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Exogenous Acetylsalicylic Acid Stimulates' Physiological Changes to Improve Growth, Yield and Yield Components of Barley under Water Stress Condition

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Abstract
To assay the effect of acetylsalicylic acid pretreatment on enhancement of yield and yield components of barley (Reihan-03) under water stress condition, an experiment was conducted as a spilt-plot arrangement based on randomized complete block design with four replications at the Research Field of Shahid Bahonar University of Kerman, Iran, in 2012-2013. Irrigation condition was arranged in main plots (normal irrigation and withholding irrigation at flowering) and acetylsalicylic acid (0, 0.5, 1 mM) in sub-plots. Water stress caused a significant reduction in relative water content, fertile spike/m^2, kernels per spike, 1000 kernel weight, grain yield and biomass and increased electrolyte leakage of plasma membranes, proline content, catalase and also guaiacol peroxidase enzymes activity of flag leaf. Results showed that acetylsalicylic acid application brought on the increased levels of water stress tolerance in barley. Acetylsalicylic acid pretreatment increased antioxidant enzymes activity, relative water content, grain yield, biomass as well as proline content of flag leaf and reduced electrolyte leakage. The concentration of 1 mM acetylsalicylic acid was more effective. In conclusion, seed priming with acetylsalicylic acid increased the tolerance of barley to water stress condition via maintaining cellular membrane integrity and neutralizing or scavenging of reactive oxygen species.

Keywords: Acetylsalicylic acid; Antioxidant enzymes; Barley; Drought stress; Electrolyte leakage; Proline

Abbreviations: Acetylsalicylic acid—ASA; Catalase—CAT; EL—Electrolyte leakage; Guaiacol peroxidase—GPX; ROS—Reactive oxygen species; RWC—Leaf relative water content; SA— Salicylic acid

Introduction

Drought is one of the most serious world-wide problems for agriculture, which determines the success or failure of plant's establishment. The effects of water deficiency depend on several factors such as its intensity and duration, phonological phase of growth and genetic resistance capacity of plants. Water stress affects different aspects of plant growth (morphology, physiology and anatomy) and causes many changes such as decrease or delay in germination, aerial organ growth reduction and decrease in biomass and the rate of growth (Huang 1997). Increasing evidence suggest that drought stress induces oxidative stress through the production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals during stress conditions (Shi *et al.* 2007; Kabiri *et al.* 2014). To control the ROS level, plants have evolved an antioxidant enzymatic defense system comprising of enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR) which are responsible for scavenging excessively accumulated ROS in plants under stress conditions (Shi *et al.* 2007). Electron leakage in the electron transport system in chloroplast and mitochondria is the main source of ROS. ROS are highly toxic and can damage many important cellular components such as lipids, proteins, DNA and RNA (Smirnoff 1993). At the germination

stage, drought could decrease shoot and root length (Okcu *et al.* 2005); at the vegetative period, it would decrease shoot growth and cause dehydration on cell and osmotic imbalance (Schuppler *et al.* 1998). Hanan (2007) reported that germination percentage in barley seed was decreased under drought conditions. Seed priming treatments such as osmopriming, hydropriming and hormonal priming have been employed to accelerate the germination, seedling growth and yield in most of the crops under normal and stress conditions (Basra *et al.* 2003). Although, the mechanism of seed priming treatments is not fully understood, it has been observed that physiological and biochemical changes take place during the seed treatments (Basra *et al.* 2005). The benefits of seed priming have been reported, including improving stand establishment in arid and semi-arid conditions, increasing seed vigor, improving dormancy breakdown, or increasing productivity (Hussein *et al.* 2007).

Aspirin, a trade name for acetylsalicylic acid (ASA), is a derivative of salicylic acid (SA) and when applied exogenously, it undergoes spontaneous hydrolysis and converts to SA (Popova *et al.* 1997). SA is an endogenous growth regulator of phenolic nature, which participates in the regulation of physiological processes in plants. SA could be included in the category of phytohormones (Raskin 1992). Exogenous application of SA may influence a range of diverse processes in plants, including seed germination, stomatal closure, ion uptake and transport, membrane permeability, photosynthetic and growth rates (Raskin 1992; Senaratna *et al.* 2000). There are experimental data indicating the participation of SA in signal transduction for gene expression during leaf senescence in Arabidopsis (Larkindale and Knight 2002). Moreover, SA might serve as a regulator of gravitropism, inhibition of fruit ripening (Srivastava and Dwivedi 2000) and of other processes. It is now clear that SA provides protection against a number of biotic and abiotic stresses (Horvath *et al.* 2007; Gue *et al.* 2007; Zhou *et al.* 2009). The alleviation of oxidative damage and increasing of resistance to the environmental stresses are often correlated with an efficient antioxidative system.

