chemical is very effective in the stomach release gastric juices that speed up digestion; it is also good way to get rid of pain due to rheumatism and arthritis; and it is considered as a spice due to terpenic compounds isolated from its seeds and, used in preparation of various dishes (Emami & Hosseini., 2008). In aromatic plants, growth is influenced by various environmental factors such as water stress (Burbott & Loomis., 1969). Water deficit is one of the most common environmental stresses that limit plant productivity. Understanding the cellular processes that ameliorate the consequences of water loss is clearly important (Neill *et al*., 2003). One of the biochemical changes occurring when plants are subjected to this harmful stress condition, is accumulation of reactive oxygen species (ROS) which causes oxidative damage in plants (Smirnoff., 1993). Free radicals are toxic to living organisms unless remove 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 (Tian & Li., 2006). To control the ROS level, plants have evolved an antioxidant defense system comprising of enzymes such as superoxide

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dismutase, catalase and ascorbate peroxidase as well as non-enzymatic constituents such as anthocyanin, flavonoids and polyphenol compounds, which are responsible for scavenging excessively accumulated ROS in plants under stress conditions (Shi *et al*., 2007). The regulation of these antioxidant constituents by an exogenous substance might mediate the plant tolerance to drought stress. Among different strategies which were used to cope with drought stress, priming is an easy, low cost and low risk technique and this approach has recently been used to overcome drought stress (Nasibi *et al*., 2011). L-arginine (Arg) 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). It has been suggested that both endogenous and exogenous arginine have roles in plant stress responses, such as drought and salinity (Zeid., 2009; Nasibi *et al*., 2011). However, there are few researches on the effect of exogenous arginine as a precursor of these compounds in the possible antioxidative responses of plants against drought stress. The objective of the present experiment was to investigate the effects of arginine pretreatment on alleviation of oxidative damages induced by drought stress. Comparing these responses can be useful in understanding the physiological and biochemical mechanisms of this compound in plants which have to cope with drought stress.

2. Material and Methods

2.1. Plant Material

Ajwain (*Trachyspermum ammi*) were grown from seeds in plastic pots contains sand and compost until the seeds were germinated. The seedlings were irrigated with water daily and half-strength Hoagland's solution once a week. After five weeks of growth, the uniformly seedlings were transferred to bottles containing Hoagland's solution aerated with air pump and, were treated with 0 (as a control), 10 and 20 µM arginine (arginine was added to nutrient solution). After 24 h, plants were subjected to drought stress. Polyethylene glycol (PEG_{6000}) compound has been used to simulate osmotic stress effects in vitro for plants maintain uniform water potential throughout the experimental period. For this purpose, seedlings were placed in aerated bottle containing distilled water served as a control and Polyethylene glycol of 13.5% and 17% (W/V) strengths to achieve osmotic stress levels of -0.3 and -0.5 MPa. These concentrations of arginine and PEG were optimized in preliminary experiment (Nasibi *et al*., 2013b). After 72 h, the shoots of plants were gathered and, immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

2.2. Leaf Relative Water Content (RWC)

RWC was calculated as follow: RWC= [(fresh weight-dry weight)/ (saturated weight – dry weight)] ×100 (Wheutherley., 1950).

2.3. Electrolyte Leakage

The electrolyte leakage was determined as described by Ben Hamed *et al*., (2007). Shoot samples (0.2 g) were placed in test tubes containing 10 mL of double distilled water. The tubes were incubated in a water bath at 32 °C for 2 h and the initial electrical conductivity of the medium (EC_1) was measured by an EC meter (Metrohm, Filderstadt, Germany). The samples were autoclaved at 121 °C for 20 min to release all the electrolytes, cooled at 25 °C and then the final electrical conductivity (EC_2) of each was measured. The electrolyte leakage (EL) was calculated by using the following formula:

$EL= (EC_1/EC_2) \times 100$

2.4. Determination of Soluble Sugar Content

Frozen samples (0.1 g) were grinded and extracted with 2.5 mL of 80% (v/v) ethanol at 90 °C for 60 min, followed by centrifugation at 10000×g at 4 °C for 10 min. The process was repeated for complete extraction. Total soluble sugar content was determined using anthrone reagent and glucose as standard (Roe., 1955). Results are expressed as mg soluble sugar per gDW^{-1}.

