International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Role of Brassinosteroids in Osmolytes Accumulation under Salinity Stress in *Zea mays* Plants

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Abstract: ***The accumulation of osmoprotectants is a typical response to salt stress which enables the plants to grow under stress conditions. These are the nontoxic osmoprotectants that are collectively known as compatible solutes. Brassinosteroids (BRs) are the steroidal 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 (HBL) and 24-epibrassinolide (EBL) on osmolytes (proline, glycine betaine, mannitol and total sugars) content of 30 and 60 days old plants of Zea mays subjected to salt stress (0, 40, 60, 80, 100 mM). The seeds of Zea mays var. DKC 9106 were pre-soaked in different concentrations of HBL (10^{-10}, 10^{-8}, 10^{-6} M) and EBL (10^{-10}, 10^{-8}, 10^{-6}M) for 12 hours. Finding of present study revealed that application of HBL and EBL both enhanced the osmolytes accumulation in salt stressed plants of Zea mays which was found to be provide tolerance against salinity stress.***

Keywords: Brassinosteroids, Osmolytes, Salt stress, Maize

1. Introduction

Plants as a sessile organism, often exposed to various abiotic and biotic stresses like salinity, drought, flooding, high or low temperature, UV-radiations, herbicides, metal toxicity and pathogen, which adversely affected the crop production and yield [1, 2]. Among these salinity stress is a major constraint to agricultural yield and production. Salinity at higher levels affects the plants growth by causing hyperosmotic and hyperionic stress. Hyperosmotic stress reduced the water absorption capacity of root systems and increased the loss of water from the leaves which results in growth retardation [3], whereas hyperionic stress includes the accumulation of Na^+ and Cl^- ion to such a toxic level that it interfere with the other important cellular functions [4].

Plants exposed to salt stress undergo various changes to acclimatize the environment. The ability of plants to tolerate salt depends on the ion homeostasis, specific proteins, osmolytes production, toxic radicals scavenging and water transport [5, 6]. Among these, accumulation of osmoprotectants enables the plants to grow under stress conditions by maintaining water balance between plant cell and environment [7]. These are nontoxic and collectively known as compatible solutes and accumulated in large amount without disturbing the cell metabolism. It includes mainly amino acids and their derivatives (proline and glycine betaine), polyamines, quaternery compounds, amines and polyols (mannitol, sorbitol, trehalose, fructans) [8, 9] etc. Accumulation of these compatible solutes provide protection to the membrane structure, cytoplasm and chloroplasts from Na^+ ion damage, scavenging ROS, stabilizing the proteins, increase resistance to all stresses and overall providing physiological stability to plants under stressful conditions [10, 11, 12].

BRs are the first natural steroidal plant hormones having growth promoting activity [13]. It has ability to conquer the various environmental stresses such as thermal, drought, heavy metals, infection, pesticides and viruses including salt stress by activating the different mechanism [14, 15, 16, 17, 18, 19, 20, 21]. BRs regulate various physiological processes such as cell differentiation, cell elongation, pollen tube development, differentiation of vascular bundles and enhancement of enzymatic and photosynthetic activities [22].

The present study was undertaken to study the effect of HBL and EBL on osmolytes (proline, glycine betaine, mannitol and total sugar) content of *Zea mays* plants under salt stress.

2. Material and Methods

2.1 Seed Treatments and Growth Conditions

Certified seeds of maize (var. DKC 9106) were surface sterilized with 0.03% mercuric chloride for 2 min followed by repeated rinses with sterile distilled water. Seeds were soaked in aqueous solution of HBL (10^{-10}, 10^{-8}, 10^{-6}M) and EBL (10^{-10}, 10^{-8}, 10^{-6}M) for 12 hours. The field area was divided into randomised blocks and then salinised with different concentrations of NaCl (0, 40, 60, 80 and 100 mM). Plants harvested on 30th and 60th day and used for the estimation of proline, glycine betaine, mannitol and total sugar content.

2.2 Proline Content

The proline content was estimated by following the method given by Bates, [23]. 0.5 g of shoot material was homogenized in 10 ml of 3% aq. sulphosalicylic acid. 2ml of filtrate was taken and mixed with 2ml of each glacial acetic acid and acid ninhydrin. After one hour of incubation at

100^0C, the reaction was terminated by placing the test tubes in ice bath. Stir well for 20- 30 sec followed by adding 4ml toluene. The absorbance of upper phase was determined spectrophotometrically at 520 nm. The proline concentrations were determined by running a series of standard with pure proline in a similar way.

