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Alteration of antioxidant machinery by 28-homobrassinolide in *Brassica juncea* L. under salt stress

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ABSTRACT

Induction of oxidative stress occurs under salt stress, which enables the production of ROS. Plants have evolved an elaborate system of antioxidants which help to scavenge these indigenously generated reactive oxygen species. Brassinosteroides (BRs) are sixth class of plant hormones which influence number of physiological and morphological processes in plants and play diverse role in plant growth and development. The present work was conducted to study the effect of 28- homobrassinolide (homoBL) on lipid peroxidation and antioxidants (superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase) content of 30 day old plants of Brassica juncea L. subjected to180 mM salt stress. The seeds of Brassica juncea var. RLC-1 were pre-soaked in different concentrations of 28-homoBL (10^{-6}, 10^{-9}, 10^{-12} M) for 8 hours. Finding of the present study revealed that application of 28-homoBL enhanced the antioxidants level in salt stressed plants of Brassica juncea L. which was found to be provide tolerance against extreme salt stress.

Keywords: *Brassica juncea*, Salt stress, 28-homobrassinolide, Superoxide Dismutase, Lipid peroxidation

Abbreviations: *BRs: Brassinosteroides; 28-homoBL: 28-homobrassinolide; TCA: trichloroacetic acid; NaoH: sodium hydroxide; MDA: Malondialdehyde; SOD: superoxide dismutase; H_2O_2: hydrogen peroxide; POD: peroxidase; CAT: catalase; APOX: ascorbate peroxidase; GR: glutathione reductase; NBT: nitro blue tetrazolium;. ROS: reactive oxygen species; TBA: thiobarbituric acid.*

INTRODUCTION

Nature nurtures all living plants as true mother by providing all required resources of life like air, light, water, temperature and soil in apposite and ample quantity. Finest level of these factors helps the plants to grow in healthy conditions. Adversities in these environmental conditions results into major loss in productivity. Among these salt stress is a major constraint to agricultural yield. Extreme salt stress at higher level affects the plant growth by hyperosmotic and hyperionic stress. Extreme changes in salt stress causes overproduction of reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO.). Plants possess antioxidant defense machinery that protects against oxidative stress damages (Gill and Tuteja, 2010).To increase plant productivity under stress extensive use of growth regulators is a common practice all over the world. Several plant hormones are also implicated in modulating the responses to various stresses, including ethylene (Vahala *et al*., 2003), abscisic acid (Kovtun *et al*., 2000), salicylic acid (Metwally *et al*., 2003) and brassinosteroids (Ozdemir *et al*., 2004). Brassinosteroids (BRs) are class of plant polyhydroxy steroids that have been recognized as a new class of phytohormones, play a crucial role in plant growth and development. BRs can protect plants from various biotic and abiotic stresses, such as those caused by salt stress (Alyemeni *et al*., 2013;Fariduddin *et al*., 2014). Antistress properties of different active forms of BRs have been suggested by various workers as salt stress (Ali *et al*., 2008), cold stress (Hu *et al*., 2008), heat stress (Ogweno *et al*., 2008), and heavy metal stress (Rady, 2011). BRs found to

play crucial role in protecting plants from adverse effects of salt stress by inducing BRs mediated up and/or down regulation of specific genes (Divi *et al*., 2010; Chung *et al*., 2014).The present study was undertaken to study the effect of 28-homoBL on growth, lipid peroxidation and antioxidant (SOD, POD, CAT, APOX and GR) activities of *B. juncea* L. plants under temperature stress.

MATERIALS AND METHODS

Plant material and growth conditions: Seeds of *B. juncea* L. cultivar(RLC-1) were procured from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana, India. Seeds were surface sterilized with 0.01% $HgCl_2$ and rinsed 5-6 times with double distilled water. The sterilized seeds were soaked for 8h in different concentrations of 28-homobrassinolide (Sigma-Aldrich. USA) (10^{-6}, 10^{-9} and 10^{-12}M). The treated seeds were propagated in triplicate in cemented pots under natural field conditions. 3 kg soil was added to each pot and 1 L solution of 180 mM NaCl was added to each pot at the time of sowing. Plants were sampled on the 30th day after sowing for measuring morphological and biochemical parameters.

Morphological parameters:
Morphological data in terms of shoot length, fresh and dry weights was measured on 30th days after sowing.

