The Effect of Application of Humic Acid Foliar on Biochemical Parameters of Pistachio under Drought Stress

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Abstract: The aim of the present study was to evaluate the resistance of pistachio plants (Iranian Badami cultivar) to drought stress via different treatments of humic acid. A total of 108 one–year–old pistachio seedlings were used in a 4×3 factorial experiment based on a completely randomized design (CRD) with 3 replicates and 3 plants for each replicate. The two factors involved were drought stress and humic acid. The former was used in three levels, namely D1) 80% of field capacity (control), D2) 40% of field capacity (medium stress), and D3) 20% of field capacity (severe stress); the latter in four levels of H1) 0, H2) 500, H3) 1000, and H4) 1500 ppm, respectively. Traits measured in this experiment included leaf chlorophyll content, relative water content (RWC), proline amino acid, abscisic acid hormone (ABA) in fresh leaf, and root dry weight. The results of this investigation revealed that drought stress (severe type) increased proline amino acid and ABA. Whereas, it decreased root dry weight, relative water content (RWC), and leaf chlorophyll content ($P \leq 0.05$). The effect of different levels of humic acid on all measured parameters was significant ($P \leq 0.05$). According to the results, humic acid treatments resulted in a reduction of proline amino acid and ABA in comparison to those of plants without humic acid treatments. Conversely, leaf chlorophyll content, root dry weight, and relative water content (RWC) were increased by humic acid treatments in combination with drought stress (severe stress).

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1. Introduction

Drought is one of the most destructive factors affecting plant growth and productivity. Such factors affect many physiological processes in plants. Numerous efforts have been made to improve productivity and performance of crops under drought stress (Cattivelli et al., 2008). Drought resistance of plants can be increased by appropriate application of some substances (Farooq et al., 2009). These which are usually used for reducing environmental stresses include proline amino acid, ABA, glycine betaine, soluble sugars, humic acid, potassium, and similar ions (Serraj & Sinclair, 2002; Yamada et al., 2005; Hussain et al., 2008). These substances are non-toxic for plants in appropriate concentrations and can protect them against environmental stresses by interference in metabolic activities and photosynthetic process through osmotic adjustment, removal of oxygen radicals, stabilization of cell membranes, protection of enzymes and proteins, and involvement in stomatal opening and closure, (Bohnert & Jensen, 1996; Farooq et al., 2009). It was reported that unfavorable effects of environmental stresses can be reduced by means of the humic acid (Nardi et al., 2002). Research studies showed that it could be used as a growth regulator of hormone level to improve the plant growth and enhance stress tolerance (Piccolo et al., 1992). It can stimulate shoot and root growth, improving resistance to environmental stress in plants (Goatley & Schmidt, 1990). There were reports that unfavorable effects of environmental stresses could be reduced by the use of humic acid (Zhang & Ervin, 2004; Nardi et al., 2002). Humic substances are known to increase the plants' tolerance towards different stresses like water deficiency (Natalia, et al., 2006). Some investigations reported the positive effects of the humic acid on increasing the photosynthesis, the rooting, and drought resistance (Liu et al., 1998; Zhang & Ervin, 2004).The present study was carried out to investigate the effects of humic acid on tolerance of pistachio against drought stress.

1.0 Materials and Methods

The experiment was conducted during 2010 growing season at institute of soil and water in Karaj, Iran (latitude 35.55 N, longitude 50.54 E). A total of 108 one–year–old pistachio plants of Iranian *Badami* cultivar were used in a factorial experiment based on a completely randomized design (CRD) with 3 replicates and 3 plants for each replicate. A pistachio seedling that was cultivated for the sake of the experiment in a 5 Kg pot soil had a sandy texture with 10% silt. The two factors involved were drought stress in three levels of D1 (80% of field capacity as control), D2 (40% of field capacity as medium stress), and D3 (20% of field capacity as severe stress), respectively; and humic acid in four levels of H1: 0, H2: 500, H3: 1000, and H4: 1500 ppm, respectively. The field capacity was measured using pressure plate device. In order to apply the drought stress, the pistachio plants were subjected to different levels of it for 60 days, then, sprayed by the humic acid solution five times a day for four weeks to study the effects of the humic acid.

