

Brassinosteroid (24-epibrassinolide) enhances growth and alleviates the deleterious effects induced by salt stress in pea (*Pisum sativum* L.)**M. A. Shahid*¹, M. A. Pervez¹, R. M. Balal², N. S. Mattson³, A. Rashid⁴, R. Ahmad⁵, C. M. Ayyub¹ and T. Abbas¹**¹Institute of Horticultural Sciences, University of Agriculture, Faisalabad (38040) Punjab, Pakistan²Department of Horticulture, University College of Agriculture, University of Sargodha, Sargodha (Punjab) Pakistan³Department of Horticulture, Cornell University, Ithaca, New York, USA⁴Directorate of Research, Innovation and Commercialization, University of Agriculture, Faisalabad (38040) Punjab, Pakistan⁵Department of Crop Physiology, University of Agriculture Faisalabad (38040) Punjab, Pakistan***Corresponding author: adnanshahid@uaf.edu.pk****Abstract**

The response of pea (*Pisum sativum* L.) cv. Climax seeds imbibed with 24-epibrassinolide (EBL) and sodium chloride (NaCl) prior to sowing was evaluated. Soaking of seeds in two different concentrations of EBL (5 and 10 μ M) for 4 hours, caused an increase in germination, embryo axis length and most of the aspects of shoot and root growth at seedling stage, maturity stage (90 DAS) along with seed yield at the time of harvest. Both the EBL treatments (5 and 10 μ M) improved the above mentioned attributes but maximum improvement was observed in response to EBL concentration of 10 μ M with respect to the control. At seedling stage, EBL (10 μ M) significantly enhanced the fresh and dry biomass, seedling height (shoot + root), photosynthesis rate (Pn), stomatal conductance (g_s), total chlorophyll contents (Chl), proline contents, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), nitrate reductase activity (NRA) and nitrite reductase activity (NiRA) as compared to control (water soaked alone). Similarly, at maturity stage the plants grown from seeds pre-imbibed in EBL (10 μ M) also exhibited the augmentation in dry biomass, physiological aspects (Pn, g_s and Chl), enzymatic activities (NRA, NiRA, SOD, POD, CAT), leaf proline contents, nodule number and nodule dry biomass in comparison to water imbibed control. Seed attributes like seed yield, seed number and seed protein contents also showed the improvement in response to EBL (10 μ M) at the time of harvest. Although, plants subjected to saline stress exhibited a reduction in all the morpho-physiological and enzymatic attributes (NRA and NiRA) but proline contents and enzymatic activities of antioxidants were enhanced in response to NaCl stress. However, deleterious effects induced by salinity were reduced if seeds were treated with EBL before or after NaCl imbibitions.

Keywords: Pea, 24-epibrassinolide, salt stress, NaCl, photosynthesis, stomatal conductance.**Abbreviations:** EBL- 24-epibrassinolide, DAS- days after sowing, Pn- photosynthesis rate, g_s- stomatal conductance, Chl- chlorophyll contents, SOD- superoxide dismutase, POD- peroxidase, CAT- catalase, NRA- nitrate reductase activity, NiRA- nitrite reductase activity.**Introduction**

Plants exhibit several adaptive strategies in response to various abiotic stresses such as salt, water, cold and heat stress, which ultimately affect plant growth and yield (McCue and Hanson, 1990). It is well documented that beside other stresses, exposure to saline conditions limits plant growth and productivity (Abbas et al., 2010; Bahantana and Lazarovitch, 2010). To cope with these stresses, plants adapt various changes in physio-morphological and enzymatic processes (Camara-Zapata et al., 2004; Vinocur and Altman, 2005). The extent to which salinity is increasing in arid and semi-arid lands has become an issue of great concern in agriculture due to gradual declines in crop productivity in saline soils. In arid and semiarid regions, soil

salinization occurs when there is insufficient irrigation, regular use of saline water or with cultural practices such as excessive fertigation. It is estimated that worldwide 831 x 10⁶ hectare of land are affected by salinity (Beltran and Manzur, 2005). Salinity disrupts plant morpho-physiological processes due to osmotic disturbance and ionic stress. (Vinocur and Altman, 2005). Resultantly the osmotic disturbance can create a water deficient condition called physiological drought (Munns, 2002). Salt stress can restrict photosynthesis by decreasing green pigments (Sudhir and Murthy, 2004) suppressing rubisco activity (Soussi et al., 1998) and reducing stomatal conductance, thus affecting internal CO₂ availability (Bethkey and Drew, 1992). Salt

