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ALUMINUM INDUCED ENZYMATIC DISORDER AS AN IMPORTANT ECO BIOMARKER IN SEEDLINGS OF *LENS CULINARIS* MEDIC.

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Abstract

This article discusses the Al (Aluminum) induced disorder on the activities of nitrate and nitrite reductase (NR), protease (PA) and proline contents of seedlings of *Lens culinaris* as some important eco-biomarkers. The seedlings were cultured hydroponically in the nutrient solution with or without $AlCl_3$. $6H_2O$ (pH = 4.0) for 15 days. The relative toxicity of $Al^{3+}$was found to be directly related with Al concentration in the nutrient medium. The reduction in the seedlings growth may be attributed with the poor root growth which in turns related with an inhibition in the cell division. Al treatments for 15 days increased the nitrate reductase activities in the seedlings while protease activity was decreased. Increase in the proline contents may cause a substantial shield to the enzymes against the detrimental effects of the tense components Al. The essential electrolyte like sodium (Na) and potassium (K) contents were found to be decreased, accredited to the rupturing of cell membrane. These results suggest that inhibition of the root growth by Al, closely related to the metabolic changes including an increase in nitrate reductase activity and decline in proteases activity in of the seedlings.

Key words: Al, *Lens culinaris,* Nitrate reductase, Protease, Proline.

Introduction

Toxic action of environmental pollutants like Aluminum causes a serious disorder in physiology as well as in the morphology of vegetative organism. As metals are persistent, non-biodegradable enters in the plant through biological processes and transferred to their various parts like root, stem leaves and buds. A uniform decrease in root and shoot elongation was marked primary morphological sign of Al injury (Akmal *et al*., 2005; Andrew *et al*., 1973; Ayala-Silva & Al-Hamdani, 1997; Azmat *et al*., 2007). The seedling growth in terms of root and its cation i.e., K ion concentration was monitored in the two species, *Pinus radiata* (D. Don) and *Eucalyptus mannifera* sub sp. *mannifera* (Mudie) to demonstrate the ability of eucalypt species to maintain vigorous growth in Al concentration and low in Ca and P, whereas *Pines species* found to be fail to maintain its growth in acid soil (Huang & Bachelard, 1993).

Al induced changes in growth are the results of direct and instant injury of the metabolism, photoprotective system, water relation, carbohydrates contents, mineral nutrition, organic acid metabolism and nitrogen metabolism (Azmat *et al*., 2007 and Azmat & Hasan, 2008). Proline, an effective singlet oxygen quencher, accumulates heavily in a number of plants under strain, providing the plants defense against injury by ROS. Proline plays a vital role in osmoregualation, protection of enzymes, and stabilization of the machinery of protein synthesis. Al toxicity results in the elevation of free proline and total free amino acid contents in the shoot of two cultivars followed by no effect on the percentage of free proline in total amino acid in the shoot and recommended that proline gathering is just a symptom of Al damage. Nitrate reductase activity in the leaves of 2 cultivars is greatly decrease by Al (Toioka, 2007) may be related with the detoxification of Al in maize plant. Alteration in protease activity, gelatinase profile and protein oxidation in the sunflower cotyledons may be related to plant adaptation with changed environmental conditions. Proteolysis is also allied to oxidative stress results by ROS (O_2^-, H_2O_2, and OH) whereas oxidative stress can modified the protein which is characterized for the production of carbonyl groups in the molecules (Azmat *et al.*, 2007 : Palma *et al*., 2002; Umebese & Motajo, 2008).

The aim of the present study was the characterization of the impact of aluminum on some biomarkers like proline, protease, nitrate and nitrite reductase enzymes. The paper will also discuss the plant growth and electrolyte under Al stress.

Materials and Methods

Ten to fifteen healthy seeds of *Lens culinaris* were surface sterilized with 0.1% Mercuric Chloride and germinated in natural environment in Petri dishes in darkness containing Whatman no. 1 filter paper moist with Hoagland nutrient solution for 15 days (Azmat *et al*., 2007). After 48 hours of germination, seeds were transferred to pots containing Hoagland nutrient solution. Aluminum was given in form of Aluminum chloride ($AlCl_3.6H_2O$) at increasing concentration viz., (20, 30, 90, 100, 150 mg L^{-1}).

Observations of foliar architecture, blade and petiole epidermal and number of stomata in *Lens culinaris* leaves tissues were examined using light microscopy by clearing the leaves using 10% KOH solution. All stomata and leaf pubescence within the eyepiece grid (SC) were counted and compared with the leaves of control plant as described by Azmat *et al*. (2009).

