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EXOGENOUS APPLICATION OF ALA REGULATES GROWTH AND PHYSIOLOGICAL CHARACTERS OF *Leymus chinensis* (TRIN.) TZVEL. UNDER LOW TEMPERATURE STRESS

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ABSTRACT

A pot experiment was conducted to assess regulation of growth and physiology of *Leymus chinensis* plants by exogenous application of 5-Aminolevulinic acid (ALA) at various concentrations (10, 50 and 100 mg/L) exposed to low temperature stress. In control distilled water was applied as spray under both normal and low temperature stress. Experiment was laid out using completely randomized design with three replications. Results revealed that low temperature stress impaired plant growth and photosynthetic pigments while elevating the accumulation of malondialdehyde (MDA), osmolytes and enzymatic antioxidants of *L. chinensis* plants. However, application of ALA improved the plant height, leaf area, plant fresh and dry weight, root activity, chlorophyll *a*, chlorophyll *b* and total chlorophyll content of *L. chinensis* plants when compared with control under low temperature stress. Treatment with ALA at a concentration of 10 and/or 50 mg/L was found better pertaining to growth while 100 mg/L was better regarding biosynthesis of photosynthetic pigments. Furthermore, ALA treatment enhanced the accumulation of soluble proteins, soluble sugars and free proline while reducing the MDA content and 10 and/or 50 mg/L ALA concentration was superior. Application of ALA also boosted the enzymatic antioxidants viz. super oxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate (APX) and glutathione reductase (GR) as compared to control at low temperature and application of 10 mg/L performed better. It may be concluded that treatment of ALA was helpful in the attainment of tolerance against low temperature stress manifested through improved growth, biosynthesis of photosynthetic pigments, higher levels of osmolytes and antioxidant enzymes, and reduced MDA content of *L. chinensis* plants.

Key words: *Leymus chinensis*; 5-Aminolevulinic acid (ALA); low temperature; growth; osmolytes; antioxidants.

INTRODUCTION

Leymus chinensis (Trin.) Tzvel, also known as sheep grass is an important forage grass which is perennial in nature having high productivity, protein content and high palatability (Wang *et al*., 2009). It also has high adaptability to various environmental conditions (Chen *et al*., 2013) and can survive at extremely low temperature, drought and high salt stress (Ma and Liang, 2007; Wang *et al*., 2008). It is one of the grassland communities in Eurasian steppe region and is widely distributed in eastern Eurasian steppe, including the western North Korea, outer Baikal area of Russia, the Northeast Plain, Mongolia, the Northern Plain and Inner Mongolian Plateau of China (Bai *et al*., 2004). In China it extends up to 220,000 km^2. The grasslands of China play an important role in the conservation of soil and water resources, and support the livestock farming, particularly in the northern China (Bai *et al*., 2010).

In the recent decades the over use of land and grazing in the grasslands coupled with abiotic stresses such as drought, salinity, heavy metal stress and temperature extremes have made the condition worse leading to the degradation of grasslands. Among all low temperature stress is one of the most adverse abiotic stresses which impart very complex effects on plants limiting their growth, development and survival (Baruah *et al*., 2011). Cold stress induces oxidative damage by the production of reactive oxygen species (ROS) in cells, viz. superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen (O^*) (Dai *et al*., 2009). Low temperature causes the activity of antioxidant to be slowed down which results in the over production and accumulation of ROS in plant cells (Lukatkin, 2002). This is associated with the exaggerated oxidative damage to proteins and nucleic acid, and membrane lipid peroxidation which is indexed through increased levels of malondialdehyde (MDA) content (Zhang *et al*., 2011). This is associated with membrane

injury manifested by leakage of cellular ions and organic substances (Zauralov and Lukatkin, 1996).

Plants respond to abiotic stresses in many ways to combat with damaging effects. Osmolytes are accumulated in plants in response to abiotic stresses which aid the plants to attain some degree of stress tolerance. Compounds such as proline, soluble sugars and proteins and some amino acids act as osmolytes in plants and maintain the tissue water status through osmotic adjustment and protect the biological membranes and organic molecules from injurious effects of ROS (Hincha *et al*., 2006; Klerk and Pumisutapon, 2008). Many sugars such as sugar alcohols are synthesized in plants exposed to abiotic stresses (Peterbauer and Richter, 2001). Furthermore, plants induce anti-oxidative defense system to scavenge the ROS under stressed conditions (Khan *et al*., 2015). The enzymatic anti-oxidative system includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate (APX) and glutathione reductase (GR) which aids in the defense against oxidative stress (Smeets *et al*., 2009).

