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EFFECT OF ASCORBIC ACID APPLICATION ON PHYSIOLOGY OF WHEAT UNDER DROUGHT STRESS

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Drought is one of the most devastating environmental stresses which adversely affect a multitude of plant metabolic processes. Grain crops including wheat are badly affected by drought. It has been reported that ascorbic acid is a powerful antioxidant which protects plants from the oxidative damage caused by the harmful reactive oxygen species produced under drought stress. Two wheat (*Triticum aestivum* L.) genotypes, a drought resistant variety Chakwal-86 and a drought sensitive genotype 6544-6 were grown in a hydroponics culture. Drought was developed by using PEG_{8000}. Overall, ascorbic acid application as seed priming, as foliar spray and by rooting medium helped the wheat seedlings under drought to overcome adverse effects of oxidative stress by maintaining growth, relative water content, cell membrane stability, osmotic adjustment through proline accumulation and by enhanced activity of antioxidant enzymes, however, rooting medium treatment proved to be the most effective mode of application.

Keywords: Ascorbic acid, vitamin C, wheat, antioxidants, relative water content, cell membrane stability, proline, glycine betaine,

INTRODUCTION

Wheat is a leading crop among all the cereal crops of the world, being the third most produced cereal after maize and rice. It is widely adapted to different soil and climatic conditions so is grown in almost all parts of the world. In order to fulfill the increasing food demand of the growing population, wheat production must increase at an annual rate of 2% (Gill *et al.*, 2004). However, biotic and abiotic stresses are the main hurdles against its increase in production. Drought is a major abiotic stress which affects wheat production to a great extent (Shao *et al.,* 2009). Drought or water deficit is defined as the absence of adequate moisture necessary for normal growth.

Relative water content (RWC) of well hydrated tissues is usually between 85-95% (Pardo, 2010) which varies in different species. Severe loss of water causes cell membranes to dry up, become porous and lose their proper functioning (Levitt, 1980). Soil water potential decreases under water deficit conditions, so plant reduces its osmotic potential (OP) in order to absorb water and maintain turgor. This is achieved through accumulation of a variety of inorganic and organic osmotica including compatible solutes such as proline and glycine betaine etc and is referred to as osmotic adjustment (Turner, 1979).

According to Foyer and Noctor (2000), drought stress causes an imbalance in light capturing and its utilization during photosynthesis. The uncaptured or excess energy is harmful for photosystem II because of over-reduction of the reaction centre (Deming and Adams, 1992) leading to dissipation of free radicals of reduced oxygen in the chloroplasts (Smirnoff, 1993). These free radicals are commonly called as 'reactive oxygen species' (ROS) or 'active oxygen species' (AOS). The injury caused by these ROS such as hydrogen peroxide (H_2O_2) is known as oxidative stress and is one of the major damage to plants exposed to stresses such as drought (Tartoura, 2010). Various antioxidants such as SOD (Super oxide dismutase), POD (Peroxidase), CAT (Catalase) etc. are produced in response to the ROS to protect plants against oxidative damage (Ashraf, 2009). The activity of antioxidants generally increases under stress conditions (Leprince *et al.*, 1994). The elevated level and high activity of antioxidants provides resistance to plants against stress (Casano *et al.*, 2001). A non enzymatic antioxidant, Vitamin C (L-ascorbic acid) is a ubiquitious molecule in eukaryotes. It is a small water-soluble vitamin like C_6 sugars. Being a powerful antioxidant, it scavenges and controls the concentration of H_2O_2 in plants (Sairam *et al.*, 1998) with the help of an enzyme ascorbate peroxidase (APX). This enzyme transfers electrons from ascorbate to H_2O_2 producing dehydroascorbate and water as products (Raven, 2000). Scientists have been testing the role of ascorbic acid application in protecting various crop species under drought stress (Amin *et al.*, 2009; Dolatabadian *et al.*, 2010; Hussein and Khursheed, 2014). In the present study we determined the effect of exogenous application of ascorbic acid as seed priming, foliar spray and in the rooting medium to wheat grown under drought stress The objective of the study was to

assess its effectiveness in helping plants maintain growth and development under drought stress.