2.5. Estimation of Chlorophyll Content

Chlorophyll content was determined using the methods of Lichtenthaler (1987). In this method, Chlorophyll was extracted in the 80% acetone. Extracts were centrifuged at 3000 g and, the absorbance of the supernatant was measured at 663.2, 646.8 and 470 nm with a spectrophotometer (Cary 50; Varian Instruments, Walnut Creek, USA).

2.6. Determination of Anthocyanin Content

For determination of anthocyanin content, frozen tissue samples (100 mg) were soaked immediately in 10 mL of acidified methanol (methanol: HCl 99:1 (v/v)). Tissues were crushed using a glass pestle and kept at 25 °C for 24 hour in the dark. The extract was then centrifuged at 4000 × g for 5 min at room temperature and absorption at 550 nm of the supernatant was read by an UV-VIS spectrophotometer (Cary50, Germany). For the calculation of the amount of anthocyanin, the ex-

tinction coefficient of 33000 $M^{-1}Cm^{-1}$ was used (Wagner., 1979).

2.7. Estimation of Flavonoids Content

To determine the content of flavonoids, 0.1 g of leaf tissue were extracted in 15 mL glass centrifuge tubes containing 10 mL of acidified ethanol (ethanol: acetic acid, 99:1 (v/v)). The samples were gently boiled for 10 min in a water bath at 80 °C and brought up to volume. Absorbance was measured at three wavelengths: 270, 300 and 330 nm with UV-VIS spectrophotometer (Krizek *et al*., 1998).

2.8. Determination of Polyphenol 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 a milligram on a dry weight (Gao *et al*., 2000).

2.9. Enzyme Extraction and Activity Determination

Frozen shoot samples (0.5 g) were homogenized in 2.5 ml of 50 mM phosphate buffer (PH=7) containing 1mM ethylenediamine tetra acetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride (PMSF) and 1% polyvinyl pyrrolidone (PVP). The homogenate solution was centrifuged at 20000×g for 20 min at 4 °C and, the clear supernatant was used directly for the assay of enzyme activity and estimation of protein. The supernatant was used for measurement of total soluble protein according to Bradford (1976) using bovine serum albumin as standard.

2.10. Phenylalanine Ammonia-lyase (PAL) Activity Assay (EC 4.3.1.5)

PAL activity was assayed according to the method of D'cünha (1996). The reaction mixture contained 100 mM Tric-HCl buffer (pH 8.5), 1 mM 2-mercaptoethanol, 50 mM 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 0.5 mL 6 M HCl and absorbance of the supernatant was measured at 290 nm. One unit of enzyme represents the conversion of 1 µmol substrate to cinammic acid per min.

2.11. Polyphenol Oxidase (PPO) Activity Assay (EC1.14.18.1)

The PPO activity was assayed as per the procedure of Haplin and Lee (1987). The reaction mixture consisted 1.5 ml of 0.1 M Sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. 0.01ml Catechol was added to the reaction mixture to start the reaction. PPO activity was expressed as change in absorbance at 412 nm.

3. Results

3.1. Leaf Relative Water Content (RWC)

The results analysis of variance showed that drought stress caused a significant ($p \leq 0.01$) reduction in RWC (Table 1). Pretreatment with Arg markedly alleviated the adverse effects of water stress. The highest and lowest RWC was recorded for control and -0.5 MPa treatments, respectively (Table 2). This treatment (-0.5 MPa) reduced RWC by 41.9% compared with control (Table 2).

3.2. Electrolyte Leakage (EL)

Drought stress caused an increase in EL to inter cellular space and Arg pretreatment reduced this leakage at all levels of drought stress (Table 1). Increasing the drought stress from 0 to -0.5 MPa led to an increment of EL. Highest level of osmotic potential (-0.5 MPa) caused an increase of 84.7% in EL as compared with control (Table 2).

3.3. Soluble Sugar Content

The effect of different levels of drought stress on soluble sugar content was significant (Table 1). The response of soluble sugar content to the interaction of Arg concentrations and drought levels were different, so that the highest concentration of this trait belonged to -0.5 MPa (9.37) and both concentrations of Arg caused an increasing of soluble sugar content compared with control (Table 2).

3.4. Chlorophyll (Chl) and Carotenoids Content

Drought had a significant effect on Chla, total Chl and carotenoids content of *Trachyspermum ammi* (Table 1). Drought stress at the level of -0.5 MPa reduced Chl a, b, total Chl and carotenoids by approximately 24.7, 33.33, 27.5 and 21.8% compared with control respectively. Pretreatment of plants with Arg was more effective under severe drought stress (Table 2).

