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Effect of exogenous application of 24-epibrassinolide on growth, protein contents, and antioxidant enzyme activities of *in vitro*-grown *Solanum tuberosum* L. under salt stress

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Abstract Brassinosteroids, such as 24-epibrassinolide (EBR), are some of the biologically most active growth regulators that specifically modulate plant responses to abiotic stress. In this study, the ameliorative effect of EBR on growth, protein contents, and antioxidant enzymes was investigated in two *Solanum tuberosum* L. cultivars, Cardinal and Desiree, growing *in vitro* under salinity stress. EBR (0, 1, or 2 μM) was exogenously applied in two ways, *i.e.*, EBR pretreatment of nodal explants for 8 h (PT) and addition of EBR directly into the Murashige and Skoog (MS) basal medium (IM) followed by explant inoculation. These explants were subjected to salt stress (0, 40, 60, or 80 mM) for 30 d. The growth of plants subjected to NaCl stress was substantially reduced. EBR pretreatment (both PT and IM) alleviated the harmful effects of salt stress for all the measured morphological and biochemical parameters. In general, the quantity of total soluble proteins, superoxide dismutase (SOD), and peroxidase (POD) increased in plants treated with EBR or NaCl alone but were reduced when both were supplemented together. Therefore, exogenous application of EBR not only played a role in terms of *in vitro* potato growth but also significantly affected the tested biochemical parameters.

Keywords Brassinosteroids · 24-epibrassinolide · NaCl stress · Peroxidase · Potato

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Introduction

Abiotic stresses such as salinity, drought, temperature extremes, heavy metals, UV radiation, and nutrient deficiency impair crop growth and productivity and hence threaten global food security (Witcombe *et al.* 2008). Among these, salinity has affected more soils worldwide. It is reported that about 20% of the world's crops on irrigated land are affected by salt stress (Ejaz *et al.* 2012). The reasons include poor drainage, flooding of salt water from coastal land, low quality irrigation water, and accumulation of salts in dry areas (Kijne 2006). Salt stress not only impedes seed germination, but also changes the anatomy and physiology of plants. These circumstances favor the enhanced production of reactive oxygen species (ROS). ROS including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical ($OH^{\bullet}$) are produced as by-products during cellular metabolism and are fairly regularly removed by antioxidant enzyme activities. Stress conditions lead to disruption of the equilibrium between ROS synthesis and scavenging. Enhanced production of ROS negatively affects cell membrane and cellular functions by causing damage to oxidizing proteins and nucleic acids (Wahid and Ghazanfar 2006). The antioxidant system generally consists of two principle players, the non-enzymatic ones including carotenoids, flavonoids, and α-tocopherol; and the enzymatic ones such as superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, and glutathione reductase (Mittler *et al.* 2004). Therefore, the biochemical ways and means to control and/or scavenge the overproduction of ROS may potentially be exploited to increase a plant's ability to withstand saline conditions (Gill and Tuteja 2010). A better understanding of these facts may pave the way towards a precise breeding approach for increasing stress tolerance in plants. A number of culture techniques which include field screening and pot experiments have already been employed

to study several parameters for stress tolerance (Hayat *et al.* 2001; Fariduddin *et al.* 2009; Piñol and Simón 2009). However, the physical and chemical properties of soil and environmental fluctuations make the selection of salt-tolerant varieties a real challenge in such studies. An *in vitro* approach offers better prospects by circumventing the abovementioned limitations (Queirós *et al.* 2007; Karan and Subudhi 2012).

Polyhydroxysteroids, a relatively new class of phytohormone, includes brassinosteroids (BRs) such as 24-epibrassinolide (EBR). They cause morphological and physiological responses in plants at micromolar to nanomolar concentrations, and improve plant growth and yield (Rao *et al.* 2002). Key roles of BRs include modulating cell division, stem elongation, xylem differentiation, leaf development, and reproductive development (Clouse and Sasse 1998); and ethylene biosynthesis, overproduction of DNA, RNA and protein, and changes in the level of endogenous growth regulators such as abscisic acid (ABA; Bajguz 2000). Considerable attention has been given to EBR for its positive effect during stress tolerance in a wide variety of plants such as *Chlorella vulgaris* (Bajguz 2000), *Vigna radiata* (Fariduddin *et al.* 2004), *Oryza sativa* (Özdemir *et al.* 2004), *Cucumis sativus* (Yu *et al.* 2004), *Brassica juncea* (Sharma and Bhardwaj 2007; Ali *et al.* 2008a), *Triticum aestivum* (Ali *et al.* 2008b), *Glycine max* (Zhang *et al.* 2008), and *Vigna unguiculata* (El-Mashad and Mohamed 2012). Its exogenous application has enhanced the growth and yield of many plants by modulating protein content, antioxidant enzyme activities, seed germination, seedling growth, proline content, lipid peroxidation, photosynthetic capacity, and water relations (Özdemir *et al.* 2004; Yu *et al.* 2004; Sharma and Bhardwaj 2007). Although enhanced plant growth has been observed in many plants in response to BRs in field trials during stress, no study has yet been undertaken of potato *in vitro*. In view of this background, the aim of the present investigation was to determine a possible ameliorative effect of EBR on the morphological and biochemical aspects of *in vitro*-grown cultures of potato under salt stress. In addition, the best method of EBR application that could facilitate the alleviation of salt stress was also examined.

