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THE RELATION BETWEEN TANNIN, PHYTOCHELATINS, GLUTATHIONE AND PEROXIDATION IN TWO CULTIVARS OF SORGHUM (*SORGHUM BICOLOR*) EXPOSED TO ALUMINUM

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ABSTRACT

The objective of the present investigation was to study the role of tannin, glutathione and nonprotein thiols, and related enzymes in sorghum grown at aluminum (Al) stressed levels. Two sorghum cultivars 132 (high tannin) and 552 (low tannin) were selected. Plants were cultivated in four concentrations (0, 10, 20 and 30 mg l^{-1}) of $AlCl_3$ in a mixture of river sand and peat. The exposure to Al increased allocation to tannins, glutathione and nonprotein thiols (NP-SH), which are potential defensive compounds. In roots of both cultivars the production of NP-SH was coupled with a decrease in total glutathione. The concentration of glutathione in leaves of cultivar 132 tended to be lower, but in cultivar 552, it tended to be higher. Aluminium enhanced the level of lipid peroxidation in the leaves of cultivar 552 higher than in cultivar 132. Glutathione is a substrate for NP-SH synthase so that the depletion of glutathione could be ascribed to the Al-induced NP-SH synthesis in the roots of both cultivars. Although the synthesis of NP-SH, glutathione and lipid peroxidation could be sensitive to changes in tannins, the amount of glutathione, lipid peroxidation and NP-SH are useful for Al resistance. Studies have shown that phenylalanine-ammonia lyase (PAL) activity is stimulated by Al which is the key enzyme in the general phenylpropanoide pathway. The capacity of the two cultivars in antioxidant and NP-SH in new and old leaves was different; in cultivar 552, the antioxidant compounds such as glutathione with a rapid turnover and high cumulative could be cost effective than tannin for the leaves as tannin was low and stable. In cultivar 132, the amount of tannin was high; therefore high cumulative of glutathione in leaves is not necessary. The surface of roots was darker and seemed to be from Al-stimulated efflux of organic acids. The threshold concentration level of Al for acute toxicity in cultivar 132 was higher than cultivar 552 suggesting that tannins are normally involved in Al sequestration under conditions of subtoxic exposure. The localization of NP-SH in the roots could provide an effective means of restricting Al to this organ by chelations in cultivar 132 seedlings. Al treatment increased total tannin content mediated by enhanced activities of PAL. In cultivar 552 glutathione contents may have been consumed for two strategies: the

maintenance of regular redox potential, and the precursor for NP-SH. This was a predictable result because it is known that Al acts as a strong sink for thiols, which increase the demand for sulfate absorption. The transformation of glutathione in cultivar 552 was much parallel to the rate of synthesis. However in cultivar 132 the rate of transformation was higher than the rate of synthesis. Thus, the imbalance between MDA and PAL generation, and the scavenging of compound tannin in leaves may reflect the different defense strategy in the two cultivars of sorghum studied.

Keywords: tannin, nonprotein thiols, malondialdehyde, phenylalanine-ammonia lyase.

INTRODUCTION

Most strategies of plant tolerance to Al exposure are based on the reduction, by various mechanisms, of the cytosolic concentration of free Al. In this way, the plant cell avoids cumulative in the cytosol by compartmentalizing Al in subcellular compartments, although this distribution has not been clearly established yet (Delhaize et al. 1995). It is known for years that roots usually are the most Al sensitive organs and that Al causes fast inhibition of the elongation of the main axis (Juan et al. 2002). Extensive membrane damage, peroxidation of membrane lipids, and loss of cell compartmentation are relatively late occurring events in the Al induced root syndrome and cannot be made responsible for fast root growth inhibition (Kinraide et al. 1999).

Several investigations described an Al triggered-increase of organic acid anion release from root tips of Al tolerant plant. The citrate seems to be the most common organic acid anion exudated by Al tolerant species or varieties (Delhaize et al. 1993). Root exudation of phenolic compounds has been described by many authors. Phenolics can reverse the toxic effects of Al on hexokinase (Mamoudou et al. 2002). However, at equimolar concentrations they are less efficient than citrate in complex of Al. This is especially important for simple tannins like catechol at low pH, where H^+ efficiently competes with Al^{3+} for the binding sites in 1:1 complexes (Juan et al. 2002). Therefore, tannins site in themselves are considered important for complexation of Al in acid environment. Recent investigations found Al induced exudation of the flavonoid type tannins catchin and quercetin from 10 mm root tips in an Al resistant maize variety (Boots et al. 1998). The Al induced exudation of catechin reached rates above 100 nmol per tip h^{-1} while that of citrate did not exceed 1 nmol per tip h^{-1}, a rate similar to those reported for citrate exudation in maize by other author (Conæeicao et al. 2006). Another mechanism of tolerance involves Al complexation by organic molecules. The nonprotein thiols (NP-SH) are small metal binding peptides with the structure $(\gamma\text{-glu-cys})_n\text{-gly}$, in which n varies from 2 to 11. The synthesis from glutathione, homo–glutathion, hydroxymethyl-glutathione is catalysed by a transpeptidase, named phytochelatin synthase (PCs) (Cobbett et al. 2000). PCs syntheses have been shown to be activated by a broad range of metals and metalloids, in particular Cd, Ag, Zn, Cu

and Au. The capacity to synthesize NP-SH is supposed to be presented in all higher plants. However, the Cu and Zn exposure levels that are minimally required to induce PCs at considerable concentrations in plant cells are far above the normal nutritional requirements, or even close to the toxicity thresholds (Grill et al. 1987). On the other hand, there is convincing evidence that PCs are essential for normal constitutive tolerance to several non essential metals, particularly Cd (De Knecht et al. 1995). First disruption of the PCs gene in *S. pombe* resulted in hypersensivity to Cd. Second, expression of PCs cDNA from wheat, Arabidopsis dramatically increased Cd tolerance in *Saccharomyces cerevisiae*. In addition, tomato cell lines selected for hypertolerance to Cd exhibited enhanced PC synthesis under Cd exposure, due to increase (γ-glutamyl-cysteine) synthetase (Clemens et al. 1999).

A common consequence of most abiotic and biotic stresses is that they result, at some stages of stress exposure, in an increased production of reactive species. The successive reduction of molecular oxygen to H_2O yields the intermediates O_2^-, HO and H_2O, which are potentially toxic because they are relatively reactive compared with O_2. Reactive oxygen species may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury (Kessler et al. 2003). As a consequence, tissues injured by oxidative stress generally contain increased concentrations of carboxylated proteins and malondialdehyde and showed an increased production of ethylene. The control of oxidant levels is achieved by antioxidative systems (Chien et al. 2001). These defense systems are composed of metabolites such as ascorbate, glutathione, tocopherol, tannin and enzymatic scavenger of activated oxygen such as SOD, peroxidases, etc. (Balestrasse et al. 2001).

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Chien et al. 2001). Polyphenols are plant secondary metabolites consisting condensed tannin. They are of *OH radical scavengers because phenolic groups are excellent nucleophiles and are also able to quench lipid peroxidation, which acts as chain break antioxidant. The antioxidant activity of several tannins involving the prevention of *OH formation and lipid peroxidation has been correlated with their iron chelating properties (Chardonnens et al. 1999). Tannin chelates metal due to its ten galloyl groups and it diminishes intestinal non-heme iron absorption.

