Short- and Medium-Term Effects of Aluminium Toxicity and Resistance in Common Bean (*Phaseolus vulgaris* L.)

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades eines

Doktors der Gartenbauwissenschaften
- Dr. rer. hort. –

genehmigte Dissertation

von

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Geboren am 19.11.1971 in Santa Fé de Bogotá, Colombia

2007

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Tag der Promotion: 22. November 2007

Dedicated to

El esfuerzo de mis padres Maria Teresa y Fabio Armando
El amor de mis hermanos Carlos Armando, Alvaro José y Diego
Las enseñanzas y el recuerdo del Abuelo Carlos, Colega (q.e.p.d)
La energía de la abuela Olga
La música del abuelo Otón
Y al cariño y alegría de la abuela Blanca (q.e.p.d)

ABSTRACT

Common bean (*Phaseolus vulgaris* L.) needs significant improvement in aluminium (Al) resistance to reduce the dependence of small farmers on lime and fertilizer inputs on acid soils. Knowledge on the mechanisms of Al toxicity can contribute to the development of rapid and reliable screening procedures needed for accelerated breeding for Al resistance. The initial response of plants to Al is an inhibition of root elongation. Therefore, the present study focused on the temporal and spatial effects of Al on root growth, Al accumulation and cellular localization, and Al exclusion mediated by the release of organic acid anions.

The response of root elongation to Al in Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) common bean genotypes was characterized by a similar initial period (4 h) of Al sensitivity followed by a contrasting recovery period (8-25 h). Whereas the transition zone (TZ, 1-2 mm) was the most Al-sensitive apical root zone, Al applied to the elongation zone (EZ, 2-10 mm) also induced inhibition of root elongation. The initial enhanced inhibition of root elongation was related to high Al accumulation in the epidermal and outer cortical cells in the root apices in both genotypes, more in Quimbaya than in VAX-1, due to its higher content of unmethylated pectin and thus higher negativity of the cell wall (CW). The observed recovery of root elongation-rates of both genotypes was significantly negatively correlated with the free apoplastic and the stabile-bound, non citrate-exchangeable CW-Al representing the most important Al fraction in the root apex (80 %), but not with the symplastic and the labile-bound, citrate-exchangeable CW-Al. Sustained Al injury in genotype VAX-1 after 24 h Al treatment was related to the penetration of Al across the cortex and the endodermis up to the central cylinder.

The initial genotype-independent Al injury was due to the absence of citrate exudation from the root tips in both genotypes. Thereafter (5–9 h), in both genotypes root elongation recovered due to an Al-enhanced exudation of citrate from the internal organic acid content. In VAX-1 citrate efflux could be sustained during this period by a down-regulation of the activity of NADP-isocitrate dehydrogenase (NADP-ICDH) thus reducing the cytosolic turnover of citrate and a low but constant citrate synthase (CS) activity. In Quimbaya, the citrate efflux was sustained by both a lower NADP-ICDH activity and a greater internal citrate pool in spite of a decreased CS activity. The recovery of the CS activity after 25 h Al treatment allowed sustaining an enhanced capacity to exude citrate and to restore the internal organic acid pool, leading to decreasing Al contents and thus recovery of root growth in Quimbaya. In VAX-1, a decreased CS activity coupled with an exhausted internal citrate pool resulted in lower rates of citrate secretion, resumption of Al accumulation and, consequently, to severe inhibition of root elongation.

In conclusion, the study shows that in common bean the TZ is the most Al-sensitive apical root zone, however, Al applied to the entire EZ is also root growth-inhibiting. Thus the entire 10 mm root apex requires protection against Al injury in the Al-resistant genotype Quimbaya. Common bean proved to be a typical Pattern II plant species characterized by a most Al-sensitive response to short-term Al supply and then recovery from Al injury through the release of particularly citrate. Since citrate could only be released in response to Al after a lag phase of 4 hours in spite of high citrate concentrations in the root apex, this suggests that Al resistance in common bean requires the expression/activation of a citrate permease. Thereafter, the continuous release of citrate and maintenance of the cytosolic citrate concentration depends on the up-regulation of citrate synthesis and down-regulation of its degradation.

Key words: aluminum toxicity, aluminum fractionation, citrate exudation.

KURZFASSUNG

Die Verbesserung der Aluminium (Al) Resistenz in Bohnen leistet einen wichtigen Beitrag zur Verringerung der Abhängigkeit von Kleinbauern von Kalk- und anderen Düngern auf sauren Böden. Das Wissen um die Mechanismen der Al Toxizität kann dazu beitragen, schnelle und zuverlässige Selektionsmethoden zu entwickeln, die benötigt werden, um den Züchtungserfolg im Hinblick auf Al Resistenz zu beschleunigen.

Die erste Antwort der Pflanzen auf Al ist die Hemmung des Wurzelwachstums. Die vorliegende Arbeit beschäftigt sich daher mit den zeitlichen und räumlichen Effekten von Al auf Wurzeln, der Akkumulation von Al und der Lokalisierung im Gewebe, sowie mit Ausschlussmechanismen, die durch die Ausschüttung von organischen Säuren vermittelt werden.

Die Antwort des Wurzelwachstums auf Al in den Genotypen Quimbaya (Al-resistent) und VAX-1 (Al-sensitiv) zeichnete sich aus durch eine ähnlich verlaufende Anfangsphase (4h) der Al Sensitivität, gefolgt von einer Erholungsphase, die sich zwischen den Genotypen unterschied (8-25h).

Die Transitionszone (TZ, 1-2 mm) war die am meisten Al empfindliche Wurzelzone, jedoch rief auch die Zugabe von Al zur Elongationszone (EZ, 2-10 mm) eine Verminderung des Wurzelwachstums hervor.

Die anfangs verstärkte Absenkung des Wurzelwachstums war mit einer hohen Al Akkumulation in den Zellen der Epidermis und des äußeren Kortex in den Wurzelspitzen beider Genotypen verbunden, zeigte sich jedoch stärker in Quimbaya als in VAX-1, aufgrund dessen höheren Gehaltes an unmethyliertem Pektin, und somit höherer Negativität der Zellwand (CW). Die beobachtete Reduktion des Wurzelwachstums in beiden Genotypen war signifikant negativ korreliert mit dem Al in der Fraktion des freien apoplastisch und des fest gebundenen, nicht mit Citrat austauschbaren Zellwand Aluminiums, welches die wichtigste Al Fraktion in der Wurzelspitze darstellt (80%), aber nicht mit dem symplastischen und der lose gebundenen, Citrat austauschbaren Zellwand-Aluminiums. Die weitere Verletzung durch Al in VAX-1 nach 24 h Al Behandlung war verbunden mit dem Eindringen des Aluminiums durch den Kortex und die Endodermis bis in den Zentralzylinder.

Der anfängliche, vom Genotypen unabhängige Schaden durch Al entstand durch die fehlende Citrat- Exudation in den Wurzelspitzen beider Genotypen. Danach (5-9 h) erholte sich das Wurzelwachstum in beiden Genotypen, ausgelöst durch eine durch Al angeregte Ausschüttung von Citrat aus den internen Vorräten an organischen Säuren, was zu reduzierten Citrat- und Al Gehalten führte. In VAX-1 konnte der Citratefflux während dieser Zeit durch ein Herunterschrauben der NADP-Isocitratdehydrogenaseaktivität erreicht werden, wodurch der cytosolische Gesamtumsatz von Citrat reduzierte, und eine niedrige, aber konstante Aktivität der Citratsynthase erreicht wurde. In Quimbaya wurde die Citratausschüttung durch eine niedrigere NADP-ICDH-Aktivität und einen größeren internen Citratvorrat trotz einer verringerten CS Aktivität aufrechterhalten. Die Wiedereinsetzen der CS Aktivität nach 25h Al Behandlung erlaubte es, eine erhöhte Kapazität beizubehalten, um Citrat auszuschütten, und um die internen Vorräte an organischen Säuren zu erneuern, was zu niedrigeren Al Gehalten und somit zu einer Erholung des Wurzelwachstums in Quimbaya führte. In VAX-1 resultierte eine verringerte CS Aktivität, verbunden mit erschöpften internen Citratvorräten, zu einer Wiederaufnahme der Al Akkumulation und einer nachfolgenden schweren Hemmung des Wurzelwachstums.

Als Fazit dieser Arbeit kann gesagt werden, dass in Bohnen die TZ die am meisten Al sensitive Zone der Wurzelspitze ist. Dennoch bewirkt auch die Zugabe von Al auf die gesamte EZ eine Wachstumsreduzierung. Die gesamte 10 mm Wurzelspitze benötigt also einen Schutz vor einer Verletzung durch Al im Al resistenten

Genotypen Quimbaya. Die Ergebnisse dieser Untersuchungen zeigen, dass in Bohnen die EZ die am meisten sensitive apikale Wurzelzone ist; jedoch führt Al auch in der gesamten EZ zu einer Hemmung des Wurzelwachstums. Somit benötigt die gesamte 10-mm Wurzelspitze einen Schutz vor Verwundung durch Al im Al resistenten Genotypen Quimbaya. Bohnen sind bewiesenermaßen typische Pattern II Pflanzen, charakterisiert durch eine sehr sensitive Antwort auf Kurzzeit Applikation von Al und anschließender Erholung der Verletzung durch die Ausschüttung insbesondere von Citrat, aber auch anderen organischen Säuren. Citrat wurde trotz hoher Konzentrationen in der Wurzelspitze erst nach einer Verzögerung von 4h als Antwort auf Al entlassen, was darauf schließen lässt, dass Al Resistenz in Bohnen eine Aktivierung der Citratpermease verlangt. Demnach hängen die kontinuierliche Ausschüttung von Citrat und die Beibehaltung der cytosolischen Citratkonzentrationen von der Hochregulierung der Citratsynthese und der Herabregulierung deren Degradation ab.