Barely (*Hordeum vulgare* L.) is recognized as one of the most economic and important cereals in the world. By area and production barley is the fourth most important cultivated crop, following, wheat, rice and maize. It can be grown in a wide range of environmental conditions and give satisfactory yields in areas that are not suitable for growing most of the other cereal crops due to problems of biotic and abiotic stress (Abdel-Ghani *et al.* 2005). The aim of the present study was to determine the effect of ASA on tolerance of barley plants to water stress. Studying these responses can be useful in understanding the physiological and biochemical mechanisms of SA in plants which have to cope with water stress.

Materials and Methods

In order to evaluate the effect of ASA on yield, yield components and some biological characters of barley, an experiment was conducted in Kerman (30° 20′ N and 57° 10′ E with 1750 m above sea level). The experiment was carried out as a spilt-plot arrangement based on randomized complete block design with four replications. The

irrigation at two levels, control (normal irrigation) and water stress (withholding at flowering), was arranged in the main plots and ASA at three levels (0, 0.5, 1 mM) in the sub-plots. Each plot consisted of six rows with two meters long and a row spacing of 30 cm. Based on the soil test, 150 kg/ha nitrogen using urea (at sowing and tillering time), 150 kg/ha triple super phosphate (before sowing) and 100 kg/ha potassium sulphate (before sowing) was added to the plots. The seeds of Reihan-03 cultivar were collected from the Seed and Plant Improvement Institute, Karaj, Iran. The seeds were treated with 0.5 and 1 mM of acetylsalicylic acid at room temperature (25ºC) for 24 hr and distilled water was used as the control. The pretreated seeds were sown by hand. Surface irrigation was done using siphon. Normal irrigation was performed every 10 days until the end of the growing season. For the water stress treatment, plots were irrigated every 10 days until the flowering time. In order to control broadleaf weeds, 2,4-Dichlorophenoxyacetic acid (2,4-D) herbicide was applied at the tillering stage.

Leaf relative water content (RWC): In order to measure the relative water content of flag leaves, five flag leaves from each plot were selected at the middle of grain filling period and after water withhold; the leaves were then put in zip pack and transferred to the laboratory immediately. After that, their fresh and dry weights were measured with a digital balance (Sartorius Model: LIBROR AEL-40 SM with 0.00001 g precision). To estimate the saturated weight, the flag leaves were put in distilled water for 20 hr. To measure dry weight, the leaves were put in oven at 70^{0C} for 48 hr. Leaf relative water content (RWC) was calculated as follows (Wheutherley 1950):

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

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 hr and the initial electrical conductivity of the medium (EC_1) was measured by an EC meter (Winlab Data Windaus). 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 rate (EL) was then calculated by using the formula:

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

Proline: Free proline content was determined at 520 nm according to the procedure of Bates *et al.* (1973).

Enzymes extraction and activity: 500 mg leaves were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% soluble polyvinyl pyrrolidone (PVP), 1 mM ethylene diamine tetra acetic acid (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). All of the procedures were done at 4°C. The homogenate was centrifuged at 20,000×g for 20 min, and the supernatant was used to assay the activity of enzymes and protein content.

Catalase (CAT) activity (EC 1.11.1.6): CAT activity was determined by the spectrophotometer

following the decrease of absorbance of H_2O_2 within 30 s at 240 nm (Dhindsa *et al.* 1981). The 3 ml reaction solution consisted of 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 100 ml of enzyme extract. Addition of H_2O_2 started the reaction and the decrease in absorbance was recorded after 30 s.

