Turk J Bot
32 (2008) 345-352

Research Article

The Effect of Aluminium on Oxidative Stress-Related Enzyme Activity in Two Clover Species

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Received: 04.01.2008
Accepted: 18.07.2008

Abstract: The impact of aluminium (Al) stress on the production of hydrogen peroxide and on the activity of antioxidant enzymes involved in oxidative metabolism, such as ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT), were studied in seedlings of 2 clover species (*Trifolium alexandrinum* L. and *Medicago sativa* L.). A uniform decrease in germination and root elongation were detected as the primary signs of Al injury. A significant increase in hydrogen peroxide content was also related to an increase in Al uptake by the roots of the 2 clover species, especially at relatively highly toxic Al concentrations (2-6 mM) for 7 days. APX-, POD-, CAT-, and H_2O_2-consuming enzymes were activated and followed similar patterns of expression in each species, exhibiting a relationship between their elevated activity and inhibition of root growth. Furthermore, it was shown that clover roots of the Al-tolerant (*M. sativa*) species accumulated more Al than the Al-sensitive (*T. alexandrinum*) cultivar during long-term Al treatment. Proline content increased more in *M. sativa* than in *T. alexandrinum*, especially at relatively highly toxic Al levels after 7 days of exposure.

Key Words: Aluminium, Egyptian clover (berseem), antioxidant enzymes, root growth, Al uptake, proline

Introduction

Due to the rapid increase of acid soil, Al toxicity is a major agricultural problem in several countries across the globe. Industrial pollution is a major concern near the aluminium factory in Ngha Hammadi, Upper Egypt. Pollutants discharged from this factory accumulate in soil and are available for uptake by plants. The inhibition of root elongation is a general and very sensitive response by numerous plant species to the presence of soluble forms of Al in acid soils (pH < 5.0). Al is reported to alter plasma membrane properties by enhancing the peroxidation of phospholipids and proteins (Cakmak & Horst, 1991), alter the cation-exchange capacity of cell walls (Horst, 1995), interfere with signal transduction (Jones & Kochian, 1995), and bind directly to DNA or RNA, etc. Indeed, the accumulation of proline may be part of a general adaptation to several stresses, including exposure to Al. Proline stabilises cellular structure as well as scavenges free radicals (Hare & Cress, 1997). A common feature of several stresses (including Al toxicity) is enhanced production of active oxygen species (AOS), which are generally considered harmful to plant cells (Richards et al., 1998; Tamas et al., 2004); however, it has been recently recognised that hydrogen peroxide plays a central role in several physiological processes, such as defence reaction, stomatal closure, programmed cell death, peroxisome biogenesis, cell wall cross-linking, and lignin synthesis (Van Breusegem et al., 2001; Neill et al., 2002). More recently, hydrogen peroxide was also identified as an expression of the signal molecule of several genes in plants (Desikan et al., 2000). Since some of these Al-induced genes are encoded for antioxidant enzymes (ascorbate peroxidase, peroxidase, and catalase), it has been suggested that there is a strong connection between Al stress and oxidative stress in plants (Cakmak & Horst, 1991; Richards et al. 1998; Simonovicova et al., 2004). The key role of antioxidant enzymes is to reduce or scavenge reactive oxygen species, such as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals. The resulting hydrogen peroxide is removed

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through the activity of the Asada-Halliwell scavenging cycle in chloroplasts and in cytoplasm by ascorbate peroxidase (APX) (Asada, 1992). Peroxidases (POD) participate in lignin biosynthesis, cell wall cross-linkage, IAA degradation, and disease resistance, and convert hydrogen peroxide to water (Asada, 1992; Siegel, 1993).

The objective of this study was to assess the accumulation of Al in the emerged roots of 2 clover species that exhibit differential sensitively to Al and to determine which was more tolerant of Al toxicity. Additionally, we investigated the role of antioxidant enzymes, including APX, POD, and CAT, and hydrogen peroxide-consuming enzymes as possible mechanisms for Al stress adaptation in clover, which is known as an Al-tolerant species capable of surviving in aluminium-polluted environments.