MATERIALS AND METHODS

The experiment was carried out under hydroponics culture in the wire-house of the Department of Botany, University of Agriculture Faisalabad during the winter 2010-2011. Seeds of the drought tolerant wheat cultivar, Chakwal-86 and a drought sensitive genotype, 6544-6 were obtained from the Department of Plant Breeding and Genetics, University of Agriculture Faisalabad. A completely randomized design with three replicates was employed.

Seeds of the cultivar/genotype were sown in Petri plates kept in a growth chamber. Seven days old germinated seedlings were transferred into plastic tubs each containing 4 liter of Hoagland's nutrient solution. There were 24 tubs each having 20 seedlings, 10 of each genotype. After transplantation, the seedlings were allowed to establish in the nutrient solution for one week. Drought stress was developed in the medium by dissolving 20 % PEG_{8000} in the Hoagland's nutrient solution in three equal doses with an interval of one day until the final concentration of 20%. This optimum level of PEG at which plants suffered stress but did not show permanent wilting was found in an initial experiment (results not shown). A week after imposing drought stress, optimized levels of ascorbic acid for each mode of application (seed priming, foliar spray and in the rooting medium) determined in an experiment (results not shown) were applied in the following manner:

Out of the 24 tubs, 3 were with Hoagland's nutrient solution only, 3 with PEG dissolved in Hoagland's nutrient solution, 3 with 0.5 mM ascorbic acid dissolved in Hoagland's nutrient solution, 3 with PEG and 0.5 mM ascorbic acid dissolved in Hoagland's nutrient solution, 3 with 1 mM ascorbic acid applied as a foliar spray to the seedlings, 3 with PEG and 1 mM ascorbic acid applied as a foliar spray to the seedlings, 3 with seedlings from seed primed with 1mM ascorbic acid, 3 with PEG and seedlings from seed primed with 1 mM ascorbic acid. For priming treatment, seeds were soaked for 10 h in the ascorbic acid solution. For control, seeds were soaked in distilled water. For foliar spray, equal volume of ascorbic acid solution was sprayed twice (with an interval of one week) to each set of seedlings. Fresh Hoagland's nutrient solution was replaced every week and the solution was kept well aerated by using an electric pump. Data for the following parameters were recorded five weeks after transplantation of the seedlings (Three samples from each tub were used for analysis of all parameters):

The samples were harvested and oven-dried at 65°C for 72 h to record dry biomass.

Relative water content (RWC) was determined using the following equation.

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

Where, FW= Fresh weight, DW= Dry weight, TW= Turgid weight

Cell membrane stability (CMS) was calculated as reciprocal of relative cell injury (Blum and Ebercon 1981) using the following formula:

$$CMS\ \% = [\{1-T_1/T_2\}/\{1-C_1/C_2\}]\times 100$$

Where, T1= Initial EC (electrical conductivity) of stress sample, T2= Final EC of stress sample, C1= Initial EC of control sample, C2= Final EC of control sample

Leaves were frozen in a -80^0 freezer for the determination of osmotic potential, antioxidant enzymes, ascorbic acid, proline and glycine betaine. Osmotic potential of the cell sap was measured using an osmometer (Wescor 5500). Total soluble protein content was determined using the method of Bradford (1976). The activity of SOD was determined using the method of Giannopolitis and Ries (1977) with some minor modifications. The activities of CAT and POD enzymes were determined according to Chance and Maehly (1955) with some modifications. The activity of APX was determined following Asada and Takahashi (1987) with some minor modifications. Ascorbic acid content of frozen leaf material was determined according to Mukherjee and Choudhuri (1983) by grinding the leaves in 6% trichloro acetic acid.

The level of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) concentration was determined according to Yagi (1982). The absorption coefficient was calculated at 155 mmol cm^{-1} and expressed as nmol/MDA/g fresh weight.

$$MDA\ level\ (nmol) = (A532nm-A600nm)/1.56 \times 10^5$$

Where MDA = malondialdehyde content

Hydrogen peroxide (H_2O_2) content was determined according to Velikova *et al*., (2000). Glycine betaine content was determined according to Grieve and Gratan (1983) while the proline content was determined according to Bates *et al*., (1973) using a standard curve and the following equation:

µmole proline/g fresh weight

= (µg proline/ml x ml of toluene/ 115.5)/ g of sample

The data obtained were subjected to analysis of variance using COSTAT software. LSD was calculated to see the differences among the means (Steel *et al*., 1997).

