

Full Length Research Paper

Protein and polyphenol profile changes in soybean roots under aluminum stress

Dechassa Duressa*, Khairy Soliman and Ernst Cebert

Department of Natural Resources and Environmental Sciences, Alabama A&M University, Normal, AL 35762, USA.

Accepted 16 April, 2020

It is well documented that aluminum (Al) toxicity is the most important constraint of crop production on acid soils. Chelation of Al in the rhizosphere with root secreted organic acid anions is a common mechanism of Al tolerance in most plants including soybean. Phenolic substances are recently implicated as additional physiological mechanism of plant Al tolerance. This study was undertaken to investigate the role of phenolics in soybean Al tolerance and to examine protein expression changes in soybean roots under Al stress. An Al-tolerant soybean genotype PI 416937 and Al-sensitive Young were used in the study. Protein and polyphenol profile changes in response to Al stress, were examined in roots tips of hydroponically grown plants 72 h post treatment. Al significantly increased total phenol exudation from roots of the Al tolerant genotype PI 416937, whereas, flavonoid content did not vary with treatment. Al also altered the expression level of several proteins in genotypic and non genotypic specific manner. This is the first study to show that polyphenol is involved in soybean Al tolerance. Future research should consider quantification of individual flavonoid compounds in root tissue as well as culture solution, and sequencing and functional annotation of Al regulated proteins.

Key words: Soybean, aluminum tolerance, protein expression, phenolics.

INTRODUCTION

Aluminum (Al) toxicity is a major constraint of crop production on acid soils. In view of the fact that 40% of world's arable land is acidic (Kochian et al., 2004) Al toxicity remains as a major hurdle for increasing world food production especially in developing tropical and subtropical regions, where increase in food production is much needed. Aluminum reduces crop yield through root growth inhibition and impairment in nutrient and water uptake (Route et al., 2001).

Plants have some degree of tolerance to Al toxicity with tolerance level varying with species and among cultivars within species. The tolerance mechanisms are divided into exclusion and internal detoxification. Aluminum exclusion mechanisms include chelation and detoxification of aluminum at root-soil interface with organic ligands primarily citrate, malate and oxalate and also phosphate, phenolics, and polypeptides (Kidd et al., 2001; Mau et al., 2001; Kochian et al., 2004; Zheng et al., 2005) and reducing Al uptake by possessing low cell wall cation

exchange capacity (Eitcha et al., 2005; Hossian et al., 2006; Liu et al., 2008; Yang et al., 2008). Internal detoxification involves binding of Al in root cytoplasm with organic ligands and subsequent sequestration of non-toxic form of Al in leaf and root vacuoles (Kochian et al., 2004; Watanabe et al., 2006; Morita et al., 2007).

The detoxification of aluminum with organic acid anions is widely documented and it is the well known physiological mechanism of Al tolerance in plants. However, Al binding with organic acid anions alone does not explain all the variation in Al tolerance trait (Nian et al., 2004; Piñeros et al., 2005). Some agronomic plants with high degree of Al tolerance such as rice and signal grass do not employ organic acid anions as Al detoxification mechanism (Wenzle et al., 2001; Ma et al., 2002). This has led to the conclusion that multiple Al tolerance mechanisms exist in plants in species and genotype specific manner.

Phenolic compounds particularly flavonoid type phenolics have recently been implicated as a new class of plant metabolites involved in plant Al tolerance (Manu et al., 2001; Kidd et al., 2001; Barcelo' et al., 2002; Kochian et al., 2004; Tolrá et al., 2005; Morita et al., 2008). Phenolics impart Al tolerance by dual mechanisms

*Corresponding author. E-mail: dech_1@yahoo.com.

of anti-oxidant scavenging and Al chelation (Kidd et al., 2001; Kochian et al., 2004). Because of their high affinity for aluminum at neutral pH, phenolic compounds could contribute to internal aluminum detoxification mechanism. For example, a tea plant with very high internal Al tolerance level employs catechin- a flavonoid type phenolics to sequester Al in leaf vacuole (Mortia et al., 2008). In *Rumex acetosa L.*, Al induces high shoot levels of catechol, catechin and rutin as probable internal Al tolerance mechanism (Tolrá et al., 2005). At lower pH such as in rhizosphere, H⁺ competes with Al³⁺ for binding sites in phenolics molecules decreasing the effectiveness of phenolics in Al chelation. This is the case for simple phenolics such as catechol. On the contrary, flavonoid-type phenolics effectively out competes H⁺ and form very stable complex with Al under acidic condition making them a relevant chemical species for external Al detoxification (Kidd et al., 2001). Furthermore, research by Kidd et al. (2001) in maize demonstrated that flavonoid phenolics-catechin is exuded at much higher rate than citrate in response to Al in dose-dependent manner.