Materials and Methods

Plant Materials and Stress Conditions

Seeds of 2 clover (*Trifolium alexandrinum* L., *Medicago sativa* L.) species were chosen in this study due to their tolerance to aluminium toxicity. These seeds were sterilised with 5% sodium hypochlorite for 10 min and then rinsed 3 times with distilled water. The seeds were then germinated on filter paper fully moistened (15 ml) with solutions containing 0.2 mM $CaCl_2$ (pH 4.0) [control (no Al)], or in 0.2 mM $CaCl_2$ containing 0.5, 1, 2, 4, and 6 mM $AlCl_3$ solution (pH 4.0) [Al-treated]. The imbibed seeds were incubated at 25 °C in darkness for 3 and 7 days. After 3 days the percentage of germination was calculated, and after 3 and 7 days root length of the seedlings was measured. Then the seedlings were used for further analysis. Each experiment was repeated 3 times with 3 replicates.

Aluminium Uptake

Haematoxylin staining was used for the determination of Al uptake (Ownby, 1993). Freshly harvested roots were washed with distilled water for 15 min and then stained with a 0.2% haematoxylin and 0.02% KIO_3 solution for 15 min at room temperature. After washing with distilled water for 15 min, 15 root tips (1.5 cm) were excised and soaked in 3 ml of 1 M HCl for 1 h. The optical density of the released stain was measured at 490 nm.

Determination of H_2O_2 Content

Hydrogen peroxide content was determined according to Velikova et al. (2000). Seedlings [emerged shoot and root from seed (FW: 500 mg)] were homogenised in an ice bath with 5 ml of 0.1% (W/V) trichloroacetic acid. The homogenate was centrifuged at 12,000 × g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of KI. The absorbance of the supernatant was measured at 390 nm. The content of hydrogen peroxide was expressed as mM g FW^{-1}.

Extraction of Protein

Total protein was extracted by the method of Anderson et al. (1995). Seedlings (FW: 1 g) were homogenised in 1 ml of 0.1 M potassium phosphate (pH 7.8) containing 1 mM EDTA. For analysis of ascorbate peroxidase, 50 mg of frozen seedling was homogenised with 500 µl of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM ascorbate (ASA) and 1 mM EDTA. The homogenate was mixed well in a pre-cooled mortar and pestle. The homogenate was centrifuged at 12,000 × g for 15 min at 4 °C. Total soluble protein content was determined by Lowry's method (Lowry et al., 1951), using bovine serum albumin as the standard.

Enzymatic Activity Assays

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano & Asada (1987) using UV-Vis spectrophotometry. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM hydrogen peroxide (H_2O_2), and 0.5 mM ASA and enzyme extract, in a total volume of 3 ml. The reaction was started by adding H_2O_2 and was conducted at 22 °C. The oxidation rate of ASA was determined by the decrease in absorbance at 290 nm for 3 min (40-s lag period), using the extinction coefficient of 2.8 mM cm^{-1}.

Peroxidase (POD; EC 1.11.1.7) activity was determined using the method of Srinivas et al. (1999), following the formation of tetraguaiacol as measured by the absorbance at 470 nm and using an extinction coefficient of 26.6 mM cm^{-1} to calculate the amount of tetraguaiacol. The 3-ml reaction mixture contained 20 mM phosphate buffer (pH 6.0), 5 mM 2-methoxy phenol (guaiacol), and 1 mM hydrogen peroxide (with an

appropriate aliquot of enzyme extract, and the reaction was carried out for 3 min. One unit of peroxidase activity represents the amount of enzyme that catalyses the oxidation of 1 µmol of guaiacol in 1 min.

Catalase (CAT; EC 1.11.1.6) activity was assayed by measuring the initial rate of the disappearance of hydrogen peroxide using the method of Velikova et al. (2000). Three millilitres of catalase assay reaction mixture contained 10 mM potassium buffer (pH 7.0), with an appropriate aliquot (100 µl) of enzyme extract, and 33 mM hydrogen peroxide. The decrease in hydrogen peroxide was followed as a decline in optical density at 240 nm, and the activity was calculated using the extinction coefficient of 40 mM cm^{-1} of hydrogen peroxide.