RESULTS AND DISCUSSION

Drought stress caused a significant reduction in shoot and root dry weight, RWC, OP and CMS, however the reduction was relatively lower in the drought resistant variety. A significant increase in O.P, H_2O_2, MDA, total soluble protein content, G.B, proline and activity of SOD, POD, CAT and APX was observed under drought with relatively higher effects on the drought resistant variety. The analyses of variance of the parameters studied in the experiment are given in Table 1 and effect of drought and ascorbic acid

(AsA) application are presented in Figs 1-5. Generally the effect of all different modes of AsA application used in the present study improved drought resistance of wheat seedlings, however application of ascorbic acid in the root growth medium (nutrient solution) caused relatively higher effects.

Drought stress is known to suppress plant growth in terms of lower plant fresh and dry biomass production. In the present investigation, exogenous application of AsA application enhanced growth of wheat seedlings under drought conditions which was observed in terms of increased shoot and root dry weight. Increase in plant biomass by seed priming with ascorbic acid was found effective by Razaji *et al*. (2014). However in the present study rooting medium application was the most effective mode compared to seed priming and foliar spray (Fig. 1). Higher growth in ascorbic acid treated plants under drought stress may be attributed to increase in cell division and cell expansion (Pignococchi and Foyer, 2003).

Maintenance of high RWC under limited water is considered a resistant mechanism against drought stress (Ritchie *et al*., 1990)). In the present study, RWC of both wheat genotypes decreased under drought conditions. Chakwal-86 showed relatively lesser reduction in RWC under drought stress.

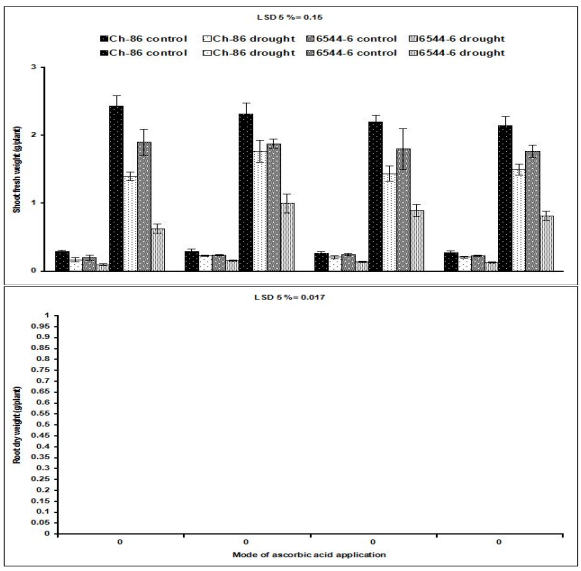

Figure 1. Comparison of shoot and root dry biomass of drought stressed and non-stressed 6 week old plants of two wheat genotypes, Chakwal-86 (Ch-86) and 6544-6 with ascorbic acid application in different modes i.e 0..5 mM in the rooting medium, 1mM as foliar spray and 1mM as priming or soaking treatment (mean+S.E.).

Table 1. Mean squares from analyses of variance of the data for shoot and root dry biomass, cell membrane stability (CMS), leaf osmotic potential (OP), relative water content (RWC), leaf total soluble protein content and activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes, ascorbic acid, glycine betaine (GB), proline content, H_2O_2, and MDA contents of leaves of drought stressed and non-stressed 6 week old plants of two wheat genotypes (Chakwal-86 and 6544-5) with ascorbic acid application in different modes.

