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ORIGINAL ARTICLE

Brassinosteroid (Brassinolide): An Alleviating Option Under Salinity Stress in Mungbean

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ABSTRACT
Brassinosteroids are widely used to overcome various abiotic stresses including salinity (NaCl) stress in plants. The presence of salinity in the soil above a particular level is proposed to check not only morphological characteristics but also metabolic and biochemical activities. The present study investigates the role of brassinolide (BL) in inducing tolerance against salinity stress in mungbean plants taken in net house and growth chamber. Brassinolide (BL) supplementation reduced the inhibitory effect of salinity stress on plant growth and increased antioxidant enzyme activities in mungbean genotypes. Plants exposed to salinity (NaCl 100mM) exhibited a significant decline in fresh weight, dry weight, number of leaves plant^{-1}, nitrate reductase and catalase activities with an increase in hydrogen peroxide content in two salinity sensitive mungbean genotypes (HUM-12, HUM-16). However, the follow up treatment with BL from 0.01 to 0.05mM in combination with induced salinity (NaCl 100mM) and 0.05mM alone significantly improved the above parameters. The results of present study demonstrate the protective role of Brassinolide (BL) against imposed salinity stress in mungbean.
***Key words**: Brassinosteroids, soil salinity, catalase activity, hydrogen peroxide content etc.*

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INTRODUCTION

Providing food and nutritional security to escalating human population is a major challenge. These challenges became the major factors with the certainty in changing climate scenario and continuously increasing human population. An estimated growth in current world population of 7.3 billion is expected to reach 8.5 billion by 2030, 9.7 billion in 2050 and 11.2 billion in 2100. (UN DESA report, "World Population Prospects: The 2015 Revision")[1]. Promoting food/grain legume crops, might be an important amendment in the global cropping pattern through which India can meet food and nutritional security challenges to exploding human population effectively. Further, soil salinization and increasing use of poor quality of water for irrigation limiting growth and productivity of plants in many areas of the world therefore, salinity becomes major abiotic stress that effects metabolic pathways and molecular or gene networks. The arable land is continuously transforming into saline land due to natural salinity and human interferences. These transformations expected to have devastating global effects, resulting in up to 50% land loss by 2050 [2,3] Salt stress imposes substantial adverse effects on the performance and physiology of the crop plants, which eventually leads to plant death as a consequence of growth arrest and metabolic damage [4]. Many types of stress symptoms and deficiencies in plants, like potassium (K) deficiency [5], osmotic stress [6] and soil sodicity, alkalinity and other soil problems had been aroused in plants due to high levels of soluble salts including chlorides of sodium, calcium, and magnesium. The plant growth declines at higher concentrations of soluble salts which leads to hyperosmolality and imbalance of nutrients in plant systems [7]. Salt stress and many other biotic and abiotic stresses, lead to the

production of reactive oxygen species (ROS) like superoxide (O_2^-), hydroxyl (OH^-) free radicals, hydrogen peroxide (H_2O_2), and free singlet oxygen [8] [9].

Mungbean is generally known as a salt sensitive crop. There is a considerable growth reduction in mungbean is observed in terms of reduction in seed germination, fresh and dry biomass and various other physiological traits [10]. To minimize the adverse effects of salinity on the normal functioning of plants various plant breeding and physiological approaches are applied. Moreover, plant growth regulators both natural and synthetic, are widely applied exogenously to agricultural crops as a means of crop improvement [11]. Thus, the study was considered to appraise the effect of brassinolide, applied exogenously on salt sensitive mungbean genotypes HUM-12 and HUM-16 to mitigate salinity stress.

