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Effects of 5-aminolevulinic acid on osmotic adjustment and antioxidant system in mung bean under chilling stress

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Abstract

The present study was carried out to determine the effect of chilling stress on mung bean [*Vigna radiata* (L.) Wilczek], and the possible mediatory role of applied 5-aminolevulinic acid (ALA) on osmotic adjustment and antioxidant system. The increased accumulation of superoxide radical, hydrogen peroxide, and malondialdehyde during chilling stress indicates an enhanced oxidative stress. The chilling stress induced an enhancement of reduced or oxidized glutathione and ascorbic acid, and improvement of superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR), and ascorbate peroxidase (APX) activities, but the activity of catalase (CAT) declined. Exogenous ALA enhanced the content of glutathione and ascorbic acid and elevated the antioxidant enzyme activities (POD, SOD, GR, and APX), which improved reactive oxygen species scavenging. In addition, an increased content of osmoprotectants including soluble sugars, soluble proteins, and proline contributed to ROS detoxification and enzyme/protein stabilization. Thus, ALA had a positive effect in improving the chilling resistance of mung bean plants.

Additional key words: ascorbate peroxidase, catalase, glutathione reductase, H₂O₂, malondialdehyde, peroxidase, superoxide dismutase, *Vigna radiata*.

Introduction

Temperature is an important factor for growth, development, productivity and geographical distribution of many plants (Nahar *et al.* 2015). Chilling stress is a major abiotic stress of crop production in Northeast China. Chilling stress exposure has been shown to enhance production of reactive oxygen species (ROS) and oxidative stress occurs (Nahar *et al.* 2015). The ROS, which include superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), hydroxyl radical (•OH), and singlet oxygen (¹O₂), cause damage to structural proteins, nucleic acids, enzymes, cell membranes, and other essential molecules involved in plant metabolism (Sharma *et al.* 2012, Nahar *et al.* 2015). Plants have developed mechanisms to tolerate environmental stress conditions through various physiological adaptations, including non-enzymatic and enzymatic ROS scavenging pathways (Hossain *et al.* 2010, Sharma *et al.* 2012, Nahar *et al.* 2015). Non-enzymic components of the antioxidative

defense system include reduced ascorbate (AsA) and reduced glutathione (GSH) as well as osmotic adjustment substances as proline, soluble sugars, and soluble proteins which protect membrane integrity and cellular components from dehydration (Özlem and Ekmekçi 2011). The enzymatic antioxidants comprise superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), *etc.* (Gill and Tuteja 2010, Hossain *et al.* 2010). These enzymes, through step-by-step reaction, scavenge ROS with AsA and GSH as electron acceptors (Gill and Tuteja 2010). Nahar *et al.* (2015) has established that low temperature stress increased H₂O₂ and MDA content. Exogenous spermidine (Spd) in low temperature treatment increases the content of AsA and GSH, decreases the content of oxidized ascorbate (DHA) and oxidized glutathione GSSG, and improves activity of APX and GR.

Various strategies are being employed in order to minimize the adverse effects of environmental stresses in plants.

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Abbreviations: ABA - abscisic acid; ALA - 5-aminolevulinic acid; APX - ascorbate peroxidase; AsA - reduced ascorbate; CAT - catalase; DHA - oxidized ascorbate; DHAR - dehydroascorbate reductase; DTNB - dithionitrobenzoic acid; GA - gibberellin; GR - glutathione reductase; GSSG - oxidized glutathione; GSH - reduced glutathione; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; O₂^{•-} - superoxide radical; •OH - hydroxyl radical; ¹O₂ - singlet oxygen; PBS - phosphate buffer saline; PGRs - plant growth regulators; POD - peroxidase; ROS - reactive oxygen species; Spd - spermidine; SOD - superoxide dismutase.

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Exogenously applied plant growth regulators (PGRs) is an effective, facile, and practical technique to enhance tolerance of crops, and this approach has been used widely in recent years. One of the PGRs is 5-aminolevulinic acid, or 5-amino-4-oxo-pentanoic acid (ALA), which has a relative molecular mass of 131, and it is an essential precursor of tetrapyrrole compounds including chlorophyll, heme, and phytochrome (Balestrasse *et al.* 2010) and its formation may be the rate limiting step. Hotta *et al.* (1997a,b) observed that low dosage of ALA has plant growth regulator properties, such as promoting chlorophyll biosynthesis and enhancing photosynthesis (Memon *et al.* 2009), responding to environmental stresses (Korkmaz and Korkmaz 2009, Korkmaz *et al.* 2010, Zhang *et al.* 2012, Dan *et al.* 2013, Fu *et al.* 2016), and promoting recovery of growth after herbicide applications (Zhang *et al.* 2008). High dosages of ALA can cause the accumulation of several chlorophyll synthesis intermediates, but also production of ROS leading to oxidative stress (Balestrasse *et al.* 2010).

Mung bean is indigenous to tropical areas and regarded as sensitive to various abiotic stresses, especially chilling stress. At present, the research on the influence of chilling stress on the growth and development of mung bean is mainly concentrated on the seedling stage (Kaur *et al.* 2015, Nahar *et al.* 2015). To our knowledge, the ALA mediated role of osmotic adjustment substances and antioxidant system in mitigating chilling-induced oxidative stress in mung bean has not been reported previously. Therefore, in the present study, an effort has been made to find the impact of foliar application of ALA on mung bean growing under chilling stress.