Biochemical parameter
Estimation of protein content:

Fresh leaf tissues 0.5 g were homogenized in 100mM potassium phosphate buffer, pH -7.0 using a prechilled mortar and pestle. Total protein was extracted by10% trichloroacetic acid (w/v) and kept at 4 °C for precipitation. After that it was centrifuged at 5000 g_n and collected the residue containing precipitate of protein. The precipitate were dissolved in 0.1 N NaOH and again centrifuged. The supernatant was collected and used for protein estimation with the help of Lowry *et al*., (1951). Absorbance of protein was estimated at 750 nm using bovine serum albumin as standard and expressed per gm fresh weight.

Lipid peroxidation:
Lipid peroxidation was determined in terms of MDA content described by Heath and Packer (1968). 1 mL of extract was added to 2 mL of a reaction solution containing 20 % TCA (w/v) and 0.5% TBA (v/v). The solution was placed in a water bath at 95 ºC for 30 min and then transferred to an ice water bath. After this solution was centrifuged at 10,000 g_n for 10 min and the absorbance of the supernatant was recorded at 532 and 600 nm.

Estimation of Superoxide Dismutase (EC 1.15.1.1):
SOD activity was determined using the method of Kono (1978). 3 mL reaction mixture was prepared containing 50 mM sodium carbonate (w/v), pH 10.2, 750 µL NBT (w/v), 0.1 mM EDTA (w/v), 1 mM hydroxylamine (w/v), 0.03% triton-X-100(v/v) and 70 µL enzyme extract. Absorbance of SOD was recorded at 560 nm for 2 min.

Estimation of Guaiacol Peroxidase (EC 1.11.1.7):
POX activity was measured according to the method of Putter (1974) by taking 3 mL of reaction mixture containing 50 mM phosphate buffer (w/v), PH 7.0, 20 mM guaiacol (v/v), 12.3 mM H_2O_2(v/v)and 100 µL enzyme extract. POX activity was determined by measuring the absorbance at 436 nm and using extinction coefficient of 25 mM^{-1} cm^{-1}.

Estimation of Catalase (EC 1.11.1.6):
CAT activity was measured according to Aebi (1984) by taking 3 mL reaction mixture containing 100 mM phosphate buffer (v/v), pH 7.0, 150mM H_2O_2(v/v) and 100 µL enzyme extract. The reaction was started by addition of H_2O_2 and CAT activity was measured as decrease in absorbance at 240 nm for 30 sec. Enzyme activity was computed by using an extinction coefficient 6.93 × $10^{-3}$$mM^{-1}$ cm^{-1}.

Estimation of Ascorbate peroxidase (EC 1.11.1.11):
Activity of APOX was measured following the method of Nakano and Asada (1981) by monitoring the rate of decrease in absorbance at 290 nm for 1 min. The reaction mixture contained 50mM phosphate buffer (w/v), pH 7.0, 5.0mMascorbate (w/v), 1.0mM H_2O_2(v/v) and 100 µL enzyme extract. Enzyme activity was calculated by using an extinction coefficient 2.8 $mM^{-1}cm^{-1}$.

Estimation of Glutathione Reductase (EC 1.8.1.7):
Activity of GR was measured according to Carlberg and Mannervik (1975). The reaction mixture contained 1.5 mL of 50 mM phosphate buffer (w/v), pH 7.0, 3 mM EDTA (w/v), 0.1 mM NADPH (w/v), 1 mM GSSG (w/v) and 600 µL enzyme extract. Activity of GR was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH at 340 nm for 1 min.

RESULTS

Morphological analysis:
Growth in length and biomass in terms of fresh and dry weight in plants of *B.juncea* L. were reduced by 180 mM salt treatment (Figure 1A, B, C). However, on average, the levels of salt sensitivity were higher under extreme conditions. Where38 % decrease in shoot length and 23 %, 46% decline in fresh and dry weights (Figure 1B, C) was observed as compared to control plants respectively.

