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Effect of 24-epibrassinolide on aluminium stress induced inhibition of seed germination and seedling growth of *Cajanus cajan* (L.) Millsp.

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Abstract

The effect of 24-epibrassinolide on seed germination and seedling growth of Cajanus cajan (L.) Millsp. subjected to aluminium stress was investigated. 24-EBL was found to reduce the impact of Al stress on seed germination. Further, the application of 24-EBL resulted in removal of the inhibitory influence of Al on seedling growth. Epibrassinolide application caused enhancement of proline in Al stressed Cajanus seedlings. Further, the supplementation of brassinosteroids to Al stress treatment increased the activities of antioxidative enzymes such as 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 24-EBL on the inhibition of germination and seedling growth of C.cajan by reducing the oxidative stress.

Keywords: *Aluminium, 24-epibrassinolide, Cajanus cajan, Germination, Seedling Growth, Antioxidative enzymes*

1. Introduction

Metal toxicity is considered to be one of the main constraints for agricultural production. Metals are continuously released to the soil by natural weathering of rocks and by industrial activities such as mining, combustion fuels, urban waste disposal etc. Al is a light metal that makes up 7% of the earth crust, occurring in the form of harmless oxides and Al silicates. If the soil becomes acidic, Al is solubilised into toxic forms like Al^{3+} [1]. Al^{3+} interfering with cell division in root tips and lateral roots, reduce DNA replication by increasing the rigidity of DNA double helices decreases root respiration, interference with enzyme activities and interfering with uptake and transport of several essential nutrients like Ca, Mg, K,P and Fe [2, 3].

Recently increasing interest has been developed in utilizing plant growth regulators with multiple functions in agriculture management to regulate plant growth and enhance resistance to various environmental stresses. Brassinosteroids are a group of naturally occurring plant steroid hormones that influence different physiological plant processes at very low concentrations [4, 5]. Apart from plant growth stimulation, brassinosteroids have the ability to confer tolerance in the plants against biotic and abiotic stresses [6]. Brassinosteroids are involved in protecting plants from a broad spectrum of stresses such as drought, high temperature, chilling, salinity, and heavy metals also from the toxic effects of pathogen infection, pesticides and herbicides [7, 8].

Pigeon pea [*Cajanus cajan* (L.) Millsp. is a multi use legume crop in world, It is the main source of proteins, vitamins for much poorest population and plays important role in reducing malnutrition for millions of people all over the world [9]. The present study is to assess the effect of 24-EBL on seed germination, seedling growth, proline content and activities of certain antioxidative enzymes of *Cajanus* seedlings subjected to Al stress.

Materials and Methods

Chemicals and Plant material

The bioactive brassinosteroid- 24-EBL (EBL) was employed in the present study was purchased from CID tech. Research Inc, Mississauga, Ontario, Canada. The seeds of red gram [*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 chosen 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:

1) Distilled water (control)
2) 7.5 mM Al^{+3}solution (Metal stress control)
3) 0.5µM, 1µM and 2 µM 24-EBL
4) 7.5 mM Al^{+3}supplemented with 0.5µM, 1µM and 2 µM 24-EBL

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. However, further 3 ml of test solutions were added on the 4th 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 7th day, seedling growth was recorded in terms of seedling length, fresh weight and dry weight. The seedlings were carefully removed from petri dishes and the water adhering to them was removed with the help of blotting paper. The length and fresh weights of the seedling were recorded. 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. [10]. 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. [11].

Catalase (CAT, E.C.1.11.1.6.)

Catalase activity was determined following Aebi [12]. 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 ^{0}C.

Peroxidase (POD, E.C.1.11.1.7)

Peroxidase activity was assayed by employing the procedure of Kar and Mishra [13]. 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 ^{0}C, 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 [14]. 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)

APX activity was assayed by the method of Nakano and

Table-1: Effect of 24-epibrassinolide on germination of *Cajanus cajan* seeds subjected to Aluminium stress (Results expressed as % seed germination)

Treatment	36 Hours	48 Hours	60 Hours
Control	15	43	85
0.5µM EBL	15	47	86
1.0 µM EBL	18	48	83
2.0 µM EBL	19	57	89
Al^{3+}(7.5 mM)	09	31	80
Al^{3+}+0.5µM EBL	16	33	88
Al^{3+}+1.0 µM EBL	18	53	91
Al^{3+}+2.0 µM EBL	22	59	93

The data presented above are Mean ± S.E. (n=5). Al: Aluminium. EBL= 24-epibrassinolide.