In soybean, Al tolerance is quantitatively inherited, suggesting multiple tolerance mechanisms (Bianch-Hall et al., 2000; Nian et al., 2004). Cellular response to Al toxicity involves cascade of events ranging from induction of Al tolerance genes, proteins and metabolites that ultimately detoxify aluminum. Duressa (2009) analyzed soybean genome for Al tolerance and identified putative Al tolerance genes. Zhen et al. (2007) profiled the proteome of an Al tolerant soybean genotype BX10 and detected some proteins with probable role in Al tolerance mechanism. The objectives of the present study were to:

(1) explore if phenolics play a role in soybean Al detoxification mechanism, (2) compare the proteome profile of Al-tolerant and Al-sensitive soybean genotypes under Al stress. Magnesium (Mg) treatment was included in the phenolics experiment to test if the Al toxicity ameliorative effect of Mg (Silva et al., 2001) operates through stimulation of biosynthesis and secretion of phenolics as Al detoxification mechanism.

MATERIALS AND METHODS

Plant culture and experimental design

Two soybean genotypes PI 416937 (Al-tolerant) and Young (Al-sensitive) were used in the study. Seeds were germinated in germination paper at 26°C for 72 h in an incubator. Three-day-old seedlings were transferred to hydroponic pot of about 4 L capacity filled with 800 µM CaCl₂ (control) or 800 µM CaCl₂ plus 10 µM Al (+Al) or 800 µM CaCl₂ plus 10 µM Al plus 50 µM Mg (Al +Mg) treatments, respectively. Fifteen seedlings per pot and three replicates per treatment of each genotype were grown in Conviron Environmental Chamber (Conviron, Inc.) at 28°C and 16 h light / 20°C and 8 h dark cycles for 72 h. At the end of the treatment period, 1 cm tap root tips in phenolics experiment and 1 cm tap and lateral root tips in proteome experiment were harvested on ice and stored at -70°C until further processing. In the phenolics

experiment, 3 intact seedlings were removed from each experimental unit at the end of 72 h treatment period and set up in 50 ml centrifuge tubes filled with 4 ml treatment solutions (control or +Al or Al + Mg) and set for additional 24 h at 20°C in the same growth chamber covered with aluminum foil to avoid treatment solution evaporation but with holes in the center for seedling insertion. Afterwards, the solutions were collected and stored at -70°C until phenol extraction.

Proteome analysis

Protein extraction and concentration assay

Plant culture, experimental design and sampling were as described above except magnesium treatment was not included. Protein was extracted using phenol following the procedure of Hurkman and Tanaka (1986) with some modification. Briefly, 2 g root tissue was homogenized in 6 ml extraction buffer using mortar and pestle. The homogenate was transferred to 15 ml tubes and centrifuged for 15 min at 4000 rpm. Water saturated phenol was then added to supernatant of the homogenate at 1:2 (phenol: homogenate ratio) and the mixture shaken vigorously for 10 min and centrifuged again for 15 min at 4000 rpm.

The supernatant after the centrifugation step was discarded and the phenol and interface were collected and washed with wash buffer (0.4 M Tris HCl, 0.6 M NaCl, 5 mM EDTA, 3% mercaptoethanol) 3 times at 1:1 sample to wash buffer ratio with centrifugation for 10 min at 4000 rpm after each wash and discarding the supernatant. Samples were then precipitated for several hours at -20°C in 0.1 M of ammonium acetate (NH₄OAc) solution prepared in methanol-solvent. Protein pellet was dried in Speed Vac and the resulting powder stored at -20°C until gel analysis was done. Protein concentration of extracts was determined using Bradford method (Bradford, 1976) with bovine serum albumin as standard.