Guaiacol peroxidase (GPX) activity (EC1.11.1.7): GPX activity was measured using guaiacol as a substrate. Reaction mixture (3 ml) contained 25 µl of enzyme extract, 2.77 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of 1% H_2O_2 (V/V) and 0.1 ml of 4% guaiacol (V/V). The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 3 min. One unit of enzyme activity was defined as the amount that causes a change of 0.01 in absorbance per minute (Zhang *et al.* 2005).

Statistical analysis: Computations and statistical analyses were done using SPSS (ver. 14). The data means were compared using Duncan's multiple ranges test ($p \leq 0.05$).

Results

Based on the analyses of variance, the effect of water stress, ASA and their interaction on relative water content, proline concentration, CAT and GPX enzymes activity, yield and yield components (fertile spike per m^2, kernel per spike and 1000 kernel weight) and biomass were significant (Table 1). Mean comparisons showed that withholding irrigation caused a significant reduction in RWC at the flowering stage (Figure 1). Pretreatment with ASA markedly alleviated the effects of water stress and also ameliorated RWC in both conditions (normal irrigation and water stress) significantly.

Water stress increased (55%) the electrolyte leakage from leaf cells (Figure 2). Under water stress condition, the application of ASA caused a reduction of 40% in electrolyte leakage compared to non-primed plants (Figure 2). As shown in Figure 2, the highest concentration of ASA (1 mM) was more effective under the stress condition.

The effect of water stress on proline content of barley was shown in Figure 3. On the basis of these results, water stress caused an increase of 60% in proline content. ASA had a significant effect on proline content under stress condition. When plants pretreated with ASA, the amount of proline was increased approximately 25% compared to the control (Figure 3). Also the highest concentration of ASA was more effective. The application of ASA had no effect on proline content under non-stress condition (Figure 3).

The effect of water stress on CAT and GPX activity in barley plant leaves, either with or without ASA pretreatment, was assayed. As it is shown in Figures 4 and 5, the enzymes activity of CAT and GPX were higher in the stressed plants. Pretreatment of plants with 0.5 and 1 mM ASA increased the activity of CAT and GPX, in those plants which were under water stress. This may be related to the key role of these enzymes in ROS detoxification under these conditions.

Results showed that water stress caused a significant reduction in fertile spike per m^2, grain per spike, 1000 grain yield, grain yield and

biomass compared to the control treatment (Tables 1 and 2). All the concentrations of ASA caused an increase in yield and yield components of barley especially under stress condition, however, the highest concentration of ASA was more effective (Table 2). Application of ASA had no significant effect on grain yield and biomass under normal condition; but under water stress condition ASA caused an increasing of 14% and 5% in these traits at the flowering stage, respectively (Table 2).

Based on our results, the concentration of 1 mM ASA caused an increase of 3% and 5% in fertile spikes per m^2, 5% and 20% in grain per spikes and 5% and 15% in 1000 grain weight under control and water stress conditions, respectively (Table 2).

Discussion

Water deficit is one of the most important environmental factors that regulate plant growth and development, and limit plant production. Plants can respond and adapt to water stress by alerting their cellular metabolism and invoking various defense mechanisms. Survival under this stressful condition depends on the plants' ability to perceive the stimulus, generate and transmit the signal and initiate various physiological and biochemical changes. Understanding plant responses to water stress has great importance and is also a fundamental part for making the crops tolerant to this stress factor (Bohner and Jensen 1996). 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 ASA was used as an important signal molecule for modulating plant responses to water stress that participates in the regulation of physiological processes, to study the role of this hormone in some physiological characters under this stress. The results showed that water stress decreased RWC (Figure 1). Similar results obtained in barley (Kocheva *et al.* 2005), wheat (Lei *et al.* 2007) and rice (Hsu and Kao 2003) under drought stress. Lower water uptake by roots may have resulted in the decrease of RWC. The decrease in water potential gradient between roots and their surrounding media adversely affects RWC. The application of ASA under water stress condition was more effective than the normal irrigation. These results were in agreement with the findings of Singh and Usha (2003). The increase in RWC may be related to the role of ASA in accumulation of compatible osmolytes in plants, which were subjected to drought stress (Singh and Usha 2003).

