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EFFECT OF BRASSINOSTEROIDS ON SEED GERMINATION AND SEEDLING GROWTH OF RADISH (*RAPHANUS SATIVUS* L.) UNDER ARSENIC TOXICITY STRESS

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ABSTRACT

The effect of brassinosteroids on germination and seedling growth of radish (*Raphanus sativus* L.) under arsenic toxicity was investigated. Exogenous application of brassinosteroids substantially removed the inhibiting effect of arsenic toxicity on seed germination. The application of brassinosteroids removed the toxic impact of arsenic on seedling growth. The amelioration of arsenic stress by brassinosteroids found associated with increased levels of nucleic acids, soluble proteins as well as the osmoprotectant proline. Brassinosteroid feeding resulted in reduced membrane peroxidation (measured as MDA content) in seedlings challenged with elevated levels of arsenic. Further, the application of brassinosteroids to radish seedlings growing under toxic levels of arsenic was resulted in increased activity of antioxidant enzymes such as catalase, superoxide dismutase and peroxidase. The results of the study clearly demonstrated the protective role of brassinosteroids in radish seedlings against the arsenic toxicity.

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INTRODUCTION

Arsenic is a toxic metalloid (Rathinasabapathi *et al*., 2006), and it is ubiquitous in many environments and highly toxic to all forms of life. Arsenic occurs predominantly in inorganic form as arsenate (AsV) and arsenite (AsIII) (Tripathi *et al*., 2007). Though the main source of arsenic is geological, anthropological activity like burning of fossil fuels, mining processes and use of arsenic based pesticides is contributing substantially to the arsenic contamination (Bissen and Frimmel. 2003). Arsenic is analogous to phosphate as both are placed in same group in the periodic table Va and have similar electron configuration, chemical properties and compete for the same uptake carriers in the root plasmalemma (Ullrich-Eberius *et al*., 1989; Meharg and Hartley-Whitaker., 2002).
Brassinosteroids (BRs) comprises a class of plant-specific steroidal hormones (Bajguz and Hayat 2009; Clouse 2011). Brassinosteroids are considered as plant hormones with pleiotropic effect as they regulate various aspects of plant growth and development, including cell elongation, photomorphogenesis, xylem differentiation, seed germination, leaf bending and epinasty, flowering, senescence, abscission and photosynthesis (Rao *et al*., 2002; Yang *et al*. 2011).

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Moreover, brassinosteroids were shown to ameliorate various environmental stresses such as drought stress, high temperature stress, chilling stress, salinity stress and heavy metal stress (Vardhini *et al*. 2010; Vriet *et al*. 2012). The present study was carried out to investigate the impact of brassinosteroids on germination of seeds and seedling growth of radish (*Raphanus sativus* L.) under high levels of arsenic.

MATERIALS AND METHODS

24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) were purchased from CID tech research Inc, Mississauga, Ontario, Canada. Seeds of radish (*Raphanus sativus* L.) Pusa Chekthi variety were obtained from National Seed Corporation, Hyderabad, India. Preliminary experiments were conducted employing different concentrations of arsenic solutions viz., 10 µM, 25 µM, 50 µM, 100 µM, 200 µM, 400 µM. Arsenate was applied as sodium arsenate ($Na_2HAsO_4./7H_2O$). 50 µM of arsenic solution was selected as metal stress concentration where growth was considerably but not completely inhibited. Radish seeds were surface sterilized with 0.5% (v/v) sodium hypochlorite solution from commercially available 4% NaClO and washed thoroughly with several changes of sterile distilled water. They were soaked for 24 h either in: (i) distilled water (control) (ii) 0.5, 1.0 and 2.0 µM concentrations of EBL/HBL (iii) 50µM

arsenic solution (stressed control) (iii) 50µM arsenic solution supplemented with 0.5, 1.0 and 2.0 µM EBL/HBL. 20 seeds for each treatment were distributed in separate petri plates (15 cm diameter) provided with whatman No.1 filter papers and 5ml of respective test solution was added. The seeds were allowed to germinate in the dark at 25±1 ^{0}C. The number of seeds germinated was recorded at the end of 24, 36 and 72 h under safe green light. After 72 h, 5 seedlings were retained in each plate and 5 ml test solution was added and allowed to grow. On 7th day growth of the seedlings was recorded in terms of length, fresh weight and dry weight. 7-day old seedlings were thoroughly homogenized in 70% (v/v) ethyl alcohol and were stored in deep freezer at -20°C and used for the extraction of soluble proteins and nucleic acids. However 7 day old fresh seedlings were used for estimation of free proline, MDA and enzyme assays.

