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Oxidative biomarkers in leaf tissue of barley seedlings in response to aluminum stress

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ABSTRACT

Cellular responses to Al-stress in *Hordeum vulgare* seedling bioassay were evaluated with an objective to identify the possible biomarkers in leaf tissue that would be best suited to biomonitor aluminum (Al) in the environment. Germinating seeds were treated with different concentrations of $AlCl_3$ at pH 4.5 for 12 h. Al-uptake and accumulation in root and leaf, generation of reactive oxygen species (ROS: $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$), cell death, activity of antioxidant enzymes: catalase, superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, lipid peroxidation, protein oxidation, DNase activity and DNA damage were measured in leaf tissue of the seedlings on day 6 after treatment. The above parameters assessed in leaf tissue that followed a dose-response exhibited significant correlation with concentration of Al^{3+} in experimental solution as well as in root tissue. The findings underscored the sensitivity as well as potential of *Hordeum vulgare* seedling bioassay for biomonitoring of Al in the ambient environment.

1. Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust after oxygen and silicon. Owing to its wide use, Al is one of the most sought after metals. With the increasing global demand for Al, anthropogenic activities related to bauxite-mining, Al-refining and smelting are steadily increasing. India ranks sixth in bauxite mining and eighth in Al production in the world. Environmental pollution due to Al has been a looming problem in the state of Orissa, India that accounts for 51 percent bauxite deposits and has been a major multinational hub for bauxite and aluminum-related mining and industrial activities (Kuo, 2008). Al-toxicity has been a primary factor limiting crop productivity in acid soils, which comprise up to 40 percent of the total world's arable lands in tropics and subtropics (Kochian et al., 2004). Obviously, it becomes imperative that the ambient environments at each stage of processing or production of Al is subjected to regular environmental surveillance and monitoring, necessary for impact assessment environmental pollution. One of the growing problems in environmental management is how best to monitor and evaluate the consequences of widespread release of chemicals from industrial processes to natural and managed ecosystems. Biological monitoring through analysis of diversity of microbes, lower and higher plant groups proved useful and cost effective (Prasad, 2001; Gjorgieva et al., 2011). Higher plants, in particular, have been employed for biomonitoring and ecotoxicological assessment of metal pollution (Bargagli, 1998; Hagemeyer, 2004). Considerable work has been done to understand Al-tolerance or toxicity in plants (for review see Delhaize and Ryan, 1995; Matsumoto, 2000). A number of plants such as barley (Tamas et al., 2003), cedar (Hirano and Hijii, 2000), maize (Boscolo et al., 2003), onion (Achary et al., 2008), pea (Yamamoto et al., 2001), pumpkin (Dipierroa et al., 2005), radish (Zhang and Zhou, 2005), rice (Sharma and Dubey, 2007), soybean (Zhen et al., 2007), tobacco (Yin et al., 2010), tomato (Zhou et al., 2009), etc. have been employed to evaluate Al-phytotoxicity. In most of the above studies, roots or underground parts have been used for analysis of Al-toxicity that underscored the involvement of reactive oxygen species (ROS). Furthermore, plants in response to Al-stress generate ROS mediated through the oxidative burst that involved plasma membrane bound NADPH-oxidase and/or cell wall bound NADH-peroxidase (Achary et al., 2008). Information on plant-responses to Al-stress analyzed in leaf or aerial parts having potential for application in environmental biomonitoring and ecotoxicological assessment, however, is scarce. We present here cellular responses to Al-stress evaluated on the bases of Al-uptake, cell death, generation of ROS ($O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$), lipid-peroxidation, protein-oxidation, antioxidant enzymes and nuclease activities and DNA damage; all assessed in leaf tissue of growing seedlings of *H. vulgare* L.

2. Materials and methods

2.1. Assay system

Seeds of hulled six rowed barley (*H. vulgare* L. variety BH, $2n=14$) were used as the assay system. Healthy hand picked seeds were first surface sterilized by

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soaking in one percent sodium hypochlorite solution for 5 min followed by soaking in 95 percent ethanol for 5 min. The seeds were finally washed in sterile water twice before being used in the experiments.

