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REGULAR ARTICLE

EFFECTS OF ALUMINIUM EXPOSURES ON GROWTH, PHOTOSYNTHETIC EFFICIENCY, LIPID PEROXIDATION, ANTIOXIDANT ENZYMES AND ARTEMISININ CONTENT OF *ARTEMISIA ANNUA* L.

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SUMMARY

Acid soils are widely distributed at a global scale; under acidic conditions, the solubility of aluminium (Al) increases and the elevated concentration of toxic Al^{3+} in soil solution seriously limits crop production. There is no information on the effects of aluminium (Al) on *Artemisia annua* L., which is a most important antimalarial plant in the recent time being artemisinin; responsible for its antimalarial activity. In this report, we describe the effects of Al contamination on growth, photosynthetic efficiency, membrane damage, antioxidant enzyme activities and changes in artemisinin content in *A. annua*. Al addition to the soil medium significantly reduced the yield and growth of the plants. Lower values of net photosynthetic rate, stomatal conductance, internal CO2 and total chlorophyll content were observed as a result of different Al concentrations applied. The activities of nitrate reductase (NR) and carbonic anhydrase (CA) were also found to be hampered by Al exposure to the plants. Al enhanced lipid peroxidation rate (TBRAS content) and activated the activities of catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) enzymes in the treated plants. The elicitation in the endogenous ROS levels, due to the Al treatments, was also noticed in the present study. Furthermore, enhanced artemisinin content and yield was obtained at 0.10mM concentration of soil applied Al. Our study provides evidence that excess Al in soil hamper the growth and yield, slow down the activities of NR and CA, induce lipid peroxidation and antioxidant enzymes but a low level of Al-toxicity can induce artemisinin content in *A. annua* plants.

Key words: Aluminium (Al); *Artemisia annua* L.; Artemisinin; Lipid peroxidation; Reactive oxygen species (ROS)

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1. Introduction

Malaria is, next to HIV/AIDS and tuberculosis, one of the most destructive diseases, today the world has to deal with. *Artemisia annua* L. is an aromatic plant which has been used for centuries in Chinese traditional medicine for the treatment of fever and malaria (Klayman, 1985). Being the world's most severe parasitic infection, malaria threatens more than one-third of the global population, killing approximately two million people annually (Snow et al., 2005). Despite tremendous efforts for the control of malaria, the global morbidity and mortality have not been significantly changed in the last 50 years (WHO, 2006). Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge, has become increasingly popular as an effective and safe alternative therapy against malaria (Abdin et al., 2003). Artemisinin and its derivatives are effective against multi-drug resistant, *Plasmodium falciparum* strains mainly in Southeast Asia and more recently in Africa, without any reputed cases of resistance (Kremsner and Krishna, 2004). Artemisinin, along with taxol, is considered one of the novel discoveries in the recent medicinal plant research. Its isolation and

characterization has increased the interest in *A. annua* worldwide (Efferth et al., 2001; Ferreira et al., 2005). Since the non-natural chemical synthesis of artemisinin is very costly therefore the intact plant remains the only viable source of the artemisinin production, and the enhanced production of the artemisinin content in the whole plant is highly desirable (Abdin et al., 2003; Aftab et al., 2010a, c, d).

Aluminium (Al) is the most abundant metal and the third most common element in the earth's crust. Al toxicity is the primary factor limiting crop productivity in acidic soils, which comprise large areas of the world's land, particularly in the tropics and subtropics (Foy et al., 1984). It is also an important factor limiting crop production in many developing countries. The first visible symptom of Al toxicity is the inhibition of root growth, which can occur within 1–2 h after exposure to Al (Ryan et al., 1993). As soil becomes more acidic, phytotoxic forms of Al are released into soil to levels that affect root system, plant growth and seed yield. Physiological mechanisms of Al toxicity and of root growth inhibition include alterations of the plasma membrane properties (Cakmak and Horst, 1991), modifying of the cation-exchange capacity of the cell wall (Horst 1995), interfering with signal transduction (Jones and Kochian, 1995) and binding to polynucleotides (DNA or RNA). Direct evidence has been demonstrated that the root apex is the primary site of Al-induced root growth inhibition. Al can interact with a number of extracellular and intracellular substances like interaction within the root cell walls, disruption of plasma membrane and plasma membrane transport system, interaction with symplastic constituents such as calmodulin (Kochian, 1995). Al ions have been shown to cause increased peroxidation of membrane lipids. For example, Yamamoto et al. (1996, 1997) observed increased levels of lipid peroxidation in *Nicotiana tabacum* cells in the presence of Al. They suggested that lipid peroxidation might be the primary lesion involved in Al induced inhibition of growth. Furthermore, recent studies with *Pisum sativum* roots suggested that lipid peroxidation is a relatively early symptom induced by the accumulation of Al (Yamamoto et al., 2001). Al toxicity increases the activity of superoxide dismutase and peroxidase (Cakmak and Horst, 1991). Several Al-induced genes, including peroxidase (POX) and superoxide dismutase (SOD), have also been found to be induced by oxidative stress (Richards et al., 1998). An important response to stress by aerobic cells is the production of reactive oxygen species (ROS), like superoxide radical, hydroxyl radical, singlet oxygen and toxic hydrogen peroxide molecules (Breusegem et al., 2001; Kovacik et al., 2008; Aftab et al., 2010b, c). These ROS produced in the cell are detoxified by both non enzymic and enzymic antioxidant system. ROS if not detoxified cause serious damage to proteins, lipids and nucleic acids (Alscher et al., 1997).

