RESEARCH PAPER

Intra-cellular distribution and binding state of aluminium in root apices of two common bean (*Phaseolus vulgaris*) genotypes in relation to Al toxicity

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Running title: Intra-cellular distribution of Al and its relationship to Al toxicity

Abstract

The role of the intra-cellular distribution and binding state of aluminium (Al) in Al toxicity, using Al exchange and Al fractionation methodologies, were studied in two common bean (*Phaseolus vulgaris* L.) genotypes differing in Al resistance. These two genotypes are characterized by a similar initial period (4 h) of Al sensitivity followed by a contrasting recovery period (8-24 h). A higher initial Al accumulation in Quimbaya (Al-resistant) in the 5 mm root apex compared to VAX-1 (Al-sensitive) could be related to its higher content of unmethylated pectin and thus higher negative charge of the cell walls. The binding state and cellular distribution of Al in the root apices revealed that the root elongation-rate was significantly negatively correlated with the free apoplastic and the stable-bound, not citrate-exchangeable cell-wall Al representing the most important Al fraction in the root apex (80%), but not with the symplastic and the labile-bound, citrate-exchangeable cell-wall Al. It is postulated that the induced and sustained recovery from the initial Al stress in the Al-resistant genotype Quimbaya requires reducing the stable-bound Al in the apoplast thus allowing cell elongation and division to resume.

Key words: aluminum toxicity, apoplast, cell wall, compartmentation, root apex

Abreviations

CW	Cell wall
CEZ	Central elongation zone
DM	Degree of methylation
DTZ	Distal transition zone
EZ	Elongation zone
Al_{mono}	Monomeric aluminium
PEM	Pectin methylesterase
PCV	Pyrocatechol violet
SYM	Symplast
TZ	Transition zone
WFSF	Water free space fluid

Introduction

Aluminium (Al) toxicity is a major factor limiting plant growth especially on acid soils in the tropics and subtropics (von Uexküll and Mutert 1995). Common bean (*Phaseolus vulgaris* L.) growing areas of about 40% of Latin America and 30 to 50% of central, eastern, and southern Africa are affected by Al toxicity resulting in yield reduction from 30 to 60% (CIAT 1992). The primary effect of Al is an inhibition of root growth (Foy 1988), an effect that can be seen within hours of Al treatment (Llugany et al. 1995, Blamey et al. 2004). The major site of Al perception and response is the root apex (Ryan et al. 1993), and particularly the distal part of the transition zone (DTZ, 1-2 mm) is the most Al-sensitive apical root zone (Sivaguru and Horst 1998, Kollmeier et al. 2000). In common bean in contrast to maize (*Zea mays* L.), however, Al applied to the elongation zone (EZ) contributed to the overall inhibition of root elongation by Al (Rangel et al. 2007). Also, common bean differs from most other plant species, particularly cereals, through a lag phase after the beginning of Al treatment before Al resistance mechanisms are expressed (Cumming et al. 1992, Rangel et al. 2007). This is typical for a pattern II response to Al treatment (Ma et al. 2001) characterized by an Al-induced delayed (several hours) exudation of organic acid anions, particularly citrate in common bean (Mugai et al. 2000, Ma et al. 2001, Shen et al. 2002, Rangel and Horst 2006, Stass et al. 2007).

The role of the root exudation of organic acid anions in reducing Al uptake/binding in the root apoplast thus enhancing Al resistance is widely accepted particularly in pattern I plant species (Ma et al. 2001, Ryan et al. 2001, Kochian et al. 2004, Delhaize et al. 2007). However, the role of symplastic lesions of Al toxicity and of sequestration of Al by organic ligands as a mechanism of Al resistance are still issues of debate (Vázquez et al. 1999, Illes et al. 2006). Thus, there is a need to better understand the kinetics of Al accumulation in root apices and its

distribution at a cellular and tissue level in relation to genotypic differences in Al resistance particularly in pattern II plant species such as common bean.

Aluminium accumulates in roots with a rapid initial phase (accumulation of easily exchangeable Al in the apoplast) followed by a lower linear rate (metabolism-dependent binding of Al into the apoplast and transport of Al into the symplast, Zhang and Taylor 1989, 1990). In the apoplast, the negative charge of the cell wall (CW) established by the pectin content and its degree of methylation is a major determinant of this initial Al accumulation (Blamey et al. 1990, Grauer and Horst 1992, Schmohl and Horst 2000, Schmohl et al. 2000) and Al injury (Schmohl et al, 2000, Eticha et al. 2005*a*, Horst et al. 2007) through altering CW characteristics and functions, such as extensibility, porosity, hydraulic conductivity, displacement of ions from critical sites (Rengel 1990, Blamey et al. 1993, Mimmo et al. 2003, Sivaguru et al. 2006, Horst et al. 2007) and/or disrupting the CW-plasma membrane-cytoskeleton continuum (Sivaguru et al. 1999, Horst et al. 1999).

There is no doubt that Al can enter the symplast (Tice et al. 1992, Lazof et al. 1994, Vázquez et al. 1999, Eticha et al. 2005*b*). Taylor et al. (2000) using the model giant algae *Chara corallina* showed that Al can be transferred from the apoplast to the symplast. However, the low rates of transport observed through the plasma membrane will favor the accumulation of Al in the apoplast (Rengel and Reid 1997). Therefore, interactions of Al with the CW and plasma membrane will necessarily precede any transport into the symplast, these interactions being potentially harmful (see above, Delhaize and Ryan 1995). According to the above scenario, internalization of Al in the symplast (Vázquez et al. 1999, Illes et al. 2006) appears to be a mechanism of Al resistance rather than of Al toxicity.

The main objective of the study was to elucidate the role of the intra-cellular distribution and binding state of aluminium (Al) in relation to Al toxicity in two common bean genotypes differing in Al resistance. These two genotypes appeared to be particularly suitable for this

study because they are characterized by a similar initial period of Al sensitivity followed by a contrasting recovery period (Rangel et al. 2007).

Materials and methods

Plant material and growth conditions

Seeds of the Al-resistant common bean genotype Quimbaya, large seeded Andean cultivar, and an Al-sensitive genotype VAX-1, small seeded Mesoamerican advanced line (Rangel et al., 2005) kindly supplied by the Bean Outcome Line of CIAT (International Center for Tropical Agriculture, Cali, Colombia) were germinated between filter-paper and foam sandwiches soaked with tap water in an upright position. Uniform seedlings were transferred to 18 l pots with constantly aerated simplified nutrient solution (Rangel et al. 2005). Plants were cultured in a growth chamber with controlled environmental conditions of a 16/8 h light/dark regime, 27/25^{o}C day/night temperature, 70% relative air humidity, and a photon flux density of 230 µmol m^{-2} s^{-1} photosynthetic active radiation at the plant level (Sylvania Cool White, 195 W, Philips, Germany).

After 24 h the pH of the solution was lowered gradually from 5.6 to 4.5 and kept constant throughout the treatment period using an automatic pH titration device with 0.1 M HCl/KOH. Plants were treated with 0 or 20 µM $AlCl_3$ for up to 24 h. Mononuclear Al (Al_{mono}) concentrations were measured colorimetrically using the pyrocatechol violet method (PCV) according to Kerven et al. (1989). Nominal 20 µM Al supply resulted in 16 ± 2 µM Al_{mono} after 24 h. Then the roots were washed in distilled water and the root tips (5 mm length) were harvested for pectin and Al determinations.

Effect of Al on root growth

Two hours before Al treatment tap roots were marked 3 cm behind the root tip using a fine point permanent marker (Sharpie blue, Stanford) which did not affect root growth during the experimental period. Afterwards, the plants were transferred to simplified nutrient solution (Rangel et al. 2005) containing 0 or 20 µM $AlCl_3$. Root elongation was measured at 4, 8 and 24 h of Al treatment using a 1 mm scale.

Determination of pectin and its degree of methylation

Pectin and its degree of methylation was determined in 25 (5 mm) root tips per sample, which were excised and collected in 96% (v/v) ethanol in Eppendorf vials. Root samples were thoroughly homogenized in ethanol using a mixer mill (MM200; Retsch, Haan, Germany) at a speed of 30 oscillations per second for 3 min. The homogenization was repeated twice. Cell-wall material was prepared as alcohol-insoluble residue after repeated washing with ethanol, modified after Schmohl and Horst (2000). After every ethanol addition, the samples were centrifuged at 23000 g for 10 min and the supernatant was discarded. The remaining CW was dried using a centrifugal evaporator (RC10-22T, Jouan SA, France), weighed, and hydrolysed according to Ahmed and Labavitch (1977) extending the incubation time to 10 min in concentrated H_2SO_4 and the hydrolysis completed overnight by a stepwise dilution with double-deionized water. The uronic acid content was determined colorimetrically according to Blumenkratz and Asboe-Hansen (1973) using a microplate spectrophotometer (µQuant™, Bio-Tek Instruments, Winooski, Vermont, USA). Galacturonic acid was used as a calibration standard, thus the root pectin content is expressed as galacturonic acid equivalents (GalA).