Source	df	Shoot dry wt	Root dry wt	CMS	OP	RWC	TSP	APX	SOD
Main effects									
Genotype (G)	1	0.0100**	0.03000*	1695.10**	0.54**	15.88ns	0.16**	1.31**	75.61**
Drought (D)	1	0.0400**	0.00200*	11726.65**	1.90**	1391.20*	0.11 **	0.48 **	975.70 **
AsA	3	0.0001ns	0.00100ns	326.40*	0.14**	184.50ns	0.03**	0.11 **	120.37**
Interaction									
G × D	1	0.0021ns	0.00060ns	305.02ns	0.010ns	152.66ns	0.01ns	0.15*	2.02ns
G × AsA	1	0.0007ns	0.00009ns	360.64*	0.004ns	67.56ns	0.02*	0.12**	1.22ns
D × AsA	3	0.0100**	0.00030ns	636.54**	0.057ns	102.12ns	0.02*	0.29**	120.2**
G × D × AsA	3	0.0020ns	0.00020ns	71.35ns	0.032ns	64.67ns	0.004ns	0.064*	17.81

Source	df	CAT	POD	AsA	GB	Proline	H_2O_2	MDA
Main effects								
Genotype	1	22.500**	1594.60**	121.94**	979.15**	17970.30**	0.08ns	3.80 ns
Drought	1	0.064ns	161.95 **	99.67**	4661.17**	111059.2**	0.70**	120.46**
AsA	3	2.640ns	209.42**	93.76**	576.61*	934.32ns	0.26**	11.45**
Interaction								
G × D	1	13.37*	0.71ns	0.99ns	103.99ns	4739.63**	0.002ns	0.645ns
G × AsA	1	2.74ns	72.56*	17.01*	147.45*	441.71ns	0.03ns	2.813ns
D × AsA	3	7.02*	11.74ns	50.71**	404.08**	999.63ns	0.21**	15.88**
G × D × AsA	3	4.52ns	20.59ns	17.08*	129.43*	119.58ns	0.11**	0.93ns

*, **= significant at 0.05 and 0.01 levels, respectively ns= non-significant

This may have been due to maintenance of lower osmotic potential through osmotic adjustment. Ascorbic acid application slightly improved RWC of both genotypes (Fig. 2).

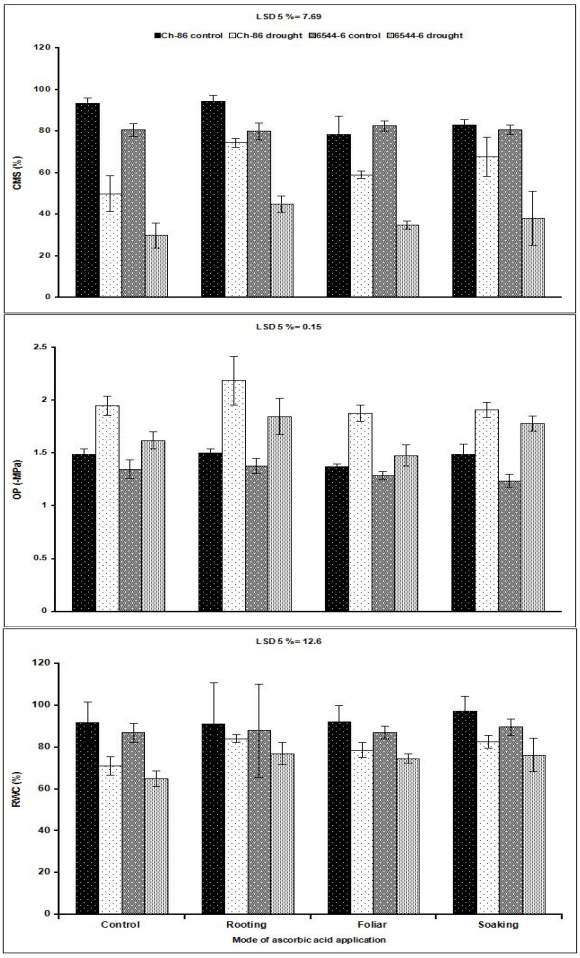

Figure 2. Comparison of cell membrane stability (CMS), leaf osmotic potential (OP) and relative water content (RWC) of drought stressed and non-stressed 6 week old plants of two wheat genotypes, Chakwal-86 (Ch-86) and 6544-6 with ascorbic acid application in different modes i.e 0..5 mM in the rooting medium, 1mM as foliar spray and 1mM as priming or soaking treatment (mean + S.E.). Stars show significant effect of ascorbic acid.