The aim of the present study was to determine the impact of Al toxicity on growth of the plants, photosynthetic efficiency, enzyme activities, membrane damage and antioxidant enzymes. The changes in artemisinin content and yield due to Al toxicity were also recorded in the present research.

2. Materials and Methods

Growth conditions and Al treatments

Seeds of *Artemisia annua* L. were initially surface sterilized with 95% ethyl alcohol for 5 min and then washed thoroughly with double distilled water. Prior to seed sowing, 5.0 kg homogenous mixture of soil and farmyard manure (4:1) was filled in each pot. Physico-chemical characteristics of the soil were: texture-sandy loam, pH (1:2) – 8.0, E.C. (1:2) -0.48 m mhos/cm, available N, P and K – 97.46, 10.21 and 147.0 mg kg^{-1} soil, respectively. Then seeds were sown at a depth of 2 cm in earthen pots (25 cm diameter × 25 cm height) containing sandy-loam soil. Before seed sowing, Al was applied at the rates of 0, 0.01, 0.10, 1.00 and 10.0mM from $AlCl_3$. The experiment was conducted according to a simple randomized complete block design using different concentrations of Al. Each treatment was replicated five times. The pots were watered as and when required and plants were

grown under naturally illuminated environmental conditions.

Analyses of growth and yield attributes

Growth and biochemical attributes of the *Artemisia annua* L. were determined at flowering stage i.e. 150 days after sowing (DAS). The plants from each treatment were harvested with the roots carefully and shoot and root lengths were recorded. Plants were washed with tap water to remove adhering foreign particles. The plants were dried at 80°C for 48 h, and dry weights were recorded individually of shoots, roots and total leaves.

Photosynthetic measurements and chlorophyll content

Net photosynthetic rate (P_N), stomatal conductance (gs) and internal CO_2 (c_i) were measured on sunny days at 1100 hours using fully expanded leaves of *A. annua* with the help of an IRGA (Infra Red Gas Analyzer, LI-COR 6400 Portable Photosynthesis System, Lincoln, Nebraska, USA). Before recording the measurement, the IRGA was calibrated and zero was adjusted approximately every 30 min during the measurement period. Each leaf was enclosed in a gas exchange chamber for 60 s. All the attributes measured by IRGA were recorded three times for each treatment.

Total chlorophyll content in fresh leaves was estimated by the method of Lichtenthaler and Buschmann (2001). The fresh tissue from interveinal leaf-area were ground using a mortar and pestle containing 80% acetone. The absorbance of the solution was recorded at 662 and 645 nm for chlorophyll estimation using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan).

Determination of nitrate reductase (NR) and carbonic anhydrase (CA) activities

Nitrate reductase (E.C. 1.6.6.1) activity in the leaf was determined by the intact tissue assay method of Jaworski (1971). Chopped leaf pieces (200 mg) were incubated for 2 h at 30° C in a 5.5 mL reaction mixture, which contained 2.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.2 M potassium nitrate, and 2.5 mL of 5% isopropanol. The nitrite formed subsequently was colorimetrically determined at 540 nm after azocoupling with sulphanilamide and naphthylene diamine dihydrochloride. The NR activity was expressed as nM NO_2 g^{-1} FW h^{-1}.

Carbonic anhydrase (E.C. 4.2.1.1) activity was measured in fresh leaves using the method as described by Dwivedi and Randhawa (1974). Two hundred mg of fresh leaf pieces were weighed and transferred to petri plates. The leaf pieces were dipped in 10 mL of 0.2 M cystein hydrochloride solution for 20 min at 4°C. To each test tube, 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.022% bromothymol blue were added. The reaction mixture was titrated against 0.05 N HCl using methyl red as indicator. The enzyme was expressed as µM CO_2 kg^{-1} leaf FW s^{-1}.

Lipid peroxidation rate (TBRAS content)

Oxidative damage to leaf lipids was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst (1991). TBARS were extracted from 0.5 g chopped fresh leaves, ground in 5mL of 0.1% (w/v) trichloroacetic acid (TCA). Following the centrifugation at 12000×*g* for 5 min, an aliquot of 1 mL from the supernatant was added to 4mL of 0.5% (w/v) TBA in 20% (w/v) TCA. Samples were incubated at 90 °C for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 10000×*g* for 5 min, and absorbance of the supernatant was read at 532 nm on a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. TBARS content was expressed as nmol g^{-1} fresh weight.

