Hayati, Maret 2003, hlm. 30-33 Vol. 10, No. 1
ISSN 0854-8587

Aluminum Tolerance in Soybean: Protein Profiles and Accumulation of Al in Roots

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Diterima 10 Juli 2002/Disetujui 22 Oktober 2002

Four soybean genotypes were evaluated for their tolerance to aluminum toxicity using solution culture with pH 4.5. Based on the difference of root length, two genotypes were selected as Al-sensitive genotype (Lumut) and Al-tolerant genotype (Yellow Biloxi). The magnitude of the difference in Al tolerance between two genotypes was evidenced by the fact that at 0.7 mM Al the root length of Lumut was inhibited by 58%, whereas in Yellow Biloxi the inhibition was only 19.6%. Moreover, the hematoxylin staining indicated that Lumut accumulated more Al in the roots than Yellow Biloxi. This finding supports the proposed hypothesis of exclusion mechanisms of Al in the roots of tolerant soybean genotype. Characterization of total protein in the root tips (0.5-0.8 cm in length and adjacent 2 cm) by SDS-PAGE revealed the difference of protein profiles. In 0.7 mM Al exposure, only root tips of tolerant genotype could express a new protein band with molecular weight of approximately 79.8 kD, the protein of which disappeared when Al was removed from the treatment media. This phenomenon was found only in the protein characterization of root meristem with 0.5-0.8 cm in length, and not in the adjacent 2 cm of the root. Accumulation of these proteins in the presence of Al and their subsequent disappearance after removal Al from the growth medium suggested a possible involvement in Al resistance. These results are consistent with the hypothesis that these proteins are synthesized in the root tip region (0.5-0.8 cm) where the early effects of Al toxicity are often observed.

INTRODUCTION

The effort to increase soybean production through extensification program in Indonesia faces various soil problems, especially soil acidity and aluminum toxicity. Aluminum toxicity is a potential limiting factor for soybean in acid soil in many parts of the world (Foy *et al*. 1993). The primary effect of Al is to inhibit root growth with subsequent effect on nutrient and water uptake. Cell division and root elongation is affected within hours of Al exposure (Ryan *et al.* 1993), and the primary target site of Al toxicity in soybean appears to be the root apex (Lazof *et al.* 1994).

Although many of the toxic effects of Al on plant growth and function have been described (Foy *et al*. 1978, Taylor 1988, 1991, Matsumoto 1991), little is known about the physiological and biochemical mechanisms of Al tolerance in roots, especially in soybean. In wheat, several exclusion mechanisms have been proposed, including alkalization of rhizosphere, efflux of chelator ligands and selective permeability of plasma membrane (Taylor 1988, 1991). Basu *et al*. (1994) pointed out that the root tip is the tissue of choise for investigating mechanisms of Al resistance. It is likely that resistance is mediated by one or more proteins. These proteins could be membrane proteins that actively export Al, enzymes involved in the synthesis or export of chelator ligands, or enzymes responsible for the synthesis of cellular components that have properties that confer Al resistance (Taylor 1991). Basu *et al.* (1994) have identified a 51-kD polypeptide (or polypeptides) in microsomal membrane preparation that appeared only in the Al-resistant wheat cultivar upon exposure to Al.

In this experiment, we investigate the physiological basis of the differential Al tolerance of soybean through the study of protein profiles in root tips and the accumulation of Al in the roots of soybean.

MATERIALS AND METHODS

Plant Materials and Seedlings Growth. Two Al-tolerant genotypes (Yellow Biloxi and Slamet) and two Al-sensitive genotypes (Lumut and Arksoy) were used in this experiment. These genotypes were selected from 400 accessions for Al tolerance by Sopandie *et al*. (1996). Soybean variety of Slamet was selected from several pure breeding lines developed by Sunarto (1993).

In all experiments, seeds were surface-sterilized in 0.1% (w/v) benlate for 10 min and soaked for 3 h in double-distilled water, rinsed and germinated on nylon mesh suspended over 8 L of aerated water for 3 days. Seedlings were then grown for 2-3 days in an aerated nutrient solution containing (1/3 strength concentration): $Ca(NO_3)_2$ 1.5 mM, KCl 1.0 mM, NH_4NO_3 1 mM, $MgSO_4$ 0.4 mM, KH_2PO_4 1 mM, $MnSO_4$ 0.5 ppm, $CuSO_4$ 0.02 ppm, $ZnSO_4$ 0.05 ppm, H_3BO_3 0.5 ppm, $(NH_4)_2Mo_7O_{24}{\cdot}4H_2O$ 0.01 ppm, Fe-EDTA 0.068 ppm (pH 4.5). For Al exposure, 3-d-old seedlings were transferred to the same solutions with Al ranging from 0 to 0.9 mM (pH 4.5) and grown for 7 d. Terminal 0.5-0.8 cm (root tips, RT region) and the adjacent 2 cm (R region) of the roots were excised and immediately frozen in liquid nitrogen and stored at -50°C for later use.

