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EFFECTS OF ALUMINUM ON NUCLEOLI IN ROOT TIP CELLS, ROOT GROWTH AND THE ANTIOXIDANT DEFENSE SYSTEM IN *VICIA FABA* L.

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The effects of different concentrations of Al (10 μM, 50 μM, 100 μM) on nucleoli in root tip cells, root growth, antioxidant enzyme activity and malondialdehyde (MDA) content were investigated in hydroponically grown *Vicia faba* L. Aluminum significantly inhibited root growth of *V. faba* treated with 50 μM and 100 μM Al. In the nucleolus in root tip cells, some particulates containing argyrophilic proteins were extruded from the nucleus into the cytoplasm, and some were scattered in the nucleus after Al stress. Superoxide dismutase (SOD) activity in leaves and roots exposed to different concentrations of Al was mostly higher than in the control. Seedlings exposed to 100 μM Al showed significantly higher peroxidase (POD) activity in roots than in the control. POD activity increased much more in roots than in leaves. Catalase (CAT) activity was lower in roots than in leaves. Malondialdehyde (MDA) content in leaves and roots of plants exposed to 50 μM and 100 μM Al was significantly higher than in the other groups and the control at 6 to 9 days of treatment. These results suggest that alterations in nucleoli and altered antioxidant enzyme activity and MDA content in *V. faba* can serve as useful biomarkers for detection of Al toxicity. The mechanisms of Al toxicity and tolerance in *V. faba* are briefly discussed.

Key words: *Vicia faba* L., nucleoli, aluminum (Al), antioxidant enzymes, malondialdehyde.

INTRODUCTION

Aluminum (Al), a light metal, makes up 7% of the earth's crust and is the third most abundant element after oxygen and silicon (Ma et al., 2001). Rapid industrial development has made environmental pollution a serious problem around the world. Aluminum toxicity is a major growth-limiting factor for crop production in acid soils (Liu et al., 2008). Normally, Al exists in oxide and aluminosilicate forms, harmless to plants. In acid solution (pH 5 or pH 5.5), the toxicity of dissolved Al will be a strong factor limiting plant growth (Liu et al., 2004; Wang, 2006).

Increased A1 concentrations may cause growth inhibition related to reduction of mitotic activity, progressively decreasing the mitotic index, inducing c-mitosis, anaphase bridges, chromosome stickiness, and poisoning the nucleolus (Liu et al., 1993; de Campos and Viccini, 2003; Grant and Owens, 2006).

Plants have protective enzymatic mechanisms and non-enzymatic mechanisms to scavenge reactive oxygen species (ROS) and alleviate their deleterious effects (Pandhair and Sekhon, 2006). The antioxidant enzymes include CAT, POD and SOD (Vitória et al., 2001; Zhang et al., 2005). Aluminum induces the oxidative stress of lipid peroxidation (Kaneko, 2007). Malondialdehyde (MDA) is one of the end products of lipid peroxidation damage from free radicals.

For scientists working in the field of environmental mutagenesis, *Vicia faba* L. is ideal for screening and monitoring of genotoxic agents according to the standard protocol for plant assays established by the International Program on Chemical Safety (IPCS) and the World Health Organization (WHO) (Soliman and Ghoneam, 2004). It is well known as an excellent model plant and a useful biomarker for detection of heavy metal pollution (Duan et al., 2000). There are a few reports on the effects of Al on the nucleolus, antioxidant enzyme system and MDA content in *V. faba*. Here we assessed the toxic effects of Al by examining nucleoli, plant growth and antioxidant enzyme activity in *V. faba*.

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MATERIALS AND METHODS

CULTURE CONDITIONS AND ALUMINUM TREATMENT

Vicia faba seeds were soaked in distilled water and allowed to germinate and unfold the second pair leaves (3–4 cm) at 25°C. Then the seedlings were grown in containers with 2 L Hoagland's nutrient solution spiked with 10 μM, 50 μM and 100 μM Al for 9 days in a greenhouse with controlled temperature (18–20°C), relative humidity (60%) and supplementary lighting (14 h photoperiod). The Hoagland's solution consisted of 5 mM Ca $(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 50 μM H_3BO_3, 1 mM $MgSO_4$, 4.5 μM $MnCl_2$, 3.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 mM $(NH_4)_6$ Mo_7O_{24} and 10 μM FeEDTA (Stephan and Prochazka, 1989). Hoagland's nutrient solution without Al was used for the control. The solutions were aerated by pump, connected to the containers with pump lines. In each treatment group, ten treated seedlings were observed for growth, and their antioxidant enzyme activity and MDA concentration were measured at the end of each three-day interval. All treatments were done in three replicates.