Schlagwörter: Aluminumtoxizität, Aluminumfraktionierung, Citratausschüttung.

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ABBREVIATIONS

DTNB	"5,5´dithio-bis-2-nitrobenzoic acid"
ATP	adenosine triphosphate
Al	aluminium
Al_{mono}	aluminium monomeric
ANOVA	analysis of variance
AEZ	apical elongation zone
Ba	barium
BEZ	basal elongation zone
Ca	calcium
CW	cell wall
CEZ	central elongation zone
CIAT	Centro Internacional de Agricultura Tropical
Cl	chloride
CS	citrate synthase
TCA	citric acid cycle
d	days
^0C	degree Celsius
DM	degree of methylation
DTZ	distal transition zone
EZ	elongation zone
EDXMA	energy-dispersive X-ray analysis
EDTA	ethylen diamide tetra acetate
Fig	figure
GalA	galacturonic acid equivalent
g	gram
GFAAS	graphite furnace atomic absorption spectrometer
R	growth rate
ha	hectare
HPLC	high pressure liquid chromatography
H	hydrogen
Fe	iron
ICL	isocitrate lyase
kg	kilogram

l	liters
Mg	magnesium
MDH	malate dehydrogenase
MS	malate synthase
MΩ	mega Ohm
MZ	meristematic zone
µl	microliters
mg	milligram
ml	milliliters
µM	micromolar
mM	millimolar
mm	millimeters
MSD	minimum significant difference
min	minutes
M	molar (mol l^{-1})
Mo	molybdenum
HEPES	N-(2-hydroxyethyl)-piperazin-N´-2-ethansulfonacid
ICDH	NADP-Isocitrate dehydrogenase
nm	nanometer
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
N	nitrogen
n.s.	not significant
OA	organic acid
ppm	parts per million
P	phosphorous
pep	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PFK	phosphofructokinase
PVC	polyvinyl chloride
K	potassium
p	probability level
PEZ	proximal elongation zone
REGR	relative elemental growth rate

SIMS	secondary ion mass spectrometry
s	seconds
Si	silicon
Na	sodium
SD	standard deviation
SYM	symplastic
TZ	transition zone
TTC	triphenyl tetrazolium chloride
Tris	tris-(hydroxymethyl)-amino methane
dd water	ultra pure water
UV	ultraviolet light
VA	vesicle arbuscular
v/v	volume per volume
WFSF	water free space fluid
λ	wavelength
w/v	weight per volume

GENERAL INTRODUCTION

The legume family (Leguminosae) is very large with 643 genera (18000 species) grouped into 40 tribes that are found in both tropical and temperate environments. The tribe *Phaseoleae* [common bean (*Phaseolus vulgaris* L.), long-bean/cowpea (*Vigna unguiculata* L.), and soybean (*Glycine max* L.)] is by far the most important economic group, and contains 75 % of the legumes traded in the world (Broughton et al., 2003). Bean (*Phaseolus* spp. L) is one of the most ancient crops of the world, consisting of 50 to 60 known species, all originated in the Americas (Debouck, 1999). From the still existing wild forms, only five species have been fully domesticated and among them, common bean is one of the most widely grown (Debouck, 2000). From the nucleus of diversity (limits between Ecuador and Peru), two distinct gene pools have been derived: Mesoamerican (northwards) and Andean (southwards). They have been also qualified as sub-species based on the existence of geographic partial-reproductive isolation between them (Gepts, 1998).

Common bean is the most important food legume for more than 300 million people, most of them in the developing world. Therefore, common bean has been named as “the poor man’s meat”, with a yearly consumption ranging between 6 to 24 kg per person in Latin America and 12 to 66 kg per person in eastern and southern Africa (CIAT, 1999; Broughton et al., 2003). Common bean is the second source of protein in eastern and southern Africa and fourth in Latin America (65 % of all protein consumed). It is also the third most important caloric source (32 % of all calories consumed) after cassava (*Manihot esculenta* Crantz) and maize (*Zea mays* L.) (CIAT, 1999).

In year 2005, dry beans including cowpeas represented around 22.8 million tons harvested on 34.8 million hectares (ha), with an usually low average yield of 657 kg ha^{-1} (Faostat, 2005 http://faostat.fao.org/site/340/default.aspx). Singh (1999) estimated that annually 11.6 million tons of dry beans alone are produced on 14.3 million ha, with an annual production value of over US$ 10 billion. Common bean is mainly produced by resource-poor farmers on small-scale marginal farms (80 % of the area planted) in developing countries in Latin America (44 % of the world annual production) and Africa. Apart from the losses related to diseases and insect pests, drought and low availability of P, N, and toxicity of Al and/or Mn associated with low soil pH (< 5.5) lead to the low average yield registered (Thung and Rao, 1999; Wortman et al., 1998).

About 40 % of the bean-growing area in Latin America and 30 to 50 % of the bean-production areas of central, eastern, and southern Africa are affected by Al toxicity and/or P deficiency resulting in yield reduction from 30 to 60 % (CIAT, 1992). Correction of acidity-related soil constraints (i.e. low pH or Al toxicity) using lime and phosphate fertilizers, increasing the base saturation through fertilization, or increasing the organic matter content (Lopes, 1996; Baligar et al., 1997) are beyond the capacity of resource-poor farmers (Rao et al., 1993). Moreover, these practices are inefficient to correct deeper layers of the soil. Thus, development of cultivars resistant to soil acidity are ecologically friendly, energy conserving and much more economical to resource-poor farmers in the tropics than amending the soil to overcome soil acidity (reduce Al toxicity). However, it is clear from the existing information that sustainability in this system has to be achieved with the use of Al-resistant cultivars and selected agronomic measures (Rao et al., 1993). The humid tropics in Africa and the sub humid Brazilian savannah Cerrado are characteristic examples where food production has being improved by such an integrated approach (Sanchez, 1997).

Acid soils with pH below 5.5 in the surface zone occupy approximately 4000 million ha of the world's land, from which as much as 50 % are potentially arable land. They mainly occur in two extremely weathered global belts (von Uexküll and Mutert, 1995). A northern belt covering the humid northern temperate zone is predominantly comprised of organic acid soils supporting coniferous forest. A southern belt localized in the humid tropics mainly covered by savannah and tropical rain-forest on Oxisols and Ultisols, represents about 1700 million ha (von Uexküll and Mutert, 1995). Acid soils result from accelerated chemical and biological processes on a geological parent material that is low in essential mineral elements (Ca, Mg, K and Na) coupled with an intense rate of weathering. Under these conditions, loss of most nutrients by leaching and the development of a highly acidic soil profile, dominated by end-products of mineral weathering: kaolinite and the oxides and hydrous oxides of Fe and Al, determine an inherent infertility (Rao et al., 1999). Except for extreme situations, pH per se rarely has a direct effect on plant growth. Thus, poor crop growth on acid infertile soils usually occurs by a combination of factors such as: high levels of Mn and Al, low saturation of bases (Ca, Mg, and often K), low or unavailable soil-P, low levels/availability of micronutrients, especially Mo, and low populations of rhizobia and VA-mycorrhizae (Howeler, 1991; von Uexküll and Mutert, 1995; Marschner, 1995). However, when the pH drops below 5, Al is solubilized into the soil solution and becomes the single most important factor, being a major constraint for crop production on 67 % of the total acid soil area (Foy, 1976; Horst, 1995; Eswaran et al., 1997). The chemistry of Al in solution is complex because Al hydrolyses in a pH-dependent manner into various complexes with hydroxyl groups. Although the toxicity of these soluble Al species varies considerably, under acidic conditions particularly Al^{+3} rather than $Al(OH)^{+2}$ and $Al(OH)_2^+$ is the most phytotoxic Al species

(Kinraide, 1991; Delhaize and Ryan, 1995; Kochian et al., 2004). Under alkaline conditions, the aluminate ion ($Al(OH)_4^-$) is the most important Al species (Martin, 1988). However, the formation of polymeric Al species (Al_{13}) is also possible (Parker et al., 1989; Kinraide, 1990 and 1997). Although, the toxicity of Al under alkaline conditions in nutrient solution it is well recognized (Parker et al., 1989; Kinraide, 1990, 1997; Kopittke et al., 2004; Stass et al., 2006), the determination of the most phytotoxic Al species (Stass et al., 2006) or its appearance in soil solution remains a matter of debate (Larsen et al., 1995; Kerven et al., 1995).

Aluminium primarily affects the plant roots and with the exception of cassava (Asher et al., 1980) there is no specific leaf symptom associated with Al toxicity. However, given sufficient exposure, Al induced symptoms of drought stress and P, Ca, Fe and Mg deficiencies (Furlani and Clark, 1981, Foy, 1988). Aluminium-injured roots are characterized by swollen root apices and poor or no root-hair development (Horst, 1987; Blancaflor et al., 1998). Root tips and lateral roots become thickened due to an uneven and radial expansion of cortical cells (Ciamporova, 2002), giving the whole root system a coralloid appearance with many small stubby and brittle lateral roots but no fine branching (Furlani and Clark, 1981). Moreover, destruction of the root epidermal and outer cortical cells resulted in disintegration of the outer root-surface area (Lee and Foy, 1986).