Brassinosteroids (BRs) are recognized as group of naturally occurring plant growth regulators that regulate plant growth and productivity. These are steroidal lactones widely distributed in plant kingdom. Exogenous application of BRs increased tolerance to low and/or high temperature stress [12]; [13] and heavy metal stress, drought stress [14], salinity[15] and water logging [12]. A vast array of mechanisms like cell division, cell elongation, vascular differentiation, germination, rhizogenesis, flowering, and modulation of gene expression are promoted by BRs [16]. Exogenous application of BRs promote leaf photosynthesis by positively regulating synthesis and activation of a variety of photosynthetic enzymes including Rubisco[17]. However, an insufficient literature is present regarding the use of Brassinosteroids in mungbean to mitigate salinity stress. Thus, there is demand of climate change scenario to find out such crop production techniques and information that neutralize abiotic stresses like salinity stress in mungbean with exogenously applied plant growth regulators. Thus, present research work was designed with an objective to investigate the effects of brassinolide (BL) on seedlings, morpho-physiological and biochemical parameters in mungbean cultivars (HUM-12, HUM-16) under salinity stress.

MATERIAL AND METHODS

The present investigation was conducted at Department of Plant Physiology, Institute of agricultural sciences, Banaras Hindu University, Varanasi, India during *kharif* season 2014. The whole investigation was taken out in petriplates and experimental pots in growth chamber and net house (i.e. controlled condition) with two salt sensitive mungbean genotypes (HUM-12, HUM-16). Soil samples collected from experimental pots for different physico-chemical properties of the soil revealed that the soil of pots was sandy loam in texture having pH of 7.11, EC of 0.25 dSm^{-1}, bulk density of 1.49 g cm^{-3} and water holding capacity of 39.46%. The experiment was laid out in Complete Randomised Design with different Brassinolide concentrations 0.01mM, 0.05mM in combination with induced salinity stress 100mM NaCl and 0.05mM alone with treatments **T_0**- control, **T_1**- 100mM (NaCl), **T_2**- 100mM (NaCl) + 0.05mM (BL), **T_3**- 100mM (NaCl) + 0.01mM (BL), **T_4**- 0.05mM (BL). The seeds of two genotypes were surface sterilized with 0.1% $HgCl_2$ (Mercuric chloride) for 5 min and then rinsed five to six times with sterilized distilled water. To each Petri plate 0.01mM, 0.05mM of Brassinolide (BL) and NaCl 100mM individually and in combination with above concentrations of BL were added having 10 seeds in each petriplate. Same treatments were taken in experimental pots in net house also. All the morphological parameters (fresh weight, dry weight, number of leaves $plant^{-1}$) and biochemical parameters (catalase activity, hydrogen peroxide content, nitrate reductase activity) were recorded at 5, 10, 15 and 30 DAS in growth chamber and net house and evaluated as per standard procedure.

Catalase (CA) activity was determined by [18]. Fresh leaf material about (200) mg was grind with 10 mL of phosphate buffer and centrifuged at 10,000 rpm for 30 min at 4°C. The enzyme extract was collected at low temperature; the enzyme activity was recorded by taking 1.25 mL H_2O_2, 0.5 mL enzyme extract, 3.25 mL PO_4 buffer. The reaction mixture was withdrawn and poured into 4 mL potassium dichromate acetic acid and kept on water bath for 10 min and colour intensity was measured after cooling of 570 nm. The results were expressed as enzyme unit g^{-1} fresh weight. Hydrogen peroxide level was determined by method described in [19]. For the determination of H_2O_2 level, the absorbance was measured at 415 nm and concentration was calculated using an extinction coefficient of 1.878 nM^{-1} cm^{-1}

The data obtained on various parameters was subjected to Analysis of Variance as described by (Cochran and Cox) [20] to identify the effects of treatments on the basis of critical difference.

RESULTS AND DISCUSSION

Number of leaves $plant^{-1}$

Maximum numbers of leaves was recorded in plants treated with 0.05mM BL treatment as compared with control and lowest number was observed in 100mM NaCl treatment. However, treatment 0.05mM BL + 100mM NaCl induced more leaf number and reduced effects of salinity stress in both genotypes when compared with 100Mm NaCl treatment. Treatments with 0.05mM BL + 100mM NaCl and 0.01mM BL +