2.2. Experimental solution, seed germination, treatment and seedling growth

Stock solution (50 mM) of $AlCl_3$ (Qualigens, Mumbai) was prepared by dissolving in distilled water at pH 4.0. Experimental solutions of $AlCl_3$ at 0, 2.5, 5 and 10 mM were prepared by diluting the stock in sterile tap water. The pH was adjusted to 4.5 ensuring that during treatments, the metal in soluble form (Al^{3+}) was available for plant-uptake (Kochian et al., 2004). To start with, seeds pre-soaked for 15 h were surface sterilized and set for germination on moist filter paper placed in Petri plates kept in dark at 24 ± 1 °C under aseptic condition. After 30 h when seeds germinate, 50 germinating seeds were treated with the experimental solution (100 mL) containing Al^{3+} at different concentrations in Petri plates for 12 h. While treatment, care was taken not to use any filter paper to avoid adsorption of Al^{3+} onto the filter paper limiting the availability of the metal ion for root uptake. Treatments were terminated by washing the seeds thoroughly for 30 min with five changes of sterile water and placed again on moist filter paper in Petri plates facilitating seedling growth under cool fluorescent light at an intensity of 200 μmol m^{-2} s^{-1} having 12/12 h light/dark cycle. Seedlings were grown under aseptic condition and maintained at constant moisture by adding equal volumes of water into the Petri plates, daily. On day 6 after treatment, seedling height was recorded by measuring the length of the first leaf from 15 randomly chosen seedlings per treatment. Leaves and roots from growing seedlings of *H. vulgare* seedlings were then excised and processed for biochemical, histochemical and metal analyses described as follows.

2.3. Determination of Al concentration in experimental solution, root and leaf tissue

Experimental solutions prepared at 0, 2.5, 5 and 10 mM solutions of $AlCl_3$ were acidified by HNO_3 (two percent, v/v) and the actual concentrations of Al^{3+} were determined by Atomic Absorption Spectrophotometry (Shimadzu AA-6300, Japan) at 309.3 nm using nitrous oxide-acetylene flame. Root and leaf samples excised from seedlings were dried to constant weight. Samples weighing in between 0.8 and 1 g taken in silica dishes were ashed using microwave (Milestone, Italy) at a temperature ramp of 7 °C/min. Samples were heated at two stages, first at 400 °C for 2 h, and then at 800 °C for 2 h. The resulting ash residue was dissolved in a solution containing 3 mL each of 16 N HNO_3 and 11.5 N HCl and 1 mL of H_2O_2 (30 percent, v/v), heated on a sand bath for 2 h under reflux mode. The solution was transferred to a 10 mL volumetric flask and made up to volume by adding two percent (v/v) mixture of HNO_3 and HCl. Determination of Al in the solution was carried out by using a simultaneous solid state detector inductively coupled plasma optical emission spectrometer (ICP-OES, model ACTIVA, Horiba Jobin Yvon SAS, France). Intensity of emission was measured at two different wavelengths at 308.215 nm and 396.152 nm. The calibration of the instrument was carried out using ICP-OES standard solution from E-Merck (Germany). Concentration of Al in plant tissue was expressed in μg g^{-1} DW.

2.4. Histochemical visualization and quantification of Al-uptake in root

Root uptake of Al^{3+} was determined by hematoxylin staining assay (Ownby, 1993). Following treatment with Al^{3+} the intact roots were washed thoroughly in running tap water for 10 min and then stained with a solution containing 0.2 percent hematoxylin (Qualigen, Mumbai) and 0.02 percent KIO_3 (Merck, Mumbai) for 15 min at room temperature. After staining, the roots were washed in distilled water for 10 min. Representative stained roots from treatment groups were macro-photographed for the record. At this step, 10 root tips of equal length (10 mm) from each treatment were excised and immersed in 4 mL of 1 N HCl (Qualigens, Mumbai) for 1 h. Absorbance of the resulting clear solution was measured at 490 nm using a UV–Visible spectrophotometer (Model GS5701, ECIL, India).

2.5. In-leaf estimation and visualization of ROS ($O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$)

Cellular generation of $O_2^{\bullet-}$ (Kiba et al., 1997), H_2O_2 (Loreto and Velikova, 2001) and $^{\bullet}OH$ (Halliwell et al., 1987) were measured using a UV–Visible spectrophotometer and visualized histochemically in leaf (Romero-Puertas et al., 2004) as described below. For measurement of $O_2^{\bullet-}$, three 6-day-old leaves of equal length weighing about 150 mg FW were immersed in 6 mL of the reaction mixture containing 50 mM Tris–HCl buffer pH 6.4, 0.2 mM nitro blue tetrazolium (NBT), 0.2 mM NADH and 250 mM sucrose, with or without 1 mM salicylic hydroxamic acid (SHAM, Acros, USA, a specific inhibitor of cell wall-bound NADH-peroxidase) or 10 mM imidazole (IMZ, SRL, Mumbai, a specific inhibitor of plasma membrane-bound NADPH-oxidase); vacuum-infiltrated for 10–15 min and illuminated at 200 μmol m^{-2} s^{-1} for 24 h to develop color, characteristic of blue monoformazan precipitation. The absorbance of blue monoformazan formed as a result of reduction of NBT in the reaction mixture was measured at 530 nm using an extinction coefficient of (ε=12.8 mM^{-1} cm^{-1}) and expressed in μmoles g^{-1} FW. Furthermore, the stained leaves were bleached in 95 percent (v/v) ethanol at 90 °C for 10 min to localize cellular $O_2^{\bullet-}$ generation and were macro-photographed against a white fluorescent light background using a Nikon Coolpix digital camera.