For the determination of the degree of methylation (DM), the CW was prepared in the same way as for pectin determination. Methanol was released from the CW by saponification according to Fry (1988), modified after Wojciechowski and Fall (1996). After addition of 2

units of alcohol oxidase (EC 1.1.3.13 from *Piccia pastoris* Sigma, Deisenhofen, Germany) the complex of formaldehyde with Fluoral-P (15 mg ml^{-1}) (Molecular Probes, Leiden, The Netherlands) was measured fluorometrically (excitation λ = 405 nm, emission λ = 503 nm).

Aluminium exchange from intact root tips

For the exchange (desorption) of Al from the root tips, roots of twelve seedlings were quickly washed with double-deionized water, then 5 mm root tips were excised with a razor blade and placed in filter units with a pore size of 0.45 µm (GHP Nanosep® MF Centrifugal Device, Pall Life Sciences, Ann Arbor, USA). Loosely bound Al was exchanged with 500 µl of 50 mM $BaCl_2$, for 15 min. Root tips were briefly washed in 500 µl of double-deionized water and then transferred for 15 min to 500 µl of 33 mM Na_3-citrate (pH 5.8) and the filtrate collected in a new vial. Preliminary experiments had shown that longer incubation periods did not release more Al in either fraction. Desorption experiments were conducted at 4°C to minimize loss of Al from the symplast (Zhang and Taylor 1989). Thereafter, the root tips were washed and transferred into a new Eppendorf vial for Al determination.

Aluminium fractionation

For the determination of apoplastic and symplastic Al fractions in the root tips, the apoplastic and symplastic saps from root tips were collected according to the method described by Yu et al. (1999) and modified by Wang et al. (2004). Briefly, freshly excised 5 mm root tips from twenty five seedlings were arranged in a filter unit (Ultrafree-MC, 0.45 µm; Millipore, Bedford, MA) with the cut ends facing down, and the water free-space fluid (WFSF) collected by centrifugation (4000 g) at 4°C for 15 min. After collecting the WFSF, the root tips were frozen at -20°C. The first symplastic-Al fraction (SYM-1) was recovered from the frozen-

thawed samples by centrifugation (4000 g) at 4°C for 15 min. The residue was then transferred to Eppendorf vials and homogenized in 500 µl of ethanol with a mixer mill (MM200; Retsch, Haan, Germany) at a speed of 30 oscillations per second for 3 min. All further centrifugation steps were conducted at 23000 g (4°C) for 5 min. After centrifugation, supernatant and pellet were separated and the pellet suspended again in 500 µl of ethanol. The complete process was repeated twice and both supernatants combined. The supernatants representing the second symplastic-Al fraction (SYM-2) were evaporated in a centrifugal evaporator (RCT 10-22T; Jouan, Saint-Herblain, France) for later Al determination. Subsequently, the pellet consisting of the CW was desorbed at room temperature with 500 µl of 33 mM Na_3citrate (pH 5.8) for 15 min. After centrifugation, the supernatant containing the labile-bound CW Al fraction was analysed for Al. The pellet was washed with double-deionized water, centrifuged and the supernatant discarded. Thereafter, the pellet containing the stable-bound CW Al fraction was dried in a centrifugal evaporator (RCT 10-22T; Jouan, Saint-Herblain, France) for later determination of Al.

Determination of Al

For Al determination, roots, CW material, and the SYM-2 fractions were digested in 500 µl ultra-pure HNO_3 (65%) overnight. Digestion was completed by incubation in a water bath at 80°C for 20 min. $BaCl_2$-exchangeable, citrate-exchangeable, WSWF, and SYM-1 Al fractions were directly measured using a Unicam 939 QZ graphite furnace atomic absorption spectrophotometer (GFAAS; Analytical Technologies Inc., Cambridge, UK) at a wavelength of 308.2 nm and with an injection volume of 20 µL. When required, the samples were diluted with double-deionized water.

Statistical analysis

Each experiment had a completely randomized design with four replicates. The ANOVA procedure of the statistical program SAS 9.1 (SAS Institute, Cary, NC, USA) was used for analysis of variance. Means were compared using the Tukey test. *, **, *** denotes significant differences at $p < 0.05$, 0.01, and 0.001, respectively; n.s. denotes not significant.

Results

Effect of Al on overall root elongation

In presence of Al root elongation of both genotypes was severely inhibited (60-65%) 4 h after the beginning of the Al treatment (Fig. 1a). After 8 h Al treatment, both genotypes recovered, Quimbaya more than VAX-1. Whereas this recovery continued in Quimbaya until the root-elongation rate nearly reached the level of the control (without Al), VAX-1 was increasingly damaged by Al after 24 h of Al treatment which is reflected by the highly significant genotype x time interaction.

Effect of Al treatment on Al content in root tips

The decrease in root-elongation rate after 4 h Al supply in both genotypes was associated with an increase in Al content in the root tips (Fig. 1b). Recovery of root-elongation rates after 8 h of Al treatment was accompanied by reduced Al contents in the root tips. This decrease continued during further recovery in the Al resistant genotype (Quimbaya), while Al contents increased again in the Al-sensitive genotype (VAX-1) after 24 h of Al treatment (highly significant genotype x time interaction). Aluminium contents per unit root tip length (5 mm)

after 4 and 8 h of Al treatment were significantly higher in Quimbaya than in VAX-1 (86%). When the Al contents were expressed on a root tip fresh weight basis (nmol per mg root tip; data not shown), this difference was somewhat lower (about 70%) due to a higher mass of the root tips of Quimbaya (9.55 ± 1.1 mg) as compared with VAX-1 (8.70 ± 2.4 mg).

Fig. 1

Determination of pectin and its degree of methylation

Since binding of Al to CW is mainly due to pectins, the pectin content and its degree of methylation was determined in the CW isolated from 5 mm root tips. Constitutively, Quimbaya had significantly higher CW pectin contents than VAX-1 (Fig. 2a) whereas the DM did not differ between the two genotypes before the start of the Al treatment (Fig 2b). Aluminium treatment increased the pectin contents in both genotypes independent of Al treatment duration (Fig. 2a) while the DM decreased after 4 h Al treatment in both genotypes (Fig. 2b). However, while recovery of root growth in Quimbaya at longer Al-treatment duration was reflected by increased DM up to the initial value, it remained at the lower level in VAX-1. The resulting content of unmethylated pectin (Fig. 2c) which is a measure of the negativity of the CW was consistently higher (31%) in Quimbaya than in VAX-1. This genotypic difference was smaller (17%) but still significant when the pectin contents were expressed on a CW mass basis (nmol per mg CW, data not shown) due to a higher mass of CW recovered from the twenty five root tips of Quimbaya (4.5 ± 0.2 mg) as compared to VAX-1 (3.6 ± 0.1 mg). After 24 h Al treatment which did not affect the dry mass of CW per root tip, the content of unmethylated pectin decreased again corresponding to the observed recovery in DM to the level observed prior to the Al treatment in Quimbaya but not in VAX-

1. This is reflected by the significant genotype x time interaction observed in DM and unmethylated pectin.

Fig. 2

Binding state of Al in the root tips

The recovery from initial Al stress leading to genotypic differences in Al resistance might be related to changes and differences in the binding state and compartmentation of Al in the root apices. Therefore, in a first approach root apices were subjected to a fractionated desorption of Al in order to differentiate between loosely and firmly bound Al. Excised root tips were incubated for 15 min each in 50 mM $BaCl_2$ and then in 33 mM Na_3citrate in order to release from the apoplast free and exchangeable bound Al, and Al weakly bound to the unmethylated pectin, respectively. The Al that was not released from the root tips was considered as non-exchangeable (symplastic and more strongly bound apoplastic Al). $BaCl_2$ was not able to release any detectable amounts of Al from the root tips (data not shown). Incubation in Na_3citrate released between 10 and 30% of the total Al in the root tips depending on the genotype and the Al-treatment period (highly significant genotype x time interaction, Fig. 3). In both genotypes the decrease of root elongation after 4 h Al treatment (see Fig. 1a) was characterized by substantial Al accumulation in both Al fractions (Fig. 3). During the recovery from Al injury after 8 h, Al contents decreased more in the non-exchangeable than in the Na_3citrate-exchangeable fraction. The Al contents of both fractions were higher in Quimbaya than in VAX-1 during the first 8 h. After 24 h Al treatment the picture changed. Whereas in Quimbaya the Al contents continued to decrease in both fractions, they increased again only in the non-exchangeable fraction in VAX-1, leading to a highly significant genotype x time interaction.