Determination of Proline Content

Proline was extracted according to the method of Bates (1973). Seedlings (FW: 0.5 g) were homogenised in 10 ml of 3% aqueous sulfosalicylic acid. After a 3-h extraction period the homogenate was centrifuged at 1500 × g for 10 min. The proline extract was treated with 2 ml of freshly prepared acidic ninhydrin and 2 ml of glacial acetic acid in the test tube for 1 h at 100 °C. The reaction was terminated in an ice bath and the mixture was extracted with 4 ml of toluene. The chromophore-containing toluene absorbance was photometrically determined at 520 nm using toluene for a blank. Acidic ninhydrin was prepared by warming 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid, with agitation until dissolved. At 4 °C, the reagent remained stable for 24 h. Using the extinction value we determined the proline concentration from the standard curve (Bates, 1973), on a fresh weight basis. Three independent measures per regimen were carried out, and each concentration group was assayed in triplicate.

Statistical Analysis

The present study compared data of Al treated and untreated (control) plants using the least significant difference (LSD) test at 5% (*) and 1% (**) levels (Snedecor & Cochran, 1980). The data were analysed using 2-way ANOVA. Subsequent comparison between the 2 clover species and between the 2 (3 and 7 days) periods was made with the STATISTICA computer software program (Stat Soft, Inc).

Results

Al Uptake and Root Growth Inhibition

One of the very early symptoms of Al toxicity was inhibition of germination and root length, which were accompanied by an increase in Al uptake by root tips at higher Al concentrations in both species we tested. After 3 days of Al treatment, germination (Figure 1) decreased proportionally to Al concentration, and at the 6-mM concentration, for both cultivars it was only 60% of that of the control. Moreover, root length significantly decreased as Al concentration (0.5-6 mM) increased in both of the clover species (Figure 2). After 7 days of Al treatment root length of the 2 clover species was significantly inhibited and at the 6-mM concentration root length of *T. alexandrinum* and *M. sativa* seedlings was 2.9% and 8.3% that of the controls (Figure 2). Figure 3 shows that increasing the Al concentration reduced FW of seedlings. This was more significant (ANOVA $P < 0.001$) in *T. alexandrinum* than in *M. sativa*, especially at the 0.5-mM and 1-mM Al concentrations after 7 days of treatment. Al uptake continuously rose as the concentration of Al increased (Figure 4). This increase was more significant (ANOVA $P < 0.001$) in *M. sativa* than in *T. alexandrinum*. At the 6-mM $AlCl_3$ concentration a large increase in Al uptake by the root tips was also detected, which was generally more pronounced in *M. sativa* than in *T. alexandrinum* (Figure 4). These results indicate a positive correlation between Al concentration, and Al uptake, seedling fresh weight, and root length.

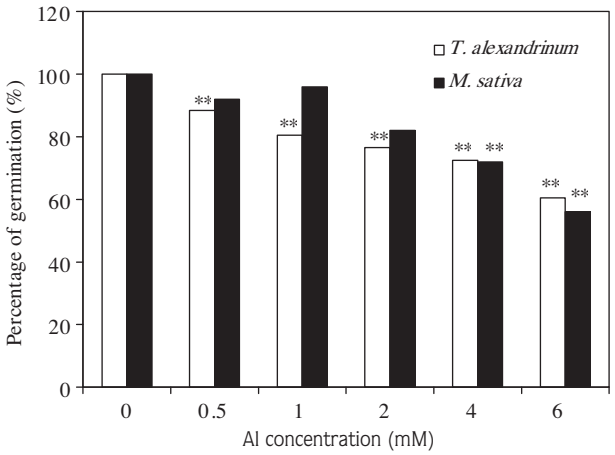

Figure 1. Percentage of germination of the seeds of 2 clover species germinated on filter paper moistened with different concentrations of Al for 3 days.
*Significant at P = 0.05. **Significant at P = 0.01.