Nucleic acids

DNA and RNA fractions in the ethyl alcohol homogenate were separated by the method of Ogur and Rosen (1950). DNA was estimated by the procedure of Burton (1968) employing diphenylamine reagent and RNA was quantified by the method of Schneider (1957) using orcinol reagent.

Soluble proteins

Soluble proteins in alcohol homogenate (extract in case of enzyme assay) were precipitated by using 20% (w/v) trichloroacetic acid. The precipitate was dissolved in 5 ml of 1% (w/v) sodium hydroxide and was centrifuged at 4000 rpm for 10 min. The supernatant was used for estimation of proteins by Lowry *et al.,* (1951) method

Free proline

The amount of proline content was estimated as described by Bates *et al*., (1973). Seedling material (0.5 g) was homogenized with 10 ml of 3 % (w/v) sulfosalicylic acid and the homogenate was filtered through whatman No. 2 filter paper. The supernatant was taken for proline estimation. The reaction mixture was composed of 2 ml of plant extract, 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid and heated in a boiling water bath for one hour. The reaction was terminated in an ice bath followed by addition of 4 ml of toluene. The contents were shaken vigorously and then allowed to separate into phases. The chromophase containing upper toluene phase was carefully taken out with the help of a pipette and the absorbance was taken at 520 nm in a UV-visible spectrometer (SCHIMADZU UV-1800, Japan). The amount of proline present was quantified with the help of proline standard graph.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content following the method of Heath and Packer (1968). Seedlings (1.0 g) were homogenized with 3 ml of 0.5% thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid. The homogenate was incubated at 95 ºC for 30 min and the reaction was stopped in ice. The samples were centrifuged at 10,000 × g for 5 min, the absorbance of the resulting supernatant was recorded at 532 nm and the value for the non-specific absorbance at 600 nm was subtracted. The absorbance coefficient of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Antioxidant enzymes

Fresh seedling material (200 mg) was homogenized with sodium phosphate buffer at pH 7.0 for Catalase, Peroxidase and at pH 7.8 for Superoxide dismutase activities. The supernatant was used to measure the activity of the enzymes.

Catalase (CAT, EC; 1.11.1.6) activity was assayed by the method of Barber (1980). Enzyme extract (0.5 ml) was added to 2 ml of hydrogen peroxide and 3.5 ml of phosphate buffer (pH 7.0). The reaction was stopped after incubation by adding 10 ml of 2% (v/v) concentrated sulphuric acid, and the residual hydrogen peroxide was titrated against 0.01 M $KMnO_4$ until a faint purple color persisted for at least 15 sec. The activity of the enzyme was expressed as enzyme units.

Superoxide dismutase (SOD, E.C; 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) of Beauchamp and Fridovich (1971). Three ml of reaction mixture contained 40 mM phosphate buffer (PH=7.8), 13 mM methionine, 75 µM nitroblue tetrazolium, 0.1 mM EDTA, 0.1 ml of enzyme extract and 2 µM riboflavin. Riboflavin was added at the end. After mixing the contents, test tubes were shaken and placed 30 cm below light source consisting of two 15 watt fluorescent tubes. The reaction was started by switching on the lights. The reaction was allowed to take place for 30 minutes and was stopped by switching off the lights. A tube with protein kept in the dark served as blank, while the control tube was without the enzyme and kept in the light. The absorbance was measured at 540 nm. The activity of superoxide dismutase is the measure of NBT reduction in light without protein minus NBT reduction in light with protein. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light.

Peroxidase (POD, EC; 1.11.1.7*)* activity was assayed adopting the method of Kar and Mishra (1976). To 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 7.0), 1.0 ml of 0.01 M pyrogallol and 1.0 ml of 0.005 M H_2O_2 were added. After incubation, the reaction was stopped by adding 1.0 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm. The enzyme activity was expressed in absorbance units.