Nitrate Reductase in leaves extract by using phosphate buffer containing KNO_3, were treated with Sulphanilamide and (1-Napthyl)-ethylene diamine dihydrochloride. Incubate at room temperature for 20 minutes. Absorbance of the complex was observed at 542 nm (Bordon, 1984).

Nitrite Reductase was observed through phosphate buffer with sodium nitrite. Incubate at room temperature at 30°C in water bath. Transfer the tubes in boiling water bath 2 min. Then add Sulphanilamide and (1-Napthyl)-ethylene diamine dihydrochloride. Make up the volume with distilled water. Optical Density of the complex was observed at 540nm (Ramarao *et al*., 1983).

Protease was determined by treating the seedling with 1% NaCl in phosphate buffer. Extract was treated with 1% Casein and 40% TCA solution, and then Folin Phenol reagent was added to it as described by Ainous (1970) and absorbance was recorded on Spectrophotometer at 570nm.

The method developed by Bates *et al*. (1973) was used for the quantification of proline. 0.5 g of plant material (root and shoot) was homogenized using 10 ml of 3% aqueous sulphosalicylic acid. The homogenate was filtered through Whatmann No.1 filter paper and mixed with 2 mL of acid ninhydrin (1.25 g of ninhydrin + 30 mL of glacial acetic acid + 20 mL of 6 M phosphoric acid) and 2 mL of glacial acetic acid. The sample was heated for one hour at 100°C in a water bath and followed by addition of 4 mL of toluene. This solution was mixed well and read at 520 nm in a UV-visible spectrophotometer.

Na and K were observed on Flame Photometer by dry ash method using HCl and HNO_3; extract was prepared in deionized water

Statistics: The data obtained through three replicate were subjected to statistical analysis, ± standard error obtained from the one-way analysis of variance followed by Tukey's honestly significant difference analysis performed by SPSS Version 12.0 (SPSS, Chicago, IL, USA.).

Results and Discussion

Present investigations were pertaining to the physiological and biochemical parameters of *L. cularinus* in relation to the phytotoxicity of Al in 15 days old seedlings. Relative toxicity (RT) of Al on germination of seed, fresh and dry weight of root and shoot were reported in the Table 1 ($p<0.05$). Results reported in the Table 1 reflects that the RT increases with elevated concentration of the Al in contrast to non treated ones which showed that Al toxicity is a probable growth restraining factor for the specie under investigation. The symptoms of Al was not easily identifiable physically, foliar indication be similar to that of P insufficiency (Azmat *et al*., 2007) due to which feat, small, dark green, leaves development, purpling of stems and leaf veins were observed. Yellow and demise of leaves showed that the Al is biotoxic for the root and the shoot growth of *L. cularinus* as reported earlier by (Akmal *et al*., 2005). The same phenomena were observed in present investigation. Primary indication of Al noxious was an reticence of the root elongation (Andrew *et al*., 1973) with new branching pattern with stunt roots at different concentration of Al in contrast to the untreated ones (Fig. 1). The roots of *L. cularinus* demonstrated greater mark of cellular damage than other parts of plants, predominantly in root tips and in lateral roots which become thickened and turn brownish black with fine branching system (Fig. 1). This may be related with the adaption for survival strategy of the seedlings under stress. The roots were turned black and stout which was related to the direct toxicity of Al towards roots (Azmat & Hasan, 2008). In *L. cularinus* more pronounced abnormality was the damaging of cell wall of roots due to which absorption of nutrient from the solution was affected.

Al induced disorder in electrolyte metabolism: The two important electrolytes Na & K were analyzed under Al stress and results are presented in the Table 2 for contents of root and shoot at various applied concentration of Al in the Hoagland medium. Results reported in the Table 2 support the cell wall damaging (Fig. 1) specially at 100ppm where a significant decrease in the Na and K contents were observed attributed with the leakage of these ions due to the damage of cell wall of roots. The contents of both electrolytes in shoot were decreased with an increase in Al concentration as compared to control while at 90ppm some survival of specie was marked. The decrease in the concentration of two important electrolytes with P deficiency (Azmat & Hasan, 2008) may results in the reduction of shoot growth. Al has been prove to obstruct with cell wall damaging which ultimately affect the cell division in plants roots that result in the decrease in the water supply to plants. Al causes extensive root damage leading to reduced ion and water uptake. Al decreases the Na and K contents and perturbs the deposition of polysaccharides in cell wall by altering the function of certain enzymes (Azmat & Hasan, 2008). Present investigation suggests that Al is highly reactive that may bind to the cell wall, plasma membrane surface, cytoskeleton and the nucleus. Root cell wall specially root epidermal and cortical cells may be of primary sites of Al tie.