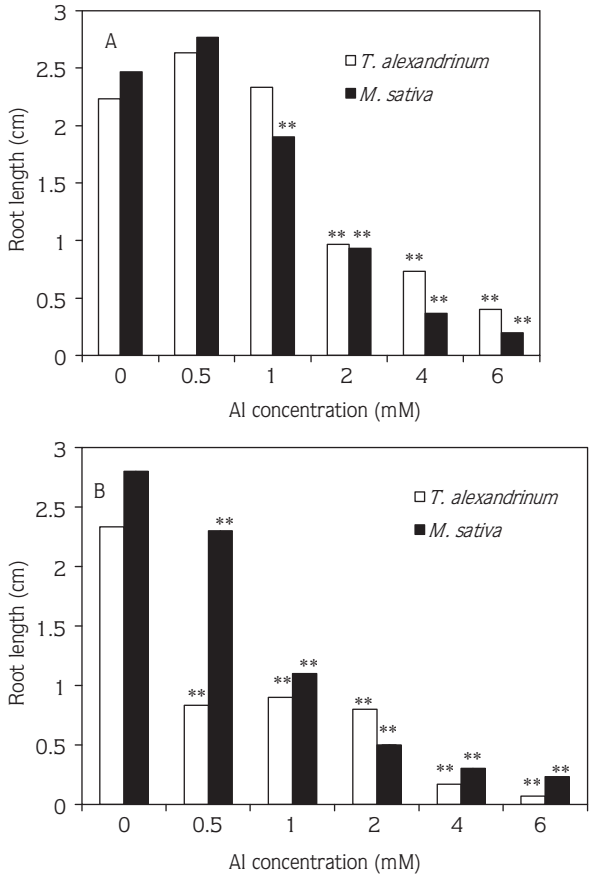

Figure 2. Inhibition of root growth of 2 clover species exposed to different concentrations of Al for 3 (A) and 7 (B) days. *Significant at P = 0.05. **Significant at P = 0.01.

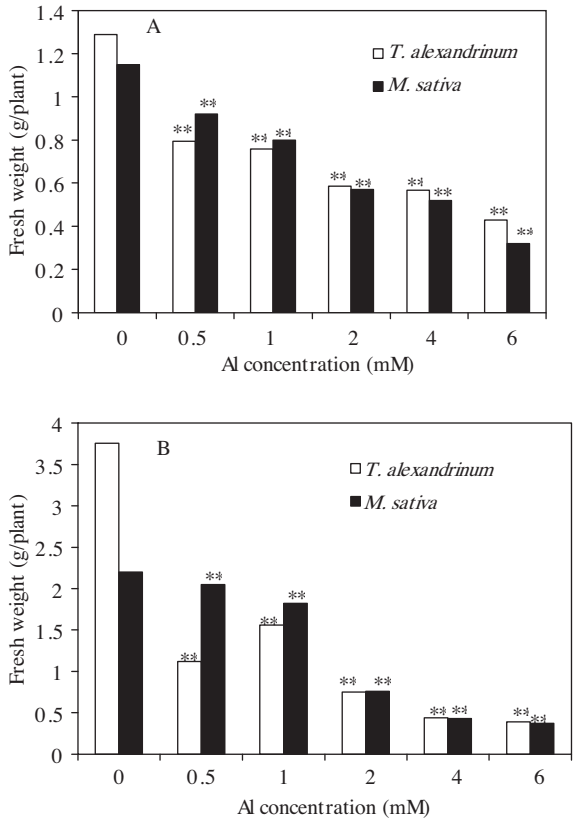

Figure 3. Fresh weight of clover seedlings exposed to Al concentrations for 3 (A) and 7 (B) days. *Significant at P = 0.05. **Significant at P = 0.01.