2.3 Glycine betaine content

Glycine betaine was determined by following the method described by Grieve and Grattan, [24]. Dry plant material was ground in 10 ml of distilled water and filtered. 1 ml of the extract was taken and mixed with 1 ml of 2M HCl. Then 0.5 ml of this above mixture was taken and 0.2 ml of potassium tri-iodide solution was added into it.

The contents were shaken and cooled in an ice bath for 90 min. 2.0 ml of ice cooled distilled water and 20 ml of 1,2-dichloromethane were added to the mixture. Two different layers were formed in the mixture and optical density of the lower layer was measured at 365 nm.

2.4 Mannitol content

For mannitol estimation, plant extract was prepared according to the method given by Sanchez, [25].

0.1 ml of extract was taken and 0.5 ml of formate (0.5 M) was added to it. To this solution, 0.3 ml of 5 mM sodium periodate was added. The contents were vortexed and 0.3 ml of a solution containing 0.1M acetylacetone, 2M ammonium acetate and 0.02 M sodium thiosulphate were added into it. Mixture was heated in boiling water bath upto 2 min and then cooled under running tap water and the absorbance was measured at 412 nm.

2.5 Total sugar content

Total sugars were estimated according to the method purposed by Scott and Melvin, [26]. To the 25 mg of plant sample, 1.25 ml of 2.5 N HCl was added and cooled it to room temperature. Na_2CO_3 was added to neutralize it and made final volume to 25 ml. Then 4 ml of anthrone reagent was added to 1 ml of supernatant. It was heated for 8-10 minutes in boiling water bath. After cooling the reaction mixture, the optical density of dark green colour was measured spectrophotometricallyat 630 nm

3. Results (Tables)

Table1: Effect of HBL and EBL on proline content (µ mol g^{-1} FW) of 30 days old plant of *Zea mays* subjected to salt stress.

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**16.90±0.721**	**20.50±2.39**	**19.84±2.733**	**22.00±1.553**	**22.60±1.478**
10^{-10}M HBL	20.93±1.774	27.88±2.356	24.88±2.396	26.23±2.141	27.16±3.580
10^{-8}M HBL	20.07±2.686	16.76±1.855	29.43±1.730	24.63±2.086	26.10±1.570
10^{-6}M HBL	33.86±2.444	24.15±4.374	23.11±1.922	25.25±1.118	19.43±2.890
Treatment Dose Treatment× Dose F-ratio 5.0639* 25.012* 9.7901*					
10^{-10}M EBL	21.61±1.54	28.87±2.136	16.31±1.241	27.44±2.427	25.96±2.614
10^{-8}M EBL	16.90±1.501	25.77±1.667	24.58±2.654	26.10±1.570	23.70±2.345
10^{-6}M EBL	18.30±.703	19.78±1.832	26.44±3.413	19.43±2.890	22.62±2.516
Treatment Dose Treatment× Dose F-ratio 10.363* 5.390* 4.896*					

*Indicate statistically significant differences from control at p≤0.05

Table 2: Effect of HBL and EBL on glycine betaine content (µ mol g^{-1} FW) of 30 days old plant of *Zea mays* subjected to salt stress.

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**12.04±1.704**	**19.63±5.470**	**15.81±2.785**	**24.90±3.695**	**28.62±2.94**
10^{-10}M HBL	18.16±3.787	15.97±0.328	29.26±1.128	42.34+1.932	44.59±2.031
10^{-8}M HBL	12.05±2.715	26.91±2.475	29.62±1.466	37.03±2.03	48.40±1.180
10^{-6}M HBL	20.29±2.875	23.58±4.477	24.27±2.667	36.78±1.826	33.31±2.101
Treatment Dose Treatment× Dose F-ratio 112.91* 37.90* 17.99*					
10^{-10}M EBL	15.30±2.884	19.24±1.735	36.86±3.371	29.00±3.712	41.01±2.218
10^{-8}M EBL	22.20±2.653	26.02±1.704	20.80±0.498	38.31±4.327	48.09±3.739
10^{-6}M EBL	18.25±1.128	31.96±4.495	46.01±3.544	23.99±1.317	42.18±1.398
Treatment Dose Treatment× Dose F-ratio 176.96 * 25.24* 10.914*					

*Indicate statistically significant differences from control at p≤0.05

Table 3: Effect of HBL and EBL on mannitol content (µ mol g^{-1} FW) of 30 days old plant of *Zea mays* subjected to salt stress.