Materials and methods

Plants and chilling stress: Mung bean [*Vigna radiata* (L.) Wilczek] cv. Lvfheng 2 was used for chilling stress experiments at National Coarse Cereals Engineering Research Center. Ten seeds were sown in plastic pots (9.0 cm lower inside diameter, 13.0 cm upper inner diameter, and 11.0 cm height) filled with mixed air-dried soil + Vermiculite + Perlite (2:1:1, v/v/v) and placed in a growth chamber under day/night temperatures of 25/18 °C, a 12-h photoperiod, an irradiance of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a 75 % relative humidity. The plants were sprayed with 0.5 mM ALA solution (Balestrasse *et al.* 2010) and distilled water for control at cotyledon stage. About 48 h after ALA treatment, the plants were exposed to chilling stress conditions for 0, 6, 12, 24, 48, and 60 h, respectively. For temperature treatments, the indoor controlled growth chambers was already set at low temperature treatment of 5 °C and 25 °C as control. The leaves were harvested directly into liquid nitrogen and stored at -40 °C for further use. This experiment was set up in a completely randomized design with four replications.

Reactive oxygen species: The production rate of $\text{O}_2^{\cdot -}$ was appraised according to Yang *et al.* (2010). Leaf tissues (100 mg) were ground in a K-P buffer solution (pH 7.8),

and centrifuged at 5 000 g. To monitor the nitrite formation from hydroxylamine, the supernatant was mixed with 10 mM hydroxylamine hydrochloride and extraction buffer, and incubated at 25 °C for 20 min. After 20 min, 7 mM naphthylamine and 17 mM sulfanilamide were added and the mixture was incubated again at 25 °C for 20 min. Absorbance was measured at 530 nm (UV-3600 Plus, Shimadzu, Kyoto, Japan).

The content of H_2O_2 was determined according to the method of Yu *et al.* (2003) with some modifications. Fresh leaf tissues (200 mg) were ground in ice cold acetone. The reaction mixture consisted of 1.0 cm^3 supernatant, 0.1 cm^3 of 5 % (m/v) titanous sulfate, and 0.2 cm^3 of ammonia. After centrifugation at 3 000 g for 10 min, 5.0 cm^3 of 18.4 mM H_2SO_4 was added. Absorbance was measured at 415 nm (UV-3600 Plus).

Lipid peroxidation: Lipid peroxidation in leaves was measured by estimating the content of thiobarbituric acid (TBA) reactive substances, which determined malondialdehyde (MDA) content (Kaur *et al.* 2015). A leaf sample (0.1 g) was ground in 5 cm^3 of phosphate buffer (pH 7.8) and centrifuged at 12 000 g for 20 min. To a 1 cm^3 aliquot of the supernatant, 1 cm^3 of phosphate buffer saline (PBS) (pH 7.8), and 2 cm^3 of 20 % (m/v) trichloroacetic acid containing 0.5 % (m/v) TBA were added and incubated in boiling water for 15 min. Then the mixture was quickly cooled in an ice bath and was centrifuged at 1 800 g for 10 min. The absorbance of the supernatant was read at 532 nm and 600 nm (UV-3600 Plus). The MDA content was calculated from the extinction of absorbance of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Osmolytes determination: Total soluble sugar content of each sample was determined following the method of Buyse and Merckx (1993). Fresh leaf samples (0.1 g) were homogenized in 10 cm^3 of 80 % (v/v) ethanol and extracted in a water bath at 80 °C for 15 min. The supernatant was centrifuged three times (10 000 g, 20 min). The combined liquid supernatants were concentrated in 25 cm^3 tubes as a mother liquor. Total soluble sugar content was measured at 630 nm by the colorimetry of sulfuric acid-anthrone method (UV-3600 Plus).

The protein content of each sample was determined following the method of Bradford (1976) using bovine serum albumin as a protein standard. Leaves were homogenized in distilled water and centrifuged at 4 000 g for 15 min. The supernatant and 5 cm^3 of Coomassie Brilliant Blue solution were placed in tubes. Formation of the product was recorded after 2 min at a wavelength of 595 nm (UV-3600 Plus).

Proline content was appraised according to Bates *et al.* (1973). Leaves (0.5 g) were homogenized in 3 % (m/v) sulfosalicylic acid and centrifuged at 3 000 g for 10 min. Then, 2.0 cm^3 of supernatant was mixed with 2.0 cm^3 of acid ninhydrin and 2.0 cm^3 of glacial acetic acid. After incubating the mixture at 100 °C for 15 min and subsequent cooling, 5.0 cm^3 of toluene was added, and absorbance was read at 520 nm (UV-3600).

Non-enzymatic antioxidants: The estimation of AsA and oxidized ascorbate (DHA) was done according to the method of Zhang and Kirkham (1996). Plant tissue was homogenized in 5 % (m/v) phosphoric acid, and the homogenate was centrifuged at 22 000 g and 4 °C for 15 min. The mixture contained 0.5 cm³ of the enzyme extract, 1.0 cm³ of absolute ethanol, 0.5 cm³ of 0.4 % H₃PO₄-ethanol, 1.5 cm³ of 5 % phosphoric acid, 0.5 cm³ of 0.03 % (m/v) FeCl₃-ethanol and 1.0 cm³ of 0.5 % (m/v) phenanthroline-ethanol. The mixture was boiled for 90 min in a constant temperature water bath (30 °C) and then cooled to room temperature. The absorbance was read at 530 nm (*UV-3600 Plus*).

