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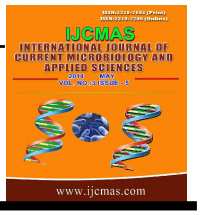

Original Research Article

Amelioration of aluminium toxicity on seed germination and early seedling growth of Pigeon Pea [*Cajanus cajan* (L.) Millsp.] by 28-Homobrassinolide

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Aluminium, 28-homo-brassinolide, *Cajanus cajan*, Germination, Antioxidative enzymes

A B S T R A C T

The effect of 28-homobrassinolide on seed germination and seedling growth of pigeon pea [*Cajanus cajan* (L.) Millsp.] under aluminium toxicity was studied. 28-HBL reduced the impact of Al stress on seed germination. Application of 28-HBL removed the inhibitory influence of Al on seedling growth. Homobrassinolide application enhanced proline in Al stressed *Cajanus* seedlings. Further, the supplementation of 28-HBL to Al stress treatments increased the activities of antioxidative enzymes *viz.*, catalase [EC 1.11.1.6]; peroxidase [EC 1.11.1.7]; superoxide dismutase [EC 1.15.1.1] and ascorbate peroxidase [EC 1.11.1.11]. The present studies demonstrated the ameliorating ability of 28-HBL on the Al induced inhibition of germination and seedling growth of *C.cajan* by reducing the oxidative stress.

Introduction

Aluminium is the third most abundant element in earth's crust. Aluminium toxicity is manifested in acidic conditions in which phytotoxic form of Al 3^+ predominates. Open cast bauxite mining and refinement also significantly aggrevating aluminium toxicity. Excess of aluminium is a major soil constraint to food and biomass production (Vitorella, 2005). It is estimated that 40% of the arable soils of the world are acidic and therefore aluminium poisoining is vary important agricultural problem. Aluminium toxicity severely impairs root growth and interfere with water and mineral nutrient uptake.The use of plant growth promoting substances potentially improve the plant growth and productivity in metal challenged habitats. The prospects of employing plant growth regulators in mitigating the metal toxicity are immense**.**

Brassinosteroids are new group of phytohormones with significant growth promoting activity (Rao et al., 2002; Bajguz, 2007). Brassinosteroids are regarded as plant growth regulators with pleiotropic effects as they influence

diverse development process such as seed germination, plant growth, rhizogenesis, flowering, senescence, and abscission. In addition, Brassinosteroids also confer resistance to the plants against abiotic stresses (Sasse, 2003; Vardhini et al., 2006). The present study aimed to explore the possibility of ameliorating aluminium toxicity in pigeon pea [*Cajanus cajan* (L) Millsp] plant by employing 28-homobrassinolide a bioactive brassino steroid.

Materials and Methods

Chemicals and Seed material

28-Homobrassinolide (HBL) was purchased from CID tech. Research Inc, Mississauga, Ontario, Canada.The seeds of pigeon pea [*Cajanus cajan* (L.) Millsp.] were procured from National Seed Corporation, Hyderabad, India.

Aluminum (Al^{+3}) in the form of aluminum sulphate ($Al_2 (SO_{4)3} .16H_2O$) was used for the studies. Preliminary experiments were conducted employing different concentrations of Al and 7.5 mM of Al was choosen as metal stress concentration, where seedling growth was found inhibited considerably but not completely.

Seed Germination and Seedling growth

Seeds of pigeon pea were surface sterilized with 0.5% (v/v) sodium hypochlorite from commercially available (4% $NaOCl_2$) and washed thoroughly with several changes of sterile distilled water. They were soaked for 24 hours in either: i) Distilled water (control) ii) 7.5 mM Al^{+3}solution (Metal stress control) iii) 0.5µM/1µM/ 2 µM 28-HBL iv) 7.5 mM Al^{+3}supplemented with 0.5µM/ 1µM / 2 µM 28-HBL.

Twenty seeds from each treatment were placed in each of 9 cm sterile petri dishes layered with Whatman No.1 filter paper. The petri dishes were supplied with 5 ml of respective test solutions. The seeds were allowed to germinate in dark at 20 $\pm 1^0$C. 3 ml of test solutions were added on the 4^{th} day of the experiment. Number of seeds germinated was recorded at the end of 36, 48 and 60 hours under safe green light. Emergence of radicle was taken as the criteria for germination.

Growth parameters

On 7^{th} day, seedling growth was recorded in terms of root and shoot length and their fresh weight. Root and shoot parts of seedlings were dried in oven at 110^0 C for 24 hours and their dry weights were recorded.

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. The test tubes containing above mixture were 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. The amount of proline present was quantified with the help of proline standard graph.

Antioxidant Enzymes

Upper part of 7-day-old seedling material (1 g) was homogenized in 50 mM Tris–HCl (pH 7.5) with addition of 40 mM phenyl methyl sulfonyl fluoride (PMSF) and 2 % (w/v) polyvinylpolypyrrolidone (PVPP). The extract was centrifuged at 15,000g for 20 min and the resultant supernatant was used for measuring the following enzyme assays (except for c-GCS). The amount of protein in the enzyme extract was calculated according to Lowry et al. (1951).