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Seedlings for hematoxylin staining were surface-sterilized in 0.01% chlorox and soaked for 3 h in double-distilled water, rinsed and germinated as above for 24 h. Seedlings were then transferred to small grid and cultured for 1-2 d in an aerated nutrient solutions containing: $MgCl_2$ 2.5 mM, KNO_3 6.5 mM, NH_4NO_3 0.4 mM, $(NH_4)_2SO_4$ 0.1 mM, $CaCl_2$ 5 mM (pH 6.5). Approximately 10-15 seedlings per grid were transferred when the primary root was 2 cm long. For Al exposure, seedlings were transferred to the same solutions with Al addition (0; 0.07; and 0.09 mM; pH 4.5) and grown for 17 h.

Hematoxylin Staining of Intact Root Systems. Seedlings from each Al treatment and controls were removed after imposition of Al treatment, rinsed in 300 ml distilled water for 30 min and then stained for 15 min in 200 ml hematoxylin stain (hematoxylin 2%; $NaIO_3$ 0.2%) prepared as described by Polle *et al*. (1978). After staining roots were washed in aerated distilled water for a further 30 min and then examined.

Extraction of Root Tips Proteins. Preparation and extraction of root-tips proteins were carried out following procedures described by Picton *et al*. (1991). Frozen root-tips were ground to a powder in liquid nitrogen and homogenized in two volumes of extraction buffer (NaCl 300 mM, Triton X-100 2%, EDTA 1 mM, EGTA 1 mM, ascorbic acid 5 mM, DTT 100 mM, PMSF 1 mM, and leupeptin 10 ug/ml). The homogenate was incubated at 25°C for 20 to 30 min and then protamine sulphate was added to 2 mg/ml and incubated continued for a further 10 min at 30°C. Cell debris was pelleted at 13 000 g (5 min, 4°C) and the supernatant transferred to fresh tubes. Proteins were precipitated overnight at -20°C following the addition of 5 volumes of 10% w/v TCA, 10 mM DTT in ice-cold aceton. TCA insoluble material was pelleted at 13 000 g (30 min, 4°C) and the pellet was washed twice with 10 m*M* DTT in acetone. Finally, the pellet was dried under room temperature and resuspended in 50 µL of buffer sample (Basu *et al*. 1994) consisting of 0.125 M Tris-Cl, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol.

Polyacrylamide Gel Electrophoresis. Protein samples were mixed with 50 µL of sample buffer and 5-8 µL blue bromophenol and incubated in boiled water for 3 min. SDS-PAGE gels (12.5%) with 6% stacking gel were run at 15 mA per slab for 10-12 h in a buffer of 25 mM Tris, 186 mM glycine, 1% w/v SDS. Following electrophoresis, gels were stained overnight with Commasie blue and destained for 2 d.

RESULTS

Root Growth. After 4 days grown in nutrient solutions containing 0.7 mM Al, the growth increment (final length minus initial length) of primary roots of Al-sensitive genotypes was reduced by 35 to 58% (average 46.5%), while in the tolerant genotypes the inhibition was only 20 to 21% (average 19.6%; Table 1 & Figure 1). The root increment was strongly inhibited by Al, the magnitude of the inhibition was much higher than that of root length. The inhibition of Al on root growth (root increment and root length) of Al-sensitive soybean genotypes was more severe than that of Al-tolerant soybean genotypes (Table 1 & 2). Root dry matter was affected to the same extent in two soybean genotypes by this short-term Al exposure (data not shown). From these results, we selected 0.7 mM was an appropriate concentration of Al to be used to observe protein difference in the root tips of soybean.