Table 1. Relative toxicity of Al on germination and biomass of root and shoot of *L. cularinus* ($p<0.05$).

Al (ppm)	Germination (RT %)	FW of shoots (RT %)	FW of roots (RT %)	DW of shoots (RT %)	DW of roots (RT %)
20	0.166 ± 0.02	0.159 ± 0.02	0.306 ± 0.11	0.0516 ± 0.03	0.142 ± 0.02
30	0.333 ± 0.03	0.179 ± 0.01	0.335 ± 0.12	0.053 ± 0.02	0.095 ± 0.01
90	0.305 ± 0.02	0.289 ± 0.03	0.553 ± 0.03	0.055 ± 0.01	-3.238 ± 0.03
100	0.388 ± 0.01	0.284 ± 0.02	0.546 ± 0.02	0.363 ± 0.01	0.476 ± 0.01
150	0.444 ± 0.01	0.178 ± 0.01	0.34 ± 3.00	0.350 ± 0.02	0.238 ± 0.08

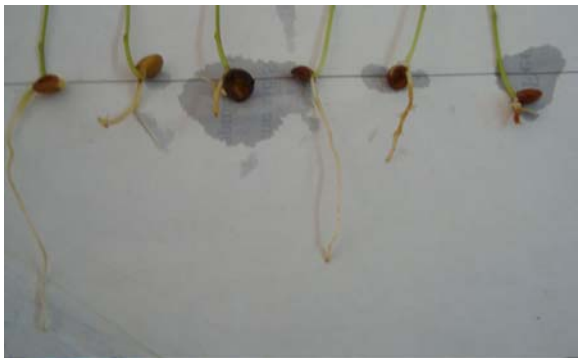

Fig. 1. Effect of aluminium on root branching system at various applied concentration.

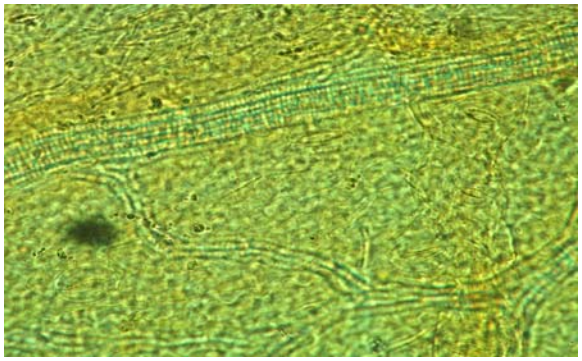

Fig. 2. Photo camera microscopy of leaves showing stomata of non Al treated plants.

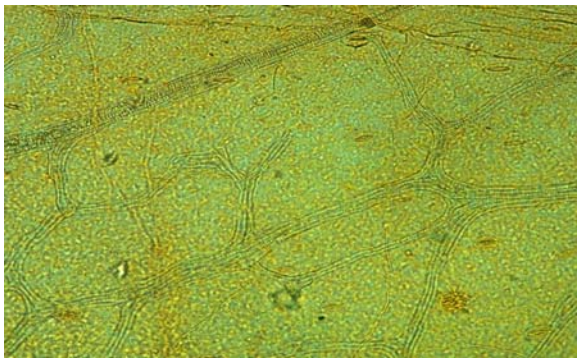

Fig. 3. Photo camera microscopy of leaves showing increased stomatal dennsity of Al treated plant.

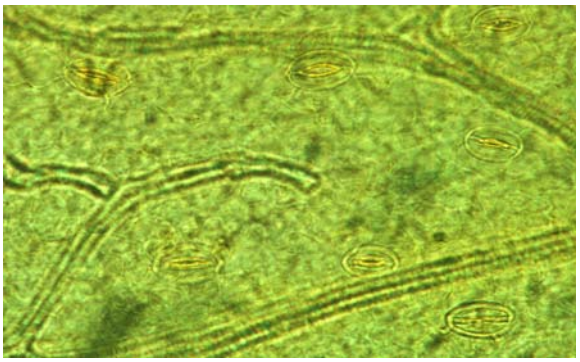

Fig. 4. Photo camera microscopy of leaves showing increased size of stomata of Al treated plant(100ppm).