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	12.32±0.64	12.59±1.194	29.37±2.610	39.88±5.291	36.64±3.124
10^{-10}M HBL	19.97±1.399	25.11±3.520	37.23±3.649	46.50±3.149	36.84±3.540
10^{-8}M HBL	15.84±0.745	25.28±2.927	45.91±2.322	56.36±3.550	33.58±2.812
10^{-6}M HBL	17.33±0.621	15.23±1.547	39.30±2.033	40.21±4.930	50.30±2.590
Treatment Dose Treatment× Dose F-ratio 88.205* 12.93* 4.960*					
10^{-10}M EBL	14.96±2.579	29.38±1.572	37.09±3.471	38.36±4.416	53.60±2.997
10^{-8}M EBL	27.36±3.670	31.67±1.889	35.13±2.309	50.90±5.226	56.60±3.20
10^{-6}M EBL	33.41±4.130	19.16±5.078	26.89±2.886	46.58±4.931	58.91±4.212
Treatment Dose Treatment× Dose F-ratio 123.46* 12.903* 2.388					

*Indicate statistically significant differences from control at p≤0.05

Table 4: Effect of HBL and EBL on total sugar content (µ mol g^{-1} FW) of 30 days old plant of *Zea mays* subjected to salt stress

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**1.543±0.045**	**1.687±0.095**	**1.428±0.054**	**1.745±0.051**	**2.137±0.057**
10^{-10}M HBL	1.640±0.051	2.424±0.286	1.661±0.045	1.944±0.029	3.160±0.075
10^{-8}M HBL	1.738±0.057	2.718±0.091	1.849±0.061	2.455±0.123	3.518±0.172
10^{-6}M HBL	1.905±0.080	1.915±0.035	1.565±0.032	2.029±0.129	3.014±0.125
Treatment Dose Treatment× Dose F-ratio 119.29* 51.542* 6.163*					
10^{-10}M EBL	2.075±0.110	3.537±0.288	1.900±0.073	1.741±0.251	2.578±0.166
10^{-8}M EBL	2.484±0.242	2.611±0.132	1.678±0.144	2.459±0.235	3.283±0.217
10^{-6}M EBL	1.560±0.201	2.279±0.171	1.811±0.022	2.656±0.083	2.763±0.296
Treatment Dose Treatment× Dose F-ratio 384.57* 53.367* 42.660*					

***Indicate statistically significant differences from control at p≤0.05**

Table5: Effect of HBL and EBL on proline content (µ mol g^{-1} FW) of 60 days old plant of *Zea mays* subjected to salt stress.

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**27.73±4.967**	**46.06±4.561**	**39.23±5.108**	**56.31±6.511**	**60.61±6.162**
10^{-10}M HBL	45.25±1.276	64.60±4.366	46.01±3.790	74.21±3.478	59.73±6.217
10^{-8}M HBL	35.50±2.754	56.60±3.271	61.00±1.159	49.97±6.569	74.61±2.628
10^{-6}M HBL	28.29±3.429	44.63±2.623	64.42±4.384	64.71±3.908	73.91±2.475
Treatment Dose Treatment× Dose F-ratio 13.592* 11.899* 4.956*					
10^{-10}M EBL	42.80±3.696	60.63±5.748	48.48±4.444	74.15±2.899	63.59±2.976
10^{-8}M EBL	33.66±3.484	55.96±1.820	37.31±3.693	76.48±7.626	73.54±3.111
10^{-6}M EBL	26.83±3.339	62.26±3.712	57.38±4.400	62.37±3.903	63.56±3.859
Treatment Dose Treatment× Dose F-ratio 49.188* 11.672* 2.306					

*Indicate statistically significant differences from control at p≤0.05

Table 6: Effect of HBL and EBL on glycine betaine content (µ mol g^{-1} FW) of 60 days old plant of *Zea mays* subjected to salt stress