DETERMINATION OF ANTIOXIDANT ENZYME ACTIVITY AND MDA CONCENTRATION

Fresh roots or leaves from each treatment were homogenized with mortar and pestle in 0.05 M sodium phosphate buffer (pH 7.8) at the end of each three-day interval of Al treatment. The homogenate was centrifuged at 10,000 rpm for 20 min and the supernatant was used for SOD, POD and CAT determinations. These steps were carried out at 4°C (Guo et al., 2004; Meng et al., 2007).

SOD activity was estimated according to the method of Zhang et al. (2005), with our modifications. The reaction mixture consisted of 54 mL methionine, 2 mL nitroblue tetrazolium chloride (NBT), 2 mL EDTA-Na_2 and 2 mL riboflavin. An appropriate quantity of enzyme extract was added to the reaction mixture. The reaction was initiated by placing the tubes below two 15 W fluorescent lamps for 15 min, and was stopped by keeping the tubes in the dark for 10 min. Absorbance was recorded at 560 nm. One unit of SOD enzyme activity was defined as the quantity of SOD enzyme required to produce 50% inhibition of NBT reduction under the experimental conditions, and specific enzyme activity was expressed as units per g fresh weight.

The reaction mixture in a total volume of 50 mL 0.1 M sodium phosphate buffer (pH 6.0) containing 19 μL H_2O_2 (30%) and 28 μL guaiacol was prepared immediately before use. Then 1 mL enzyme extract was added to 3 mL reaction mixture. The increase in absorbance was measured at 470 nm at 0.5 min intervals up to 2 min using a UV-Vis spectrophotometer (UV-2550, Shimadzu Japan). Enzyme specific activity is defined as units (one peroxidase activity unit defined as number of 0.1 changes in absorbance at 470 nm per minute) per g fresh weight.

CAT activity was assayed according to the method of Zhang et al. (2005). CAT activity was determined with a UV-Vis spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) at 25°C in 2.7 mL reaction mixture containing 1.5 mL 0.05 M sodium phosphate buffer (pH 7.8), 1.0 mL deionized water and 0.3 mL 0.1 M H_2O_2 prepared immediately before use, and then 0.2 mL enzyme extract was added. CAT activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. Activity was expressed as units (one catalase activity unit defined as number of 0.1 changes in absorbance at 240 nm per minute) per g fresh weight.

Lipid peroxidation level was expressed as malondialdehyde (MDA) content according to Zhang et al (2005). Fresh samples from each treatment were homogenized in 5 mL 10% trichloroacetic acid (TCA) with mortar and pestle. Homogenates were centrifuged at 4000 × g for 20 min. To each 2 mL aliquot of supernatant, 2 mL 0.6% thiobarbituric acid (TBA) in 10% TCA was added. The mixtures were heated in water for 15 min and then quickly cooled in an ice bath. After centrifugation at 4000 × g for 10 min, the absorbance of the supernatant was recorded at 532 nm and 450 nm. Lipid peroxidation was expressed as MDA content in nM per g fresh weight.

CYTOLOGICAL STUDY

The seeds were soaked in distilled water and allowed to germinate at 25°C, producing roots reaching ~2 cm in length. After that they were treated in Petri dishes with different concentrations of Al in solution (10 μM, 50 μM and 100 μM) for 24 h, 48 h and 72 h. Distilled water was used for the control. The test liquids were changed regularly every 24 h. Ten root tips in each treatment group were cut and fixed in 95% ethanol:acetic acid 3:2 for 4 h and hydrolyzed in 1 M hydrochloric acid: 95% ethanol:99.8% glacial acetic acid (5:3:2) for 4–5 min at 60°C. For observation of changes in the nucleolus, 10 root tips were squashed in 45% acetic acid, dried, and two days later stained with silver nitrate (Liu and Jiang, 1991).