Materials and Methods

Procurement of plant material and disinfestation Healthy tubers of potato cultivars Cardinal and Desiree were obtained from the Seed Centre, University of the Punjab, Lahore. They were placed in sterilized sand in a glasshouse in mid-October. After 2 wk, 10-cm long shoots were cut and used as explants. The excised shoots were thoroughly washed with a household detergent (Unilever Karachi, Pakistan) to get rid of adhering particles. Shoots were then rinsed with distilled water several times, and placed for 5–10 min in a 500-mL Erlenmeyer flask containing a solution of 0.7% sodium hypochlorite (NaOCl; Unilever) and 0.1% Tween-20. Shoots were then washed three times with sterile water in laminar air-flow cabinet to remove traces of NaOCl. Shoot induction and maintenance was carried out on Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium prepared manually from individual reagents (Sigma-Aldrich®, St. Louis, MO). The medium was supplemented with 30 g L^{-1} sucrose and 0.7% (*w*/*v*) agar (Agar Technical No. 3; Oxoid™, Thermo Fisher, Hampshire, UK). The pH of the medium was adjusted to 5.7 prior to autoclaving for 15 min at 121°C (103.42 kPa). Ten-milliliter medium was then poured into 25 × 160-mm pre-autoclaved culture tubes. The chlorinated ends of shoots were trimmed and placed as 8-mm single-node cuttings in each culture tube and incubated at 25 ± 2°C with a 16-h photoperiod (40 μmol m^{-2} s^{-1} photon flux density, cool-white fluorescent light, Philips, Karachi, Pakistan) after closing with polypropylene sheets of appropriate size.

Treatment outline and experimental design *In vitro*-grown 30-d-old plants were removed from culture vessels, and nodal segments (1-cm long) were cut for pretreatment with 24-epibrassinolide (EBR: Sigma-Aldrich®) solutions. A 4 × 3 × 2 factorial combination was used involving NaCl, EBR, and method of treatment, respectively. In the first method of EBR treatment (PT), nodal segments were kept for 8 h in the filter-sterilized EBR solutions (0, 1, or 2 μM) on an orbital shaker. Control nodal segments were pretreated in the same manner with autoclaved distilled water. All nodal explants were then cultured in MS medium containing 0, 40, 60, or 80 mM NaCl. Ten culture vessels were used for each treatment. In the second method (IM), the same EBR and NaCl concentrations were added directly in the MS medium prior to placing the nodal explants on the media. MS media with abovementioned NaCl treatments were autoclaved and cooled to around 55°C before the addition of filter-sterilized EBR. The specific levels of EBR and pretreatment duration in this study were selected on the basis of pilot experimentation (Khalid and Aftab, unpublished). The culture vessels were kept at 25 ± 2°C with a 16-h photoperiod (40 μmol m^{-2} s^{-1} photon flux density, cool-white fluorescent light; Philips) for 30 d. The experiment was repeated twice over a period of 8 mo with the same number of replicates for each experiment. Data were pooled for subsequent analysis.

Morphological and biochemical analysis After 30 d, results were recorded for various growth and biochemical parameters including shoot length and number, root length and number, the number of nodes and leaves, fresh weight (FW), protein content, and superoxide dismutase (SOD) and peroxidase (POD) activities. Morphological parameters were measured, and 1 g of plant material from each sample was ground in liquid nitrogen using a mortar and pestle to obtain a fine

powder. Two milliliters of 0.1 M phosphate buffer containing 0.1 g polyvinypolypyrrolidone and 0.01 mL Triton were added to make a slurry that was then centrifuged at 15,400*g*. The supernatant was collected and used as a crude enzyme extract for further estimation.

For quantitative estimation of protein, the Biuret method of Racusen and Johnstone (1961) was employed with slight modifications. To test tubes (15 × 150 mm) containing 2 mL of Biuret reagent (Sigma-Aldrich®), 0.2 mL crude enzyme extract (experimental samples) or 0.2 mL distilled water (control) was added. Test tubes were vortexed and kept for 15 min at 25 ± 2°C to complete the reaction. The absorbance was measured at 545 nm. The protein contents were calculated using a standard curve prepared from bovine serum albumin (Merck, Kenilworth, UK).

Determination of superoxide dismutase (SOD; E.C 1.15.1.1) activity was carried out following Maral *et al.* (1977) with some modifications. Briefly, to 3 mL of reaction mixture (50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 μM Nitroblue tetrazolium, 0.1 mM ethylenediaminetetraacetate, and 2 μM riboflavin; all reagents from Sigma-Aldrich®), 15 μL of crude enzyme extract was added to a 15 × 150-mm test tube (experimental samples), or 15 μL of distilled water was added (control). Both experimental samples and controls were vortexed and then irradiated with 40-W fluorescent cool-white light (40 μmol m^{-2} s^{-1} photon flux density) for 10 min. The absorbance was measured at 560 nm, and SOD activity was calculated by using the formula:

$$\%\,inhibition = \frac{Absorbance\,of\,control\,sample - Absorbance\,of\,experimental\,sample}{Absorbance\,of\,experimental\,sample} \times 100$$

Determination of peroxidase (POD; EC 1.11.1.6) activity was based on Racusen and Foote (1965). For experimental samples, 10-μL crude enzyme extract was added to 0.1 M Tris-HCL buffer (pH 7.2) containing 1% guaiocol (Sigma-Aldrich®). For control samples, 10-μL distilled water was added to the buffer. Before the addition of 0.3% H_2O_2, the experimental and control samples were left for 30 min. The absorbance was measured at 470 nm. Calculation for enzyme content is as follows;

$$\text{Peroxidase content}\left(\text{mgg}^{-1}\text{of tissue}\right) = \frac{\text{A} \times \text{df}}{\text{EU} \times \text{Wt} \times 1000}$$

where

A = absorbance, df = dilution factor, EU = extract used, and Wt = fresh weight of the sample tissue.