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**23.57±1.767**	**25.01±2.068**	**35.14±2.424**	**29.02±5.248**	**47.84±1.278**
10^{-10}M HBL	42.96±3.358	34.92±7.346	58.57±4.598	43.66±4.507	58.67±4.261
10^{-8}M HBL	32.90±5.360	58.35±4.592	62.30±3.543	65.20±2.637	73.51±2.385
10^{-6}M HBL	24.95±1.464	49.31±5.511	48.28±4.344	54.37±4.573	64.73±2.892
Treatment Dose Treatment× Dose F-ratio 29.377* 47.361* 6.387*					
10^{-10}M EBL	44.68±2.610	46.78±3.878	50.07±4.808	43.08±3.342	61.53±2.461
10^{-8}M EBL	22.39±1.654	46.64±3.176	45.31±2.648	28.40±4.518	53.88±2.20
10^{-6}M EBL	20.66±2.328	30.06±5.794	39.22±0.704	36.13±2.197	49.41±3.104

Treatment Dose Treatment× Dose F-ratio 41.70* 23.042* 4.953*					

*Indicate statistically significant differences from control at p≤0.05

Table 7: Effect of HBL and EBL on mannitol content (µ mol g^{-1} FW)of 60 days old plant of *Zea mays* subjected to salt stress.

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**25.41±2.647**	**54.44±2.234**	**40.95±5.750**	**42.21±3.784**	**59.56±5.424**
10^{-10}M HBL	30.25±1.732	56.32±3.605	58.89±2.351	65.84±4.72	66.33±3.452
10^{-8}M HBL	36.25±1.732	67.16±4.102	59.75±5.893	75.87±2.834	76.61±1.836
10^{-6}M HBL	29.02±4.394	59.36±7.809	56.64±3.704	62.57±5.114	62.81±4.915
Treatment Dose Treatment× Dose F-ratio 76.898* 19.442* 5.252*					
10^{-10}M EBL	32.84±2.930	45.15±2.707	50.33±0.671	64.51±1.709	60.65±3.675
10^{-8}M EBL	28.39±4.285	45.69±3.640	56.68±3.901	69.54±4.21	71.92±1.820
10^{-6}M EBL	44.43±2.909	56.32±3.605	48.00±4.785	59.96±4.42	66.39±3.932
Treatment Dose Treatment× Dose F-ratio 153.19* 22.087* 10.637*					

*Indicate statistically significant differences from control at p≤0.05

Table 8: Effect of HBL and EBL on total sugar content (µ mol g^{-1} FW) of 60 days old plant of *Zea mays* subjected to salt stress.

BRs Treatment	0 mMNaCl	40mM NaCl	60mM NaCl	80mM NaCl	100mM NaCl
0M	**2.579**±0.143	**3.785±0.145**	**3.254±0.143**	**4.486±0.236**	**3.058±0.108**
10^{-10}M HBL	3.228±0.241	3.344±0.264	4.261±0.222	5.484±0.238	5.185±0.092
10^{-8}M HBL	3.250±0.145	3.817±0.117	6.714±0.203	7.663±0.181	4.457±0.118
10^{-6}M HBL	3.355±0.213	5.185±0.219	5.625±0.119	4.933±0.064	3.650±0.142
Treatment Dose Treatment× Dose F-ratio 125.04* 105.79* 22.61*					
10^{-10}M EBL	3.247±0.173	4.287±0.175	3.820±0.528	6.581±0.225	4.049±0.090
10^{-8}M EBL	3.940±0.393	4.834±0.438	5.349±0.373	5.450±0.263	3.903±0.473
10^{-6}M EBL	4.294±0.179	4.047±0.553	2.454±0.230	5.566±0.264	4.249±0.338
Treatment Dose Treatment× Dose F-ratio 27.313* 14.75* 4.991*					

***Indicate statistically significant differences from control at p≤0.05**

4. Results and Discussion

Salt stress imposition resulted in increase of osmolytes content (proline, glycine betaine, mannitol and total sugar content) in both 30 and 60 days old *Zea mays* plants. In 30 days old plants, maximum proline content was recorded in 100 mM salt stressed plants (22.60 µ mol g^{-1} FW) in comparison to control plants (16.90 µ mol g^{-1} FW). Further treatment of HBL (10^{-8} M) along with NaCl (60mM) showed the maximum increase of proline content (1.48 times) as compared to NaCl alone. Similarly application of EBL (10^{-10}M) in conjunction with NaCl (40mM) enhanced the proline content 1.40 times as compared to NaCl alone (Table 1). However in 60 days old plants, osmolytes content were found to increase as compared to 30 days old plants. Application of HBL (10^{-8}M) and EBL (10^{-8}M) both ameliorated the toxic effect of salt stress by enhancing the proline content (1.23 & 1.35 times respectively) under 100 mM and 80 mM NaCl alone respectively (Table 5).