100mM NaCl were found statistically at par to some extent, but were significantly differ from treatment with 0.05mM BL. Comparison among different treatments clearly showed significantly increased in leaf number in plants treated with BL in both the genotypes at 30 DAS. The highest number of leaves was found at 30 DAS as compared to 15 DAS in pot culture. BL appeared to have diluting effect in salt stressed environment so that there was resumption of growth as evident from the table of present investigations.
Salinity stress results in two phase systems, first specific ion effect and second non specific ion effects. Non specific effects may leads to osmotic stress which may resist plant to uptake water and specific ion effect may leads to specific ion toxicity in shoots. Therefore, in plants treated with salinity stress may undergo both the phases and resulted in less growth of shoot and ultimately reduced number of leaves. But BL treated plants showed resumption in growth and restored the level of chlorophylls in leaves. In a study by Krishna, (2003) [21] the effect of BR on barley leaf cell ultrastructure was examined under salt stress. Leaf segments were pre-incubated in either BR solution or water and then incubated in 0.5 M NaCl solution in the presence or absence of BR. BR had no effect on the leaf cell ultra-structure under normal conditions. However, damages imposed by salt stress on nuclei and chloroplasts were significantly reduced by BR treatment. Therefore it may be sufficient to say that BRs may act as really steroids for growth and development of plants.

Table 1. Effect of Brassinolide on number of leaves plant^{-1} of mungbean (*Vigna radiata L.*) genotype (HUM-12 and HUM-16) under induced salinity at different stages of growth.

TREATMENTS	HUM-12				HUM-16			
	5 DAS	10 DAS	15 DAS	30 DAS	5 DAS	10 DAS	15 DAS	30 DAS
T_0 (Control)	2.00	3.67	12.67	19.33	2.00	3.33	9.67	16.33
T_1 (100mM NaCl)	1.67	2.33	4.00	5.00	2.00	3.03	5.03	5.33
T_2 (100mM NaCl + 0.05mM BL)	2.00	3.67	8.33	11.00	2.40	3.00	7.67	12.67
T_3 (100mM NaCl + 0.01mM BL)	2.00	2.67	4.67	9.00	2.35	3.00	5.00	9.33
T_4 (0.05mM BL)	2.33	3.00	11.00	15.33	2.00	3.67	9.00	16.33
SEm±	**0.21**	**0.54**	**0.89**	**1.43**	**0.26**	**0.42**	**0.61**	**1.20**
CD 5%	**1.12**	**1.16**	**2.82**	**4.51**	**1.13**	**1.18**	**1.94**	**3.79**

Total fresh weight (g)

The reduction in total fresh weight of seedlings (stem, leaves, roots) with respect to control was evident in NaCl stress treatment. The fresh weight of seedlings was reduced in NaCl (100mM) treatment as compared to control. Application of either 0.01mM or 0.05mM BL concentrations with salinity stress significantly increased the fresh weight of plants when compared with NaCl treated plants in HUM-12 genotype. Both the treatments 0.01mM and 0.05mM BL showed the neutralizing effect of salinity stress when compared with salinity treated plants. But the maximum total fresh weight was recorded in plants treated with BL 0.05mM as compared to control. Further, the highest total fresh weight per plant was found at 30 DAS as compared to 15 DAS in net house. From the table it was observed that the two genotypes HUM-12 and HUM-16 responded similarly to salinity stress and BL treatments. The different level of salinity significantly affected the growth attributes by reducing the biomass and length of root and shoot. The promotion of growth by BR under salt stress conditions was associated with enhanced levels of nucleic acids and soluble proteins (Anuradha and Rao, 2001) [22].

Table 2. Effect of Brassinolide on **fresh weight plant^{-1}** (leaves and stem) of mungbean (*Vigna radiata L.*) genotype **HUM-12 and HUM-16** under induced salinity at different stages of growth.

