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EXOGENOUS APPLICATION OF BRASSINOLIDE AMELIORATE CHILLING STRESS IN *LEYMUS CHINENSIS* (TRIN.) TZVEL. BY MODULATING MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TRAITS

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Abstract

The effect of exogenously applied brassinolide (BR) at various concentrations *viz*. 0.01, 0.1 and 1.0 mg/l on growth and physiological attributes of *Leymus chinensis* (Trin.) Tzvel. was studied at low temperature stress in a pot culture. Foliar application of BR exerted an ameliorative effect on plant height, leaf area, plant fresh and dry weight, chlorophyll a and b, total chlorophyll, carotenoids and chlorophyll a/b ratio, while, further boosting the accumulation of proline, soluble proteins and sugars, exaggerating the activity of antioxidant enzymes and reducing the accumulation of Malendialdehye (MDA). And the highest value was obtained by treatment with 0.1 mg/l BR. The application of BR enhanced the growth and development of *L. chinensis* under low temperature stress by improving the biosynthesis of photosynthetic pigments and reducing MDA accumulation by modulating the osmolyte contents and activity of antioxidant enzymes.

Introduction

China is rich in natural grasslands covering an area of 41% of the total area and considered integral renewable resources. *Leymus chinensis* (Trin.) Tzvel. is commonly found in grasslands which is called sheep grass containing high nutritious value rich in protein, carbohydrate and mineral contents. Moreover, it is a perennial grass with rhizomes lying horizontally in soil and has good palatability and high forage value (Huang *et al*. 2002). Nonetheless, the grasslands of China are under threat of degradation due to increased land use, grazing pressure over time and abiotic stresses. The degradation of grasslands has been rapidly aggravated during the recent years and has been reported nationally up to 90% (Bai *et al*. 2004).

Low temperature stress hampers the growth and development of plants by lowering enzyme activity (Li *et al*. 2012), membrane rigidification, destabilization of proteins structure, stabilization of secondary structure of RNA, accumulation of reactive oxygen species (ROS) (Ruelland *et al*. 2009), impairment of photosynthetic machinery and membrane damage (Strauss and Van Heerden 2011). Under low temperature stress, plants modify the lipid composition of biological membranes like enhancement in unsaturated fatty acid and phospholipid content in order for stress mitigation (Moellering *et al*. 2010).

Brassinosteroids (BRs) are a novel class of plant hormones that are polyhydroxysteroids and are involved in the growth and development of plants (Asami *et al*. 2000, Fukuta *et al*. 2002). BRs have been known to play stress protective roles in plants against diverse variety of stresses (Dhaubhadel *et al*. 1999, Ozdemir *et al*. 2004). In addition, studies have revealed that BRs enhance the accumulation of osmolytes and elevate the activity of antioxidant enzymes and

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ultimately results in reduced synthesis and accumulation of ROS in plant cells. Scavenging of ROS by improved antioxidants activity reduces the accumulation of MDA which is the outcome of lipid peroxidation (Yun and Hua 2008). Therefore, understanding the stress mitigatory role of BRs, the present study was undertaken to study the possibility of exogenously applied BR on morphological, physiological and biochemical attributes in *L. chinensis* plants under low temperature stress.

Materials and Methods

Seeds of *Leymus chinensis* (Trin.) Tzvel. were collected from natural community of the Ecological Experimental Station of *Leymus chinensis* in Xilingole grassland in late July 2013. The seeds were stored in a cloth bag after drying at room temperature and placed at 4°C refrigerator. The present experiment was conducted in Physiological and Biochemical Laboratory, College of Agronomy and Biotechnology, Southwest University, Chongqing, China in 2014. Sowing of seed was carried out in a greenhouse incubator (light 10 h/30°C; dark 14 h/20°C). The experimental area situated between latitudes 29° 49′ 32″ N, longitudes 106° 26′ 02″ E and altitude 220 m. After one week seedlings were transplanted to sand culture in 20 cm diameter pots. Hoagland nutrient solution was poured everyday in the evening. When attained the height of 18 - 21 cm, thinning was made and 25 seedlings were kept in each pot. The foliar application of BR was done on leaves with three different concentrations *viz*. 0.01, 0.1 and 1.0 mg/l while distilled water was sprayed as control. Seedlings were exposed to normal and low temperature stresses (CK_2). Seedlings were kept at room temperature (control (CK_1) 20°C/15°C (day/night), light 10 hrs) and in greenhouse incubator at low temperature (5°C/1°C (day/night), light 10 hrs), four days after the second spraying. There were five treatments having three replications. Each morphological, physiological and biochemical traits were determined 7 days after treatments.