Antioxidant enzymes assay

Catalase activity was measured according the methods given by Chandlee and Scandalios (1984) with a small modification. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.4 mL of 15 mM H_2O_2 and 0.04 mL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme

activity was expressed in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Peroxidase was assayed by the method of Kumar and Khan (1982). Assay mixture of POX contained 2 mL of 0.1M phosphate buffer (pH 6.8), 1 mL of 0.01M pyrogallol, 1 mL of 0.005M H_2O_2 and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25 ^oC after which the reaction was terminated by adding 1 mL of 2.5N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a reagent blank prepared by adding the extract after the addition of 2.5N H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One unit of the enzyme activity corresponded to an amount of enzyme that change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Superoxide dismutase activity was assayed as described by Beauchamp and Fridovich (1971). The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1M methionine, 2×10^{-5}M KCN and 5.6×10^{-5}M nitroblue tetrazolium salt (NBT) dissolved in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8) and 3mL of the reaction medium was added to 1 mL of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40W fluorescent tubes in a single row. The reaction was initiated at 30 ^oC for 1 hour. Identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity was expressed as U mg^{-1} protein. One unit (U) is defined as the amount of change in the absorbance by 0.1 h^{-1} mg^{-1} protein.

Endogenous ROS determination

The content of H_2O_2 in the leaves was determined according to the method of Mukherjee and Choudhuri (1983). Fresh leaves of *A. annua* (0.5 g) were homogenized using a cold mortar and pestle in pre-cooled acetone (5 mL) and the homogenate was centrifuged at 12,000g for 5 min. One mL of the supernatant was mixed with 0.1 mL of 5% $Ti(SO_4)_2$ and 0.2 mL 19% ammonia. After a precipitate was formed, the reaction mixture was centrifuged at 12,000g for 5 min. The resulting pellet was dissolved in 3 mL of 2 M H_2SO_4 and the absorbance was read at 415 nm using a spectrophotometer. The H_2O_2 concentration was calculated according to a standard curve of H_2O_2 ranging from 0 to 10 µM.

Singlet oxygen (O_2^-) production was measured as described by Able et al. (1998) with some modifications. The reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbo-xanilide) in the presence of O_2^- was monitored. *A. annua* leaves (0.5 g, FW) were homogenized with 1 mL of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 12,000g for 15 min. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 100 µL supernatant and 0.5 mM XTT. The reduction of XTT was determined by measuring the absorbance at 470 nm in a spectrophotometer. The quantity of O_2^- was determined using the molar extinction coefficient (ε) 2.16×10^4 M^{-1} cm^{-1}.

Artemisinin extraction and estimation

Dry leaf material (1 g) was used for the estimation of artemisinin modified to a compound Q_{260} and quantified using HPLC method (Zhao and Zeng, 1986). Standard curve was prepared using 1 mg of standard artemisinin dissolved in 1 mL of HPLC-grade methanol to make the stock solution. One g dry material was taken for extraction of artemisinin. It was extracted with 20 mL petroleum ether in shaker at 70 rpm for 24 h. After 24 h, solvent was decanted and pooled and 20 mL of petroleum ether added again and this step was repeated thrice. Petroleum ether fractions were pooled and concentrated under reduced pressure and residues defatted with CH_3CN (10 mL×3). Precipitated fat was filtered out and filtrate concentrated under reduced pressure. Residues were dissolved in 1 mL of methanol. 100 µL aliquot of each sample of each treatment was taken and to this 4 mL of 0.3% NaOH was added. The samples were incubated in shaking water bath at 50˚C for 30 min, thereafter cooled and neutralized with glacial acetic acid (0.1 M in 20% MeOH). The pH of the solution was maintained at 6.8. Derivatized artemisinin was analyzed and

quantified through reverse phase column (C18; 5 µm; 4.6 mm; 250 mm) using premix methanol: 10 mM K-Phosphate buffer (pH, 6.5) in the ratio of 60:40 as mobile phase at constant flow rate of 1 mL/min, with the detector set at 260 nm. Artemisinin was quantified against the standard curve of artemisinin, obtained from Sigma–Aldrich, USA.

Statistical analysis

Each pot was treated as one replicate and all the treatments were replicated five times. The data was analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values were statistically compared by Duncan's Multiple Range Test (DMRT) at *p<0.05 % level using different letters.

3. Results

The presence of Al in the soil medium significantly lowered the values for growth attributes (Shoot and root lengths, shoot and root dry weights and dry leaf yield). Shoot length noted in 10.0mM Al treated plants was 77.6% lower than the control plants showing the reduced growth under toxic levels of Al (Table I). The root length was most severely affected by Al toxicity compared to other growth attributes. In 10.0mM Al treated plants, the root length was 103.5% reduced compared to untreated plants. Shoot and root dry weights were also significantly reduced by different Al concentrations. At 10.0mM Al (the highest applied concentration), the shoot and root dry weights of the plant were reduced by 88.2% and 130.2% respectively (Table I). The dry leaf yield was reduced by 86.3%, compared to control, when the plants were growth in 10.0mM concentration of Al (Table I).

A reduced photosynthetic activity was observed in the Al treated plants. In comparison to control, at 10.0mM Al concentration, the net photosynthetic rate was 79.4% lower showing the degree of damage to the leaf tissues (Fig. 1a). Both, stomatal conductance and internal CO_2 was observed the lowest (110.8 and 71.2% below the control, respectively) at the highest applied concentration of Al (Fig. 1b and c). Chlorophyll content was also reduced in Al stressed plants and the most toxic effect was noted at 10.0mM concentration of Al at which the content was 98.6% less than that of untreated plants (Fig. 1d).