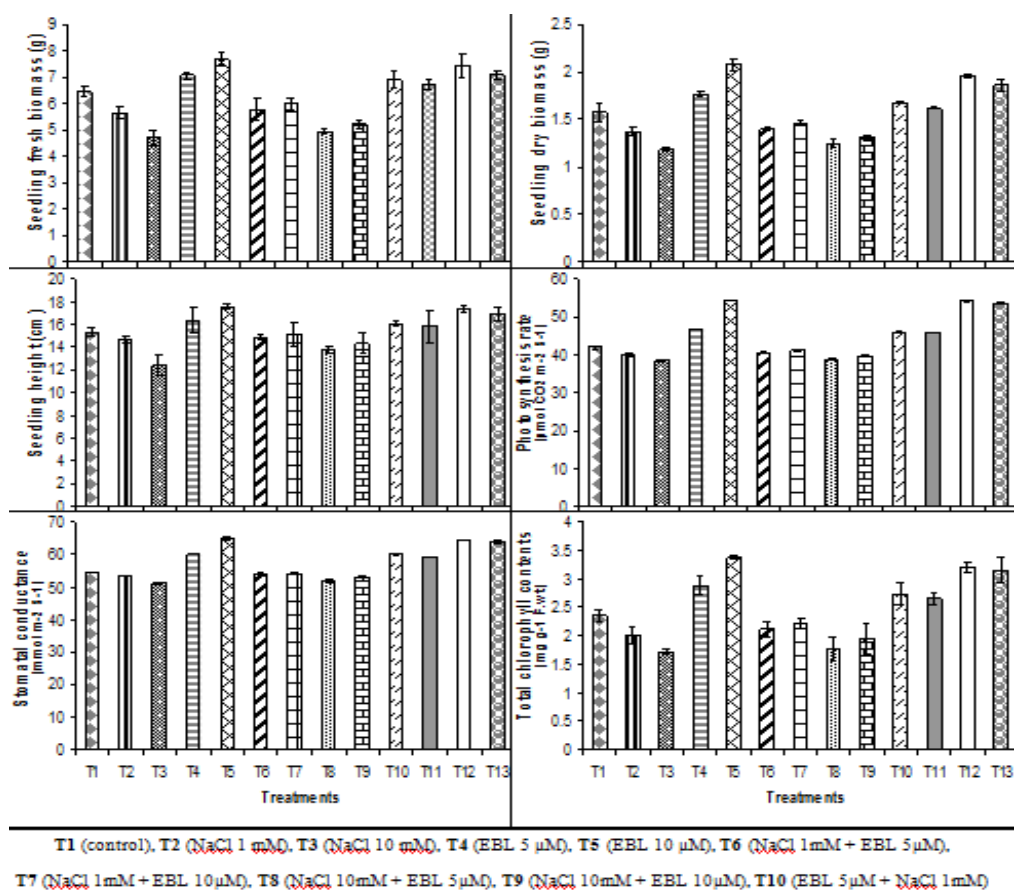


Fig 1. Effect of NaCl and EBL on plant biomass and physiological attributes at seedling stage

stress can reduce activity of various enzymes involved in nitrogen metabolism thus reducing plant nitrogen status (Soussi et al., 1998; Munns et al., 2006). Salt stress causes increase in reactive oxygen species (ROS) which thereby accelerate toxic reactions like lipid peroxidation, protein degradation and DNA mutation (McCord, 2000). To alleviate these oxidative effects, plants generate different kinds of antioxidants like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Noreen and Ashraf, 2009). Overall, the combination of osmotic stresses causes the reduction in photosynthesis and nitrogen status which ultimately lead to poor growth and reduced crop yield. Various mechanical, biological and chemical approaches are being practiced to overcome the threats generated by salinity. Many compounds are being used to cope with the toxic effects of salinity including ascorbic acid (Khafagy et al., 2009), proline (Hoque et al., 2007), glycinebetaine (Arafa et al., 2009; Abbas et al., 2010), silicon (Ashraf et al., 2010) etc. but brassinosteroids (BRs) are considered the most prominent among rest of others (Rao et al., 2002). Brassinosteroids are capable of enhancing plant defense system against environmental stresses such as water, salt, heat and cold stress etc. (Ali et al., 2007; Hasan et al., 2008; Farooq et al., 2009). Membrane stability and osmotic adjustment are two mechanisms through which BRs are reported to enhance abiotic stress tolerance (Wang and Zeng, 1993). Brassinosteroids are considered a category of steroids which

act as a plant hormone and are distributed through various vegetative and reproductive parts of a plant (Bajguz and Tretyn, 2003). This hormone controls and regulates various physiological processes in plants including cell differentiation, cell elongation, pollen tube development, swelling of cells, differentiation of vascular bundles, reassembling of nucleic acid to form proteins and acceleration of enzymatic as well as photosynthetic activities (Sasse, 2003; Yu et al., 2004). Growth and productivity of crops is directly related to germination percentage, root growth and seed/fruit setting and BRs induce significant improvement in these attributes (Kamuro and Takatsuto, 1999). A significant mitigating role of BRs has been identified in plants exposed to various biotic and abiotic stresses (Ali et al., 2007; Hasan et al., 2008). However a prominent role of BRs against salt stress has been experimented in various plant species (Sasse, 2003; Shahbaz et al., 2008; Fariduddin et al., 2009). Pea (*Pisum sativum*) is a vital cool season vegetable crop, cultivated across the globe including China, India, USA, France, and Egypt. It is utilized for various purposes like fresh peas, dry pulses and edible podded type. Nutritional value of peas cannot be denied as these are an excellent source of protein, carbohydrate (Hussein et al., 2006), water-soluble fibers, vitamins (vitamin B₁), and antioxidants (Mukerji, 2004). Salinity and its allied factors limit the production and growth of various legumes including pea (Najafi et al., 2007). The aim of this work was