Statistical analysis Data were analyzed for two quantitative factors (NaCl and EBR) and one qualitative factor (method of EBR treatment) using analysis of variance (ANOVA). The dependant variables included root number and length, shoot number and length, number of nodes and leaves, FW, protein, SOD, and POD. A full factorial multivariate analysis as mentioned above was performed (along with the preparation of three dimensional graphs) using SPSS 20.0 (Sajid and Aftab 2009).

Results

Morphological parameters Compared to control potato seedlings (without EBR), treatment with various concentrations of NaCl (40, 60, or 80 mM) adversely affected all the studied growth parameters (Table 1). Individual treatments with EBR increased all growth parameters significantly (Table 2; Figs. 1–7). Although both treatment methods also had a significant effect on most growth parameters (except for root length in both cultivars), PT with 1 μM EBR was found to be better for Cardinal and IM containing 2 μM EBR better for Desiree. Mixed results were observed as far as interaction between NaCl, EBR, and method of treatment were concerned. When size was measured, NaCl was found to have a strong effect that drove the morphological parameters (Table 2).

Biochemical attributes In this study, NaCl stress led to alteration in protein contents and levels of antioxidant enzyme activities (Figs. 8–10). An increase in the protein contents was generally observed when either EBR or NaCl were applied alone in comparison with the tested controls. In Cardinal, the quantity of protein was 0.196 and 0.087 mg g^{-1} in non-treated and pretreated control plants, respectively, which increased up to 0.386 and 0.217 mg g^{-1} at 1 μM EBR, and 0.294 and 0.138 mg g^{-1} at 2 μM EBR. A similar trend was observed in Desiree where this value reached 7.133 and 6.737 mgg^{-1} at 1 and 2 μM EBR. The combination of NaCl and EBR led to decreased total proteins in both treatments (Fig. 8). The results were statistically significant among comparisons of cultivars, media, and methods of treatment (Table 2).

Exogenous application of either EBR or NaCl resulted in an overall enhancement of antioxidant enzyme activities.

Table 1 Effects of exogenous application of EBR on morphological parameters of potato cultivars Cardinal and Desiree in response to NaCl stress