The most widely recognized symptom of Al toxicity is inhibition of root elongation (Foy, 1988) which can be measured within 30 to 90 min after exposure of the roots to excess Al supply (Llugany et al., 1995; Sivaguru and Horst, 1998). The rapidity of the root-growth inhibition suggests that Al quickly disrupted root cell-expansion and elongation (Horst and Klotz, 1990) rather than cell division (Horst, 1995; Frantzios et al., 2001). However, reduced cell division will contribute to growth inhibition at later stages (Horst et al., 1983; Kochian, 1995; Silva et al., 2001; Barceló and Poschenrieder, 2002). Considering that Al interferes with a range of physical and cellular processes before growth is impeded (Rengel, 1996), it has been hypothesized that Al toxicity could result from complex interactions of Al with apoplastic (Horst, 1995; Blamey, 2001), plasma membrane (Zhao et al., 1987; Wagatsuma et al., 1995; Ishikawa and Wagatsuma, 1998), and symplastic targets (Kochian, 1995, Rengel, 1996; Barceló and Poschenrieder, 2002; Kochian et al., 2005). Although, the physiological mechanisms of Al-induced inhibition of root elongation are still not well understood, considerable progress, summarized in a number of reviews, has been made during the last two decades based on physiological studies combined with molecular-assisted breeding and biotechnological approaches (Taylor, 1991, 1995; Kochian, 1995; Delhaize and Ryan, 1995;

Matsumoto, 2000; Barceló and Poschenrieder, 2002; Kochian et al., 2005; Vitorello et al., 2005; Horst et al., 2007). According to these reviews, it is generally accepted that Al perception and response mechanisms occurs mainly at the root apex (Horst, 1995; Taylor, 1995). This conclusion is based on the fact that Al accumulates predominantly in this zone (Delhaize et al., 1993a, Sivaguru and Horst, 1998), and Al-induced callose formation, a sensitive indicator of Al injury and a reliable parameter for the classification of genotypes of different plant species for Al resistance (Wissemeier et al., 1987; Zhang et al., 1994; Wissemeier and Horst, 1995; Horst et al., 1997; Eticha et al., 2005a), is induced primarily in outer cortical cells of the root apex (Sivaguru and Horst, 1998). Our previous studies have shown that genotypic differences in Al resistance of common bean could be observed based on inhibition of root elongation in nutrient solution after 36 h at 20 µM Al supply as a parameter for Al injury (Rangel et al., 2005, Manrique et al., 2006). As in maize, short-term Al supply (4 h) led to maximum accumulation of callose in the root apex of common bean. However, no relationship was observed between Al-induced callose contents and root-growth inhibition, hampering the use of this parameter as a screening tool for Al resistance in common bean (Rangel et al., 2004). Cumming et al. (1992) proposed that Al resistance is an inducible trait in common bean, requiring a period of stress before a resistance mechanism is "switched on". It thus appears that the Al-perception and response mechanism in common bean could differ from those of maize or wheat. Detailed studies on the kinetics of the response of root-growth rates to exposure to Al offer the possibility to differentiate between constitutive or inducible Al-resistance mechanism and to verify whether this is consistent across genotypes (Parker, 1995). Similarly, a quantitative description of spatial growth profiles (Peters and Bernstein, 1997) coupled with physiological, morphological and cytological studies allowed improving our understanding on the physiology of growth regulation and its modification under Al-stress conditions, and the important role of the root apex in the Al-perception and response mechanism (Ryan et al., 1993; Ishikawa and Evans, 1995; Baluška et al., 1990, 1996; Kollmeier et al., 2000).

For the manifestation of Al toxicity, both in roots or cell suspensions, it is necessary that Al uptake occurs (Taylor, 1991; Horst, 1995, Kochian, 1995). Up to date, there is no consensus whether symplastic or apoplastic lesions are the primary target of Al toxicity. However, it is generally agreed that Al is accumulated in roots with a rapid initial phase and a lower rate thereafter (Zhang and Taylor, 1989, 1990). The negativity of the cell wall established by the pectin content and its degree of methylation is a major determinant of this initial Al accumulation (Blamey et al., 1990; Schmohl and Horst, 2000, Schmohl et al., 2000, Eticha et al. 2005b, Horst et al., 2007) leading to Al injury through altering cell-wall characteristics and functions (Blamey, 2001; Sivaguru et al., 2006). There is no doubt that Al can enter the symplast (Tice et al., 1992; Lazof et al., 1994; Vázquez et al., 1999; Taylor et al., 2000; Eticha et al., 2005c). 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 cell wall and plasma membrane will necessarily precede any transport into the symplast, these interactions being potentially harmful (Delhaize and Ryan, 1995). Dicotyledons are characterized by higher pectin contents in the cell wall than grasses (Carpita and Gibeaut, 1993), leading to higher rates of Al accumulation (Schmohl and Horst, 2000; Marienfeld et al., 2000). In common bean, genotypic differences in Al resistance have been observed using Al accumulation after long-term (between 24 and 72 h) Al treatment (Lee and Foy, 1986; Massot et al., 1999, Mugai et al., 2000; Shen et al., 2002a, Rangel et al., 2004). However, detailed studies on the kinetics of the Al accumulation and its distribution at the cellular and tissue level in relation to genotypic differences in Al resistance of common bean has been not conduct so far.

Wide genetic variation, both within and between species, in the Al resistance of plants exist, suggesting that Al-resistant species or cultivars possess several mechanisms for detoxifying Al (Taylor, 1991; Horst, 1995, Matsumoto, 2000; Kochian et al., 2004; Vitorello et al., 2005). As proposed by Taylor (1991), the resistance mechanism identified can be separated into those that allow the plant to tolerate Al within the cells and strategies involved in exclusion of Al from the root apex. In general, plant species that developed mechanisms of cellular tolerance are called Al includers or accumulators and those that developed strategies to minimize the uptake of Al into the root apex are called Al excluders (Watanabe and Osaki, 2002). In Al accumulating plants, such as *Camellia sinensis* and *Hydrangea macrophylla*, Al is complexed with organic ligands, mainly organic acids, and readily translocated from roots to shoots, and then stored in specialized cells such as leaf epidermal cells (Ma et al., 1997, 1998; Watanabe and Osaki, 2002). Additionally, there is increasing evidence that phenols may be involved in the internal sequestration of Al (Barceló and Poschenrieder, 2002). In Al excluders, other mechanism of internal tolerance such as compartmentation in the vacuole (Vázquez et al., 1999; Vázquez, 2002), accelerated turnover of root epidermal cells (Delisle et al., 2001) have been also observed.

As mentioned above, Al binds readily to binding sites in the apoplast and the plasma membrane leading to the impairment of root growth and functions. Therefore, it has to be postulated that reduced binding of Al in the apoplast is a requisite for Al resistance (Horst et al., 2007). Several mechanism of Al exclusion have been postulated (Taylor, 1991), such as plant-induced pH barrier in the rhizosphere (Degenhardt et al., 1998); exudation of phosphate (Pellet et al., 1995, 1997); reduced binding capacity of the cell wall (Schmohl and Horst, 2000; Eticha et al., 2005b); enhanced accumulation of Al in the root cap mucilage forming a diffusion barrier to Al or concentrated organic acids that chelate Al (Henderson and Ownby, 1991; Horst et al., 1992; Archambault et al., 1996); binding of Al by excreted polypeptides (Basu et al., 1999); and exudation of organic acid anions (Ma et al., 2001; Kochian et al., 2004). Since the early 90´s, accumulating evidence has shown that organic acids play an important role in detoxifying Al both internally and externally (Ma, 2000; Ma et al., 2001; Ryan et al., 2001; Ma and Furukawa, 2003). Both, the kind and the amount of exuded organic acid contribute to the Al-detoxification capacity (Zheng et al., 1998a). Consequently, the organic acids were classified as strongly (citrate, oxalate and tartrate), moderately (malate, malonate and salicylate) and weakly (succinate, lactate, formate and acetate) Al-detoxifying compounds according to the stability of the Al complex (Hue et al., 1986). Excretion of organic acids that chelate Al have been reported in common bean, wheat, maize, soybean, rye, among others (for reviews, see Ma et al., 2001, Ryan et al., 2001, Ma and Furukawa, 2003; Kochian et al., 2004). However, the patterns of release vary between plant species (Ma et al., 2001). In several plant species such as wheat, activation of organic acid efflux (i.e. malate) occurs rapidly without any discernable delay after exposure to Al (Ryan et al., 1995). In some other plant species, a lag phase in the secretion of organic acids has been observed, suggesting the requirement of gene induction and protein synthesis either for organic acid transport or synthesis (Ma et al., 2001, Ryan et al., 2001; Kochian et al., 2004). In common bean, Al stress enhanced exudation of citrate (Miyasaka et al., 1991; Mugai et al., 2000; Shen et al., 2002a, Rangel and Horst, 2006; Stass et al., 2007). However, neither the pattern of citrate exudation nor the influence of Al on the metabolism of citrate synthesis has been characterized so far.

First attempts to determine resistance to Al in beans dated from the mid 70´s (Howeler, 1991, Foy et al., 1972). Experiments conduced at Carimagua, Colombia in the mid 70's classified cowpea as more tolerant than black seeded beans, maize and soybeans as intermediate and non-black seeded beans as highly sensitive of soil acidity (Howeler, 1991). Another screening of 60 bean accessions in strongly acid soil in Quilichao, Colombia, corroborated that black seeded beans had not only higher yields at different low lime levels, but also a higher acid-soil tolerance index than beans of other colors. From those experiments, a critical Al-saturation level of about 8 to 10 % was determined, and confirmed in snap beans (Howeler, 1991). Foy and his collaborators (1972) tentatively associated the better performance of certain snap bean varieties grown on an acid Bladen soil with Al tolerance. Further experiments in nutrient solution confirmed that the Bladen soil-tolerant variety Dade was much more tolerant to Al (8 ppm) than the Bladen-sensitive variety Romano. Experiments conducted by Lee and Foy (1986) with the same cultivars, indicated that Dade had a higher potential for Al chelation and detoxification than Romano. Thung et al. (1987) screened large number of bean cultivars for both tolerance to high Al and low P, and found that those cultivars tolerant to both stresses tended to originate from acid-soils areas in Brazil (cultivars Carioca, Brasil 349 and Iguacu). Genotypic differences in seed yield of 5000 common bean germplasm accessions and breeding lines have been observed in field screening on Al-toxic soils that were amended with or without lime (65 % Al saturation) (Rao et al., 2004). Significant genotypic differences in Al resistance in common bean were also reported based on Al-inhibited root elongation in nutrient solution (Massot et al., 1999; Rangel et al., 2005; Manrique et al., 2006). However, in spite of some progress in the identification and breeding of bean genotypes for acid, Al-toxic soils, common bean is still among the plant species least adapted to soil acidity, generally, and Al toxicity, specifically. Consequently, improving Al resistance of common bean to reduce the dependence of small farmers on lime and nutrient inputs (CIAT, 1999) remains a major challenge. Knowledge on the mechanisms of Al toxicity can contribute to the development of more accurate and simpler screening procedures through the identification of physiological/molecular markers for Al resistance.