Table-2: Effect of 24-epibrassinolide alone treatments and in combination with Al^{+3} stress on *Cajanus cajan* 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 EBL	6.17±0.50	6.49±0.39	1136.20±6.0	355.5±14.7	1125.1±18.0	325.23±54.0
1.0 µM EBL	7.68±0.44	7.69±0.12	1336.94±5.0	402.0±7.0	1340.2±12.0	412.12±45.0
2.0 µM EBL	8.83±0.71	8.87±0.74	1528.83±8.4	465.6±13.6	1537.2±52.0	461.56±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 EBL	2.49±0.38	3.80±0.68	439.23±1.22	138.0±12.0	658.2±14.0	197.23±12.2
Al^{3+}+1.0 µM EBL	3.085±0.58	3.75±0.07	680.39±10	207.0±10.1	745.2±28.0	220.23±67.0
Al^{3+}+2.0 µM EBL	5.19±0.73	5.40±0.10	911.66±8.45	288.0±14.3	940.3±9.1	289.0±23.45

The data presented above are Mean ± S.E. (n=5). Al: Aluminium. EBL= 24-epibrassinolide.

Table-3: Effect of 24-epibrassinolide 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 EBL	1.47±0.13	1.55±0.80	0.030±0.006	1.777±0.34	5.50±0.51
1.0 µM EBL	1.91±0.16	2.88±1.27	0.032±0.006	2.213±0.97	6.25±0.79
2.0 µM EBL	2.11±0.27	3.43±1.60	0.036±0.007	2.315±0.47	6.54±0.74
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 EBL	2.61±0.12	2.32±1.28	0.024±0.006	2.870±0.67	8.54±0.44
Al^{3+}+1.0 µM EBL	2.83±0.23	3.13±2.13	0.026±0.006	2.980±0.94	9.21±0.25
Al^{3+}+2.0 µM EBL	3.20±0.08	3.40±2.26	0.027±0.007	3.179±0.14	9.14±0.47

The data presented above are Mean ± S.E. (n=5). Al: Aluminium. EBL= 24-epibrassinolide.

Asada [15]. 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). 24-EBL reduced the toxic effects of Al^{3+} on seed germination. The percentage of seed germination increased with an increase in 24-EBL concentration. In stressed seedlings supplemented with 24-EBL, the percentage of seed germination approached that of unstressed control treatments, indicating the stress alleviation capability of 24-EBL.

Aluminium toxicity caused substantial reduction in the seedling length (Table 2). Al toxicity impaired the growth in terms of length, fresh and dry mass of root and shoot (Table 2). The impact of Al toxicity was more pronounced

on root growth. Exogenous applications of 24-EBL improved the seedling growth in seedlings challenged with toxic levels of Al. 24-EBL 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 [16] 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 [17].Similarly exogenous application of BRs improved the growth of Ni- stressed maize seedlings [18] and Cr-stressed radish seedlings [19] and Zn stressed radish seedlings [20].

The proline content increased in the seedling subjected to Al stress (Table 3). The level of proline in stressed control was higher when compared to unstressed control seedlings. The treatment of seedlings with 24-EBL further enhanced the proline content in 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 [21]. Increase in proline content may be due to the stimulation of Delta-1, Pyroline 5-carboxylate synthase (P5CS) responsible for proline biosynthesis under stressed conditions [22]. An increase in proline content under metal stress due to brassinosteroids application was also reported by Choudary et al. [19] and Ramakrishna and Rao [23].

The activity of antioxidant enzymes (SOD, CAT, POD and APX) increased in seedlings subjected to Al stress. Supplementation of 24-EBL further enhanced the activity in stressed seedlings compared to control and stressed control (Table 3). Al toxicity cause oxidative damage to the plant system and act as catalyst in ROS production [24]. These ROS like superoxide radical (O_2^-), hydroxyl radicals (OH^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) produced in cells under stress are detoxified by enzymatic antioxidant system. ROS if not detoxified causes serious damage to proteins, lipids and nucleic acids. In order to scavenge ROS and to counter oxidative 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 [25]. CAT and peroxidases further breakdown H_2O_2 to H_2O and O_2.

The increased activity of APX as observed in the seedlings reflecting elevated H_2O_2 detoxification. Similarly, Behnamnia et al. [26] reported that 24-epibrassinolide increased the APX activity under drought stress in *Lycopersicon esculentum*.

The study clearly showed that application of 24-EBL to stressed seedlings enhanced the antioxidative defense system in plants. The production of H_2O_2 which diffuses across the plasma membrane is toxic as it acts as both an antioxidant as well as reductant [27]. CAT and POD further breakdown H_2O_2 to H_2O and O_2. However, supplementation of 24-EBL to Al stressed seedlings increased the activities of all the enzymes (CAT, POD, SOD and APX) implying the protective role of BRs in scavenging ROS (Table 3). The studies of obtained are in consistence with the studies carried out by Hayat et al. [28] and Ozdemir et al. [29] who reported that the 24-EBL application could alleviate abiotic stress by improving the activation of antioxidant enzymes.

Conclusion

The present investigation revealed the ability of 24-EBL to alleviate Al stress. The stress alleviation was associated with increase in seed germination and growth. The results further reveal that 24-EBL strongly protect the stressed seedlings by increasing antioxidant enzyme activities, thereby limiting ROS levels and improving tolerance. Thus it can be inferred that 24-EBL acts as growth promoter and inhibit aluminium toxicity.

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