1.1. Extraction and Measurement of Proline

Samples of fresh leaves were collected one week after the last spray. The ninhydrin method (Bates et al., 1973) was applied for extraction and measurement of the proline content. Meanwhile, the fresh leaf samples (0.5 g) were briefly frozen in the temperature -80 ℃ and placed in 5 ml sulfo-salicylic acid 3% to be homogenized. The obtained homogeneous solution was centrifuged at 10000 r/min pm. Then, 2 ml of the supernatant were mixed with two ml of the acetic acid and 2ml of ninhydrin reagent. The resulting solution was boiled at 100 °C for 30 minutes. After cooling in an ice bath, 6 ml of toluene was added to it. Afterwards, the mixture was transferred to a separator funnel. During mixing, cromofer containing toluene was separated, and the absorbance at 520 nm in a spectrophotometer was compared with the control containing toluene. Subsequently, the proline concentration was specified using a standard curve.

1.2. Extraction and Measurement of ABA

According to the procedures introduced by Isogani et al. (1967) for extraction and measurement of ABA, the following method was applied. In brief, 2 g of the frozen leaf samples were crushed in a mortar and poured into a 50-ml tube. Then, 40 ml of 80% ethanol containing 0.25 mg l^{-1} hydroxide toluene and 0.5 mg l^{-1} sodium ascorbate was added to it and it was maintained in the dark at 4 ºC for 16 hr in order for ABA to be dissolved in the solution. Subsequently, the solution was filtered through Whatman filter paper. The extra methanol was removed from the solvent using rotary evaporator at 35 ºC. At this stage, phosphate buffer solution was added to the residue solution (4-5 ml) and the pH value was adjusted to 8.5 via KOH. After that, the solution was rinsed twice by adding the ethyl acetate, and afterward, divided into two parts. Subsequent to vortex of the solution, ethyl acetate was removed, and what was remained of it in the solution was also removed by rotary evaporator, then, the pH value of remaining solution was adjusted to 2-3 by means of hydrochloric acid. Again, the solution was rinsed twice with ethyl acetate where the remaining ethyl acetate was completely evaporated by rotary evaporator at 35ºC. Immediately after that, the remaining solution was dissolved in 4 ml methanol (HPLC grade) and filtered through disposable polytetrafluoroethylen filter. The obtained solution was loaded onto a HPLC system equipped with a c18 column, using a flow rate of 0.8 ml/sec, 0.1 solutions of acetic acid, and 80% methanol for HPLC ratio of 50:50. The amount of ABA was determined by means of the standard samples.

1.3. Determination of Leaf Relative Water Content (RWC)

The leaf samples were collected one week after the last spray, immediately measured for their fresh weight (FW), then kept in distilled water for 4 hr, and measured in terms of turgid weight (TW). They were then dried in an oven for a week at 70 °C to determine the dry weight (DW), and finally the relative water content (RWC) of the leaf samples was calculated according to the following equation (Adriano et al., 1982):

$$(RWC) = 100\times (FW-DW)/ (TW-DW);$$

where FW hydrating is the fresh weight, DW is the dry weight, and TW the turgid weight after re-hydrating the leaves.

1.4. Measurement of Leaf Chlorophyll Content

For measuring the leaf chlorophyll content three of the middle leaves were initially selected. Then, three interpretations of the various parts of each leaf were carried out by chlorophyll meter (SPAD-502), and the average reading of the three leaves recorded.

1.5. Measurement of Root Dry Weight

At the end of the experiment, the roots were cleaned from the mud, their fresh weight was immediately measured, then they were oven dried at 70 ºC for a week, and subsequently weighed.

1.6. Statistical Analysis

The statistical analysis was performed using Microsoft Excel (Microsoft office 2007 package) and SAS software (SAS Institute Inc., 1990) where the means were compared using Duncan's Multiple Range Test (DMRT) at $P\leq 0.05$.

2. Results

The results as to the interaction of different levels of the humic acid and drought stress on the leaf proline amino acid was indicative of a significant effect on the leaf's free proline content $P \leq 0.05$ (see Table 1). The highest amount of proline was recorded at 1500 ppm for the humic acid in combination with the severe stress group (H_4D_3). On the contrary, the lowest amount was observed in 500 ppm for the humic acid and the control treatment (H_2D_1).