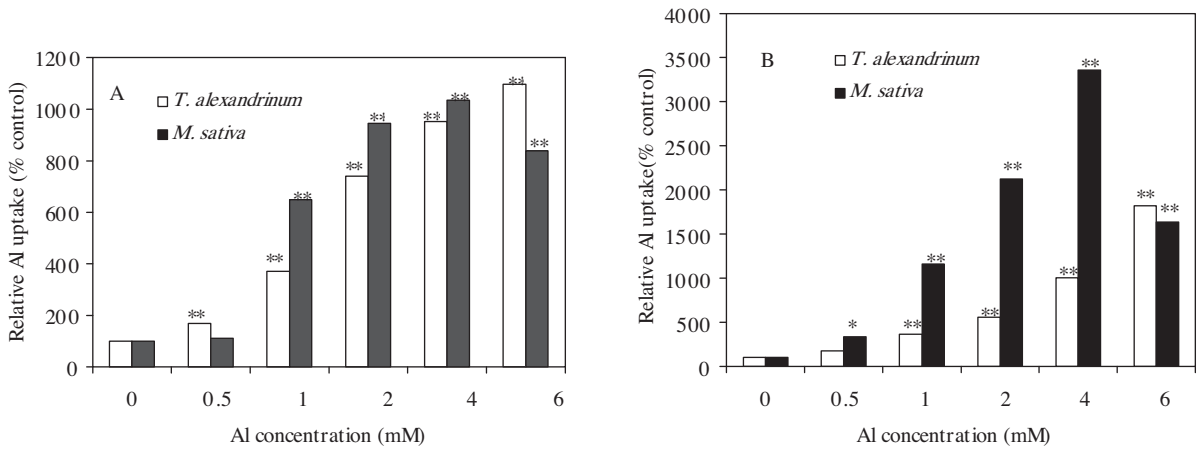

Figure 4. Al uptake by the root tips of 2 clover species (15 tips) exposed to different concentrations of Al for 3 and 7 days. *Significant at P = 0.05. **Significant at P = 0.01.

H_2O_2 Production

After 3 days of Al treatment production of H_2O_2 increased as Al concentration increased, reaching a maximum at 6 mM Al (10-fold that of the control seedlings) in both clover species (Table 1). This increase was more significant (ANOVA $P < 0.001$) in *T. alexandrinum* than in *M. sativa*, at each Al concentration. Al induced significantly (ANOVA $P < 0.001$) greater H_2O_2 production after 7 days than after 3 days at high Al (4 mM and 6 mM) concentrations. Generally, after 7 days H_2O_2 content increased significantly more (ANOVA $P < 0.05$) in *M. sativa* than in *T. alexandrinum* at high Al (4 mM and 6 mM) concentrations.

Enzyme Activity

To examine the activation of 3 oxidative stress-related enzymes, the same Al concentration range was applied, and the Al dose dependency of enzyme expression was evaluated after 3 and 7 days in both clover species. APX and POD activity of both species increased significantly (ANOVA $P < 0.001$) at high toxic Al levels (2, 4, and 6 mM) after 3 days of Al exposure (Table 2); however, CAT activity was significantly greater (ANOVA $P < 0.05$) in *T. alexandrinum* than in *M. sativa* at the same Al levels after 3 days of Al treatment. After 7 days of Al treatment significant activation of other oxidative stress-related enzymes could also be detected. This activation was more significant (ANOVA $P < 0.001$) after 7 days of germination than after 3 days. APX, POD, and CAT activity increased more in *M. sativa* than in *T. alexandrinum* at different Al concentrations. Protective mechanisms against Al stress seem to be conferred by the activation of APX, POD, and CAT.

Table 1. Hydrogen peroxide content of 2 clover species exposed to different Al concentrations for 3 and 7 days.
*Significant at P = 0.05. **Significant at P = 0.01.

Species	Treatment Al (mM)	3 days	7 days
		H_2O_2 (mM/g of FW)	H_2O_2 (mM/g of FW)
T. alexandrinum	0	0.17	0.12
	0.5	0.15	0.14
	1.0	0.24*	0.22
	2	0.56**	0.38
	4	1.34**	1.83**
	6	2.14**	4.14**
LSD at 5%		0.08	0.66
LSD at 1%		0.12	0.93
M. sativa	0	0.12	0.19
	0.5	0.16	0.23
	1.0	0.20*	0.20
	2	0.62**	0.76
	4	0.83**	2.12**
	6	1.01**	5.90**
LSD at 5%		0.07	0.90
LSD at 1%		0.10	1.28

Total Soluble Proteins and Proline Content

To investigate the influence of Al concentration on total soluble proteins and proline accumulation (Table 3), after 3 and 7 days of Al treatment, total soluble protein content and proline accumulation of the 2 clover seedlings were measured. We observed that both parameters increased as Al concentration increased, reaching the highest level at the 6-mM Al concentration (Table 3). This increase was more significant (ANOVA $P < 0.001$) in *M. sativa* than in *T. alexandrinum*, especially at high Al levels (4 mM and 6 mM). Furthermore, both clover cultivars contained significantly higher (ANOVA $P < 0.001$) total soluble protein and proline levels after 7 days than after 3 days of Al exposure (Table 3).