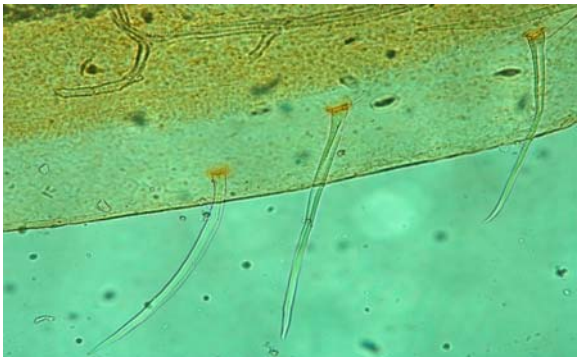

Fig. 5. Photo camera microscopy of leaves showing closed stomata and appearance of marginal hairs of Al treated plant (100ppm).

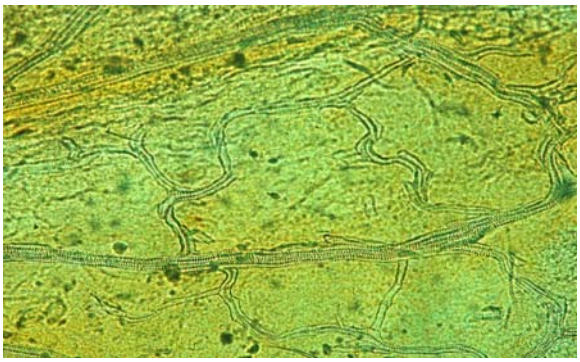

Fig. 6. Photo camera microscopy of leaves showing closed stomata and appearance of small wavy veins of Al treated plant(100ppm).

Table 2. Al toxicity on the essential electrolytes of *L. cularinus* (p<0.01).

Al (ppm)	Sodium (Na)		Potassium (K)	
	Shoots	Roots	Shoots	Roots
0	117.9 ± 21.2	540.7 ± 36.2	77.8 ± 16.5	199.2 ± 22.5
20	118.0 ± 18.2	553.8 ± 35.2	77.9 ± 14.3	219.7 ± 23.1
30	123.0 ± 17.3	534.3 ± 36.3	51.8 ± 12.3	134.9 ± 21.6
90	115.3 ± 17.5	530.0 ± 36.2	106.9 ± 18.2	128.1 ± 26.3
100	108.8 ± 18.2	517.2 ± 39.3	85.5 ± 14.6	117.6 ± 21.3
150	82.7 ± 15.6	439.6 ± 38.3	135.5 ± 21.6	189.8 ± 22.2

Table 3. Al toxicity on some biomarkers of *L. cularinus* (p<0.05).

Al^{+3} (ppm)	Nitrate reductase (µg/ml)	Nitrite reductase (µg/ml)	Protease (mg/mL)	Proline 3 (mg/mL)
0	0.071 ± 0.01	0.095 ± 0.02	--	--
20	0.083 ± 0.02	0.156 ± 0.03	81.83 ± 19.6	74.967 ± 17.9
30	0.09 ± 0.03	0.497 ± 0.03	81.83 ± 18.8	74.967 ± 19.6
90	0.10 ± 0.01	0.093 ± 0.02	77.78 ± 15.9	78.571 ± 15.3
100	0.095 ± 0.01	0.502 ± 0.03	77.51 ± 12.9	78.211 ± 14.6
150	0.111 ± 0.03	0.703 ± 0.04	77.24 ± 14.6	78.571 ± 19.6

Al induced disorder in eco biomarkers: Accumulation of the free proline, protease, nitrate reductase and nitrite reductase in response to Al toxicity in the *L. cularinus* were studied as important biomarkers and results were reported in the Table 3. Results showed an increase in the proline, nitrate and nitrite reductase activity with the decrease in the protease activity. An increase in osmoprotectant proline activity directly related with the Al concentration. As proline is extremely deliberated fragment in the environment of the plant retort to aboitic strain, such as salinity, water deficiency, low and high temperature or heavy metal stresses. Proline increases the metal tolerance in stressful climate (Chen & Chiu. 2004; Azmat *et al.,* 2009). It is a compatible solute, shown surprising property when experience stress environment. Proline addition may be recognize by enhance synthesis, liberated from macromolecules or decrease degradation (Costa & Morel. 1994) proposed that elevated level of proline in higher plants due to inhibition of proline oxidation. A direct relation was observed in the present investigation with proline, total amino acids and total proteins and Al concentration (Azmat *et al*., 2007). Increase in protein contents under the Al stress in the *L. cularinus* indicates that it may contain larger proportion of proline. Proline may bind with metal due to its chelating capability provide the strength against free radicals. A converse liaison among biomass and proline suggest that proline might be produced at the cost of matter in favor of the growth of seedlings which results in the reduction of root and shoot length (Table 1).