In sorghum cultivars, tannin is an abundant component as high as 8-15% of dry weight, which prevents damage from Al stress. In our previous chemical study carried out on two cultivars of sorghum revealed that tannin in cultivar 132 was 10% higher than cultivar 552, and leaf toughness in cultivar 552 was higher than cultivar 132. Polyphenols compounds and specialized condensed tannins play an important role in plant defense by the oxidation of endogenous tannin compound into quinines. Tolerance to Al in sorghum may be defined as the ability to survive in a soil that is toxic to other plants. The question is whether this means is only a single biochemical or molecular change required to produce tolerance to Al. The

main goal of this study was to test the hypothesis that nonprotein thiols have greater activities or condensed tannin, involved in the detoxification of Al. We compared the oxidative activities of two cultivars and the relationship between glutathione due to Al induced NP-SH syntheses, oxidative stress, and the key enzymes malondialdehyde (MDA) and phenylalanine-ammonia lyase (PAL) involved in Al stress. The study was carried out in the leaves and roots of two cultivars of sorghum.

MATERIALS AND METHODS

Plant material

The experiments were carried out between 21 April and 18 September 2007 in outdoor under natural daylight at the University of Bu Ali Sina, Hamedan. The temperature during growth season was 20±5°C. Seeds of two sorghum cultivars (552 and 132) were obtained from Seed Research Center of Isfahan. The seeds were sterilized for 20 min in a 10% sodium hypochlorite solution. They were cultivated in cycle pots with surface area of 1015 cm^2 and depth of 60 cm.

The medium culture used was river sand and peat in 3:1 ratio, respectively. Two small holes were made in pots, the top hole for nutrient feeding, and the bottom one for drainage of excess nutrient solution. The Hoagland's nutrient solution was added to each pot once in two week (Hogland 1950). Simultaneously, the $AlCl_3$ was applied in four concentrations (0, 10, 20 and 30 mg l^{-1}) as treatments according to thresholds fixed by Ahn et al. (2002).The plant samples were harvested at 50 days after sowing.

Sample preparation

After the incubation period, all seedlings were immersed in cold 10 mM $CaCl_2$ for 10 min to remove the adhering Al in root surfaces, after which followed by washing of the roots with distilled water. The plant samples were then separated into roots, stems and leaves, and each tissue was subdivided into two parts. One was immediately weighed and frozen in liquid nitrogen and kept at -50°C for analysis of total nonprotein thiols, glutathione, tannin, and the other was for analysis of Al concentrations. The fresh leaves and roots were also sampled to determine melondialodehyde (MDA) and phenylalanine-ammonia lyase activities. Leaf area per plant was determined using leaf area meter (LI 3100; Li-Cor, Lincoln, NB, USA). The samples were placed in an oven at 60 °C for 4 days then the dry weight was measured separately.

Determination of total nonprotein thiols (NP-SH)

Total nonprotein thiols (NP-SH) was extracted and assayed according to the method suggested by De Voss et al. (1992). In short, total nonprotein thiols was extracted by homogenizing 0.5 g frozen plant materials with 2 ml of a 5%, 5-sulphosalicyclic

acid with 6.3 mM diethylenen triaminepentaacetic (DTPA) at 0 °C (using mortar, pestle and quartz sand). The homogenate was centrifuged at 12,000 revolutions per minute (r.p.m.) at 4 °C for 10 min. Clear supernatants were collected and immediately used for the assay of total nonprotein thiols compound. The concentrations of total nonprotein thiols compound were determined using Ellmans reagent (DTNB). The supernatant at 300 µl volume was mixed with 630 µl of 0.5 M K_2HPO_4 and final pH recorded at 7.5, and the absorbance measured at 412 nm (A_{412}) on a spectrophotometer (Perkin Elmer, Lambda 45, uv/vis D6484. USA). After the addition of 25 µl of DTNB solution (6.3 mM DTNB in 0.143 M K_2HPO_4 and 6.3 mM DTPA, pH 7.5), the A_{412} was measured again after 2 min (ϵ_{DTNB}=13600 mol l^{-1} cm^{-1}). The increase in absorbance was corrected for the absorbance of DTNB. Aluminium did not affect the increase in absorbance. The concentrations of Al were determined by an atomic absorption spectrophotometer.

$$\text{SH concentration (sample)} = \frac{\text{Total vol.}}{\text{Sample vol.}} \times \frac{\text{OD412nm}}{13600}$$

$$= \frac{100 \times \text{OD412nm}}{13600}$$

Determination of total glutathione

Total glutathione (GSH+GSSG) was extracted and assayed according to the method reported by De Voss et al. (1992). Frozen plant materials were homogenized in 0.1M sodium phosphate, 0.005 EDTA buffer (pH=8) and 25% metaphosphoric acid (used as protein precipitatant). The homogenate was centrifuged at 12,000 r.p.m. at 4 °C for 15 min to obtain supernatant for total GSH determination. Total GSH was determined fluorometrically and fluorescence intensity was recorded at 420 nm after excitation at 350 nm on spectrophotometer.

Determination of phenylalanine-ammonia lyase activity (PAL)

Activity of PAL was measured according to the method of Assis et al. (2001) with slight modifications. A 1 ml of enzyme extract was incubated with 2 ml of borate buffer (50 mM, pH 8.8) and 1 ml of L-phenylalanine (20 mM) for 60 min at 37°C. The reaction was stopped with 1 ml of 1M HCl. The assay mixture was extracted with 3 ml of toluene by vortexing for 30 sec. The absorbance of toluene phase containing trans-cininamic acid was measured at 290 nm. Enzyme activity was expressed as nmol trans-cinnamic acid released h^{-1} g^{-1} of fresh weight (FW).

Determination of tannins compounds

Total condensed tannin was determined with acid butanol assay (Hagerman et al. 1998). In a screw cap culture, 6 ml of the acid butanol reagent was added to a 1 ml aliquot of the sample. An iron reagent at 0.2 ml was added and the sample was vortexed. With the cap loosely placed on the tube, the latter was placed in a boiling water bath for 50 min. The tube was cooled and the absorbance, read at 550 nm. Then the absorbance of a blank containing only sample solvent, acid butanol and iron was subtracted from the sample absorbance. The purified tannin mg g^{-1} was used for standard curves.

Determination of lipid peroxidation

The level of lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) reactive metabolites chiefly malomdialdehyde (MDA) as described previously (Heath and Packer, 1968). Plant tissues (0.2 g) were extracted in 2 ml of 0.25% TBA made in 10% TCA. The extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10,000 r.p.m. for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation is expressed as nmol of MDA formed using an extinction coefficient of 155 mM cm^{-1}.

$$\text{Total MDA (nmol } g^{-1} \text{ FW)} = \text{Amount of ext. buffer (ml)} \times \text{amount of supernatant (ml)} \times \frac{[Abs532 - Abs660]}{155} \times \frac{103}{\text{Amount of sample (g)}}$$

Statistical analyses

Two factorial ANOVA was used to compare total glutathione and nonprotein thiols of the leaf and root parameters in two cultivars using the SPSS software (Version 133).