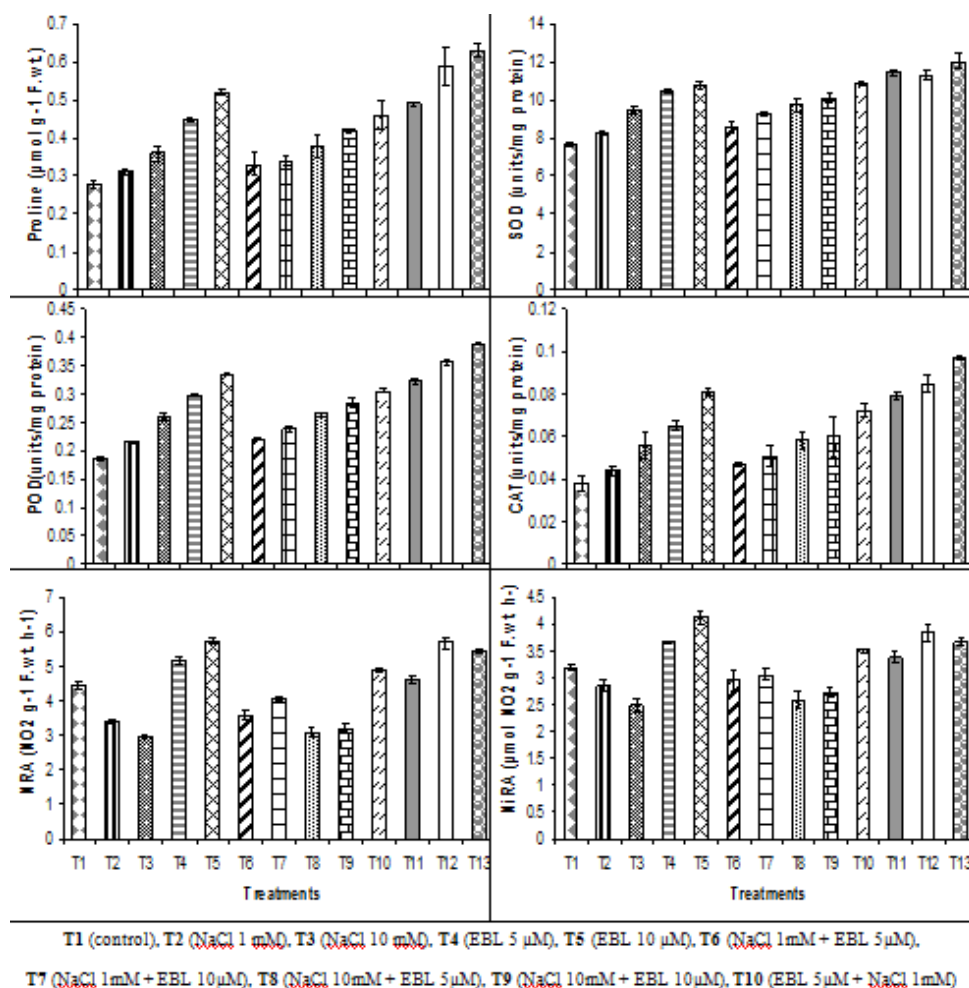


Fig 2. Effect of NaCl and EBL on proline and enzymatic attributes at seedling stage

to investigate the potential of BRs to reduce toxic effects associated with physiological changes in *Pisum sativum* seeds subjected to saline stress. The hypothesis tested is that 24-epibrassinolide (EBL) regulates photosynthesis, stomatal conductance, ionic contents, osmolytes (proline) and antioxidant enzyme activities which will ultimately induce tolerance in plants against salt stress.

Materials and methods

Plant material and experimental treatments

The seeds of pea (*Pisum sativum* L.) cv. Climax were obtained from Ayyub Agricultural Research Institute, Faisalabad (AARI). Seeds of uniform size were washed with distilled water after surface sterilizing with 10% sodium hypochlorite solution. The treatments were applied in three sets of seeds treated on the same day. Seeds of the first set were imbibed in double distilled water (control), two different concentrations of 24-epibrassinolide (5 μM or 10 μM), or NaCl (1 mM or 10 mM) for 4 hours. There were fifty seeds per treatment (ten seed pot⁻¹ considered as one replicate). These two EBL levels were screened in an optimization experiment in plastic trays (data not shown

here). The seeds treated with these two levels gave healthy seedlings with high biomass. The second set of seeds was subjected to salinity by imbibing them in NaCl (1 or 10 mM) for 2h and then shifted to EBL (5 μM or 10 μM) solutions for an additional 2 hrs. The third set of seeds were first imbibed in EBL solution (5 μM or 10 μM) for 2 h then shifted to NaCl solution (1 or 10 mM) for 2 h. The seeds were then grown in plastic pots (9 L volume), filled with fine sand as growth medium. Ten seeds per pot were sown and then thinned out to six per pot. The plants were harvested at seedling stage and mature plant stage (90 days after sowing) to analyze various morpho-physiological biochemical and yield attributes. There were five pots per treatment where each pot represented one experimental unit.

Determination of germination percentage and embryo axis length

After disinfecting the seeds with 10% sodium hypochlorite, these were treated with NaCl (1 & 10 mM) and 24-epiBL (5 & 10 μM) solutions and then grown in Petri dishes on Whatman filter paper (20 seeds per Petri dish). The treatments were replicated five times and placed in growth

chamber at 20 °C. Germination was calculated for five days. The germination percentage was calculated as under;

$$\text{Germination \%} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

Embryo axis length (plumule + radical) internodal distance was determined with the help of graded scale in centimeters (cm).

Measurement of growth attributes

Three plants were harvested randomly from five replicate at seedling as well as at mature stage (90 days after harvesting). Dry mass was determined after drying at 75°C for 24 hours. The number of nodules per plant was counted after washing roots in tap water while nodule fresh weight per pot was measured after separating them from plants. Nodule mass was measured after drying at 75°C for 24 hours

Determination of enzymatic attributes

Three plants per treatment were harvested at both growth stages for estimation of following enzymatic attributes

Nitrogenase

Fresh nodule samples were utilized to estimate the nitrogenase activity by the procedure described by Hardy et al. (1968).

Superoxide dismutase, catalase and peroxidase

For estimating antioxidant activities, fresh leaves (0.5 g) were ground in an ice-cooled tissue grinder in 5 mL of 50 mM cooled phosphate buffer (pH 7.8). The homogeneous mixture was centrifuged at 15000 g for 20 min at 4 °C. The supernatant was used for determining the activities of the following enzymes

Superoxide dismutase

The SOD activity was determined by the method of Giannopolitis and Ries (1977)

Catalase and peroxidase

Catalase (CAT) and peroxidase (POD) activities were measured by the procedure of Chance and Maehly (1955) with some alteration. Variations in absorbance of the reaction solution at 470 nm were calculated after every 20 seconds. One unit POD activity was assigned as an absorbance change of 0.01 units per min. The activity of each enzyme was expressed on the basis of protein content.

Nitrate reductase

Nitrate reductase activity (NRA) of pea was calculated by the protocol of Sym (1984).

Nitrite Reductase

Nitrite reductase activity (NiRA) of pea was calculated by the protocol of Ramarao et al. (1983).