TREATMENTS	HUM-12				HUM-16			
	5 DAS	10 DAS	15 DAS	30 DAS	5 DAS	10 DAS	15 DAS	30 DAS
T_0 (Control)	0.28	0.52	3.40	5.97	0.31	0.47	5.17	6.30
T_1 (100mM NaCl)	0.13	0.26	1.77	2.03	0.18	0.35	1.57	2.53
T_2 (100mM NaCl + 0.05mM BL)	0.27	0.42	2.17	5.43	0.21	0.42	2.53	5.63
T_3 (100mM NaCl + 0.01mM BL)	0.31	0.39	2.07	5.23	0.18	0.32	1.80	5.30
T_4 (0.05mM BL)	0.34	0.52	3.87	6.40	0.25	0.50	3.87	6.30
SEm±	**0.05**	**0.01**	**0.47**	**0.41**	**0.02**	**0.03**	**0.47**	**0.69**
CD 5%	**0.02**	**0.04**	**1.08**	**1.29**	**0.07**	**0.10**	**1.48**	**2.17**

Table 3. Effect of brassinolide on **fresh weight plant^{-1}** (roots) of mungbean (*Vigna radiata L.*) genotype **HUM-12 and HUM-16** under induced salinity at different stages of growth**.**

TREATMENTS	HUM-12				HUM-16			
	5 DAS	10 DAS	15 DAS	30 DAS	5 DAS	10 DAS	15 DAS	30 DAS
T_0 (Control)	0.40	0.46	1.87	3.50	0.50	0.15	1.97	3.07
T_1 (100mM NaCl)	0.13	0.19	0.50	1.40	0.14	0.18	0.37	1.13
T_2 (100mM NaCl + 0.05mM BL)	0.23	0.42	1.80	2.30	0.57	0.67	1.77	2.37
T_3 (100mM NaCl + 0.01mM BL)	0.30	0.33	1.74	2.13	0.30	0.37	1.50	2.63
T_4 (0.05mM BL)	0.70	0.56	2.37	3.87	0.70	0.80	2.00	3.47
SEm±	**0.07**	**0.16**	**0.13**	**0.25**	**0.07**	**0.14**	**0.17**	**0.31**
CD 5%	**0.21**	**0.04**	**0.40**	**0.58**	**0.23**	**0.10**	**0.30**	**0.17**

Total dry weight (g)

Data recorded at various periods of growth as influenced by treatments are presented in Table. It was observed that the total dry weight increased with the crop age and conspicuous increase was observed between 15 and 30 DAS in BL treated genotypes. The variations in total dry weights of different treatments indicates the effect of salinity stress and exogenously applied brassinolide and also clearly characterize the ameliorating effect of BL on salinity stress treated plants. The increased total dry weight was found in control plants of both the genotypes as compared to other treatments at 5 DAS whereas, at subsequent stages, increased total dry weight was recorded with 0.05 mM BL treatment. Further, the lowest dry weight was recorded in salinity stress (NaCl 100mM) treatment. This observation was in agreement with (Al-mutawa, 2003) [23] who found negative correlation between shoot and root dry matter, and concentration of NaCl. Seeds treated with brassinolide BL 0.05mM with NaCl 100mM enhanced dry weights, minimized the damaging effects on seedlings and were found vigorous and robust which ultimately results in increased dry weights. Both the treatments 100mM NaCl + 0.05mM BL and100mM NaCl + 0.01mM BL were found statistically at par compared with control. But the highest dry weight was found at 30 DAS as compared to 15 DAS in plants raised in pots in net house in HUM-12 genotype, similar trends of increasing and decreasing total dry weights were also found in HUM-16 genotype. The decreased in dry weight in salinity stressed plants might be due to accumulation of Na^+ and Cl^- ions in shoots and roots which may leads to osmotic stress and ultimately lowered the water potential of plants where as BL treated plants may reduces these ion effects and causes the sequestration of these toxic ions in vacuoles. Moreover, it may leads to activation of certain channels at plasma membranes which maintains the water potential in the plant cell.