NaCl (mM)	EBR conc. (μM)		No. of roots		Root length (cm)		No. of shoots		Shoot length (cm)		No. of nodes		No. of leaves	
			Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
0	0	NT	12.8 ± 0.58	7.8 ± 1.46	5.47 ± 0.36	5.90 ± 0.36	1.4 ± 0.24	1.2 ± 0.2	6.39 ± 0.64	6.80 ± 0.61	11.6 ± 1.07	12.0 ± 0.32	14.6 ± 1.16	15.0 ± 0.55
		PT	6.0 ± 0.707	6.6 ± 0.51	6.74 ± 0.43	7.16 ± 0.90	1.8 ± 0.2	1.2 ± 0.2	7.78 ± 0.72	11.0 ± 1.09	9.8 ± 0.66	7.4 ± 1.02	12.4 ± 0.74	13.5 ± 1.28
	1	IM	14.8 ± 1.15	16.6 ± 0.8	6.58 ± 0.68	6.24 ± 0.18	2.8 ± 0.37	1.4 ± 0.24	16.89 ± 1.09	10.24 ± 1.02	12.8 ± 0.96	13.4 ± 1.32	16.4 ± 1.02	17.0 ± 1.14
		PT	6.2 ± 1.46	6.8 ± 0.54	7.26 ± 0.9	8.16 ± 0.89	2.4 ± 0.6	1.4 ± 0.2	10.92 ± 0.97	13.5 ± 1.96	12.6 ± 0.81	9.4 ± 0.67	15.8 ± 0.66	15.9 ± 0.84
	2	IM	14.4 ± 1.24	16.2 ± 0.86	6.07 ± 0.54	7.48 ± 0.91	2.2 ± 0.37	1.2 ± 0.24	13.77 ± 0.69	10.48 ± 0.6	12.4 ± 1.02	12.2 ± 0.37	15.2 ± 1.15	15.8 ± 0.4
		PT	7.0 ± 1.34	7.8 ± 0.66	7.36 ± 0.76	8.66 ± 0.85	2.4 ± 0.24	1.6 ± 0.2	9.88 ± 1.89	11.0 ± 1.58	13.8 ± 1.31	10.6 ± 1.07	16.9 ± 1.28	16.8 ± 0.93
40	0	NT	3.8 ±	1.8 ± 1.56	4.42 ± 0.83	3.14 ± 0.49	1.4 ± 0.24	1.0 ± 0.2	4.37 ± 0.86	2.70 ± 0.74	8.2 ± 1.15	7.0 ± 0.73	11.6 ± 1.12	11.0 ± 0.91
		PT	1.6 ± 0.4	1.4 ± 0.24	4.4 ± 0.87	2.0 ± 0.38	1.6 ± 0.24	0.8 ± 0.2	3.0 ± 0.311	2.35 ± 0.7	7.2 ± 1.15	6.7 ± 0.50	11.0 ± 0.87	9.9 ± 0.51
	1	IM	6.0 ± 1.04	4.8 ± 0.24	5.54 ± 1.81	5.16 ± 1.21	2.2 ± 0.37	1.4 ± 0.4	6.87 ± 0.19	4.96 ± 0.22	10.4 ± 1.07	10.8 ± 0.49	13.8 ± 1.46	14.6 ± 0.24
		PT	4.4 ± 0.81	3.1 ± 0.58	6.0 ± 0.87	3.66 ± 0.39	3.2 ± 0.37	1.1 ± 0.2	7.06 ± 1.71	4.05 ± 0.42	11.0 ± 2.46	9.8 ± 1.02	11.2 ± 3.67	12.4 ± 1.01
	2	IM	4.8 ± 1.01	4.0 ± 0.87	5.41 ± 1.63	3.68 ± 1.09	2.6 ± 0.5	1.2 ± 0.37	6.20 ± 0.95	4.94 ± 0.45	7.8 ± 2.17	11.4 ± 1.09	11.0 ± 2.25	15.0 ± 1.87
		PT	4.8 ± 0.86	2.8 ± 0.37	6.02 ± 0.36	3.32 ± 0.38	3.2 ± 0.2	1.0 ± 0.2	7.26 ± 1.65	4.01 ± 0.42	7.9 ± 0.58	8.8 ± 0.93	11.6 ± 0.83	11.8 ± 0.97
60	0	NT	1.8 ± 0.37	0.6 ± 0.86	2.96 ± 0.29	1.72 ± 0.44	1.8 ± 0.48	0.8 ± 0.4	1.76 ± 0.51	1.66 ± 0.87	6.8 ± 0.58	5.8 ± 0.81	9.2 ± 0.86	9.6 ± 0.83
		PT	1.2 ± 0.58	0.8 ± 0.2	3.46 ± 1.8	1.28 ± 0.37	1.6 ± 0.4	0.8 ± 0.2	1.62 ± 0.23	1.83 ± 0.52	4.6 ± 0.5	5.3 ± 0.51	8.4 ± 0.77	8.6 ± 0.37
	1	IM	2.6 ± 0.87	1.4 ± 0.87	3.88 ± 0.49	3.22 ± 0.92	3.2 ± 0.37	2.0 ± 0	1.88 ± 0.48	3.18 ± 0.38	7.4 ± 1.74	7.0 ± 0.58	10.0 ± 1.87	12.0 ± 0.51
		PT	2.6 ± 0.74	1.0 ± 0	4.9 ± 1.5	2.54 ± 0.31	4.2 ± 1.06	1.9 ± 0.31	2.32 ± 0.31	2.89 ± 0.36	8.0 ± 0.83	6.7 ± 0.58	12.1 ± 0.67	11.8 ± 0.37
	2	IM	3.8 ± 0.58	2.6 ± 0.92	5.44 ± 0.37	2.64 ± 0.02	2.4 ± 0.24	1.6 ± 0.48	2.14 ± 0.6	3.02 ± 0.09	7.8 ± 1.15	6.8 ± 0.58	10.6 ± 1.29	10.6 ± 1.12
		PT	3.6 ± 1.58	0.9 ± 0.37	3.48 ± 1.65	2.17 ± 0.92	2.6 ± 0.67	1.4 ± 0.24	2.70 ± 0.4	2.56 ± 0.4	7.9 ± 0.51	6.4 ± 0.70	10.6 ± 0.4	9.2 ± 0.54
80	0	NT	1.4 ± 0.67	ND	1.66 ± 0.71	ND	1.0 ± 0	1.4 ± 0.4	0.78 ± 0.17	0.72 ± 0.5	3.8 ± 0.37	3.4 ± 0.81	7.4 ± 0.51	6.6 ± 1.41
		PT	1.0 ± 0.77	ND	2.36 ± 2.12	ND	1.4 ± 0.4	1.2 ± 0.2	0.91 ± 0.22	0.68 ± 0.14	3.4 ± 0.81	2.9 ± 9.37	6.4 ± 1.12	5.9 ± 0.49
	1	IM	2.0 ± 0.44	ND	2.97 ± 0.23	ND	1.6 ± 0.4	2.2 ± 0.66	1.69 ± 0.28	1.26 ± 0.08	4.6 ± 0.67	4.0 ± 0.31	8.4 ± 0.5	8.6 ± 0.6
		PT	2.6 ± 1.43	ND	3.48 ± 1.65	ND	3.0 ± 0.54	1.8 ± 0.37	1.74 ± 0.21	1.12 ± 0.19	4.6 ± 0.24	3.9 ± 0.32	8.8 ± 0.2	7.6 ± 0.51
	2	IM	1.8 ± 0.37	ND	3.02 ± 0.31	ND	1.4 ± 0.24	1.6 ± 0.24	1.66 ± 0.19	1.0 ± 0.05	5.0 ± 0.71	3.6 ± 0.24	8.6 ± 0.67	7.8 ± 0.66
		PT	2.6 ± 1.16	ND	2.7 ± 1.21	ND	2.8 ± 0.2	1.3 ± 0.24	1.69 ± 0.24	0.9 ± 0.08	3.8 ± 0.2	3.8 ± 0.51	8.7 ± 0.24	6.9 ± 0.71

Results of all the parameters are means from 30 replicate cultures

Table 2 Multivariate full factorial analysis between fixed factors and dependent variables of *S. tuberosum*

