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Research article

Aluminum stress inhibits root growth and alters physiological and metabolic responses in chickpea (*Cicer arietinum* L.)

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ABSTRACT

Chickpea (*Cicer arietinum* L.) roots were treated with aluminum (Al^{3+}) in calcium chloride ($CaCl_2$) solution (pH 4.7) and growth responses along with physiological and metabolic changes were investigated. Al^{3+} treatment for 7d resulted in a dose dependent decline of seed germination and inhibition of root growth. A significant ($p \leq 0.05$) decline in fresh and dry biomass were observed after 7d of Al^{3+} stress. The root growth (length) was inhibited after 24 and 48 h of stress imposition. The hydrogen peroxide (H_2O_2) levels increased significantly ($p \leq 0.05$) with respect to control in Al^{3+} treated roots. The hematoxylin and Evans blue assay indicated significant ($p \leq 0.05$) accumulation of Al^{3+} in the roots and loss of plasmamembrane integrity respectively. The time-course evaluation of lipid peroxidation showed increase in malondialdehyde (MDA) after 12, 24 and 48 h of stress imposition. Al^{3+} treatment did not alter the MDA levels after 2 or 4 h of stress, however, a minor increase was observed after 6 and 10 h of treatment. The proton (^1H) nuclear magnetic resonance (NMR) spectrum of the perchloric acid extracts showed variation in the abundance of metabolites and suggested a major metabolic shift in chickpea root during Al^{3+} stress. The key differences that were observed include changes in energy metabolites. Accumulation of phenolic compounds suggested its possible role in Al^{3+} exclusion in roots during stress. The results suggested that Al^{3+} alters growth pattern in chickpea and induces reactive oxygen species (ROS) production that causes physiological and metabolic changes.

1. Introduction

About 40% of the global arable land is acidic (Xia et al., 2010). Acid soils have high concentrations of aluminum (Al) and toxic levels of manganese (Mn) along with low phosphorus (P), nitrogen (N) and molybdenum (Mo) contents (Barceló et al., 1996; Kidd and Proctor, 2001). Al is present in the form of aluminosilicates in the soil, which transforms into toxic trivalent (Al^{3+}) cation under acidic condition (Ma et al., 2001). Al^{3+} primarily targets plant root and inhibits growth (Kochian et al., 2005; Ma, 2007). It also elevates the levels of reactive oxygen species (ROS), which targets the plasmamembrane and interacts with lipid components to initiate lipid peroxidation (Yamamoto et al., 2001). Al^{3+} do not participate in redox reactions; rather it causes iron (Fe) mediated loss of plasmamembrane integrity, which leads to cell death (Yamamoto et al., 1997, 2001). ROS mediated cell death under Al^{3+} stress was previously reported in wheat (Delisle et al., 2001). Studies have shown that oxalate oxidase (*OxO*) gene that produces hydrogen peroxide (H_2O_2) and results in the initiation of the cell death process (Matsumoto and Motoda, 2012). Further, Al^{3+} induces superoxide semi-reduce ion in the membrane, which increases the rate of lipid peroxidation (Exley, 2004). In tobacco BY2 cells, oxidative stress is induced as a result of elevated production of superoxide radical ($O_2^{\bullet-}$) (Kawano et al., 2003). In *Arabidopsis*, prolonged exposure of Al^{3+} increases the peroxidase and superoxide dismutase mRNA levels and the overexpression of peroxidase (*NtPOX*) and glutathione-*S*-*transferase* (*parB*) in tobacco have suggested that ROS are strongly induced under Al^{3+} treatment and an efficient antioxidant defense metabolism is a key factor in controlling oxidative damage (Ezaki et al., 2001). High concentration of ROS depletes ATP production as a result respiration is inhibited and plants show poor growth pattern (Yamamoto et al., 2002). Thus, Al^{3+} alters the cellular redox homeostasis and exerts oxidative stress by elevating the ROS production.

