Exogenously-applied 5-aminolevulinic acid modulates some key physiological characteristics and antioxidative defense system in spring wheat (*Triticum aestivum* L.) seedlings under water stress

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**A B S T R A C T**

Aminolevulinic acid, one of the key precursors involved in chlorophyll biosynthesis, was applied exogenously to assess its role in improving seedling drought stress tolerance in two wheat cultivars (Shafaq-2000 and Auqab-06). Varying levels (0, 50, 100 and 150 mg L⁻¹) of 5-ALA were foliarly applied to one month old seedlings. Data showed that plant growth (shoot and root fresh and dry weights), chlorophyll *a* and *b* contents, chlorophyll *a/b* ratios, and leaf and root *P* of seedlings of both wheat cultivars decreased considerably at both drought stress regimes i.e. 80% and 60% of field capacities. In contrast, water-use efficiency (WUE) calculated as *A/E* and proline contents accumulated considerably under drought conditions in both wheat cultivars. However, none of the two water regimes altered sub-stomatal CO₂ concentration (*Ci*), photosynthetic rate (*A*), stomatal conductance (*gₛ*), transpiration rate (*E*) and *Ci/Ca* ratio (*Ca*; ambient CO₂ concentration), glycine betaine (GB) contents, leaf and root *N*, *Ca*²⁺, and *K*⁺ concentrations, activities of enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and total soluble proteins in the seedlings of both wheat cultivars. 5-ALA enhanced shoot and root fresh and dry weights, chlorophyll pigments, GB contents, root *K*⁺, and *N* (leaf & root) in both wheat cultivars at the different water stress regimes. Of all ALA levels, 50 and 100 mg L⁻¹ were more effective in improving seedling growth. However, shoot and root fresh weights, shoot dry weights, chlorophyll *a* and *b* contents, *A*, *E*, *gₛ*, *Ci*, and *Ci/Ca*, and proline contents were not affected by ALA treatments. Of the wheat cultivars, cv. Shafaq-2000 showed significantly higher shoot fresh and dry weights, chlorophyll *b* contents, *gₛ*, and *Ci*, while cv. Uqab-06 was relatively better at proline accumulation, leaf and root and root *N* concentrations. Overall, foliar-applied 5-ALA improved growth, chlorophyll *a* and *b* contents, GB, root *K*⁺, leaf and root *N* contents in both wheat cultivars at different water stress regimes, while all other attributes were not affected significantly.

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1. Introduction

Water deficiency or drought stress is one of the significant environmental factors that considerably limits crop productivity in most parts of the world (Jaleel et al., 2007; Ashraf, 2010). Optimum quantity of water is required at all plant growth stages including seedling, vegetative and reproductive to counter osmotic effects due to drought-induced imbalances in uptake of inorganic nutrients (Na, Ca, K, N & P) and generation of a variety of reactive oxygen species (ROS) (Jabeen et al., 2008; Ashraf, 2009). Drought stress also causes considerable reductions in chlorophyll and carotenoid contents (Anjum et al., 2003b; Farooq et al., 2009) thereby affecting plant growth and development negatively. Almost all plants exhibit drought tolerance, but the extent varies among species and even among the cultivars/lines within the same species (Ashraf, 2010). However, if such variation is exploited judiciously to generate tolerant lines/cultivars of different crops of considerable commercial values or if meaningful technologies for growing plants under water deficit conditions are adopted, the crops can then survive well under a water limited environment (Jaleel et al., 2007; Nakayama et al., 2007; Jabeen et al., 2008; Sharma et al., in press).

