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Effect of elevated Al and pH on the growth and root morphology of Al-tolerant and Al-sensitive wheat seedlings in an acid soil

Efecto de la elevación de Al y pH en el crecimiento y la morfología de la raíz de plantas de trigo tolerantes y sensibles al Al en un suelo ácido

Efeito do aumento do teor de Al e do pH no crescimento e na morfologia da raiz de plantas de trigo tolerantes e sensíveis ao Al num solo ácido

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ABSTRACT

Aluminium ion (Al^{3+}) toxicity and hydrogen ion (H^{+}) activity are the major constraints for plant growth in acid soil. This study was undertaken to determine the effect of pH and Al on the growth response and changes in root morphology of Al-tolerant (ET8) and Al-sensitive (ES8) wheat seedlings. Different levels of $AlCl_3$ and $CaCO_3$ were added to the soils to manipulate soil pH and extractable Al. The results showed that the bulk soil pH remained constant at pH 4.1 with further applications of $AlCl_3$, and that the seedlings died at the 200 mg $AlCl_3$/kg treatments. The ET8 seedlings responded better than the ES8 seedlings in both low and high Al and pH. The ET8 seedlings had higher root surface areas and root tip numbers than the ES8 seedlings in the Al treatment. In contrast, the ES8 had higher root diameters than the ET8 seedlings due to the elevated Al supply. Apoplast Al increased with the increase of soil available extractable Al, and declined with the decrease of soil extractable Al. The ET8 seedlings accumulated more Al in their apoplast than the ES8 seedlings. This study concluded that accumulation of Al in the apoplast is also involved in Al tolerance mechanism with the addition of organic acid exudation.

Abbreviations: ALMT1, Aluminium activated malate transporter; **PCV**, Pyrocathecol Violet; **NSW**, New South Wales.

RESUMEN

La toxicidad del ión aluminio (Al^{3+}) y la actividad del ión hidrógeno (H^{+}) son los factores que más limitan el crecimiento de las plantas en un suelo ácido. Este estudio se llevó a cabo para determinar el efecto del pH y el Al sobre la respuesta en el crecimiento y los cambios en la morfología de la raíz de plantas de trigo tolerantes (ET8) y sensibles (ES8) al Al. Se añadieron diferentes cantidades de $AlCl_3$ y $CaCO_3$ al suelo para producir variaciones en el pH y en el Al extraíble del suelo. Los resultados mostraron que el pH neto del suelo permaneció constante en un valor de 4,1 con aplicaciones adicionales de $AlCl_3$ y que las plantas murieron con los tratamientos realizados con 200 mg $AlCl_3$/kg. Las plantas ET8 respondieron mejor que las ES8 bajo condiciones tanto altas como bajas de Al y pH. Para el tratamiento realizado con Al, las plantas ET8 presentaron mayor área superficial de raíces y mayor número de raicillas que las plantas ES8. Por el contrario, las plantas ES8 mostraron mayores diámetros de raíz que las plantas ET8 debido a la elevada disponibilidad de Al. El Al apoplástico se incrementó con el aumento de Al extraíble disponible en el suelo y se hizo menor con la disminución de Al extraíble. Las plantas ET8 acumularon más Al en su apoplasto que las plantas ES8. Este estudio concluye que la acumulación de Al en el apoplasto también está implicado en el mecanismo de tolerancia al Al con la adición de exudación ácida orgánica.

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RESUMO

A toxicidade do ião Alumínio (Al^{3+}) e a atividade do ião hidrogénio (H^{+}) são os fatores que mais limitam o crescimento das plantas em solos ácidos. Este estudo foi realizado para determinar o efeito do pH e do alumínio na resposta ao crescimento e nas alterações da morfologia das raízes de plantas de trigo tolerantes (ET8) e sensíveis (ES8) ao Al. Adicionaram-se quantidades diferentes de $AlCl_3$ e $CaCO_3$ ao solo para produzir variações no pH e no Al extraível do solo. Os resultados mostraram que o pH do solo permaneceu constante num valor de 4,1 com aplicações adicionais de $AlCl_3$ e que as plantas morreram nos tratamentos realizados com 200 mg $AlCl_3$/kg de solo. As plantas ET8 responderam melhor que as ES8 quer sob condições de concentrações elevadas quer baixas de Al e quer a valores elevados quer baixos de pH. Para o tratamento realizado com Al, as plantas ET8 apresentaram uma maior área superficial e um maior número de raízes que as plantas ES8. Pelo contrário, as plantas ES8 apresentaram maiores diâmetros radiculares que as plantas ET8 devido à elevada disponibilidade de Al. O Al apoplástico aumentou com o aumento do Al extraível disponível no solo e diminuiu com a redução de Al extraível. As plantas ET8 acumularam mais Al no seu apoplasto que as plantas ES8. Este estudo concluiu que a acumulação de Al no apoplasto também tem implicações no mecanismo de tolerância ao Al através da exsudação de ácidos orgânicos.

KEY WORDS
Apoplast Al, genotypic variation, lime

PALABRAS CLAVE
Al apoplástico, variación genotípica, cal

PALAVRAS-CHAVE
Al apoplástico, variação genotípica, calcário

1. Introduction

Low pH and high concentrations of toxic Al are the major causes for poor plant growth in acid soils (Bose et al. 2010). Generally, Al^{3+} activities reduce plant growth in low pH soil and therefore it is necessary to study Al^{3+} stress in combination with low pH soil (Lazof and Holland 1999). It is also important to know how plants respond under low Al^{3+} activities and high pH, in order to gain a deeper understanding of Al-tolerance mechanisms. Although some research has examined plant growth response under Al^{3+} activities with low pH soils, few experiments have been conducted under low Al^{3+} activities and high pH conditions (Ma et al. 2003).

Plant species and different genotypes within species respond differently to Al^{3+} toxicity (Iqbal 2012a). For example, Al-tolerant wheat (ET8) seedlings release 10 times more malate from the root tips than the Al-sensitive wheat (ES8) seedlings when exposed to toxic levels of Al^{3+} (Delhaize et al. 1993). This released malate chelates Al^{3+} in the rhizosphere of ET8 seedlings and enables the ET8 seedlings to grow better than ES8 seedlings (Ryan et al. 1995). This malate exudation has been quantified for ES8 and ET8 genotypes in solution culture using excised root tips of wheat seedlings (Kataoka et al. 2002). However, solution culture experiments avoid the chemical and biological complexities that occur in soil (Schefe et al. 2008), and thus soil-grown experiments with high Al^{3+} are needed to verify the genotypic variation.