However, the results associated with the effects of different levels of the humic acid and drought stress interaction on ABA of the leaf was statistically significant ($P \leq 0.05$), in that both the highest and the lowest amount of ABA were attributable to the treatments of H_4D_2 and H_3D_1, respectively (see Table 1).

Nonetheless, in terms of the effects of different levels of humic acid and drought stress on the leaf relative water content (RWC), the statistical analysis exhibited a significant difference between the former and the latter, so that treatments of H_4D_3 together with H_2D_2 had the highest and H_2D_1 the lowest relative water content, respectively (see Table 1).

As far as the effects of different levels of the humic acid and drought stress on the leaf chlorophyll content were concerned, the results obtained on the chlorophyll content (see Table 1) indicated significant differences among different treatments ($P \leq 0.05$). That is, the highest chlorophyll content was observed in H_2D_2 and the lowest in both H_4D_2 and H_4D_3.

With respect to the effects of different levels of the humic acid and drought stress on the root dry matter, as observed in Table 1, the drought stress in combination with humic acid caused significant differences in various treatments ($P \leq 0.05$). Thus, the highest amount of the root dry matter was observed in H_2D_1 and H_2D_2 while the lowest amount was observed in H_4D_2, H_4D_3 treatments, respectively.

3. Discussion

One of the common responses of plants to the change in external osmotic pressure is accumulation of metabolites which are soluble and do not disrupt normal plant metabolism (Orcutt and Nilsen, 2001; Wahid and Close, 2007). Based on the results of the current study, the pistachio plant accumulated amino acid of the proline and ABA in response to the drought stress, thereby increasing their resistance to drought (Jianhua et al., 2006). The positive role of the proline was reported in mediating the osmotic pressure of drought stress on petunia (Yamada et al., 2005) and arabidopsis (Kiyosue et al., 1996). It was also reported that the proline accumulated under several types of environmental stresses such as drought stress (Singh et al., 1973; Boggess et al., 1976; Alexieva et al., 2001), low temperature (Chu et al., 1974), and salinity (Chu et al., 1976). The proline amino acid content showed a decrease amid the humic acid application under drought conditions. The results indicated that using the humic acid brought about resistance against drought stress (Zhang & Schmidt, 1997; Ervin et al., 2003; Tewfik, 2008). The hormone-like role of humic acid could be stated as one of the reasons. Zhang and Ervin (2004) extracted cytokinin and gibberellic acid from the humic acid and also showed that spraying the humic acid on the leaves of the lawn would increase its resistance to drought. The other probable reason for proline enhancement could be attributed to ABA hormone. Furthermore, Stewart (1980) stated that exposure of the plant to ABA stimulated the synthesis of some proteins which improve the plant resistance to stress. The hormone probably involved in the synthesis of proline from glutamic acid.

The present study tried to show that the pistachio plant increased its level of ABA to cope with drought stress. ABA is defined as a stress-induced hormone because of its rapid accumulation in response to stresses and its mediation of many stress responses that help the plants survive the stresses (Jianhua et al., 2006). It is worth noting that abscisic acid hormone controls processes such as stomatal closure, seed dormancy, fruit and leaf abscission, and retarded growth during stress (Schroeder et al., 2001). Under environmental stress, the ABA level increases rapidly in plants. This rapid change causes the plant to close its stomata so as to reduce water loss and enhance the plant resistance against drought stress (Artega, 1999). When plant is exposed to water stress, ABA is synthesized in root tips and transported bidirectionally to leaves to control closing of stomata (Davis & Jones, 1991; Jianhua et al., 2006; Tawfik, 2008).

According to the results of the study, the leaf chlorophyll exhibited a reduction by increasing drought stress. However, the most important physiological effect of water stress is limitation of photosynthesis. Severe drought conditions would limit the phenomenon due to a decline in Rubisco activity. Shortage of water stress causes a reduction in photosynthesis, in turn, through reduction in leaf area, closing of stomata and reduction in CO_2 fixation efficiency due to partial stomatal closure and decrease of leaf chlorophyll. It was maintained that the rate of leaf chlorophyll decreased as the drought stress in *Pistacia khinjuk*, *Pistacia mutica* (Ranjbarfardooei et al., 2000), and almond (Rouhi, 2007) increased. Still, another reason for reduction in the rate of the leaf chlorophyll could be some growth regulators like ABA which lead to stimulation of chlorophyllous activity under stress conditions (Sanchez et al., 2008).