Chlorophyll content

Chlorophyll concentration (Chl) was estimated following the protocol of Arnon (1949). Chlorophyll a, b and total chlorophyll was recorded using the following formulae.

$$\begin{aligned} \text{Chl a} &= [12.7 (\text{OD } 663) - 2.69 (\text{OD } 645)] \times \frac{\text{Volume}}{1000} \times \text{Weight} \\ \text{Chl b} &= [22.9 (\text{OD } 645) - 4.68 (\text{OD } 663)] \times \frac{\text{Volume}}{1000} \times \text{Weight} \\ \text{Total Chl} &= \text{Chl a} + \text{Chl b} \end{aligned}$$

Gas exchange measurements

Photosynthesis rate (Pn) and stomatal conductance (gs) were measured from the second fully expanded young leaves (five randomly selected plants per treatment were measured) using an open system LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddesdon, England). All these calculations were recorded at day time from 10.00 to 12.00 a.m. with molar flow of air per unit leaf area 403.3 mmol m⁻² s⁻¹, atmospheric pressure 99.9 kPa, water vapor pressure into chamber ranged from 6.0 to 8.9 mbar, PAR (photosynthetic active radiation) at leaf surface was maximum up to 1711 μmol m⁻² s⁻¹, temperature of leaf ranged from 28.4 to 32.4°C, ambient temperature ranged from 22.4 to 27.9 °C ambient CO₂ concentration was 352 μmol mol⁻¹

Proline contents

Proline contents were estimated according to the method of Bates et al. (1973).

Seed protein

Seed protein contents were analyzed by the method of Lowry et al. (1951).

Yield components

At the end of the experiment (120 DAS), 15 plants (5 plants from each of three pots) from each treatment were harvested randomly and the number of pods per plant was counted. Number of seeds per pod was noted as the average seed count for thirty five pods from each treatment. The pods from five plants per replication were harvested to calculate the seed yield. After measuring the above mentioned yield attributes, seeds from pods of every plant treatment⁻¹ were taken and 1000 uniform sized seeds treatment⁻¹ were separated and weighed by using a digital balance.

Experimental design and statistical analysis

A Completely Randomized Design (CRD) with three imbibitions solutions (distilled water, NaCl, or EBL) and at two concentrations in varying sequences were combined to form 13 treatments used in this experiment. Each treatment contained five pots (experimental units) and for yield attributes five plants were harvested per pot. For enzymatic and gas exchange attributes (photosynthesis and stomatal conductance), plant material was taken from five plants treatment⁻¹. Collected data was analyzed statistically by using package Statistix version 8.1 (Analytical Software, Tallahassee, Florida).

Table 1. Effect of NaCl and EBL on germination (%), embryo axis length (cm), plant biomass (g), nodule number, nodule fresh/dry weight (g) and nitrogenase activity (nmC_2H_4 (g nodule f.m.)⁻¹ h⁻¹) on *Pisum sativum*, cv. Climax after 90 days of sowing

Treatments	Soaking duration (hours)	Germination %	Embryo axis length	Dry mass	Nodule number	Nodule fresh mass	Nodule dry mass	Nitrogenase activity
Control	4	97.8 ± 0.28	4.66 ± 0.45	2.19 ± 0.01	30.92 ± 0.77	272.83 ± 1.48	55.47 ± 2.08	475.70 ± 3.01
NaCl (1 mM)	4	94.0 ± 1.99	4.20 ± 0.54	1.80 ± 0.04	27.30 ± 0.37	248.67 ± 2.03	48.22 ± 1.31	461.44 ± 2.10
NaCl (10 mM)	4	86.7 ± 4.08	3.59 ± 0.37	1.55 ± 0.05	20.45 ± 0.61	240.93 ± 1.55	41.00 ± 1.15	444.89 ± 2.75
EBL(5µM)	4	98.0 ± 0.71	5.00 ± 0.31	2.39 ± 0.04	40.93 ± 0.65	319.33 ± 2.60	62.33 ± 1.76	484.93 ± 5.58
EBL(10µM)	4	99.6 ± 0.37	5.45 ± 0.52	2.66 ± 0.05	46.68 ± 0.74	336.67 ± 2.73	66.67 ± 2.85	529.63 ± 4.28
NaCl (1mM) + EBL (5µM)	2 + 2	94.5 ± 1.47	4.40 ± 0.38	2.07 ± 0.06	29.81 ± 0.24	236.33 ± 1.45	41.33 ± 0.88	471.07 ± 1.02
NaCl (1mM) + EBL (10µM)	2 + 2	95.2 ± 1.90	4.65 ± 0.42	2.12 ± 0.07	33.59 ± 0.52	240.11 ± 0.59	45.33 ± 0.88	482.76 ± 4.09
NaCl (10mM) + EBL (5µM)	2 + 2	86.9 ± 2.09	3.84 ± 0.35	1.82 ± 0.03	23.11 ± 0.37	228.33 ± 1.45	32.33 ± 1.76	422.19 ± 1.70
NaCl (10mM) + EBL (10µM)	2 + 2	90.6 ± 1.71	4.18 ± 0.56	1.97 ± 0.03	26.96 ± 1.83	232.67 ± 2.19	37.00 ± 1.15	432.90 ± 1.56
EBL (5µM) + NaCl (1mM)	2 + 2	97.7 ± 0.66	4.87 ± 0.32	2.18 ± 0.02	35.23 ± 0.23	306.00 ± 3.79	55.00 ± 2.31	472.26 ± 2.52
EBL (5µM) + NaCl (10 mM)	2 + 2	96.6 ± 1.41	4.72 ± 0.39	2.04 ± 0.03	32.18 ± 0.44	297.67 ± 5.04	43.67 ± 1.33	451.96 ± 2.07
EBL (10mM) + NaCl (1 mM)	2 + 2	98.8 ± 0.60	5.27 ± 0.62	2.55 ± 0.06	44.42 ± 0.33	324.67 ± 2.33	62.00 ± 2.08	496.38 ± 3.87
EBL (10mM) + NaCl (10 mM)	2 + 2	98.1 ± 0.44	4.99 ± 0.38	2.25 ± 0.07	42.10 ± 1.79	290.33 ± 7.22	50.67 ± 1.20	476.00 ± 4.16
LSD at <i>P</i> =0.05		4.92	1.28	0.178	4.67	10.17	6.91	25.56

Each value is the mean of five replications (One replicate = one pot with five plants) ± standard error, LSD is Least Significant Difference test

Table 2. Effect of NaCl and EBL on chlorophyll 'a' (mg g⁻¹ F.wt.), chlorophyll 'b' (mg g⁻¹ F.wt.), photosynthesis rate ($\mu\text{mol CO}_2$ m⁻² s⁻¹), stomatal conductance (mmol m⁻² s⁻¹) and proline ($\mu\text{mol g}^{-1}$ F.wt.) content of (*Pisum sativum*), cv. Climax after 90 days of sowing