Plant species and genotypes also respond differently to soil pH. One solution culture study confirmed that ES8 seedlings grew better at pH 5.5, whereas ET8 seedlings grew better at pH 4.2 (Babourina et al. 2006). Another soil-grown experiment showed that the ET8 seedlings responded better than the ES8 seedlings irrespective of the native soil pH (Uddin and Iqbal 2012). This different growth response between ES8 and ET8 may be due to pH differences in both solution and soil (Stewart and Lieffers 1994). The better growth response of ET8 compared to ES8 at low pH may also be associated with the loosening

of pectin bonds in an acidified medium (Cleland 2002). However, it is unclear how these two genotypes behave in high pH soils with respect to amendment with lime. Therefore, genotypic variation with respect to high pH soil amended by lime will be considered in this study.

It was found from my previous study (Iqbal 2012b) that $AlCl_3$ application to soil reduced the bulk soil pH and increased extractable Al. Also, the root length of both ET8 and ES8 seedlings decreased with increased extractable Al in the bulk soil. However, no genotypic variation was studied specific to $CaCO_3$ supply in the previous study. Therefore, root morphological changes and genotypic variation in relation to $CaCO_3$ supply was examined in this study. The aims of this experiment were therefore to compare the growth response of ET8 and ES8 wheat seedlings in relation to a spectrum of pH levels and Al concentrations and to identify root morphological characteristics that might explain the difference in Al tolerance. The hypothesis of this study was that the ET8 seedling would respond better than the ES8 seedlings with respect to $CaCO_3$ supply.

2. Materials and Methods

2.1. Soil and plants

A Podosol according to the Australian classification (Isbell 2002) and Podzol according to the World Reference Base for Soil Resources (IUSS Working Group WRB 2006) was used in this study. It had an initial pH of 4.5 and an extractable Al of 4.98 mg/kg, with both measurements being made in 0.01 M $CaCl_2$. The Al-tolerant (ET8) and Al-sensitive (ES8) wheat genotypes were used in this experiment. Other properties of the soil were described in **Table 1**.

2.2. Plant genotypic characteristics

Al-tolerant (ET8) and Al-sensitive (ES8) wheat (*Triticum aestivum* L.) genotypes were used in this experiment. These genotypes were near-isogenic (over 95%) lines differing in Al tolerance at the *Alt* locus (Ahn and Matsumoto 2006). However, the lines were obtained by eight fold backcrossing and differed in the Al-tolerance conferred by the single *Alt1* gene (Ryan et al. 1997). They were derived from a cross between the Al-tolerant Brazilian cultivar Carazinho and the Al-sensitive cultivar Egret, with the resulting progeny backcrossed eight times to Egret or derivates of Egret and recurrent selection (Delhaize et al. 1993; Fisher and Scott 1987). Also, ES8 and ET8 differed in their Al tolerance due to *TaALMT1* gene (Sasaki et al. 2004).

Table 1. Properties of soils used in this experiment

Soil type	Podzol
Collection site	Frankston, Victoria, Australia
GPS location	38°14´S 145°22´E
pH buffering capacity	0.24 cmol/kg/pH
EC_s(1:5)	0.038 mS/cm
Water content at Ψ_m = -33kPa	13% w/w
Olsen-P	1.4 mg/kg
Particle size distribution	Sand 95%, silt 0.6% and clay 4.4%
Total N	0.05%
Total C	0.15%
NH_4-N	0.9 mg/kg
NO_3-N	1.6 mg/kg

2.3. Experimental design and pre-incubation procedure

The experiment had a completely randomised design with 13 treatments, comprising 8 $AlCl_3$ and 5 $CaCO_3$ rates, in combination with the 2 genotypes of ET8 and ES8, with all treatment combinations replicated 3 times. The eight levels of $AlCl_3$ were 0, 50, 100, 200, 300, 400, 600 and 800 mg/kg and the five levels of $CaCO_3$ were 0, 130, 250, 500 and 1000 mg/kg. The $CaCO_3$ was directly applied to soil in a powder form and mixed within the soil before pre-incubation. $AlCl_3$ was added as a stock solution to soil and the soil was pre-incubated at 30 ^{0}C for 7 days before sowing (Iqbal 2012b).

2.4. Seed germination and plant sowing

Uniform-size seeds were selected for germination. The seeds were germinated on moist paper towel in the dark at 25 ^{0}C for 70 h. Eight holes (1.0 cm depth) were made in the soil in each plastic cups which contained 200 g pre-incubated soil. Then, eight uniform pre-germinated seeds of ES8 or ET8 were placed carefully in these holes in each cup. The germinated seeds were sown in the same way with their radicals pointing downwards and then they were gently covered with the same treated soil. After sowing, each cup was covered by filter paper for first two days to avoid disturbance of top soil. Deionised (DI) water was sprayed from top on the filter paper. The soil was kept at field capacity (15% w/w) by weighing pots during incubation and the growing period of wheat plants. Basal nutrients were not applied to the soil and so the seedling growth relied only on seed reserves.

2.5. Plant growth condition

Plants were grown in a growth cabinet with day/night temperatures of 20/18 ^{0}C, with 10 h of dark and 14 h of light conditions and an average light intensity of 210 µM photons/m^2/s. All cups were re-randomised within the growth chamber on alternate days during the incubation and the growing period for the wheat seedlings, to minimize positional effects.

2.6. Plant harvest

Plants were harvested 6 days after sowing. Whole plants with roots and surrounding soil were removed from each cup by gentle agitating to provide minimum disturbance to the roots and shoots. Intact plants were then lifted gently from the soil and shaken lightly to remove bulk soil from the roots. Collected bulk soil was air-dried and stored in a controlled temperature (25 ^{0}C) room until analysis. Shoots and roots were separated and the shoots were dried at 70 ^{0}C in an oven for a minimum of 3 days before analysis. Roots were washed three times by deionised water to remove adhered soil from the external root surfaces. Then the roots were submerged in 50-ml vials containing 20 ml of 50 mM $BaCl_2$ solution that had been chilled to 4 ^{0}C for 45 minutes. All vials were shaken gently for 45 minutes at the chilling temperature of 4 ^{0}C to desorb apoplast Al in the 50 mM $BaCl_2$ solution (Iqbal et al. 2010). After the desorption of this apoplast Al, all tubes were stored in the freezer until measurements of apoplastic Al in the solution were made. The root length was then measured using a root scanner. After measuring the root length, roots were washed by deionised water and dried at 70 ^{0}C in an oven for minimum 3 days before analysis.

2.7. Analytical procedure

Bulk soil pH was determined in 0.01M $CaCl_2$ solution after overnight (17 h) shaking. Extractable Al in this 0.01M $CaCl_2$ extract was determined using the PCV method. The desorbed apoplast Al was also determined using this PCV method with the standard solutions made up in 50 mM $BaCl_2$ solution to maintain similar ionic matrix for the measurement. Root and shoot samples were cut into small pieces and digested in a mixture of concentrated nitric and perchloric acid (4:1) with stepwise heating using a Tecator DS 400 digestion system, until 230 ^{0}C was reached, and then held for 20 minutes. The Al concentration in the digest was determined calorimetrically by using the PCV method (Kerven et al. 1989) using a Cary 50 Bio, UV-visible spectrometer, with the pH in each sample being adjusted to 2.0 prior to the colour development step.