Measurement of morphological traits was made by uprooting the seedlings from pots. Seedlings were rinsed with tap water followed by 2 - 3 times rinsing with distilled water and plant height was measured from the tip of the stem to parietal lobe at the base of plants. Adhered water was absorbed from the seedlings by using filter paper. Leaf area was determined with MSD-971 scanner. Fresh weight of seedlings was taken and then dried in an oven at 105°C for 30 minutes followed by drying at 65°C till constant weight was sustained.

Photosynthetic pigments, namely chlorophyll *a* and *b*, and carotenoids were measured following the procedure of Arnon (1949). Soluble proteins were determined by using coomassie brilliant blue method (Bradford 1976). Quantification of soluble sugars was made by anthrone color method (Zhu *et al*. 2012). The root activity was measured using the triphenyl tetrazolium chloride (TTC) method of Higa *et al*. (2010). The malondialdehye (MDA) content was assessed by using thiobarbituric acid (TBA) assay (De-Vos *et al*. 1991). Proline content was determined following ninhydrin method (Bates *et al*. 1973). The activities of antioxidant enzyme *viz*. superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) were determined following the method of Parida (2004).

The data collected from the experiment was analyzed statistically by using Microsoft Excel and statistical software program SPSS19.0.

Results and Discussion

Low temperature stress CK_2 caused substantial reduction in plant growth. Considerably low plant height, leaf area, and fresh and dry weight of *L. chinensis* plants were observed under low temperature stress in comparison with control CK_1. However, exogenous application of BR assisted in the mitigation of deleterious effects of low temperature stress on growth of

L. chinensis. There was 59.4, 109.7, 181.6 and 69.8% increase in plant height, leaf area, fresh and dry weight, respectively, by the application of 0.1 mg/l BR at low temperature stress against CK_2 (Table 1). Chlorophyll *a* and *b*, total chlorophyll and chlorophyll *a/b* ratio were noticeably reduced while carotenoid content increased under low temperature stress. Treatment with 0.1 mg/l BR at low temperature stress increased the synthesis of chlorophyll *a* (183.4%), chlorophyll *b* (8.74%), carotenoids (55.9%), total chlorophyll content (114.6%) and chlorophyll *a/b* ratio (160.7%) than CK_2 (Table 2).

Table 1. Effect of BR on morphological attributes of *L. chinensis* under low temperature stress.

Treatments	Plant height (cm)	Leaf area (cm^2)	Fresh weight (mg/plant)	Dry weight (mg/plant)
Control (CK_1)	26.97 ± 1.97 a A	2.054 ± 0.234 a A	680.3 ± 57.3 a A	207.7 ± 13.2 a A
Low temp. stress (CK_2)	15.93 ± 2.04 c C	0.556 ± 0.020 d C	211.2 ± 23.2 d D	58.3 ± 60.0 d D
Stress + 0.01 mg/l BR	24.63 ± 0.15 a A	0.807 ± 0.009 c C	561.2 ± 37.0 b BC	87.7 ± 65.0 b BC
Stress + 0.1 mg/l BR	25.40 ± 1.11 a A	1.166 ± 0.043 b B	594.7 ± 37.6 b AB	99.0 ± 40.0 b B
Stress + 1 mg/l BR	20.03 ± 1.36 b B	0.748 ± 0.073 cd C	451.8 ± 12.4 c C	72.3 ± 67.0 c CD

Values are mean ± SE. Values followed by different lower case letters within each column are significantly different according to Duncan's multiple range test ($p < 0.05$). Values followed by different capital letters within each column are extremely significantly different according to Duncan's multiple range test ($p < 0.01$).

Root activity of *L. chinensis* was much lowered at low temperature stress. However, accumulation of MDA, free proline, soluble proteins and soluble sugars were substantially enhanced under low temperature conditions. Foliar application of 0.1 mg/l BR noticeably increased the root activity (28.96%) at low temperature than CK_2. Furthermore, lesser MDA accumulation (43.80%) and more soluble protein (14.35%) and soluble sugars (11.62%) accumulation was noticed at low temperature by treatment with 0.1 mg/l BR compared with control. However, free proline enhanced 135.2% at 0.01 mg/l BR application than CK_2 (Table 3). Activity of antioxidant enzymes was boosted under low temperature stress which was further improved by treatment with BR to *L. chinensis* plants. Nevertheless, increased activity of SOD (28.22%), POD (71.88%), CAT (82.28%) and APX (56.47%) was perceived by treatment with 0.1 mg/l BR. However, GR (273.2%) activity increased in by 0.01 mg/l BR treatment than (CK_2) which was statistically similar with 0.1 mg/l BR treatment (Table 4).