Two-dimensional (2-D) gel electrophoresis

First dimensional analysis or Iso-electro focusing (IEF) of the protein samples was performed using 7 cm long immobilized pH gradient IPG gel strips pH 3-10 (Bio-rad). The IPG strips were rehydrated overnight in 500 µl per lane rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT and, 0.2% bio-lyte ampholytes). Protein samples were dissolved in rehydration buffer by mixing well with tiny spoon at a protein: buffer ratio of 1 ml to 10 mg and kept at room temperature for 1 h and centrifuged for 3 min at 1600 rpm in 1.6 ml centrifuge tubes and the supernatant transferred to fresh tube and the bottom precipitate discarded. Approximately 500 µg protein was loaded per IPG strip. Protein and rehydration buffer loaded IPG strips were kept at room temperature for 12 h in closed trays for rehydration. Iso-electric focusing (IEF) conditions for IPG strips were starting voltage of 0, end voltage of 4,000 V, Volt-hr of 8-10,000 V-hr and rapid ramp at 20°C (Bio-rad Catalog # 163-2099).

After focusing, proteins were reduced by equilibrating IPG strips with 1 ml per IPG strip fresh equilibration buffer I (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 5% glycerol and 2% (w/v) DTT) for 10 min, and 1 ml per IPG strip fresh equilibration buffer II (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 5% glycerol, and 2.5% (w/v) iodoacetamide, and 1 µl bromophenol blue) for 10 min. Equilibrated IPG gel strips were stored at -70°C until second dimension gel electrophoresis. Gradient gel was used for second dimension electrophoresis. Electrophoresis was done at 75 volt for 30 min and then 120 volts for 4 h. Gels were stained with Comassie blue and scanned with 2400 dpi hap scanjet 5500 c. Gel images were digitalized using Ludesi Pro software (ludesi.com) and up-and-down regulated protein spots were detected by comparing protein spot volume

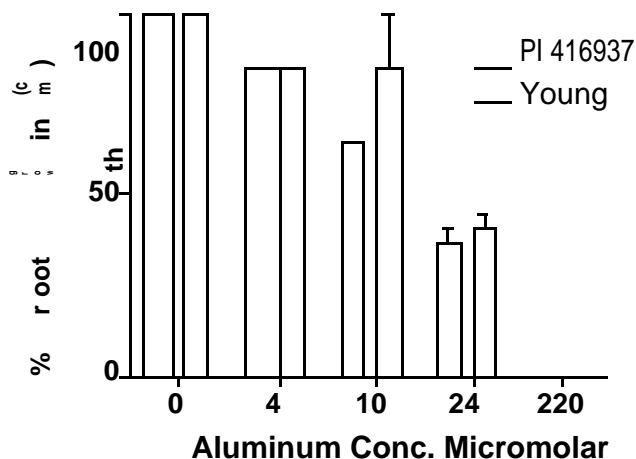


Figure 1. Aluminum dose effect on root growth of Al-tolerant soybean line PI 416937 and Al-sensitive cultivar Young in 800 µM CaCl₂ hydroponic medium. Data are mean ± SD.

changes between control and Al treated samples of each genotype.

Sample preparation for phenol extraction

One-hundred milligram root tissue was ground using mortar and pestle under liquid nitrogen. Ground samples were extracted with 80% acetone (100 mg tissue/ ml acetone) for 1 h. Extracts were filtered using Fisher brand 25 mm syringe filters (0.22 µm, PVDF filters Cat. no. 09 – 730 - 25) using disposable LS 1 ml syringe part # S7510-1. Filtered samples were evaporated at 45°C in Speed Vac from 1 to 0.1 ml (Sun and Liu, 2006). The 0.1 ml liquid-sample was resuspended in 0.4 ml deionized water and stored at -70°C until phenol quantification was done. Hydroponic sample solutions were processed in the same fashion with the exception that 0.5 ml sample solution was extracted with 0.5 ml 80% acetone.

Quantification of flavonoid

Flavonoid content was determined using the procedure in Sun and Liu (2006) with slight modification. Briefly, 25 µl sample, 125 µl water and 7.5 µl of 5% (v/v) NaNO₂ were pipetted into 96-well tray in that order and incubated for 6 min. To this mixture, 15 µl of 10% (w/v) AlCl₃ was added and samples incubated for 5 min. Finally, 50 µl 1 M NaOH and 27.5 µl deionized water were added and absorbance read at 510 nm using Synergy HT micro plate reader (Biotek, Inc). Catechin standards of concentrations 0 mg/ml, 0.025 mg/ml, 0.5 mg/ml, 1 mg/ml and 5 mg/ml were prepared in 16% (v/v) acetone from 10 mg/ml stock solution which is also prepared in 16% acetone, blank was 16% (v/v) acetone. Standard curve was constructed using the relationship between absorbance and concentration of the catechin dilutions and samples flavonoid concentrations were extrapolated from the curve. Data was analyzed with statistical analysis software (SAS) and for significant effects means were separated using Tukey test.