2.8. Statistical analysis of data

The experiment was set up in a completely randomised design consisting of eight $AlCl_3$ and five $CaCO_3$ treatments with three replicates. Soil data were analysed by a one-way analysis of variance for the effect of $AlCl_3$ and $CaCO_3$ applications to the soil. Six replicates were used for this analysis as there were no effects expected from the wheat genotypes. Seedling growth and composition data were analysed by a two-way analysis of variance for the main effects and interactions between $AlCl_3$ and $CaCO_3$ applications and wheat genotypes. All statistical analyses were conducted using Genstat 5th ed for Windows (Lawes Agricultural Trust, UK).

3. Results

3.1. Effect of $AlCl_3$ and $CaCO_3$ supply on soil pH and extractable Al

The bulk soil pH declined with increased application of $AlCl_3$ up to 200 mg/kg, but then did not decline further with following $AlCl_3$ applications. In contrast, the addition of $CaCO_3$ linearly increased the bulk soil pH values (**Figures 1a and 1b**).

The extractable Al in bulk soil increased markedly with increasing rates of $AlCl_3$. In contrast, the extractable Al in bulk soil declined with the increasing amounts of $CaCO_3$ and no extractable Al was detected in the 500 and 1000 mg $CaCO_3$/kg treatments (**Figures1c and 1d**).

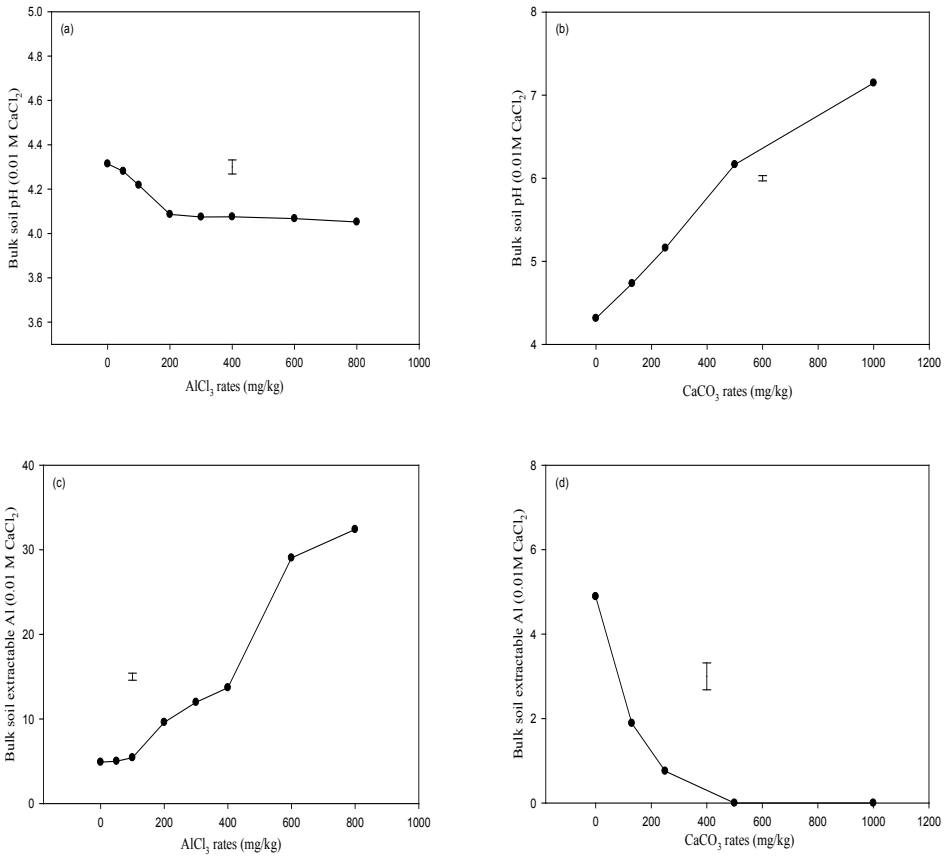

Figure 1. Effect of $AlCl_3$ and $CaCO_3$ addition on bulk soil pH (**a** and **b**) and extractable Al in bulk soil (**c** and **d**). Data were means of six replicates. $AlCl_3$ and $CaCO_3$ treatments were highly significant ($P < 0.001$) for both measurements. Vertical bars represent LSD ($P = 0.05$) for $AlCl_3$ and $CaCO_3$ separately.

Regarding the $AlCl_3$ and $CaCO_3$ applications, a close relationship was found between bulk soil pH and extractable Al in bulk soil. The bulk soil pH decreased exponentially with the increased concentration of extractable Al in bulk soil. The extractable Al in bulk soil varied from 5 to 72 mg/kg, and the bulk soil pH from 4.4 to 4.1 with $AlCl_3$ addition. In contrast, the concentrations of extractable Al in bulk soil decreased from 1.8 to 0 mg/kg and the bulk soil pH increased from 4.9 to 7.2 with $CaCO_3$ applications (**Figure 2**).

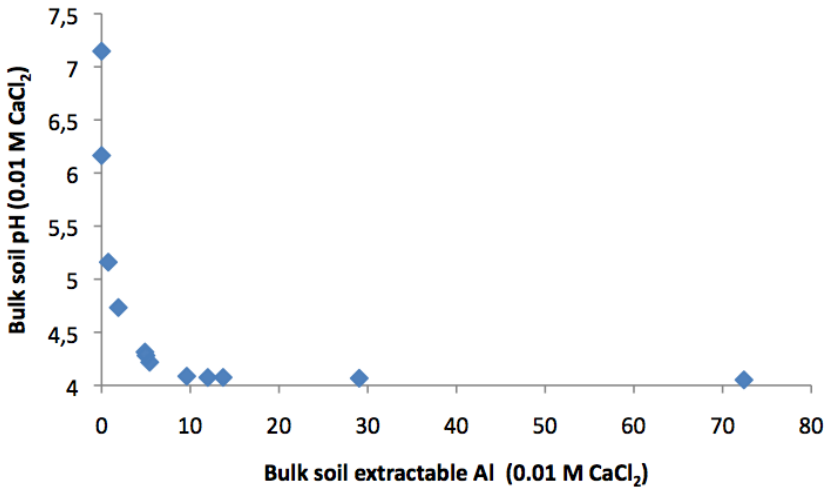

Figure 2. Relationship between bulk soil pH and extractable Al in bulk soil with $AlCl_3$ and $CaCO_3$ applications. Extractable Al in bulk soil reduced to 0 mg/kg, when 500 mg $CaCO_3$/kg was applied to the soil.