5-Aminolevulinic acid (ALA) is a vital precursor in the biosynthesis of tetrapyrrols including heme and chlorophyll pigments (von Wettstein *et al*., 1995). It improves the chlorophyll content, photosynthetic rate and gas exchange capacity of plants under low temperature (Wang *et al*., 2004). Application of ALA relieves the damaging effects of abiotic stresses by improving the plant water relations, antioxidant system, and chlorophyll content while tumbling the ROS production and accumulation (Naeem *et al*., 2010; Zhang *et al*., 2012). Chilling stress increased the accumulation of proline which was further increased by the application of ALA to the pepper seedlings (Korkmaz *et al*., 2010). Mediation of physiological processes by ALA has been found to be associated with gene expression for antioxidants to cold stress in soybean plants (Balestrasse *et al*., 2010).The present study was conducted to determine the regulatory mechanism of ALA on morphology and physiology of *L. chinensis* under low temperature stress and to determine the extent of stress tolerance by the exogenous application of ALA.

MATERIALS AND METHODS

A pot experiment was carried out in the Physiological and Biochemical Laboratory, College of Agronomy and Biotechnology, Southwest University, during 2014. *L. chinensis* seeds were collected from Ecological Experimental Station of the natural distribution of community, Inner Mongolia, China in late July, 2013 and dried at room temperature. Seeds were stored in an airy cloth bag after drying and placed at 4℃ in refrigerator. Seed were sown in greenhouse incubator (light 10 h / 30 ℃; dark 14 h / 20 ℃). Seedlings were transplanted to sand culture after one week in pots (34 cm diameter and 24 cm depth). After transplanting a quantitative Hoagland nutrient solution was poured every five days. The pots were kept moistened during the experimental period by pouring water after every two days. When *L. chinensis* were grown to 18-21 cm, thinning was done and 25 plants were kept in each pot. The ALA was applied as spray on leaves of *L. chinensis* plants in different concentrations viz. 10, 50 and 100 mg/L under low temperature and in control distilled water was applied as spray under both normal and low temperature conditions. After spray seedlings were exposed to normal and low temperature. Pots were kept at room temperature (20 ℃ / 15 ℃ (day / night), light 10 hours) and in greenhouse incubator at low temperature (5 ℃ / 1 ℃ (day / night), light 10 hours), for exposing the seedlings to normal and low temperature. After four days second spray of ALA was carried out. In all the experiment consisted of five treatments viz. T_1 (normal temperature + distilled water spray), T_2 (low temperature + distilled water spray), T_3 (low temperature + 10 mg/L ALA spray), T_4 (low temperature + 50 mg/L ALA spray) and T_5 (low temperature + 100 mg/L ALA spray). The experiment was laid out using randomized complete block design with three replications. After 7 days of treatment morphological, physiological and biochemical attributes were measured (Jin-huan *et al*., 2015).

Morphological traits were measured by uprooting the *L. chinensis* plants from pots. Plants were rinsed first with tap water and then 2-3 times with distilled water. Plant height was determined by measuring the plants from tip of the stem to parietal lobe at the base plants. Water adhered to plants was absorbed by using filter paper. MSD-971 scanner was used to determine the leaf area. Plants were weighed to determine the fresh weight and then dried by placing in oven at 105 °C for 15 minutes followed by drying at 65 °C till constant weight to obtain the seedling dry weight.

Measurement of photosynthetic pigments viz. chlorophyll *a*, chlorophyll *b* and total chlorophyll content was made by using the method of Wellburn (1994). Soluble protein content was determined by using coomassie brilliant blue method (Bradford, 1976). Soluble sugars were quantified by anthrone color method (Li *et al*., 2008). Root activity was determined by using TTC method of Higa *et al*. (2010). Thiobarbituric acid (TBA) assay was used to assess the MDA content (De-Vos*et al*., 1991). Proline content was determined by ninhydrin method (Bates *et al*., 1973). Antioxidants viz. superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) were determined by using method described by Parida (2004).

The analysis of data collected was done by using Microsoft Excel and statistical software program SPSS19.0 and comparison of treatments was done by using Duncan's multiple range test.