The total Al contents in the root tips were only loosely related to the root elongation rates when calculated across genotypes and Al treatment duration (r^2 = 0.22*). However, in Quimbaya the increase in root elongation during the recovery from initial Al stress was highly significantly related to both Na_3citrate-exchangeable and non-exchangeable Al (Fig. 4). This was also true for VAX-1 for the recovery period of 4-8 h after Al treatment. However, the severe inhibition in root elongation after 24 h Al treatment in VAX-1 appears to be mainly due to an increase in non-exchangeable Al rather than Na_3citrate-exchangeable Al.

Fig. 3

Fig. 4

In order to better characterize the intra-cellular distribution of Al in the root apices in addition to the binding state, the root tips were subjected to a more differentiated fractionation procedure (Fig. 5). The Al contents in the WFSF fraction were initially two times higher in Quimbaya than in VAX-1 which was due equally to both a greater WFSF volume (estimated on the basis of the recovered amount of WFSF by centrifugation) and Al concentration. The WFSF Al fraction decreased over time in Quimbaya because of decreasing Al concentration rather than volume, whereas the level remained constant in VAX-1, leading to comparable Al contents in both genotypes after 24 h Al treatment (highly significant genotype x time interaction). The SYM-1 Al fraction had a similar order of magnitude as the WFSF Al fraction. Again, the Al contents were higher in Quimbaya than in VAX-1. This fraction did not change with the Al treatment duration in either genotype. The SYM-2 Al fraction which was about twice as high as the two previous fractions was significantly higher after 24 h Al supply to Quimbaya, too. It increased up to 8 h after Al treatment and then remained at this higher level in both genotypes. The only fraction which was consistently higher in the Al-

sensitive VAX-1 was the labile-bound CW Al fraction. This fraction remained stable in Quimbaya but steadily increased in VAX-1 up to 24 h Al treatment. The stable-bound CW Al fraction was quantitatively the most important Al fraction. Initially (up to 8 h Al treatment) the Al contents were higher in Quimbaya but readily decreased with time, whereas in VAX-1 the contents drastically increased after 24 h of Al treatment.

Fig. 5

The stable-bound CW Al fraction represented about 80% of the total Al content in both genotypes (Fig. 6). Whereas the relative contribution of this fraction to the total Al content decreased in Quimbaya with time, in VAX-1 this fraction first decreased (8 h) but then increased again after 24 h Al treatment. The second greatest Al fraction was the symplastic fraction (combining the two symplastic Al fractions). This fraction became increasingly greater with Al treatment duration in Quimbaya, while in VAX-1 this was only the case up to 8 h. Later, this fraction decreased again. The relative contribution of the WFSF (smallest fraction) and the labile-bound CW Al fractions did not vary much over time. However, the latter was greater in VAX-1 (8%) than in Quimbaya (4.5%).

As in the Al-exchange experiments (see Fig. 4), the correlations between root-elongation rate and apoplastic (WFSF and CW Al fractions combined) and symplastic Al contents were calculated separately for each genotype because of the highly significant genotype x time interaction for most Al fractions (see Fig. 5). In both genotypes, root-elongation rate was negatively related to the Al content of the apoplast (Fig. 7a). This is clearer in Quimbaya with a continuous recovery from first Al injury at 4 h than in VAX-1, where the initial recovery after 8 h is followed by severe Al injury after 24 h Al treatment. Symplastic Al was not related to root elongation rate in either genotype. There was even a tendency of a positive correlation.

Fig. 6

Fig. 7

In order to clarify whether the recovery of root elongation from initial Al stress in Quimbaya can be related to changes in specific apoplastic Al fractions, correlation coefficients were calculated (Fig. 8). The WFSF and the stable-bound CW, but not the labile-bound CW Al fractions showed a highly-significant negative relationship with the enhanced root-elongation rate during the Al treatment.

Fig. 8

Discussion

The results from this study clearly provide additional physiological and biochemical evidence needed to substantiate the previous finding that Al resistance in common bean is an Al-inducible trait involving a lag phase of 4-6 hours in contrast to many other plant species (Cumming et al. 1992, Rangel et al. 2007; Fig. 1a).

Aluminium treatment resulted in rapid Al accumulation (after 4 h, Fig. 1b) more in the Al-resistant genotype Quimbaya than in the Al-sensitive genotype VAX-1 leading to severe decrease in the root-elongation rates in both genotypes (Fig. 1a). Aluminium is accumulated by roots with a rapid initial phase and a lower rate, thereafter (Zhang and Taylor 1989, 1990). The primary binding site of Al is likely the pectic matrix of the CW with its negatively charged carboxylic groups having a particularly high affinity for Al (Blamey et al. 1990, Chang et al. 1999). Short-term Al accumulation by roots is closely related to the pectin content. This may explain the differences in initial Al accumulation between monocots and

dicots (Schmohl and Horst 2000, Horst et al. 2007) with their higher CW pectin content (Carpita and Gibeaut 1993). In fact, the factor responsible for Al binding to pectin is not the pectin content but its negative charge determined by its DM which is controlled by pectin methylesterase (PME) (Bordenave 1996, Gerendás 2007). The role of the CW pectin-content and its DM in Al resistance has been demonstrated in maize (Schmohl et al. 2000, Eticha et al. 2005*a*), potato (*Solanum tuberosum* L., Schmohl et al. 2000, Horst et al. 2007) and common bean (Stass et al. 2007) using different experimental approaches. Eticha et al. (2005*a*) showed that the higher Al accumulation into the root apex in the maize cultivar Lixis was related to its higher content of low-methylated pectin and thus higher negativity of the cell wall compared to the cultivar ATP-Y. Therefore, it appears reasonable to assume that the higher Al accumulation of the common bean genotype Quimbaya in the root apex compared to VAX-1 (Fig. 1) is due to its higher content of unmethylated pectin (higher negative charge of the CW, Fig. 2).

However, in contrast to maize where a higher negative charge of the CW contributes to genotypic Al sensitivity (Eticha et al. 2005*a*) the common bean genotype Quimbaya is equally Al-sensitive as VAX-1 after 4 h of Al treatment in spite of its higher CW negativity (Fig. 2c) and Al accumulation even after 8 h of Al treatment (Fig. 1b), indicating possible genotypic differences in Al compartmentation and/or binding in the root apex.

It has been argued that the strong binding of Al in the CW represents a detoxification mechanism in squash (Le Van et al. 1994). However, the recovery from initial Al injury in both genotypes during 4-8 h of Al treatment and after 24 h Al treatment in the Al-resistant genotype Quimbaya were negatively correlated to the citrate non-exchangeable Al fraction of the root apices (Fig. 4) and more specific to the stable-bound Al CW fraction (Fig. 8). This suggests that the strong binding of Al to the pectic matrix of the CW is a main factor of Al toxicity and not a resistance mechanism in common bean. In contrast to the stable-bound CW Al fraction, there was no indication that the labile-bound (citrate-exchangeable) Al fraction

was related to Al-induced inhibition of root elongation (Fig. 8). This was unexpected, because in maize this fraction appeared to contribute to explaining silicon (Si)-mediated amelioration of Al toxicity (Wang et al. 2004). However, there seems to be a principal difference between monocots and dicots in Al binding to CWs, which is not surprising given the difference in CW composition (see above). This is well illustrated by the fact that treatment of CW with 50 mM $BaCl_2$ removed about 20% of the CW-bound Al in maize (Wang et al. 2004), and nearly all Al adsorbed on wheat CW could be exchanged with 2.5 mM $CaCl_2$ (Zheng et al. 2004). In contrast, $BaCl_2$ was unable to exchange any Al in common bean even after only short-term Al treatment (Stass et al. 2007, this study). The significant negative relationship between root elongation and citrate-exchangeable Al from intact root tips of genotype Quimbaya (Fig. 4a) might be explained by the contribution of free apoplastic Al to this fraction (Fig. 8).