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 (Halliwel and Gutteridge 1984). This is a consequence of an oxidative burst leading to lipid peroxidation, membrane permeability and cell injury (Scandalius 1993). As shown in Figure 2, electrolyte leakage increased in plants that were subjected to water stress. When plants were pretreated with ASA, electrolyte leakage decreased in the water stress condition, and this effect is very important for water stress tolerance. Maintaining the integrity of cellular membranes under stress conditions is considered as an integral part of salinity and drought tolerance mechanisms (Shakirova *et al.* 2003; Agarwal *et al.* 2005;

Stevens *et al.* 2006). Several studies showed that electrolyte leakage in the susceptible plants were more than resistant plants (Juan *et al.* 2005; Wang *et al.* 2009; Kabiri *et al.* 2014). In previous studies, pretreatment with SA evidenced by a reduction in the level of lipid peroxidation and leakage of electrolytes from plant tissues as well as by more intensive growth processes as compared to the control plants (Hayat and Ahmad 2007).

Under normal conditions, the total amount of ROS formed in the plants is determined by the balance between the multiple ROS producing pathways and the ability of the enzymatic and non-enzymatic mechanisms to deal with them. Under stress conditions, ROS formation is higher than the ability of plants to remove it, and this could result in oxidative damages (Laspina *et al.* 2005). Proline 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). Proline is known as non-enzymatic antioxidant for scavenging of ROS (Shi *et al.* 2007). Pireivatlou *et al.* (2010) demonstrated that proline content was accumulated in wheat cultivars under drought stress. The SA greatly improves the dehydration tolerance through the increment of proline content.

In barley plants that are under water stress, CAT and GPX activities were increased when compared with the control plants (Figure 3 and 4). Therefore, we can assume that the plant antioxidant machinery was effectively struggling against stressful condition. Relatively higher activities of ROS-scavenging enzymes have been reported in tolerant genotypes when compared to susceptible ones, suggesting that the antioxidant system plays an important role in plant tolerance against environmental stresses (Shi *et al.* 2007). In many studies, it was found that the function of ASA alleviation of oxidative stress was attributed to the induction of various ROS-scavenging enzyme activities (Clark *et al.* 2002; Agarwal *et al.* 2005). This effect was observed in the high concentration of ASA (1 mM).

In fact, water stress decreased grain yield of barley remarkably via the reduction of yield components. Researches have declared that the reduction of grain yield is related to the decline of photosynthesis under drought stress (Uhart and Andrade 1995). Zarea-Feizabady and Ghodsi (2004) and Plaut *et al.* (2004) illustrated that under drought stress condition, the yield of wheat decreased due to the reduction of spikes per surface unit and grains per spikes. Also Abbate *et al.* (1998) reported that there was a significant correlation between spikes per surface unit and total yield. The reduction of 1000 grain weight was due to the restriction of remobilization in treatments under water stress condition. In addition, water stress reduced assimilates allocation through disturbance in absorption and translocation of photosynthetic substances; then finally caused the changes in yield components and decreased grain yield (Zarea-Feizabady and Ghodsi 2004).

Seed priming of wheat at the concentration of 0.5 mM SA (Senaratna *et al.* 2000), alfalfa at 0.01 mM SA (Drazic *et al.* 2006) and barley at 1 mM SA (El-Tayeb 2005) have resulted in the increase of plant growth through the enhancement of cell

Table 1. Mean squares of relative water content (RWC), electrolyte leakage (EL), catalase activity (CAT), guaiacol peroxidase activity (GPX), proline, yield and yield components and biomass of barley under different irrigation and ASA treatments.

SOV	df	RWC	EL	Proline	CAT activity	GPX activity	Fertile spike/m^2	Grain per spike	1000 grain weight	Grain yield	Biomass
Replication	3	87.56^{ns}	29.6^{ns}	53.24^{ns}	0.00088^{ns}	0.129^{ns}	752.2^{ns}	21.35^{ns}	33.2^{ns}	1012.67^{ns}	1356.58^{ns}
Irrigation (a)	1	1185.24**	198.57**	8814.25**	6.196**	538.59**	1690.23*	327.28**	542.83**	63541.5**	1058963.5**
E (a)	3	12.36	45.22	56.24	0.00014	0.0682	235.21	3.24	7.5	1103.25	15462.3
ASA (b)	2	988.35**	189.5*	202.23*	0.068**	6.442**	1168.23*	101.2**	55.58*	1006.2**	13681.9*
a*b	2	896.25*	205.34**	253.67*	0.0295**	7.147**	1145.23*	98.95*	637.52**	876.6*	200873.25**
E (b)	12	7.38	21.42	34.23	0.000356	0.051	86.2	0.86	3.5	232.3	1057.1

**, * and ^{ns} denote significant differences at 0.01 and 0.05 probability levels and not significant, respectively.

Table 2. The effect of ASA pretreatment on yield and yield components of barley under normal and stress conditions.