3.2. Effect of $AlCl_3$ and $CaCO_3$ supply on shoot growth

The plant height reduced with increasing $AlCl_3$ additions to the soil. However, the seedlings did not grow with 200 mg $AlCl_3$/kg soil or higher. This indicated that wheat seedlings were not able to survive under extremely Al-toxic conditions. However, wheat seedlings were able to survive under moderately acidic conditions as seedlings survived and grew with the 100 mg $AlCl_3$/kg soil treatment. Plant height showed an asymptotic response to increasing lime applications. The plant heights of the two genotypes varied with $AlCl_3$ and $CaCO_3$ additions. The plant height was consistently higher with ET8 than ES8 for the different rates of $AlCl_3$ and $CaCO_3$ application (**Figures 3a and 3b**; **Tables 2 and 3**).

The shoot dry weight declined significantly ($P < 0.05$) with increasing rates of $AlCl_3$ application. In contrast, shoot dry weight increased significantly ($P < 0.05$) at 130 mg $CaCO_3$/kg treatment and remained steady for the rest of the lime treatments. The shoot biomass was also consistently higher in ET8 seedlings than ES8 seedlings with the different rates of $CaCO_3$ and $AlCl_3$ application (**Figures 3c and 3d**; **Tables 2 and 3**).

Table 2. Significance levels from the analysis of variance for the main effects and interaction terms for plant height and shoot dry weight, for $AlCl_3$ rate and genotypes, and $CaCO_3$ rate and genotypes

Source of variation	Plant height	Shoot dry weight
$AlCl_3$ experiment		
Genotype (G)	***	***
$AlCl_3$ (Al)	***	***
G×Al	n.s.	n.s.
$CaCO_3$ experiment		
Genotype (G)	***	***
$CaCO_3$ (Ca)	**	***
G×Ca	n.s.	n.s.

Where n.s., ** and *** represent probability of > 0.05, ≤ 0.01 and ≤ 0.001. Values are means of three replicates.

Table 3. Main-effect means for shoot dry weight and plant heights, for genotype, $AlCl_3$ and $CaCO_3$ treatments, where the interactions with genotypes were not significant ($P > 0.05$)

Treatments	Plant height (cm)	Shoot dry weight (mg/plant)
Al experiment		
Genotypes		
ES8	6.11	4.97
ET8	7.65	6.25
LSD ($P = 0.05$)	0.56	0.53
P value	*<0.001*	*<0.001*
AlCl3 addition (mg $AlCl_3$/kg)		
0	8.97	6.97
50	7.36	6.13
100	4.31	3.73
LSD ($P = 0.05$)	0.68	0.59
P value	*<0.001*	*<0.001*
Lime experiment		
Genotypes		
ES8	10.36	7.92
ET8	10.79	9.06
LSD ($P = 0.05$)	0.32	0.39
P value	*0.010*	*<0.001*
$CaCO_3$ addition (mg $CaCO_3$/kg)		
0	8.97	6.97
130	10.62	8.68
250	10.94	8.51
500	11.20	9.14
1000	11.17	9.07
LSD ($P = 0.05$)	0.51	0.62
P value	*<0.001*	*<0.001*

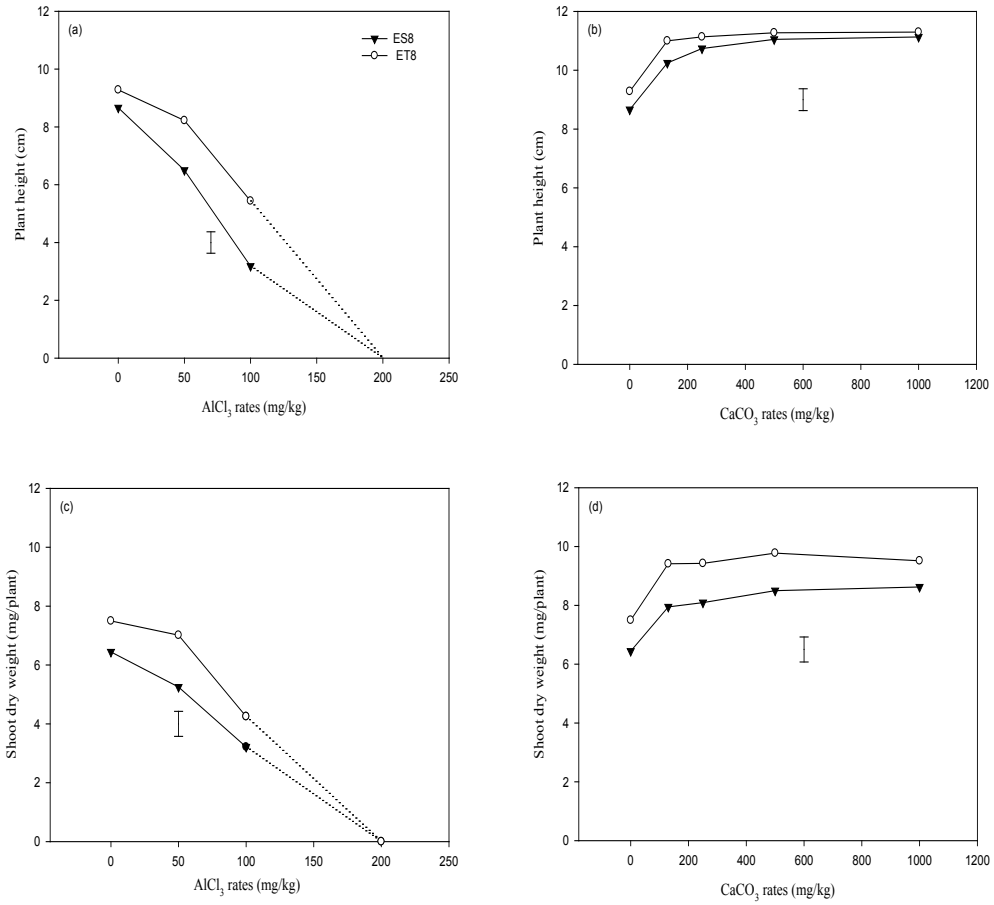

Figure 3. Effect of $AlCl_3$ and $CaCO_3$ addition on plant height (**a** and **b**) and shoot dry weight (**c** and **d**) after 6 days. Seedlings died with the 200 mg $AlCl_3$ treatment and the data were not included in the analysis. Vertical bars represent LSD (P= 0.05) for G × Al and G × Ca interactions separately.

3.3. Effect of $AlCl_3$ and $CaCO_3$ supply on root growth

Root length decreased as $AlCl_3$ addition increased. In contrast, root length increased significantly (P < 0.05) with the 130 mg $CaCO_3$/kg treatment and remained similar for the remaining $CaCO_3$ rates compared with the control. The two genotypes responded differently to $AlCl_3$ and $CaCO_3$ applications. The mean root lengths of ET8 seedlings were higher than the ES8 seedlings with the 50 and 150 mg $AlCl_3$/kg treatments, but similar to the nil $AlCl_3$ treatment, resulting in the significant genotype × $AlCl_3$ interaction. In contrast, the mean root length was consistently higher for the ET8 seedlings than the ES8 seedlings within the range of $CaCO_3$ applications, as there was no interaction between genotype and $CaCO_3$ application (**Figures 4a and 4b**; **Table 4**).