The fractionated extraction procedure allowed to separate operationally defined apoplastic and symplastic Al fractions (Fig. 5). Among the 5 fractions the WFSF Al and the stable-bound CW Al-fractions are expected to best represent *in vivo* compartmentation of Al, the first because it is recovered by centrifugation from the root tips without destroying the compartmentation, the latter because it is expected to most slowly react during the extraction steps. These two fractions showed a close negative relationship with root elongation-rate reflecting recovery from initial Al stress particularly in genotype Quimbaya (Fig. 8). It is difficult to decide whether the symplastic and the labile-bound CW Al fractions under or overestimate the *in vivo* compartmentation. During the extraction process particularly during the recovery of the cell sap, organic ligands may mobilize labile-bound CW Al or symplastic Al is bound by CW due to a higher Al-binding strength of CW compared to symplastic ligands (Rengel 1996). In spite of these uncertainties the fractionated extraction procedure has proven to contribute to the understanding of Si amelioration of Al toxicity (Wang et al. 2004), Si-accumulating and Si-excluding plant species in relation to their resistance against plant pathogens (Heine et al. 2005, 2007), and Al accumulation of plant species like hydrangea

(*Hydrangea macrophylla* L.) and buckwheat (*Fagopyrum esculentum* Moench), which accumulate up to 70% Al in the symplast (B. Klug, personal communication) compared to 6-15% in common bean (Fig. 6).

The symplastic Al fraction neither reflect the recovery from initial Al stress in genotype Quimbaya nor the enhanced Al sensitivity of VAX-1 after the temporary recovery period at 8 h Al treatment (Fig. 6). However, the trend of increasing symplastic Al contents with the recovery and the significantly higher symplastic Al contents in Quimbaya compared to VAX-1 (Fig. 5) seems to indicate that transport of Al into the symplast is not a prerequisite for Al toxicity. Higher symplastic Al contents may rather be indicative of enhanced/acquired Al resistance which is in line with the observations by Vázquez et al. (1999) who ascribed internalization of Al into the symplast contributing to Al tolerance in an Al-tolerant maize genotype, and may indicate that also in common bean. Al internalization into endosomal/vacuolar compartments may contribute to the recovery from initial Al stress as reported for *Arabidopsis* (Illes et al. 2006). However, it is rather unlikely that this can explain enhanced Al resistance because of the quantitatively small Al fraction in the symplast (Fig. 6).

The transitory (VAX-1) or sustained (Quimbaya) recovery from initial Al-induced inhibition of root elongation (Fig. 1a) typical for pattern II plant species (see introduction) is related to a decrease in Al contents of the root tip (Fig. 1b), particularly in the apical 2 mm region (Rangel et al. 2007). The close negative correlation of root-elongation rate and Al contents of the WFSF and the stable-bound CW fraction (Fig. 8) suggests that the recovery from initial Al stress is related to the expression of an Al exclusion mechanism. This is in agreement with previous studies indicating that citrate exudation is a mechanism of Al resistance in common bean (Miyasaka et al. 1991, Mugai et al. 2000, Shen et al. 2002, Rangel and Horst 2006). Evidence for the effects of organic acid secretion in Al resistance is substantial, but the mode of action remains not well understood (Kinraide et al. 2005). Wehr et al. (2002) showed that citrate and malate were able to remove Al from artificial Al-pectate gels suggesting that

exudation of organic acids would remove Al bound to pectin and this could alleviate toxicity. However, the decrease of the Al content of the stable-bound CW Al fraction with increasing Al treatment duration as shown in Fig. 8 by root-released citrate appears to be improbable because this fraction is defined as citrate non-exchangeable. It thus appears that once Al is firmly bound it is unlikely to be released by the citrate exuded from the cells, unless the citrate concentration in the apoplast is much higher than the concentration used for the exchange (33 mM). Therefore, it is more likely that citrate released into the apoplast reduces the binding of Al in the apoplast by complexing Al and decreasing the strength of Al binding, thus preventing the strong binding of Al to the CW (Zheng et al. 2004). This allows resuming cell division and cell elongation, and explains reduction of the Al contents in the root apex through dilution by growth.

In conclusion, the results support the view that in common bean inhibition of root elongation cannot be explained by enhanced Al accumulation in the symplast. The present study indicates that the inhibition of root elongation is induced by apoplastic Al and that the induced and sustained recovery from the initial Al stress in the common bean genotype Quimbaya is mediated by reducing the stable-bound Al in the apoplast thus allowing cell elongation and division to resume

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Figure legends

Figure 1. Effect of Al treatment on the root-elongation rate (a) and total Al contents (b) of the root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl and 8 µM H_3BO_3 without (Control) or with 20 µM Al for up to 24 h, pH 4.5. Bars represent means ± SD, n = 4. For the ANOVA ***, ** denotes levels of significance at $P < 0.001$ and 0.01. Means with the same letter are not significantly different between times within each genotype, capital letters for Quimbaya and small letters for VAX-1; * denotes significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Figure 2. Total cell-wall pectin-content (a) its degree of methylation (b) and unmethylated pectin content (c) in 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 without (Control) or with 20 µM Al for up to 24 h, pH 4.5. Bars represent means ± SD, n = 4. For the ANOVA *, **, *** denote levels of significance at $P < 0.05$, 0.01 and 0.001, n.s. = not significant. Means with the same letter are not significantly different between times within each genotype, capital letters for Quimbaya and small letters for VAX-1; * denotes significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Figure 3. Citrate-exchangeable (a) and non-exchangeable (b) Al contents in 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, 8 µM H_3BO_3 and 20 µM Al for up to 24 h, pH 4.5 Excised root tips were incubated for 15 min each, first in 50 mM $BaCl_2$ and then in 33 mM Na_3Citrate. Bars are means ± SD, n = 4. For the ANOVA *** denotes a level of significance at $P < 0.001$. Means with the same letter are not significantly different between times within each genotype, capital letters for Quimbaya and small letters for VAX-1; * denotes significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Figure 4. Relationship between root-elongation rate and citrate-exchangeable (a) or non-exchangeable (b) Al contents of root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, 8 µM H_3BO_3 and 20 µM Al for up to 24 h, pH 4.5. Excised root tips were incubated for 15 min each, first in 50 mM $BaCl_2$ and then in 33 mM Na_3Citrate. For the ANOVA *, *** denote levels of significance at $P < 0.05$ and 0.001.

Figure 5. Aluminium contents of different cell compartments in 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, 8 µM H_3BO_3 and 20 µM Al for up to 24 h, pH 4.5. Bars represent means ± SD, n = 4. For the ANOVA **, *** denote levels of significance at $P < 0.01$ and 0.001. n.s. = not significant. Means with the same letter are not significantly different between times within each genotype, capital letters for Quimbaya and small letters for VAX-1; * denotes significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Figure 6. Relative distribution of Al contents of different cell compartments in the 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, 8 µM H_3BO_3 and 20 µM Al for up to 24 h, pH 4.5. The size of each pie chart represents the sums of all Al fractions. Means with the same letter are not significantly different between times in each fraction (Tukey test $P < 0.05$).

Figure 7. Relationship between root-elongation rate and apoplastic (a) or symplastic (b) Al contents of root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, 8 µM H_3BO_3 and 20 µM Al for up to 24 h, pH 4.5. For the ANOVA *, *** denote levels of significance at $P < 0.05$ and 0.001.

Figure 8. Relationship between root-elongation rate and the Al contents of three different apoplastic fractions in 5 mm root tips of genotype Quimbaya (Al-resistant) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, 8 µM H_3BO_3 and 20 µM Al for up to 24 h, pH 4.5. For the ANOVA *** denote levels of significance at $P < 0.001$.