Catalase (CAT, E.C.1.11.1.6.) activity was determined following Aebi (1974). The reaction mixture consisted of 50 mM phosphate buffer, 0.1mM H_2O_2 and enzyme extract. The rate of H_2O_2 decomposition at 240 nm was measured spectrophotometrically and calculated using a molar extinction coefficient of 45.2 mM^{-1} cm^{-1}. One unit of catalase activity was assumed as the amount of enzyme that decomposed 1 µmol of H_2O_2 per mg of soluble protein per minute at 30 ^0C.

Peroxidase (POD, E.C.1.11.1.7) activity was assayed by employing the procedure of Kar and Mishra (1976). To 0.5 ml of enzyme extract, 2.5 ml of 0.1 M phosphate buffer (pH 7), 1 ml of 0.01 M pyrogallol and 1 ml of 0.005 M H_2O_2 were added. A blank was prepared with 0.5 ml of enzyme extract, 3.5 ml of 0.1 M phosphate buffer and 1 ml of 0.005 M H_2O_2. After 5 minutes of incubation at 25 ^0C, the reaction was stopped by adding 1 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm against a blank. The enzyme activity was expressed as change in absorbance Units mg^{-1} protein min^{-1}.

Superoxide dismutase (E.C 1.15.1.1):

SOD 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.

Ascorbate peroxidase (APX; E.C 1.11.1.11) was assayed by the method of Nakano and Asada (1981) The reaction mixture contained 1.5 ml of 50 mM sodium phosphate buffer (pH 7), 0.2 mM EDTA, 0.5 ml of 0.5 mM ascorbic acid, 0.5 ml 0.5 mM H_2O_2 and 0.5 ml of enzyme sample. The activity was recorded as the decrease in absorbance at 290 nm for 1 minute and the amount of ascorbate oxidized was calculated from the extinction coefficient of 2.6 $mM^{-1}cm^{-1}$.

Results and Discussion

Al^{3+} toxicity inhibited the seed germination in *C.cajan* (Table 1).However application of 28-HBL reduced the toxic effects of Al^{3+} on seed germination. The promotion of seed germination by 28-HBL was found to be dose dependent. At 2 µM concentration, HBL not only negated the toxic influence of Al on seed germination but also further promoted seed germination.

Aluminium toxicity resulted in suppression of seedling growth (Table 2). Al toxicity reduced the growth in terms of length, fresh and dry weight of root and shoot (Table 2). The impact of Al toxicity was much more pronounced on root growth. However application of 28-HBL improved the seedling growth in seedlings challenged with toxic levels of Al. 28-HBL at 2µM concentration, caused a considerable increase in seedling growth under Al stress and restored the growth to the level of unstressed control seedlings. The growth promoting effects of BRs on seedlings under stress conditions might be attributed to their involvement in cell elongation and cell cycle progression (Gonzalez-Gracia et al.2011) as well as regulation of genes encoding xyloglucan endotransglucosylase/hydrolase (XTHs), expansions, glucanases, sucrose synthase and cellulose synthase or by activating the H-ATPase activity (Ashraf et al.2010).Similarly exogenous application of BRs improved the growth of Ni-stressed maize seedlings (Bhardwaj et al.2007) and Cr- stressed radish seedlings (Choudary et al.2011) and Zn stressed radish seedlings (Ramakrishna and Rao, 2013).

The levels of proline increased in the seedlings under Al stress (Table 3). Feeding of seedlings with 28-HBL further enhanced the proline content in Al stressed seedlings. Though a minor constituent of the amino acid pool, proline as an osmolyte act as cellular protector in several plant species in response to abiotic stress and scavenge ROS (Ahraf and Fooland 2007). Such an increase in proline levels under metal stress due to brassinosteroids application was also observed by Choudary et al, (2011) and Ramakrishna and Rao (2012).

Due to Al toxicity, the activity of antioxidant enzymes- SOD, CAT, POD and APX were found increased in pigeon pea seedlings (Table 3). Application of 28-HBL caused further increase in the activities of the oxidizing enzymes in seedlings growing under Al toxicity. Al toxicity cause oxidative damage to the plant system by activating the production of reactive oxygen species (Shah et al 2001). These ROS like superoxide radical (O_2^-), hydroxyl radicals (OH^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) generally are detoxified by enzymatic antioxidant system. ROS if not detoxified causes serious damage to macro molecules such as proteins, lipids and nucleic acids. In order to scavenge ROS and to combat oxiadative stress, plants evolved an efficient antioxidant defense system.SOD constitutes the first line of defense against ROS in plants. This enzyme catalyzes the detoxification of O_2- to H_2O and O_2 (Alscher et al.2002). CAT and POD further breakdown H_2O_2 to H_2O and O_2. Further, Ascorbate peroxidase (APX) which uses reduced ascorbate as a reductant in the first step of ascorbate-glutathione cycle is recognized as important peroxidase in H_2O_2 detoxification. Application of HBL to Al