The root dry weight declined as the $AlCl_3$ application increased. The root dry weight was consistently greater in the ET8 seedlings than the ES8 seedlings for the different levels of $AlCl_3$ application (**Figure 4c**; **Table 4**), as the main effect for $AlCl_3$ addition was significant and there was no interaction between genotypes and $AlCl_3$ applications.

Table 4. Significance levels for the main effect and interaction means for root measurements, with the genotypes, $AlCl_3$ and $CaCO_3$ treatments

Source of variation	Root length	Root dry weight	Root surface area	Root average diameter	Root tips count number
$AlCl_3$ experiment					
Genotype (G)	***	**	***	*	***
$AlCl_3$ (Al)	***	***	***	***	*
G×Al	***	n.s.	n.s.	**	**
$CaCO_3$ experiment					
Genotype (G)	***	**	n.s.	n.s.	***
$CaCO_3$ (Ca)	***	***	***	*	***
G×Ca	n.s.	n.s.	n.s.	n.s.	*

Where n.s.,*, ** and *** represent probability of > 0.05, ≤ 0.05, ≤ 0.01 and ≤ 0.001, respectively. Values are means of three replicates.

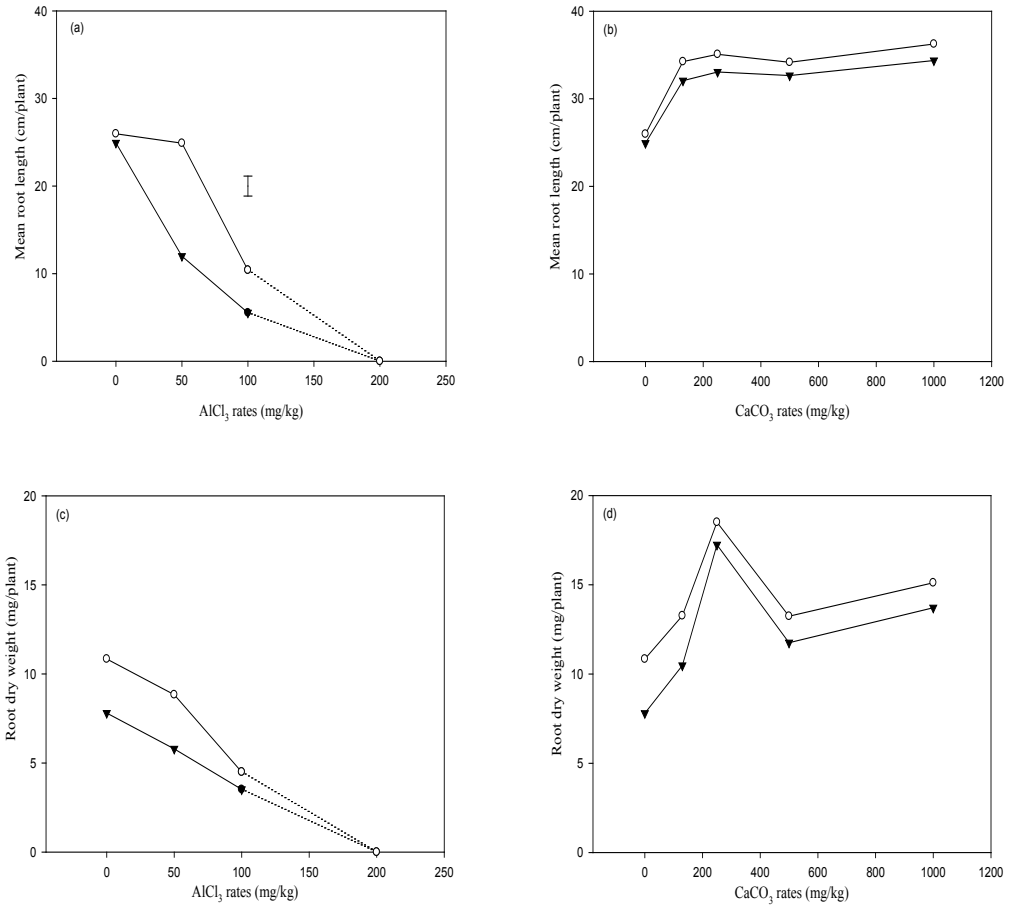

Figure 4. Effect of $AlCl_3$ and $CaCO_3$ addition on mean root length (**a** and **b**) and root dry weight (**c** and **d**). Seedlings died with the 200 mg $AlCl_3$ treatment and the data were not included in the analysis. Vertical bar represents LSD (P= 0.05) for the G × Al when this interaction was significant. The absence of bars indicates that the interaction was not significant.

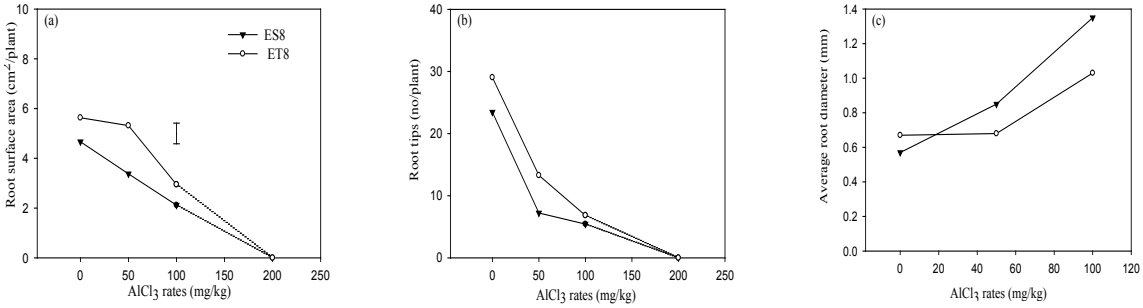

Figure 5. Effect of $AlCl_3$ addition on root surface area (**a**) root tip number (**b**) and average root diameter (**c**). Vertical bar represents LSD (*P*= 0.05) where the G × Al interaction was significant. No bars are presented if the G × Al interactions were not significant (*P* > 0.05).

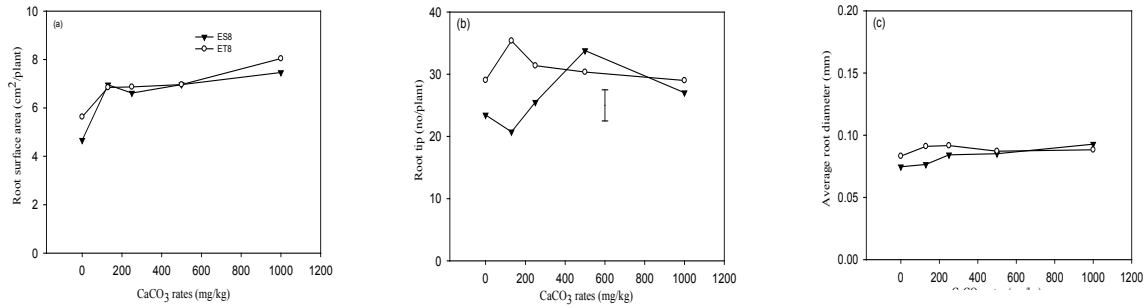

Figure 6. Effect of CaCO3 addition on root surface area (**a**) root tip number (**b**) and average root diameter (**c**). Vertical bar represents LSD (*P*= 0.05) where the G × Ca interaction was significant. No bars are presented if the G × Al interactions were not significant (*P* > 0.05). LSD values were presented Table 5.