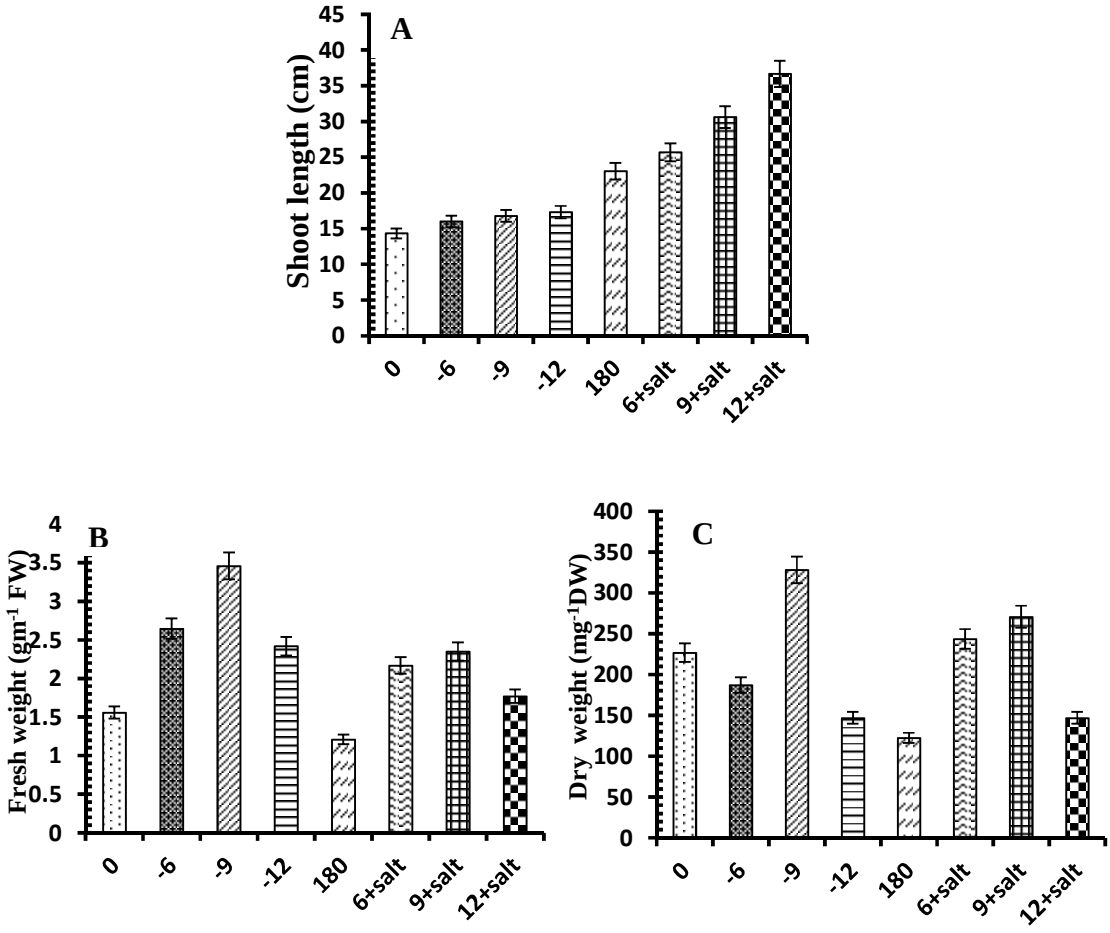

Figure 1- Effect of 28-Homobrassinolide on Shoot length (A), Fresh weight (B), Dry weight (C), on 30-day old plants of *B. juncea* L. under (180 mM) NaCl stress [Bars represent the SE (n=3)]

Biochemical analysis:
Higher rate of lipid peroxidation (MDA) leads to imbalance of membrane stability. Salt irrigation (180 mM) to thirty day old plants of *B. juncea* L. enhanced the 323%MDA content (Figure 2 B).Impact of 180 mM NaCl was much higher on production of H_2O_2 through oxidation of plasma membrane. Exogenous application of 10^{-9}M 28-homoBL at seed priming level improve membrane stability by 29 % under control conditions.