Table.1 Effect of 28-homobrassinolde on germination of *Cajanus cajan* seeds subjected to Aluminium toxicity (Results expressed as % seed germination)

Treatment	36 Hours	48 Hours	60 Hours
Control	15	43	85
0.5µM HBL	14	48	87
1µM HBL	17	51	89
2µM HBL	20	61	86
Al (7.5 mM)	9	31	80
Al+0.5µM HBL	17	37	86
Al+1µM HBL	19	52	88
Al+2µM HBL	20	58	91

The data presented above are Mean ± S.E. (n=5). **Al^{+3}**: 7.5mM Aluminium; HBL= 28-homobrassinolide

Table.2 Effect of 28-homobrassinolide alone treatments and in combination with Al^{+3} stress on *Cajanus* seedling growth

Treatment	Root length (cm)	Shoot length (cm)	Root FW (mg)	Root DW (mg)	Shoot FW (mg)	Shoot DW (mg)
Control	5.12±0.70	5.50±0.51	884.22±3.98	267.2±5.05	952.3±12.0	275.22±12.0
0.5µM HBL	6.28± 0.24	6.51±0.54	1141.34±9.2	372.2±10.2	1129.4±45.0	327.50±13.3
1.0 µM HBL	8.10± 0.26	7.91±0.89	1408.12±11.6	427.9±12.1	1376.1±23.0	428.32±42.0
2.0 µM HBL	9.30± 1.09	8.88± 0.45	1601.23±23.1	486.9±14.3	1541.3±27.0	490.83±12.0
Al^{3+}(7.5 mM)	0.50±0.24	3.11±0.59	87.77±7.54	27.0±6.0	487.0±23.0	151.25±23.0
Al^{3+}+0.5µM HBL	2.87±0.87	4.20±0.91	504.11±0.86	157.9±7.2	710.1±11.2	210.98±17.1
Al^{3+}+1.0 µM HBL	4.23±0.12	4.82±0.29	755.32±0.92	232.0±30.1	823.3±19.7	245.15±25.1
Al^{3+}+2.0 µM HBL	5.28± 0.34	5.91±0.78	929.23±0.11	294.0±12.2	994.7±10.0	301.72±15.6

The data presented above are Mean ± S.E. (n=5). **Al^{+3}**: 7.5mM Aluminium; HBL= 28-homobrassinolide.

Table.3 Effect of 28-homobrassinolide alone treatments and in combination with Al^{+3} stress on content of free proline and antioxidant enzyme activities (CAT: catalase, POD: peroxidase, SOD: super oxide dismutase and APX: ascorbate peroxidase)

Treatments	Free Proline (mg g^{-1}FW)	CAT (U mg^{-1} protein min^{-1})	POD (U mg^{-1} protein min^{-1})	SOD (U mg^{-1} protein min^{-1})	APX (µmol ASA mg^{-1} protein min^{-1})
Control	1.25±0.20	0.84±0.70	0.028±0.007	1.208±0.08	5.39±0.35
0.5µM HBL	2.11±0.27	1.72±0.56	0.031±0.008	1.689±0.48	5.84±0.17
1.0 µM HBL	1.93±0.12	2.65±0.70	0.032±0.005	1.911±0.65	6.33±0.51
2.0 µM HBL	2.07±0.13	2.07±0.13	0.038±0.008	2.373±0.60	6.72±0.42
Al^{3+}(7.5 mM)	2.50±0.16	1.82±0.73	0.021±0.004	2.516±0.25	7.83±0.48
Al^{3+}+0.5µM HBL	2.54±0.12	1.82±0.57	0.024±0.005	3.013±0.44	8.49±0.50
Al^{3+}+1.0 µM HBL	2.98±0.13	2.35±1.06	0.027±0.002	3.050±0.14	9.28±0.61
Al^{3+}+2.0 µM HBL	3.15±0.07	2.78±1.41	0.029±0.007	3.428±0.14	9.69±0.52

The data presented above are Mean ± S.E. (n=5). **Al^{+3}**: 7.5mM Aluminium; HBL= 28-homobrassinolide.

stressed seedlings enhanced the APX activity, indicating the efficient scavenging of H_2O_2 thereby presenting the H_2O_2 –mediated cell damage. Similarly, Bajguz (2010) observed that exogenous application of brassinosteroid increased the activity of APX in *Chlorella* cultures under heavy metal stress. The study clearly showed that application of 28-HBL to stressed seedlings enhanced the antioxidative defense system in plants.

The results of the study revealed the capability of 28-homobrassinolide is counteracting the toxicity of aluminium on seed germination and seedling growth. The alleviation of Al toxicity stress by 28-HBL was found associated with furthering the enzymatic antioxidant defense mechanism.

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