RESULTS

Cultivars dry weight (DW) and leaf expansion

A gradual decrease in total dry weight was observed with the increase in Al concentration (Table 1, 2 and 3; $P<0.001$). In two cultivars under high concentration of Al, the dry weights of root, leaf (Figure 1) and leaf area (Figure 2) were significantly decreased ($P<0.001$). When exposed to low Al concentration (10 mg ^{1}), the leaf dry weight in cultivar 552, and the leaf area in cultivar 132 were

significantly increased (Table 2; f (3, 3) = 7.38 P<0.001; Figure 1; Table 2). Leaf expansion in cultivar 132 (6.43 mm d^{-1}; Table 2) was higher than in cultivar 552 (5.1 mm d^{-1}; Table 3). When compared the mean values, Al was observed to significantly decrease DW of roots, and the decrease in cultivar 552 was most remarkable (Figure 1; Table 2 and 3; P<0.001). In cultivar 132, the leaves dry weight decreased by 29% when treated with 30 mg l^{-1} Al, whereas the equivalent reduction for roots was 37% in comparison to cultivar 552 where the respective decrease in the dry weights of the leaves and root were about 36% and 46%.

Table 1. F-values from analysis of variance for the effects of Al and cultivars on leaf area (LA), leaves dry weight (Dry. L), roots dry weight (Dry. R), leaf area (L.A.), phytochelatin (PCs), total glutathione (GSH), tannin (Tannin), phenylalanine-ammonia lyase (PAL) and lipid peroxidation (MDA) in the leaf.

Factor	D.F.	Dry.L	Dry.R	L.A.	PCs	GSH	Tannin	PAL	MDA
Treatment	3	2.3	3*	2.1	4.1**	2	2.7	1.1	1.6
Cultivar	1	6.3***	5**	11***	4.2**	7***	9***	5 **	3.2*
Al	3	12***	9***	10***	14***	17***	14***	8***	7***
Cultivar*Al	3	7**	2	4.3**	7***	8***	6***	4**	4.2**
Error	22								

D.F., degree of freedom; F-values with their probability: ***P<0.001, ** P<0.01, * P<0.05

Total glutathione (GSH)

Concentrations of total glutathion in leaves and roots were significantly different depending upon cultivars and Al treatment (Table 2 and 3; Figure 3). With increase of Al concentration, the amount of glutathion in leaves (41%) and roots (123%) of cultivar 132 was significantly decreased (Table 2 and 3). Whereas in cultivar 552, Al stress caused a significant decrease in roots by about 109% and in leaves by about 39% increased (Figure 3). The amount of glutathion in cultivar 552 was higher than cultivar 132. (f (3,3)=7.2; P<0.001; Figure 3; Table 2 and 3). The amount of glutathione in new leaves of cultivar 552 was higher than cultivar 132. In new leaves of the two cultivars, Al also increased the total glutathione; and the increase in cultivar 552 was higher than cultivar 132.

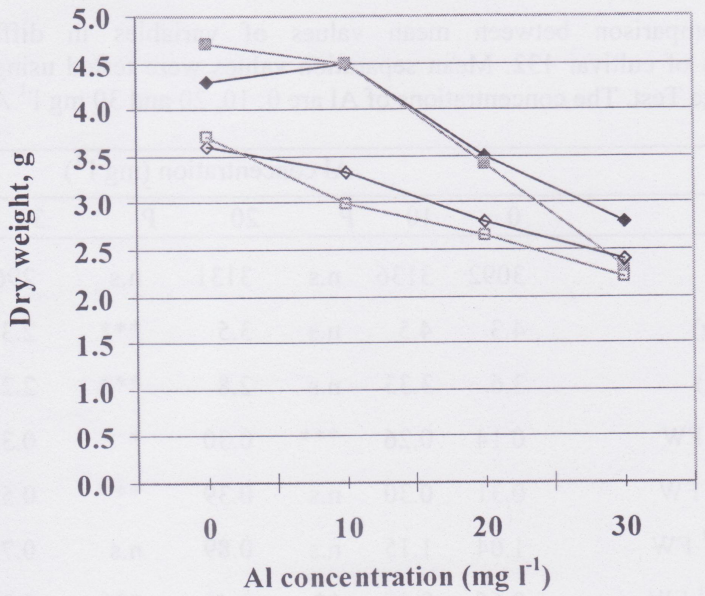

Figure 1. Changes in dry weights of the leaves of cultivars 132 (-◆-) and 552 (-■-), and the roots of cultivars 132 (-◇-) and 552 (-□-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

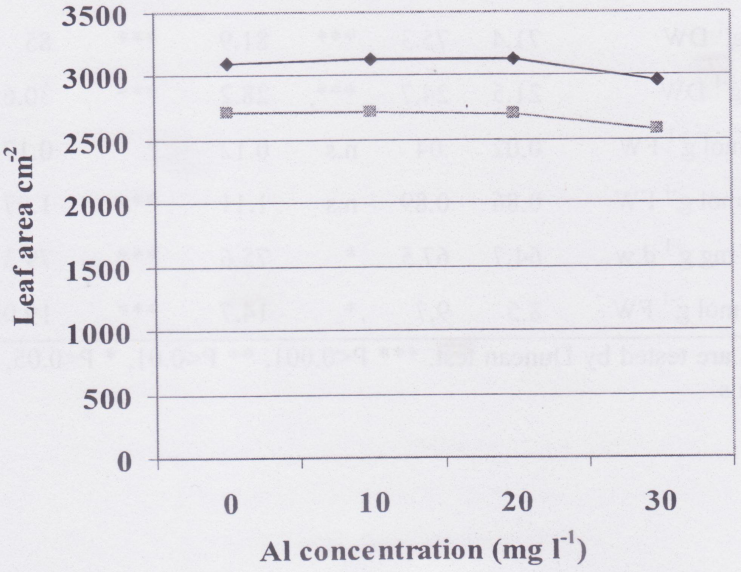

Figure2. Changes in the leaf area of cultivars 132 (-◆-) and 552 (-■-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

Table 2. Comparison between mean values of variables in different Al concentrations of cultivar 132. Mean separation values were tested using Duncan Multiple Range Test. The concentrations of Al are 0, 10, 20 and 30 mg l^{-1} $AlCl_3$.