Discussion

The effect of Al toxicity on germination has not been adequately investigated. It is known that germination and subsequent emerged young roots are very sensitive to Al (Figures 1-3). After 7 days Al treatment resulted in retarded germination and root length inhibition in both clover species, which was concentration dependent (corresponding values declined from 100% to 30% of the control, according to Al concentration). Highly toxic Al concentrations (> 1 mM) were required to inhibit the growth of the emerging roots of germinating wheat seeds (Delima & Copeland, 1990). In the case of the emerging young roots of the 2 clover species in the present study, more Al accumulated in the root tips of *M. sativa* than in those of *T. alexandrinum* (Figure 4). Tamas et al. (2004) reported that emerging young barley roots are probably more capable of reducing the entry of Al into cells than

Table 2. APX, POD, and CAT activity of the seedlings of 2 clover species exposed to different Al concentrations for 3 and 7 days. *Significant at P = 0.05. **Significant at P = 0.01.

Species	Treatment Al (mM)	3 days			7 days		
		APX (mM/g of FW)	POD (mM/g of FW)	CAT (mM/g of FW)	APX (mM/g of FW)	POD (mM/g of FW)	CAT (mM/g of FW)
T. alexandrinum	0	6.28	2.54	0.75	8.87	5.64	0.83
	0.5	11.53**	3.80	0.95*	15.28**	12.78	0.89
	1.0	17.76**	6.84**	1.04**	15.84**	14.59**	1.27**
	2	15.45**	9.11**	1.24**	16.08**	16.35*	1.30**
	4	24.34**	15.50**	1.47**	18.17**	90.14**	1.39**
	6	28.00**	12.83**	1.97**	22.46**	134.20**	2.33**
LSD at 5%		0.83	1.751	0.153	1.02	7.930	0.127
LSD at 1%		1.19	2.488	0.217	1.45	11.270	0.181
M. sativa	0	13.34	22.77	0.89	15.03	27.44	0.88
	0.5	15.56**	58.04	0.84	18.33**	31.95	1.04*
	1.0	18.30**	36.23	0.72*	18.27**	40.97	1.26*
	2	16.70**	117.32**	0.69**	19.02**	71.42	1.46*
	4	30.49**	162.66**	1.20**	40.65**	260.65**	2.23**
	6	42.29**	267.16**	2.19**	52.43	563.85**	5.97**
LSD at 5%		0.88	58.749	0.171	0.81	63.400	0.162
LSD at 1%		1.25	83.513	0.257	1.15	90.13	0.230

Table 3. Total soluble protein and proline contents of 2 clover species exposed to different Al levels for 3 and 7 days. *Significant at P = 0.05. **Significant at P = 0.01.

Species	Treatment Al (mM) (mM/g of FW)	3 days		7 days	
		T. soluble protein (mM/g of FW)	Proline (mM/g of FW)	T. soluble protein (mM/g of FW)	Proline
T. alexandrinum	0	0.14	1.71	0.13	0.62
	0.5	0.19*	2.04*	0.13*	1.17**
	1.0	0.21*	2.51**	0.14	1.27*
	2	0.24**	2.67**	0.13	1.32**
	4	0.46**	3.83**	0.23**	2.02**
	6	1.05**	6.98**	0.52**	1.84**
LSD at 5%		0.03	1.02	0.007	0.83
LSD at 1%		0.05	1.45	0.01	1.19
M. sativa	0	0.22	1.53	0.25	1.45
	0.5	0.39**	2.48**	0.26*	1.66
	1.0	0.42**	2.71**	0.25	1.89**
	2	0.59**	2.38**	0.40**	2.04**
	4	1.53**	5.56**	0.76**	2.52**
	6	1.42**	8.06	0.71**	4.06**
LSD at 5%		0.12	0.81	0.02	0.88
LSD at 1%		0.17	1.15	0.03	1.25