Fig 1. Effect of different concentrations of Al on net photosynthetic rate (a), stomatal conductance (b), internal CO_2 (c) and total chlorophyll content (d) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (┬) show SE

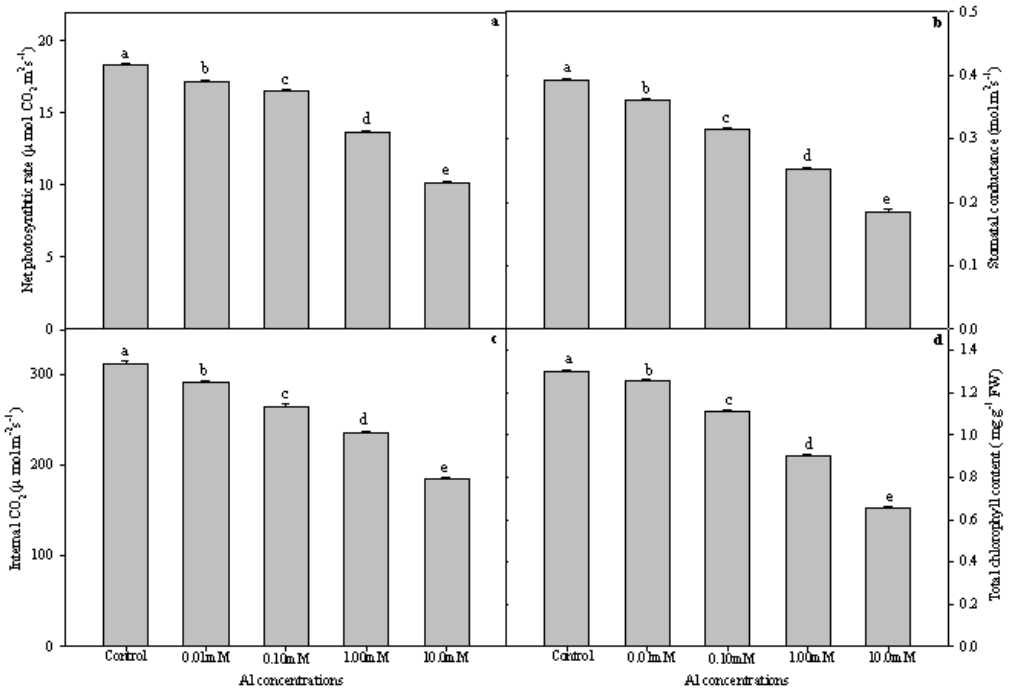
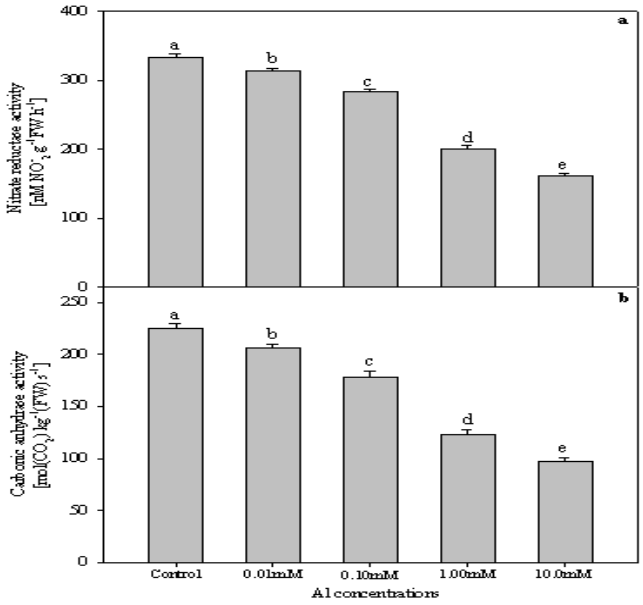


Fig. 2. Effect of different concentrations of Al on NR (a) and CA (b) activities in leaves of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (┬) show SE



The Al stress decreased the activities of nitrate reductase (NR) and carbonic anhydrase (CA). The inhibitory effect of the metal was proportionate to the concentration applied and the highest applied concentration (10.0mM Al) decreased the activities of NR and CA by 106.4 and 131.8%, respectively compared to control (Fig. 2a and b).

Unlike the above parameters the values of TBRAS content and antioxidant enzymes significantly enhanced in the plants subjected to Al stress. The TBRAS content was measured as an indicator of oxidative stress/membrane damage. In the leaves of the plants treated with different concentrations of Al, TBRAS content was increased progressively. The highest content was noted when the plants were supplied with 10.0mM of Al through soil medium (Fig. 3a). There was a high CAT activity noted in the plants receiving different Al treatments having a most pronounced effect on 10.0mM concentration of Al (Fig. 3b). Compared to control, the activity of POX and SOD were also significantly increased in the Al treated plants and the application of 10.0mM Al to the *A. annua* plants showed highest activity of POX and SOD (Fig 3b and c).