	Al concentration (mg l^{-1})						
Factors	0	10	P	20	P	30	P
Leaf area cm^{-2}	3092	3136	n.s	3131	n.s	2964	***
Dry weight leaf (g)	4.3	4.5	n.s	3.5	***	2.3	***
Dry weight root(g)	3.6	3.33	n.s	2.8	***	2.2	***
PCs leaf µmol g^{-1} FW	0.14	0.26	***	0.30	*	0.35	**
PCs root µmol g^{-1} FW	0.31	0.30	n.s	0.39	**	0.52	***
GSH leaf µmol g^{-1} FW	1.04	1.15	n.s	0.89	n.s	0.73	**
GSH root µmol g^{-1} FW	0.66	0.57	**	0.46	***	0.38	***
PAL leaf µmol g^{-1} FW	0.76	1.36	**	1.73	***	1.97	***
PAL new leaf µmol g^{-1} FW	0.83	1.24	***	1.19	n.s	1.37	**
MDA leafµmol g^{-1} FW	18.5	27.3	***	31.2	***	33.03	***
MDA root µmol g^{-1} FW	5.8	11.6	***	13.3	***	15.5	***
Tannin leaf mg g^{-1} DW	71.4	75.3	***	81.9	***	85	***
Tannin root mg g^{-1} DW	21.5	24.7	***	28.2	***	30.6	***
PCs new leaf µmol g^{-1} FW	0.02	.04	n.s	0.12	*	0.17	*
GSH new leaf µmol g^{-1} FW	0.86	0.89	n.s	1.11	***	1.07	*
Tannin new leaf mg g^{-1} d.w	64.7	67.5	*	75.6	***	79.3	**
MDA new leafµmol g^{-1} FW	8.5	9.7	*	14.7	***	19.03	***

Mean values are tested by Duncan test. *** $P<0.001$, ** $P<0.01$, * $P<0.05$, and not significant, n.s.

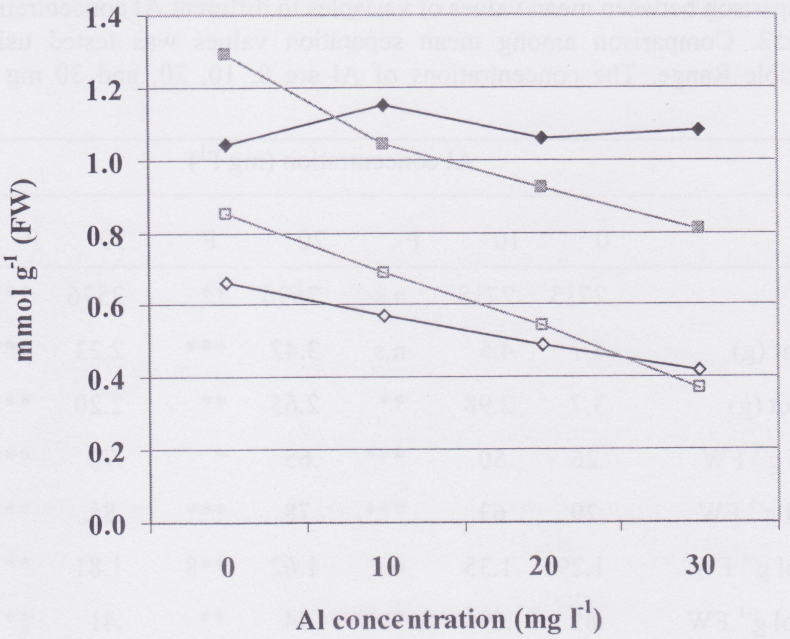

Figure 3. Glutathion content in the leaves of cultivars 132 (-◆-) and 552 (-■-), and roots of cultivars 132 (-◇-) and 552 (-□-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

Total nonprotein thiols (NP-SH)

There was a strong positive correlation between the concentration of total nonprotein thiols and Al concentration in the roots of two cultivar (R^2=0.91; f(2, 32)= 9.35; P<0.001; Table 1). The nonprotein thiols in roots and leaves of cultivar 552 significantly was increased with increasing Al concentration, root (f(3,3)= 14.26; P<0.001; Figure 4; Table 2 and 3), and leaf (f(3,3)= 7.5; P<0.001; Figure 4). The nonprotein thiols in root cultivar 132 increased consistently with the concentration of Al supplied (f(3,3)= 8.3; P<0.001; Figure 4; Table 2 and 3). In control cultivars only small amounts of nonprotein thiols were present. The level of nonprotein thiols was increased by 136% in the roots and about 73% in the leaves of cultivar 552, and Al caused a significant increase in roots and leaves nonprotein thiols by about 115%, and 37%, respectively.

Table 3. Comparison between mean values of variables in different Al concentration of cultivar 552. Comparison among mean separation values was tested using Duncan Multiple Range. The concentrations of Al are 0, 10, 20, and 30 mg l^{-1} $AlCl_3$.

	Al concentration (mg l^{-1})						
Factors	0	10	P	20	P	30	P
Leaf area cm^{-2}	2713	2718	n.s	2690	**	2576	**
Dry weight leaf (g)	4.7	4.5	n.s	3.42	***	2.23	***
Dry weight root (g)	3.7	2.98	**	2.65	**	2.20	***
PCs leaf µmol g^{-1} FW	.26	.50	***	.65	*	.76	***
PCs root µmol g^{-1} FW	.29	.63	***	.78	***	.85	***
GSH leaf µmol g^{-1} FW	1.29	1.35	n.s	1.62	**8	1.81	**
GSH root µmol g^{-1} FW	.85	.69	*	.54	**	.41	***
PAL leaf µmol g^{-1} FW	.46	.79	***	1.5	***	1.29	n.s
PAL new leaf µmol g^{-1} FW	.91	.84	n.s	1.11	***	1.14	n.s
MDA leafµmol g^{-1} FW	22.9	29.6	***	34.2	***	36.3	**
MDArootµmol g^{-1} FW	3.21	7.4	***	10.7	***	11.8	n.s
Tannin leaf mg g^{-1} DW	35.8	39.1	n.s	43	*	54.2	***
Tannin root mg g^{-1} DW	4.8	7.2	**	9.3	n.s	11.3	**
PCs new leaf µmol g^{-1} FW	.11	.21	n.s	.39	*	.34	*
GSH new leaf µmol g^{-1} FW	1.21	1.05	***	0.94	*	0.85	*
MDA new leaf µmol g^{-1} FW	16.3	21.7	n.s	25.5	**	28.7	*
Tannin new leaf mg g^{-1} DW	32.7	38.4	n.s	41.2	**	49.6	***

Mean values are tested by Duncan test. *** P<0.001, ** P<0.01, * P<0.05, and not significant, n.s.