Therefore, the present study focuses on three areas, which are considered to particularly contribute to quantify genotypic differences in Al resistance and to develop quick screening techniques for Al resistance in common bean:

(i) The temporal and spatial effect of Al on root growth and accumulation of Al in the root apex (Chapter 1).

(ii) The role of cellular distribution of Al in Al toxicity and resistance (Chapter 2).

(iii) The characterization of the role of organic acid anion efflux and the synthesis of organic acid in Al resistance (Chapter 3).

CHAPTER 1:

Localized aluminium sensitivity of root apices of two common bean (*Phaseolus vulgaris* L.) genotypes with contrasting aluminium resistance

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(To be submitted)

Abstract

The initial response of plants to aluminium (Al) is an inhibition of root elongation. In the present study, short and medium-term effects of Al treatment (20 µM) on root growth and Al accumulation of two common bean (*Phaseolus vulgaris* L.) genotypes, VAX-1 (Al-sensitive) and Quimbaya (Al-resistant) were studied. Root elongation of both genotypes was severely inhibited during the first 3 to 4 h of Al treatment. Thereafter, both genotypes showed gradual recovery. However, this recovery continued in genotype Quimbaya until the root-elongation rate reached the level of the control (without Al), while the genotype VAX-1 was increasingly damaged by Al after 12 h of Al treatment. Short-term Al treatment (90 µM Al) to different zones of the root apex using agarose blocks corroborated the importance of the transition zone (TZ, 1 to 2 mm) as a main target of Al. However, Al applied to the elongation zone (EZ) also contributed to the overall inhibition of root elongation. Enhanced inhibition of root elongation during the initial 4 h of Al treatment was related to high Al accumulation in root apices in both genotypes (Quimbaya > VAX-1). Recovery from Al stress was not only reflected by decreasing Al contents especially in the TZ, but also in the EZ. After 24 h of Al treatment the high Al resistance of Quimbaya was reflected by much lower Al contents in the entire root apex. The results confirmed that genotypic differences in Al resistance in common bean are build up during medium-term exposure of the roots to Al. For this acquisition of Al resistance, the activation and maintenance of an Al exclusion mechanism especially in the TZ but also in the EZ appears to be decisive.

Introduction

Toxicity of aluminium (Al) in acid soils in the tropics is a serious problem and correcting soil acidity by amending the soils with lime is difficult and prohibitively expensive for most small farmers (Rao et al., 1993). Common bean (*Phaseolus vulgaris* L.) needs significant improvement in Al resistance to reduce farmer’s dependence on lime and fertilizers (Rao, 2001). Genotypic differences in seed yield of 5000 common bean germplasm accessions and breeding lines have been observed in field screening on Al-toxic soils that were amended with or without lime (65 % Al saturation) (Rao et al., 2004). Significant genotypic differences in Al resistance in common bean were also reported based on Al-inhibited root elongation in nutrient solution (Massot et al., 1999; Rangel et al., 2005; Manrique et al., 2006).

Reduction of root growth is the most widely recognized symptom of Al toxicity (Foy, 1976). It can be measured from 30 to 90 min after Al exposure in maize (*Zea mays* L.) (Llugany et al., 1995; Sivaguru and Horst, 1998). But more frequently, genotypic differences in Al resistance entailed measurements of root growth between 24 and 72 h (Furlani and Clark, 1981; Horst et al., 1983; Bennet and Breen, 1991a). Detailed studies on the kinetics of the response of root-growth rates to exposure to Al offer the possibility to differentiate between constitutive or inducible Al-resistance mechanism and to verify whether this is consistent across genotypes (Parker, 1995). Evidence of root acclimation to Al stress has been observed in maize (Llugany et al., 1995; Barceló and Poschenrieder, 2002), cowpea (*Vigna unguiculata*) (Horst et al., 1983) and yellow lupin (*Lupinus luteus* L.) (Grauer and Horst, 1990). Cumming et al. (1992) proposed that Al resistance is an inducible trait in common bean requiring a period of stress before a resistance mechanism is “switched on”. In fact, these authors observed an initial decline in the root elongation of an Al-resistant cultivar, followed by a substantial increase after 24 h of Al exposure, while the Al-sensitive cultivar showed a steady decline in the elongation rate over the experimental time. Resistance to Al might be achieved by chelation or detoxification of Al by organic acids, either within the plant (Al tolerance) or in the rhizosphere by root exudation (Al exclusion) (Foy, 1988; Taylor, 1988). The Al-stimulated exudation of citrate was first reported in common bean (Miyasaka et al., 1991). Since then, Al-induced secretion of organic anions has been reported in several plant species or cultivars (For more detailed information, see Ma et al., 2001, Ryan et al., 2001; Kochian et al., 2004). Lower Al contents were observed in root tips of Al-resistant compared with Al-sensitive common bean cultivars after 1 or 3 d of Al treatment, respectively (Mugai et al., 2000; Shen et al., 2002a). The lower Al contents were related to a higher capacity to exude citrate in response to Al treatment.

Two patterns of organic anion secretion have been identified. In wheat, buckwheat (*Fagopyrum esculentum* Moench), tobacco (*Nicotiana spp*., L.), and maize the activation of the organic acid anion-efflux is rapid and occurs without any discernible delay after exposure to Al (Pattern I). In chakod (*Cassia tora* L.), soybean (*Glycine max* L.), triticale (x *Triticosecale*) and rye (*Secale cereale*), a lag phase was observed between the addition of Al and the start of citrate release (Pattern II). Subsequently, the exudation is enhanced with time (Ma et al., 2001; Ryan et al., 2001).

In plants, growth is confined to distinct zones along which diverse spatial patterns of growth intensity exist (Erickson and Sax, 1956). Hence, a physiological study about regulation of growth and its modification under stress condition requires a detailed quantitative description of spatial growth profiles (Peters and Bernstein, 1997). In maize, specialized zones of the root apex have been classified on a millimeter scale based on physiological (Ishikawa and Evans, 1993), morphological (Ishikawa and Evans, 1995) and cytological (Baluška et al., 1990 and 1996) approaches. These studies allowed improving our understanding of the important role of the root apex in the Al-perception and response mechanism. Bennet and Breen (1991a, b) hypothesized a major role of the root cap in Al-triggered signal perception and transduction. Later, Ryan et al. (1993) showed that not the root cap but the root meristem was the most Al-sensitive zone in maize. Further research work by Sivaguru and Horst (1998) and Kollmeier et al. (2000) presented evidence that the distal transition zone (DTZ) is the most Al-sensitive zone of the root apex in maize. In this zone, Al accumulation was greatest (Sivaguru and Horst, 1998) and caused severe changes in the organization of microtubules and actin microfilaments leading to root-growth inhibition that could possibly be mediated by the interaction of Al with the apoplastic side of the cell wall-plasma membrane-cytoskeleton continuum (Horst et al., 1999; Sivaguru et al., 1999). Application of Al to DTZ and not to the EZ significantly reduced the root elongation, indicating the presence of Al-induced signal transduction between the DTZ and the EZ which could be mediated by auxin (Kollmeier et al., 2000).

In our previous studies, we found significant genotypic differences in Al resistance of common bean in nutrient solution based on inhibition of root elongation after 36 h at 20 µM Al supply as a parameter for Al injury (Rangel et al., 2005). Callose formation was found to be a sensitive marker of Al stress (Wissemeier et al., 1992) and a reliable parameter for the classification of maize genotypes for Al resistance (Eticha et al., 2005a). In common bean, short-term Al supply (4 h) led to maximum accumulation of callose in the root apex. However, no relationship was observed between Al-induced callose contents and root-growth inhibition, hampering the use of this parameter as a screening tool for Al resistance in common bean (Rangel et al., 2004). It thus appears that the Al-perception and response mechanism in common bean could differ from maize. Therefore, a better understanding of the temporal and spatial effect of Al on root growth and of the accumulation of Al is necessary to quantify genotypic differences in Al resistance and to develop quick screening techniques for Al resistance in common bean.

Materials and methods

Plant material and growth conditions

Seeds of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) were germinated between filter-paper/styrofoam sandwiches soaked with tap water, in an upright position. Three days-old uniform seedlings were transferred to 18 l pots with constantly aerated simplified nutrient solution containing 5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 (Rangel et al., 2005). This solution allowed optimum root elongation for 3 days at least. After 24 h of root growth, the pH of the solution was decreased from 5.6 to 4.5 within 24 h in steps of 0.3 units using an automatic pH titration device. The pH was controlled in each pot by adding 0.1 M HCl or 0.1 M KOH. All experiments were conducted under controlled environmental conditions in a growth chamber with a 16/8 h light/dark regime, 27/25 ^{o}C day/night temperatures, 70 % relative air humidity and a photon flux density of 230 µmol m^{-2} s^{-1} (photosynthetic active radiation) at the plant-canopy level (Sylvania Cool White, 195 W, Philips, Germany).