Fig. 3. Effect of different concentrations of Al on TBRAS content (a), CAT (b), POX (c) and SOD (d) activities in leaves of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (┬) show SE

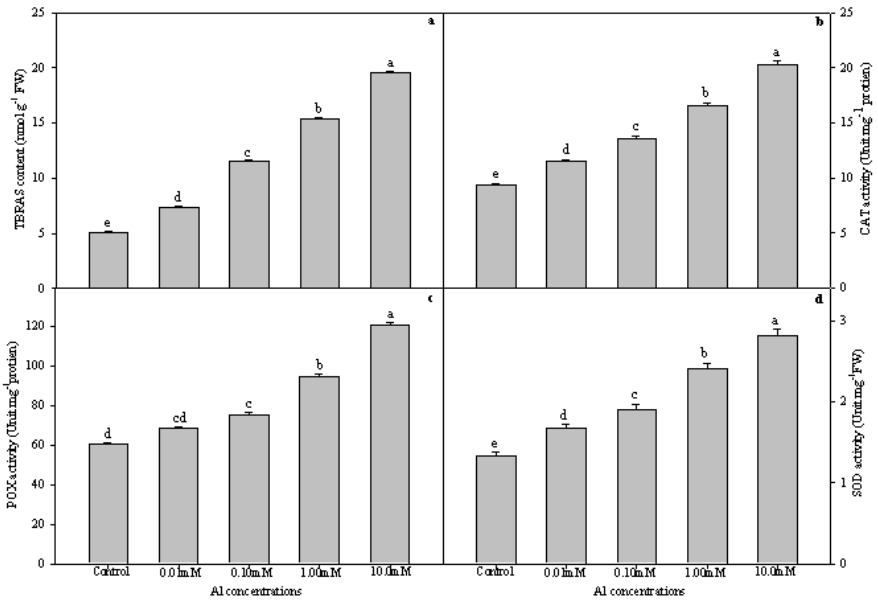
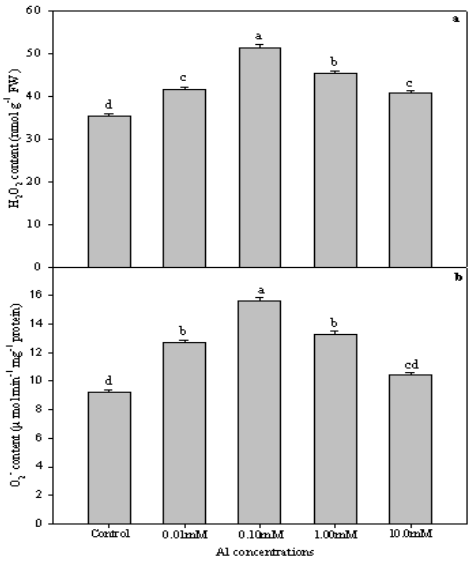


Fig. 4. Effect of different concentrations of Al on H_2O_2 (a) and O_2^- (b) contents of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (┬) show SE



The level of endogenous H_2O_2 and O_2^- was measured in order to determine the internal ROS status by soil applied Al in *A. annua* plants. Both the previous contents were found higher upto 0.10mM of Al, as compared to control, suggesting the increment in ROS level of the cells. At 0.10mM Al, the increase in internal H_2O_2 and O_2^- was 45.3 and 69.5%, respectively, compared to control showing the sharp elicitation in the both contents when 0.10mM Al was applied (Fig. 4a and b).

The artemisinin content was higher when 0.10mM Al was applied (51.8% more), as compared to untreated plants, however a decrease in artemisinin content was noted when further higher doses of Al were applied (Fig. 5a and 6). The artemisinin yield was 24.9% high, compared to control in the 0.10mM Al treated plants, while it decreased sharply when high levels of Al were given to the plants (Fig.5b).

Fig. 5. Effect of different concentrations of Al on artemisinin content (a) and artemisinin yield (b) of *Artemisia annua* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (┬) show SE