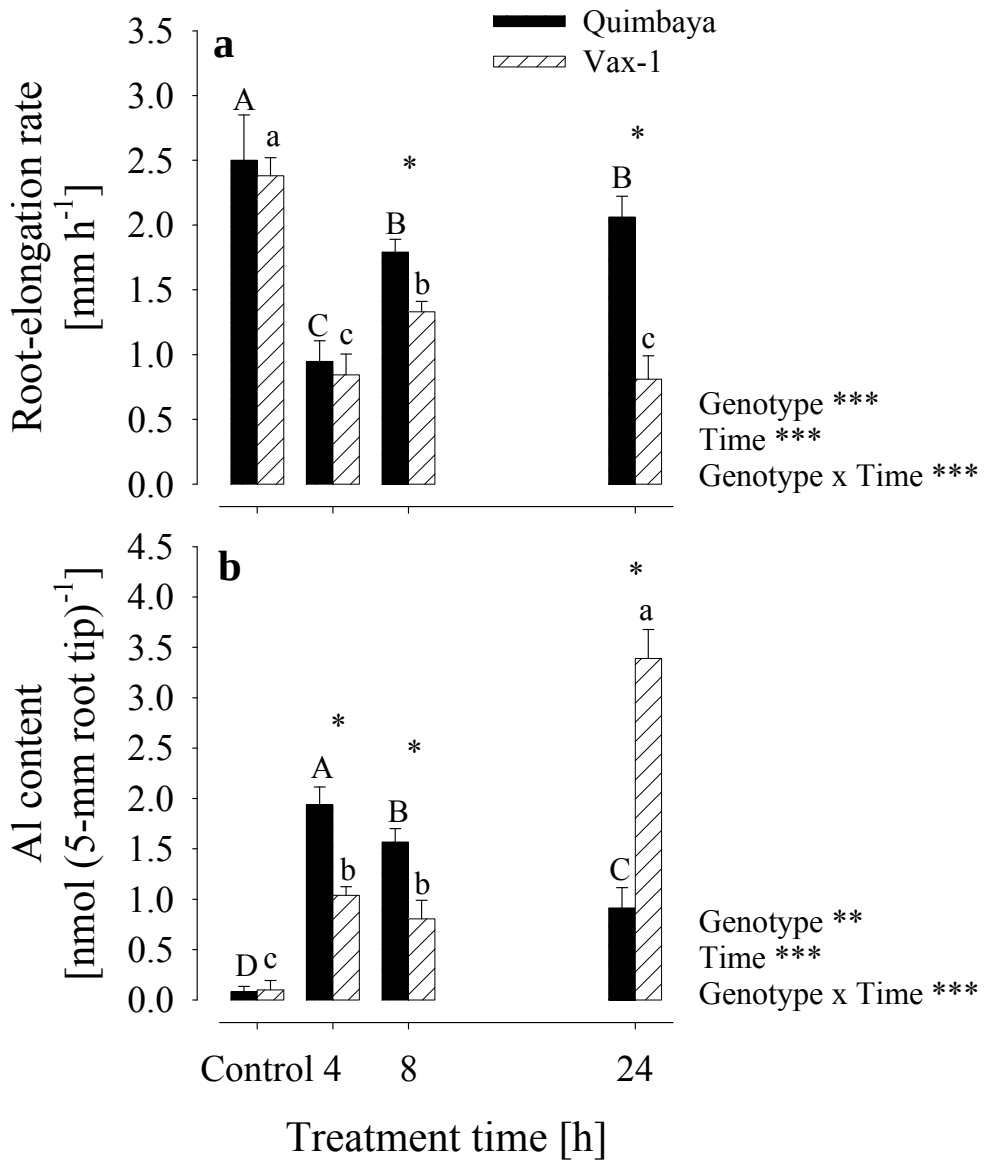

a
Quimbaya
Vax-1
Root-elongation rate [mm h-1]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
a
*
B
b
*
B
c
C
c
Genotype ***
Time ***
Genotype x Time ***
b
Al content [nmol (5-mm root tip)-1]
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
a
*
A
*
B
b
b
C
D
c
Genotype **
Time ***
Genotype x Time ***
Control
4
8
24
Treatment time [h]

Fig. 1

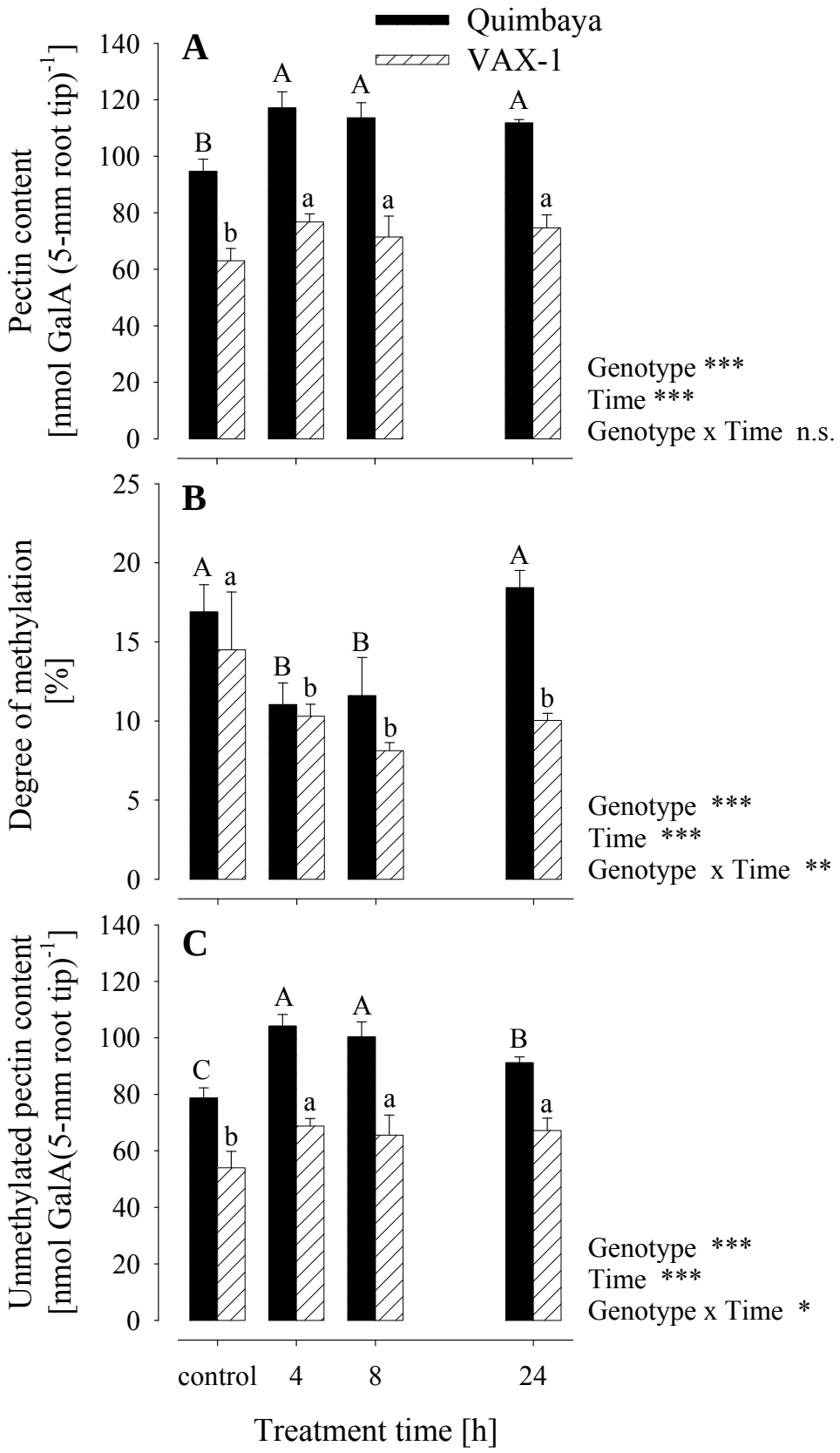

Quimbaya
VAX-1
A
Pectin content
[nmol GalA (5-mm root tip)$^{-1}$]
Genotype ***
Time ***
Genotype x Time n.s.
B
Degree of methylation
[%]
Genotype ***
Time ***
Genotype x Time **
C
Unmethylated pectin content
[nmol GalA(5-mm root tip)$^{-1}$]
Genotype ***
Time ***
Genotype x Time *
control
4
8
24
Treatment time [h]