Treatments	Soaking Duration	Chlorophyll a	Chlorophyll b	Photosynthesis rate	Stomatal conductance	Proline
Control	4	1.29 ± 0.02	0.64 ± 0.06	53.89 ± 1.23	67.10 ± 1.19	0.43 ± 0.02
NaCl (1 mM)	4	0.82 ± 0.04	0.42 ± 0.03	47.22 ± 1.18	50.45 ± 0.44	0.46 ± 0.03
NaCl (10 mM)	4	0.53 ± 0.05	0.34 ± 0.04	40.56 ± 0.55	42.26 ± 1.49	0.52 ± 0.02
EBL(5µM)	4	1.55 ± 0.04	0.79 ± 0.02	60.55 ± 1.23	72.20 ± 0.94	0.60 ± 0.02
EBL(10µM)	4	1.92 ± 0.06	1.02 ± 0.05	69.45 ± 0.97	84.05 ± 2.32	0.78 ± 0.03
NaCl (1mM) + EBL (5µM)	2 + 2	0.93 ± 0.06	0.49 ± 0.02	42.00 ± 0.98	55.68 ± 1.08	0.47 ± 0.02
NaCl (1mM) + EBL (10µM)	2 + 2	1.11 ± 0.04	0.53 ± 0.03	44.96 ± 0.65	60.13 ± 0.62	0.53 ± 0.02
NaCl (10mM) + EBL (5µM)	2 + 2	0.58 ± 0.04	0.39 ± 0.02	33.41 ± 0.27	44.08 ± 0.36	0.55 ± 0.04
NaCl (10mM) + EBL (10µM)	2 + 2	0.76 ± 0.04	0.42 ± 0.02	38.52 ± 0.75	46.46 ± 0.71	0.59 ± 0.03
EBL (5µM) + NaCl (1mM)	2 + 2	1.40 ± 0.04	0.72 ± 0.03	57.52 ± 0.47	70.56 ± 0.39	0.62 ± 0.03
EBL (5µM) + NaCl (10 mM)	2 + 2	1.34 ± 0.03	0.68 ± 0.02	54.93 ± 0.17	68.53 ± 1.25	0.73 ± 0.03
EBL (10mM) + NaCl (1 mM)	2 + 2	1.80 ± 0.08	0.99 ± 0.03	65.60 ± 1.20	80.19 ± 1.25	0.85 ± 0.05
EBL (10mM) + NaCl (10 mM)	2 + 2	1.73 ± 0.04	0.81 ± 0.06	60.96 ± 1.01	75.77 ± 1.12	1.04 ± 0.04
LSD at <i>P</i> =0.05		0.228	0.116	4.99	6.97	0.09

Each value is the mean of five replications (One replicate = one pot with five plants) ± standard error based on Least Significant Difference test (LSD)

Results

Germination and embryo axis length

The plants developed from seeds treated with 24-epibrassinosteroid (EBL) exhibited the highest germination percentage however the higher EBL concentration (10 μ M) induced highly efficacious effect than its lower concentration (5 μ M) in terms of high germination percentage i.e. 99% as compared to control (97%) (Table 1). On the other hand plants developed from NaCl treated seeds exhibited the lowest values for germination percentage. Both NaCl treatments (1 & 10 mM) caused the decrease in seed germination percentage but the salinity level 10 mM induced more drastic effects in terms of less germination (86%). This deleterious effect of salt stress on germination was surmounted by 10 μ M of EBL, applied before or after salt stress (NaCl) treatment. EBL when applied alone gave the excellent performance in terms of maximum embryo axis length, (Table 1). Among the EBL concentrations, 10 μ M exhibited the highest incremental trend in embryo axes length by 17% than control.

Effect of 24-epibrassinolide and NaCl at Seedling stage

Salt stress exerted an inhibitory effect on seedling fresh/dry biomass and physiological attributes. The seedlings grown from NaCl-treated (1 or 10 mM) seeds exhibited a striking decrease in fresh and dry biomass, seedling height, photosynthesis rate, stomatal conductance, total chlorophyll contents, proline, NRA and NiRA while on the other hand antioxidant enzymes (SOD, POD and CAT) increased in response to salt stress (Fig. 1). But the EBL treatments (5 or 10 μ M) expeditiously mitigated the injurious effects of salt stress when seeds were imbibed EBL before or after NaCl treatment. Pre soaking of seeds with EBL (10 μ M) gave the seedlings with high fresh biomass (19%), dry biomass (31%), seedling height (14%), photosynthesis rate (29%), stomatal conductance (18%), total chlorophyll contents (43%), proline contents (85%), SOD (39%), POD (79%), CAT (113%), NRA (28%) and NiRA (29%) with respect to control (water soaked alone). Generally the seeds pre soaked with EBL showed better performance than those pre treated with NaCl (Fig. 1)

Effect of 24-epibrassinolide and NaCl at maturity stage

Growth, nodule and nitrogenase attributes

The plants developed from seeds treated with EBL exhibited the highest dry biomass at 90-day stage after sowing. EBL treatment (10 μ M) produced plants with a greater dry biomass than its lower concentration (5 μ M), representing a 21% increase as compared to control (water soaked alone) (Table 1). Both the NaCl treatments (1 and 10 mM) caused reductions in dry mass but more drastic effects were seen with 10 mM which reduced dry biomass by 29% compared with control. This deleterious effect of salt stress was surmounted by 10 μ M of EBL, applied before or after salt stress (NaCl) treatment. Similarly both NaCl treatments (1 & 10 mM) caused reductions in nodule number, nodule fresh and dry mass and nodule nitrogenase activity (NA) but reductions were enhanced with 10 mM NaCl. The higher NaCl concentration reduced the nodule number by 33%, nodule fresh mass by 11%, nodule dry mass by 26% and nitrogenase activity by 6% as compared to control (soaked in

water alone) while this injurious effect was mitigated by EBL treatments. EBL application of 10 μ M incremented the nodule number, nodule fresh/dry mass and NA by 50, 23, 20 and 11% respectively in relation to control (Table 1).