Dependant variables	Source	Sum of squares		df		Mean square		*F*		Significance		Partial Eta squared	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
Root number	Model	1097	14947	23	23	477	694	130	301	0.000	0.000	0.811	0.909
	NaCl (A)	7766	10805	3	3	2588	3601	705	1668	0.000	0.000	0.753	0.878
	EBR (B)	367	611	2	2	183	305	50	141	0.000	0.000	0.126	0.289
	EBR treatment (C)	781	871	1	1	781	871	212	403	0.000	0.000	0.234	0.367
	AB	60	600	6	6	10	100	2	46	0.000	0.000	0.023	0.286
	AC	1896	1447	3	3	632	482	172	223	0.000	0.000	0.426	0.491
	BC	48	233	2	2	24	116	6	53	0.001	0.000	0.019	0.134
	ABC	52	378	6	6	8	63	2	29	0.026	0.000	0.020	0.201
	Total	29490	25524	720	720								
	Corrected total	13528	16450		719								
	R squared	0.811	0.909	719									
	Adj R squared	0.805	0.906										
Root length	Model	2003	5487	23	23	87	238	17	138	0.000	0.000	0.366	0.821
	NaCl (A)	1595	5029	3	3	531	1676	106	975	0.000	0.000	0.315	0.808
	EBR (B)	151	53	2	2	75	26	15	15	0.000	0.000	0.042	0.043
	EBR treatment (C)	3	1	1	1	3	1	0.716	0.698	0.398	0.404	0.001	0.001
	AB	29	134	6	6	4	22	0.984	13	0.435	0.000	0.008	0.101
	AC	110	135	3	3	36	45	7	26	0.000	0.000	0.031	0.102
	BC	40	27	2	2	20	13	4	7	0.017	0.000	0.012	0.022
	ABC	71	378	6	6	11	17	2	10	0.026	0.000	0.020	0.082
	Total	22478	14160	720	720								
	Corrected total	5475	6684		719								
	R squared	0.366	0.821	719									
	Adj R squared	0.345	0.815										
Shoot number	Model	423	104	23	23	18	4	24	12	0.000	0.000	0.444	0.292
	NaCl (A)	56	27	3	3	18	9	24	9	0.000	0.000	0.096	0.097
	EBR (B)	213	7	2	2	106	3	139	9	0.000	0.000	0.287	0.028
	EBR treatment (C)	48	1	1	1	48	1	63	4	0.000	0.000	0.083	0.007
	AB	50	17	6	6	8	2	10	7	0.000	0.000	0.086	0.064
	AC	24	7	3	3	8	6	10	6	0.000	0.000	0.044	0.027
	BC	15	17	2	2	7	8	9	23	0.000	0.000	0.028	0.063
	ABC	16	26	6	6	2	4	3	12	0.002	0.000	0.029	0.096
	Total	4626	1704	720	720								
	Corrected total	953	359		719								
	R squared	0.444	0.292	719									
	Adj R squared	0.426	0.268										
Shoot length	Model	13816	10984	23	23	600	477	216	215	0.000	0.000	0.877	0.877
	NaCl (A)	10949	9970	3	3	3649	3323	1315	1498	0.000	0.000	0.850	0.866
	EBR (B)	1050	381	2	2	525	190	189	85	0.000	0.000	0.352	0.198
	EBR treatment (C)	219	40	1	1	219	40	79	18	0.000	0.000	0.102	0.025
	AB	809	129	6	6	134	21	48	9	0.000	0.000	0.295	0.077
	AC	226	410	3	3	74	138	27	61	0.000	0.000	0.105	0.210
	BC	123	23	2	2	61	11	22	5	0.000	0.005	0.060	0.015
	ABC	436	29	6	6	72	4	26	2	0.000	0.042	0.184	0.019
	Total	34793	27156	720	720								
	Corrected total	15747	12528	719	719								
	R squared	0.877	0.877										

Table 2 (continued)

Dependant variables	Source	Sum of squares		df		Mean square		*F*		Significance		Partial Eta squared	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
	Adj R squared	0.873	0.873										
Nodes	Model	6448	7180	23	23	280	477	56	127	0.000	0.000	0.651	0.809
	NaCl (A)	5564	5817	3	3	1854	1939	373	794	0.000	0.000	0.617	0.774
	EBR (B)	373	436	2	2	186	218	37	89	0.000	0.000	0.098	0.204
	EBR treatment (C)	168	273	1	1	168	273	33	112	0.000	0.000	0.046	0.139
	AB	150	265	6	6	25	44	5	18	0.000	0.000	0.042	0.135
	AC	90	289	3	3	30	96	6	39	0.000	0.000	0.025	0.146
	BC	76	19	2	2	38	9	7	4	0.001	0.018	0.022	0.011
	ABC	24	78	6	6	4	13	0.808	5	0.564	0.000	0.007	0.044
	Total	52968	492380	720	720								
	Corrected total	9908	8880	719	719								
	R squared	0.651	0.809										
	Adj R squared	0.639	0.802										
Leaves	Model	5620	6777	23	23	244	294	36	110	0.000	0.000	0.544	0.786
	NaCl (A)	4751	5757	3	3	1583	1919	233	721	0.000	0.000	0.502	0.757
	EBR (B)	280	372	2	2	140	186	20	70	0.000	0.000	0.056	0.168
	EBR treatment (C)	244	217	1	1	244	217	36	81	0.000	0.000	0.049	0.105
	AB	92	280	6	6	15	46	2	17	0.036	0.000	0.019	0.132
	AC	35	64	3	3	11	21	1	8	0.155	0.000	0.008	0.034
	BC	30	14	2	2	15	7	2	2	0.103	0.067	0.007	0.008
	ABC	185	70	6	6	30	11	4	4	0.000	0.000	0.038	0.037
	Total	98004	103572	720	720								
	Corrected total	10336	8627	719	719								
	R squared	0.544	0.786										
	Adj R squared	0.529	0.778										
Fresh weight	Model	10	19	23	23	0.459	0.838	108	408	0.000	0.000	0.782	0.931
	NaCl (A)	6	14	3	3	2.23	2.35	528	2351	0.000	0.000	0.695	0.910
	EBR (B)	0.744	1.64	2	2	0.372	0.823	88	401	0.000	0.000	0.202	0.536
	EBR treatment (C)	1.	0.741	1	1	1.34	0.741	317	361	0.000	0.000	0.313	0.342
	AB	0.303	1.95	6	6	0.051	0.326	11	158	0.000	0.000	0.094	0.578
	AC	0.416	0.265	3	3	0.139	0.088	32	42	0.000	0.000	0.124	0.156
	BC	0.699	0.130	2	2	0.349	0.065	17	31	0.000	0.000	0.192	0.083
	ABC	0.359	0.061	6	6	0.060	0.010	14	4.9	0.000	0.000	0.109	0.041
	Total	35	46	720	720								
	Corrected total	13	20	719	719								
	R squared	0.782	0.931										
	Adj R squared	0.775	0.929										
Protein	Model	104	19151	23	23	4.5	832	107	241	0.000	0.000	0.781	0.889
	NaCl (A)	62	6265	3	3	20	2088	496	606	0.000	0.000	0.681	0.723
	EBR (B)	8.5	405	2	2	4.2	202	101	58.8	0.000	0.000	0.227	0.145
	EBR treatment (C)	10	4254	1	1	10.9	4254	262	1235	0.000	0.000	0.274	0.640
	AB	8.7	456	6	6	1.45	76	34.8	22	0.000	0.000	0.231	0.160
	AC	9.8	7591	3	3	3.28	2530	78.2	734	0.000	0.000	0.252	0.760
	BC	1.4	19	2	2	0.729	9.75	17.3	2.8	0.000	0.060	0.048	0.008
	ABC	2.08	159	6	6	0.348	26.6	8.29	7.7	0.000	0.000	0.067	0.063
	Total	383	63494	720	720								
	Corrected total	133	21548	719	719								