The osmotic potential (OP) of both wheat genotypes in the present study decreased considerably under drought stress. Chakwal-86 showed relatively lower OP compared to that of genotype 6544-6. Application of ascorbic acid further decreased OP of the drought stressed plants of both genotypes (Fig. 2). Drought reduced osmotic potential of wheat leaves, shows that the leaves maintained turgor through osmotic adjustment. Osmotic adjustment or active lowering of cell osmotic potential under drought has a positive correlation with plant productivity under water deficit (Ludlow and Muchow, 1990) because decrease in OP improves water uptake of cells by increasing their solute concentration thereby increasing turgor, stomatal conductance and hence net photosynthesis (Gupta and Berkowitz, 1987).

Cell membrane stability (reciprocal of cell membrane injury) is considered as measure of tolerance to stress (Blum and Ebercon, 1981) In the present study, cultivar Chakwal-86 maintained higher CMS under drought stress which reflects its higher drought tolerance compared to the genotype 6544-6. Application of ascorbic acid in the present study increased CMS of both wheat genotypes subjected to drought especially when applied in the rooting medium (Fig. 2). On application of ascorbic acid, the cultivar chakwal-86 showed higher CMS than that of the genotype 6544-6. Maintenance of CMS under drought stress by application of ascorbic acid may have been due to its antioxidant property which prevented membrane damage by oxidants.

Drought stress resulted in a marked increase in H_2O_2 levels in both wheat genotypes (Fig. 3).

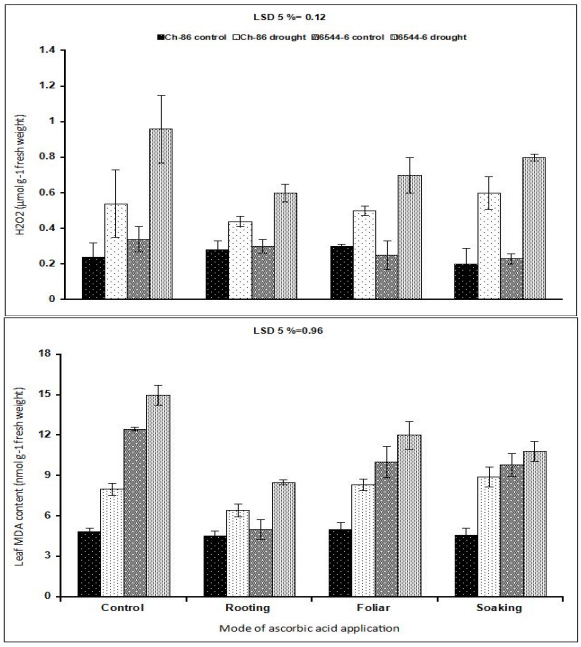

Figure 3. Comparison of H_2O_2 and leaf MDA contents of drought stressed and non-stressed 6 week old plants of two wheat genotypes, Chakwal-86 (Ch-86) and 6544-6 with ascorbic acid application in different modes i.e 0.5 mM in the rooting medium, 1mM as foliar spray and 1mM as priming or soaking treatment (mean + S.E.). Stars show significant effect of ascorbic acid.

The H_2O_2 level has been reported to increase under drought due to glycolate oxidase reaction of photorespiration in peroxisomes (Corpas *et al*., 2001). In the present study, drought-induced increase in the levels of H_2O_2 was higher in genotype 6544-6 than that of cultivar Chakwal-86. Drought tolerant wheat cultivars showed lower contents of H_2O_2 in an earlier study as well (Khanna-Chopra and Selote, 2007; He *et al*., 2011). Moussa and Abdel-Aziz (2008) also reported an increase in H_2O_2 content in maize under drought stress. However, the amount of H_2O_2 produced in drought tolerant cultivars was less compared to the drought sensitive ones. The reactive oxygen species (ROS) such as H_2O_2 are very harmful for plant membranes primarily due to their ability to initiate a variety of oxidative chain reactions on unsaturated fatty acids (Smirnoff, 2000; Mittler, 2002). In the present study, ascorbic acid application helped both the wheat genotypes to tolerate stress by reducing their H_2O_2 concentration when subjected to drought. This could be due to the antioxidant properties of ascorbic acid and its H_2O_2 scavenging ability.