Quantification of total phenol

Total phenolics was determined in same extracts as in flavonoid using the 96-well plates and Biotek Synergy HT plate reader. To a 12.5 µl sample, 50 µl deionized water, 12.5 µl Folin-Ciocalteu

colorimetric reagent, and 125 µl of 7% Na₂CO₃ were added in that order. Samples were then incubated for 1 h and absorbance read at 710 nm, blank was 16% acetone. Standard curve was constructed from gallic acid dilution series of 0, 0.025, 0.25, 0.5 and 1 mg/ml that were titrated from 10 mg/ml stock solution and samples total phenol concentrations were extrapolated from the curve. Data was analyzed with statistical analysis software (SAS) and for significant effects means were separated using Tukey test.

RESULTS AND DISCUSSION

Aluminum dose effect on soybean root growth

To identify an optimum dose of aluminum that best distinguish between the tolerant and sensitive genotypes and establish a protocol for the research, an aluminum dose experiment was conducted at Al concentrations of 0, 4, 24 or 220 µM using plant growth conditions described under material and methods. Tap root length of approximately 15 seedlings of each genotype was measured in cm before and after 72 h treatment period in hydroponics and the difference between, after and before treatment measurements was taken. Root elongation of tap roots were calculated as percentage of control as follows:

$$\text{Root growth (\%)} = \frac{\text{Root length after treatment (cm)} - \text{root length (cm) before treatment}}{\text{Final root length control (cm)} - \text{initial root length control (cm)}}$$

The results revealed that 10 µM Al produced the largest difference in root growth between the two genotypes (Figure 1). Beyond that dose, the difference between Al tolerant and sensitive genotypes disappeared and at 220 µM Al the root growth of both genotypes was nearly completely inhibited. Silva et al. (2001) observed a large difference in root growth between PI 416937 and Young at 1.5 µM Al activity in similar culture medium. The 10 µM Al dose was used in the present study. This dose was calibrated based on actual plant response without the necessity of estimating Al activity with chemical speciation software as is done by most authors and could be adapted by other investigators using dilute calcium chloride hydroponic medium for soybean Al toxicity studies.

Aeration experiment

Hydroponic experiments involve growing plants in liquid medium that restrict oxygen circulation that might have detrimental effect on root growth. Cognizant of this fact, many researchers provide supplementary aeration in experiments involving hydroponic system. In the growth chambers used in the current experiment, an in-built air pump lines were lacking and any other convenient way of aerating large number of pots containing the liquid medium was not available.

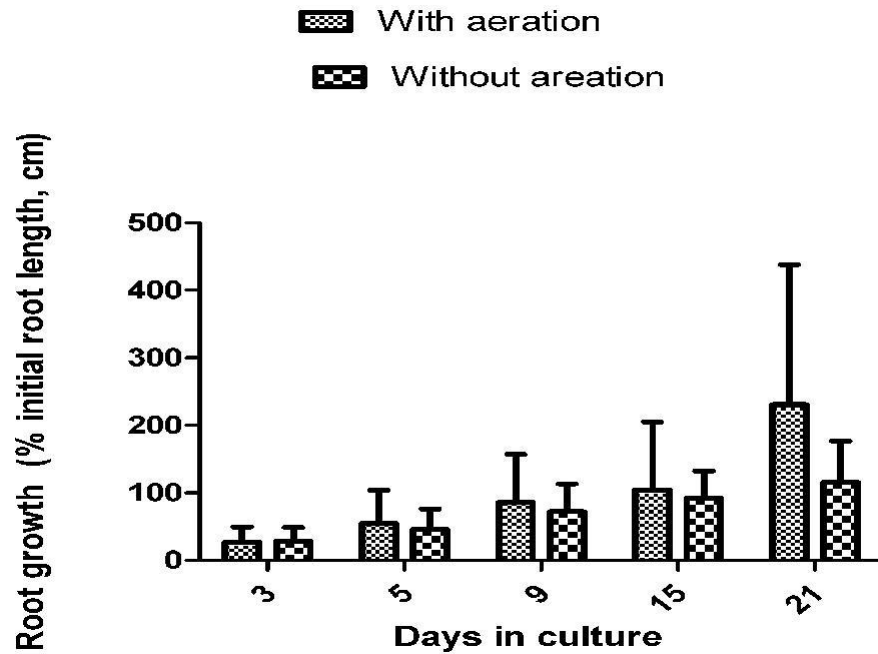


Figure 2. Soybean root growth as affected by aeration status of hydroponic medium. Data are mean \pm SD.