RESULTS

Plant growth and development of *L. chinensis* was impaired by low temperature stress. Conversely, treatment with ALA improved the plant growth under stressed condition. Plant height, leaf area and plant fresh weight was severely affected by low temperature stress, however, plant dry weight was not affected. Application of ALA amended the plant growth and development at low temperature. The ALA treatment at a concentration of 50 mg/L enhanced the plant height (20.95%) and leaf area (154.73%), while, plant fresh weight (61.01%) and dry weight (38.46%) of *L. chinensis* was bettered by 10 mg/L ALA application when compared with control under cold stress (Table 1).

Biosynthesis of photosynthetic pigments of *L. chinensis* was rigorously hampered by cold stress which was improved by ALA application. It was noticed that chlorophyll *a*, chlorophyll *b* and total chlorophyll biosynthesis lowered by cold stress; however, surprisingly chlorophyll *a*/*b* ratio (14.72%) was improved by low temperature as compared to control in which the *L. chinensis* plants grew at normal temperature. Improvement in photosynthetic pigments viz. chlorophyll *a*, *b* and total chlorophyll was perceived by ALA treatment. It was found that biosynthesis of chlorophyll *a* (45.07%) boosted most by ALA treatment with 50 mg/L as compared to control at low temperature. Chlorophyll *b* (68.57%) and total chlorophyll content (53.95%) was elevated by treatment of ALA at a concentration of 100 mg/L when compared with at low temperature. However, it was perceived that chlorophyll *a*/*b* ratio was not affected by ALA treatment when compared with control under low temperature stress (Table 2).

Low temperature stress resulted in lowering of root activity and soluble protein content while increased the production and accumulation of MDA, soluble sugar and proline content of *L. chinensis* plants compared with control. Treatment with ALA exalted the root activity (61.89%), soluble protein (55.92%) and proline content (64.70%) as compared to control under low temperature stress and it was realized that 50 mg/L concentration better in this respect. A decrease in MDA accumulation (7.49%) and increase in soluble sugars (28.08%) was noticed by ALA treatment when compared with control at low temperature, while application of 10 mg/L of ALA proved most beneficial (Table 3).

A boost up in enzymatic antioxidants activity of *L. chinensis* plants was observed at low temperature than plants grown at normal temperature. Exaggeration in the levels of antioxidants was caused by low temperature, which was further elevated by ALA treatment. It was noticed that application of 10 mg/L ALA proved most beneficial in enhancing the activity of SOD (32.02%), POD (94.10%), CAT (239.63%), APX (476.53%) and GR (129.41%) at low temperature when compared with control. However, the effect of 50 mg/L ALA application was similar on GR activity (129.41%) of *L. chinensis* plants (Table 4).

Table 1. Morphological attributes of *L. chinensis* in response to ALA under low temperature stress

Treatments		Plant height (cm)	Leaf area (cm^2)	Fresh weight (g/plant)	Dry weight (g/plant)
Temperature	**ALA concentration (mg/L)**				
Control CK1	0	26.51 ± 0.30^{a}	2.33 ± 0.38^{b}	0.135 ± 0.030^{b}	0.027 ± 0.007^{b}
Low temperature CK2	0	19.86 ± 1.52^{c}	1.48 ± 0.36^{d}	0.118 ± 0.020^{bc}	0.026 ± 0.002^{b}
Low temperature	10	22.85 ± 1.21^{abc}	2.12 ± 0.06^{bc}	0.190 ± 0.007^{a}	0.036 ± 0.005^{a}
Low temperature	50	24.02 ± 2.72^{ab}	3.77 ± 0.05^{a}	0.098 ± 0.015^{bc}	0.021 ± 0.005^{b}
Low temperature	100	21.02 ± 2.77^{bc}	1.75 ± 0.29^{cd}	0.086 ± 0.021^{c}	0.019 ± 0.002^{b}

Values in the table are means of at least three replicates ± SE. Values followed by the same letter within columns are not significantly different according to LSD test ($P < 0.05$).

Table 2. Photosynthetic pigments of *L. chinensis* in response to ALA under low temperature stress.