Drought induced production of oxidants causes formation of MDA which is a byproduct of oxidative membrane damage (Moller *et al*., 2007). It is produced due to lipid peroxidation of membranes caused by the ROS such as H_2O_2 (Sairam *et al*., 1998; Borsani *et al*., 2001) and can be used as an indicator to assess the amount of oxidative damage (Zhang *et al*., 2007). In the present study, drought stress caused a significant increase in the leaf MDA content in both wheat genotypes (Fig. 3). Shao *et al*., (2009) also found high MDA content in drought stressed wheat. This increase in MDA under drought has also been observed in other plant species (Moran *et al*., 1994; Guha *et al*., 2010). In the present study, application of ascorbic acid ameliorated the adverse effects of drought by reducing the MDA content.

In the present study, drought stress significantly enhanced the total soluble protein and activity of the enzymatic antioxidants, APX (ascorbate peroxidase), POD (peroxidase), CAT (catalase) and SOD (superoxide dismutase) in leaves of both genotypes (Fig. 4). Earlier studies also report that drought stress significantly increased the activity of SOD and APX in the leaves of maize plants (Moussa and Abdel-Aziz, 2008). APX uses ascorbate as an electron donor in the first step of the ascorbate–glutathione cycle and is considered the most important plant peroxidase in H_2O_2 detoxification (Noctor and Foyer, 1998). Both catalase and the ascorbate-glutathione cycle are very important in H_2O_2 scavenging. In the present study, Chakwal-86 showed higher antioxidant activity compared to that of genotype 6544-6. Drought resistant genotypes of wheat in earlier studies also showed higher activity of SOD, POD, CAT and APX under drought stress compared to those of the sensitive ones (Khanna-Chopra and Selote, 2007). Ascorbic acid application improved the activity of these enzymes.