The branching pattern of roots, an increase in number of stomatas, development of marginal hairs and wavy veins system (Figs. 2-6) with an increase proline activity related to the approach acclimatize by the plants to manage nitrate, nitrate and protease activity under Al toxicity. As proline has multiple functions such as osmoticum, scavenger of free radicals, protective role of cytoplasmic enzymes, source of nitrogen and carbon for post-stress growth, stabilizer of membranes, machinery for protein synthesis and a sink for energy to regulate redox potential (Palma *et al.,* 2002). Proline acts as a cytoplasmic osmoticum as it accumulates to a higher degree under stress conditions, which may play an adaptive role for any stress tolerance. Similar results were reported in screening of rice genotypes, chickpea genotypes and sugarcane genotypes under salinity (Rout & Shaw, 1998; Rai *et al*., 2004; Chien-The *et al*., 2004).

Proteases are vital for surviving cells and take part in the plant cell adjustment to ecological environment. Al is protease inhibitor in *L. cularinus*. Decrease in the protease activity under the Al stress and increase in proline contents showed that Al affects the plant defense system which may be attributed with the visual reduced morphological growth symptoms (Fig. 1). The results suggested that inhibition in the proteases was the effects of Al contamination in the seedlings which stop the hydrolysis of proteins, that was previously reported as high protein under Al stress by Azmat *et al*. (2007). Therefore, it appears that the adaptation to high Al concentration may involve in the inhibition of hydrolysis of some proteins. Because hydrolysis of proteins by proteases releases amino acids for storage and/or transport and for osmotic adjustment during stress in seedlings (Palma *et al*., 2002) which also supported by Figures (1-6) which that showed an increase in the number of stomata with large size and marginal hairs development for the detoxification of the Al in seedlings (Zengin & Munzuroglu, 2005). Increase in proline contents may accompanied with the reduced absorption of Al in seedlings as reported in the Table 2 where an increase in concentration of both electrolyte was observed at 90ppm of metal (p<0.05). The physiological effect of Al on the activities of nitrate and nitrite reductase seedlings showed that nitrate and nitrite activities increases with an increase in the dose of metal. These results suggest that the stimulation (branching pattern) of root growth by Al might be closely related to metabolic changes including the increase in nitrate reductase activity in the leaves and roots (Toioka *et al*., 2007). As reported earlier that nitrate reductase is a vital enzymes of nitrogen absorption in plants, catalyzing the transfer of two electrons from nicotinamide-adenine dinucleotide phosphate [NAD(P)H] to nitrate to yield nitrite which also catalyzes the NAD(P)H-dependent reduction of nitrite to NO (Wang *et al*., 2010 ; Hua-Hua *et al*., 2010).

Additionally, Al stimulates NO production and NR activity (Pallavi & Dubey 2005). The NO production was markedly related with the increase in nitrate and nitrite activity and reactive oxygen species. An increase in both activities under Al stress showed that Al toxicity results in free radical generation which damages the plant defense system (p<0.01). Nitrite was ineffective to stimulate stomatal closure in epidermal cells of shoots as open stomatas, which were observed in the leaves of seedlings (Figs. 2-6). The increased activities of NR enzymes under

Al stress may be related with the inhibition of protease activity by NO radical. Metal induced ROS and an increase in nitrate and nitrite activity in seedling results in simultaneous increase of NO which activate a hypersensitive cell demise in *L. cularinus* and plant experienced more rapid death as compared to control one.

Conclusion

It was concluded that many of the biological activities of the plant were altered *via* the Al toxicity and Al solubilized in acidic soil is extremely toxic in terms of root elongation, and is believed to be the primary factor inhibiting plant growth. However the nutrient deficiencies associated with the Al toxicity in acid soil need to be addressed in developing new Al stress tolerant plant lines. These technologies will prove useful in environmental cleanup procedures as well as in restoration of soil fertility. These measures in the field of research can be able to solve the problem of food scarcity due to abiotic stress and thus give food security to the malnourished population in the developing third world countries.

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