Treatments		Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total chlorophyll (mg/g)	Chlorophyll *a*/*b*
Temperature	**ALA concentration (mg/L)**				
Control CK1	0	3.13 ± 0.11^{a}	1.27 ± 0.01^{a}	4.58 ± 0.04^{a}	2.65 ± 0.08^{c}
Low temperature CK2	0	2.13 ± 0.01^{d}	0.70 ± 0.01^{e}	2.91 ± 0.02^{d}	3.04 ± 0.03^{a}
Low temperature	10	2.29 ± 0.01^{c}	0.79 ± 0.02^{d}	3.16 ± 0.03^{c}	2.91 ± 0.05^{b}
Low temperature	50	3.09 ± 0.02^{a}	1.10 ± 0.02^{c}	4.08 ± 0.03^{b}	2.57 ± 0.03^{c}
Low temperature	100	2.82 ± 0.01^{b}	1.18 ± 0.07^{b}	4.48 ± 0.21^{a}	2.45 ± 0.02^{d}

Values in the table are means of at least three replicates ± SE. Values followed by the same letter within columns are not significantly different according to LSD test ($P < 0.05$).

Table 3. Root activity, MDA, soluble protein, soluble sugars and free proline of *L. chinensis* in response to ALA under low temperature stress

Treatments		Root activity (μg/g*h)	MDA (nmol/g)	Soluble protein (mg/g)	Soluble sugars (mg/g)	Proline content (mg/g)
Temperature	ALA concentration (mg/L)					
Control CK1	0	159.35±7.27^{a}	19.58±0.75^{c}	26.54±2.78^{b}	18.58±0.93$^{c\ B}$	0.07±0.04^{d}
Low temperature CK2	0	101.36±6.36^{c}	23.22±1.13^{a}	21.37±1.62^{c}	25.11±0.92$^{b\ AB}$	0.17±0.01^{c}
Low temperature	10	137.17±2.05^{b}	21.48±0.82^{b}	27.88±1.48^{b}	32.16±4.62$^{a\ A}$	0.22±0.01^{b}
Low temperature	50	164.10±6.63^{a}	23.68±0.81^{a}	33.32±2.85^{a}	24.48±0.32$^{b\ B}$	0.28±0.03^{a}
Low temperature	100	144.07±4.88^{b}	24.27±1.03^{a}	21.86±2.23^{c}	21.27±2.57$^{bc\ B}$	0.23±0.01^{ab}

Values in the table are means of at least three replicates ± SE. Values followed by the same letter within columns are not significantly different according to LSD test ($P < 0.05$).
MDA = Malondialdehyde

Table 4. Antioxidant enzymatic activities of *L. chinensis* in response to ALA under low temperature stress

Treatments		SOD (U $g^{-1}FW^{-1}$)	POD (U $g^{-1}min^{-1}$)	CAT (U $g^{-1}min^{-1}$)	APX (U $g^{-1}min^{-1}$)	GR (U $g^{-1}min^{-1}$)
Temperature	ALA concentration (mg/L)					
Control CK1	0	982.92±5.45^{c}	1041.17±121.21^{c}	37.85±15.48^{d}	6.35±1.82^{d}	0.17±0.09^{c}
Low temperature CK2	0	984.60±33.60^{c}	1112.60±38.01^{c}	71.51±7.60^{c}	14.06±3.13^{c}	0.26±0.02^{bc}
Low temperature	10	1297.73±86.67^{a}	2020.98±64.70^{a}	128.55±20.87^{a}	36.61±1.02^{a}	0.39±0.06^{a}
Low temperature	50	1131.34±9.17^{b}	1572.23±16.32^{b}	95.88±14.23^{b}	24.35±2.67^{b}	0.39±0.08^{a}
Low temperature	100	1060.39±22.85^{bc}	1113.72±7.91^{c}	55.00±21.05^{cd}	24.32±0.73^{b}	0.36±0.03^{ab}

Values in the table are means of at least three replicates ± SE. Values followed by the same letter within columns are not significantly different according to LSD test ($P < 0.05$).
SOD = Superoxide dismutase, POD = Peroxidase, CAT = Catalase,
APX = Ascorbate peroxidase, GR = Glutathione reductase

DISCUSSION

Low temperature stress causes the plants to lower their growth and development through impairment of physiological and biochemical events taking place within the plant body. The activity of photosynthetic pigments and enzymes is slowed down when the plants are exposed to cold stress which is further encountered by oxidative stress as a result of increased production and accumulation of ROS and reduced activity of antioxidant enzymes. All these physiological events are dependent on gene expression which is stimulated by plant growth regulators (Bajguz, 2000). In present study a decline in plant growth and development of *L. chinensis* was perceived by low temperature stress, however, application of ALA recovered the plant height (20.95%), leaf area (154.73%), plant fresh weight (61.01%) and dry weight (38.46%) as compared to control (Table 1). At low temperature the plants often experience the reduction in CO_2 assimilation in leaves and accumulation of sugars in the roots due to lowered sugar translocation from source to sink which leads to lowered plant growth and development (Klimov *et al*., 2002). Treatment of plants with ALA at low temperature stress improved the plant growth and development by improving the photosynthetic rate i.e. 50%-200% as compared to control which was attributable to enhanced biosynthesis of heme containing chlorophyll content (Wang *et al*., 2004). Naeem *et al*. (2010) reported results similar to our study that application of ALA improved the fresh and dry weight of shoots and roots of *Brassica napus* plants exposed to salinity stress. Similarly, Zhang *et al*. (2008) reported an improvement in the shoot length (8%), shoot fresh weight (24%-27%) and root fresh weight (18%-25%) by ALA treatment of extremely herbicide stressed plants of *B. napus* as compared to control.