Drought tolerance is a very complicated trait due to convoluted interactions between the stress factor and various molecular, and physiochemical mechanisms affecting plant development (Razmjoow et al., 2008). Water stress at elevated level results in the suppression of CO₂ assimilation and it interferes with the normal physiological functions of the plant, thereby causing poor growth or death of the plant (Jabeen et al., 2008; Sharma et al., in press). Drought stress causes more damage to plants during the early growth stages as it affects both the expansion and elongation of plant cells (Anjum et al., 2003a; Shao et al., 2008; Wu...
et al., 2008). This is because during drought stress the incomplete counteraction of ROS results in marked oxidative damage (Ashraf, 2009; Sharma et al., in press). The detoxification of ROS is correlated with high production of antioxidants both enzymatic such as superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR), catalase (CAT), ascorbate peroxidase (APX), and mono-dehydroascorbate reductase (MDAR) as well as non-enzymatic antioxidants such as carotenoids, anthocyanins, phenolics, glycinebetaine, proline, phenols, flavonoids and ascorbic acid (Ashraf, 2009).

The compound 5-ALA is an important biosynthetic precursor of all tetrapyrroles such as vitamin B12, billiens, heme, chlorophyll and other specialiized machinery in plants and animals (Rebeiz et al., 1984; Von Wettstein et al., 1995). Several researchers (Al-Khateeb et al., 2006; Zhang et al., 2006; Maruyama-Nakashita et al., 2010; Naeem et al., 2010) have suggested that this compound plays a significant role in regulating chlorophyll biosynthesis, antioxidant metabolism, ion uptake/accumulation, photosynthesis, carbon and nitrogen fixation, fruit formation and yield (Al-Khateeb et al., 2006; Zhang et al., 2006; Maruyama-Nakashita et al., 2010; Naeem et al., 2010).

Wheat (Triticum aestivum L.) is one of the crops that are severely affected by drought stress. Different plant growth regulators (PGRs) or their analogs are being used as foliar sprays in order to ameliorate the harmful effects of drought stress on different crops, e.g. number of researchers have recommended ALA as an effective agent to alleviate the effect of stressful environments on different crops, e.g. Hotta et al. (1998) in rice, Al-Khateeb (2006) in barley, Youssef and Awad (2008) in date palm and Zhang et al. (2008) in Brassica rapa. The present study was conducted to investigate if 5-ALA could be used to ameliorate the harmful effects of drought stress on two selected wheat cultivars. The parameters measured in the present study included chemical constituents such as inorganic nutrients and physiological ones such as gas exchange attributes.

2. Materials and methods

2.1. Location of research site

The experiment was conducted at the GC University Faisalabad, Pakistan during 2012. A versatile environmental growth chamber (Company; SANYO; model, MLR-251H) was used to grow the wheat seedlings under optimal conditions. The temperature was set at 25 °C (7.30 am – 6.30 pm) and 20 °C (6.30 pm to 7.30 am) and the light duration 11 h (7.30 am – 6.30 pm) and relative humidity 55% (7.30 pm to 6.30 am) and 65% (6.30 pm to 7.30 am).

2.2. Plant material and substrate

Seeds of wheat (T. aestivum L.) cultivars Shafaq-06 and Uqab-2000 were collected from the AARI (Ayub Agricultural Research Institute), Faisalabad, Pakistan. Each plastic pot contained 400 g of soil. The soil used for the experimentation was analyzed for different physico-chemical properties that are as follows: pH, 7.8; Ee, 2.8 ds m-1; and Na, K and Ca contents 14.0, 29.0 and 10 meq L-1, respectively. Per plastic pot, 10 seeds of each cultivar were sown initially. After one week of sowing, 100% germination was observed and seedlings were irrigated as per plant requirement for two weeks. Seedlings of uniform size were maintained up to 3 in each pot.

2.3. Drought stress treatment

Three drought stress levels i.e., control (normal watering), 80% (mild drought stress), and 60% (high drought stress) were applied on the basis of soil field capacity. After 14 days of drought stress treatments, five levels of ALA (no spray), water spray, 50, 100 and 150 mg L-1 were applied as a foliar spray to plants under drought and non-drought stress conditions. After 14 days of ALA spray, data were collected for.