Al Accumulation. Hematoxylin staining of the two genotypes revealed their differential tolerance to Al, with Lumut (sensitive) tips staining much more intensely than Yellow Biloxi (tolerant) after Al treatment (Figure 2). It was obviously that the concentration of 0.09 Al with 17 h of time exposure was required to assess differential tolerance of the two genotypes. The results revealed that both Yellow Biloxi and Lumut gave similar response in Al accumulation at 0.07 mM Al (data not shown).

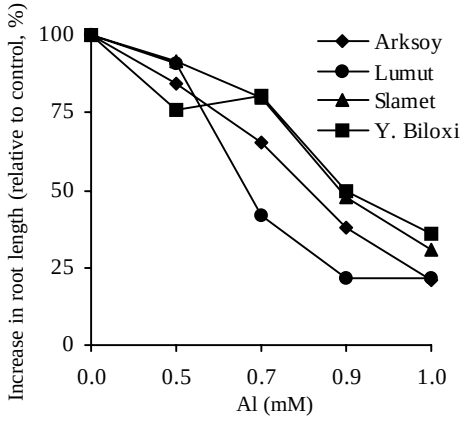

Figure 1. Effect of Al on root elongation of different soybean genotypes.

Table 1. Root increment (cm/day) of several soybean genotypes grown under control and Al stress condition

Soybean genotypes	Control	0.7 mM Al	% Inhibition
Tolerant			
Yellow Biloxi	1.15	0.93	19.1
Slamet	0.90	0.72	20.0
Average	1.03a	0.83a	19.6b
Sensitive			
Lumut	0.86	0.36	58.1
Arksoy	0.83	0.54	34.9
Average	0.85b	0.45b	46.5a

Seedlings were subjected to Al for 4 days. Different letters within columns indicate significant difference at 5% level (based on contrast orthogonal test)

Table 2. Root length (cm) of several soybean genotypes grown under control and Al stress condition

Soybean genotypes	Control	0.7 mM Al	% Inhibition
Tolerant			
Yellow Biloxi	15.05	13.48	10.4
Slamet	12.31	11.04	10.3
Average	13.68a	12.26a	10.4b
Sensitive			
Lumut	13.04	9.51	27.1
Arksoy	11.82	9.81	17.0
Average	12.43a	9.66b	22.1a

Seedlings were subjected to Al for 4 days. Different letters within columns indicate significant difference at 5% level (based on contrast orthogonal test)

Root tip proteins. SDS-PAGE analysis showed that protein profiles differ between root tips 0.5-0.8 cm and the next 2 cm of the root. Electrophoregram showed that root tips of Yellow Biloxi expressed a new protein band with molecular weight approximately 79.8 kD (Figure 3). The protein appeared when seedlings were subjected to Al for 48 or 72 h. On the other hand, Lumut had no specific protein expression in the RT region. Recovery test showed that synthesis of the protein 79.8 kD was regulated by Al. Analysis of protein of the R region showed that the two genotypes revealed the difference in protein profiles. At least five bands were detected in polyacrylamide gel for Lumut and six for Yellow Biloxi. Although both genotypes differ in response to Al treatments, only RT region (0.5-0.8 cm) appeared responsible to distinguish between sensitive and tolerant soybeans.

DISCUSSION

The physiological basis of Al-toxicity and genotypic tolerances in Al-tolerance for many plants have been documented, such as in rice (Sivaguru & Paliwel 1993), wheat (Miyasaka *et al*. 1989, Delhaize *et al*. 1993, Delhaize & Ryan 1995), and sorghum (Baligar *et al*. 1995). In soybean, however, as far as known, the experimental work addressing the physiological basis of Al tolerance in roots, is still limited. Our results (Table 1 & 2, Figure 1 & 2) indicated that in Al-tolerant soybean genotype the higher of Al tolerance seems to have emanated from Al exclusion, as evidenced by the lower Al accumulation in roots. This result strongly supports the previous results (Anwar 1995, Supijatno *et al.* 1995, Sopandie *et al.* 1996, Sopandie 1999, Sopandie *et al.* 2000a) in which Yellow Biloxi had a lower content of Al in its root tissue than Lumut. The previous experiment revealed that a lower accumulation of Al in roots of Al-tolerant genotype Yellow Biloxi was associated with their ability to increase pH in the media (Supijatno *et al.* 1995, Anwar 1995) as well as to release citric acid and malic acid from their roots (Sopandie 1999, Kasim *et al*. 2000), thereby minimizing the uptake of Al and reducing Al toxicity. Moreover, a visual detection using hematoxylin staining method (Fatimah 1997, Sopandie *et al.* 2000a) showed that the higher accumulation of Al in roots of sensitive Lumut was caused by a higher penetration of Al into their roots as compared to that of Al-tolerant Yellow Biloxi. In the present study, a lower accumulation of Al in roots of Al-tolerant Yellow Biloxi is likely associated with protein accumulation in roots, which was induced by Al stress.