Glutathione was assayed by an enzymic recycling procedure according to Grace and Logan (1996), in which it was sequentially oxidized by dithionitrobenzoic acid (DTNB) and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GR. Fresh leaf tissues (1 g) was ground in liquid nitrogen using a mortar and a pestle in 5 cm³ of EDTA-trichloroacetic acid and then centrifuged at 20 000 g for 15 min. The reaction mixture consisted of the aliquot of the supernatant, 1 nM NaOH, and DTNB. The reaction was initiated by the addition of 0.2 unit of GR (from yeast, *Boehringer*, Mannheim, Germany) in a total volume of 2 cm³, and a change in absorbance at 412 nm was monitored at 30 °C (*UV-3600 Plus*).

Antioxidant enzymes: Mung bean leaves (0.5 g) were homogenized in 10 cm³ of chilled/ice cold 50 mM PBS extraction buffer (pH 7.0) using a mortar and a pestle, and centrifuged at 11 500 g and 4 °C for 15 min. The supernatant was used for enzyme activity assays.

To measure SOD (EC 1.15.1.1) activity, the method of El-Shabrawi *et al.* (2010) was followed. The reaction mixture contained 2.4 cm³ of 50 mM PBS (pH 7.8), 0.2 cm³ of 13 mM methionine, 0.1 cm³ of EDTA, 50 mm³ of the enzyme extract, and 0.2 cm³ of 25 mM nitroblue tetrazolium (NBT). The reaction was started by adding 0.2 cm³ of 2 mM riboflavin followed by exposure to a 300 µmol m⁻² s⁻¹ fluorescent radiation for 15 min. A change in absorbance was read at 560 nm (*UV-3600 Plus*) and the total SOD activity of the samples was assayed by measuring its ability to inhibit the photochemical reduction of NBT. One unit (U) of SOD activity was defined as the amount of SOD required to inhibit NBT by 50 % in 1 min.

Peroxidase (EC 1.11.1.7) activity was measured adopting the method of Polle *et al.* (1994) by estimating the amount of enzyme that oxidized guaiacol at 470 nm during the linear phase of the reaction. For the POD assay, a 3.0 cm³ of reaction mixture contained 0.1 M H₂O₂ in 50 mM PBS (pH 7.8) and 20 mM guaiacol. The reaction was initiated by adding 0.2 cm³ of the crude enzyme extract. A change in absorbance was read at every 1 min for 5 min at 470 nm on a spectrophotometer (*UV-3600 Plus*).

Catalase (EC 1.11.1.6) activity assessment was made using the method of Cakmak and Horst (2010) by monitoring a change in absorbance at 410 nm (during decomposition of H₂O₂). The reaction mixture contained

50 mM phosphate buffer (pH 7.0) and 0.1 cm³ of the enzyme extract. The reaction was initiated by adding 200 mM H₂O₂. The change in absorbance was recorded at 410 nm for 1 min (*UV-3600 Plus*). The activity was calculated using the coefficient of absorbance of 39.4 M⁻¹ cm⁻¹.

The activity of APX (EC 1.11.1.11) was assayed by measuring a decrease in absorbance at 290 nm (*UV-3600 Plus*) for 1 min following the method of Nakano and Asada (1981). The reaction solution contained 50 mM PBS (pH 7.0), 15 mM AsA, 100 mM H₂O₂, 0.1 mM EDTA, and the enzyme extract (a final volume of 700 mm³). The reaction was started by adding H₂O₂.

The activity of GR (EC 1.6.4.2) was determined by the method of Foyer and Halliwell (1976) by monitoring glutathione dependent oxidation of NADPH at 340 nm (*UV-3600 Plus*). The assay mixture contained 20 mM EDTA-Na₂, 5 mM GSSG, 50 mmol dm⁻³ PBS (pH 7.8), and the enzyme extract. The reaction mixture was placed in a water bath at 25 °C for 5 min, and then 10 mM NADPH was added to initiate the reaction.

Statistical analysis: Statistical and correlation analysis were conducted using the *SPSS* (21.0) software. All data of treatment and control were analyzed by one-way analysis of variance (*ANOVA*) with Duncan's multiple range test at $\alpha = 0.05$. The figures were drawn by the *OriginPro 9.1* software (*OriginLab*, Northampton, MA, USA).

Results

Chilling stress induced dramatic accumulation of O₂⁻ and H₂O₂. The ROS in mung bean had a significantly positive correlation with stress duration ($P < 0.05$). The rate of O₂⁻ generation and the content of H₂O₂ reached their maximum values when plants were exposed to chilling stress for 60 h, chilling stress caused a 2.91- and 1.42-fold enhancement in the above indicators related to controls, respectively. Application of ALA significantly reduced O₂⁻ and H₂O₂ content in mung bean leaves, compared with the plants under chilling environment alone. Accumulation of O₂⁻ and H₂O₂ gradually declined with prolonged recovery time, but it was always higher than in the control plants. The ALA treatment caused 20.54 and 17.88 % reduction in the above indicators compared to the chilling-treated plants. In brief, the plants supplemented with ALA could maintain lower ROS content (Fig. 1).

Exposure to 5 °C for 60 h resulted in a maximum enhancement of malondialdehyde (MDA, indicator of lipid peroxidation) content (Table 1). Chilling stress caused a 3.90-fold increase in MDA content, compared with the control plants. It was calculated that the relationship between MDA content and stress time has a quadratic equation of $y = -0.024x^2 + 3.642x + 34.966$, and R^2 was 0.993. Application of ALA significantly reduced MDA content in mung bean leaves, compared with the plants under chilling stress alone.