2.4. Plant growth

Dry and fresh weights (roots and shoots) of the wheat seedlings were recorded individually after 28 days of drought treatment. Fresh shoots and roots of two plants per pot were measured and placed in an oven at 60 °C for 3 days and after which time their dry weights were measured.

2.5. Gas exchange characteristics

Photosynthetic rate (A), transpiration rate (E), stomatal conductance (g), sub-stomatal CO2 concentration (C), C/Ca ratio and water-use efficiency (calculated as A/E) were measured using a portable photosynthesis apparatus [IRGA: CI-301 PS; CID, INC, USA]. Measurement period was at 10.00 to 11.00 a.m.

2.6. Chlorophyll contents

Fresh leaf tissue (0.5 g) was triturated in 10 mL of acetone (80%) solution. The aliquots were kept overnight at 4 °C and then chlorophyll content was estimated spectrophotometrically by recording optical densities at 663 and 645 nm. Then chlorophylls a and b, and chlorophyll a/b ratio were determined following Arnon (1949).

2.7. Inorganic nutrients

The plant material (0.1 g) was digested in 10 mL Pyrex glass vials with 2 mL digestion mixture [14.0 g LiSO4 (BDH Chemicals Ltd. Poole England) + 0.42 g Se + 350 mL H2O2 (35% A.R. Grade extra pure)] and 0.5 mL perchloric acid (HClO4), kept on a hot plate enclosed in a fume hood. Temperature of the hot plate was maintained at 350 °C. The digestion of the material was considered complete when the material became colorless. Final volume of the extract was raised to 50 mL with distilled H2O and filtered. Then filtrate was used for analyzing potassium (K+), calcium (Ca2+), phosphorus (P) and nitrogen (N).

2.8. Leaf free proline content

Leaf samples (0.5 g) were triturated with 3% sulfosalicylic acid (MP, Biomedicals, Inc.) solution and proline concentration was determined at 520 nm following Bates et al. (1973).

2.9. Leaf glycinebetaine (GB) content

The dry leaf material (500 mg) was extracted in toluene (0.5%) and kept at 4 °C over-night. After centrifugation, filtrate (0.1 L) was mixed with 0.1 L 2 N H2O2. Then, 0.5 mL of this solution was taken and treated further following Grieve and Grattan (1983). The OD of the lower layer was measured at 365 nm spectrophotometrically.

2.10. Total soluble proteins

Fresh leaf (0.5 g) was extracted in 10 mL; 50 mM potassium phosphate buffer, centrifuged and protein contents of the extract were determined at 595 nm following Bradford (1976).

2.11. Activities of antioxidant enzymes

Fresh leaf tissues (500 mg) were ground in 10 mL phosphate buffer [pH 7.8; 50 mM] in a pestle and mortar. The extract was centrifuged at 15,000 × g for 15 min at 4 °C and the supernatant was separated in autoclaved Eppendorf tubes. This extract was used for analyzing activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) enzymes. The activity of SOD enzyme per unit was calculated on the basis of the amount of enzyme that inhibited 50% of NBT.
photoreduction (Giannopolitis and Ries, 1977). For POD and CAT determination, we followed the protocol advised by Chance and Maehly (1955). The activities of all these enzymes were calculated on the basis of total soluble proteins/sample.

2.12. Statistical analysis

Analysis of variance of the data was performed using the MSTAT computer package and values for error mean squares along with significance levels were determined (MSTAT Development Team, 2013).

3. Results

Shoot fresh weight (SFW) of seedlings of both wheat cultivars decreased considerably at both drought stress regimes i.e. 80% and 60% of field capacities (Fig. 1A). Foliar-applied varying levels of ALA had a non-significant effect on SFW at different water stress regimes. Cv. Shafaq-2000 was relatively better than cv. Uqab-06 under drought stress conditions.