Partial elongation of 1 mm root zones

Assessment of spatial growth patterns of individual root sections was based on experiments (Erickson and Sax, 1956; Peters and Bernstein, 1997) where the first 10 mm of the root apex was marked with consecutive black and red dots at 1 mm intervals using a fine brush and Indian ink (Pelikan, Hanover, Germany). The process of marking did not affect root growth during the experimental period. Subsequently, plants were transferred to simplified nutrient solution containing 0 or 20 µM $AlCl_3$. The distances between the dots were measured after 4 h, and then the elongation rates of each specific root zone were calculated and plotted as a continuous curve against the distance from the root apex. Measurements were made with a precision of 25 µm at a 40-fold magnification against a 1 mm scale using a stereo microscope (Askania GSZ 2T, Rathenow, Germany).

In addition, relative elemental growth rates (REGR) were calculated as the derivative of the fifth-order sigmoidal function of growth-rate profiles (Erickson and Sax, 1956; Silk, 1984; Peters and Bernstein, 1997) and the spatial organization of the root apex determined following the methodology used by Ishikawa and Evans (1993). Briefly, the elongation zone (EZ) was divided arbitrarily into six sub-zones: meristematic zone (MZ), transition zone (TZ), apical elongation zone (AEZ), central elongation zone (CEZ), basal elongation zone (BEZ) and proximal elongation zone (PEZ) based on rates of elongation relative to the peak rate in the CEZ.

Localized effect of Al in 1 mm PVC blocks or 20 mm plastic cylinder blocks

Local Al treatments were performed in low gelling-temperature agarose (1 % w/v, Fluka, Deisenhofen, Germany) dissolved in simplified nutrient solution containing 0 or 200 µM Al. Nominal 200 µM Al added to cooled agarose yielded 90 ± 6 µM Al_{mono} following the procedure described by Sivaguru and Horst (1998) and the aluminon method according to Kerven et al. (1989).

Aluminium was applied to specific 1 mm apical root zones using a polyvinylchloride (PVC) block system previously described by Sivaguru and Horst (1998). Briefly, 5 uniform seedlings were mounted on the PVC blocks with different apical root positions placed into the horizontal 1 mm slit, which was vertically sealed with vaseline. Thereafter, agarose was poured into the horizontal slit using a fine-tipped Pasteur pipette just before solidification. Subsequently, the PVC blocks were placed in a growth chamber in an upright position, under the conditions described above. The whole-root system was kept moist during all manipulations by soaking the plant-supporting filter-paper/styrofoam layer with nutrient solution, covering the roots with moistened filter paper, and repeatedly spraying nutrient solution with a hand sprayer. Root elongation rate was calculated from the measurements of root lengths at every hour for up to 6 h as described above.

Alternatively, Al was applied to 20 mm apical root zones using a plastic-cylinder block system. Each block consisted of five vertical cylinders (20 mm length x 0.5 mm width) mounted on plant-supporting filter-paper/styrofoam layer previously soaked with simplified nutrient solution. Five uniform seedlings were placed at different apical root positions into the 20 mm chambers, and sealed at the base with vaseline. In contrast to the 1 mm PVC block system, the apical root zone in consideration was located outside the Al-treated zone. Thereafter, the Al-containing agarose was poured into the cylinder using a fine-tipped Pasteur pipette, and the plants were covered with a second filter-paper/styrofoam layer. Afterwards, each block system was placed in an upright position into plastic pots containing enough nutrient solution to cover the base of each block, and kept in a growth chamber under the conditions described above. Root elongation rates were calculated from measurements of root length after 4 h of Al treatment.

Effect of Al on root growth

Tap roots of 3 cm behind the root tip were marked at 2 h before the Al treatment using a fine point permanent marker (Sharpie blue, Stanford) and this did not affect root growth during the experimental period. Afterwards, the plants were transferred to simplified nutrient solution (see above) containing 0 or 20 µM $AlCl_3$ for up to 24 h. Root elongation was determined every hour during the first 10 h and then at 12, 16, 20 and 24 h of Al treatment using a 1 mm scale.

Determination of Al in 5 mm and 2 mm root segments

For Al analysis, roots of 4 plants per replicate were rinsed with distilled water and 5 mm (primary root) or 2 mm segments along the first 10 mm of root tips (primary plus the two longest basal roots) were excised using a razor blade, placed in separate Eppendorf reaction vials (four 5 mm and twelve 2 mm root sections per vial) and digested in 500 µl ultra-pure HNO_3 (65 %) by overnight shaking on a rotary shaker (Heidolph, Reax 20, Germany). Preliminary studies did not reveal any differences between primary and basal roots. The digestion was completed by heating the samples in a water bath at 80 ^{o}C for 20 min. Then 1.5 ml of ultra-pure deionized water (18.2 MΩ; E-pure, D4642; Barnstead, Dubuque, IA, USA) was added after cooling the samples in an ice-water bath. Samples were diluted and measured with a Unicam 939 QZ graphite furnace atomic absorption spectrometer (GFAAS; Analytical Technologies Inc., Cambridge, UK) at a wavelength of 308.2 nm with an injection volume of 20 µl.

At harvest, the culture solutions were filtered immediately through 0.025 µm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Mononuclear Al (Al_{mono}) concentrations were measured colorimetrically using the pyrocatechol violet method (PCV) according to Kerven et al. (1989). Nominal 20 µM Al treatments resulted in 16 ± 2 µM Al_{mono} after 24 h.

Statistical Analysis

Each experiment had a completely randomized design with eight (root elongation), four (Al contents), six (partial elongation rate) and four (spatial sensitivity) independent replicates for each treatment. Analysis of variance was performed using the ANOVA procedure of the statistic program SAS 9.1 (SAS institute, Cary NC, USA) and means were compared using the Tukey test. *, **, ***, ns, denote significant differences at $p < 0.05$, 0.01, and 0.001, or non significant, respectively.

Results

Effect of Al on partial elongation of specific 1 mm root zones

To obtain information on partial growth patterns along the 10 mm root apex, elongation growth of individual segments was measured by identifying the main zone of Al-induced growth inhibition. Previous experiments had shown that there was no observable root elongation beyond the 10 mm zone (data not shown). In the absence of Al, the growth patterns of both genotypes were similar (Fig. 1), however, the genotype x segment interaction was highly significant. The calculated relative elemental growth rates (REGR) suggested that the EZ extended from 1.7 or 1.5 mm to 10.5 or 8.9 mm behind the root tip in Quimbaya and VAX-1, respectively. The peaks of maximum relative elongation rate were located at 3.9 mm (31 % h^{-1}) in Quimbaya and 3.3 mm (41 % h^{-1}) behind the root tip in VAX-1.

Based on the position of maximum relative elongation, the EZ was divided arbitrarily on a millimeter scale in both genotypes. The pattern of root-growth inhibition induced by 4 h of Al supply was similar in both genotypes (no significant genotype x treatment x segment interaction) and resulted from a general inhibition along the entire EZ without shortening the growth zone. The inhibition was greater in the CEZ where the maximal rate of relative elongation was decreased to 15 % h^{-1} in both genotypes. Although the genotype x treatment interaction was not significant, there was a trend showing that Quimbaya was less severely inhibited by Al in the EZ (40 %) than VAX-1 (60 %), which was particularly Al-sensitive in the TZ (88 %).

Effect of localized application of Al on root elongation

Application of Al for 2 h to individual 1 mm root zones of the 5 mm root apex significantly inhibited root elongation in both genotypes (Fig. 2). Aluminium inhibited root elongation when applied to all segments except the apical segment. Calculated across the genotypes (not shown), the Al treatment x segment interaction was significant, suggesting that Al was particularly toxic when applied to the 1-2 mm zone.