3.4. Effect of $AlCl_3$ and $CaCO_3$ supply on the root morphology of the wheat genotypes

The root surface area declined with increased $AlCl_3$ application but increased with the initial rate of 130 mg $CaCO_3$/kg by 29%, compared to the control. The root surface area was higher in the ET8 seedlings than the ES8 seedlings for both the $AlCl_3$ and $CaCO_3$ applications (**Figures 5a and 6a**; **Tables 4 and 5**). However there was no interaction for root surface area between genotypes and $AlCl_3$ or $CaCO_3$ applications.

The number of root tips was reduced as $AlCl_3$ application increased. The two genotypes responded differently to the $AlCl_3$ treatments. The number of root tips for the ET8 seedlings was higher than of the ES8 seedlings for the nil and 50 mg $AlCl_3$/kg treatments, but did not differ with the 100 mg $AlCl_3$/kg treatment, resulting in the significant genotype by $AlCl_3$ interaction. Similarly the root tip number of the genotypes was affected by $CaCO_3$ addition in different ways. The root tip number was higher for ET8 at the lower rates of $CaCO_3$ application, but the number did not differ between ES8 and ET8 at the higher rates of $CaCO_3$ application, resulting in the significant interaction for root tip number between genotypes and $CaCO_3$ application (**Figure 5b**; **Tables 4 and 5**).

The average root diameter increased as $AlCl_3$ application increased. There were differences between the genotypes, but only with the 100 mg $AlCl_3$/kg treatment in which ES8 produced thicker roots than ET8, resulting in the significant genotype by $AlCl_3$ interaction (**Figure 5c; Table 3**). In contrast, root diameter increased gradually between the nil $CaCO_3$ rate and the 1000 mg $CaCO_3$/kg treatments. Root diameter did not differ between ES8 and ET8 with different rates of $CaCO_3$ supply (**Figure 6c**; **Tables 4 and 5**).

Table 5. The main effect means for root morphology measurements with $AlCl_3$, $CaCO_3$ and genotype treatments

Treatments	Root surface area (cm^2/plant)	Root tips (count/plant)	Average root diameter (mm)
$AlCl_3$			
Genotypes			
ES8	3.3	12.0	0.92
ET8	4.6	16.4	0.79
LSD (P = 0.05)	0.3	3.6	0.09
P value	*<0.001*	*0.001*	*0.014*
$AlCl_3$ addition (mg $AlCl_3$/kg)			
0	5.1	26.2	0.61
50	4.3	10.3	0.76
100	2.5	6.1	1.18
LSD (P = 0.05)	0.4	6.9	0.05
P value	*<0.001*	*0.020*	*<0.001*
Lime			
Genotypes			
ES8	6.5	13.9	0.63
ET8	6.8	31.1	0.66
LSD (P = 0.05)	0.4	3.6	0.03
P value	*0.101*	*<0.001*	*0.10*
$CaCO_3$ addition (mg $CaCO_3$/kg)			
0	5.1	26.2	0.61
130	6.9	23.5	0.62
250	6.7	17.8	0.67
500	6.9	16.9	0.64
1000	7.7	24.8	0.70
LSD (P = 0.05)	0.6	5.8	0.06
P value	*<0.001*	*0.001*	*0.03*

3.5. Effect of $AlCl_3$ and $CaCO_3$ supply on apoplast, root and shoot Al concentrations and total Al uptake by the seedlings

The concentration of apoplast Al increased as $AlCl_3$ application increased. The concentration was higher in ET8 than ES8, but only for the 50 mg $AlCl_3$/kg treatment, which resulted in the significant interaction for apoplast Al concentration between genotypes and $AlCl_3$ application (**Figure 7**). In contrast, the apoplast Al concentration declined as $CaCO_3$ supply increased. In addition the concentration was consistently higher in ET8 than ES8 across all rates of $CaCO_3$ (**Tables 6 and 7**). Thus there was no interaction between genotypes and $CaCO_3$ application for this measurement.

The root Al concentration increased as $AlCl_3$ application increased. The root Al concentrations did not differ between the ET8 and ES8 seedlings under different levels of $AlCl_3$ addition (**Tables 6 and 7**), as there was no significant genotype main effect for shoot Al concentration, nor was there any interaction for root Al concentration between genotype and $AlCl_3$ application.

The shoot Al concentration increased as $AlCl_3$ application increased. At 100 mg $AlCl_3$/kg treatment, shoot Al concentration was 1.5 times higher compared to the control. The two genotypes did not differ for shoot Al concentration (**Tables 6 and 7**), nor was there any interaction for shoot Al concentration between genotype and $AlCl_3$ application.

The total Al uptake by wheat seedlings did not increase as $AlCl_3$ application increased. However ET8 seedlings did take up more total Al than the ES8 seedlings across the $AlCl_3$ treatments (**Tables 6 and 7**). There was no interaction between the genotypes and $AlCl_3$ application.

Table 6. Significance levels for the main effect and interaction means for root measurements, with the genotypes, $AlCl_3$ and $CaCO_3$ treatments

Source of variation	Apoplast Al	Root Al concentration	Shoot Al concentration	Total Al uptake
$AlCl_3$ experiment				
Genotype (G)	***	n.s.	n.s.	**
$AlCl_3$ (Al)	**	***	*	n.s.
G×Al	**	n.s.	n.s.	n.s.
$CaCO_3$ experiment				
Genotype (G)	***	NA	NA	NA
$CaCO_3$ (Ca)	***	NA	NA	NA
G×Ca	n.s.	NA	NA	NA

Where n.s.,*, ** and *** represent probability of > 0.05, ≤ 0.05, ≤ 0.01 and ≤ 0.001, respectively. 'NA' indicates no measurements were undertaken. Values are means of three replicates.