The content of osmolytes, soluble sugars, soluble proteins, and proline increased under chilling stress by 9.1 - 103.6, 12.2 - 139.9, and 39.2 - 236.2 %, respectively,

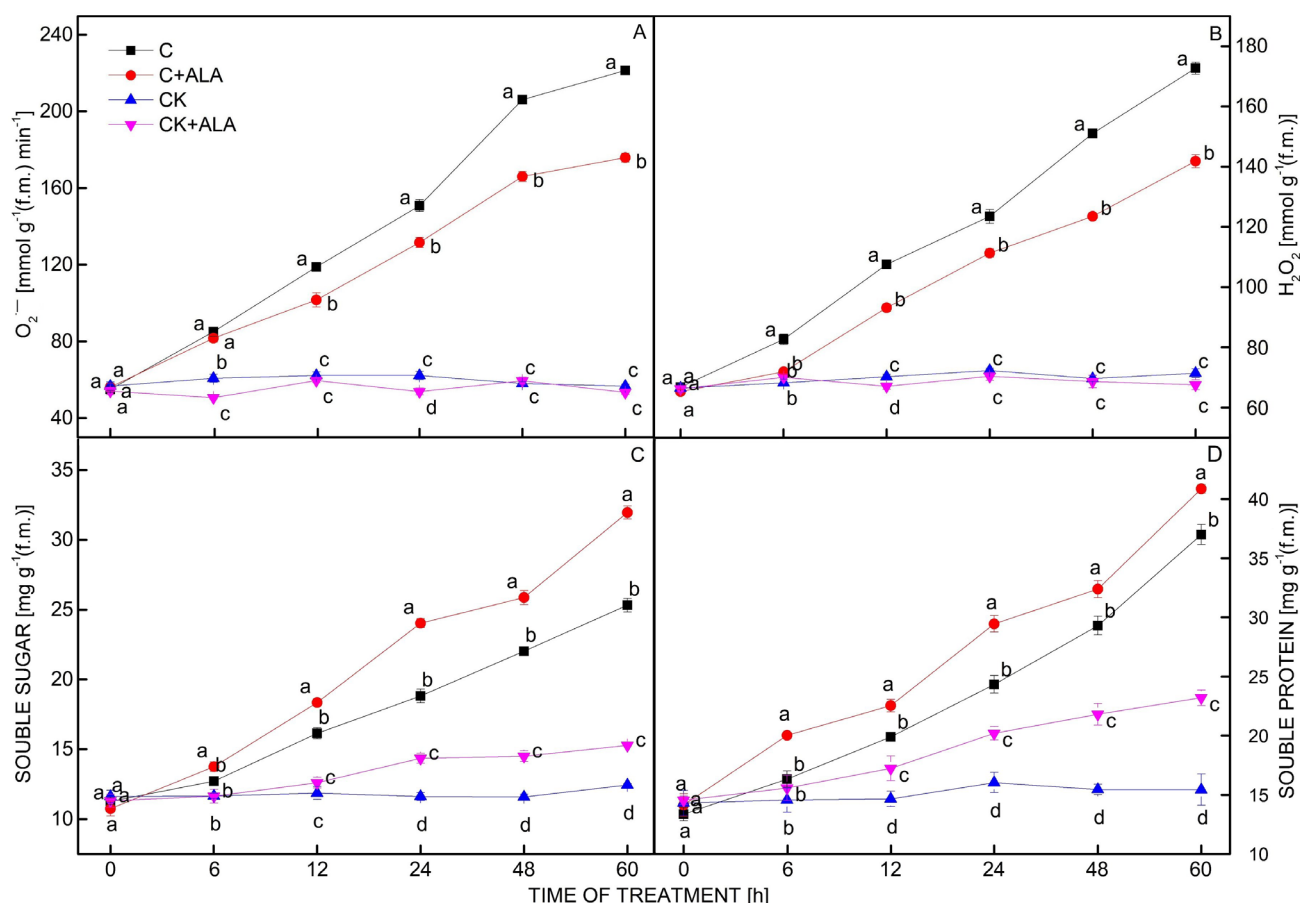


Fig. 1. Effect of 0.5 mM 5-aminolevulinic acid (ALA) on superoxide radical ($O_2^{\cdot-}$) generation (A), hydrogen peroxide (H_2O_2) (B), soluble sugar (C), and soluble protein (D) content in mungbean leaves under chilling stress. C - chilling stress, C+ALA - chilling stress + ALA, CK - control conditions, CK+ALA - control conditions + ALA. Means \pm SEs, $n = 4$, different letters indicate significant differences between the treatments at $P \leq 0.05$ determined by Duncan's multiple range test.

Table 1. Effect of 0.5 mM 5-aminolevulinic acid (ALA) on lipid peroxidation (expressed as malondialdehyde content) [$nmol\ g^{-1}(f.m.)$] in mung bean leaves under chilling stress. C - chilling stress, C+ALA - chilling stress + ALA, CK - control conditions, CK+ALA - control conditions + ALA. Means \pm SEs, $n = 4$. Different letters indicate significant differences ($P < 0.05$) determined by Duncan's multiple range test.