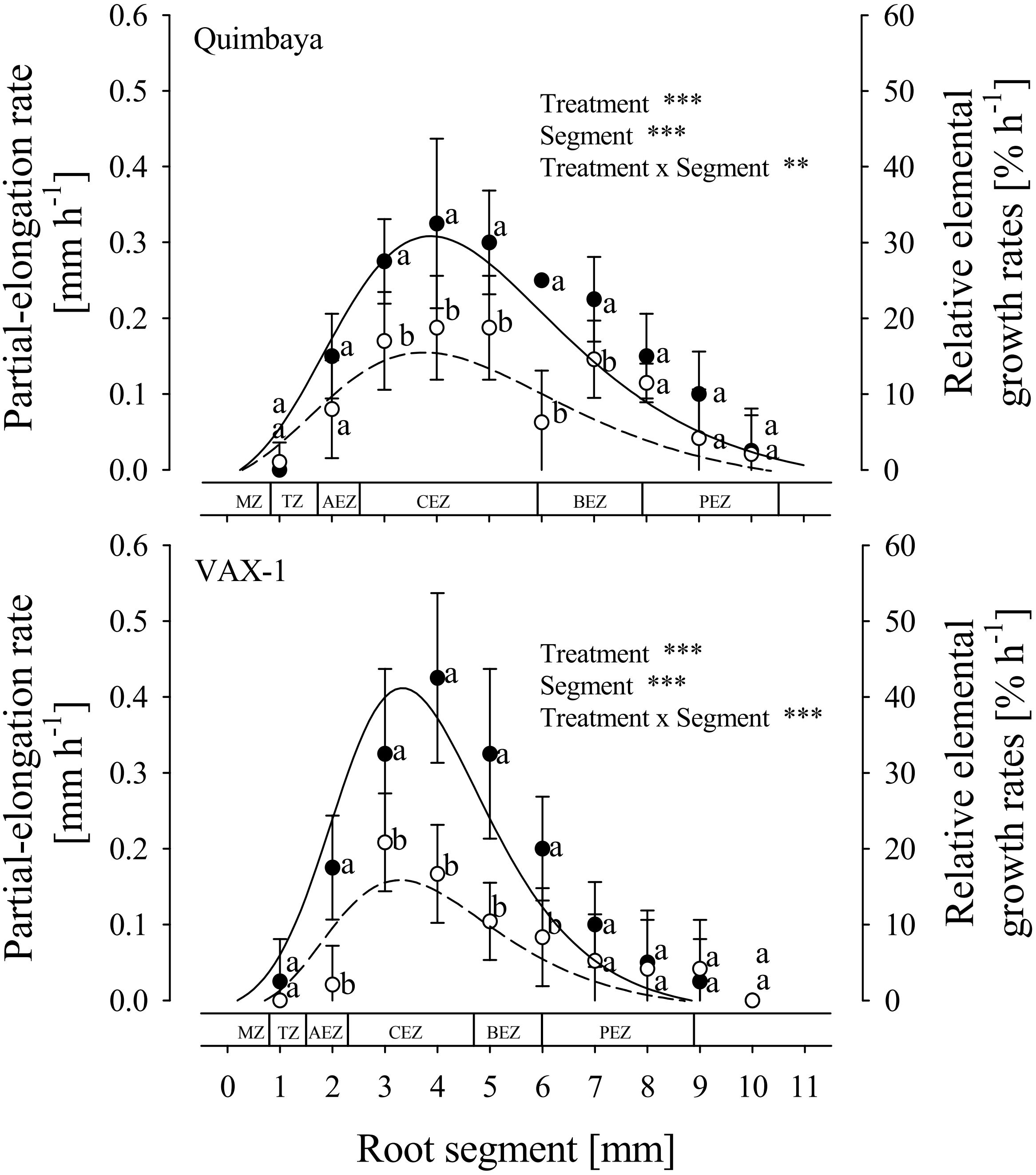

Figure 1. Effect of Al supply (20 µM Al) on the partial elongation rates of apical 1-mm root segments (dots ± SD, n = 6) and calculated relative elemental growth rates (% of total root elongation rate in mm h^{-1}, lines) for 0 µM Al (filled dots, solid line) and 20 µM Al (open dots, dashed lines) of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution for up to 4 h, at pH 4.5. Means with the same letter are not significantly different between Al treatments within individual sections (Tukey test, $p < 0.05$). The REGR profiles were generated as a derivative of the fifth-order sigmoidal function of growth-rate profiles. For the ANOVA, **, *** denote levels of significance at $p < 0.01$ and 0.001. In each figure, the arbitrarily defined sub-zones (MZ, TZ, AEZ, CEZ, BEZ, and PEZ) of the elongation zone are indicated.

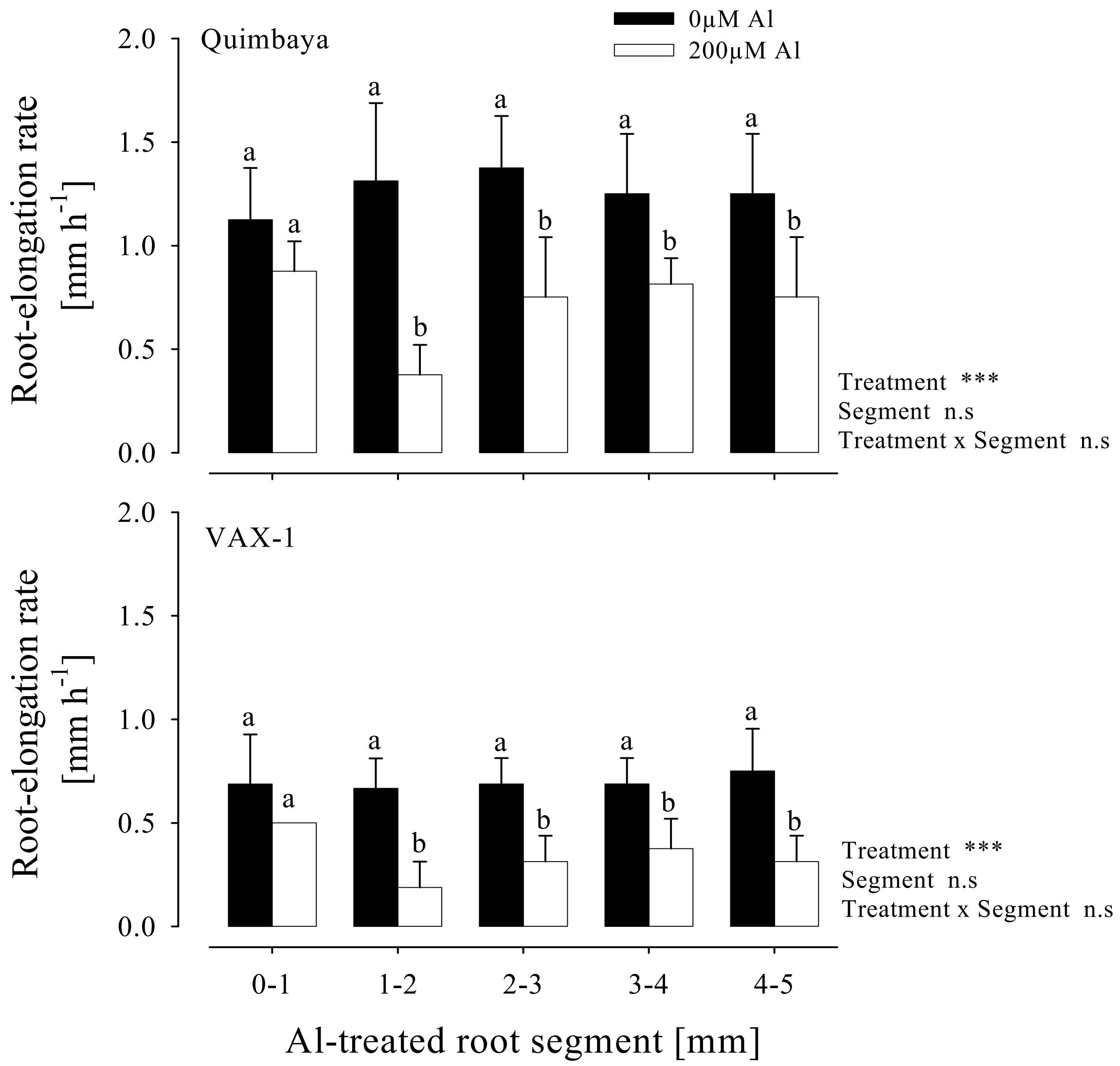

Figure 2. Effect of Al supply (200 µM Al) to individual 1-mm apical root segments for 2 h in agarose medium, pH 4.5 (PVC-block technique) on the root elongation-rate of common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive). Bars represent means ± SD of four replicates. For the ANOVA, *** denote a level of significance at $p < 0.001$; n.s = not significant. Means with the same letter are not significantly different between Al treatments in each cultivar (Tukey test, $p < 0.05$).

This study was extended using a different experimental approach where increasing parts of the 10 mm root apex contributing to root elongation (compare Fig. 1) were not treated with Al (Fig. 3). Aluminium significantly inhibited root elongation even if Al was not applied to the 0-6 mm root zone (both genotypes) or even the 0-8 mm zone (VAX-1). Particularly in VAX-1 (significant difference between segments and Al treatment x segment interaction), the comparison of means indicate that Al applied to the 0-2 mm segment was most toxic, although basal root segments also contributed to the inhibition of the overall root elongation.