Table 7. Main effect means for Al concentrations and total uptake of Al with $AlCl_3$, $CaCO_3$ and genotype treatments

Treatments	Apoplast Al (µg/g r.dwt)	Root Al concentration (µg/g r.d.wt)	Shoot Al concentration (µg/g r.d.wt)	Total Al uptake µg/plant
$AlCl_3$				
Genotypes				
ES8	78	95	17	0.60
ET8	130	95	17	0.86
LSD (*P* = 0.05)	20	24	4	0.17
P value	*<0.001*	*0.98*	*0.84*	*0.006*
$AlCl_3$ addition (mg AlCl3/kg)				
0	72	56	14	0.59
50	91	96	16	0.76
100	142	132	21	0.83
LSD (*P* = 0.05)	25	30	2	0.21
P value	*0.002*	*<0.001*	*0.02*	*0.48*
Lime				
Genotypes				
ES8	23	-	-	-
ET8	35	-	-	-
LSD (*P* = 0.05)	8	-	-	-
P value	*<0.001*	-	-	-
$CaCO_3$ addition (mg CaCO3/kg)				
0	72	-	-	-
130	51	-	-	-
250	45	-	-	-
500	33	-	-	-
1000	0	-	-	-
LSD (*P* = 0.05)	13	-	-	-
P value	*<0.001*	-	-	-

'-' indicates measurements not undertaken.

4. Discussion

4.1. Solubility of Al ions and their effect on soil pH

The extractable Al in the bulk soil increased from 4.9 to 5.2 mg/kg with the addition of 100 mg $AlCl_3$/kg to the podzolic soil. At 200 mg $AlCl_3$/kg, the extractable Al in the bulk soil reached 9.6 mg/kg (**Figure 1c**) which was too toxic for the wheat seedlings as both ET8 and ES8 seedlings died with this treatment. Thus, less than 200 mg $AlCl_3$/kg was used for the other experiments (Iqbal et al. 2010; Iqbal 2012b). This indicates that the level of added Al needs to be between 0 to 200 mg $AlCl_3$/kg in other experiments resulting in an extractable Al concentration between 4.5 and 6.0 mg/kg, for the short term experiments with ES8 and ET8 seedlings.

The bulk soil pH declined to 4.1 with the addition of $AlCl_3$ and then remained constant with further applications (**Figure 1a**). One study speculated that below pH 4.5, the bulk of Al ions are present as aluminium hexahydrate ion [$(Al.6H_2O)^{3+}$], which is usually designated as Al^{3+}(Naramabuye and Hynes 2006). This Al^{3+} becomes more soluble in low pH enabling it to react with water in the soil solution to form aluminium hydroxide ($Al(OH)^{+2}$) releasing H^+ ions which lower the soil pH (Serrano 2003).

It appears that adding further $AlCl_3$ from 100 to above 200 mg/kg to this Podosol soil saturated this reaction. As a result, pH did not decline further with additional $AlCl_3$. The likely reason is that $AlCl_3$ in water forms a series of hydrous oxides (Faust and Hunter 1967) and the amphoteric nature of these hydrous oxides and the combinations with soil results in the buffering to pH 4.1. The hydrogen ion concentration remains constant during the dissolution of the hydroxide due to amphoteric nature of hydrous oxides (Robinson and Britton 1931) which buffers the pH.

Extractable Al declined as pH increased with the addition of $CaCO_3$ (**Figure 1d and Figure 2**). The $CaCO_3$ increases soil pH by providing carbonate (CO_3^{2-}) ions that react with H^+ ions from water to form bicarbonate (HCO_3^-) releasing OH^- ions. The bicarbonate ions react with additional H^+ ions to form H_2O and CO_2. The pH increases as the H^+ concentration declines (Edmeades et al. 1990). As pH increases, Al^{3+} ions sequentially dissociate, releasing OH^- ions in place of OH_2 groups, resulting in formation of the increasing insoluble monomers $AlOH^{2+}$, $Al(OH)^+_2$ and $Al(OH)_3$. This results in the reduction of $CaCl_2$ extractable Al (**Figure 1d**). The decline in the concentration of Al^{3+} means that the soil should become less Al toxic for the wheat seedlings.

4.2. Genotypic differences due to elevated Al

The 6 day old ET8 seedlings grew better and produced more shoot biomass than the ES8 seedlings under the Al^{3+} toxic conditions in this study (**Figure 3a and Table 3**). The highly significant main effect for genotypes indicates that the ET8 seedlings produced 1.1 mg shoot dry matter more than the ES8 seedlings over the nil, 50 and 100 mg $AlCl_3$/kg treatments. The Al toxicity level corresponds to a $CaCl_2$ extractable Al concentration ranging from 4.9 to 5.2 mg Al/kg. With the 200 mg $AlCl_3$/kg treatment, the extractable Al concentration rose to 9.6 mg Al/kg, which was too toxic and the seedlings of both genotypes died in this treatment, as discussed above.

The data for shoot height and root length confirm the greater tolerance of ET8 seedlings to toxic Al concentrations resulting from the 50 and 100 mg $AlCl_3$/kg treatments. However, there were highly significant genotype ×Al interactions for both measurements (**Tables 2 and 4**), which resulted from similar shoot height and root length responses from the two genotypes, with the nil $AlCl_3$ treatment (**Figures 3c and 4a**). Thus, under the conditions of this experiment, there was a greater reduction in ES8 shoot biomass relative to ET8 in soil with the nil added Al, where the extractable Al concentration was 4.8 mg/kg, than the reduction in shoot height or root length for ES8 relative to ET8.

Many studies explain why the ET8 is more Al-tolerant than the ES8. One study suggested that the Al^{3+} dependent efflux of malate from root apices is a mechanism for Al-tolerance in ET8. The malate anions protect the sensitive root tips by chelating the toxic Al^{3+} cations in the rhizosphere to form non-toxic complexes

(Zhang et al. 2001). Their findings also provided evidence that the higher Al^{3+}-induced malate efflux in ET8 than ES8 is due to the activation of both malate-permeable and cation channels for sustained malate release. Later, another study reported that the ET8 had higher H^+-ATPase activities in the plasma membrane resulting in increased transport of H^+ through the plasma membrane in ET8 compared with ES8. This higher H^+-ATPase activity and associated increase in H^+ transport through the plasma membrane in ET8 is also thought to contribute to the difference in Al-tolerance (Ahn et al. 2004). Thus, the higher malate exudation from the ET8 seedlings (Delhaize et al. 1993) via malate-permeable channels is accompanied by the increased zeta potential of the plasma membrane from enhanced H^+-ATPase activity in ET8, compared with ES8. This indicates that the *ALMT1* locus, which was identified as being responsible for the difference in Al-tolerance between ES8 and ET8 (Delhaize et al. 1993) is potentially pleiotropic, having multiple effects from this single gene locus. This was also confirmed by others, and their findings suggested that the *Alt1* locus may control more than the malate channels in the plasma membrane of ET8. They also suggested that the ET8 had higher Al-induced signalling capacity in its root vacuoles than the ES8, and this also contributed to the greater Al-tolerance in ET8. This proposed mechanism for ET8 was that the Al^{3+} induced the opening of slow Al channels into the vacuole, enabling Al to be sequestered in the root vacuole (Wherrett et al. 2005). Thus, these are a range of proposed mechanisms that contribute to the differential response between ES8 and ET8 that may help ET8, and assist the Al-tolerance in ET8.