3.5. Estimation of Anthocyanin, Flavonoids, Polyphenol Compounds, Phenylalanine Ammonia-lyase (PAL) Activity and Polyphenol Oxidase (PPO) Activity

As shown in Table 1, the effect of osmotic potential on flavonoids (270, 300 and 330nm) and total phenol compounds was significant. Results showed that drought stress induced by PEG, caused the reduction of anthocyanin, but pretreatment with Arg increased this pigment under stress conditions (Table 2). At the con-

centration of 20 µmol Arg, anthocyanin content was increased by approximately 57.3% compared to non-primed seedlings under -0.5 MPa drought stress.

Flavonoids contents were increased by increment of drought stress especially at the highest level of PEG (Table 2). As expected, spectrophotometer measurements at all three wavelengths (270, 300 and 330nm) revealed that Arg application in plants under the mentioned levels of drought stress increased the flavonoids contents (Table 2). The data indicated that drought treatments at the potentials of -0.3 and -0.5 MPa caused an increment of 52.5 and 68.3% respectively compared with control (Table 2). Pretreatment of ajwain plants with Arg had a significant effect on these compounds only under drought conditions (Table 2). The effect of drought stress on PAL and PPO activities in ajwain plants, either with or without Arg pretreatment was investigated (Table 1). The effect of water deficit on PAL activity is shown in table 2. Based on results, the highest level of drought stress led to a remarkable increase in PAL activity. The highest amount of this enzyme belonged to -0.5 MPa (15.9) and application of Arg increased the activity of PAL in drought stressed plants (Table 2). On the basis of these results, increasing drought levels caused a remarkable raise in PPO activity (Table 2). The difference in this enzyme's activity was statistically significant between control and both concentrations of Arg (Table 2).

4. Discussion

Water is a major limiting factor in crop production in arid and semi-arid areas of the world; So that drought stress is one of the most devastating environmental stresses. Drought stress influences on plants and results in morphological, physiological, molecular and, growth mutations. Water stress affects plants in different levels from a cell to a plant colony. It reduces plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism and growth promoters (Farooq *et al*., 2008). The reaction of plants to water stress differ significantly at various organizational levels depending upon intensity and duration of stress as well as plant species and, its stage of growth (Chaves *et al*., 2002). Stress factors are well-known to cause a shift in antioxidant balance in plant cells. This shi ft is due to an increase in the rate of generation of ROS, which induces lipid peroxidation in the membrane structures of the cells.

Table 1 Mean squares for relative water content (RWC), electrolyte leakage (EL), soluble sugar content, chlorophyll a (Chl a), chlorophyll b (Chl b), total Chlorophyll (Chl T), carotenoids content, anthocyanin content, flavonoids content (270, 300 and 330 nm), total phenols content, Phenylalanine ammonia-lyase activity (PAL) and Polyphenol oxidase activity (PPO) of *Trachyspermum ammi*.

		MS						
SOV	DF	RWC	EL	soluble sugar content	Chl a content	Chl b content	Chl T content	carotenoids content
Arginine	2	425.42**	18.11**	16.683**	5.832**	6.652**	24.7**	1.703**
Drought	2	937.212**	780.703**	43.486**	3.497**	0.167^{ns}	5.19**	0.368**
Arginine × Drought	4	141.08**	3.161**	2.125^{ns}	2.379**	1.981**	7.88**	0.191*
Error	18	6.25	0.32	1.974	0.095	0.081	0.18	0.051

**, * and ns denote significant differences at 0.01, 0.05 % levels, and not significant respectively.

		MS						
SOV	DF	anthocyanin content	flavonoids (270nm)	flavonoids (300nm)	flavonoids (330nm)	total phenols content	PAL activity	PPO activity
Arginine	2	1.0011**	0.147^{ns}	0.178**	23.488^{ns}	0.0709**	0.385**	0.0142^{ns}
Drought	2	0.0045^{ns}	262.004**	192.26**	174.27**	2.876**	106.95**	4.636**
Arginine × Drought	4	0.2315^{ns}	0.0837^{ns}	0.109**	13.438^{ns}	0.019^{ns}	0.197**	0.0599**
Error	18	0.1091	0.0481	0.0148	10.572	0.0067	0.026	0.0073

**, * and ns denote significant differences at 0.01, 0.05 % levels, and not significant respectively

Table 2 The effect of arginine (Arg) pretreatment on relative water content (RWC), electrolyte leakage (EL), soluble sugar content, chlorophyll a (Chl a), chlorophyll b (Chl b), total Chlorophyll (Chl T), carotenoids content, anthocyanin content, flavonoids content (270, 300 and 330 nm), total phenols content, Phenylalanine ammonia-lyase activity (PAL) and Polyphenol oxidase activity (PPO) of *Trachyspermum ammi* under different levels of drought stress.