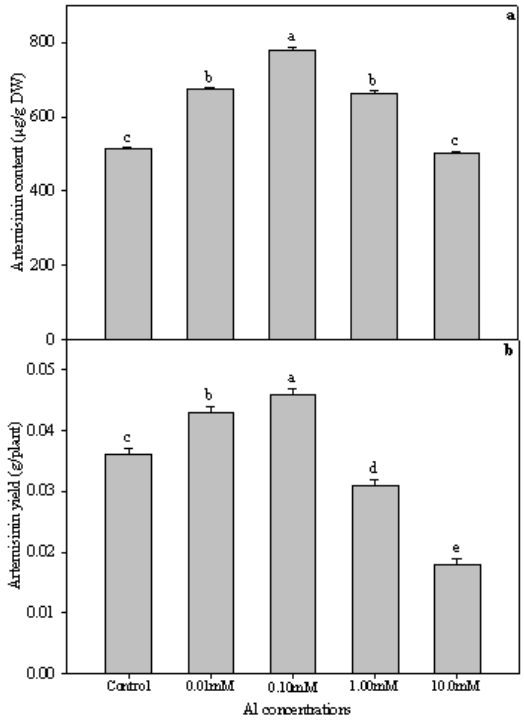
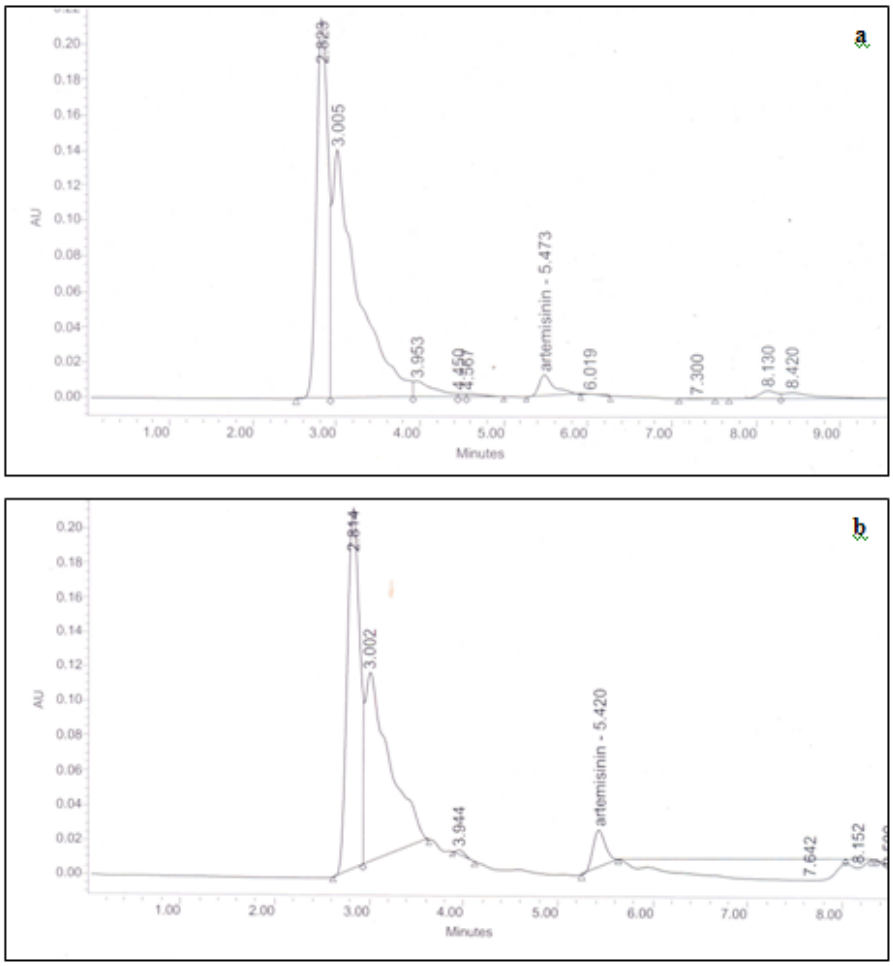


Fig. 6. HPLC chromatogram showing peaks of artemisinin in control (a) and 0.10mM Al treated plants (b) of *Artemisia annua* L.



4. Discussion

The ill effects generated by Al toxicity impaired the growth of the plants as evident by the shoot and root lengths, shoot, root and total leaf dry masses. A gradual decrease in root and shoot elongation rate was observed with the increase in Al concentrations. The reduction in root and shoot elongation with increasing concentrations of Al has also been observed in many other crops, as the first sign of Al toxicity appears in the root system (Zaifnejad et al., 1997; Patra and Panda, 1998; Subrahmanyam, 1998). The most degenerative effects, of the different Al concentrations applied, were noted on the root growth as the roots are the easily affected region of Al toxicity. Al toxicity block the mechanism of cell division and as a result of this root become shunted and brittle, root hair development is poor and root apices become swollen and damage (Barcelo and Poschenreider, 2002; Panda et al., 2009). Detailed spatial studies have indicated that within the root, the root apex, and more specifically the distal part of the transition zone within the apex, is the primary target of Al toxicity (Sivaguru and Horst, 1998). Within this root zone, some Al can enter the cytosol of cells within minutes following Al exposure (Vazquez et al., 1999; Silva et al., 2000; Taylor et al., 2000). The promptness of the root growth inhibition upon exposure to Al indicates that Al quickly disrupts root cell expansion and elongation, prior to inhibiting cell division (Wallace and Anderson, 1984; Frantzios et al., 2001). Prolonged exposures lead to Al interactions with the root cell nuclei, resulting in disruption of cell division and the cytoskeleton (Silva et al., 2000). Also,

Al causes extensive root injury leading to poor ion and water uptake (Panda et al., 2009). The consequences of inhibited root growth were also noticed on shoot growth and biomass accumulation (Table 1). Increasing concentrations of Al reduced the height of the plant and also fresh and dry masses per plant. The observed lower values for fresh and dry masses of the plant upon Al treatments are in agreement with many researchers investigating the response of various plant species to Al stress (Barcelo and Poschenreider, 2002; Yang et al., 2007; Panda et al., 2009).