Treatments	0 h	6 h	12 h	24 h	48 h	60 h
C	33.55 \pm 0.99 ^a	56.77 \pm 1.33 ^a	73.81 \pm 1.76 ^a	113.55 \pm 4.38 ^a	146.58 \pm 3.26 ^a	169.29 \pm 3.37 ^a
C+ALA	34.06 \pm 0.60 ^a	45.94 \pm 1.76 ^b	62.97 \pm 2.73 ^b	99.10 \pm 2.67 ^b	122.32 \pm 2.84 ^b	134.71 \pm 2.84 ^b
CK	33.55 \pm 0.52 ^a	34.06 \pm 0.60 ^c	33.55 \pm 1.30 ^c	34.58 \pm 0.99 ^c	35.10 \pm 0.84 ^c	34.58 \pm 1.55 ^c
CK+ALA	34.06 \pm 1.03 ^a	34.58 \pm 1.76 ^c	30.97 \pm 0.84 ^c	26.32 \pm 0.52 ^d	25.29 \pm 0.99 ^d	15.48 \pm 1.03 ^d

Table 2. Effect of 0.5 mM 5-aminolevulinic acid (ALA) on proline content [$mg\ g^{-1}(f.m.)$] in mung bean leaves under chilling stress. Means \pm SEs, $n = 4$. Different letters indicate significant differences ($P < 0.05$) determined by Duncan's multiple range test. CK - control conditions, CK+ALA - control conditions + ALA, C - chilling stress, C+ALA - chilling stress + ALA.

Treatments	0 h	6 h	12 h	24 h	48 h	60 h
C	14.44 \pm 1.49 ^a	20.35 \pm 1.16 ^b	30.49 \pm 0.93 ^b	34.10 \pm 0.96 ^b	43.68 \pm 1.00 ^b	53.82 \pm 0.47 ^b
C+ALA	14.76 \pm 1.22 ^a	26.84 \pm 0.65 ^a	40.07 \pm 0.79 ^a	48.37 \pm 0.67 ^a	55.31 \pm 0.87 ^a	70.07 \pm 1.20 ^a
CK	15.00 \pm 0.57 ^a	14.62 \pm 0.48 ^c	14.86 \pm 0.91 ^c	15.56 \pm 1.43 ^c	16.35 \pm 2.17 ^c	16.01 \pm 1.37 ^c
CK+ALA	14.27 \pm 1.19 ^a	16.04 \pm 1.26 ^c	14.97 \pm 1.19 ^c	15.80 \pm 1.12 ^c	17.05 \pm 2.03 ^c	16.11 \pm 1.86 ^c