STATISTICAL ANALYSIS

Each treatment was made in three replicates. For statistical analysis, one-way ANOVA (Sigma Plot 13.0 software) and the t-test were used to determine significance at $p < 0.05$.

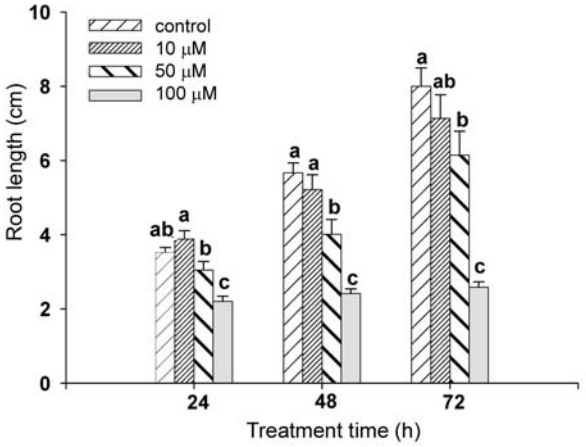

Fig. 1. Effects of Al concentration on root growth in *Vicia faba* L. Values with different letters differ significantly ($p < 0.05$, t-test).

RESULTS

The effects of Al on root growth of *Vicia faba* varied with the Al concentration and treatment time. There was significant inhibition of root growth in seedlings treated with 50 μM and 100 μM Al (Fig. 1) during the whole course of treatment. Versus the control there was hardly any root growth at 100 μM Al. At 10 μM Al there was no toxic effect.

Generally, the diploid nucleus of *V. faba* contains 1 or 2 nucleoli (Fig. 2a). The effects of Al on nucleoli varied with the Al concentration and treatment time. At 10 μM Al, from 24 h to 48 h, small particulates containing argyrophilic proteins were extruded from the nucleus into the cytoplasm (Fig. 2b). At 24 h some particulates were scattered in the cytoplasm in both the short rounded meristematic cells (Fig. 2c,d) and the long oblong root cap cells (Fig. 2e,f) after treatment with a higher concentration of Al (e.g., 50 μM Al). The nucleolar material in the cytoplasm progressively increased with increasing Al concentration (100 μM Al 24 h) and duration of treatment (Fig. 2g). At 48 h that silver-stained nucleolar material surrounded the nucleolus (Fig. 2h) and at 72 h it occurred throughout the cytoplasm (Fig. 2i).

The effects of Al on SOD activity in *V. faba* roots and leaves varied with the Al concentration. SOD activity was high in leaves exposed to 10 μM to 100 μM Al during the whole course of treatment, except for 10 μM Al on day 6 when it did not differ from the control (Fig. 3a). Versus the control, SOD activity in roots exposed to 50 μM and 100 μM Al increased significantly throughout the duration of stress exposure except for day 9 in the 50 μM Al treatment (Fig. 3b).

POD activity in leaves treated with different concentrations of Al showed no obvious changes during the course of the experiment (Fig. 3c). As compared with seedlings in the 10 μM Al treatment and the control, roots of seedlings exposed to 100 μM Al showed a significant increase of POD activity during the whole experiment (Fig. 3d). Both the controls and Al treatments showed much higher POD activity in roots than in shoots.

CAT activity in presented in Figure 3e,f. In leaves of seedlings exposed to 100 μM Al it was significantly higher during the whole course of treatment than in the 10 μM Al treatment and the control (Fig. 3e). In roots the same trend of CAT activity was found in all Al treatments (Fig. 3f). Both the controls and Al treatments showed higher CAT activity in shoots than in roots.

The effects of Al on MDA concentration are presented in Figure 4. MDA content in leaves of seedlings exposed to 10 μM Al showed no obvious changes versus the control (Fig. 4a). MDA content in leaves from the 50 μM and 100 μM Al treatment was significantly higher at days 6 and 9 than in leaves from the 10 μM Al treatment and the control (Fig. 4a). Roots from seedlings exposed to 50 μM and 100 μM Al had significantly higher MDA content than roots from the 10 μM Al treatment and the control through the whole course of treatment (Fig. 4b).