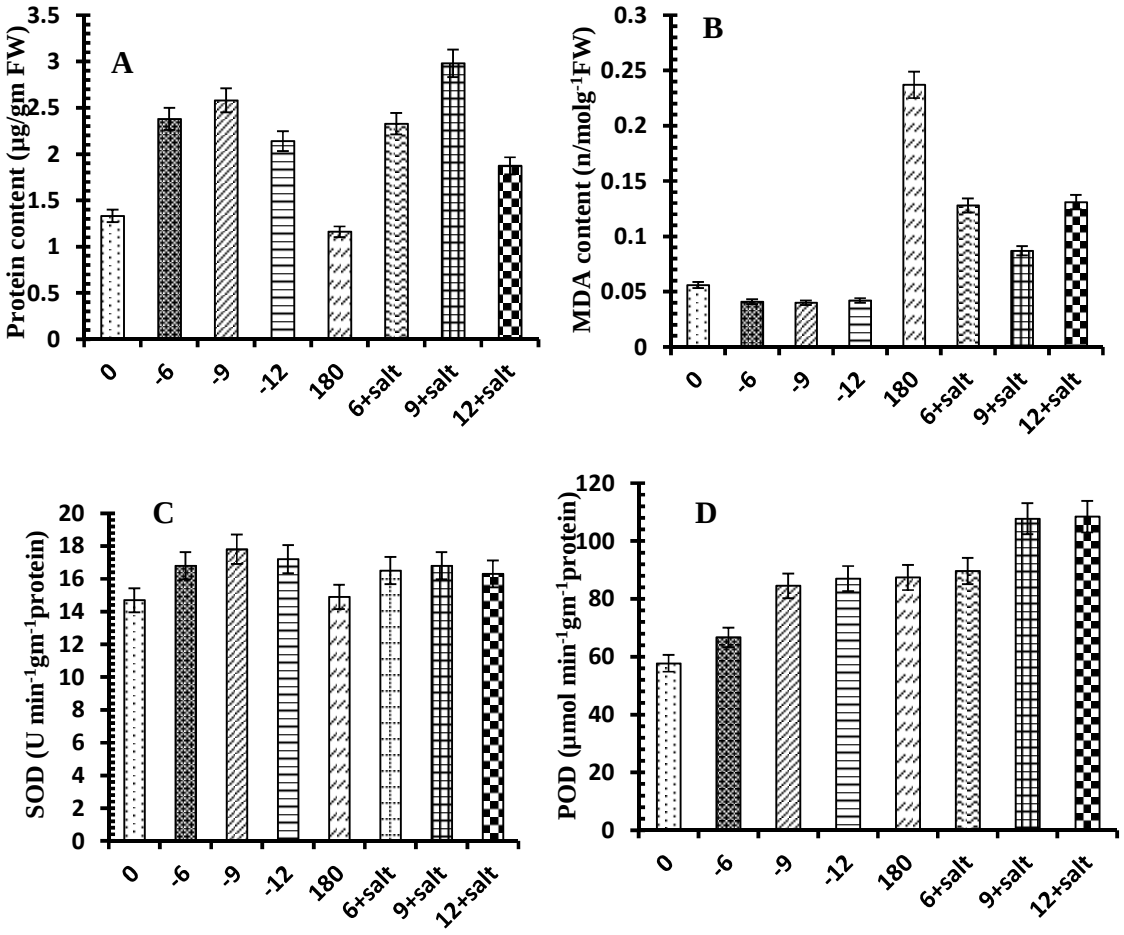

Figure 2- Effect of 28-Homobrassinolide on Protein content (A), MDA content (B), SOD activity (C), POD activity (D), on 30-day old plants of *B. juncea* L. under (180 mM) NaCl stress [Bars represent the SE (n=3)]

The SOD activity did not change in distilled water control seedlings but 1% increase in SOD activity (Figure2C) was found in 180 mM NaCl treated plants. when supplementation of salt is given with different concentrations of 28-homoBL then maximum increase 14 % was found in 10^{-9}+180mM concentration which is followed by 10^{-6} M and 10^{-12}M 28-homoBL treated plants 12 % and 10% respectively .

POD activity was found to be maximum 15% in case of 180 mM NaCl concentration (Figure2D). Which was further increased on treatment with different concentration of 28-homoBL. Maximum increase 51% was noticed in 10^{-9}M 28-homoBL concentration which is tracked by10^{-6} M 50% and 10^{-12}M 46% respectively.

However, Increase in CAT activity 6 %in plants treated with salt only was observed at 180mMNaCl(Figure 3A). Maximum enhancement in CAT activity 30 % were found in plants treated with 28-homoBL at 10^{-9}mM and grown under 180 mM of NaCl concentration which is followed by 17% and 13 % increase with 10^{-6}M and 10^{-12} M 28-homoBL treatments respectively.

The APOX activity was significantly changed by NaCl treatment 63%in plants of *B.juncea* L. (Figure 3B). However, the activity of this enzyme was always higher in 28-homoBL treated plants, regardless of the concentration given in present experiment. The activity of APOX in the 180 mM treated plants supplemented with 10^{-9} M concentration was, about three times higher than plants treated with distilled water control.