Table 2 (continued)

Dependant variables	Source	Sum of squares		df		Mean square		*F*		Significance		Partial Eta squared	
		Car	Des	Car	Des	Car	Des	Car	Des	Car	Des	Car	Des
	R squared	0.781	0.889										
	Adj R squared	0.774	0.885										
SOD	Model	23132	27200	23	23	1005	1182	328	172	0.000	0.000	0.916	0.851
	NaCl (A)	10159	826	3	3	3386	275	1105	40	0.000	0.000	0.827	0.147
	EBR (B)	378	2580	2	2	189	1290	61	188	0.000	0.000	0.151	0.351
	EBR treatment (C)	3346	19680	1	1	3346	19680	1092	2868	0.000	0.000	0.611	0.805
	AB	842	753	6	6	140	125	45	18	0.000	0.000	0.283	0.136
	AC	7318	1126	3	3	2439	375	796	54	0.000	0.000	0.774	0.191
	BC	297	491	2	2	148	245	48	35	0.000	0.000	0.122	0.093
	ABC	790	1741	6	6	131	290	43	42	0.000	0.000	0.270	0.267
	Total	71674	101478	720	720								
	Corrected total	25264	3175	719	719								
	R squared	0.916	0.851										
	Adj R squared	0.913	0.846										
POD	Model	43249	43270	23	23	1880	18813	458	463	0.000	0.000	0.938	0.939
	NaCl (A)	1530	5460	3	3	510	1820	124	44	0.000	0.000	0.349	0.162
	EBR (B)	388	10750	2	2	194	5357	47	132	0.000	0.000	0.120	0.276
	EBR treatment (C)	30828	351220	1	1	30828	351220	7513	8657	0.000	0.000	0.915	0.926
	AB	3870	24539	6	6	645	4089	157	100	0.000	0.000	0.575	0.465
	AC	2194	5490	3	3	731	1830	178	45.1	0.000	0.000	0.435	0.163
	BC	518	10691	2	2	259	5345	63	131	0.000	0.000	0.154	0.275
	ABC	3919	24549	6	6	653	4091	159	100	0.000	0.000	0.578	0.465
	Total	81442	813188	720	720								
	Corrected total	46105	460938		719								
	R squared	0.938	0.939	719									
	Adj R squared	0.936	0.937										

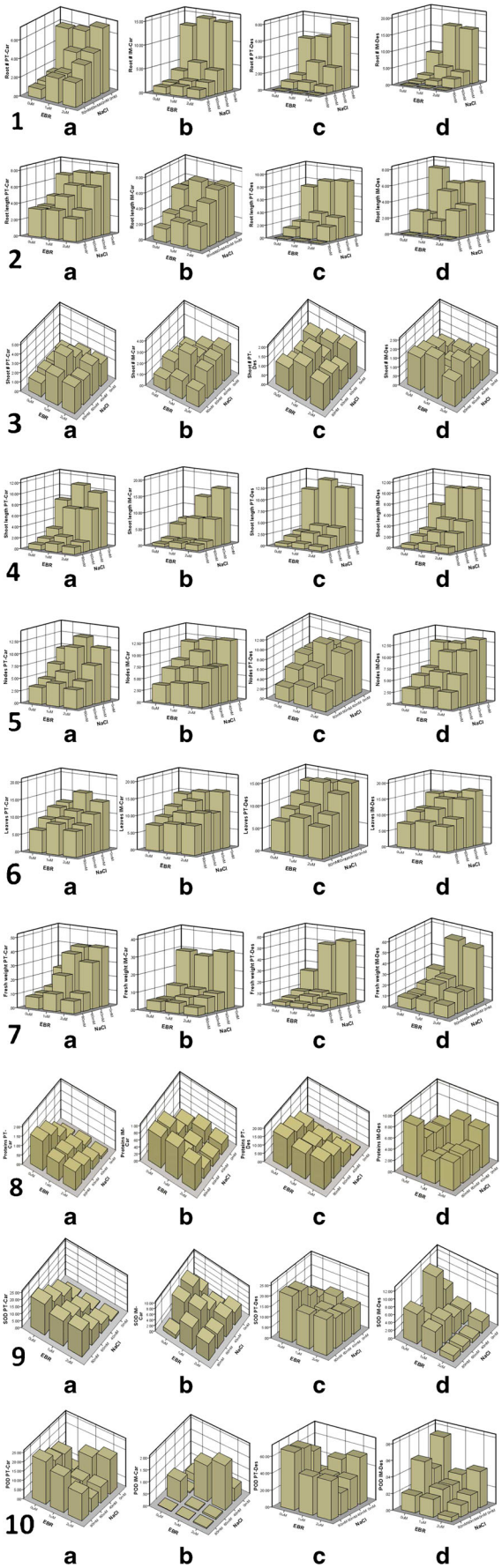
However, a reduction in the level of SOD was observed when plants were grown with both EBR and NaCl (Fig. 9). The maximum decline was observed in Desiree when plants were supplemented with 2 μM EBR and 40 mM NaCl compared with the respective controls (Fig. 9).