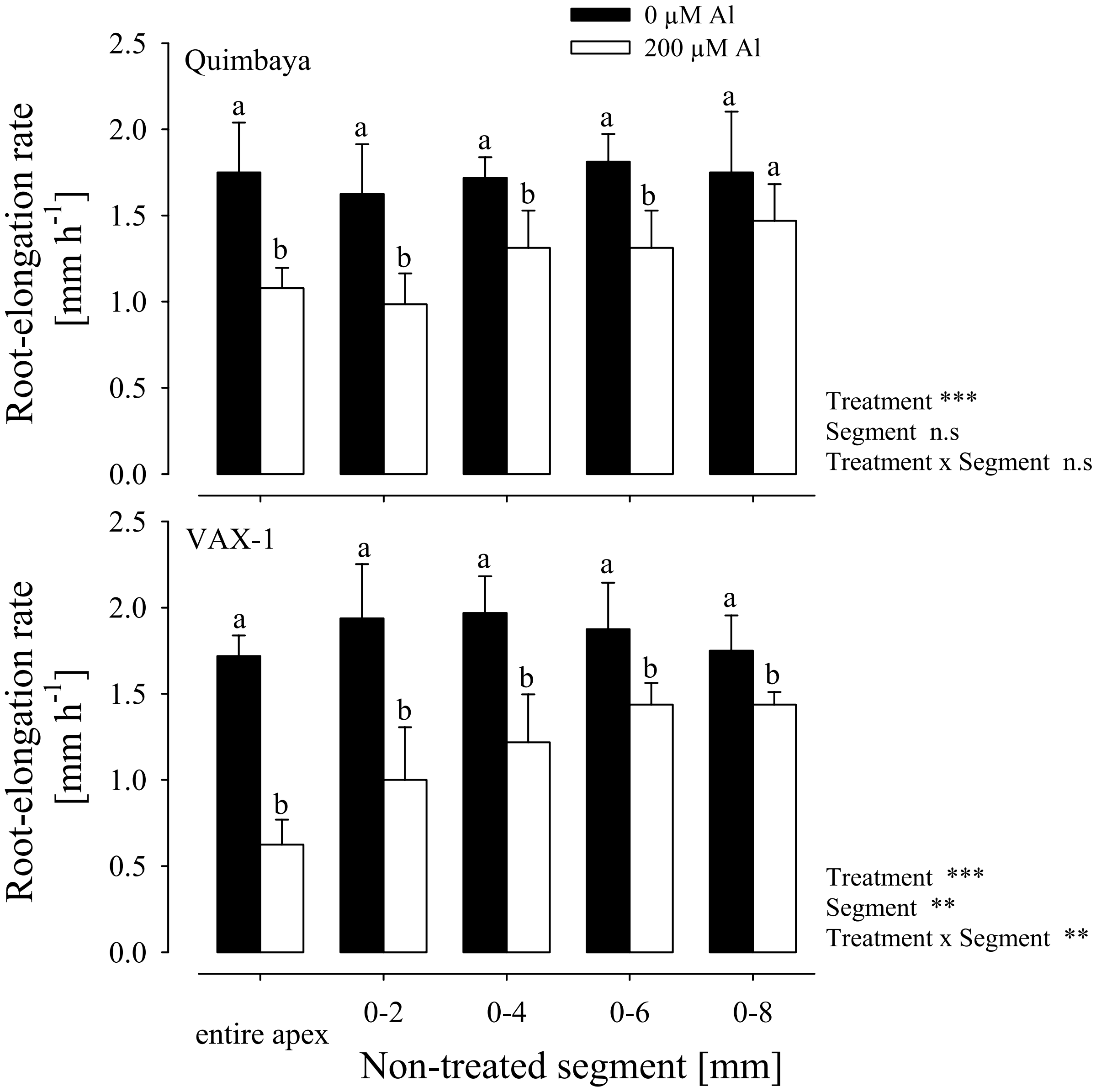

Figure 3. Effect of 4 h Al supply (200 µM Al) in agarose medium, pH 4.5 (PVC-cylinder technique) to the entire or part of the 20-mm root apex on the root elongation-rate of common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive). Bars represent means ± SD of four replicates. For the ANOVA, **, *** denote probability levels at $p < 0.01$ and 0.001; n.s = not significant. Means with the same letter are not significantly different between Al treatments in each cultivar (Tukey test, $p < 0.05$).

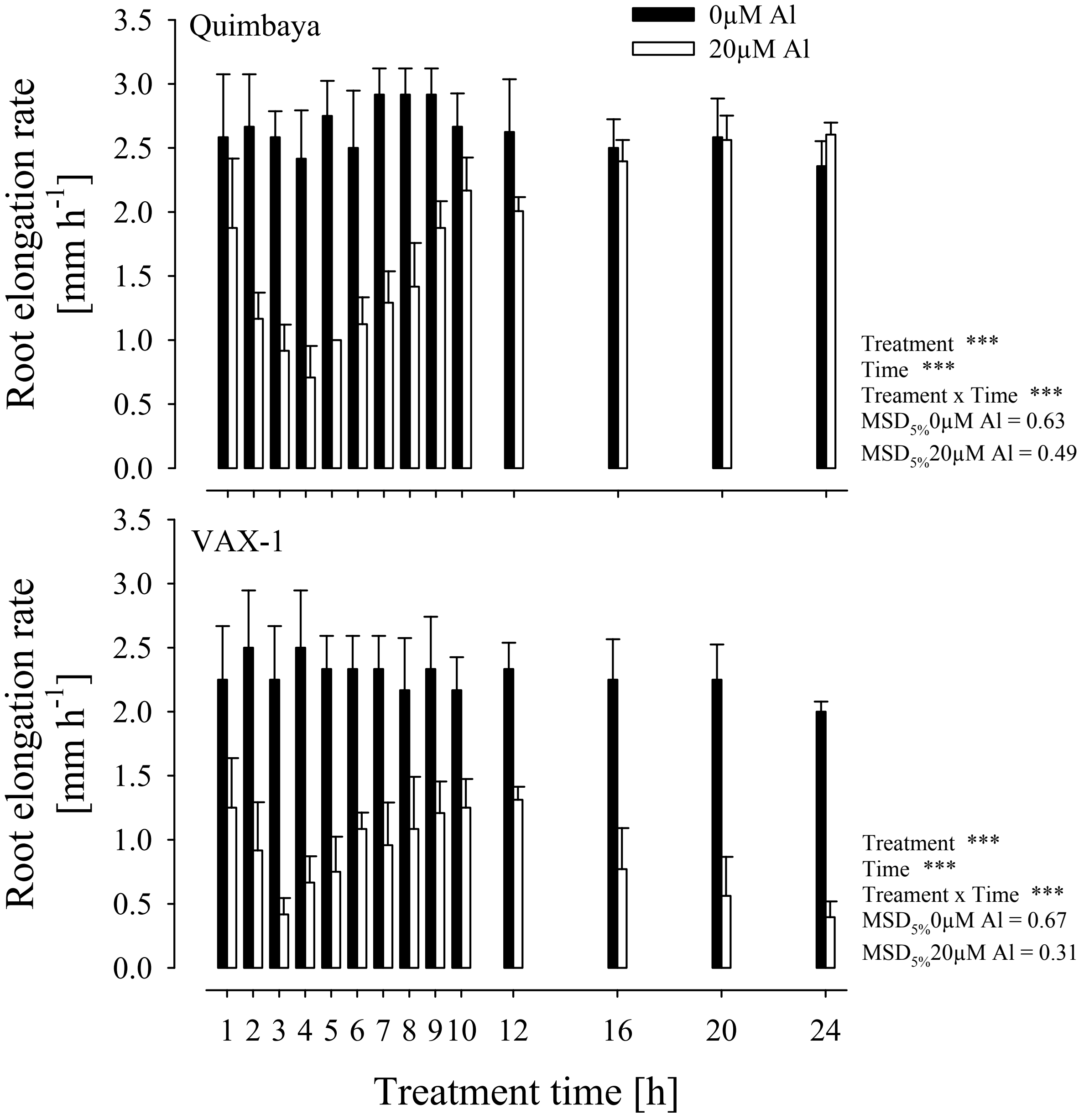

Figure 4. Root elongation rate of Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) common bean genotypes grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl and 8 µM H_3BO_3 with or without 20 µM Al for up to 24 h, pH 4.5. Bars represent means ± SD of eight replicates. For the ANOVA, *** denote a level of significance at $p < 0.001$. Minimum significant differences (MSD) according to the Tukey test.

Effect of Al on overall root elongation

In presence of Al, root elongation of both genotypes was inhibited as early as 1 h after the beginning of the Al treatment. The inhibition was enhanced up to a maximal level after 3 and 4 h for VAX-1 and Quimbaya, respectively (Fig. 4). Thereafter, both genotypes gradually recovered, but the recovery with Quimbaya was much faster than with VAX-1. Whereas this recovery continued in Quimbaya until the root elongation rate reached the level of the control (without Al), VAX-1 was increasingly damaged by Al after 12 h of Al treatment which is reflected by the highly significant Al treatment x time interaction.

The relative inhibition of root elongation more clearly visualizes the response of the genotypes to Al supply (Fig. 5). The maximum inhibition of root elongation reached about 80 % in both genotypes after 3 or 4 hours and then recovered. Whereas Quimbaya completely recovered after 24 hours, VAX-1 recovered only to 40 % after 12 h and then got increasingly damaged up to 80 % again after 24 h of Al treatment.

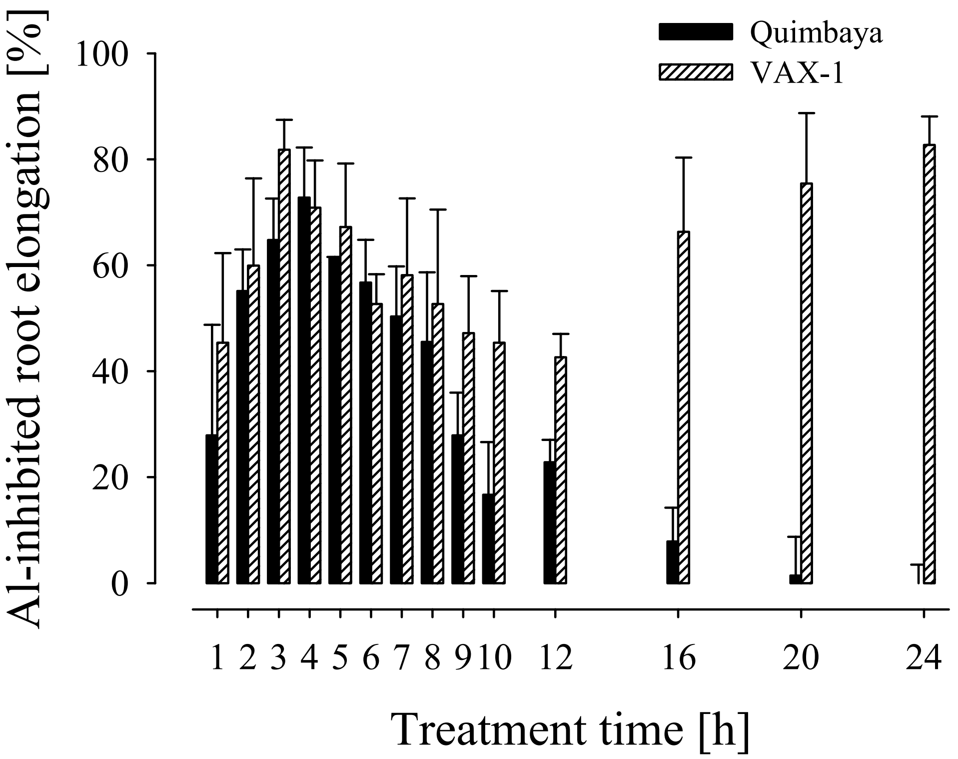

Figure 5. Effect of Al supply on root elongation of Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) common bean genotypes growing in a simplified nutrient solution for up to 24 h at pH 4.5. Bars represent means ± SD of eight replicates. For the ANOVA, *** denote a level of significance at $p < 0.001$. Minimum significant differences (MSD) according to the Tukey test.