RESULTS AND DISCUSSION

Arsenic at 50µM concentration substantially reduced the germination of radish seeds (Table-1). Similar inhibition of seed germination by arsenic was also reported due to arsenic toxicity in case of rice (Abedin, 2002) and sunflower (Imran *et al*., 2013). However, in the present study, exogenous application of brassinosteroids resulted in reducing the toxic impact of arsenic on seed germination. With the increase in the concentration of brassinosteroids, there was gradual improvement in percentage of seed germination under arsenic toxicity. At 2µM concentration both the brassinosteroids employed in the study completely eliminated the inhibitory effect of arsenic on seed germination. The growth of the radish seedlings was found severely impaired under arsenic toxicity

(Table-2). This observation was consistent with the findings of Singh *et al* (2007) and Srivastava *et al* (2013) in case of mung bean and black gram seedling growth respectively at high arsenic levels. In the present study, with the supplementation of brassinosteroids, the toxic effect of arsenic on seedling growth of radish was found reduced. The impact of added brassinosteroids on the removal of toxic influence of arsenic was found dose dependent. At 2µM concentration both the brassinosteroids completely offseted the toxicity of arsenic. In an earlier study Ramakrishna and Rao (2012, 2013) demonstrated the alleviation of zinc toxicity by brassinosteroids in case of radish seedling growth. A sharp decline in nucleic acid content was observed in radish seedlings growing under arsenic toxicity (Table-3). Supplementation of brassinosteroids improved the RNA and DNA in radish seedlings experiencing arsenic toxicity. Similar increase in DNA and RNA content was observed due to brassinosteroid application in maize plants under salinity stress by Khallal *et al* (2009). There was significant decrease in soluble protein levels in radish seedlings growing under arsenic toxicity (Table-4). Due to brassinosteroid feeding, the impact of arsenic toxicity on the protein content of radish seedlings was found reduced.

Table 1. Effect of brassinosteroids alone treatments and in combination with arsenic stress on seed germination of radish

Treatments	48 Hours	72 Hours	96 Hours
Control	40.8±0.734	79.2±1.529	94.0±0.707
0.5µM EBL	43.0±0.836	84.4±0.748	95.2±0.374
1 µM EBL	48.0±0.316	87.2±0.583	96.0±0.316
2 µM EBL	51.6±0.60	89.6±0.509	97.2±0.374
0.5 µM HBL	45.6±0.509	86.0±0.707	95.0±0.316
1 µM HBL	48.2±0.374	89.0±0.707	97.0±0.316
2 µM HBL	57.6±0.509	92.2±0.663	98.8±0.374
As (50µM)	24.4±0.748	43.4±0.748	61.6±0.927
As+0.5 EBL	29.0±0.707	57.6±0.509	75.4±1.077
As+1 µM EBL	33.6±0.509	60.4±0.927	77.0±0.707
As+2 µM EBL	44.6±0.678	68.8±0.860	81.8±0.969
As+0.5 µM HBL	32.4±0.509	62.6±1.029	78.8±0.860
As+1 µM HBL	37.4±0.678	69.8±0.860	87.8±0.583
As+2 µM HBL	49.8±0.734	77.0±0.707	90.4±1.805

The data presented above are Mean ± S.E. (n=5).
As: Arsenic, EBL: 24-epibrassinolide, HBL: 28-homobrassinolide

Table 2. Effect of brassinosteroids alone treatments and in combination with arsenic stress on seedling growth of radish

Treatments	Seedling length (cm)	Fresh weight (mg)	Dry weight (mg)
Control	9.40±0.400	379.2±2.745	31.6±0.927
0.5µM EBL	11.4±0.509	405.0±5.779	36.4±0.748
1 µM EBL	12.8±0.374	427.2±11.86	40.2±0.663
2 µM EBL	14.8±0.285	481.2±4.768	43.6±0.812
0.5 µM HBL	11.8±0.374	421.6±9.947	36.8±0.374
1 µM HBL	14.0±0.447	440.2±5.624	41.6±0.509
2 µM HBL	15.6±0.509	457.2±5.314	43.2±0.374
As (50µM)	4.62±0.303	180.2±2.615	19.4±0.519
As+0.5 EBL	5.20±0.284	202.2±4.694	21.6±0.591
As+1 µM EBL	6.92±0.101	230.8±7.282	23.0±0.707
As+2 µM EBL	7.32±0.700	219.2±12.35	25.6±0.748
As+0.5 µM HBL	6.06±0.153	236.4±7.025	22.4±0.529
As+1 µM HBL	7.92±0.086	274.4±3.544	26.4±0.509
As+2 µM HBL	8.70±0.141	306.8±6.483	28.8±0.663