Fig. 2

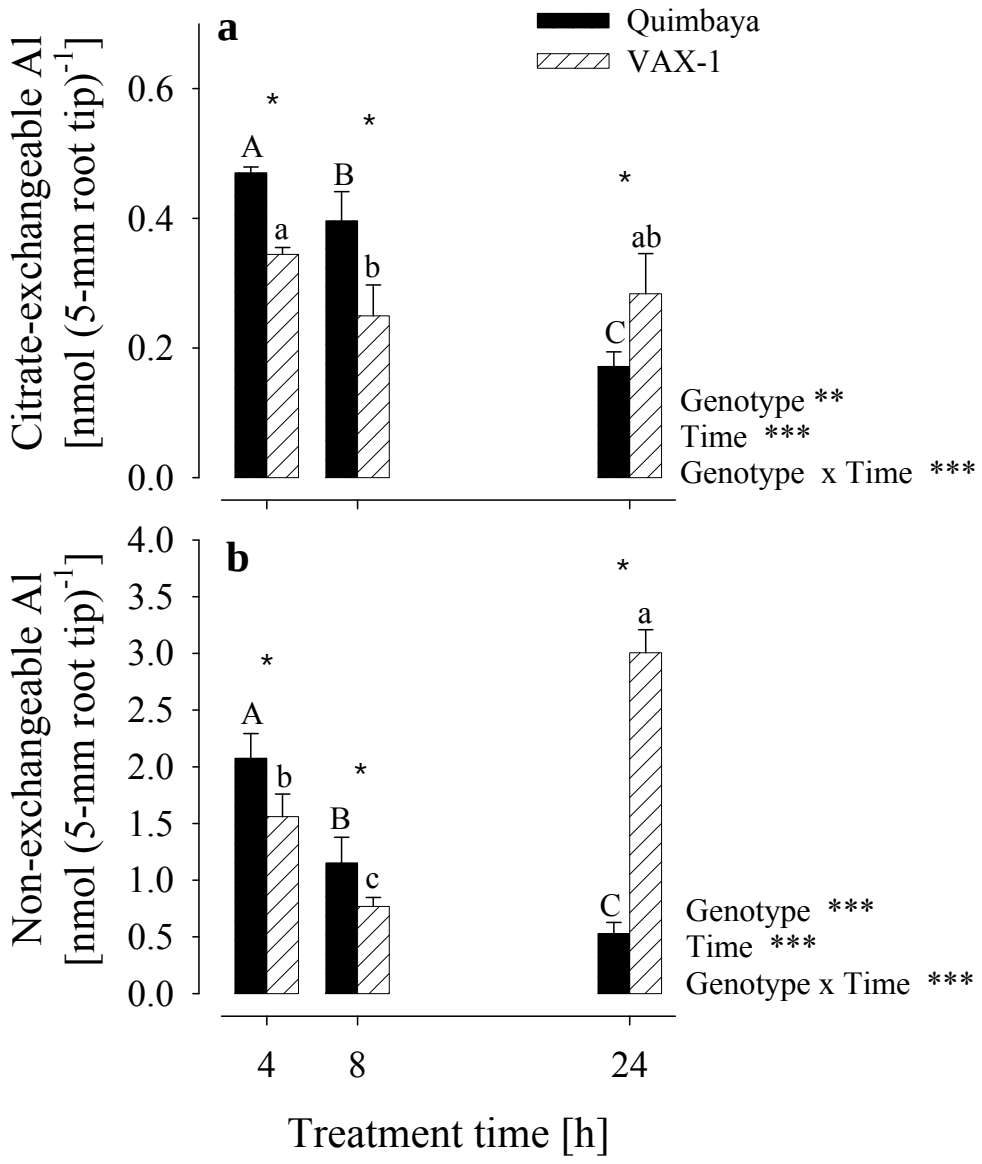

a
Quimbaya
VAX-1
Citrate-exchangeable Al
[nmol (5-mm root tip)$^{-1}$]
0.0
0.2
0.4
0.6
A
a
B
b
C
ab
*
Genotype **
Time ***
Genotype x Time ***
b
Non-exchangeable Al
[nmol (5-mm root tip)$^{-1}$]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
A
b
B
c
C
a
Genotype ***
Time ***
Genotype x Time ***
4
8
24
Treatment time [h]

Fig. 3

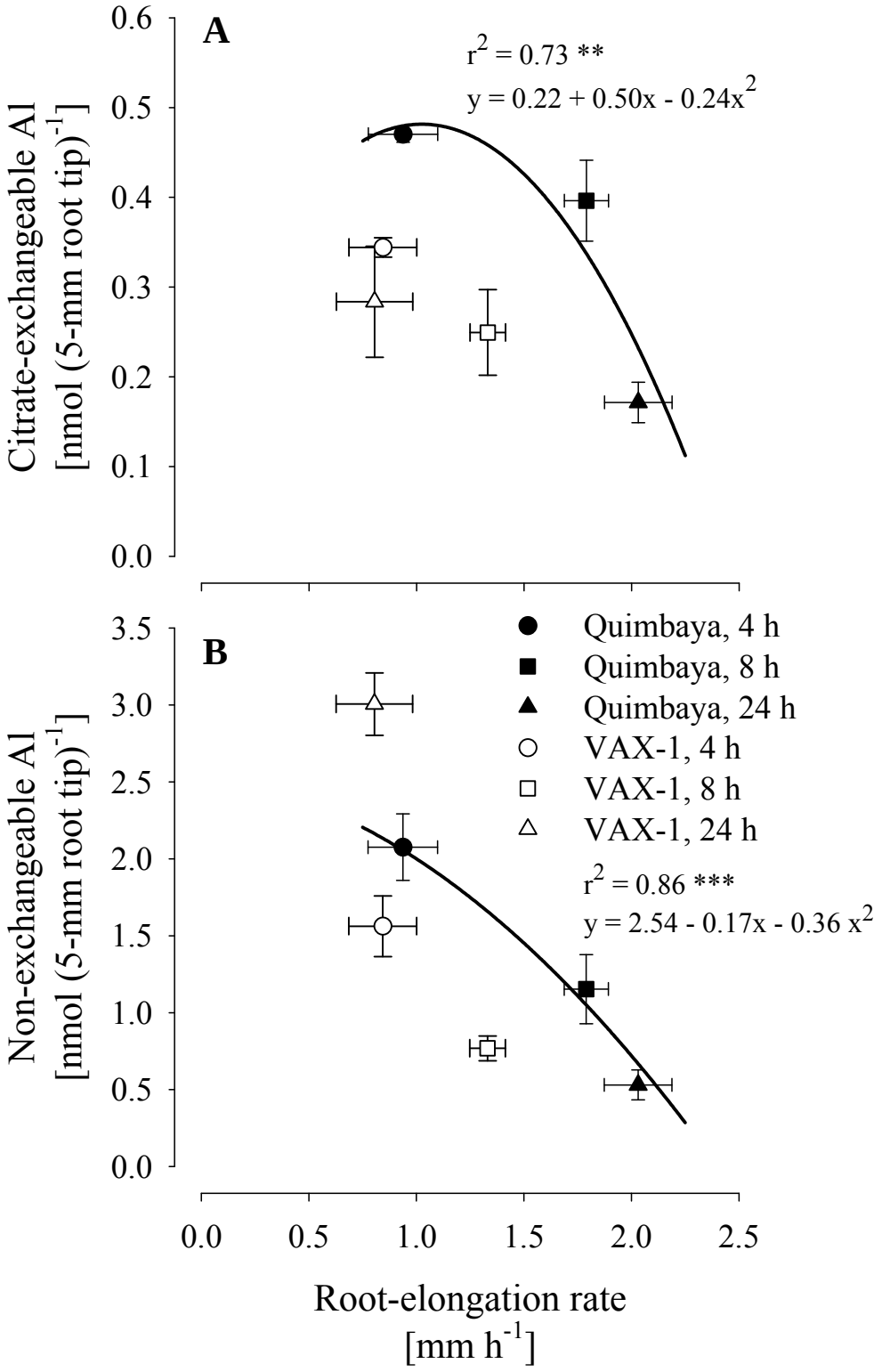
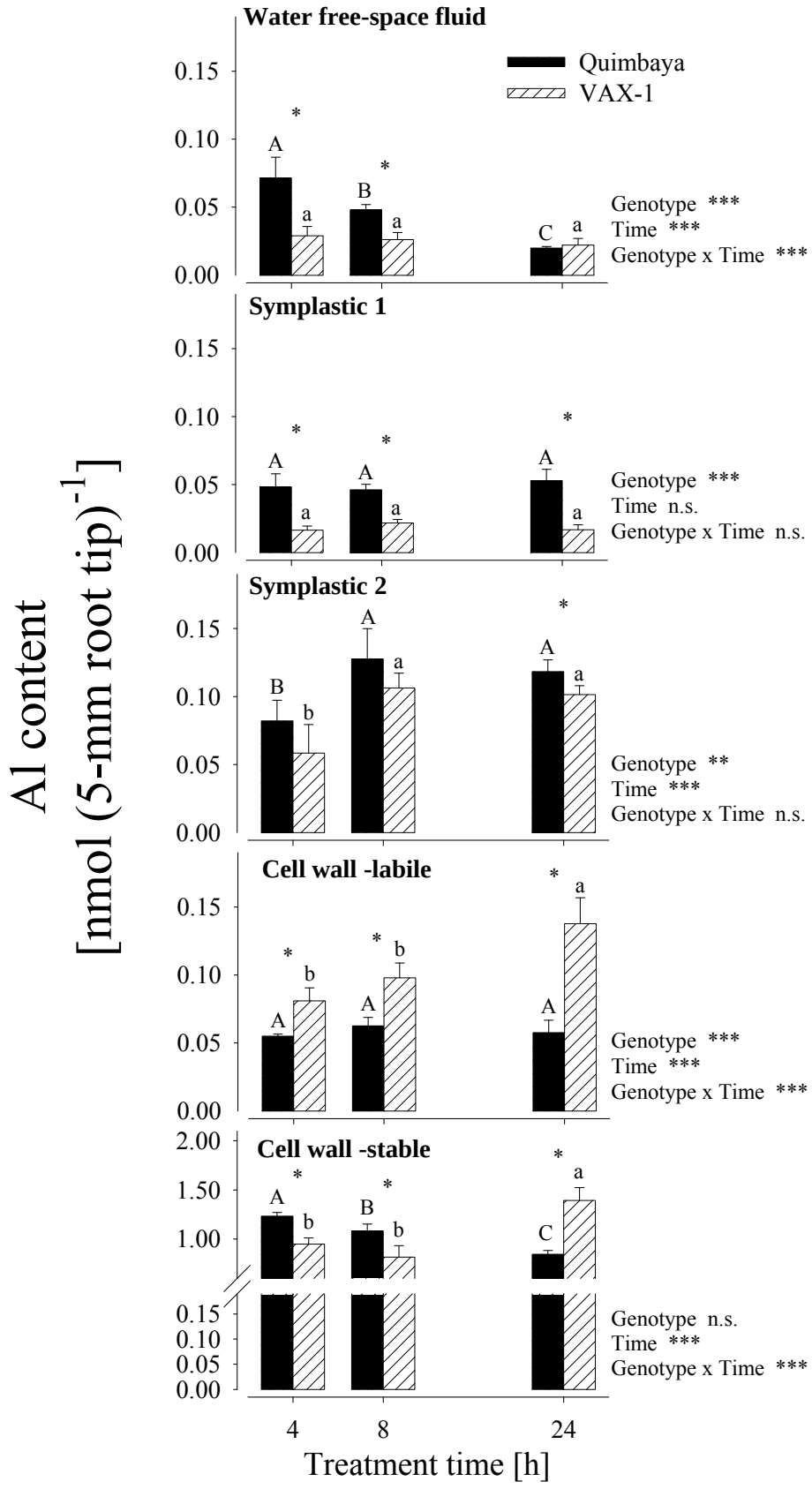