Water stress	ASA (mM)	Grain per spike	Fertile spike/m^2	1000 grain weight (g)	Grain yield (gr/m^2)	Biomass (gr/m^2)
Normal	0	45.13ab	521.25b	41.23b	816.21a	1798.24a
	0.5	47.25a	530.54a	41.42b	820.25a	1812.21a
	1	47.28a	532.17a	44.68a	825.86a	1815.53a
Irrigation withhold	0	32.37c	491.75e	30.24d	352.15c	1102.27c
	0.5	39.56b	503.23d	33.56cd	390.52bc	1132.25b
	1	41.35b	521.87bc	36.26c	408.23b	1140.86b

In each column means with different letters are significantly different based on Duncan's Multiple Range Test ($p \leq 0.05$)

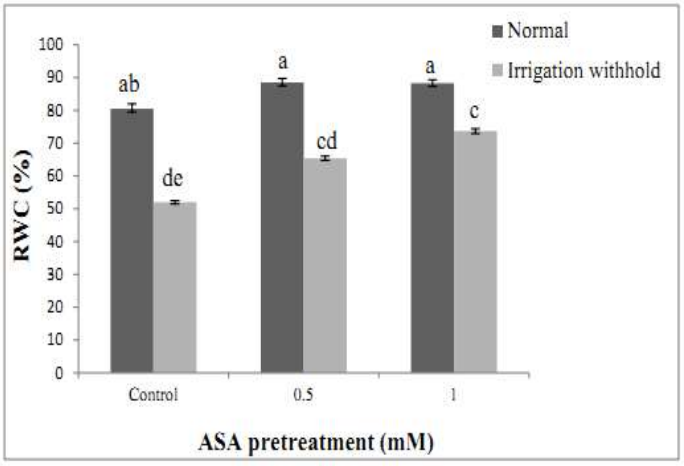

Figure 1. Effect of acetylsalicylic acid pretreatment on relative water content (RWC) of barley flag leaf under normal and water stress conditions (Means with different letters are significantly different based on Duncan's Multiple Range Test; p≤0.05).

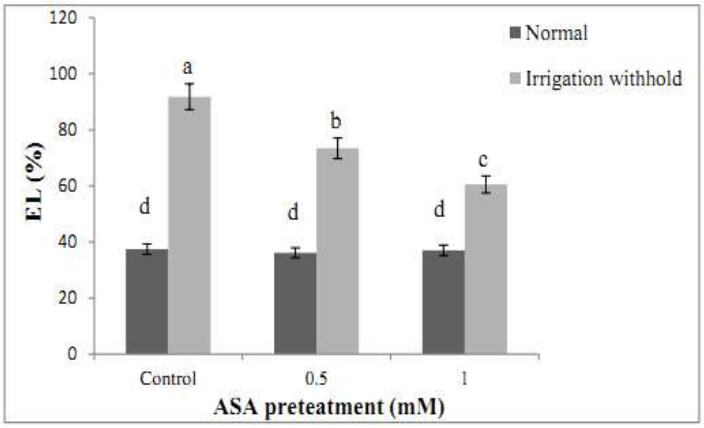

Figure 2. Effect of acetylsalicylic acid pretreatment on electrolyte leakage of barley flag leaf under normal and water stress conditions (Means with different letters are significantly different based on Duncan's Multiple Range Test; p≤0.05).