Shoot dry weight (SDW) of wheat seedlings decreased significantly ($P \leq 0.001$) under drought stress. Foliar-applied varying levels of ALA did not have a significant effect on DW of wheat seedlings under varying

Fig. 1. A–H Shoot and root fresh and dry weights, chlorophyll contents ($a$ & $b$), chlorophyll $a/b$ ratio and stomatal conductance ($g_{s}$) of drought-stressed and non-stressed plants of two cultivars of wheat (Triticum aestivum L.) subjected to foliar-applied varying levels of 5-aminolevulinic acid (ALA) (Mean ± S.E.; $n = 3$). NS, no spray; FC, field capacity; Cvs, cultivars; D, drought; ns, non-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.
water regimes. Of both wheat cultivars, cv. Shafaq-2000 was relatively higher in SDW as compared to cv. Uqab-06 under drought stress conditions (Fig. 1B). Root fresh weight (RFW) of both wheat cultivars reduced significantly (P ≤ 0.001) under drought stress. No significant change was observed in RFW due to foliage spray of varying levels of ALA. The cultivars’ response was almost the same for RFW under drought stress conditions (Fig. 1C).

Root dry weight (RDW) of wheat seedlings decreased significantly under drought stress conditions (Fig. 1D). Foliar-applied varying levels of ALA improved (P ≤ 0.05) RDW significantly under drought stress. Of all ALA levels, 100 and 150 mg L−1 were found to be most effective in promoting RDW. The cultivars’ response was almost the same for this growth attribute.

Chlorophyll a contents decreased under water-deficit conditions in both wheat cultivars. Both wheat cultivars remained similar in chlorophyll a contents. Foliar-applied different levels of ALA were found to be slightly effective in altering chlorophyll a contents in both wheat cultivars (Fig. 1E).

Chlorophyll b pigments decreased under water-deficit conditions in both wheat cultivars. Foliar-applied different levels of ALA had no significant effect in altering chlorophyll b contents in both wheat cultivars (Fig. 1F). A significant difference was observed between the wheat cultivars in terms of chlorophyll b and of both wheat cultivars, cv. Shafaq-2000 was relatively higher in chlorophyll b contents than the other cultivar.

Drought stress significantly decreased the chlorophyll a/b ratios in both wheat cultivars. The response of both wheat cultivars to drought stress and foliar-applied ALA for chlorophyll a/b ratio remained unchanged (Fig. 1G).

Both water regimes (80% and 60% field capacities) and 5-ALA did not alter photosynthetic (A) and transpiration (E) rates in the seedlings of both wheat cultivars. The response of both wheat cultivars in terms of A and E to exogenously applied stress or ALA was almost the same (data not shown).

Stomatol conductance (gsc) remained unchanged in both cultivars at both water regimes (80% and 60% field capacities) and 5-ALA applications. Of both wheat cultivars, cv. Shafaq-2000 was relatively higher in gsc under drought stress conditions (Fig. 1H).

Drought stress and 5-ALA did not alter sub-stomatomal CO2 concentration (Ci) and C/Ci in the seedlings of both cultivars (Fig. 2A & B). Of both wheat cultivars, cv. Shafaq-2000 was relatively (P ≤ 0.05) higher in both above-mentioned gas exchange attributes.

Drought stress significantly (P ≤ 0.001) increased the water use efficiency (WUE) calculated as A/E of both wheat cultivars. The response of both wheat cultivars in terms of WUE to exogenously applied stress or ALA was almost the same (Fig. 2C).

High accumulation of proline was observed in the seedlings of both cultivars under different water regimes. However, foliar-applied 5-ALA did not change proline contents in both wheat cultivars. Of both wheat cultivars, cv. Uqab-06 was relatively better in proline accumulation than the other cultivar (Fig. 2D).

Drought stress did not affect glycinebetaine (GB) accumulation in both wheat cultivars. The exogenous application of 5-ALA considerably (P ≤ 0.01) improved the GB contents in both wheat cultivars, and of all ALA levels, 150 mg/L was the most effective in improving GB accumulation. The response of both wheat cultivars remained the same in terms of GB accumulation (Fig. 2E).