A
r^2 = 0.73 **
y = 0.22 + 0.50x - 0.24x^2
Citrate-exchangeable Al
[nmol (5-mm root tip)$^{-1}$]
0.0
0.1
0.2
0.3
0.4
0.5
0.6
B
Quimbaya, 4 h
Quimbaya, 8 h
Quimbaya, 24 h
VAX-1, 4 h
VAX-1, 8 h
VAX-1, 24 h
r^2 = 0.86 ***
y = 2.54 - 0.17x - 0.36 x^2
Non-exchangeable Al
[nmol (5-mm root tip)$^{-1}$]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.0
0.5
1.0
1.5
2.0
2.5
Root-elongation rate
[mm h^{-1}]

Fig. 4


Water free-space fluid
Quimbaya
VAX-1
Genotype ***
Time ***
Genotype x Time ***
Symplastic 1
Genotype ***
Time n.s.
Genotype x Time n.s.
Symplastic 2
Genotype **
Time ***
Genotype x Time n.s.
Cell wall -labile
Genotype ***
Time ***
Genotype x Time ***
Cell wall -stable
Genotype n.s.
Time ***
Genotype x Time ***
Al content
[nmol (5-mm root tip)-1]
Treatment time [h]
4
8
24

1

2 Fig. 5

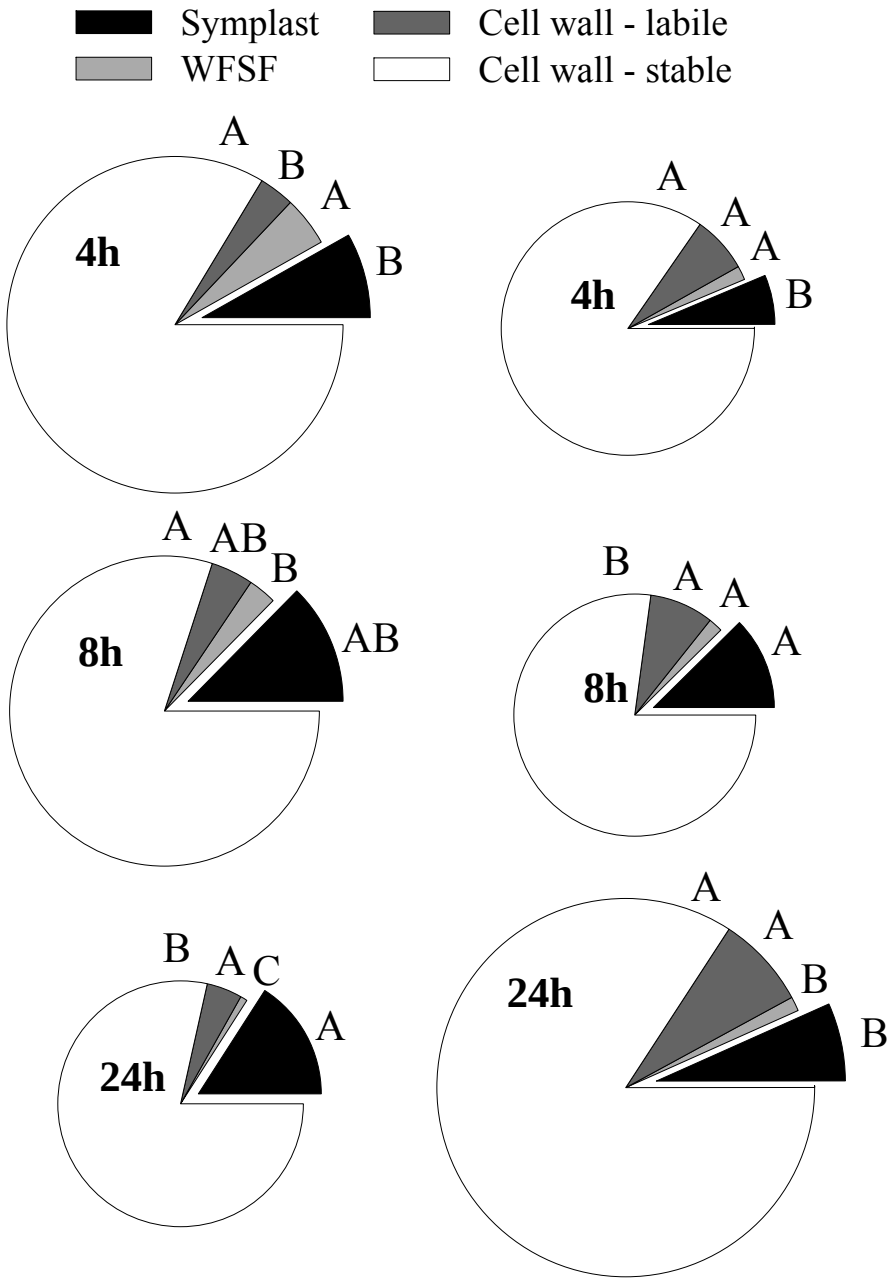

Symplast
Cell wall - labile
WFSF
Cell wall - stable
A
B
A
B
4h
A
A
A
B
4h
A
AB
B
AB
8h
B
A
A
A
8h
B
A
C
A
24h
A
A
B
B
24h
Quimbaya
VAX-1