Table 4. Effect of Brassinolide on **total dry weight plant^{-1}**mungbean (*Vigna radiata L.*) genotype **HUM-12 and HUM-16** under induced salinity at different stages of growth**.**

TREATMENTS	HUM-12				HUM-16			
	5 DAS	10 DAS	15 DAS	30 DAS	5 DAS	10 DAS	15 DAS	30 DAS
T_0 (Control)	0.08	0.10	1.83	2.67	0.04	0.07	1.73	2.53
T_1 (100mM NaCl)	0.02	0.03	0.73	1.23	0.02	0.06	0.70	1.37
T_2 (100mM NaCl + 0.05mM BL)	0.05	0.08	1.73	2.40	0.03	0.09	1.63	2.07
T_3 (100mM NaCl + 0.01mM BL)	0.03	0.07	1.60	2.33	0.02	0.05	1.67	2.23
T_4 (0.05mM BL)	0.04	0.11	2.20	3.23	0.05	0.11	2.57	3.33
SEm±	**0.01**	**0.03**	**0.15**	**0.25**	**0.01**	**0.11**	**0.12**	**0.12**
CD 5%	**0.03**	**0.04**	**0.48**	**0.78**	**0.23**	**0.03**	**0.38**	**0.17**

Hydrogen peroxide (µM g^{-1} fresh weight)

The data pertaining to hydrogen peroxide at different growth stages at periodic intervals are summarized in Table no.5 In general, plants raised in salinity stressed soil showed direct relationship between hydrogen peroxide and NaCl treatment. As hydrogen peroxide was found as potent ROS in disturbing plant various metabolic activities under different stresses Therefore, comparison among different treatments clearly revealed significantly increased hydrogen peroxide in plants treated with NaCL 100mM, where as the lowest hydrogen peroxide was obtained in BL 0.05mM treatment as compared to control in both the genotypes but the plants having salinity and BL treatments were found healthy showing the neutralizing effect of BL against salinity stress as clearly indicated in the table no. at 10, 15, and 30 DAS. The treatment 100mM NaCl + 0.05mM BL was recorded statistically at par with control at 30 DAS in both HUM-12 and HUM-16 genotypes. Seeds treated with 0.05 mM BL showed positive effects on

hydrogen peroxide (in terms of decreased hydrogen peroxide content) and further it decreased the adverse effect of salinity stress more than 0.01 mM BL in both the genotypes.

Salt stress can lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy which in turn increase the generation of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, and hydrogen peroxide. ROS can undergo a series of oxidation/reduction reactions known as the Halliwell-Asada pathway (Gratao *et al.* 2006(24). Extensive studies have shown that while high levels of ROS cause cell death, low levels of ROS have regulatory roles in plant stress responses but in present investigation there is accumulation of hydrogen peroxide in high levels under salinity stress which causes damaging effects in salinity stressed plants. Combined treated plants with BL and salinity showed the mitigating effect of BL which results in partial growth recovery in these plants due to accumulation of lower levels of H_2O_2 and hence triggers the signaling mechanism for activation of certain defense related genes like the upregulation of the catalase related genes which ultimately neutralize the effect of H_2O_2 by converting the toxic ROS H_2O_2 to H_2O and O_2 with the help of catalase enzyme.

Table 5.Effect of brassinolide on Hydrogen peroxide (μM g^{-1} fresh weight) of mungbean (*Vignaradiata L.*) genotype HUM-12 and HUM-16 under induced salinity at different stages of growth.

TREATMENTS	HUM-12				HUM-16			
	5 DAS	10 DAS	15 DAS	30 DAS	5 DAS	10 DAS	15 DAS	30 DAS
T_0 (Control)	2.453	3.250	3.777	4.703	2.463	3.140	3.343	4.117
T_1 (100mM NaCl)	6.717	8.250	9.587	11.847	6.117	5.470	6.733	8.707
T_2 (100mM NaCl + 0.05mM BL)	2.360	2.210	3.417	4.087	2.167	3.263	3.693	4.100
T_3 (100mM NaCl + 0.01mM BL)	2.500	2.147	2.877	3.997	2.010	2.153	2.507	3.757
T_4 (0.05mM BL)	1.437	1.817	1.940	2.000	1.943	1.843	1.870	2.060
SEm±	**0.027**	**0.025**	**0.160**	**0.332**	**0.024**	**0.071**	**0.151**	**0.102**
CD 5%	**0.084**	**0.078**	**0.506**	**1.047**	**0.074**	**0.224**	**0.476**	**0.322**

Catalase activity (g^{-1} fresh weight)