Photosynthesis related attributes and proline

Both NaCl treatments (5 and 10 mM) caused a significant reduction in above mentioned attributes except proline but NaCl treatment of 10 mM was the most deleterious of all other treatments showing the maximum decline in photosynthesis rate by 24%, stomatal conductance by 37%, chl "a" by 58%, chl "b" by 46% as compared to control (Table 2). EBL, NaCl or their combination increased proline content but plants germinated from seeds, pre treated with EBL and then post treated with NaCl exhibited the maximum amount of proline content. Plants from seeds pre imbibed with EBL (10 μ M) then post imbibed with NaCl (10 mM) showed the highest increase (58%) in proline contents than control. EBL (10 μ M) had maximum increment in photosynthesis rate by 28%, stomatal conductance by 25%, chl "a" by 48%, chl "b" by 37% as compared to control (Table 2).

Enzymatic attributes

The activities of nitrate reductase (NRA) and nitrite reductase (NiRA) were decreased while superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were significantly increased under NaCl stress (Table 3). The plants grown from seeds germinated with EBL showed the highest enzymatic activities than control and NaCl. EBL treatment (10 μ M) increased the NRA by 14% and NiRA by 26% as compared to control. However the plants grown from seeds pre imbibed with EBL (10 μ M) followed by post imbibition with NaCl (10 mM), exhibited the highest activities of SOD (58%), CAT (87%) and POD (10%) than other treatments (Table 3).

Yield components

Except the number of pods per plant all other yield attributes showed significant response to both NaCl and EBL application. Both pre-sowing treatments with EBL (5 & 10 μ M) resulted in greatest mass of 1000 seeds, seed yield per plant and number of seeds per pod as compared to all other treatments. The seeds pre-treated with EBL (10 μ M) exhibited increased seed yield by (19%), number of seeds (26%) and 1000 seed weight (18%) as compared to the control (Table 4). The NaCl treatments (1 & 10 mM) when applied alone, reduced yield attributes but the numbers of pods per plant remain statistically non-significant (Table 4). Among the NaCl treatments, 10 mM was more deleterious than 1 mM treatment while their effect was partially alleviated by post EBL treatments and fully overcome by pre EBL treatments. Both the NaCl treatments (1 and 10 mM) caused reduction in seed yield by 15% and 32% respectively over the control. The salt stresses significantly reduced the protein constituents of seed while the EBL treatments given before the NaCl application, replenished the loss in protein constituents. Seeds harvested from plants, germinated from seeds pre-imbibed with EBL (10 μ M) contained a 50% increase in protein concentrations as compared to the control (Table 4).

Table 3. Effect of NaCl and EBL on NRA ($\text{NO}_2 \text{ g}^{-1} \text{ F.wt. h}^{-1}$), NiRA ($\mu\text{mol NO}_2 \text{ g}^{-1} \text{ F.wt. h}^{-1}$), SOD (units/mg protein), CAT (units/mg protein) and POD (units/mg protein) content of (*Pisum sativum*),cv. Climax after 90 days of sowing

Treatments	Soaking Duration	NRA	NiRA	SOD	CAT	POD
Control	4	6.48 ± 0.05	4.15 ± 0.04	10.92 ± 1.02	0.057 ± 0.004	0.217 ± 0.03
NaCl (1 mM)	4	5.81 ± 0.08	3.55 ± 0.06	12.90 ± 0.82	0.061 ± 0.002	0.233 ± 0.02
NaCl (10 mM)	4	5.16 ± 0.11	3.02 ± 0.09	13.32 ± 0.54	0.066 ± 0.002	0.293 ± 0.02
EBL(5 μM)	4	6.77 ± 0.06	4.53 ± 0.07	15.13 ± 0.52	0.072 ± 0.008	0.343 ± 0.02
EBL(10 μM)	4	7.41 ± 0.09	5.27 ± 0.07	18.14 ± 0.26	0.085 ± 0.008	0.377 ± 0.02
NaCl (1mM) + EBL (5 μM)	2 + 2	6.13 ± 0.06	3.67 ± 0.07	13.29 ± 0.99	0.065 ± 0.002	0.243 ± 0.01
NaCl (1mM) + EBL (10 μM)	2 + 2	6.36 ± 0.04	3.93 ± 0.06	14.78 ± 0.27	0.069 ± 0.002	0.270 ± 0.02
NaCl (10mM) + EBL (5 μM)	2 + 2	5.38 ± 0.04	3.21 ± 0.05	15.14 ± 0.43	0.073 ± 0.004	0.293 ± 0.02
NaCl (10mM) + EBL (10 μM)	2 + 2	5.58 ± 0.05	3.39 ± 0.03	15.54 ± 0.32	0.085 ± 0.003	0.333 ± 0.01
EBL (5 μM) + NaCl (1mM)	2 + 2	6.61 ± 0.03	4.45 ± 0.04	16.17 ± 0.95	0.082 ± 0.002	0.363 ± 0.01
EBL (5 μM) + NaCl (10 mM)	2 + 2	6.54 ± 0.04	4.38 ± 0.08	17.30 ± 0.31	0.090 ± 0.015	0.390 ± 0.02
EBL (10mM) + NaCl (1 mM)	2 + 2	7.14 ± 0.06	5.13 ± 0.05	16.94 ± 1.21	0.094 ± 0.003	0.410 ± 0.02
EBL (10mM) + NaCl (10 mM)	2 + 2	6.97 ± 0.07	4.95 ± 0.05	18.51 ± 1.12	0.107 ± 0.017	0.437 ± 0.02
LSD at $P=0.05$		0.42	0.33	2.16	0.021	0.055

Each value is the mean of five replications (One replicate = one pot with five plants) ± standard error based on Least Significant Difference test (LSD)

Table 4. Effect of NaCl and EBL on number of pods plant⁻¹, number of seeds pod⁻¹, 1000 seed wt (g), yield plant⁻¹ (g) and seed proteins (%) of (*Pisum sativum*),cv. Climax after 120 days of sowing