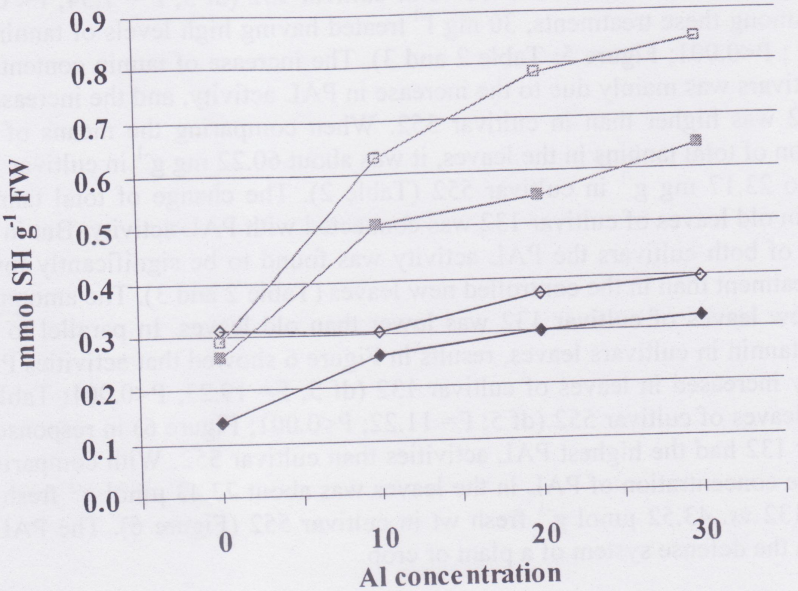

Figure 4. Phytochelatin content in the leaves of cultivars 132 (-◆-) and 552 (-■-), and roots of cultivars 132 (-◇-) and 552 (-□-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

The increase of nonprotein thiols in cultivar 552 upon Al exposure corresponded to the decrease of glutathione approximately 80% of which was accounted for by glutathione. And the increase of nonprotein thiols in cultivar 132 upon Al exposure corresponded to the decrease of glutathione approximately 58% of which was accounted for by glutathione. In spite of the presence low of Al in new leaves, a significant increase in nonprotein thiols synthesis was evident in the cultivar 552, treated with Al (Table 2 and 3).

Changes in leaf Al

The amount of Al in leaves cultivar 132 was higher than cultivar 552 (Table 4). With increase Al concentration in medium culture the amount of Al in roots and leaves of two cultivars significantly increased ($f(3,3)=21.2$; Table 4). Al concentration in the leaves remained low even in the highest 30 mg l^{-1} medium culture treatment. The Al also increased in new leaves of two cultivars under Al treatments.

Changes in tannin compounds and its relationship with PAL activity

Evidence indicates that tannin contents in cultivars significantly increased in all treatments under Al concentration, in the leaves of cultivar 552 (df 3; F= 5.13;

P<0.001; Figure 5; Table 2) and the leaves of cultivar 132 (df 3; F = 7.34; P< 001; Table 2). Among these treatments, 30 mg l^{-1} treated having high levels of tannin (F (3,5)= 8.02 ; P<0.001; Figure 5; Table 2 and 3). The increase of tannin contents in the two cultivars was mainly due to the increase in PAL activity, and the increase in cultivar 132 was higher than in cultivar 552. When comparing the means of the concentration of total tannins in the leaves, it was about 60.22 mg g^{-1} in cultivar 132 compared to 23.17 mg g^{-1} in cultivar 552 (Table 2). The change of total tannins compound in old leaves of cultivar 132 was connected with PAL activity. But in the new leaves of both cultivars the PAL activity was found to be significantly lower under Al treatment than in the controlled new leaves (Table 2 and 3). The amount of tannin in new leaves of cultivar 132 was lower than old leaves. In parallel to the increase of tannin in cultivars leaves, results in Figure 6 showed that activities PAL significantly increased in leaves of cultivar 132 (df 5; F= 19.23; P<0.001; Table 2 and 3) and leaves of cultivar 552 (df 5; F= 11.22; P<0.001; Figure 6) in response to Al. Cultivar 132 had the highest PAL activities than cultivar 552. With comparison of means the concentration of PAL in the leaves was about 73.43 µmol g^{-1} fresh wt in cultivar 132 *vs.* 43.52 µmol g^{-1} fresh wt in cultivar 552 (Figure 6). The PAL is important in the defense system of a plant or crop.

Table 4. Aluminum concentration in the roots and leaves of cultivars 132 and 552 at three days after sowing.

	Root		Shoot	
Cultivar	**132**	**552**	**132**	**552**
Control	6	7.2	2	3
AL 10 mg l^{-1}	65	59	21	18
Al 20 mg l^{-1}	86	81	35	32
Al 30 mg l^{-1}	110	98	43	41

Al concentration is expressed as mg l^{-1} dry weight. The values are the means of three independent experiments.

Change in lipid peroxidation (MDA)

The increase in MDA content was cultivar-dependent and significant at 20 and 30 mg g^{-1} Al treatments; there was more MDA in the leaves than in the roots. Results in Figure 7 showed that activities of MDA significantly increased in the leaves of cultivar 132 (df 5; f= 5.4; P<0.001), and leaves of cultivar 552 (df 5; f= 8.3; P<0.001; Figure 7; Table 2 and 3) in response to Al. MDA content in new leaves of Al treated cultivars 552 was significantly higher than cultivar 132 (Table 2 and 3).

The results showed a significant increase in MDA that was more pronounced in the leaves than in the roots of Al-treated cultivars. In high concentration Al treatments (20, 30 mg l^{-1}) MDA in leaves of cultivar 552 was increased, and consequently influenced glutathion content in cultivars 552. On the other hand, cultivar 132 have evolved different antioxidant mechanism to cope with Al stress.

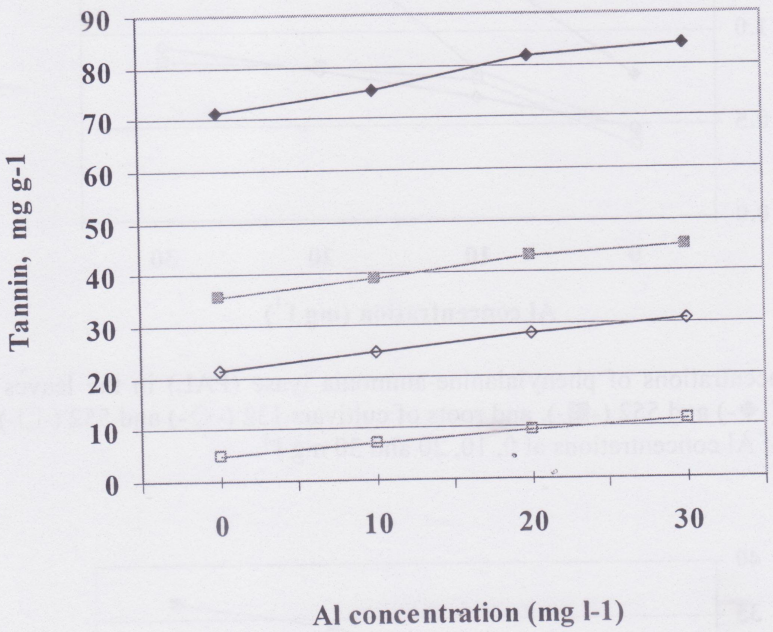

Figure 5. Tannin pool in the leaves of cultivars 132 (-◆-) and 552 (-■-), and roots of cultivars 132 (-◇-) and 552 (-□-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