Table 1. Effect of different concentrations of Al on growth and yield attributes of *Artemisia annua* L. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$). The data shown are means of five replicates ± SE

Treatments	Shoot length (cm)	Root length (cm)	Shoot dry mass (g)	Root dry mass (g)	Dry leaf yield (g)
Control	95.4±2.51[a]	31.5±0.95[a]	96.5±2.74[a]	24.8±0.87[a]	71.6±1.87[a]
0.01mM Al	88.1±2.25[b]	28.7±0.88[b]	83.8±2.47[b]	22.7±0.83[b]	65.2±1.75[b]
0.10mM Al	78.9±1.21[c]	24.8±0.91[c]	72.4±2.18[c]	17.9±0.71[c]	58.9±1.39[c]
1.00mM Al	65.3±1.36[d]	19.4±0.74[d]	62.2±2.53[d]	13.6±0.57[d]	47.1±1.48[d]
10.0mM Al	53.7±1.14[e]	15.5±0.69[e]	51.3±2.01[e]	10.5±0.45[e]	38.3±1.22[e]

In the present study, a reduced net photosynthetic rate, stomatal conductance and internal CO_2 concentration was noticed upon the exposure of toxic levels of Al. The presence of Al in the soil significantly decreased the level of chlorophyll and CA activity. The decrease in chlorophyll might be mediated through the reduced uptake of Mg as demonstrated in the studies of Vitorello and Haug (1996), that Al toxicity reduced the uptake of Mg, which is the integral part of the chlorophyll molecule. The activity of CA, that catalyzes the interconversion of CO_2 and HCO_3, is regulated by photon flux density, CO_2 concentration, availability of Zn (Tiwari et al., 2005) and expression of genes encoding CA protein (Kim et al., 1994). Al brings about ultrastructural modifications and reduced stomatal opening (Vitorello and Haug, 1996), thereby reduced stomatal conductance that resulted in decreased internal CO_2 and photosynthesis. Many studies have showed that Al inhibits CO_2 assimilation in many plant species (Pereira et al., 2000; Peixoto et al., 2002; Chen et al., 2005). Simon et al. (1994) suggested that stomatal closure is at least partially responsible for the Al-induced decrease of CO_2 assimilation rate due to the lowering of intercellular CO_2 concentration. Pereira et al. (2000) worked out that the Al-induced decrease in CO_2 assimilation is associated with structural damage to the thylakoids. Alternatively, Moustakas et al. (1995) concluded that Al causes a decline in photosynthesis in wheat (*Triticum aestivum*) as a result of the closure of PSII reaction centers and a reduction of PSII electron transport rate. Peixoto et al. (2002) suggested that a combination of factors such as reduced pigment content, impaired PSII photochemistry, and the distribution of enzymatic machinery account for the Al-induced decrease in CO_2 assimilation. It has been suggested that Al accumulates in roots where tissues are damaged, resulting in reduced sucrose utilization, which, in turn, causes leaf carbohydrate accumulation and feedback inhibition of photosynthesis (Simon et al., 1994).

It was also found that aluminium exposure elicited the changes in activity of the nitrate reductase. With increasing aluminium concentrations, the activity of nitrate reductase in leaves was reduced (Fig. 2a). The nitrate reduction response was concentration dependent and at 10.0mM of Al caused a 106.4% decrease in the enzyme activity. Lazof et al. (1996) suggested that nitrate uptake by plants could be disrupted by internal aluminium binding to membrane channel proteins or other components of nitrate transport system. Ryan et al. (1995) postulated that transport of nitrate, as well as malate and other organic anions, may occur through the same channels. Malate may

affect aluminium tolerance by chelation of aluminium ions or its transport to the vacuole. However, the mechanism(s) by which aluminium inhibits nitrate uptake is (are) still unknown. It is well documented that nitrate reductase activity is regulated by phosphorylation and dephosphorylation of enzyme protein or by binding small protein inactivator in the presence of Mg^{+2} ions (Kaiser et al., 1993; Glaab and Kaiser, 1996). It is also known that Al could interact with kinases, polymerases, calmodulin and GTP-binding regulatory proteins (Taylor, 1988; Haung et al., 1996). All these systems normally require magnesium ions and probably reduced uptake of phosphate and magnesium leads to the reduced activity of nitrate reductase enzyme.

Lipid peroxidation is an important symptom of Al toxicity, and has been reported in several species. Cakmark and Horst (1991) reported that lipid peroxidation was induced in soybean cultivar after Al treatment. Yamamoto et al. (2001) found that there was an increase in lipid peroxidation in pea plants after 4 h of Al treatment. In contrast, lipid peroxidation of maize, however, is not induced by Al treatment (Patricia et al., 2003). Therefore, the target of oxidative stress varies depending on the plant species under Al toxicity. In this study, it was noticed that lipid peroxidation rate in Al exposed plants increased in a dose dependent manner with a maximum damage to the plants treated with 10.0mM of Al. Changes in gene expression induced by toxic levels of Al were characterized by Richards et al. (1998). Most plant genes so far known to be induced by Al are either known oxidative stress genes or are induced by a range of conditions that are likely to involve oxidative stress. In addition to these commonalities one of the suggested mechanisms of Al toxicity is that it causes lipid peroxidation (Kochian, 1995; Yamamoto et al., 2001; Sharma and Dubey, 2007). Studies have also shown that Al treatment increases the activation of several oxidative stress enzyme activities (Cakmak and Horst, 1991).