Treatments	Soaking duration	Number of pods	Number of seeds pod ⁻¹	1000 seed wt	Yield plant ⁻¹	Seed proteins
Control	4	6.10 ± 0.12	6.13 ± 0.19	227.00 ± 4.04	124.33 ± 2.33	16.97 ± 1.45
NaCl (1 mM)	4	5.38 ± 0.17	5.54 ± 0.25	212.00 ± 4.04	108.00 ± 1.73	13.44 ± 0.96
NaCl (10 mM)	4	5.38 ± 0.38	4.63 ± 0.23	197.33 ± 7.26	94.33 ± 4.67	10.97 ± 0.81
EBL(5 μM)	4	5.14 ± 0.07	7.08 ± 0.13	245.33 ± 2.60	139.00 ± 2.31	21.19 ± 0.48
EBL(10 μM)	4	5.20 ± 0.09	7.73 ± 0.15	266.67 ± 2.03	147.00 ± 4.58	25.42 ± 0.36
NaCl (1mM) + EBL (5 μM)	2 + 2	5.27 ± 0.13	5.63 ± 0.41	220.00 ± 2.65	113.00 ± 1.73	14.23 ± 0.15
NaCl (1mM) + EBL (10 μM)	2 + 2	5.24 ± 0.24	6.13 ± 0.28	224.33 ± 3.48	118.00 ± 1.53	14.86 ± 0.14
NaCl (10mM) + EBL (5 μM)	2 + 2	5.38 ± 0.06	4.93 ± 0.20	203.33 ± 4.33	99.00 ± 2.31	11.22 ± 0.23
NaCl (10mM) + EBL (10 μM)	2 + 2	5.35 ± 0.08	5.33 ± 0.18	207.33 ± 8.11	103.00 ± 3.79	12.63 ± 0.16
EBL (5 μM) + NaCl (1mM)	2 + 2	5.35 ± 0.60	6.88 ± 0.10	238.33 ± 3.53	130.33 ± 1.45	21.06 ± 0.36
EBL (5 μM) + NaCl (10 mM)	2 + 2	5.34 ± 0.63	6.60 ± 0.05	229.33 ± 4.10	127.67 ± 3.84	19.22 ± 0.33
EBL (10mM) + NaCl (1 mM)	2 + 2	5.33 ± 0.67	7.63 ± 0.15	260.67 ± 4.10	144.33 ± 2.96	25.30 ± 0.48
EBL (10mM) + NaCl (10 mM)	2 + 2	5.18 ± 0.18	7.20 ± 0.30	259.00 ± 6.08	140.67 ± 4.91	24.35 ± 0.39
LSD at $P=0.05$		ns	0.35	14.57	11.98	2.20

Each value is the mean of five replications (One replicate = one pot with five plants) ± standard error based on Least Significant Difference test (LSD)

Discussion

Salt stress is important environmental problem limiting crop growth and productivity. Plants hormone like BR have a potential to alleviate the drastic effects of abiotic and biotic stresses. Pre-sowing sodium chloride treatment significantly impairs the germination percentage and embryonic axis length (Table 1). These variations in germination and embryonic axis length could be due to excessive deposition of Na⁺ and Cl⁻ ions in seed tissues that compromises the germinative metabolism by affecting mobilization of mineral and organic reserves along with the commencement of respiration. The findings are in accordance with Li et al. (2010) who reported that salt stress induces a significant reduction in germination and radical length of *Spartina alterniflora* which ultimately leads to shorter embryonic axis while similar results were also noticed by Ahmed and Khan (2010) in playa halophytes. As brassinosteroids (BRs) play key role in mitigating reactive oxygen species (ROX) produced in response to salt stress (Bajguz and Hayat, 2009) so in present study, EBL regulated the germinative metabolism by preventing seed tissues from oxidative stress due to this reason seeds pre-imbibed with EBL exhibited the excellent germination and embryonic growth as compared to those, pre-imbibed with NaCl. Findings of the present investigation showed that BRs could alleviate the adverse effects of stresses (salinity and drought) which are in accordance with results of Ali et al. (2007). Pre-sowing sodium chloride treatment significantly affected the seedlings as well as reduced the nodule number, their biomass and the nitrogenase activity at mature stage (Table 1). In this study, decline in nodule number and nodule fresh/dry biomass under salt stress is in confirmation with the reported findings (Ashraf and Bashir, 2003; Ltaief et al., 2007). Nodules of pea are noted to be differentially affected as compared with other vegetative parts as a consequence of salt stress (Cordovilla et al., 1999; Salah et al., 2009). The reason for decline in nodule biomass is possibly due to the suppression of infection threads that make dependent connection between bacteria and the host. This inhibition of infection threads results in restriction of photo assimilates mobilization which consequently limits the nodule biomass production and growth (Saadallah et al., 2001). The plants from seeds germinating with EBL showed an increase in nodule number, nodule fresh/dry matter and NA (Table 1). Likewise an increment in nodule number, their biomass and NA due to BRs was noted in *Cicer arietinum* (Ali et al., 2007). The EBL treatments, particularly pre- salt imbibitions, conspicuously reduced the negative effect of NaCl by improving the nitrogenase activity (Table 1). This might be due to the influence of EBL on gene expression (Bajguz, 2000; Khrpach et al., 2003) improving NO₃ uptake (Mai et al., 1989) and stabilizing the membrane structure (Hopkins, 1995). Photosynthesis and stomatal conductance are crucial physiological processes related to plant growth and productivity but salt stress reduces both of these attributes (Gorai et al., 2010). In the present study, NaCl stress also reduced Pn and g_s both at seedling (Fig. 1) and mature plant stage (Table 2). The plants germinated from seeds, pre-imbibed with NaCl alone, exhibited the minimum values for photosynthesis rate and stomatal conductance while the seeds imbibed with EBL resulted in the greatest values at both seedling (Fig. 1) and mature plant stage (Table 2). Similar kinds of responses have also been observed in various crops grown under saline conditions in *vinga radiata* (Hayat et al., 2010), *Salvinia natans* (Jampeetong and Brix 2009), and wheat (Zheng et al., 2009). The decline in Pn in the present