Plant growth and development is a function of better and maintained photosynthetic efficiency of plants which is checked by cold stress by its injurious effects on photosynthetic apparatus, reduced biosynthesis of photosynthetic pigments and reduced efficiency of photosynthetic apparatus (Streb *et al*., 2008; Prasad *et al*., 2011). In our study, results revealed that photosynthetic pigments of *L. chinensis* plants were substantially lowered by low temperature stress compared with plants grown at normal temperature, however, ALA application posed ameliorative effect on the biosynthesis of photosynthetic pigments under cold stress up to 45%-68% as compared to control (Table 2). Exalted levels of chlorophyll content by ALA application might be due to the increased biosynthesis of chlorophyll due to ALA which is the precursor of heme containing chlorophyll (Memon and Hou, 2009). Youssef and Awad (2008) supported our results by reporting that administering the plants with ALA improved the photosynthetic pigments

of *Phoenix dactylifera* under salinity stress. Betterment of the plants in terms of growth by ALA application under cold temperature stress may be due to improvement in the biosynthesis of chlorophyll and activity of antioxidants which in turn recuperated the photosynthetic rate (Balestrasse *et al*., 2010).

Over production of ROS occurs when the plants are exposed to abiotic stresses which cause oxidative damage to the biological membranes by lipid peroxidation manifested through increased levels of MDA content (Yun-Ying *et al*., 2009). In present study, there was an increase in MDA content when *L. chinensis* plants were exposed to low temperature stress as compared to control in which plants were grown at normal temperature, conversely, application of ALA counteracted the MDA production from 18.5% to 7.5% as compared to control (Table 3). Similar results were reported by Zhang *et al*. (2008) that ALA treatment reduced the MDA content in *B. napus* seedlings exposed to herbicide toxicity. In our study, an increased accumulation of osmolytes was noticed by cold stress which was further exalted by ALA treatment to *L. chinensis* plants as compared to control (Table 3). It has been known that plants exposed to abiotic stresses accumulate osmolytes in masses to protect the membranes and organic molecules from injurious effects of ROS and helps in the maintenance of tissue water status by osmotic adjustment (Anuradha and Rao, 2007). Zhang *et al*. (2012) reported that pretreatment of cucumber leaves with ALA enhanced the proline and soluble sugars. Also similar results were reported by Korkmaz *et al*. (2010) that chilling stress increased the accumulation of proline which was further increased by the application of ALA to the pepper seedlings.

Scavenging the ROS is of supreme significance which increase and accumulate in plant cells in masses under cold stress and aggressively perturb the plant physiological and metabolic processes. A balance between ROS production and scavenging by anti-oxidative system is necessary in such conditions to keep the plant growth in pace under stressed conditions. In our study, an increase in enzymatic antioxidants was the consequence of low temperature stress which was further aggravated by ALA application to the *L. chinensis* plants (Table 4). Antioxidants are mediated by gene expression which is further regulated by plant growth substances (Zhen *et al*., 2012). Li *et al*. (2011) reported that ALA treatment increased the activity of certain antioxidants in cucumber seedlings exposed to drought stress.

Conclusion: The growth and development of *L. chinensis* plants was impaired at low temperature as a result of decreased biosynthesis of photosynthetic pigments and enhanced lipid peroxidation as indexed by exacerbated levels of MDA. Conversely, exogenous application of ALA on *L. chinensis* plants at low temperature assisted in the accomplishment of stress tolerance through improved growth and biosynthesis of photosynthetic pigments and elevated accumulation and activity of osmolytes and antioxidant enzymes while lowering the MDA content. On the basis of these results it is recommended that ALA should be applied exogenously to *L. chinensis* plants for induction of low temperature stress tolerance.

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