Arg × Drought	RWC (%)	EL (%)	soluble sugar content (mg/gDW)	Chl a content (mg/gDW)	Chl b content (mg/gDW)	Chl T content (mg/gDW)	Carotenoids content (mg/gDW)
Control							
Control	84.83a	3.76f	4.351def	12.452abc	6.21cd	18.658b	2.617cd
-0.3 MPa	64.57c	15.93d	8.561ab	11.81d	5.086e	16.896c	2.483d
-0.5 MPa	49.27d	24.57a	9.377a	9.37e	4.147f	13.524d	2.048e
10µmol Arg							
Control	83.39a	3.3f	3.088ef	12.702ab	6.125d	18.826b	3.131ab
-0.3 MPa	74.27b	14.17e	7.09abc	12.126cd	6.908ab	19.034b	2.639cd
-0.5 MPa	68.53c	21.13b	5.88cd	12.19bcd	6.674abc	18.873b	2.922bc
20µmol Arg							
Control	86.21a	2.9f	2.63f	12.92a	6.442bcd	19.363ab	3.44a
-0.3 MPa	76.56b	13.26e	5.158cde	12.62abc	6.496bcd	19.113b	2.882bc
-0.5 MPa	76.27b	19.76c	6.807bc	12.78a	7.144a	19.923a	3.4179a

Means followed by the same letter(s) in each column are not significantly different at the 5% level.

Arg × Drought	Anthocyanin content (µmol/gDW)	Absorbance of 270nm (%)	Absorbance of 300nm (%)	Absorbance of 330nm (%)	Total phenols content (mg/gDW)	PAL activity (U/mg protein)	PPO activity (U/mg protein)
Control							
Control	1.363ab	15.4de	10.6d	12.52d	0.57e	8.5e	2.6de
-0.3 MPa	0.89bc	17.6c	17.028b	21.18abc	1.2c	12.43c	3.8b
-0.5 MPa	0.7374c	25.93a	19.46a	24.06ab	1.8a	15.9a	4.01a
10µmol Arg							
Control	1.393ab	17.6c	10.4de	20.28bc	0.6e	8.7e	2.7d
-0.3 MPa	1.414ab	17.83c	16.436c	22.33ab	1.01d	11.9d	3.6c
-0.5 MPa	1.626a	25.76ab	19.42a	24.59ab	1.6b	15.113b	3.93ab
20µmol Arg							
Control	1.429ab	15.3e	10.267e	16.06cd	0.5e	8.467e	2.487e
-0.3 MPa	1.753a	17.73c	16.93b	22.03ab	0.9d	12.01d	3.9ab
-0.5 MPa	1.729a	25.5b	19.34a	26.27a	1.65b	15.33b	3.8b

Means followed by the same letter(s) in each column are not significantly different at the 5% level.

Compounds that are able to reduce the damaging effects of various stresses are prominent in both theoretical and practical points of view. In this research Arg was used as an important signal molecule for modulating plant responses to drought stress and participates in the regulation of physiological processes, to study the role of this amino acid in some physiological parameters under this stress. Our findings showed that drought stress has negative effect on RWC and pretreatment with Arg alleviated the adverse effect of osmotic stress. Similar results were reported in wheat (Lei *et al*., 2007), rice (Hsu & Kao., 2003) and *Nigella sativa* (Kabiri *et al*., 2014) under drought stress. Increasing of RWC may be related to the role of Arg in accumulation of compatible osmolytes in plants, which were subjected to drought stress. Drought stress induced lipid peroxidation by production of ROS (Shi *et al*., 2007); thus making the membranes leaky as evinced by increased electrolyte leakage. The membrane injury was time dependent and increased with duration of stress. In the present investigation, water deficit significantly increased electrolyte leakage while Arg alleviated the adverse effect of PEG solution in ajwain plants. Several studies showed that EL in susceptible plants was higher than in resistant plants (Kabiri *et al*., 2014; Juan *et al*., 2005). A protective effect of Arg on membrane injury has been reported under drought stress (Nasibi *et al*., 2011). It has been reported that the role of Arg in prevention of lipid pe-