Aeration experiment was thus, conducted using aquarium air pumps to address the issue. Plants were grown with or without aeration in dilute calcium solution medium for 21 days. Root growth measurements were taken over time interval and mean and standard deviation calculated on the dataset. The data showed that aeration did not have any measureable impact on root growth until 21 days (Figure 2). Subsequent experiments were therefore conducted without supplementary aeration. This finding highlights in short-term hydroponic experiment which is the case in most Al toxicity studies in plants, supplementary aeration can be bypassed, although caution should be made as response could vary with plant species.

Proteome response of soybean roots to Al stress

Several proteins showed change in expression level in response to Al treatment (Figures 3, 4 and 5). The expression levels were estimated with Ludesi Pro image analyzer (Ludesi.com). A total of 26 (7 down regulated and 19 upregulated) proteins in Young (Figure 4) and 25 proteins (7 down regulated and 18 upregulated) in PI 416937 (Figure 5) showed differential expression between control and Al treated samples. Ten proteins, spot id 4, 26, 66, 71, 72, 96, 97, 107, 111, and 113 are in common between Young and PI 416937 (Figures 4 and 5); all except 26 were up regulated in both genotypes.

The pI of the proteins ranged from 4 to 8 (Figure 3), suggesting both acidic and basic proteins were among Al

responsive proteins. Molecular weight of the Al responsive proteins ranged from 10 - 40 kDa. In soybean roots under Al stress, Zhen et al. (2007) identified 39 proteins with pI values in the range of 4 - 5 in Al-tolerant soybean genotype BX10. Similarly, Yang et al. (2007) detected acidic proteins in rice roots under Al stress.

The spread of the Al responsive proteins over acidic and basic pI range in the present study reflects their uniqueness to the soybean genotypes studied. Functional analysis of Al responsive proteins in rice and tomato revealed up-regulation of primarily proteins involved in antioxidation and detoxification mechanisms (Yang et al., 2007; Zhou et al., 2009).

In Al-tolerant soybean genotype BX10, ABC transporter, heat shock proteins, glutathione S-transferase, chalcone-related synthetase, GTP-binding protein, and ATP binding protein were induced by Al stress (Zhen et al., 2007). Determination of the identity and function of the proteins regulated by Al in the current research is the subject of future study.

Root tip tissue and culture solution flavonoid and total phenol changes

Analysis of variance showed that main effects of treatment and genotype were significant ($p < 0.05$), means were therefore presented for those effects (Figures 6 and 7). Tissue total phenol was significantly lower under magnesium plus aluminum compared to control or Al only treatments. In the culture solution, total phenol was