Drought stress had a non-significant effect on leaf and root K+ concentrations in both wheat cultivars (Fig. 2F). Foliar-applied 5-ALA improved only root K+ in the seedlings of both wheat cultivars. The difference between the two wheat cultivars was non-significant.

Drought stress and 5-ALA had a non-significant effect on leaf and root Ca2+ concentrations (Fig. 2G). The difference between the two wheat cultivars was only significant for leaf Ca2+ and cv. Uqab-06 was significantly (P ≤ 0.05) better in this cation concentration.

Leaf and root P decreased significantly in both wheat cultivars under water stress regimes (Figs. 2H & 3A). Foliar-applied 5-ALA did not alter leaf and root P under varying water regimes. The response of both wheat cultivars to ALA varied significantly, and of both wheat cultivars, cv. Uqab-06 was relatively higher in leaf and root P.

Drought stress had a non-significant effect on leaf and root N concentrations. Foliar-applied 5-ALA improved leaf and root N in the seedlings of both wheat cultivars. The difference between the two wheat cultivars was significant only for root N of both wheat cultivars, cv. Uqab-06 was higher in root N concentrations. Of all ALA levels, 150 mg/L ALA was found to be the most effective in improving the accumulation of these inorganic nutrients in the seedlings of both wheat cultivars (Fig. 3B & C).

Drought stress as well as foliar-applied varying levels of ALA did not alter the total soluble proteins as well as activities of enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in both wheat cultivars (data not shown). The response of both wheat cultivars was almost the same for total soluble proteins, and activities of SOD and CAT enzymes, while cv. Shafaq-2000 was significantly higher in POD activity (Fig. 3D).

4. Discussion

It is well known that drought stress restricts production of plants in water limited environments (Ashraf, 2010; Bibi et al., 2012; Bahrami et al., 2012). However, different crops respond to water deficit conditions differently and their response differs at different stages of ontogeny. Of the various plant growth stages, the seedling stage is the most sensitive to water shortage (Bibi et al., 2012; Bahrami et al., 2012). Thus, improvement in crop growth at the seedling stage through any means could result in high crop productivity under adverse environmental cues. Shoot and root fresh and dry biomass of the seedlings of both wheat cultivars was reduced considerably under both water stress regimes (80% and 60% of field capacities). The cultivar response was almost the same for root fresh and dry weights, while in shoot fresh and dry weights, cv. Shafaq-2000 was relatively better as compared to cv. Uqab-06 under drought stress conditions.

Exogenous application of 5-aminolevulinic acid (5-ALA), a potential growth regulator, has been reported to improve growth attributes of different plant species under water-deficit conditions (Al-Thabet, 2006). Similarly, in the current study, foliar-applied 5-ALA improved shoot and root growth of wheat seedlings which is parallel to an earlier study with oilseed rape in which Naeem et al. (2010) found that foliar application of 5-ALA improved shoot and root growth. Similarly, varying levels of 5-ALA (0.06, 0.18, 0.6, 6.0 mM) applied to radish (Raphanus sativus) leaves showed that only low concentrations of this PGR were effective at increasing shoot and root dry weights of radish seedlings, but high concentration (6 mM) proved to be highly injurious to the seedlings (Hotta et al., 1997a).