As previously indicated, one of the substantial changes resulting in the drought stress is the reduction in the leaf's relative water content (Siddique et al., 2001). This parameter indicates plant capability in facing drought stress (Egilla et al., 2005). In this research, the leaf's relative water content demonstrated a reduction with an increase in the drought stress. It revealed that different levels of the drought stress and humic acid had significant influence on the leaf's relative water content. The stimulating effects of humic acid were, thus, attributed to its hormone-like properties, because some of the humic acid effects were similar to plant hormones such as auxins, cytokinins and gibberellins (Nardi et al., 2002; Pizzeghello et al., 2002; Chen et al., 2004). In their study, Gang and Evans (2000) reported that foliar spray of humic acid on seedlings of cucumber, marigold, violet, pelargonium, and henna had led to an increase in the leaf's relative water content, as well as in the fresh and dry weight of roots.

4. Conclusion

In the present study, as mentioned earlier, an investigation was conducted on the effects of the humic acid on the response by pistachio under drought stress. The results suggested that the humic acid was the most effective treatment of drought stress occurred by H_2D_1 and H_2D_2 at a concentration of 500 mg/L, which enhanced the pistachio resistance to drought stress via increasing the leaf chlorophyll content, leaf relative water content, and root dry weight as the adjustment by increasing the proline and ABA led to stomatal closure and reduction of water loss. It might be concluded that by increasing membrane stability and the leaf water content, proline might play a role in pistachio drought resistance. But, by and large, it was the humic acid which helped pistachio plants deal with the drought stress.

Table 1. Effect of different levels of humic acid and drought stress on proline, ABA, relative water content (%),Leaf chlorophyll and root dry weight in pistachio

Treatments	Proline(ml/grLF)	ABA(µmol/grLF)	relative water content (RWC) (%)	Leaf chlorophyll (SPAD)	RDW(gr/tree)
H_1D_1	43.57±7.63 bcd	12.46±1.82 cde	88.43±1.5 ab	52.11±3.59 abc	2.02±0.17 b
H_1D_2	45±5.72 bcd	12.99±1.24 bcd	87.11±1.41 ab	53.18±2.53 ab	1.95±0.2 cb
H_1D_3	50.08±6.25 b	13.55±2.25 bcd	81.63±3.18 de	50.22±2.69 cde	1.62±0.39 cd
H_2D_1	37.37±9.61 d	10.86±1.44 ef	89.91±1.35 a	54.4±2.56 a	2.43±0.52 a
H_2D_2	38.2±9.83 d	9.82±0.99 f	87.66±2.28 ab	53.34±2.39 ab	2.47±0.53 a
H_2D_3	41.13±1.2 cd	14.56±1.84 ab	82.55±3.17 d	51.03±2.27 bcd	2.29±0.52 ab
H_3D_1	38.02±5.56 d	9.93±1.09 f	89.4±1.63 a	52.8±1.85 abc	1.57±0.13 cde
H_3D_2	47.03±4.08 cd	11.72±1.31 def	87.14±2.36 ab	51.68±1.73 abc	1.33±0.13 def
H_3D_3	50.02±3.31 b	12.88±2.27 bcd	79.37±3.76 ef	49.97±2.22 cde	1.2±0.15 efg
H_4D_1	59.39±2.82 a	14.24±1.8 bc	85.9±1.22 bc	48.07±1.61 ef	1.02±0.15 fg
H_4D_2	60.8±5.02 a	16.47±0.81 a	82.88±1.48 cd	48.17±1.93 ed	0.96±0.15 fg
H_4D_3	64.51±2.67 a	16.35±1.12 a	76.48±4.2 f	45.18±1.98 f	0.88±0.16 g

*Datas with same letters had no significant differences at Duncans Multiple Range Test at (DMRT).

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