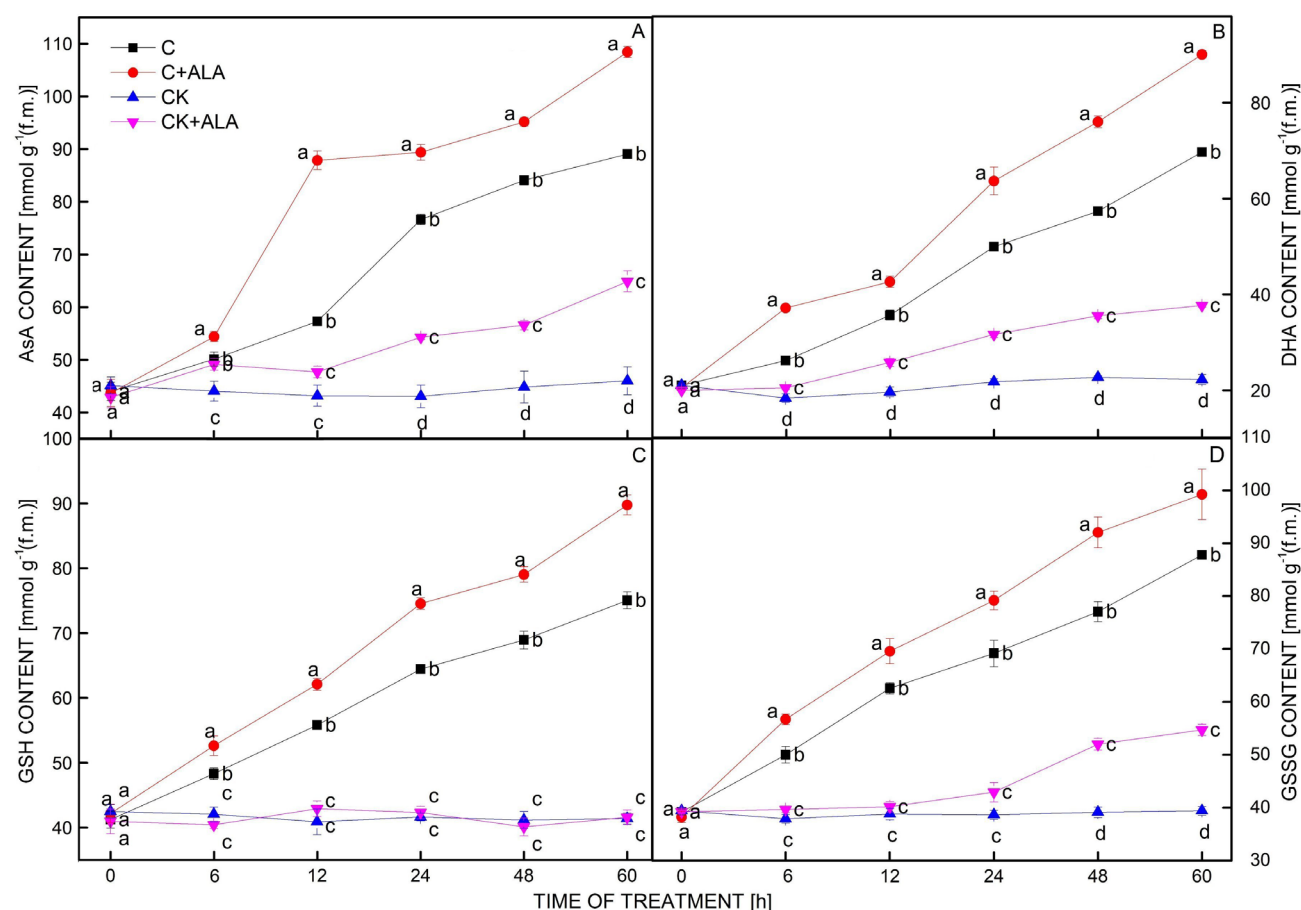


Fig. 2. Effect of 0.5 mM 5-aminolevulinic acid (ALA) on nonenzymatic antioxidants content in mung bean leaves under chilling stress. A - ascorbate (AsA), B - dehydroascorbate (DHA), C - reduced glutathione (GSH), and D - oxidized glutathione (GSSG). C - chilling stress, C+ALA - chilling stress + ALA, CK - control conditions, CK+ALA - control conditions + ALA. Means \pm SEs, $n = 4$. Different letters indicate significant differences ($P < 0.05$) determined by Duncan's multiple range test.

Table 3. Effect of 0.5 mM 5-aminolevulinic acid (ALA) on catalase (CAT) activity [$\mu\text{mol mg}^{-1}(\text{protein}) \text{min}^{-1}$] in mung bean leaves under chilling stress. C - chilling stress, C+ALA - chilling stress + ALA, CK - control conditions, CK+ALA - control conditions + ALA. Means \pm SEs, $n = 4$. Different letters indicate significant differences ($P < 0.05$) determined by Duncan's multiple range test.

Treatments	0 h	6 h	12 h	24 h	48 h	60 h
C	33.26 \pm 1.20 ^a	29.13 \pm 1.46 ^b	21.85 \pm 0.45 ^b	17.26 \pm 0.92 ^b	14.11 \pm 1.25 ^b	12.50 \pm 0.47 ^b
C+ALA	33.66 \pm 0.91 ^a	25.52 \pm 0.94 ^c	19.04 \pm 1.16 ^c	14.33 \pm 1.51 ^b	12.33 \pm 0.95 ^b	8.37 \pm 0.43 ^c
CK	33.72 \pm 0.21 ^a	32.80 \pm 0.57 ^a	33.08 \pm 0.59 ^a	32.17 \pm 0.92 ^a	33.66 \pm 1.44 ^a	33.03 \pm 0.94 ^a
CK+ALA	32.00 \pm 2.83 ^a	33.43 \pm 0.35 ^a	32.74 \pm 0.98 ^a	33.89 \pm 1.02 ^a	34.69 \pm 2.39 ^a	33.37 \pm 0.60 ^a

compared to control. The positive and strong association of osmolytes with length of stress treatment revealed the importance of osmolytes in determining stress tolerance. Content of soluble sugars, soluble proteins, and proline enhanced dramatically in ALA-supplemented plants under chilling stress, compared to chilling treatment only by 8.2 - 26.3, 10.4 - 22.7, and 26.6 - 31.9 %, respectively (Fig. 1, Table 2).

Chilling stress increased AsA and DHA content (Fig. 2). AsA and DHA content with prolonged chilling temperature treatment gradually increased and reached their maximum after 60 h. Chilling stress caused 93.5 and

213.5 % increases in the above indicators, while after ALA pretreatment they were enhanced by 135.6 and 305.0 %, respectively, compared with the control plants (Fig. 2).

Chilling stress markedly enhanced GSH and GSSG content (Fig. 2). This pattern was similar but slightly stronger in the ALA-treated plants. Compared with the control plants chilling stress for 60 h caused a 81.2 and 122.8 % increase in these indicator, while in ALA-treated plants they were increased by 116.8 and 151.7 %, respectively.

A marked decrease in CAT activity and an increase in the activities of SOD, POD, APX, and GR were observed