There is increasing evidence suggesting that oxidative stress is a key damaging factor in plants exposed to a variety of stressful conditions including metal toxicity and that plants resist oxidative stress by inducing activities of antioxidant enzymes (Cuypers et al., 2002; Verma and Dubey, 2003). Environmental stresses increase the formation of ROS that oxidize membrane lipids, protein and nucleic acids (Gong et al., 2005). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage (Sudhakar et al., 2001). All the treatments in which Al was applied had raised the activities of antioxidant enzymes. With increasing amounts of Al in the soil medium, a concomitant increase in the activities of CAT, POX and SOD were observed in the exposed plants. Similar to our results Boscolo et al. (2003), Siminovicova (2004), Sharma and Dubey (2007) and Zhen et al. (2007) also reported increase in the activities of antioxidant enzymes under Al toxicity in different plants. CAT and POX appear to play an essential protective role in scavenging process of ROS when coordinated with SOD (Jaleel et al., 2009). SOD initiates detoxification of singlet oxygen by forming H_2O_2, which is also toxic and must be eliminated by conversion to H_2O in subsequent reactions. In plants, a number of enzymes regulate intracellular H_2O_2 levels, but CAT and POX are considered the most important (Noctor and Foyer, 1988). Superoxide radicals are toxic byproducts of oxidative metabolism and can interact with H_2O_2 to form highly reactive hydroxyl radicals, which are thought to be primarily responsible for oxygen toxicity in the cell (Azevedo et al., 2005). The dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell and is catalyzed by SOD. The present observations of increased activity of antioxidant enzymes are in agreement with the fact that they remain active for scavenging of ROS.

In all adverse conditions (abiotic stresses) the over production of ROS within different cellular compartment of the plant cells is the common phenomenon (Pinheiro et al., 2004). ROS have recently been proposed as regulators of redox-dependent ion transport

across the plasma membrane during biotic and abiotic stresses, thus contributing to the plant defence system (Babourina et al., 2006). Enhanced ROS production was detected by Al treatment in various plants (Yamamoto et al., 2002; Babourina et al., 2006; Panda and Matsumoto, 2007). In the present study, H_2O_2 and O_2^- concentrations were increased significantly on applying different concentrations of Al upto 0.10mM and thereafter decreased. As the CAT, POX and SOD are known to be scavengers of ROS, when the content of ROS reaches above the threshold, they scavenge them and the observed reduction in the concentration of ROS upon further higher doses of Al is obvious. Wallaart et al. (2000) and Ferriera (2007) also reported that more ROS were accumulated when plants exposed to night frost and in case of potassium deficiency (both considered as stress conditions) in *A. annua*, respectively. We have reported in the previous work that the H_2O_2 production was significantly increased under B stress in *A. annua* plants (Aftab et al., 2010b).

A synergistic relationship between ROS production and artemisinin content was noted in the present study. The high artemisinin content was obtained when 0.01mM and 0.10mM Al was applied and at higher concentrations it was decreased (Fig. 5). Wallaart et al. (2000), Ferreira (2007), Pu et al. (2009) and Mannan et al. (2010) showed a direct relationship between the ROS and artemisinin content. Most recently, enhanced artemisinin production through invoking burst of endogenous singlet oxygen was reported (Guo et al., 2010). Presumably, a mild stress (0.01mM and 0.10mM Al) promoted the artemisinin biosynthesis, as the level of H_2O_2, which is considered to play an important role of converting dihydroartemisinic acid to artemisinin during artemisinin synthesis. In our lab study, it was observed that artemisinin content enhanced significantly upon exposure to B stress only upto 1.00mM concentration and decreased on applying further higher concentrations (Aftab et al. 2010b, c). In fact, Wallaart et al. (1999) suggested that dihydroartemisinic acid might act as a scavenger of ROS that are released in plant cells when they are exposed to oxidative stress. During the reaction, dihydroartemisinic acid hydroperoxide (DHAA-OOH) is generated that gets converted to artemisinin. In this regard, our results are in agreement with the observations recorded by Pu et al. (2009), Mannan et al. (2010) and Guo et al. (2010) who advocated that relatively high levels of ROS resulted in the enhanced the conversion of dihydroartemisinic acid to artemisinin.

5. Conclusion

From the work presented here, it is quite clear that Al toxicity resulted in poor growth and yield of *Artemisia annua* plants. The data confirm the inhibitory effect of heavy metal on NR and CA activities, which are subsequently slowed down by Al treatments. The decrease in net photosynthetic rate, stomatal conductance, internal CO_2 concentration and content of chlorophyll was proportional to the increased doses of Al, and the most phytotoxic influence was observed at the highest tested concentration (10.0mM). Treatment with Al also caused oxidative damage as evidenced by increased lipid peroxide and ROS formation. However, to cope with heavy-metal toxicity, *A. annua* plants were able to promote the activities of antioxidant enzymes. Most importantly, the artemisinin content was found to be positively upregulated by Al treatments and it was observed that a low Al concentration (0.10mM) enhanced the production of artemisinin; but the total artemisinin yield at this concentration was comparatively low. Therefore, we postulate that if, somehow, exposed plants can be protected by morphological damage, a low level of Al can be employed for obtaining higher yield also.

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