Cold stress severely affects the growth and development of plants by modifying the vital physiological processes such as reduction in photosynthesis, altered enzyme activity and over production of ROS leading to electrolyte leakage and ultimately membrane damage (Hu *et al*. 2010). Regulation of these physiological events might be dependent on gene expression which is ultimately stimulated by plant growth regulators (Bajguz 2000) exhibiting the supreme importance of plant growth regulators under stressed conditions. In the present investigation, growth and development of *L. chinensis* plants severely hampered at low temperature stress compared with normal temperature, however, exogenous BR application markedly improved the growth to a great extent. Similarly, Honnerová *et al*. (2010) also noted a considerable reduction in plant height and leaf length of low temperature stressed maize plants when compared with plants growing under normal temperature. However, foliar application of 0.1 mg/l BR considerably enhanced the growth related attributes of *L. chinensis* plants at low temperature than control (CK_2) (Table 1). Which was found consistent with Krishna (2003) who reported growth recovery of maize seedlings by treatment with BRs following low temperature stress. It has been known that

Table 2. Effect of BR on photosynthetic pigments of *L. chinensis* under low temperature stress.

Treatments	Chl *a* (mg/g)	Chl *b* (mg/g)	Carotenoid (mg/g)	Total chl (mg/g)	Chl *a/b*
Control (CK_1)	1.717 ± 0.023 a A	0.651 ± 0.011 a A	0.016 ± 0.005 d C	2.384 ± 0.032 a AB	3.638 ± 0.017 a A
Low temperature stress (CK_2)	0.631 ± 0.073 c C	0.469 ± 0.001 c C	0.034 ± 0.005 c B	1.134 ± 0.072 d D	1.346 ± 0.152 b B
Stress + 0.01 mg/l BR	1.756 ± 0.010 a A	0.486 ± 0.013 c BC	0.044 ± 0.004 b AB	2.262 ± 0.043 b B	3.617 ± 0.121 a A
Stress + 0.1 „	1.788 ± 0.003 a A	0.510 ± 0.008 b B	0.053 ± 0.004 a A	2.434 ± 0.049 a A	3.509 ± 0.059 a A
Stress + 1 „	0.767 ± 0.045 b B	0.479 ± 0.022 c BC	0.051 ± 0.003 ab A	1.297 ± 0.053 c C	1.605 ± 0.100 b B

Values are mean ± SE. Values followed by different lower case letters within each column are significantly different according to DMRT ($p < 0.05$). Values followed by different capital letters within each column are extremely significantly different according to DMRT ($p < 0.01$).

Table 3. Effect of BR on root activity, MDA, free proline, soluble protein and sugar contents of *L. chinensis* under low temperature stress.

Treatments	Root activity (μg/g/h)	MDA (nmol/g)	Free proline (μg/g)	Soluble protein (mg/g)	Soluble sugars (mg/g)
Control (CK_1)	152.33 ± 1.35 a A	16.89 ± 0.59 d D	34.36 ± 6.54 e D	10.38 ± 0.81 d D	6.91 ± 1.47 d D
Low temperature stress (CK_2)	98.25 ± 2.82 d C	34.79 ± 2.06 a A	369.56 ± 23.85 d C	21.67 ± 0.08 c C	27.79 ± 0.20 c C
Stress + 0.01 mg/l BR	106.99 ± 0.71 c C	20.61 ± 0.62 c BC	869.36 ± 85.39 a A	22.45 ± 0.14 c BC	29.73 ± 0.27 b AB
Stress + 0.1 „	126.71 ± 2.08 b B	19.55 ± 0.22 c CD	719.27 ± 124.17 b AB	24.78 ± 0.28 a A	31.02 ± 0.43 a A
Stress + 1 „	108.06 ± 5.08 c C	23.74 ± 1.64 b B	542.29 ± 65.95 c BC	23.35 ± 0.13 b B	28.82 ± 0.27 bc BC

Values are mean ± SE. Values followed by different lower case letters within each column are significantly different according to DMRT ($p < 0.05$). Values followed by different capital letters within each column are extremely significantly different according to DMRT ($p < 0.01$).

Table 4. Effect of BR on activity of antioxidant enzymes of *L. chinensis* under low temperature stress.