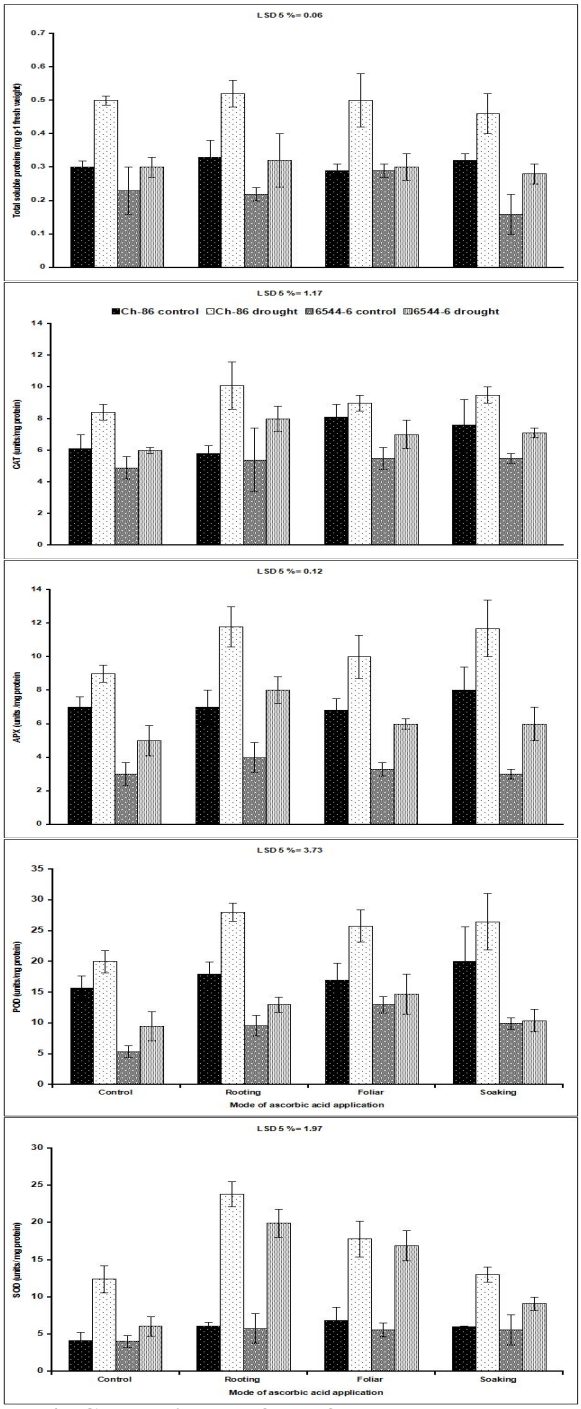

Figure 4. Comparison of leaf total soluble protein content and activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes in drought stressed and non-stressed 6 week-old plants of two wheat genotypes, Chakwal-86 (Ch-86) and 6544-6 with ascorbic acid application in different modes (mean + S.E.). Stars show significant effect of ascorbic acid.

Exogenous application of ascorbic acid is reported to be effective in mitigating the adverse effects of drought and salinity in various crop species by enhancing the activities of the enzymatic antioxidants (Athar *et al.*, 2009; Hassanein *et al.*, 2009; Dolatabadian *et al.*, 2010).

It has been observed that drought resistant species contain comparatively much higher levels of endogenous ascorbic acid. Marton *et al.*, (2010) reported that wheat seedlings contain 2.6 to 3 mg/100 g ascorbic acid while sorghum (*Sorghum bicolor*) being a relatively drought resistant species contains 15.3 mg/100 g and its concentration increases with plant growth. In the present study, drought stress decreased the endogenous ascorbic acid content of leaves of both genotypes (Fig. 5) however; the drought tolerant cultivar Chakwal-86 had higher ascorbic acid compared to that of genotype 6544-6 under drought stress. Earlier studies report decrease in ascorbic acid content under drought in other plant species as well (Sgherri and Navarri-Izzo, 1995; Zhang and Kirkham, 1996; Hong-Bo *et al*., 2005; Khanna-Chopra and Selote, 2007; Guha *et al.*, 2010). The drought induced reduction in ascorbic acid occurs because ascorbate acts a substrate for APX and is used up during the removal of H_2O_2 which was evident in the present study as a decrease in endogenous ascorbic acid content was parallel with an increase in activity of the APX enzyme. Khanna-Chopra and Selote (2007) also observed a significant decline in ascorbic acid content in wheat however, the leaves of drought acclimated plants exhibited an increase in ascorbic acid in subsequent drought stress. Foyer and Harbinson, (1994) reported natural variation in endogenous ascorbic acid content in wheat. The higher ascorbic acid content of a wheat cultivar BCH was found to be correlated with lower oxidative damage (Bartoli *et al*., 2004). It was also observed that the higher ascorbic acid content of the cultivar BCH resulted in a significant antioxidant protection to mitochondrial and peroxisomal proteins. Similarly, another wheat cultivar C306 with higher ascorbic acid content showed superior drought tolerance against oxidative damage compared to other cultivars (Sairam *et al.*, 1998).

In the present study, leaf free proline concentration of both wheat genotypes increased under drought stress (Fig. 5). These findings are in agreement with some earlier studies in which enhanced accumulation of proline under drought was observed in different plant species (Nayyer and Walia, 2003; Choudhry *et al*., 2005; Manivannan *et al.*, 2008; Anjum *et al*., 2011). Proline is one of the most important osmoprotectants produced in plants in response to stress. It can stabilize the sub-cellular structures like proteins and membranes and is also reported to scavenge free radicals (Ashraf and Foolad, 2007). In the present study cultivar Chakwal-86 showed higher leaf proline accumulation compared to genotype 6544-6. Chandrasekar *et al.*, (2000) also reported that proline concentration was higher in stress tolerant compared to susceptible cultivars of wheat. Similar results were observed in other plant species (Aziz *et al.,* 2000; Choudhary *et al.,* 2005). In the present study, ascorbic acid application in the rooting medium did not significantly increase the proline concentration of both genotypes subjected to drought stress. Amin *et al.*, (2009) found increased proline concentration in okra plants under drought stress by application of ascorbic acid.