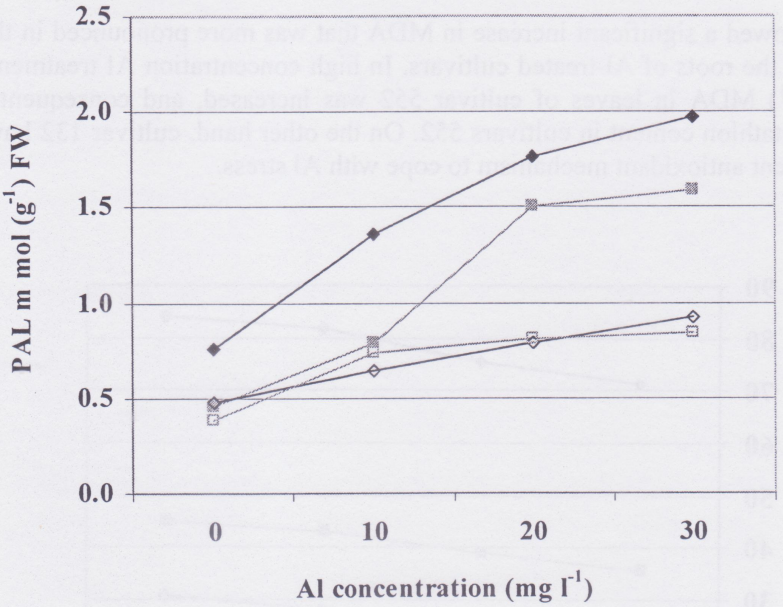

Figure 6. Concentrations of phenylalanine-ammonia lyase (PAL) in the leaves of cultivars 132 (-◆-) and 552 (-■-), and roots of cultivars 132 (-◇-) and 552 (-□-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

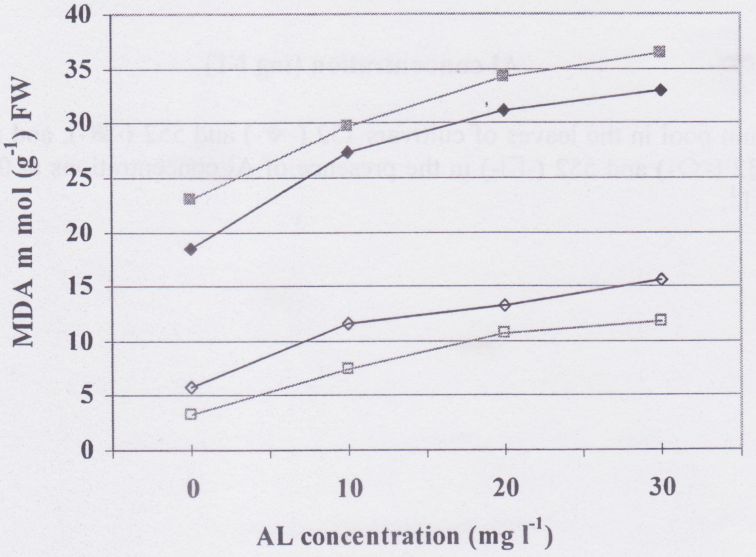

Figure 7. Levels of lipid peroxidation expressed in terms of MDA concentration in the leaves of cultivars 132 (-◆-) and 552 (-■-), and roots of cultivars 132 (-◇-) and 552 (-□-) in the presence of Al concentrations at 0, 10, 20 and 30 mg l^{-1}.

DISCUSSION

Based on previous work on Al toxicity in complete nutrient solution, cultivar 132 was selected as an Al-resistant, and cultivar 552 was selected as an Al-sensitive plants. Al decreased the dry weight of leaves, roots and leaf area in the two cultivars where the decrease in dry weight was lower in the 30 mg l^{-1} Al treatment than other Al concentrations (Table 2 and 3). This result agreed with the observation by Delheize et al. (1995). In Al stressed condition, the dry weight partitioning between roots and leaves was significantly changed. At high concentrations of Al below ground biomass was highly constrained than above ground biomass, hence, dry root weight was more affected than the dry weight of leaves (Figure 1; Table 2 and 3). Al increased allocation to tannins, glutathione, and NP-SH, which are potential defensive compounds under stress (Yoko et al. 2005). The cultivar 552 grown at 30 mg l^{-1} was moderately chlorotic, exhibiting brown lesions on the leaves and appearing noticeably smaller than the controls. Roots of Al-treated cultivar 552 generally appeared darker than control plants perhaps due to Al stimulated efflux of organic acids. However, cultivar 132 grown at 30 mg l^{-1} was observed less chlorotic, and appearing slightly smaller than controls (Delhaize et al. 1995). The Al is a toxic element, and it has different effects on the two cultivars. In low concentrations, Al stimulates the growth in some plants (Ahn et al. 2002). With increasing concentration of Al in medium culture until 10 mg l^{-1}, the dry weight of leaves and leaf area in cultivar 132 were increased, but only leaf dry weight in cultivar 552 was improved (Figure 1 and 2). At low concentration (10 mg l^{-1}) Al, leaf expansion in cultivar 132 increased more than in cultivar 552. These results matched with the results of Kinraide et al. (1999). Despite the ability of the cultivars to reduce Al toxicity, the concentration of Al in the growing medium almost multiple the concentration of Al in the roots and the leaves (Table 4).

The Al was accumulated mainly in old leaves in both cultivars during the treatment, and the amount in young leaves of the cultivars was very low (Table 4). This experiment showed that low cumulative Al in leaves of two cultivars may be a strategy to protect photosynthetic function from induced oxidative stress (Aniol et al. 1983). The cultivars can avoid Al damage when immersed in the nutrient solution present with 30 mg l^{-1} of Al. At the same time they can develop defense mechanism to cope with the affinity of Al for NP-SH. In previous work in the roots of the cultivars, the amount of Al in apical roots increased continuously, whilst the Al concentration in old leaves of cultivar 132 was higher than cultivar 552. Conversely, the Al concentration in root cultivar 132 was lower than cultivar 552, in agreement with that observed by Matsumoto et al. (2001). The high Al concentration in the roots reduced total dry weight in cultivar 552 (30%), and cultivar 132 (24%). It is likely, as previously argued, that Al is bound to the cytosolic thiols dependent enzyme and reduced sucrose synthesis (Delhaize et al. 1993). It is noted that from the result obtained in this experiment, two strategies for Al resistance in cultivars have been suggested. First, is the exclusion of Al in the roots (exclusion mechanism) where the roots of Al-treated cultivar 552 generally appeared darker than control perhaps due to root death and stimulation of the efflux

of organic acid. Second strategy, is the plant tolerance to Al once the latter enters the roots (internal tolerance mechanism) of both cultivars. Al-induced NP-SH and the central metabolism of glutathion production in roots of both cultivars have been widely considered as mechanism whereby these cultivars could acquire a degree of resistance to Al toxicity (Juan et al. 2002).