DISCUSSION

Our experiments demonstrated significant root growth inhibition in *Vicia faba* seedlings exposed to 50 μM and 100 μM Al.

The silver staining technique has been widely applied in cytological studies aimed at understanding the nucleolar cycle and nucleolar organization in both animals and plants. Nucleolar organizer regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods (Trerè, 2000). In this work, Al induced the fragmentation of particles of argyrophilic proteins, which were scattered in the nuclei, and the material was extruded from the nucleus into the cytoplasm. These effects of Al on nucleoli are more or less the same as those reported by Liu and Jiang (1991). Nucleolin is one of the main proteins in the nucleolus, and oxidative stress can induce cleavage of it (Wang et al., 2004). Al can cause a burst of reactive oxygen species. Some particles of similar silver-stained material scattered in the nuclei may have been caused by oxidative stress. The nuclear pore complex (NPC) is the most important channel for nuclear material, which is made up of many proteins (Aa et al., 2006). Due to the highly selective manner of its operation, this channel maintains the order and function of the cell

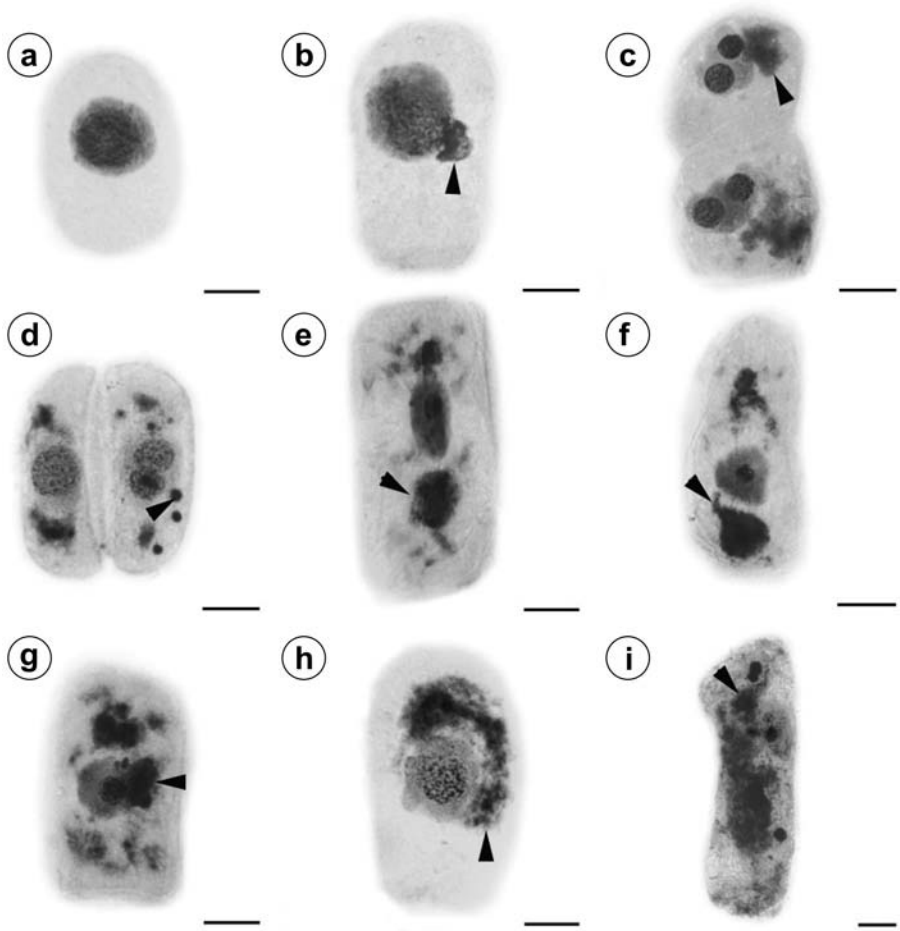

Fig. 2. Effects of Al on nucleoli in root tip cells of *Vicia faba* L. **(a)** Control cell, **(b)** Small amount of nucleolar materials extruded from nucleus into cytoplasm (10 μM Al, 24 h), **(c-f)** Some of the same silver-stained particulate materials scattered in the cytoplasm in both the short rounded meristematic cells and the long oblong root cap cells: **(c,d)** Short rounded meristematic cells (50 μM Al, 24 h), **(e,f)** Long oblong root cap cells (50 μM Al, 48 h), **(g)** Large amount of nucleolar material in cytoplasm (100 μM Al 24 h), **(h)** The same silver-stained nucleolar material surrounding the nucleolus (100 μM Al 48 h), **(i)** Nucleolar material spread thorough nearly all of the cytoplasm (100 μM Al 72 h). Arrowheads show nucleolar material extruded from nucleus into cytoplasm. Bar = 10 μm.