For measurement of H_2O_2 approximately 2 g of leaf samples were homogenized at 4 °C in 4 mL of 0.2 percent (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000*g* for 15 min and 0.5 mL of the supernatant was mixed with 1 mL of 10 mM sodium phosphate buffer pH 7.0 and 2 mL of 1 M potassium iodide. H_2O_2 content of the supernatant was measured by comparing its absorbance at 390 nm with a standard calibration curve (Fig. 5A) and expressed in nmol g^{-1} FW. For histochemical visualization of H_2O_2, three leaves of equal size were immersed in one percent solution of 3, 3′-diaminobenzidine (DAB) pH 3.8, vacuum-infiltrated for 10–15 min and then incubated at room temperature for 8 h in the absence of direct light. Leaves were then illuminated until the appearance of brown color, characteristic of polymerization reaction between DAB and H_2O_2. Following bleaching, the leaves were macro-photographed as said earlier.

For estimation of the amount of cellular hydroxyl radical ($^{\bullet}OH$), leaf samples weighing 1 g were homogenized in 10 mM sodium phosphate buffer pH 7.4 containing 15 mM 2-deoxy-D-ribose and centrifuged at 12,000*g* for 15 min. The supernatants were incubated at 37 °C for 2 h. Aliquots of 0.2 mL of the above supernatant were added to the reaction mixture containing 3 ml of 0.5 percent (w/v) thiobarbituric acid (TBA, one percent stock solution made in 5 mM NaOH) and 1 mL glacial acetic acid, heated at 100 °C in a water bath for 30 min and cooled down to 4 °C for 10 min before the measurement. The absorbance of malondialdehyde (MDA) was measured at 532 nm and concentration was calculated using an extinction coefficient (ε=155 mM^{-1} cm^{-1}) and expressed in nmoles g^{-1} FW.

2.6. In-leaf visualization and estimation of cell death

Cell death in leaf tissue was visualized histochemically as well as quantified by Evan's blue staining method (Romero-Puertas et al., 2004). Freshly excised leaves were immersed, and vacuum infiltrated in 0.25 percent (w/v) aqueous solution of Evans blue for 6 h and left overnight in the same solution. The leaves were then bleached in boiling ethanol (95 percent v/v) to remove chlorophyll. Cell death visualized as blue patches on the leaf surface were macro-photographed as said before. Furthermore, for quantification of cell death three stained leaves weighing about 150 mg from each treatment were immersed in dye-extraction solution consisting of 50 percent (v/v) ethanol in one percent (w/v) SDS for 1 h at 50 °C. Absorbance of Evan's blue released into solution was recorded at 600 nm.

2.7. Lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction (Dhindsa et al., 1981). Leaf samples weighing 500 mg from control or Al treatments were homogenized in 3 mL of twenty percent (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 10 min. One mL of the supernatant was added to reaction mixture containing twenty percent (w/v) TCA and 0.5 percent (w/v) TBA followed by 30 min incubation at 95 °C. The reaction was stopped by placing the test tubes in ice and then centrifuged at 10,000*g* for 15 min. The absorbance of the resulting supernatant was recorded at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from that recorded at 532 nm. The concentration of MDA was calculated using an extinction coefficient (ε=155 mM^{-1} cm^{-1}) and expressed in nmoles g^{-1} FW.

2.8. Protein extraction

Leaf samples weighing 1 g from each treatment were homogenized using chilled mortar and pestle in 2 mL of 50 mM Tris–HCl buffer, pH 7.2, containing 0.1 mM ethylenediamine-tetra acetic acid (EDTA), and one percent (w/v) polyvinyl-polypyrrolidone at 4 °C. For ascorbate peroxidase assay, the homogenizing solution additionally contained 5 mM ascorbate. The homogenates were centrifuged at 10,000*g* for 15 min at 4 °C and the resultant crude supernatant was collected and stored at −20 °C for estimation of protein and enzyme activities. Soluble protein content in the supernatant was determined according to the method of Bradford (1976) with bovine serum albumin as the standard.

2.9. Spectrophotometric estimation of antioxidant enzymes

Activities of antioxidant enzymes namely catalase (CAT., EC 1.11.1.6), superoxide dismutase (SOD, EC. 1.15.1.1), guaiacol peroxidase (GPX, EC. 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) were assayed by spectrophotometry described briefly as follows.

CAT activity (Aebi, 1983) was determined by measuring the decrease in absorbance at 240 nm as a result of degradation of H_2O_2 (ε=39.4 mM^{-1} cm^{-1}), which was followed in the reaction mixture containing 50 mM sodium phosphate