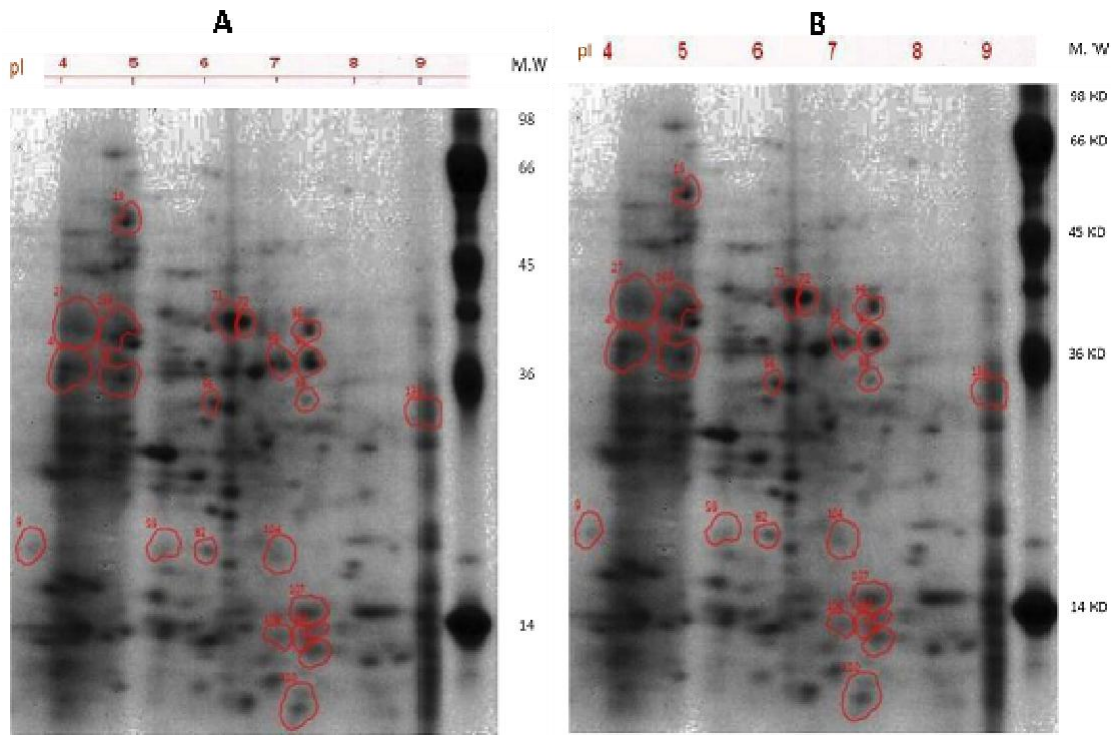


Figure 3. 2-D gel map of aluminum regulated proteins in two soybean genotypes: (A) PI 416937, (B) Young. pI = isoelectric point, M.W. = molecular weight of size marker. Marked spots are proteins that are either up-regulated or down-regulated in response to Al treatment as shown in Figures 4 and 5. Note that the protein spots shown are from reference gel for each genotype.

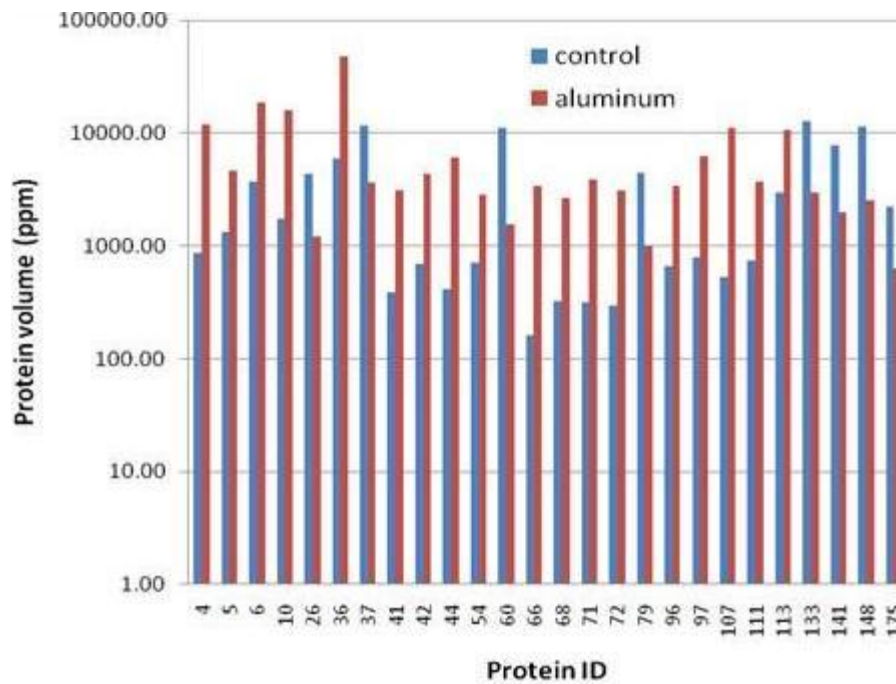


Figure 4. Spot volume (ppm) of aluminum regulated proteins in soybean genotype Young. Filter criteria: spot volume > 1000, presence \geq 100, fold change > 3. Note that when blue bar towers red the protein is down-regulated and when red bar towers blue the protein is up-regulated.