Fig. 6

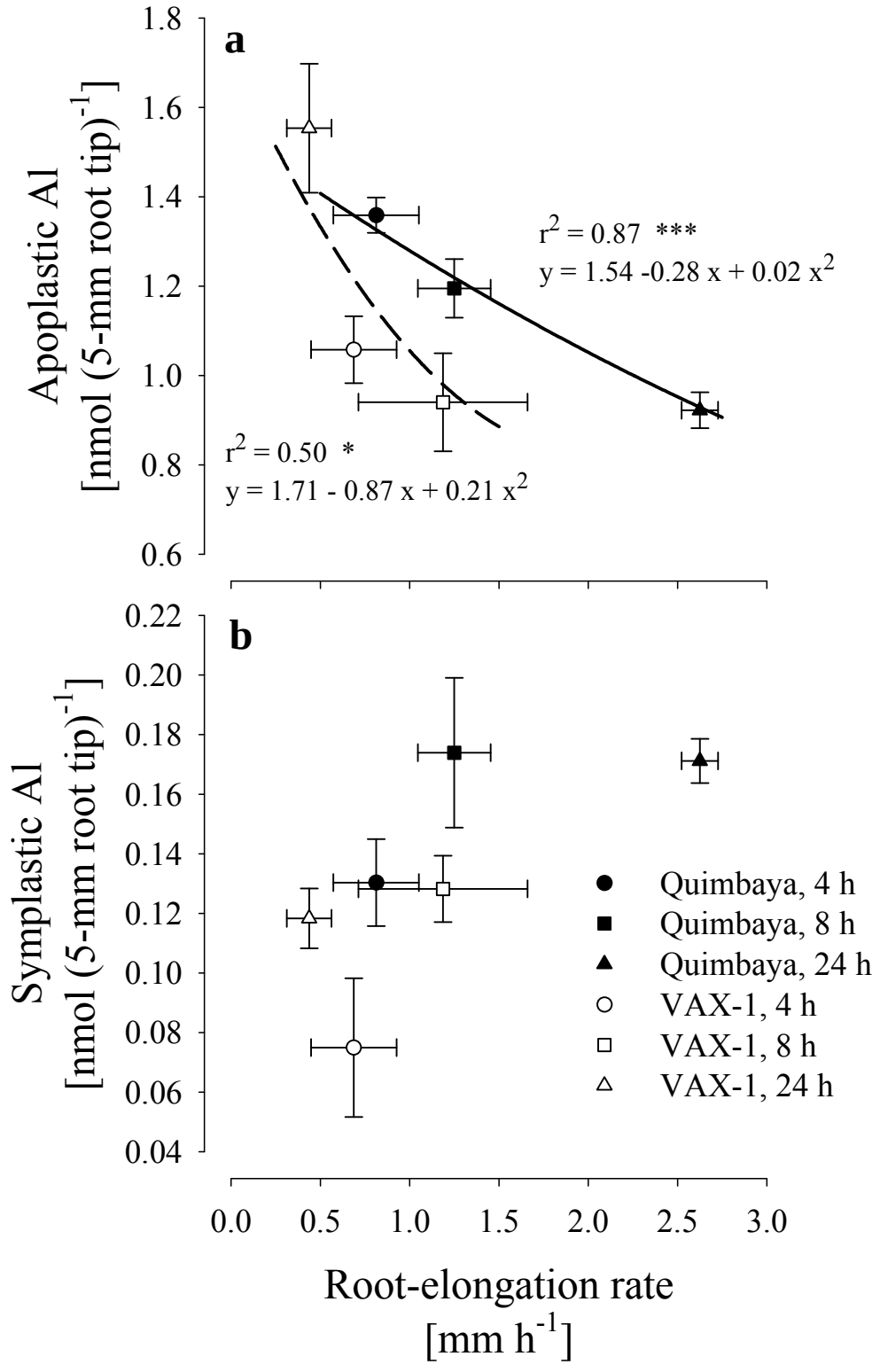
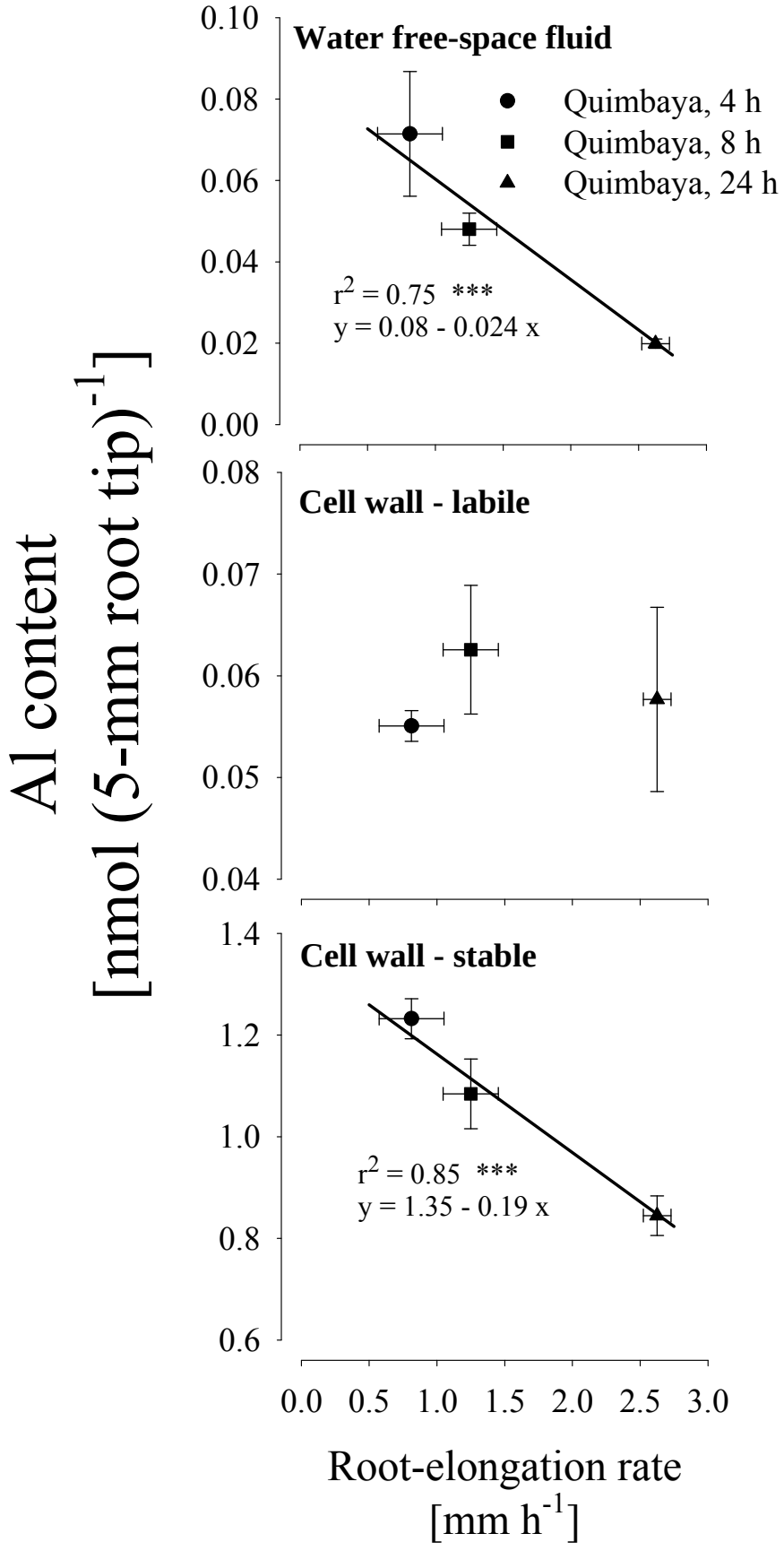

a
b
Apoplastic Al
[nmol (5-mm root tip)$^{-1}$]
Symplastic Al
[nmol (5-mm root tip)$^{-1}$]
r^2 = 0.87 ***
y = 1.54 -0.28 x + 0.02 x^2
r^2 = 0.50 *
y = 1.71 - 0.87 x + 0.21 x^2
Quimbaya, 4 h
Quimbaya, 8 h
Quimbaya, 24 h
VAX-1, 4 h
VAX-1, 8 h
VAX-1, 24 h
Root-elongation rate
[mm h^{-1}]

Fig. 7

1


Water free-space fluid
Quimbaya, 4 h
Quimbaya, 8 h
Quimbaya, 24 h
r2 = 0.75 ***
y = 0.08 - 0.024 x
Cell wall - labile
Cell wall - stable
r2 = 0.85 ***
y = 1.35 - 0.19 x
Al content
[nmol (5-mm root tip)-1]
Root-elongation rate
[mm h-1]

2

3 Fig. 8

1