4.3. Genotypic variation to high pH

The increased growth of ET8 relative to ES8 that occurred when $AlCl_3$ was added, continued with the addition of $CaCO_3$. Thus, even in the absence of toxic Al, the ET8 seedlings were consistently larger than ES8 for every growth > measurement. The increased growth was reflected in larger shoot biomass, taller shoots, larger roots, larger root biomass and in root surface areas (**Figures 3, 4 and 5a**; **Table 3**). The relative increases in growth by the ET8 seedlings, over and above that of ES8, ranged from 20% for shoot biomass, and 13% for plant height, 16% for root length, 20% for root biomass and 26% for root surface area, over the high soil pH range. Interestingly, there were no interactions between the two genotypes and the level of $CaCO_3$ application and instead there were only highly significant genotype main effect mean differences ($P < 0.001$). This means that the superior growth of ET8 over ES8 occurred over all levels of $CaCO_3$ application.

There are additional reports that Al-tolerant genotypes outperform Al-sensitive genotypes when Al-toxic soil is limed with $CaCO_3$. For example, some researchers grew Al tolerant wheat (Carazinho) in an acid soil (pH in $CaCl_2$ 4.38 with an exchangeable Al of 0.47 cmol/kg) in the field at Binnaway, NSW (Scott et al. 2001). The Carazinho variety contains the *ALMT1* gene which increases malate secretion from root apices under Al stress condition (Delhaize et al.1993). They applied lime to the fields and found that Al-tolerant genotype grew taller, was visually healthier and was slightly more advanced in plant development compared with the Al-sensitive Egret cultivar. They also speculated that malate efflux was the general mechanism of Al tolerance in wheat (Ryan et al. 1995), but the evidence from the literature that is discussed above suggests that the *ALMT1* gene has other effects in addition to malate exudation. This multi-genetic behaviour may help Carazinho to produce increased plant biomass than Egret in lime amended soil. My speculation is that ET8 seedlings are generally more vigorous than the ES8 seedlings in the presence of added lime.

4.4. Impact of elevated Al on root morphology of wheat seedlings

The increased concentration of extractable Al in the podzolic soil in this study resulted in marked changes in the root morphology of the wheat seedlings. There were highly significant main effect reductions in root surface area and root tip

numbers and increases in root diameter with the $AlCl_3$ treatments (**Table 4**). In addition there were significant main effect differences between the genotypes, are consistent with ES8's increased sensitivity to Al-toxic concentrations in the soil. The significant G × Al interaction for root surface area (**Table 4, Figure 5a**) resulted from the minimal effects of the 50 mg $AlCl_3$/kg treatment on root surface area of ET8, compared to the 30% reduction in the root surface area of ES8 with this treatment, when $AlCl_3$ increased from nil to 50 mg/kg.

Other studies in the literature confirm that Al toxicity impacts the morphology of plant roots. For example, two researchers found that increased Al supply increased the root diameter of sensitive plants (Hirano and Hijii 1998). They conducted pot experiments and grew Japanese red cedar in forest soil with applications of $AlCl_3$ as the Al source at a concentration of 5 mM and found that root diameter doubled from 0.4 to 0.9 mm in the Al treatment compared to the control. They speculated that the effects of excess Al in increasing the root diameter resulted from an increased concentration of Al in whole roots. However, the root Al concentrations in this study do not support this speculation, as root Al concentrations increased only marginally with elevated Al supply (**Figure 7a**). In contrast, elevated Al reduced the root tip numbers of wheat seedlings (**Figure 5e**). A researcher speculated in his review paper that Al supply reduces root tip numbers in sensitive species (Wright 1989). These changes in root morphology -the increase in root diameter and decrease in root tip numbers and root surface areas- are therefore symptomatic of Al toxicity in sensitive plants.

4.5. Accumulation of Al in root apoplast relates to soil available extractable Al

This study found that Al accumulated in the root apoplast as the availability of extractable Al in the soil increased. Furthermore, as the concentration of the extractable Al in the soil declined with $CaCO_3$ addition, there was a decline in apoplast Al in the roots. These results indicate that the binding of Al in the apoplast is directly related to soil available Al (**Table 7**). One author speculated that the primary binding site of Al^{3+} in apoplast is probably the pectic matrix, with its negatively charged carboxylic groups having a particularly a high affinity for Al^{3+} ions (Chang et al. 1999). Likewise, another author demonstrated that Al stress increases cell wall pectin content in common bean (Rangel et al. 2009). Thus, the increased Al^{3+} concentration in the Podosol soil in this study may have increased the pectin content in the cell walls of the wheat seedlings. This increased cell wall pectin content in turn helps to bind Al (Le et al. 1994) and increases apoplast Al.

The results also showed that the ET8 seedlings bind more Al in the apoplast than the ES8 seedlings even when the soil was amended by lime applications (**Tables 6 and 7**). This binding is reversible such that this apoplast P can be desorbed by $BaCl_2$. The higher Al binding capacity in the root apoplast of ET8 seedlings, compared to the ES8 seedlings, suggests that the 'reversible' binding of Al^{3+} ions in the apoplast might be contributing to the increased Al tolerance of the ET8 seedlings. Recently, one study reported that strongly bound Al^{3+}, which presumably is not desorbed by $BaCl_2$, contributes to Al toxicity damage (Horst et al. 2010). They speculated that this strongly bound Al, which accumulates in the root apoplast, modifies the cell wall composition and its properties. Likewise, another study suggested that the negativity of the cell wall depends mainly on the pectin content and its methylation (Eticha et al. 2005). They also demonstrated that the importance of the methylation of pectin in the cell wall in accounting for the differential Al tolerance between two maize cultivars. The cultivars did not differ in pectin content but differed in the methylation of the cell walls. The Al-sensitive cultivar had lower methylation and experienced more severe Al injury compared with the Al-tolerant maize cultivar. According to this finding, then, it is possible that ES8 has lower methylation of pectin substances in its cell walls than ET8 resulting less Al being bound in its root apoplast. This needs to be determined by further research.

5. Conclusions

Elevated Al was responsible for the toxicity of the wheat seedlings grown in an acid soil, with high rates of added $AlCl_3$ increasing soil extractable Al. In contrast, liming increased soil pH and removed Al toxicity from soil. The ET8 seedlings responded better than the ES8 seedlings in both the $AlCl_3$ and lime treatments. This indicates that the *ALMT1* gene, which is involved in Al tolerance through malate exudation, also has other multi-genetic effects involved in Al tolerance. The root surface area and the number of root tips were reduced and root thickness increased due to the effect of elevated Al. Apoplast Al increased with the increase in extractable Al in soil and declined with the reduction in extractable Al in soil with $CaCO_3$ applications. The ET8 binds more Al in its root apoplast than ES8, which is desorbed by $BaCl_2$. This difference in the reversible binding of Al in the apoplast may be involved in the increase Al tolerance of ET8.

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