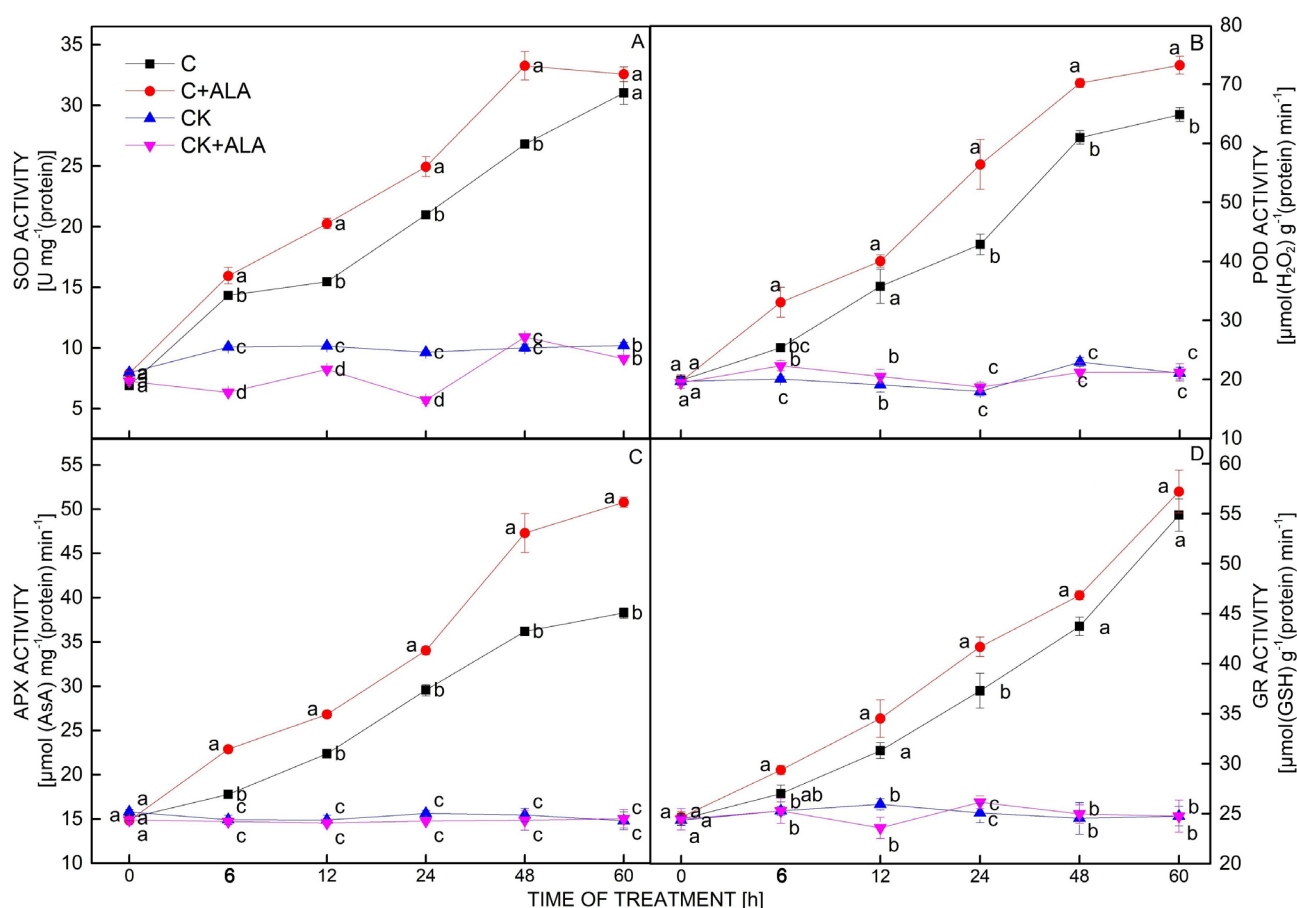


Fig. 3. Effect of 0.5 mM 5-aminolevulinic acid (ALA) on antioxidant enzyme activities in mung bean leaves under chilling stress. A - superoxide dismutase (SOD), B - peroxidase (POD), C - ascorbate peroxidase (APX), and D - glutathione reductase (GR). C - chilling stress, C+ALA - chilling stress + ALA, CK - control conditions, CK+ALA - control conditions + ALA. Means \pm SEs, $n = 4$. Different letters indicate significant differences ($P < 0.05$) determined by Duncan's multiple range test.

in chilling-affected plants (Table 3 and Fig. 3). Negative association of CAT with stress duration, and a positive and significant correlation of SOD, POD, APX and GR with stress duration were found. This pattern was similar but slightly stronger in the ALA-treated plants. The activity of CAT reached a minimum value when plants were exposed to chilling stress for 60 h, and chilling stress caused a 62.2 % its reduction compared with the control plants. Under chilling stress for 60 h, the activities of SOD, POD, APX, and GR reached their maximum values; chilling stress caused a 203.8, 208.1, 158.7, and 121.6 % increase in their activities. In ALA-treated plants, this increase was 5.0, 12.9, 32.7, and 4.3 % greater, respectively, compared with the only chilling-treated plants.

Discussion

Under optimum conditions, ROS are generated at a low level and the production and quenching of ROS in plant cells is in a dynamic equilibrium (Gill and Tuteja 2010, Sharma *et al.* 2012). However, this equilibrium may be perturbed by chilling stress, and lead to a sudden increase in their intracellular content which can cause dramatic

damage to cell structures. Lipid peroxidation is a well-known indicator for determining the extent of oxidative stress. The overproduction of ROS leads to peroxidation of membrane lipids which ultimately results in cell death (Özlem and Ekmekçi 2011, Nahar *et al.* 2015). Chilling stress induces the accumulation of $O_2^{\cdot-}$ and H_2O_2 and damage at the tissue level was previously reported in mung bean seedlings (Nahar *et al.* 2015). In our study, content of $O_2^{\cdot-}$, H_2O_2 , and MDA were increased under chilling stress which agreed with previous reports (Fig. 1 and Table 1). Various researchers have reported that exogenous PGRs induced an upregulation of nonenzymatic and/or enzymatic antioxidants and concomitantly reduced ROS and MDA content were observed in many plant species under various environmental stresses including chilling stress (Zhang *et al.* 2008, 2012, Korkmaz and Korkmaz 2009, Korkmaz *et al.* 2010, Dan *et al.* 2013, Fu *et al.* 2016). In the present study, the stressed plants of mung bean pretreated with ALA showed lower ROS and MDA content (Fig. 1 and Table 1) which was due to enhanced activity of nonenzymatic and enzymatic antioxidant defense system. This results is consistent with the study reported by Nahar *et al.* (2015) in mung bean seedlings under chilling stress. Therefore, the application of ALA

under chilling stress can help decrease membrane damage attributing to over accumulation of ROS.