Treatments	SOD (U/g/FW)	POD (U/g/min)	CAT (U/g/min)	APX (U/g/min)	GR (U/g/min)
Control (CK_1)	619.48 ± 46.83 d D	180.66 ± 25.65 d D	49.71 ± 4.93 d D	5.82 ± 0.27 d C	0.061 ± 0.0037 d D
Low temperature stress (CK_2)	758.37 ± 17.87 c C	273.40 ± 3.86 c C	139.74 ± 19.52 c C	20.01 ± 0.69 c B	0.157 ± 0.0307 c C
Stress + 0.01 mg/l BR	929.74 ± 7.74 a A	341.90 ± 18.69 b B	186.15 ± 2.69 b B	23.07 ± 1.80 b B	0.586 ± 0.0275 a A
Stress + 0.1 ,,	972.42 ± 14.18 a A	469.93 ± 16.33 a A	254.72 ± 6.80 a A	31.31 ± 1.64 a A	0.579 ± 0.0156 a A
Stress + 1 ,,	841.66 ± 25.10 b B	308.04 ± 14.75 bc BC	178.13 ± 6.01 b B	21.26 ± 0.88 bc B	0.361 ± 0.0190 b B

Values are mean ± SE. Values followed by different lower case letters within each column are significantly different according to DMRT ($P < 0.05$). Values followed by different capital letters within each column are extremely significantly different according to DMRT ($p < 0.01$). SOD = Superoxide dismutase, POD = Peroxidase, CAT = Catalase, APX = Ascorbate peroxidase, GR = Glutathione reductase.

abiotic stresses pose deleterious effects on photosynthesis by disrupting the photosynthetic machinery and inhibiting the biosynthesis of photosynthetic pigments (Prasad *et al*. 2011). In the present study, a remarkable reduction in photosynthetic pigments *viz*. chlorophyll *a, b*, total chlorophyll contents and chlorophyll *a/b* ratio was noticed under low temperature stress while, carotenoid content increased (Table 2). Decreased biosynthesis of photosynthetic pigments due to low temperature stress might be ascribed to suppress gene expression for chlorophyll synthesis (Yang *et al*. 2005). However, effect of BR is concentration dependent, showing significant stress amelioration at 0.1 mg/l application (Table 2). These results are further supported by transcriptional and translational alterations induced by BR (Bajguz 2000). At low temperature, root activity declined as compared to control; however, BR application exerted an ameliorative effect on root activity to some extent (Table 3). In addition, it has been reported that application of BR improves the root activity under stressed conditions (Yun *et al*. 2009).

Accumulation of ROS is exaggerated under low temperature stress and aggressively damages the biological membranes and organic molecules. This ultimately results in enhanced MDA accumulation which is the end product of lipid peroxidation and serves as an index of oxidative damage caused by ROS (Xu *et al*. 2009). Moreover, the plants produce osmoprotectants which protect the plants from injurious effects of ROS to biological membranes under abiotic stresses (Anuradha and Rao 2007). Free proline, soluble protein and sugar content in *L. chinensis* were enhanced at low temperature, which was further exalted by the application of BR at 0.1 mg/l concentration (Table 3). Wu *et al*. (2014) also reported an improved accumulation of proline in BR treated *Solanum melongena* plants under cold stress. Reports pertaining to accumulation of soluble proteins and sugars under stressed conditions have shown a marked increase by treatment with BR which is considered important for normal functioning of cellular processes and assists in the acquisition of stress tolerance by plants (Geetika *et al*. 2014, Wu *et al*. 2014). Plants synthesize antioxidants which scavenge the ROS and attain significant degree of tolerance against abiotic stresses (Wang *et al*. 2012). In this study, the exogenous application of BR embellished the activity of antioxidant enzymes and better response was attained by treatment with 0.1 mg/l BR (Table 4). The improved activity of antioxidant enzymes by BR to scavenge the toxic ROS may be the consequence of synthesis of antioxidants or may be the result of enzyme activation, mediated by transcriptional and translational changes in related genes (Ali *et al*. 2008). Pertaining to antioxidants, Liu *et al*. (2009) and Wu *et al*. (2014) reported enhanced antioxidants activity of *Chorispora bungeana* and *S. melongena*, respectively by treatment with BR under cold stress.

A reduction in growth, photosynthetic pigments and enhancement in accumulation of MDA, osmolytes and antioxidants was found in *L. chinensis* plants at low temperature. Conversely, exogenous application of BR to *L. chinensis* substantially increased the osmoprotectants and antioxidant enzymes activity while lowered the MDA content at low temperature. Treatment with 0.1 mg/l BR showed better results in stress mitigation. It may be assessed that application of BR aids in the acquirement of low temperature stress tolerance by improving the growth, photosynthetic pigments and antioxidants activity.

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