NP-SH increased by 136% from the first concentration to the 30 mg l^{-1} ($P< 0.001$; Figure 4; Table 2) in the roots of cultivar 552. By contrast, glutathione decreased by 109% in cultivar 552 from the first concentration to the 30 mg l^{-1} ($P< 0.001$; Figure 3). In cultivar 132, NP-SH increased by 115% from the first concentration to the 30 mg l^{-1}, ($P< 0.001$; Figure 4; Table 3) in the roots. By contrast, glutathione decreased by 123% in cultivar 552 from the first concentration to the 30 mg l^{-1} ($P< 0.001$; Figure 3) in agreement with the work by DeVos et al. (1992). In the present study the threshold Al exposure levels for root growth inhibition and cumulative NP-SH did not coincide in the two cultivars, whilst Al induced the NP-SH accumulation. However, the capacity of the two cultivars for antioxidant and NP-SH in the new and old leaves varied. The amount of NP-SH in old leaves of cultivar 552 was higher than cultivar 132 but the amounts of tannins in the old leaves of cultivar 552 was lower than cultivar 132. Following Al stress, a deep alteration of the glutathione status occurred mainly in new leaves of cultivar 552. The glutathione is used as a substrate for NP-SH production. Therefore, NP-SH may be functionally important to cultivar 552 under condition of Al stress, as in agreement with the results by Gavel et al. (2001). The threshold concentration levels of Al for cumulative NP-SH appeared in cultivar 552 were lower than cultivar 132. In cultivar 132, the induced cumulative NP-SH was not apparent until the threshold concentration level for acute toxicity had been exceeded, suggesting that tannins are normally involved in Al sequestration under conditions of subtoxic exposure (Mossor et al. 2001).

The relation NP-SH, glutathione and aluminum

In the two cultivars, the new leaf concentration of NP-SH in 30 mg l^{-1} Al was significantly higher than the control (Table 2 and 3). The localization of NP-SH in the roots could provide an effective means of restricting Al to these organs by chelations in the form of Al-NP-SH complexes, hence, the transport to the leaves was restricted (Dejene et al. 2005). The glutathione is a central metabolic, which is involved in the reaction forming of NP-SH (Grill et al. 1989). The statistical analysis showed that both glutathione and NP-SH exhibited in the two cultivars had a high degree of variability as revealed by the large error bars. This variability could have been caused by the differences in cultivar antioxidants present in the leaves. In cultivar 132 (high tannin content) seedlings, Al treatment increased total tannin content mediated by enhanced activities of PAL (Figure 5 and 6). Conversely, in cultivar 552, the total tannin was low and, consequently, the activity of PAL was also low (Table 3 and 4). In cultivar 552, the glutathione content was higher than cultivar 132, and may have been consumed by the two strategies, the maintenance of regular redox potential, and the precursor for NP-SH syntheses, which findings

are in agreement with that observed by Maier et al. (2003). The changes in both glutathione and NP-SH were dependent on the Al concentration supplied to medium culture (Figure 3 and 4). The amount of NP-SH was high in the leaves and the roots with increased concentration of Al. In cultivar 552 and at 10 mg l^{-1} Al only the glutathione was clearly increased to a level of 33% of the control compared to the extractable glutathione in the leaves of cultivar 552, which was increased to 39% of the controls with 30 mg l^{-1} Al concentration. With increase of Al concentration, the glutathion level in the leaves declined by about 41% in cultivar 132, as in agreement with that observed by Pagliari et al. (2005). The major effect observed in this research was that with 30 mg l^{-1} of Al in medium culture, the glutathion level in the roots of cultivar 552 decreased by about 109%, whilst that of cultivar 132, the decrease was about 123% (Table 2 and 3). Therefore, in cultivar 132 that is parallel to the transient depletion of glutathione, which is used as a substrate to NP-SH production, the synthesis of glutathione does not seem to occur. Also the distribution of NP-SH in roots and leaves of cultivar 132 were different, an agreement with that observed by Maier et al. (2003).

The result in cultivar 552 showed that the level of NP-SH was regulated by glutathione content and that it was parallel to the transient depletion of glutathione for NP-SH production. The synthesis of glutathione generally occurred (Table 2 and 3). The level of glutathione concentrations was induced by a treatment with Al. It also explained why in cultivar 552, the Al-treated leaves contained the increased pool of glutathion. In cultivar 552 the amount of tannin was very low, and this cultivar needed quantitatively to maintain as much tannin in reduced form. The high cost of this activity could be mitigated by using glutathione in antioxidant activity against Al impact. Glutathione can be mobile to many organs in plant, and this characteristic is very useful for an antioxidant (Peter et al. 2001; De vos et al. 1992). Glutathion also efficiently lowers the probability that Al will bind with thiols in the active sites of many photosynthetic enzymes, which would alter their functionality and inhibit photosynthesis (Matsumoto et al. 2001). The higher sensitivity of cultivar 552 towards Al, compared to cultivar 132, seemed that upon Al induction on cultivars 552 the latter could produce even more additional thiol than cultivar 132, which caused an insufficient capacity for providing thiol components (Thomas et al. 1995). This was a predictable result because it is known that Al acts as a strong sink for thiols, which increased the demand for sulfate absorption. The predominance of the glutathione, confirms the suggestion that thiols can trap Al only when they are in the reduced state (Rijstenbil et al. 1998).

The amount of glutathione in cultivar 552 was higher than cultivar 132. This difference between the two cultivars might be due to the synthesis of glutathione in leaves and roots, or recycling of glutathione in the roots and the leaves. It has been suggested that cultivar 552 with increased glutathione concentration may improve their growth and antioxidant resistance under Al excess. Therefore, glutathione regeneration by the Glutamin-Glutamat cycle is a key in antioxidation mechanism against Al stresses (Ezaki et al. 2004). The results suggested that during the first toxicity of Al, the antioxidant compounds in cultivar

552, such as glutathions, which has rapid turnover and high cumulative, to be cost effective than tannin for leaves because tannin was low and stable. In cultivar 132 where the antioxidant compounds such as tannin has low turnover and high cumulative to be cost effective than glutathion for the leaves, because from previous work the leaves toughness in cultivar 552 was found to be higher than in cultivar 132. In cultivar 552 glutathion could be mobile between roots and leaves. For that reason the amount of glutathione in new leaves of cultivar 552 was also high. In cultivar 132, tannin was not mobile between roots and leaves (Gebrehiwot et al. 2002). In cultivar 132 the new leaves concentration of NP-SH was slightly higher than the control. But in cultivar 552, total NP-SH in old leaves was significantly higher than in new leaves. Therefore, the transportation of glutathione in leaves of cultivar 552 was higher than cultivar 132 (Pagliari et al. 2005).

The amounts of NP-SH present in the root of cultivar 552 was higher than cultivar 132, and the synthesis of these compounds was rapidly induced in roots of cultivar 552 under 10 mg l^{-1} Al. The synthesis of glutathione in cultivar 132 reached a steady state under higher concentration of Al. In Al stress, the transformation of glutathione in cultivar 552 was much parallels with the rate of synthesis. However, in cultivar 132, the rate of transformation was higher than the rate of synthesis. Therefore, loss of glutathione due to Al stress in cultivar 132 was higher than cultivar 552. In cultivar 552, when the Al concentration increased, the transformation of glutathione to NP-SH was high, but in cultivar 552, when the Al concentration increased, the synthesis of tannin also increased resulting in very low transformation of glutathione to NP-SH. Similar finding was observed by Maier et al. (2003).