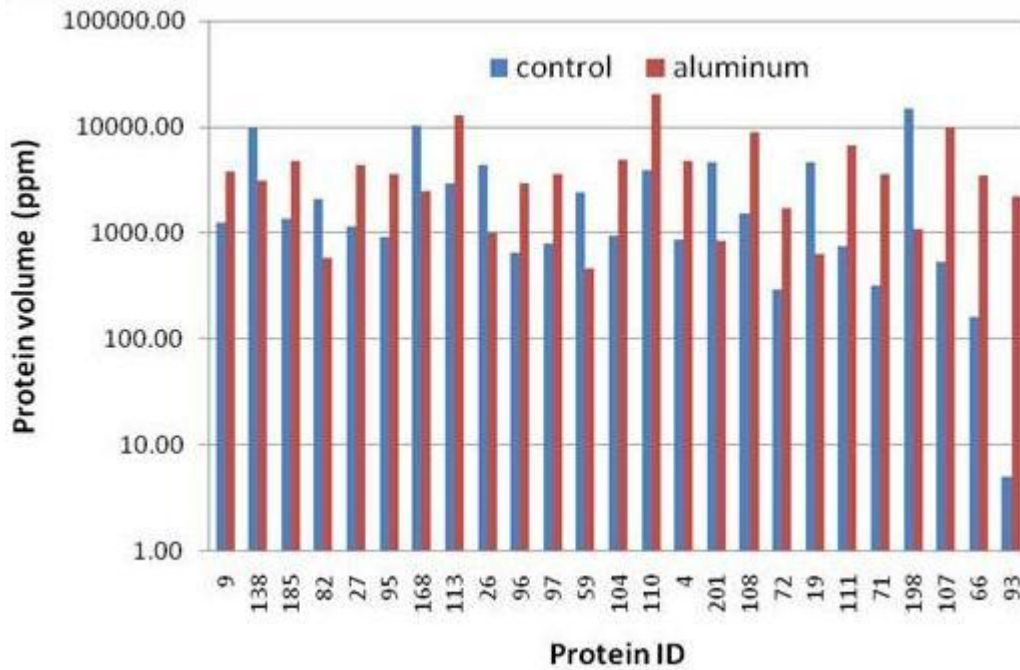


Figure 5. Spot volume (ppm) of aluminum regulated proteins in soybean genotype PI 416937. Filter criteria: spot volume >1000; presence \geq 100, fold change > 3. Note that when blue bar towers red the protein is down-regulated and when red bar towers blue the protein is up-regulated.

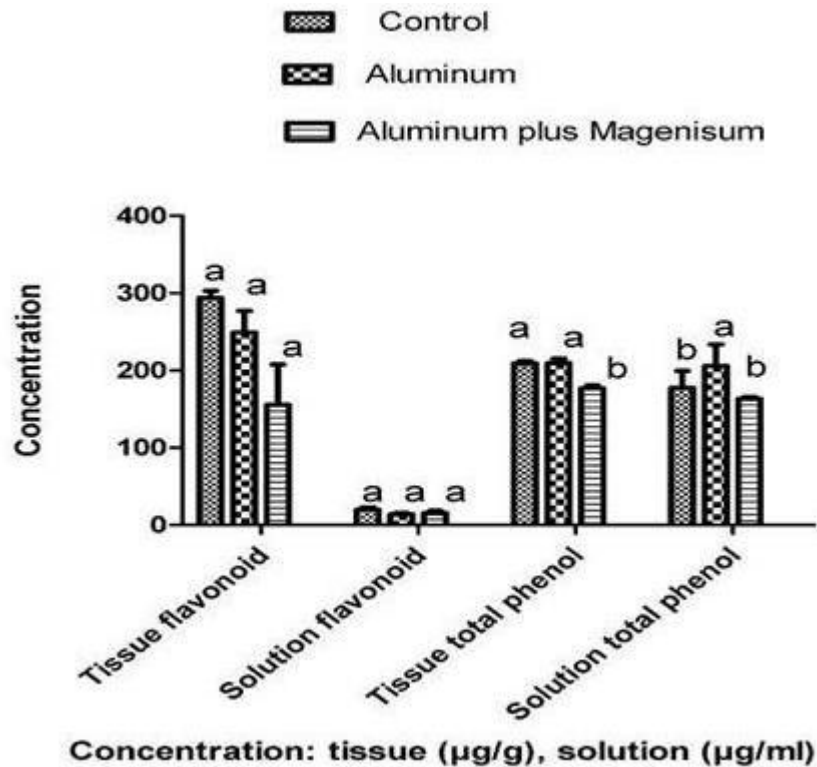


Figure 6. Concentrations of flavonoid and total phenol in soybean roots and hydroponic solution under three treatment regimes. Within a group means with same letter are not significantly different according to Tukey test ($p < 0.05$). Data are mean \pm SD.