The data presented above are Mean ± S.E. (n=5).
As: Arsenic, EBL: 24-epibrassinolide, HBL: 28-homobrassinolide

Table 3. Effect of brassinosteroids alone treatments and in combination with arsenic stress on the content of nucleic acids in radish seedlings

Treatments	DNA (µg g^{-1} FW)	RNA (µg g^{-1} FW)
Control	349.25±20.1	734.01±29.21
0.5µM EBL	360.23±20.02	740.21±32.25
1 µM EBL	387.11±23.32	746.11±30.01
2 µM EBL	460.02±21.03	770.36±23.12
0.5 µM HBL	411.26±21.14	755.42±12.36
1 µM HBL	500.53±14.42	811.13±08.25
2 µM HBL	538.34±14.23	900.56±23.32
As (50µM)	116.03±15.20	294.20±20.52
As+0.5 EBL	128.06±12.11	303.11±20.42
As+1 µMEBL	136.62±12.13	315.03±20.63
As+2 µM EBL	193.42±18.40	473.01±16.41
As+0.5 µM HBL	165.35±15.20	313.12±16.42
As+1 µM HBL	274.26±20.23	458.63±15.02
As+2 µM HBL	346.62±29.02	576.65±13.30

The data presented above are Mean ± S.E. (n=5).
As: Arsenic, EBL: 24-epibrassinolide, HBL: 28-homobrassinolide

There was a linear improvement in protein content with increasing concentration of brassinosteroids applied. Both the brassinosteroids at 2µM levels restored the soluble protein content in arsenic stressed seedlings to the levels of unstressed seedlings. Sharma *et al* (2014) found similar improvement in protein content due to brassinosteroids supplementation in case of radish plants growing under cadmium and mercury toxicity. Compared to the control, free proline levels were increased in arsenic stressed radish seedlings (Table-4). Due to supplementation of BRs to arsenic toxicity treatments, the amount of proline was further increased. It was suggested that proline synthesized during stress condition might serve as an organic nitrogen reserve that can be utilized during recovery (Trotel *et al*. 1989).

Table 4. Effect of brassinosteroids alone treatments and in combination with arsenic stress on content of soluble proteins and free proline in radish seedlings

Treatments	Soluble Proteins (mg g^{-1} FW)	Free Proline (mg g^{-1} FW)
Control	5.13±0.188	4.16±0.310
0.5µM EBL	5.61±0.251	4.88±0.215
1 µM EBL	5.96±0.209	5.28±0.257
2 µM EBL	6.60±0.277	7.62±0.233
0.5 µM HBL	5.86±0.181	5.02±0.255
1 µM HBL	6.64±0.278	6.35±0.161
2 µM HBL	7.06±0.213	8.43±0.239
As (50µM)	2.14±0.109	5.51±0.138
As+0.5 EBL	3.52±0.248	6.15±0.105
As+1 µMEBL	4.12±0.264	7.39±0.252
As+2 µM EBL	5.11±0.376	8.00±0.116
As+0.5 µM HBL	3.99±0.224	6.65±0.170
As+1 µM HBL	5.01±0.268	7.64±0.196
As+2 µM HBL	5.50±0.146	9.38±0.136

The data presented above are Mean ± S.E. (n=5).
As: Arsenic, EBL: 24-epibrassinolide, HBL: 28-homobrassinolide

Due to arsenic toxicity stress, the content of MDA steeply increased in radish seedlings (Table-5). Similar observations were made in case of wheat and black gram seedlings under arsenic stress by Chun *et al* (2007) and Srivastava and Sharma (2013) respectively. It is a well known fact that MDA levels are quantitative indices of lipid peroxidation and the consequential membrane damage and electrolyte leakage. The employment of brassinosteroids to arsenic stressed radish seedlings resulted in reduced MDA content indicating lowered lipid peroxidation. Reduced lipid peroxidation due to brassinosteroids was also reported in radish seedlings under chromium and zinc toxicity respectively by Sharma *et al* (2011) and Ramakrishna and Rao (2012).