Malondialdehyde (MDA), phenylalanine-ammonialyase (PAL) and tannin

Changes in chemical defenses including tannin and various enzyme activities, MDA and PAL, in the presence of Al in the leaves and the roots of both cultivars were investigated. MDA, the marker for lipid peroxidation, increased in cellular membranes of tissues. Results appeared that Al had induced the MDA and PAL activities in the roots and the leaves of both cultivars (Figure 6 and 7). Al increased MDA activity, which coincided with an acute decrease in glutathione in the roots (Table 2 and 3). With increasing the concentrations of Al, levels of MDA increased in the leaves of both cultivars, and that increase in MDA in cultivar 552 was higher than in cultivar 132. With increased Al concentration, the amount of tannin in the leaves of the two cultivars also increased, and the increase in cultivar 132 was higher than cultivar 552 (Singleton et al. 1999). When cultivar 552 was exposed to Al, the equilibrium between the production of tannin and glutathion was broken resulting in oxidative damage. The levels of glutathione, which are potential antioxidant compounds, markedly decreased, while MDA activity greatly increased as compared to control (Pagliari et al. 2005). Thus, the imbalance between MDA and PAL generation, and scavenging compound tannin in the leaves may reflect the defense strategy in these two sorghum cultivars, 132 and 552.

Tannins are derived from polyphenols, which is formed from phenylalanine by the action of PAL, and it is often speculated to be a key enzyme in tannin metabolism. The PAL enzyme can readily be induced by some environmental stresses (Ohi et al. 1990). Thus, we examined the possibility that PAL activity might be induced by high concentration of Al. In Figure 6, it indicates that the activity of PAL in cultivar 552 was lower than cultivar 132. With increased Al concentration in medium culture, the activity of PAL increased in cultivar 552 until reaching a maximum value, and then PAL activity decreased. But in cultivar 132 the activity of PAL recorded was higher than in cultivar 552. At the same time, the amount of tannin in cultivar 132 increased with increased concentration of Al and then reaching a maximum. These results indicate that an increase in tannin in cultivar 132 is based on an increase in the activity of PAL (Figure 5 and 6; Table 2 and 3). In cultivar 552, the enhanced formation of NP-SH during glutathion depletion was preceded by an increase in Al concentration and a subsequent lowing of PAL activity. The results matched the observation by Ohi et al. (1990). Cultivar 132 exhibited a maximum value of PAL activity, which is two times higher than cultivar 552, and the depleted glutathione was low. With the treatment 10 mg g^{-1}Al, and in the presence of tannin, the activity of PAL was not significantly altered in cultivar 552, but rather increased in cultivar 132 (Table 3 and 4). These results suggested that the tannin accumulated in cultivar 132 prior to a start of exposure to Al, plays a role in the prevention of lipid peroxidation. In the experiment, the increase in PAL activity might be controlled by either antioxidant potential of cells or activation by Al. One of the tolerant mechanisms in cultivar 132 seems to be the cumulative of antioxidant molecules such as tannin, which inhibited the peroxidation of phospholipids (Olga et al. 2003).

In addition, it has been postulated that the increase in tannin is responsible for the formation of phenoxy radicals. This experiment indicated the tannin-oxidizing Al^{3+} trait and this cation did not participate either in the response to the defense reaction neither to the hyper sensitivity in inducing resistance of cultivar 132 to Al stress. It has been well documented that Al stresses are responsible for the increase in tannin, which would be antioxidant and associated with decreased plant growth.

The changes that occurred, in the pathway of glutathione synthesis and tannin metabolism of the leaves also suggested that the whole plant improved its antioxidant defense even in those parts. It is well established that tannin has an active function in scavenging Al^{3+} (Chimi et al. 2006) as similarly observed in the present results. On the other hand, glutathione metabolism was very active with high activation of MDA (Figure 3 and 7), and total NP-SH content consequently increased at Al treatment. This showed a highly positive relationship between MDA activity and the concentration of NP-SH in cultivar 552, and suggested that an cumulative of NP-SH compounds in response to Al stress would be attributed to the activation of the glutathione metabolism (De Vos et al. 1992). This would be beneficial to achieve adaptation and tolerance to Al stress, since in cultivar 552, glutathione has been considered to be the main resource for adaptation against Al

stress. Therefore, the data obtained from the Al stressed plants suggested that the increase in MDA and PAL activation is one of the important factors of Al tolerance, which permits preservation of membrane integrity and leaf growth (Table 3 and 4). The observed increase in PAL and MDA concentration in both cultivars might indicate extensive lipid peroxidation of cell membrane. Significant decreases in dry mass occurred following in 30 mg l^{-1} Al treatment, which coincided with the increase in tannin and NP-SH concentrations (Figure 4 and 5). Polyphenols compounds are known to be antioxidant, function as precursors to structural polymers such as lignin and tannins. Comparing the two cultivars, total tannins in cultivar 132 were significantly higher than cultivar 552. Results in Table 3 and 4 showed that total tannins markedly increased in leaves in all the treated cultivars at 30 days from Al addition in medium culture, which is in agreement with those observed by Peter et al. (2001). As it seemed, induction of total tannins and changes in the contents of glutathione in the leaves of two cultivars play an important role in Al resistance, which are intimately connected with MDA and PAL (Table 3 and 4). These results agreed with the general theory that when plant cells are exposed to metals, they switch from normal primary metabolism to the multitude of secondary metabolism pathway, and followed by the activation of novel stressed-enzymes and genes (Mamoudou et al. 2002). Therefore, the potent antioxidant properties of tannin in cultivar 132 are a common response to Al resistance (Figure 4; Peter et al. 2001). The Al-treated cultivar 552 recorded the highest MDA activities, while Al-treated cultivar 132 had the highest PAL activities. It seems that there is as intensive activity of MDA coupled with the small changes in tannin during the Al stresses. The production of tannin caused an increase in the antioxidant activity, therefore, the formation of MDA in cultivar 132 was lower than in cultivar 552 (Figure 5 and 7). Also high activity of PAL in Al-stressed induce cumulative of tannins, consequently enhances the plant Al resistance (Ohi et al. 1990).

In our experiment it is difficult to determine which of these processes was responsible for increased Al stress. The higher NP-SH concentration and the decrease glutathione in roots of cultivar 552, and the higher tannins compound and higher activity of PAL, and lower activity MDA in the leaves of cultivar 132, suggested that, it is probably a combination of multi processes that were responsible in Al resistance. In cultivar 552, NP-SH has been found to be functionally important to plant under stress of Al, because under low Al concentration in cultivar 552 the synthesis was induced. But in cultivar 132, the rate of tannins production increased with an increasing Al concentration, and the NP-SH synthesis-induced glutathione depletion in the leaves of the cultivar. It was concluded that, this decrease in cultivar 132 caused by glutathione translocation from the leaves to the roots in order to make a larger amount of glutathione available for NP-SH synthesis in the roots, could consequently change the thiol metabolism in the leaves (Olga et al. 2003). When the two cultivars were analyzed, the behavior of the redox states of total glutathione and tannin were different in the roots from the leaves. This further supports the possibility of an involvement of tannins more than of glutathion in the redox reactions triggered by Al in cultivar 132. Hence, the glutathion redox state in

the leaves, and the movement from leaves to roots remained low (Tomas et al. 2001).

CONCLUSIONS

Generally, it can be said that parameters of the cellular metabolism like NP-SH, glutathione and tannin shown could be antioxidant, and it is probably a combination of multi processes that are responsible in Al resistance.

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