roxidation is related to its ability to react with lipid alcoxyl (LO·) and lipid peroxyl (LOO·) radicals and stop the chain of peroxidation (Nasibi *et al*., 2011). Soluble sugars accumulated in plants for osmotic adjustment in response to drought and salinity stress and caused the protection of macromolecules and DNA structures (Juan *et al*., 2005). Nevertheless, different results have obtained about the effect of drought and salinity stress on carbohydrate accumulation. Some researchers reported that carbohydrate contents increased under stress conditions (Jones & Turner., 1980), some believe that this trait reduced under stress conditions (Hanson & Hitz., 1982) and some others reported that soluble sugar contents remained permanent (Morgan., 1992). Our data indicated that drought stress caused the increment of soluble sugar contents, and pretreatment with Arg didn't have any effects on sugars under control condition. A possible reason for the reduction of photosynthetic pigments might be related to drought stress and this reduction dependent upon several factors such as its intensity, duration and, phonological phase of growth and genetic resistance capacity of plants. Reduction of photosynthetic pigments in drought stress could be related to degradation of chloroplast structure and, photosynthetic apparatus, chlorophyll photo oxidation, destruction of chlorophyll substrate, inhibition of Chl biosynthesis and the increase of chlorophyllase activity. In this research, drought stress caused reduction of photosynthetic pigments but Arg pretreatment increased Chl (as the main part of photosynthetic structure) and carotenoid contents in both conditions. Several reports have shown that pretreatment of plants with Arg increased chlorophyll content under abiotic stresses (Nejadalimoradi *et al*., 2014; Nasibi *et al*., 2013b). The reduction of carotenoids content under drought stress was related to the degradation of beta carotene (Sultana *et al*., 1999). Under drought stress, reduction of carotenoids could be related to the protection role of it in the photosynthetic apparatus, because carotenoids were responsible for scavenging of ROS, preventing lipid peroxidation and ultimately mitigation of oxidative stress (Koyro., 2006). When Arg applied in satisfactory concentrations may improve the antioxidative capacity of the plants and help to induce the synthesis of protective compounds (such as carotenoids). The phenylpropanoid pathway is one of the important pathways of plant secondary metabolism, which yields a variety of phenolics with structural and defense-related functions. These phenolic compounds include phenolic acid, anthocyanin and flavonoids, which act as scavengers of free radicals and other oxidative species through their hydrogen donating (antioxidant) potential (Syvacy & Sokmen., 2004). In order to control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes (PAL and PPO) and non-enzyme (anthocyanin, flavonoids and polyphenol compounds) defense mechanisms against oxidative stress. In ajwain plant, drought resulted in a decline in anthocyanin (Table 2). One of the possible reasons to explain the reduction of this compound under drought stress, is related to its antioxidant characteristics to scavenging of ROS under stress condition and also the precursor for biosynthesis of anthocyanin, flavonoids and polyphenol compounds is similar, it seems that the substrate is used to synthesis of flavonoids and polyphenol compounds rather than synthesis of anthocyanin (Sakihama et al., 2002). As shown in table 2, flavonoids contents and total phenol compounds are increased under drought stress. Enhanced the amounts of flavonoids contents and total phenol compounds were useful to maintain a cellular redox balance under stress condition. Phenylalanine ammonia-lyase (PAL) is a crucial enzyme of phenylpropanoid metabolism that catalyzes the formation of trans-cinnamic acid by L-deamination of phenylalanine. This enzyme was induced by various, biotic and abiotic stresses, which resulted in the accumulation of such phenolic compounds as phenolic acids and flavonoids (Solecka., 1997). PPO activity increased in plants which were subjected to drought stress (Table 2). Thipyapony *et al*., (2004) reported that enhanced PPO activity caused to protect the Chlorophyll contents and polyphenol compounds. Nasibi *et al*., (2013a) reported that the application of Arg increased the activities of PAL and PPO under cold stress. Arginine is the main amino acid in plants and two main pathways of its metabolism have been reported which are 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 polyamines or proline synthesis (Liu *et al*., 2006). In previous studies, the effects of nitric oxide, proline and, polyamines in the protection of plants against drought stress were reported (Nasibi *et al*., 2011). However, further studies are necessary to find the mechanism of different pathways of Arg catabolism in stressful conditions. PEG-induced drought stress could cause oxidative damage in ajwain leaves through excessive generation of ROS and exogenous Arg greatly improves the dehydration tolerance through elevated activities of antioxidant systems. Based on our results, it may be show that, application of Arg could be a method to decrease water stress damages to plants.

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