(Limón-Pacheco and Gonsebatt, 2008). We suggest that the nucleolar material extruded from the nucleus into the cytoplasm may be correlated with protein damage, causing the NPC to lose selectivity. The mechanism needs to be studied further. Our results indicated that the strongly toxic effect of Al on the nucleolus often inhibited or stopped mitosis. The evidence from the present work shows that once a large amount of nucleolar material was extruded from the nucleus into the cytoplasm, root growth in *V. faba* was markedly inhibited.

Plants, like other aerobic organisms, need oxygen for efficient production of energy. It has even been suggested that Al-enhanced oxidative stress is a decisive event for inhibition of cell growth by Al (Yamamoto et al., 2002). The presence of oxygen in the cell environment can cause continuous oxidative damage to cell structure and function (Choudhury and Panda, 2005). Aluminum toxicity can cause excessive ROS production, including superoxide radical ($O_2^{\cdot -}$), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) (Guo et al., 2006). To scavenge ROS and avoid oxidative damage, cells are able to protect themselves by engaging enzymatic and nonenzymatic mechanisms, including SOD, CAT, POD or glutathione (Apel and Hirt, 2004; Dazy et al., 2009), which may form important defense systems.

Superoxide dismutase is a crucial component of plants' antioxidative defense systems (Dazy et al., 2009; Liu et al., 2009). Increased SOD activity suggests elevated content of superoxide radical, especially at concentrations at which root growth is strongly inhibited and plasma membrane integrity lost (Šimonovicová et al., 2004). Al has been shown