investigation under NaCl could be contributed to lowered stomatal conductance which ultimately leads to decreased CO₂ to leaf mesophyll cells while non-stomatal limitation on Pn might also have occurred in leaves under stressful medium. Stomatal conductance may also be lowered due to the antagonistic effect of Na on K, as K is required for stomatal opening and closing (Ahmad and Jabeen, 2005) The results of the present study are also in accordance with the findings of Hayat et al. (2010) who found that EBL is responsible for increasing Pn and g_s in *Vigna radiata* grown under salt stress. Likewise an enhancement in Pn because of BRs was observed in cucumber, tomato and wheat (Ogwenno et al., 2008; Shahbaz et al., 2008; Xia et al., 2009). In the present study, NaCl had reduced the total chlorophyll contents in seedlings (Fig. 1) albeit chlorophyll a and chlorophyll b, at mature stage (Table 2). This reduction in chlorophyll contents may also be attributed to the reactive oxygen species (ROS) (Zhu, 2001) because salinity also causes ROS stress (Centritto et al., 2003; Dobrowski et al., 2005). However, the plants from EBL soaked seeds, showed high green pigments at both growth stages. Similar effect of BRs on chlorophyll was noticed in many other plants (Yu et al., 2004). The results of the present investigation also confirmed the findings of Ahmad et al. (2001), Fariduddin et al. (2003) and Ali et al. (2008) in *Brassica juncea*. Proline is a vital osmolyte that play a significant role in osmotic adjustment (Li et al., 2010) and its accumulation increases under environmental stresses (Cha-um et al., 2009; Pagter et al., 2009; Heidari, 2010; Hajlaoui et al., 2010). In the present study, seeds treated with NaCl gave plants with enhanced proline contents as compared to control (Table 2) but maximum proline contents was noted in plants, germinated from seeds pre treated with EBL followed by NaCl in both seedlings and mature plants. Maximum accumulation of proline in plant tissue due to EBL under stress may be associated with reduction in proline utilization due to the minimum protein formation (Viegas and Silveira, 1999), proline degradation (Hare et al., 1999) and enhancement in proline formation (Lutts et al., 1999) due to the hydrolysis of proteins (Viegas and Silveira, 1999). Reactive Oxygen Species (ROS) are generated in response to environmental stresses including salt stress (Nagesh and Devaraj, 2008) and plants have the potential to neutralize ROS by synthesizing the antioxidant enzymes like SOD, POD and CAT (Seckin et al., 2010) as well as some non-enzymatic antioxidant activities ascorbic acid, tocopherols, carotenoids and flavonoids (Schafer et al., 2002). It is evident from the present study that seeds pre treated with NaCl gave seedlings (Fig. 2) and mature plants with higher SOD, POD and CAT activities as compared to control (Table 3). The findings of this study are consistent with previous observations in *Calendula officinalis*, *Pisum sativum* and *C. maritima* exposed to salinity (Hernandez et al., 2000; Chaparzadeh et al., 2004; Amor et al., 2006). Likewise, Noreen and Ashraf (2009) analyzed the antioxidant enzyme activity in pea and found a prominent increase in SOD, POD and CAT under saline medium. The results of the present investigation are also in agreement with the findings of Heidari (2010) who found a significant increase in CAT along with other antioxidant enzymes in *Brassica napus* under salt stress. BRs have the potential to regulate the activities of various antioxidant enzymes like SOD, POD and CAT in plants grown under a stressful environment (Bajguz and Hayat, 2009). In the present investigation SOD, POD and CAT, antioxidant activities were the highest in seedlings as well as mature plants, germinated from seeds treated with EBL as compared to NaCl treatments and control (water soaked

alone) (Table 3). A similar trend has been reported in many plant species (Ozdemir et al., 2004; Ali et al., 2007; Hayat et al., 2007; Hayat et al., 2010). The results of present study are also consistent with findings of Fariduddin et al. (2009) who reported that BRs are responsible for increase in SOD, POD and CAT in *Brassica juncea* submitted to saline stress. Nitrate being the source of nitrogen (Xiao-yu et al., 2010) has a vital role in nitrogen assimilation and is the vital component of metabolic regulation (Abdelgadir et al., 2005). In the current investigation NaCl induced the reduction in NRA and NiRA while EBL ameliorated the inhibitory effect of NaCl by enhancing the activities of both these enzymes. The seedlings along with mature plants grown from NaCl treated seeds maintained the least NR and NiR activities as compared to control. The reduction in NR and NiR under salt stress was reported in many plant genotypes (Carillo et al., 2005; Nathawat et al., 2005; Debouba et al., 2006; Surabhi et al., 2008). Our results are in agreement with earlier reports. The decrease in NR and NiR activities could be due to reduced mobilization of NO_3^- and NO_2^- under salt stress and destabilization of membrane structure. The seedlings and mature plants from EBL treated seeds exhibited the highest NR and NiR activities (Table 3), it may be due to the improved NO_2^- and NO_3^- uptake (Mai et al., 1989) and a more stable membrane structure because Na and Cl cause the destruction of membranes (Hopkins, 1995) under saline conditions. The plants germinated from NaCl imbibed seeds produced seeds of the lowest weight and protein content which is ultimately responsible for decline in seed as well as pod production (Table 4). It has already been reported that salt stress causes reduction in yield of various crops (Abbas et al., 2010; Gay et al., 2010). In the present study, EBL significantly overcame the adverse effects of salt stress improving number of seeds per pod, seed weight, yield per plant and seed proteins as compared to control (Table 4). The results are also similar to the findings of Ali et al. (2007) who found significant increment in seed weight, seed protein and plant yield of *Cicer arietinum* treated with BRs.

Conclusion

On the basis of the above findings it is concluded that pea seeds, pre-imbibed with EBL resulted in seedlings/mature plants with high dry mass, nodule biomass, nodule number and physiological attributes which leads to excellent plant growth and production. However the EBL treatments especially 10 μM added prior to NaCl imbibitions significantly overcame the NaCl-induced effects by enhancing plant & nodule biomass Pn, Chl, gs, enzymatic activities and ultimately increased plant yield.

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