An increase in the activity of POD was recorded with increasing concentration of either NaCl or EBR (PT; Figs. 10*a*, 10*c*). However, their interaction led to an overall decrease in POD contents. The maximum value for protein (68.33 mgg^{-1}) in Desiree was at 80 mM NaCl. The value decreased after exogenous application of 1 or 2 μM EBR, to 45.41 and 46.01 mg g^{-1} proteins, respectively. These results revealed that while PT stimulated POD activities in both the cultivars, IM yielded mixed results (Figs. 10*b*, 10*d*). It may be observed from the above that an overall trend of the biochemical attributes in general was rather similar though the two tested potato cultivars have shown differential preference for the method of treatment. When compared statistically for the effect size, EBR and methods of treatment influenced SOD and POD more than proteins (Table 2).

Discussion

Both cultivars were significantly influenced by *in vitro* NaCl, but exhibited differential responses to various NaCl and EBR treatments, with Cardinal being comparatively salt tolerant and Desiree being moderately sensitive. These results are in line with a previous study by Shahbaz *et al.* (2008) reporting that the inhibitory effects of NaCl stress were ameliorated significantly in response to application of EBR in both wheat cultivars studied (S-24, salt tolerant and MH-97, salt sensitive). However, the salt-tolerant variety showed a better response towards EBR treatment than the sensitive one.

A couple of small-scale methods of exogenous application of EBR have already been reported including foliar application (Fariduddin *et al.* 2004), pretreatment of seeds (Hayat *et al.* 2001; Piñol and Simón 2009), and as medium constituent (Arora *et al.* 2008). Pretreatment of seeds was considered to be the preferred method in *O. sativa* (Rao *et al.* 2002; Sharma *et al.* 2013), *Medicago sativa* (Zhang *et al.* 2007), and *Zea mays* (Arora *et al.* 2008), whereas the foliar



◀ **Figures 1–10** Comparative effect of treatments (pretreated, *PT*; in medium, *IM*) *viz*-á-*viz* 24-epibrassinolide (0, 1, or 2 μM) and NaCl (0, 40, 60, or 80 mM) on root number/length (*1*, *2*), shoot number/length (*3*, *4*), number of nodes/leaves (*5*, *6*), fresh weight (g; *7*), protein (mg g^{-1}; *8*), SOD (U mg^{-1}; *9*), and POD (mg g^{-1}; *10*) in *in vitro* potato plants (cvs. Cardinal and Desiree).

application of BRs was shown to be quite useful in *Phaseolus vulgaris* (Upreti and Murti 2004), *Solanum lycopersicum* (Ogweno *et al.* 2008), *T. aestivum* (Shahbaz *et al.* 2008), and *G. max* (Zhang *et al.* 2008). Pretreatment of vegetative tissues grown *in vitro* on the other hand has not been reported so far. An *in vitro* approach provides an opportunity to manipulate cultures reproducibly under the desired set of experimental conditions.

In a study on *Hordeum vulgare*, Tabur and Demir (2009) found amelioration of the inhibitory effects of salinity stress on germination and growth of seedlings when seeds were pretreated with 3 μM EBR. In a similar study on *O. sativa*, Anuradha and Rao (2003) indicated that seed pretreatment with 3 μM EBR not only decreased the influence of salt stress but also improved plant growth and nitrate reductase activity while reducing pigment loss. Although both methods of exogenous EBR application were shown in the present study to be beneficial, pretreatment of nodal explants of Cardinal with 1 μM EBR was most effective. Interestingly, the second method (IM) with 2 μM EBR resulted in the best stress alleviation response in Desiree. These results are in line with several prior studies reporting positive role of BRs for the enhancement of growth either with or without supplemental salt in *C. sativus* (Yu *et al.* 2004), *Cicer arietinum* (Ali *et al.* 2007), *T. aestivum* (Ali *et al.* 2008b), and *B. juncea* (Fariduddin *et al.* 2009). It is, therefore, inferred that the use of low EBR concentrations generally alleviates stress in diverse plant species.