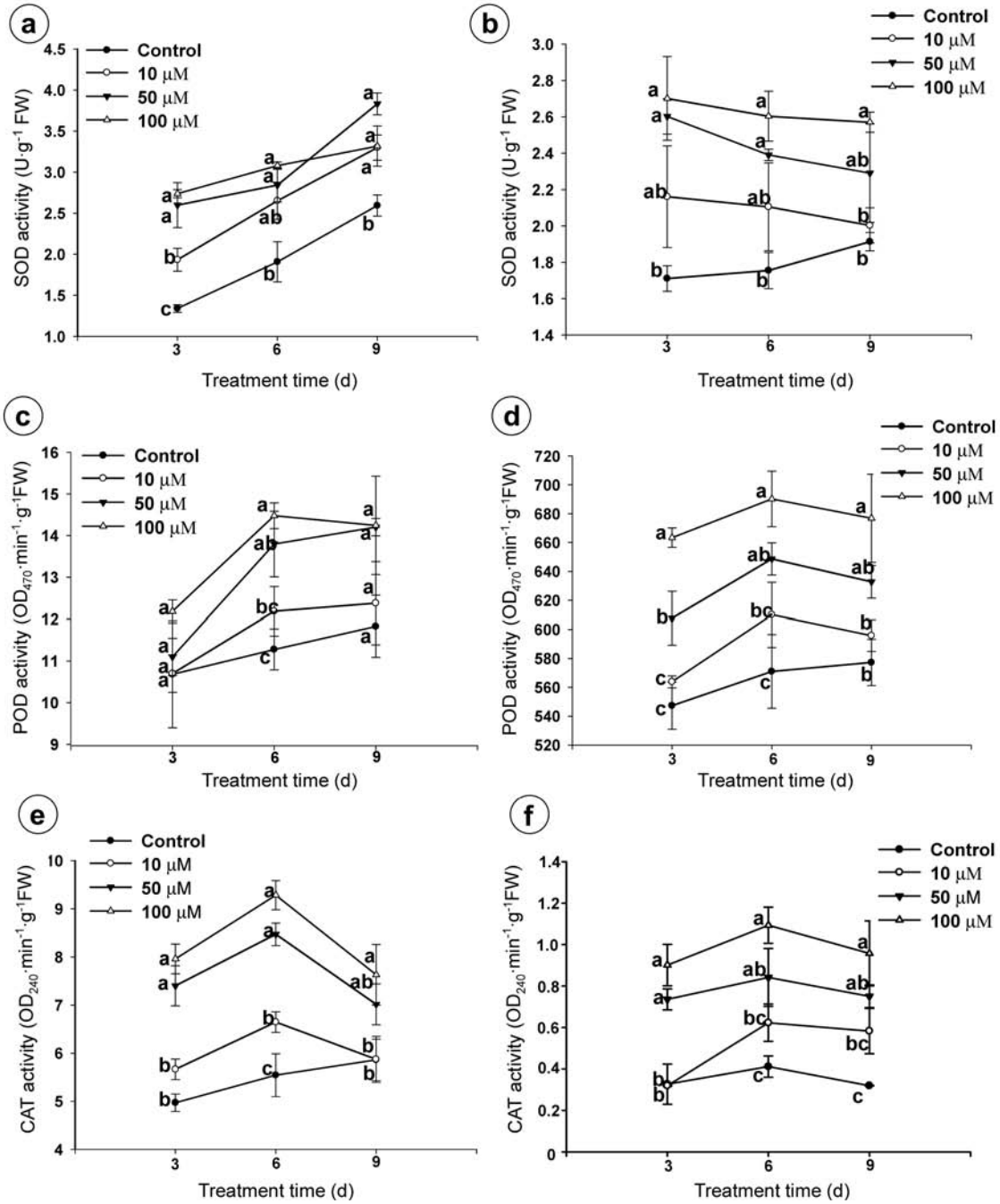

Fig. 3. Effects of Al concentration on the activity of three antioxidant enzymes in *Vicia faba* L. **(a)** SOD in leaves, showing high activity at 10 μM to 100 μM Al except for 10 μM Al on day 6, **(b)** SOD in roots, showing high activity at 50 μM and 100 μM Al except for 50 μM Al on day 9, **(c)** POD in leaves, showing no significant changes, **(d)** POD in roots, showing significantly higher activity in 100 μM Al treatment than in the 10 μM Al treatment and the control, **(e)** CAT in leaves, showing higher activity in the 100 μM Al treatment than in the 10 μM Al treatment and the control, **(f)** CAT in roots, showing the same trend as in leaves. Values with different letters differ significantly ($p < 0.05$, t-test).

to enhance SOD activity in soybean (Cakmak and Horst, 1991), *Arabidopsis* (Richards et al., 1998), sorghum (Peixoto et al., 1999) and barley (Guo et al., 2004). In this investigation, SOD activity in leaves and roots exposed to different concentrations of Al were generally higher than in the control, suggesting that enhanced SOD activity may signal oxidative stress, which triggers induction of antioxidant