the roots of older seedlings and detoxify it into non-toxic forms or store it in apoplastic spaces. Vasquez et al. (1999) suggested that Al-tolerant maize restricted soluble forms of Al to the apoplast, where it is rapidly converted to non-toxic Al-phosphate forms. Only this Al-phosphate precipitate forms a complex with haematoxylin in intercellular spaces (Ownby, 1993). The increase in haematoxylin staining and root growth inhibition after 7 days of germination in our experiments might have been the consequence of increasing phosphate release from the damaged root tissues. Production of AOS during germination and root growth is generally accepted as a developmentally regulated physiological process in plants (Chen & Schopfer, 1999; Schopfer et al., 2001). AOS production increases during biotic and abiotic stress, (Bolwell & Wojtaszek, 1997; Lin & Kao, 2001). The increase in APX activity elevated Al-enhanced H_2O_2 production in root tissues during 2 sampling days (Table 1). It has been previously suggested that accumulation of H_2O_2 caused by various environmental stresses would result in enhanced APX and CAT activity in order to protect plant cells (Mizuno et al., 1988). Dramatic enhancement of APX activity was reported to occur in comparison to POD activity in barley roots exposed to Cd (Hegedus et al., 2001). In contrast, the results of the present study show a substantially greater increase in the activity of POD than that of APX and CAT at all Al concentrations (Table 2). The role of POD in Al stress response might consist of scavenging toxic lipid hydroperoxides generated by the peroxidation of membrane lipids, as suggested by Ezaki et al. (1996), who proposed that peroxidase isoenzymes expressed in the soluble fraction of Al-stressed tobacco cells might detoxify peroxides generated by Al that pass through the membrane into the cytosol. Hegedus et al. (2001) suggested an alternative role of POD isoenzymes—participation in lignin biosynthesis to build up the physical barrier against toxic metals entering cells. The role of peroxidases in the control of cell elongation has also been proposed (Largrimini et al., 1997; Souza & MacAdam, 2001). The results of the present study confirm the report by Lin & Kao (2001) of elevated POD activity and inhibition of root growth caused by Al stress, which was suggested to be associated with cell-wall stiffening catalysed by peroxidase activity in dimerisation of ferulic acid. Increased POD activity was also described in soybean roots treated with Al (Cakmak & Horst, 1991). Ezaki et al. (2000) suggested that enhanced activity of anionic POD could act in conferring Al resistance by detoxifying AOS and restricting lipid peroxidation in membrane regions. In contrast, Jan et al. (2001) proposed that increased POD activity is part of a damage response to Al, as they showed that Al-induced POD activity in an Al-sensitive rice cultivar was unaffected by Al treatment.

In the present study the pattern of change in catalase activity differed from that of ascorbate peroxidase and peroxidase activity (Table 2). These results are in agreement with Peixoto et al. (1999), who proposed that through a decrease in CAT activity, oxidative threat and an increase in POD and GR activity may play a role in H_2O_2 detoxification. In contrast, Benavides et al. (2000) reported that the enzymes responsible for hydrogen peroxide detoxification in *Solanum tuberosum* L. are ascorbate peroxidase and catalase. Nonetheless, they suggested that ascorbate peroxidase was likely to be more important than catalase in the detoxification of H_2O_2.

The possibility that Al is detoxified by the formation of total soluble protein (Table 3) has been suggested. Many authors showed the inducible synthesis of a cytosolic Al-binding protein (Basu et al., 1999; Wu et al., 2000). An Al-induced polypeptide (TA1-18) that shows homology to a pathogenesis related (PR) protein was identified in wheat (Cruz-Ortega & Ownby, 1993). Basu et al. (1999) observed differences in the amount of polypeptide exuded in response to Al stress between wheat cultivars, and showed enhanced accumulation of 12-23-, and 43.5-kDa polypeptides in the Al-resistant Maringa wheat cv. Thus, it is hypothesised that specific proteins may be involved in Al stress defence mechanisms.

Proline may be part of a general adaptation to adverse environmental conditions, having been shown to play a role in response to several stresses, including exposure to Al (Mossor-Pietraszewska, unpublished data) (Table 3). Proline might stabilise cellular structures as well as scavenge free radicals (Hare & Cress, 1997). It is concluded that aluminium might have stimulated the production of H_2O_2 during germination of the 2 clover species seeds in the present study. APX, POD, CAT, and H_2O_2-consuming enzymes were activated and each exhibited a relationship between their elevated activity and root growth inhibition; this growth inhibition was related to aluminium uptake by the root tips. These results support the hypothesis that Al-tolerant (*M. sativa*) species appear to have strategies to protect metabolic process against the toxic effects of Al.

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