Similarly, GR activity was maximum (146%) in plants raised from 10^{-6}M 28-homBL pre-treated plants and grown under 180mM NaCl (Figure 3C) which is followed by 10^{-9} M concentration (142%) of 28-homoBL and 10^{-12}M 28-homoBL (111%) supplemented with 180 mM NaCl concentration respectively.

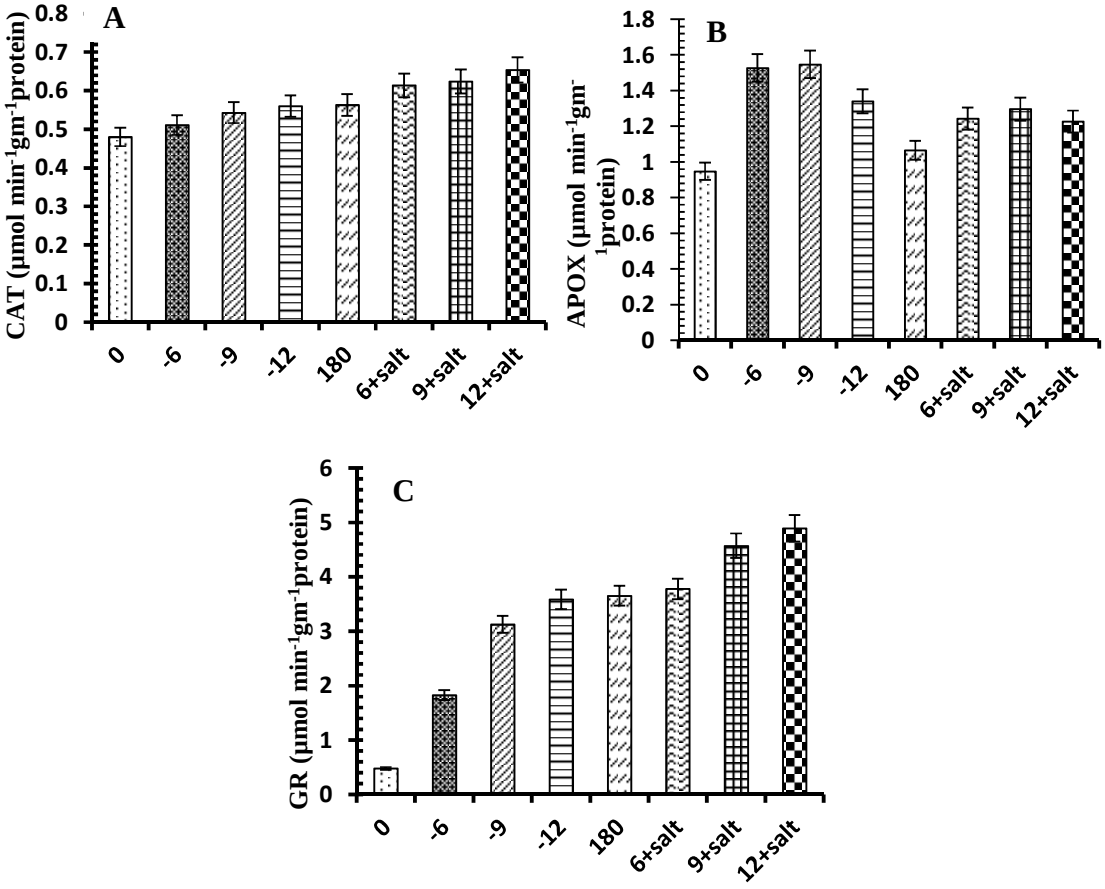

Figure 3- Effect of 28-Homobrassinolide on CAT activity (A), APOX activity (B), GR activity (C), on 30-day old plants of *B. juncea* L. under (180 mM) NaCl stress [Bars represent the SE (n=3)]