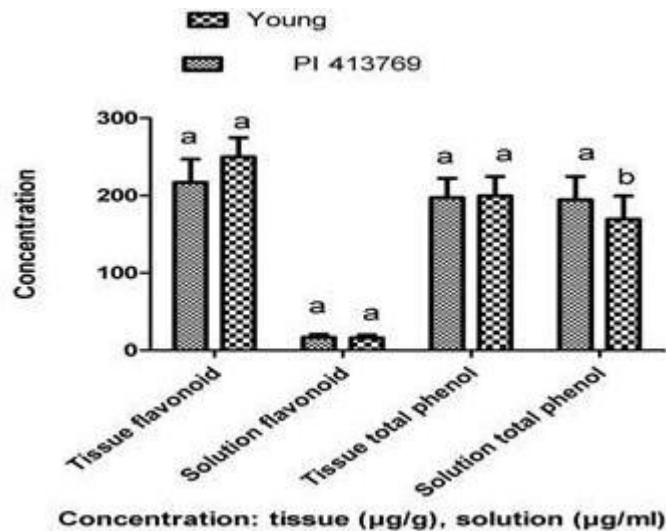


Figure 7. Tissue and culture solution concentrations of flavonoid and total phenol in hydroponic soybean averaged over three treatments (control, aluminum and aluminum plus magnesium). Within a group means with common letters are not significantly different according to Tukey test ($p < 0.05$). Data are mean \pm SD.

significantly higher in Al treated samples compared to control or aluminum plus magnesium treated samples averaged over both genotypes (Figure 6).

These observations suggest that magnesium down-regulates biosynthesis of phenolics in plants under Al stress, whereas, Al stimulates it. Study on gene expression in soybean roots under Al stress by Duressa (2009) showed that magnesium down-regulates metabolic and other genes commonly induced by Aluminum. Phenolic compounds are triggered in plants in response to biotic and abiotic stresses including aluminum (Kidd et al., 2001; Barceló et al., 2002; Shirely, 2002). And it has been amply demonstrated that magnesium ameliorates Al toxicity in soybean (Silva et al., 2001). Duressa (2009) hypothesized that the mechanism of magnesium neutralization of Al toxicity in soybean involves reduction in the energy cost of gene expression as most genes commonly induced by Al stress were down-regulated when Mg is added to Al containing culture solution. The saved energy is alternatively used for growth. The decrease in biosynthesis and exudation of phenolics under magnesium treatment in the current study conforms to that preposition. At same time, the stimulation of root phenol secretion under aluminum treatment, suggests that phenolic substances play a role in protecting soybean from Al toxicity.

Genotypic differences in tissue and culture solution flavonoid and total phenol

The genotypic effect was significant for culture solution total phenol averaged over the three treatments

($p < 0.05$). Whereas the differences in tissue and culture solution flavonoid and tissue total phenol were of no statistical significance (Figure 7). The exudation of flavonoid type phenolics in response to Al treatment have been reported in maize (Kidd et al., 2001), and that of total phenol in Norway spruce (Heim et al., 2001). In the present study, total phenol exudation was significantly higher under Al stress in the Al tolerant genotype PI 416937 (Figure 7) suggesting that this genotype probably employ exudation of phenolic substances as Al tolerance mechanism.

However, the flavonoid type phenolics, the most effective class of polyphenols in Al chelation (Kidd et al., 2001; Barceló et al., 2002) did not show difference among treatments or between genotypes. At gene expression level, however, Duressa (2009) observed the induction of a gene for isoflavonoid synthesis in Al tolerant genotype PI 416937 as a clue to the involvement of flavonoid type phenolics in Al tolerance mechanism of this genotype. Sample size probably affected the sensitivity of the statistical test to detect treatment effect for flavonoid concentration. Future research should consider large sample size and measurement of individual flavonoid compounds in tissue and culture solution to further characterize the importance of phenolics in soybean Al tolerance.

Conclusion

From the present study, we conclude that 10 μ M Al concentration is an effective dose for discriminating between Al-tolerant and Al-sensitive soybean genotypes

in dilute calcium chloride hydroponic medium and in short-term experiments soybean can be grown in hydroponic solution without aeration. In addition, Al-tolerant soybean genotype PI 416937 secretes phenolic substances to rhizosphere as a possible Al tolerance mechanism and aluminum alters the protein profile of soybean roots in genotypic and non genotypic specific manner. Future research should consider assay of individual flavonoid compounds and sequencing and functional annotation of soybean Al regulated proteins.

ACKNOWLEDGEMENT

We are grateful to USDA Plant Genetic Resources and Dr. Thomas Carter of North Carolina State University for generous gifts of the seeds of soybean genotypes used in this study. We sincerely thank Dr. Gafur Mavlonov for technical assistance in protein 2-D gel analysis. This research was supported in part by NSF/EPSCOR grant no. 05026. We owe thanks to Alabama Graduate Research Scholars Program for its financial support. Contributed by Agricultural Experiment, Station, Alabama A&M University.

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