Table 5. Effect of brassinosteroids alone treatments and in combination with arsenic stress on the content of MDA levels and activities of antioxidant enzymes in radish seedlings

Treatments	MDA (μmol g^{-1} FW)	CAT (μmol H_2O_2 mg^{-1} protein min^{-1})	SOD (U mg^{-1} protein min^{-1})	POD (U mg^{-1} protein min^{-1})
Control	7.94±0.107	12.55±0.127	42.70±0.642	0.622±0.0046
0.5µM EBL	7.72±0.086	13.76±0.161	55.54±0.499	0.635±0.0041
1 µM EBL	6.42±0.101	15.00±0.358	58.37±0.364	0.674±0.0079
2 µM EBL	5.08±0.115	16.95±0.224	61.67±0.727	0.744±0.0542
0.5 µM HBL	7.50±0.114	14.18±0.145	58.87±0.196	0.651±0.0060
1 µM HBL	6.40±0.070	17.86±0.075	61.43±0.425	0.682±0.0048
2 µM HBL	5.00±0.070	18.40±0.154	63.74±0.329	0.713±0.0075
As (50µM)	18.38±0.077	17.79±0.190	64.26±0.375	0.489±0.0097
As+0.5 EBL	12.33±0.112	19.29±0.153	65.88±0.525	0.494±0.0095
As+1 µM EBL	11.65±0.089	20.17±0.105	67.25±0.328	0.537±0.0068
As+2 µM EBL	10.05±0.090	22.18±0.270	69.39±0.287	0.577±0.0098
As+0.5 µM HBL	12.00±0.089	19.16±0.065	66.23±0.320	0.507±0.0088
As+1 µM HBL	10.54±0.178	21.74±0.246	68.20±0.299	0.560±0.0119
As+2 µM HBL	9.37±0.088	23.22±0.208	70.09±0.240	0.599±0.0065

The data presented above are Mean ± S.E. (n=5).
As: Arsenic, EBL: 24-epibrassinolide, HBL: 28-homobrassinolide.

The activity of antioxidant enzymes (CAT, SOD and POD) increased in radish seedlings subjected to arsenic stress (Table-5). It is well documented that exposure of plants to AsIII and AsV induce the production of reactive oxygen species such as superoxide (O_2^-), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (Hartley-Whitaker *et al*., 2001). The resultant oxidative stress is considered as the main driven of arsenic toxicity in plants. Several enzymes are involved in ROS defence stratagies. Highly reactive super oxide can be converted to less active, but longer-lasting H_2O_2 through the action of SOD. H_2O_2 produced in a plant cell either directly or enzymatically through enzymes such as SOD can be neutralized by catalase, an enzyme that is often induced by arsenic exposure (Duman *et al* 2010). It has been observed in the present study that exogenous supplementation of brassinosteroids to arsenic stressed seedlings accounted steep rise in the activity of antioxidant enzymes. The increase in the activities of the antioxidative enzymes due to brassinosteroids treatment well correlated with restoration of growth in arsenic stressed seedlings. The results clearly indicate that the alleviation of arsenic stress by brassinosteroids as observed in the study is being mediated by enhanced antioxidant activity. Arsenic tolerant *Pennisetum typhoides* exhibited strong enzymatic defense system as compared to arsenic susceptible *Pisum sativum* (Sharma 2013).

Conclusion

The present studies clearly demonstrated the arsenic toxicity amelioration capabilities of brassinosteroids. Higher levels of catalase, peroxidase and superoxide dismutase activities in radish seedlings due to brassinosteroids feeding might have resulted in active arsenic detoxification which further translated into restoration of growth.

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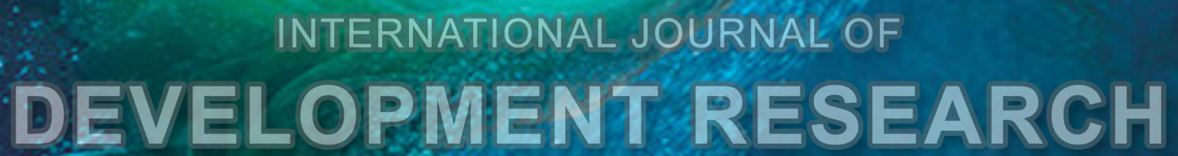

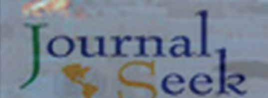

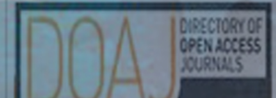

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