Catalase was found as an important antioxidant enzyme in mitigating different effects of abiotic stresses and was expressed as the amount of H_2O_2 decomposed. The decrease in antioxidant enzymes activities is a common plant stress response. In present investigation with salinity stress, a significant decrease in CAT activity with respect to control in the seedlings was observed. It was observed that treatment of mungbean seeds with BL enhanced the activity of CAT as compared to control. At the concentration of BL 0.05 mM alone the increase in activity was greater. In salinity stress (100mM NaCl) activity of CAT was recorded less as compared to control at all the three stages of germination. The decrease in CAT activity treatment with NaCl 100mM might be the result of salt-induced dehydration. When BL was added with NaCl, the activity of CAT was higher as compared to that under salt stress indicating that BL enhanced the anti oxidative metabolism in the seedlings at all the stages of growth. Combined treatment with BL and NaCl resulted in partial growth recovery as compared to the action of NaCl alone. The maximum catalase activity was found in plants treated with brassinolide BL 0.05mM + NaCl 100mM. The highest catalase activity was recorded at 15 DAS than at 30 DAS in net house grown plants, which were comparatively more in plants raised under controlled conditions in the growth chamber.

The production of reactive oxygen species (ROS) during normal respiration, photosynthesis, and nitrogen fixation [25] may acts as potent signaling molecules for plants to activate their defense responses, like activation of antioxidant enzymes activities. Inhibition of the antioxidant systems leads to disruption of the redox homeostasis [26] and oxidative stress causing degradative changes of lipids, proteins, and nucleic acids [27]. It was observed in many studies that application of BRs modifies antioxidant enzymes as well as non-enzymatic antioxidants. Therefore from table 6 it was evident that BL modifies the catalase activity under salinity stress. Similar results were also observed by (Li et al. 1998) [28] in their study when they found that maize seedlings treated with brassinolide (BL) are subjected to salinity stress, the activities of SOD, CAT, APX, as well as ascorbic acid and carotenoid content increase. BRs induce stress tolerance by triggering the accumulation of apoplast H_2O_2 which subsequently up regulates the antioxidant system [29], and may activate the transcription or post transcription activities of number of defense genes like transcription of the *CAT* gene (in case of catalase enzyme) to ensure the normal growth and development of plants.

Table 6. Effect of Brassinolide on Catalase activity (g^{-1} fresh weight) of mungbean (*Vigna radiata L.*) genotype HUM-12 and HUM-16 under induced salinity at different stages of growth

TREATMENTS	HUM-12				HUM-16			
	5 DAS	10 DAS	15 DAS	30 DAS	5 DAS	10 DAS	15 DAS	30 DAS
T_0 (Control)	0.431	0.770	0.824	0.916	0.575	0.762	0.828	0.922
T_1 (100mM NaCl)	0.366	0.472	0.524	0.593	0.365	0.436	0.516	0.536
T_2 (100mM NaCl + 0.05mM BL)	0.629	0.781	0.812	0.907	0.617	0.751	0.812	0.894
T_3 (100mM NaCl + 0.01mM BL)	0.581	0.501	0.716	0.808	0.634	0.767	0.840	0.911
T_4 (0.05mM BL)	0.687	0.727	0.860	0.863	0.779	0.820	0.961	0.978
SEm±	**0.020**	**0.060**	**0.022**	**0.126**	**0.003**	**0.007**	**0.016**	**0.011**
CD 5%	**0.063**	**0.189**	**0.068**	**0.043**	**0.010**	**0.021**	**0.051**	**0.036**

CONCLUSION

From this present study, it is possible to conclude that the application of NaCl adversely affected the growth as well as biochemical parameters of mungbean plant as compared to control. Based on study it as found that BL is a potent growth regulator which completely diluting the toxic effects of ions in plants under salinity stress. BRs application overcome the salinity stress by enhancing the antioxidant enzyme activities and thus developed the tolerance against salinity stress. The salinity effect under the field condition may not be the same due to the large variations in the environmental conditions. Therefore, further studies needed to validate these findings under field conditions.

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