Chickpea (*Cicer aerietinum* L.) is a major legume crop grown worldwide and gained tremendous importance in developing countries in terms of food security (Varshney et al., 2013). India contributes around 67% of world chickpea production with a grain

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yield of 0.8 t ha^{-1} (http://agropedia.iitk.ac.in/content/major-strategies-enhance-chickpea-productivity). Chickpea cultivation is severely affected by environmental factors like soil salinity and drought (Toker et al., 2007), however, its response to Al^{3+} stress under acidic soil conditions is not reported. Other crop plants like cowpea (*Vigna unguiculata* L. Walp.), pigeon pea (*Cajanus cajan* L. Millsp.), potato (*Solanum tuberosum* L.) and rice (*Oryza sativa* L.) are usually considered more tolerant to Al^{3+} stress (Little, 1988), while such responses of chickpea to Al^{3+} is still unknown. In the present investigation, we evaluate the effect of Al^{3+} on growth responses along with physiological and metabolic changes in chickpea. This will provide a picture on chickpea response under acidic soil conditions and also highlight the metabolites that are affected due to Al^{3+} toxicity.

2. Methods

2.1. Plant material and Al^{3+} treatment

Chickpea (*Cicer arietinum* L. cv: ICCV10) seeds were procured from Indian Agricultural Research Institute (IARI), New Delhi. Seeds were sterilized with 1% sodium hypochlorite solution for 15 min and washed thoroughly with sterile distilled water (dH_2O). Sterile seeds transferred over moistened filter paper and allowed to germinate in dark for 3d. Uniformly germinated seeds were transferred to plastic cups containing 350 ml Hoagland solution (Hoagland and Arnon, 1950) and grown for 10d under white fluorescent light with 16 h photoperiod. Prior to Al^{3+} treatments, the seedlings were transferred to 100 μM calcium chloride ($CaCl_2$, pH 4.7) for 24 h after which Al^{3+} treatment was given in the form aluminum chloride ($AlCl_3$) prepared in 100 μM $CaCl_2$ (pH 4.7) with varying concentrations (0, 10, 25, 50, 100, 250 and 500 μM).

For evaluation of effect of Al^{3+} on seed germination, sterile seeds were germinated either in sterile dH_2O ($-Al^{3+}$) or 100 μM $CaCl_2$ (pH 4.7) enriched in Al^{3+} (10, 50, 100, 250 and 500 μM). Growth experiments were performed after 7d of Al^{3+} (0, 10, 25, 50, 100, 250 and 500 μM) treatment to 10d old seedlings. Based on the screening results final concentrations of 0, 50 and 100 μM Al^{3+} were selected and stress was imposed for 24 and 48 h.

2.2. Growth analysis

Growth parameters were measured in terms of root length and fresh/dry biomass after 7d of Al^{3+} stress. For dry biomass measurement, 1 g of root tissue was weighed and dried at 80 °C for 48 h. The final dry weight was taken to measure the dry biomass. For Al^{3+} treated seedlings, the dry biomass of root was measured after 24 and 48 h of Al^{3+} treatment. Root growth (elongation) was measured after 24 and 48 h of stress imposition, using a centimeter scale. The percent (%) root growth inhibition was measured following the formula as $[(A - B)/A] \times 100$, where A is root elongation of control and B is root elongation of Al^{3+} treated seedlings.

2.3. Hydrogen peroxide production and lipid peroxidation

The hydrogen peroxide (H_2O_2) content was measured as per the method of Sagisaka (1979). Briefly, 200 mg of tissue sample was homogenized with 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 17,000 *g* for 15 min at 4 °C. The assay mixture contained 1.6 ml supernatant tissue extract, 0.4 ml 50% (w/v) TCA, 0.4 ml ferrous ammonium sulfate ($FeNHSO_4$) and 2.5 mM potassium thiocyanate (KSCN). The absorbance was recorded at 480 nm using a UV–visible spectrophotometer (Lambda 35 UV-VIS, Perkin Elmer, USA).