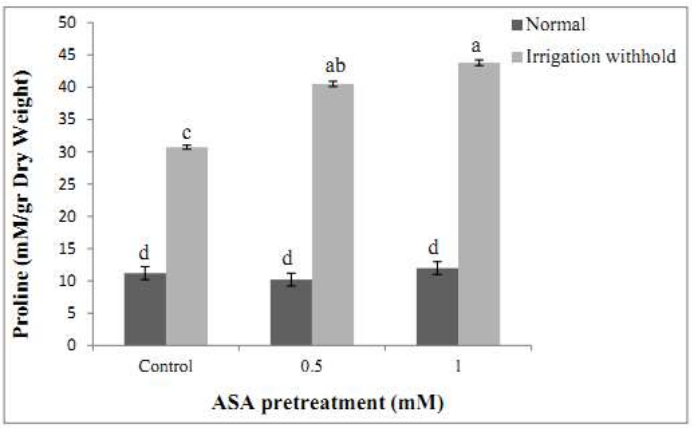

Figure 3. Effect of acetylsalicylic acid pretreatment on proline content of barley flag leaf under normal and water stress conditions (Means with different letters are significantly different based on Duncan's Multiple Range Test; p≤0.05).

division and elongation of root cells. Improvement of growth and yield may be related to the effect of SA on translocation of photosynthetic substances toward sink. Phenol compounds such as SA affect growth via the prevention of auxin oxidation (Fariduddin *et al.* 2003).

Conclusion

Water deficit had remarkably negative effect on yield, yield components and also relative water content of flag leaf. On the other hand, water stress increased proline content and electrolyte leakage. This is because the scarcity of water affected the cellular membrane structure and caused an increasing in membrane permeability to ions and macromolecules. ASA ameliorated the negative effects of water stress via the reduction of electrolyte leakage and increasing of proline content. Consequently ASA increased grain yield of barley under water stress condition. Therefore, the application of exogenous protection compounds (such as acetylsalicylic acid) can be a method to decrease water stress damages to plants.

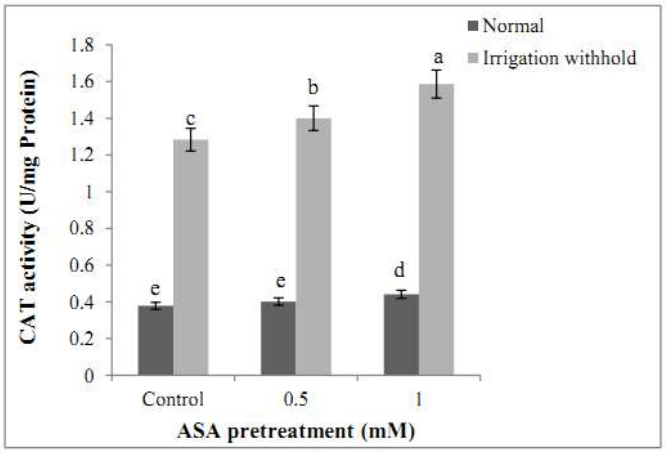

Figure 4. Effect of acetylsalicylic acid pretreatment on catalase activity of barley flag leaf under normal and water stress conditions (Means with different letters are significantly different based on Duncan's Multiple Range Test; p≤0.05).

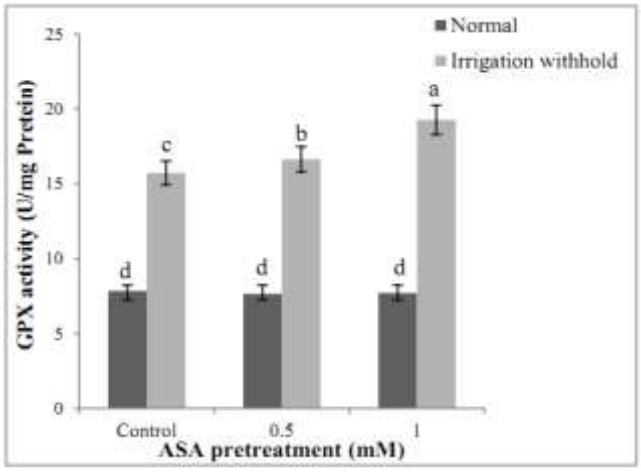

Figure 5. Effect of acetylsalicylic acid pretreatment on guaiacol peroxidase activity of barley flag leaf under normal and water stress conditions (Means with different letters are significantly different based on Duncan's Multiple Range Test; p≤0.05).

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