Previous studies in wheat have clearly demonstrated that accumulation of proteins in roots is affected by exposure to Al (Picton *et al.* 1991, Rincon & Gonzales 1991), but evident linking synthesis of specific proteins to expression of Al tolerance is lacking. Our result revealed that profiles of proteins in roots of Lumut and Yellow Biloxi were clearly different (Figure 3 & 4). SDS-PAGE analysis of root tips (0.5-0.8 cm in length) proteins revealed a band with an apparent molecular mass of 79.8 kD, which showed significant accumulation in the Yellow Biloxi following exposure to 0.7 mM Al. This Al-inducible protein occurred within 48 h of initiation of Al stress. When we removed the plants from Al-containing nutrient solutions, the protein disappeared completely by 48 h. In Lumut, no specific protein was expressed in its root tips under Al treatments. This phenomenon indicated that Lumut have no specific response to Al stress.

Figure 2. Distribution of absorbed Al in root tips of Lumut (left) and Yellow Biloxi (Right). Roots stained with hematoxylin.

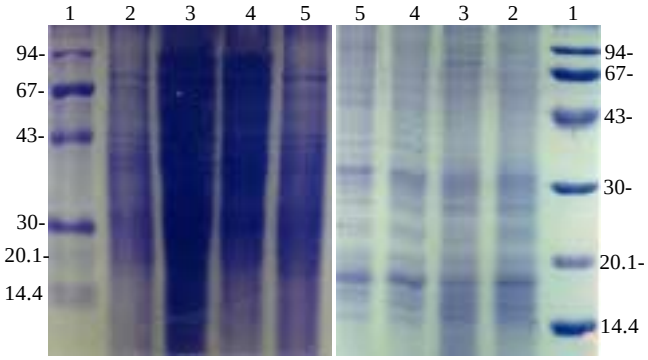

Figure 3. SDS-PAGE analysis of proteins from root tips, 0.5-0.8 cm, of Lumut and Yellow Biloxi. (1 = standard, 2 = control, 3 and 4 = 48 and 72 hours in 0.7 mM Al, 5 = 48 h after recovery).

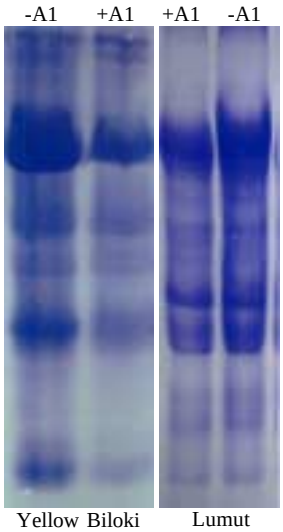

Figure 4. SDS-PAGE analysis of proteins from adjacent 2 cm of roots of Lumut and Yellow Biloxi (-Al= without Al, control; +Al = 48 h in 0.7 m*M* Al).

An investigation of spatial sensitivity in roots revealed that soybean appear to perceive Al stress in the apical regions of the root system (Ryan *et al.* 1993, Sopandie *et al.* 2000b). Our comparison of protein profiles in root tips (0.5-0.8 cm) and the next 2 cm of the root showed that specific protein was synthesized only in the root tip region. This fact suggests that this root-tip protein of Al-resistant genotipe Yellow Biloxi is strategically located to play a physiological role in Al tolerance.

A number of mechanisms have been suggested that proteins could operate either singly or together to provide Al resistance to plants (Taylor 1988, 1991). Our study showed that only in root tips of Al-resistant Yellow Biloxi a new protein was expressed as a physiological response to Al stress, although the role of this Al-inducible protein is still unknown. These proteins could be membrane proteins that actively export Al, as previously revealed by Taylor (1991) in wheat roots. In soybean, Anwar (1999) showed that the expression of gene encoding plasmamembrane-ATPase in root tips was induced by Al stress. In the present experiment, protein 79.8 kD of Yellow Biloxi might play a role in tolerance by mediating either decreased uptake or increased efflux of Al (exclusion of Al). Finally, however, purification and characterization of the protein 79.8 kD will be required to confirm a possible role in Al tolerance.

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