Chlorophylls a and b were slightly improved by the application of ALA, which is in accordance to an earlier study in which exogenous application of ALA containing Penta-Keep V increased chlorophyll a, chlorophyll b, total chlorophyll and chlorophyll a/b ratio of date palm under non-stress conditions (Awad, 2008). ALA-induced improvement in chlorophyll b and b pigments has been reported in sunflower leaves exposed to salt stress conditions (Akram and Ashraf, 2011a). Different reports have shown that 5-ALA improves net photosynthetic rate in different plants such as spinach (Nishihara et al., 2003), melon (Wang et al., 2004a), pakchoi (Wang et al., 2004b), radish (Hotta et al., 1997b; Wang et al., 2005), strawberry (Liu et al., 2006), and watermelon (Sun et al., 2009) under drought stress conditions. However, the data showed that different levels of drought stress (80 and 60% of field capacities) had no effect on photosynthetic rate (A) of the two wheat cultivars. The response of both wheat cultivars was almost the same under drought stress and ALA application, although in other studies foliar application of ALA has been reported to promote
stomatal conductance ($g_s$) in melon plants (Wang et al., 2004a) or watermelon (Kang et al., 2006). In contrast, in kudzu plants, exogenous application of 5-ALA significantly decreased sub-stomatal CO2 concentration but had no effect on transpiration rate (Xu et al., 2010). Foliar-applied 5-ALA showed non-significant effect on $E$ and $C_i$ of both wheat cultivars. Similarly, Al-Khateeb (2006) found that foliar-applied ALA did not affect net photosynthetic rate, $C_i$ and chlorophyll content, while stomatal conductance was considerably improved with increasing ALA concentration (Al-Khateeb, 2006).

It has already been observed that exogenous application of 5-ALA significantly increases nitrogen, phosphorus, potassium and calcium concentrations in different plants under stress conditions (Xu et al., 2010). For example, foliar application of 5-ALA significantly improved the growth of oilseed rape plants by increasing the accumulation of macronutrients (Na, P, K, and Ca) under stress conditions (Naeem et al., 2010). However, Al-Qurashi and Awad (2011) found that the application of 5-ALA did not affect sodium, potassium and nitrogen while the level of phosphorus increased significantly. It has been suggested

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**Fig. 2.** A–H Internal CO2 conc. ($C_i$), $C_i$/Ca and $A/E$ ratios, proline and glycinebetaine, root K+, leaf Ca$^{2+}$ and leaf P contents of drought-stressed and non-stressed plants of two cultivars of wheat (Triticum aestivum L.) subjected to foliar-applied varying levels of 5-aminolevulinic acid (ALA) (Mean ± S.E.; $n = 3$). NS, no spray; FC, field capacity; Cvs, cultivars; D, drought; ns, non-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.
that 5-ALA significantly increased the growth of young tissue-culture-derived date palm trees by increasing the chlorophyll concentration and inorganic nutrients of the date palm plants (Awad, 2008). Foliar-applied 5-ALA improved only root K⁺, while leaf K⁺, leaf and root Ca²⁺, P and N contents remained unaffected under water-deficit conditions which is in parallel to the findings of Akram and Ashraf (2011b).

Glycinebetaine (GB) contents remained constant, while proline contents increased under drought stress in the two wheat cultivars. However, ALA-treated seedlings of both wheat cultivars showed enhanced accumulation of only GB under water deficit conditions. Thus, an inconsistent pattern of accumulation of GB and proline was observed in the two wheat cultivars differing in stress tolerance under drought stress or ALA supply. In contrast to our results for GB and proline, high accumulation of both osmolytes (GB and proline) is a well known phenomenon under water deficiency as GB and proline have a considerable role in shielding membranes, proteins, as well as various key enzymes involved in the mechanism of stress tolerance (Banu et al., 2010). Very few reports can be observed from the literature on the role of ALA in altering various physio-biochemical processes involved in plant drought tolerance.

In conclusion, foliar-applied ALA improved seedling growth, chlorophyll a and b contents, GB contents, root K⁺, and leaf and root N in both wheat cultivars under different water stress regimes. Of the wheat cultivars used, cv. Shafaq-2000 displayed a relatively higher shoot fresh and dry weight, chlorophyll b contents, gₑ, and Cₑ, while cv. Uqab-06 was relatively better at proline accumulation, leaf and root P and root N concentrations. Of all ALA levels used, 150 mg L⁻¹ was effective in improving seedling growth. Overall, foliar-applied ALA at the seedling stage is effective in improving drought tolerance in wheat plants.

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