As far as the young seedlings were concerned, the increase in shoot length on EBR application may perhaps be a result of enhanced carbohydrate transport from the primary leaf to the upper region, *i.e.*, epicotyl (Nakajima and Toyama 1999). However, there are contrasting reports about the role of BRs in root development. Kartal *et al.* (2009) described a positive relationship between BRs application and root growth *via* increased mitotic activity in *H. vulgare*. On the contrary, Özdemir *et al.* (2004) reported an inhibitory effect of EBR on root growth in *O. sativa*. In addition, the response of EBR in root growth was found to be dose dependent. In two independent studies on *Arabidopsis* (Kim *et al.* 2007) and *Allium cepa* (Howell *et al.* 2007), low EBR concentrations (10^{-10} and 10^{-9} M) stimulated root growth, but inhibited root growth at higher doses (10^{-9}, 10^{-8}, and 10^{-7} M in *Arabidopsis*, and 10^{-7} M in *A. cepa*).

Stress tolerance induced by BRs appears to be a complex phenomenon and probably involves several intrinsic factors. Quantitative analysis of the total proteins in the present study

showed an increasing trend in both the potato cultivars when subjected to different NaCl concentrations. This increase was far more in Desiree compared with Cardinal. The reason might be the synthesis of some stress-related proteins (Sharma *et al.* 2013). Sajid and Aftab (2009) also described that higher amounts of proteins under stress conditions could help plants sustain growth. One of the possible modes of action may simply be to overcome an enhanced production of ROS by such upregulated proteins. It is interesting to note that studies at the gene expression level have also confirmed the association between overexpression of stress-responsive proteins (*StDREB1* gene) and stress tolerance in potato. Moreover, *StDREB1* provided protection against ROS under stress through regulation of the stress-responsive signaling pathway, *i.e.*, expressing other genes putatively associated with stress resistance, *e.g.*, *StCDPK4* and *StCDPK5* (Bouaziz *et al.* 2013). Enhancement of ROS including O_2^-, OH, H_2O_2, and $^1O_2^-$ (Munne-Bosch and Penuelas 2003) under various abiotic stresses (salt, heat, drought) is well-known. Among the biochemical defense mechanisms that many plant species have developed, antioxidant enzymes appear to be probably the most effective system at scavenging these enhanced ROS (Farooq *et al.* 2008). The role of ZmMPK5 (ABA-regulated mitogen-activated protein kinase) on antioxidants was evaluated in response to BRs application in *Z. mays* (Zhang *et al.* 2010). The accumulation of H_2O_2 was shown to upregulate the activities of antioxidant enzymes. Therefore, an upregulation of the antioxidant defense system under an enhanced ROS scenario as evident in the above study (Zhang *et al.* 2010), as well as others mentioned above including the current investigation, probably does not come as a surprise and in fact seems to hold true in many plant species.

As outlined before, both SOD and POD levels were monitored in the present investigation in order to understand their role in salinity tolerance of potato vis-á-vis EBR treatments. The results from this study only partially corroborate the findings of Shahbaz *et al.* (2008) and Liu *et al.* (2009) regarding the antioxidant enzymes SOD and POD in *T. aestivum* and *Chorispora bungeana*. Both groups had shown an increased antioxidant level in the above species under abiotic stress that rose even further with the exogenous application of BRs. As far as various salt treatments in the present study were concerned, enhanced SOD and POD levels were observed not only in line with the abovementioned studies but also in agreement with several others (Lima *et al.* 2002; Ogweno *et al.* 2008; Liu *et al.* 2009; Ejaz *et al.* 2012; Nouman *et al.* 2014). Arora *et al.* (2008) interpreted this to be a mechanism for salinity tolerance in *Z. mays* after BRs application. In the present study, however, the combination of EBR and NaCl decreased the activities of SOD and POD. The reduced activities of SOD and POD might be associated with the removal of the stressful conditions by the EBR treatments in the first place. Not surprisingly, therefore, reduction in POD activity has already been reported in EBR-treated epicotyls of *V. radiata* (Wu and Zhao 1991) and hypocotyls of *C. sativus* (Xu and Zhao 1989). Vardhini and Rao (2003) reported a decrease in POD activity after the application of BRs to *Sorghum vulgare* seeds under osmotic stress. While these findings suggest that activities of antioxidant enzymes might help plants to ameliorate the effects of salt stress, possible coexistence of additional mechanisms operating in potato may not be ruled out without further investigation. It might not be out of context here to mention the possible triggering of other phytohormones in response to EBR pretreatment in potato. Changes in the endogenous ABA levels in response to BRs treatment in *C. vulgaris* as reported by Bajguz (2000) were probably caused by the same mechanism. The interaction of BRs with gibberellins and auxins has also been reviewed in detail (Mandava *et al.* 1981; Yopp *et al.* 1981). Synergistic modes of action to enhance growth thus remain a strong possibility in potato as well.

In conclusion, a useful role of EBR in response to salinity stress in potato has been observed in this study. These results are vital not only for the understanding of the potential role of BRs in the growth and development of potato and other species but also for its use in agriculture at a larger scale. In the present investigation, salt stress markedly decreased growth in both potato cultivars. Although either method of exogenous application of EBR could potentially alleviate the inhibitory effects of stress from *in vitro*-grown potato plants, pretreatment (PT) of nodal explants with 1 μM EBR was the best choice in Cardinal. Desiree on the other hand responded best in terms of growth parameters with 2 μM EBR in medium (IM). It appears that the use of lower EBR levels in potato has greater potential for increased crop production both in saline and non-saline soils. Detailed insight of the synergistic association of EBR with other plant hormones also needs to be elucidated further. Pretreatment of vegetative parts, *i.e.*, nodes of potato, with EBR provides another method of EBR application having potential for its possible extension in the field. Pretreatment of propagules such as potato eyes is one such possibility. Although further research in this direction will answer these emerging questions, promising results in this study have provided an impetus to move forward with these studies.

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