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content in root and shoot after 0, 2, 4, 6, 8, 10, 12, 24 and 48 h, as per the method of Heath and Packer (1968). 200 mg of plant tissue was homogenized with 2 ml 1% (w/v) TCA and centrifuged at 12,000 *g* for 20 min at 4 °C. To 1 ml of the supernatant, 1 ml 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) and 100 μl of butylated hydroxytoluene (BHT, a 4% solution in ethanol) were added. The mixture was incubated at 95 °C for 30 min and centrifuged at 10,000 *g* for 15 min. The absorbance was recorded at 532 nm and corrected at 600 nm (Lambda 35 UV-VIS, Perkin Elmer, USA).

2.4. Evans blue and hematoxylin assay

The loss of plasmamembrane integrity was measured by spectrophotometric assay of Evans blue (EB) uptake as suggested by Yamamoto et al. (2001). The roots of control and Al^{3+}-treated roots were stained with EB solution [0.025% (w/v) in 100 μM $CaCl_2$ (pH 5.6)] for 10 min. The stained roots were washed thrice in 100 μM $CaCl_2$ solution till no dye elutes from the roots. The stained regions were removed with a razor blade and homogenized with 1% (w/v) sodium dodecyl sulfate (SDS) and centrifuged at 13,500 *g* for 10 min. The absorbance of the supernatant extract as measured at 600 nm (Lambda 35 UV-VIS, Perkin Elmer, USA).

The Al^{3+} uptake was measured by hematoxylin staining as suggested by Ownby (1993). Roots were washed thoroughly with deionized water and stained with 0.2% (w/v) aqueous hematoxylin solution containing 0.02% potassium iodate (KIO_3) for 15 min at room temperature. The stained roots were further washed with distilled water for 15 min and 10 root tips (~5 mm) were treated with 1 M hydrochloric acid (HCl) for 60 min. The OD of the stain released into the solution was measured at 490 nm (Lambda 35 UV-VIS, Perkin Elmer, USA). The Al^{3+} and EB uptake were determined as fold increase, calculated as: fold increase = absorbance of treated samples/absorbance of control samples.

2.5. Nuclear magnetic resonance (NMR) analysis

The sample preparation and NMR analysis of root samples were done as per the method of Kruger et al. (2008). 500 mg of frozen root sample was ground to fine powder with liquid nitrogen (LN_2) in a pre-chilled mortar pestle. After evaporation of LN_2, 1 ml ice-cold 3 M perchloric acid ($HClO_4$) was added. Sample was allowed to thaw on ice for 15 min after which 2.5 ml (5 ml/g fr wt) of 1 M $HClO_4$ was added to obtain a homogenous suspension, and mixed by thoroughly. The sample was incubated on ice for 30 min and centrifuged at 25,000 *g* for 15 min at 4 °C. The supernatant extract was retained and the pellet was re-suspended and homogenated with 2.5 ml of 1 M of $HClO_4$ and centrifuged as described above. The resultant supernatants were combined and neutralized using 2 M potassium hydroxide (KOH) to a pH of 5.6–6.0. The samples were centrifuged at 25,000 *g* at 4 °C to remove potassium perchlorate ($KClO_4$) precipitates. The sample was allowed to stand over ice for 30 min to allow further precipitation of $KClO_4$. The pH was checked to 5.6–6.0 and centrifuged at 25,000 *g* at 4 °C for precipitation of any remaining $KClO_4$, if any. The sample was freeze-dried and the dried sample was re-dissolved in 1 ml of 25 mM NaH_2PO_4 buffer (pH 7.5). The sample was centrifuged at 14,000 *g* at 4 °C and the resultant sample was freeze dried again. For NMR analysis, the dried sample was dissolved in 1 ml deuterium oxide (D_2O) containing TSP ($\delta = 0.00$ ppm). The ^1H NMR was recorded in a 400 MHz NMR spectrometer (Bruker Avance *III Nanobay*) with probe temperature adjusted to 20 °C.

2.6. Statistical analysis

The data presented are means of three replicates ± SE. The results were analyzed statistically to evaluate the significance