Thus from above results it was reported that application of BRs (HBL and EBL) enhanced the proline accumulation under stress conditions which reveals that it play an important role in osmotic adjustment. It induces the expression of genes responsible for the biosynthesis of proline and overcome the salinity stress by regulating the proline content in plants which provide protection to the sub-cellular structures by reducing the oxidative damage caused due to free radicals in response to salt stress [27, 28, 29, 30, 31]. BRs application increased the proline content in rice plants subjected to drought stress by overcoming the deleterious effects of salt [32]. Similarly Hayat *et al.* [33] also reported the enhanced proline accumulation with the treatment of BRs in *Lycopersicon esculentum* when subjected to cadmium stress.

Glycine betaine and mannitol content was also found to increase under salt stress as compared to control. In 30 days old plant, glycine betaine (2.73 folds) and mannitol content (3.23 times) was observed to be increased under 100 and 80 mM salt stress respectively in comparison to control. However supplementation of (10^{-8} M) HBL with NaCl stress maximally enhanced the glycine betaine (1.69 fold) content under 100 mM salt stress and mannitol content (1.41 times) under 80 mM salt stress as compared to NaCl alone. Similarly treatment of EBL also enhanced the glycine betaine content 1.68 times and mannitol content 1.60 times under 100mM salt stress (Table 2, 3).

In 60 days old plants, application of HBL (10^{-8}M) enhanced the glycine betaine content (2.24 folds) under 80 mM salt stress and application of EBL (10^{-10}M) increased the glycine betaine content (1.28 folds) under 100 mM salt stress. Similarly maximum increase of mannitol content was recorded 1.28 folds with the application of HBL (10^{-6}M) and 1.20 folds with EBL (10^{-8}M) application along with 100mM salt stress (Table s6, 7).

Glycine betaine and mannitol both as osmoprotectant play important role in stress alleviation. Glycine betaine occurs abundantly in chloroplast where it provides protection to the thylakoid membranes and maintains the photosynthetic efficiency [34]. Mannitol serves as free radical scavenger and also stabilizing sub cellular structures and plays role in storage of carbon and energy [35]. BRs application enhanced the accumulation of osmoprotectents which is a means to counter the adverse effect of stress [36]. In the present study, BRs application enhanced the both glycine betaine and mannitol content under stress condition. Ali and Abdel Fattah, [37] reported that BRs treatment increased the glycine betaine content under salt stress in *Phaseolus vulgaris* and *Hordeum vulgare* by activating the enzyme betaine aldehyde dehydrogenase (BADH) which catalyse the synthesis of glycine betaine from the choline and enhanced the accumulations.

Sugar content was also found to increase under salt stress in both 30 and 60 days old plants. Supplementation of 30 days old plant with HBL (10^{-8}M) plus NaCl showed the enhancement of sugars content (1.64 times) under 100 mM NaCl stress whereas application of EBL (10^{-10}M) increased the sugar content about 2.09 times under 40mM salt stress (Table 4). In 60days old plants, presoaking treatment of HBL along with NaCl stress increased the sugar content 1.70 folds and EBL application enhanced the sugar content 1.46 times both under 80 mM salt stress (Table 8). Enhancement of sugars with the application of BRs provides tolerance against salt stress. Verma *et al*. [38] reported an increased sugar level with the treatment of BRs in *Arachis hypogaea.* Similarly Vardhini *et al*. [39] also reported the increased carbohydrate fractions like reducing sugars and starch in the radish roots with the treatment of BRs.

5. Conclusion

It was concluded from present study that osmolytes 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 osmoytes accumulation and thus developed the tolerance against salinity stress.

6. Future Scope

Plants often experience various abiotic and biotic stresses like drought, high or low temperature, flooding, salinity, metal toxicity, UV-radiations, herbicides and pathogen stress which adversely affected the crop production and yield. Plants adopted various strategies to adopt the stress conditions and osmolytes accumulation is one of among defensive stratregies. Application of BRs at appropriate dose further develops the stress tolerance by enhancing the accumulation of osmolytes production which helps the plants to overcome the stress conditions. Thus our study will further helpful to study the complex and fine mechanisms of osmolytes participation in the creation of resistant plants and help to explore the fundamental signaling mechanism of BRs induced plant stress protection in abiotic stressed plants.

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