DISCUSSION

The present work showed that if based on shoot growth, fresh and dry weight of *B. juncea* L. seed application of 28-homoBL improved seedling growth (Figure 1A-C). In accordance with our results, the exogenous application of 28-homoBL has been shown to lead increase in biomass of *B. juncea*, which was also previously reported for *Saturejakhu zestanica* (Eskandari and Eskandari, 2013) and *Raphanus sativus* (Sharma *et al.*, 2010). Exogenous application of 28-homoBL show a protective effect on growth of *B. juncea* which was decreased due to salt treatment, the vigor of salt-treated *B. juncea* plants seemed deleterious without 28-homoBL. In addition, exogenous application of 28-homoBL significantly increased soluble protein content of *Brassica* plants in 28-homoBL and 28-homoBL+NaCl-treated groups compared with the control group. Bajguz (2000a) also found that BRs increased DNA, RNA and protein contents of *Chlorella vulgaris* as the number of cells increased in the medium. As salinity stress can cause membrane damage as a result of stomatal closure, decreased hydrolytic enzyme activity and increased lipid peroxidation level, it may stimulate formation of (AOS) antioxidant system, such as H_2O_2, $O^{2-}$and OH^- radicals. Among AOS, superoxide is dismutated by SOD enzyme into H_2O_2 and is further scavenged by CAT and various peroxidases. APOX and GR also play a key role by reducing H_2O_2 to water through the ascorbate–glutathione cycle (Noctor and Foyer 1998). It is widely accepted that AOS are responsible for various stress-induced damages to macromolecules and ultimately to cellular structures. Consequently, the role of antioxidative enzymes, such as POX, SOD, CAT, GR and APOX becomes very important. In this study, we were able to demonstrate that

lipid peroxidation level induced by NaCl was significantly lower in the 28-homoBL-treated *Brassica juncea* plants alone salt stress than in the plants under salt stress without 28- homo BL treatment, which revealed protection of lipid membranes from AOS-induced damage. As membrane destruction results from AOS-induced oxidative damage (McCord 2000; Jain *et al.*, 2001), the plants in 28homoBL+NaCl group might be scavenging AOS more effectively than the seedlings treated with NaCl alone. The result indicated that there was a negative relationship between SOD activity and lipid peroxidation or MDA content in *B. juncea*. as indicated in (Figure 2B-C). SOD activity increased with increasing salt stress levels. When SOD activity was high, ROS, especially superoxide radical, scavenging was done properly and thus, damage to membranes and oxidative stress decreased, leading to the increase of tolerance to oxidative stress. Salt stress increased the superoxide level in cells. If this radical is not scavenged by SOD, it disturbs vital biomolecules (Mittler, 2000). Moreover, it inactivates antioxidant enzymes which are very important for H_2O_2 scavenging such as catalases (Kono and Fraidovich, 1983) and peroxidases (Fridovich, 1989). In *B.juncea* superoxide radical production increases with the increase of salt stress. For this reason scavenging of this dangerous radical was not done perfectly. Consequently, this radical attacks vital biomolecules that mentioned before and damage to membranes happens in this cultivar. Esfandiari *et al.*, (2007), Candan and Tarhan (2003), Martinez *et al.*, (2001), Scandalios (1993), Sen Gupta *et al.*, (1993) and Zhao *et al.*, (2006) had similar findings and expressed that the increase in SOD activity and decrease in oxidative damage were closely related.

CAT is another important antioxidant enzyme (Figure 3A) that converts H_2O_2 to water in the peroxysomes (Fridovich, 1989; McCord and Fridovich, 1969). In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration (Morita *et al*., 1994). Higher activity of CAT and APX (Figure 3B) decrease H_2O_2 level in cell and increase the stability of membranes and CO_2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2 directly inhibits CO_2 fixation (Yamazaki *et al.*, 2003). In our results, CAT activity at 180 mM NaCl, in *B. juncea* was higher than 28-homoBL treated plants and exhibited a significant difference.

GR activity in *B. juncea* plants increased under salt stress (Figure 3C). 28-homoBL application with NaCl cause significant increase in GR activity in comparison to the plants applied with NaCl alone. Likewise, after brassinolide treatment, Arora *et al*., (2008).also showed increase in GR activity in leaves of a salt-sensitive maize cultivar under salt stress. Since increased GR activity enhances metal stress tolerance (Sharma *et al*. 2007), significant increase in GR activity in *B. juncea* under NaCl probably seems to be resulted from 28- homo BL treatment could alleviate the inhibitory effect of NaCl on GR activity, as well as CAT and SOD activities in the present study.

CONCLUSION

It is concluded from present study that antioxidants production during salt stress is considered as very important in view of its role in stress tolerance. Further BRs application overcome the salinity stress by enhancing the antioxidants accumulation and thus developed the tolerance. However, further studies at molecular level are needed to elucidate the complete mechanism involved in 28-homoBLinducing salt tolerance in plants

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