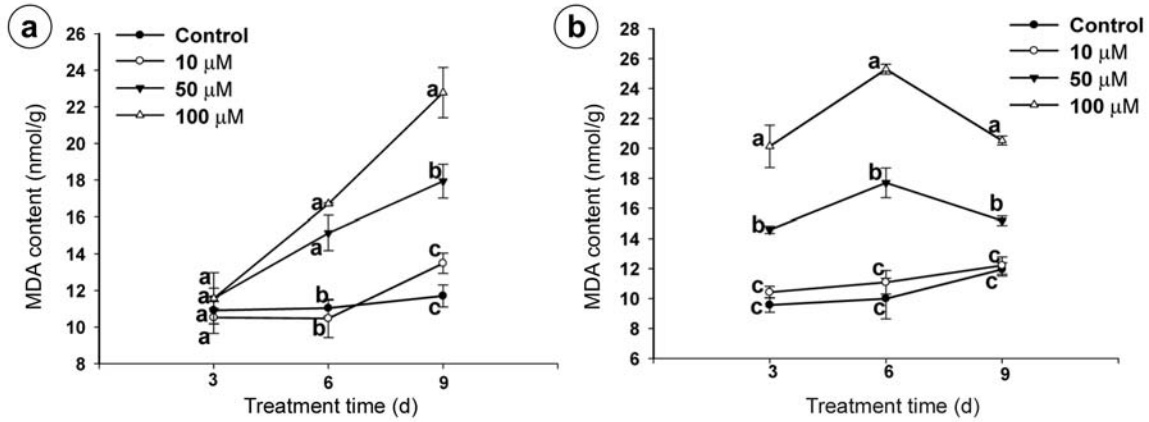

Fig. 4. Effects of Al concentration on lipid peroxidation in *Vicia faba* L. **(a)** MDA concentrations in leaves, showing higher content at days 6 and 9 in leaves exposed to 50 μM and 100 μM Al than in leaves exposed to 10 μM Al and in the control, **(b)** MDA concentrations in roots, showing higher MDA content throughout the duration of stress exposure in the 50 μM and 100 μM Al treatments than in the 10 μM Al treatment and the control. Values with different letters differ significantly ($p < 0.05$, t-test).

enzymes associated with an H_2O_2 scavenging system, particularly the ascorbate-glutathione cycle (Lee et al., 2001). Our results indicate that SOD activity in roots exposed to Al decreased with the duration of treatment. Fatima and Ahmad (2005) reported that SOD initially increased as a result of the formation of ROS (e.g., superoxide radical) under heavy metal exposure. Stroiński and Kozlowska (1997) found that SOD activity decreased due to the binding of metal ions to the active center of the enzyme at higher heavy metal concentration.

Peroxidase is also an important enzyme, able to scavenge H_2O_2, a major substance degraded by SOD. The enhanced activity of anionic POD could act to confer Al resistance by detoxifying ROS and restricting lipid peroxidation in membrane regions (Ezaki et al., 2000). In our work, roots of seedlings exposed to 100 μM Al showed significantly increased POD activity versus the control; this enhanced POD activity in *V. faba* apparently reflects its great capacity to acclimate to Al stress by rapidly engaging an antioxidative defense system. POD activity increased much more in roots than in leaves; probably the glutathione/ascorbate cycle was operating at a high rate in order to detoxify the ROS formed in the roots. Jan et al. (2001) found that Al induced POD activity in an Al-sensitive rice cultivar, whereas in an Al-tolerant cultivar it was unaffected by Al treatment, and suggested that the increased POD activity was part of a damage response to Al.

Catalase is the most universal oxidoreductase, which scavenges H_2O_2 to O_2 and H_2O. The major function of CAT is to metabolize the peroxide liberated in the peroxisome following the conversion of glycolate during photorespiration (Qureshi et al., 2007). CAT activity was lower in roots than in leaves in our experiment. Low CAT activity in roots exposed to 10 to 100 μM Al suggests that it may be a general response to the stress, apparently due to the inhibition of enzyme synthesis or to a change in the assembly of enzyme subunits (MacRae and Ferguson, 1985).

Malondialdehyde is an oxidized product of membrane lipids, and its level can show the extent of oxidative stress (Guo et al., 2004). MDA content in leaves and roots of *V. faba* seedlings exposed to 50 μM and 100 μM Al was significantly higher than in the other treatments and the control at 6 to 9 days of treatment, indicating that Al indirectly produced superoxide radicals, resulting in increased lipid peroxidative products and oxidative stress. However, there was no noticeable difference in MDA concentration between the low-dose (10 μM Al) treatment and the control. The relatively low MDA concentration suggests less oxidative stress, which may account for less inhibition of growth. The decline of MDA at day 9 in roots may reflect adaptation to stress.

In view of our findings, we suggest that the alterations in nucleoli and the changes in antioxidant enzyme levels and MDA content in *V. faba* can serve as useful biomarkers of Al toxicity. Data from these biomarkers can provide valuable information for monitoring and forecasting early effects of Al exposure in real conditions. Our laboratory results for short exposure suggest that soil enzymatic activity, microbial biomass, broad bean physiological indices and broad bean biomass might be sensitive indicators of environmental stress in a soil-aluminum-broad bean system. More research is needed to assess the long-term risks of Al contamination under field conditions.

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