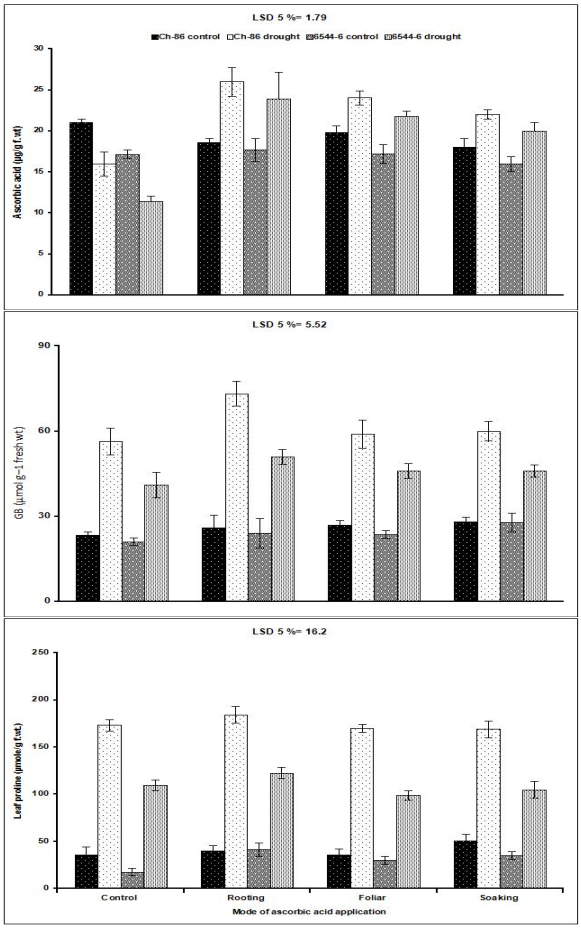

Figure 5. Comparison of ascorbic acid, glycine betaine (GB) and proline content of leaves of drought stressed and non-stressed 6 week old plants of two wheat genotypes, Chakwal-86 (Ch-86) and 6544-6 with ascorbic acid application in different modes i.e 0..5 mM in the rooting medium, 1mM as foliar spray and 1mM as priming or soaking treatment (mean + S.E.). Stars show significant effect of ascorbic acid.

Glycine betaine is a quaternary ammonium compound, commonly synthesized in xerophytes or drought tolerant plants and plays an important role in osmotic adjustment (Sakamoto and Murata, 2002). In the present study, glycine betaine content of both wheat genotypes increased significantly under drought stress (Fig. 5). The drought

tolerant cultivar Chakwal-86 accumulated significantly higher glycine betaine compared to the genotype 6544-6. Ascorbic acid had a significant increasing effect on glycine betaine content in both genotypes under stress as well as non-stress conditions.

In the present study, ascorbic acid applied to wheat seedlings especially in the rooting medium ameliorated the adverse effects of drought stress by counteracting oxidative damage and hence improving cell membrane stability, osmotic adjustment and growth compared to the non-treated plants. The application of ascorbic acid in the rooting medium was more effective because of continuous supply of ascorbic acid. The effects due to seed priming treatment are perhaps reduced with the growth of plant as the compound absorbed in the seed is gradually used up. The foliar application may be relatively less effective because ascorbic acid applied through the leaves may not reach to the target sites in appropriate concentration effective for causing a prominent change in metabolic processes involved in growth and other plant attributes. Furthermore, ascorbic acid applied as a foliar spray does not ensure a continuous supply of the compound to the plants. This may be the reason for a higher level of ascorbic acid solution (1mM) required to produce effect for foliar spray compared to rooting medium (0.5 mM) in the present study. If a significant improvement in drought tolerance of wheat by the application of ascorbic acid is proved in further experiments under soil conditions, it may be applied on commercial scale on crops to manage drought conditions or plants may be genetically improved to enhance their potential to increase the endogenous levels of ascorbic acid.

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