Osmoprotectants such as soluble sugar, soluble protein, and proline protect membrane integrity, detoxify ROS, and stabilize enzymes/proteins. Under diversified abiotic stresses, accumulation of osmotic adjustment related substances in many plant species has been correlated with stress tolerance. Özlem and Ekmekçi (2011) found an increased proline content in chickpea plant exposure to 10 °C for 7 d induced. Latef and He (2011) used tomato as a test material and reported that low temperature stress resulted in a significant enhancement of soluble sugar, soluble protein, and proline content. We also found that soluble sugar, soluble protein, and proline content increased under chilling stress. Soluble sugar, soluble protein, and proline content further enhanced in ALA-pretreated plants and then exposed to chilling stress, compared to chilling treatment only. The results of the study are consistent with previous studies (Latef and He 2011, Özlem and Ekmekçi 2011).

Plants in the long process of evolution have evolved efficient antioxidant defense systems to prevent or alleviate the damage from ROS (Gill and Tuteja 2010, Nahar *et al.* 2015). The water-soluble compounds AsA and GSH have vital roles in development of plant stress tolerance to scavenge or remove ROS. AsA can directly quench $O_2^{\cdot-}$ (Hossain *et al.* 2010), and two molecules of AsA as a substrate are utilized by APX to scavenge H_2O_2 to water, and, during this reaction with the concomitant generation of monodihydroascorbate (MDHA), which can disproportionate into DHA and AsA (Hossain *et al.* 2010, Sharma *et al.* 2012). Besides acting as a scavenger of ROS, AsA also regulates other plant metabolic processes and increases efficiency of antioxidant enzyme activities. And two molecules of GSH were utilized by DHAR to reduce DHA to AsA, and during this reaction, GSSG was generated (Gill and Tuteja 2010). Numerous studies on non-enzymatic antioxidants in plants have demonstrated that increased AsA or GSH content can effectively reduce ROS accumulation and prevent oxidative stress (Nahar *et al.* 2015). Our data revealed that chilling stress increased the AsA and DHA content in mung bean (Fig. 2) and ALA pretreatment further increased AsA and DHA content in chilling affected plants, which is one reason for reduced oxidative stress (induced by chilling stress) (Fig. 2). The trend of GSH and GSSG was similar with AsA, and again slightly stronger in the ALA-treated plants (Fig. 2). This result indicated that ALA could improve the tolerance of mung bean plants to chilling stress mainly by enhancing the content of AsA and GSH antioxidants.

Cells and/or tissues protect themselves against oxidative injury through the up-regulation of a range of antioxidants enzymes (Gill and Tuteja 2010, Sharma *et al.* 2012). In general, efficient breakdown of $O_2^{\cdot-}$ and H_2O_2 needs the action of several antioxidant enzymes acting in synchrony. SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 , whereas CAT, POD, and APX metabolize H_2O_2 . Among them, CAT has a high specificity for H_2O_2 and catalyzes the dismutation of two molecules of H_2O_2 into H_2O and oxygen (Sharma *et al.* 2012). POD

requires a reductant, since it reduces H_2O_2 to H_2O . APX is a specific peroxidase that catalyzes the destruction of H_2O_2 at the expense of oxidizing AsA to DHA. Some studies have pointed out that the activities of CAT, SOD, POD, APX, and GR in different plants are different under abiotic stresses. Hossain *et al.* (2010) described that cadmium stress causes a considerable increase in APX activity, while the activities of CAT and GR are sharply decreased. Zhou and Leul (1998) showed that freezing treatment could dramatically increase SOD activity, while reducing CAT and POD activities. Leul and Zhou (1999) demonstrated that foliar sprays of uniconazole remarkably elevates the activities of SOD, CAT, and POD. Nahar *et al.* (2015) reported that CAT activity decreases and APX and GR activities are promoted under low temperature stress. In addition, exogenous Spd application is effective at improving and restoring the activity of enzymes of the AsA-GSH cycle, including APX and GR, but it does not affect the activity of CAT. According to Latef and He (2011), low temperature stress causes a reduction of SOD, CAT, and APX activities, whereas increases the activity of POD. In mung bean in our experiment, it was found that chilling stress increased SOD, POD, APX and GR activities, and declined CAT activity (Fig. 3 and Table 3) where comparatively higher activity was observed in ALA treatment. ALA treatment significantly improved the activity of SOD, POD, APX and GR activities in the leaves of mung bean plants (Fig. 3 and Table 3), declined the content of ROS (Fig. 1), and reduced the extent of plasma membrane peroxidation (Table 1) and maintained its integrity. Therefore, it is clear that the capacity of ALA to enhance free radical scavenging could be due to increase in activity enzymatic and non-enzymatic antioxidant system. While the results were similar to previous studies, there were some differences. The differences may be because the adverse influences of chilling stress on crop plants are complex and vary with pretreatment, plant development stage, species, and duration of exposure and severity of chilling stress.

In conclusion, the results of our study indicated that chilling stress increased $O_2^{\cdot-}$, H_2O_2 and MDA content in mung bean. However, ALA pretreatment alleviate the chilling stress mostly due to enhanced ROS scavenging systems. Thus, we suggest that the applications of ALA can have practical importance in agricultural systems.

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