Aluminium contents in root apices

The Al contents of the 5-mm root apex (Fig. 6) reflected the inhibition of root elongation induced by Al (Fig. 4). Enhanced inhibition of root elongation up to 4 h of Al treatment was related to increasing Al contents in the root tips in both genotypes. However, the Al contents reached higher levels in Quimbaya. This higher Al contents did not lead to more severe inhibition of root elongation compared to VAX-1 (both absolutely and relatively, compare with Figs. 4 and 5). Subsequently, Al contents decreased in both genotypes. However, in Quimbaya the decrease continued up to 24 h while in VAX-1, the Al contents started to increase again after 10 h and reached a higher level after 24 h than after 4 h of Al treatment.

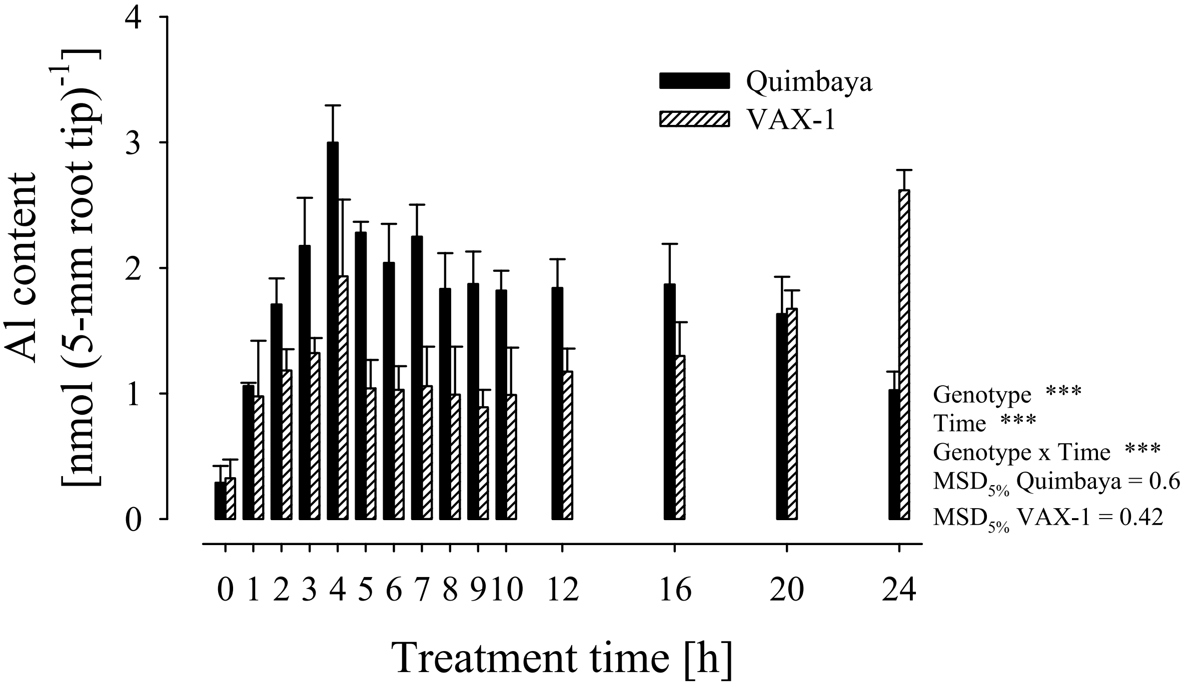

Figure 6. Effect of Al supply (20 µM Al) on Al contents of root tips (5-mm) of Quimbaya (Al-resistant) and VAX-1(Al-sensitive) common bean genotypes growing in a simplified nutrient solution for up to 24 h at pH 4.5. Bars represent means ± SD of four replicates. For the ANOVA, *** denote a level of significance at $p < 0.001$. Minimum significant differences (MSD) according to the Tukey test.

Apical root zones differ in their response to Al (see above). Therefore, the Al contents in 2 mm root zones along the root apex were determined. The distribution of Al along the 10 mm root apex clearly show that enhanced Al injury in both genotypes during the first 4 h of Al treatment were particularly due to increasing Al contents in the 0-2 mm zone (Fig. 7). The recovery in root elongation corresponded to a decrease of Al particularly in this zone. Complete recovery of Quimbaya after 12 h of Al exposure resulted also in lower Al contents in the more basal zones (Fig. 7, Quimbaya). In VAX-1, the enhanced Al injury after 12 h of Al treatment was accompanied by an increase in Al contents in all root segments (Fig. 7, VAX-1).

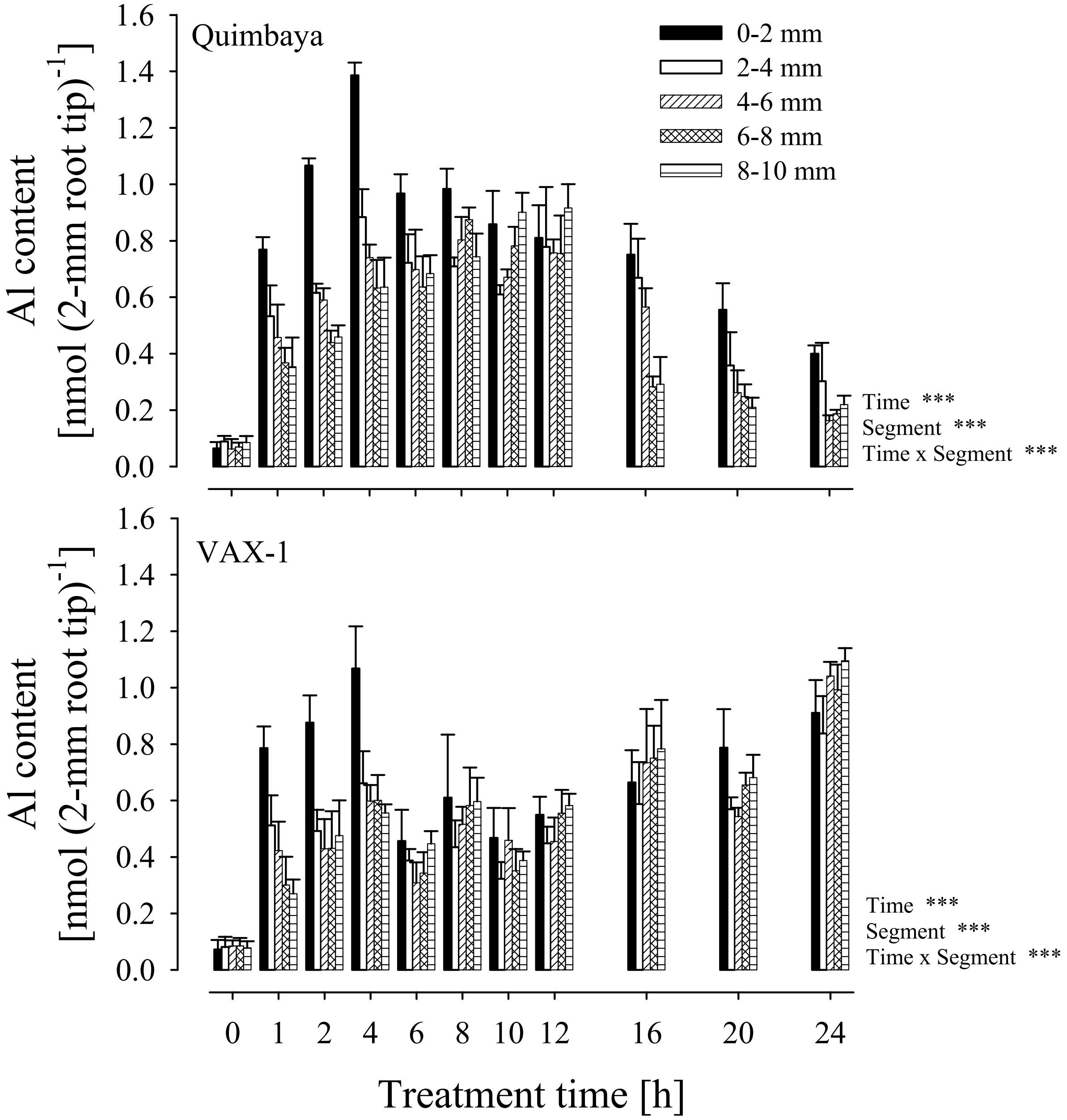

Figure 7. Effect of Al supply (20 µM Al) to the entire root apex on Al accumulation of individual 2-mm root segments of Quimbaya (Al-resistant) and VAX-1(Al-sensitive) common bean genotypes growing in a simplified nutrient solution for up to 24 h at pH 4.5. Bars represent means ± SD of four replicates. For the ANOVA, *** denote a level of significance at $p < 0.001$.

Discussion

Typically, the zones of development in roots include the cap, the apical meristem, the elongation zone and the maturation zone (Ishikawa and Evans, 1995). However, it is widely acknowledged that root growth in plants is confined to the apical regions along which diverse spatial patterns of growth intensity exist as a result of different gradients of cellular activities (Erickson and Sax, 1956; Gandar, 1983; Pritchard, 1994). These regions of growth are described in terms of growth rate (R) and relative elongation growth-rate (REGR) profiles (Silk, 1984). Although both R and REGR are true rates (the physical dimension is time^{-1}), R describes the velocity of change of material entities, i.e. fresh weight or organ length in time while REGR characterizes the distribution of growth intensities in the space (Erickson and Sax, 1956; Gandar, 1983; Peters and Bernstein, 1997). Identification and analysis of spatial growth profiles are a prerequisite for the physiological study about regulation of growth and its modification under stress condition.

In the absence of Al, the determination of REGR in common bean revealed that the pattern of elongation in both genotypes was similar (Fig. 1). However, the extension of the EZ of both genotypes differed slightly. It was larger in Quimbaya than in VAX-1. Consequently, the point of maximum elongation occurs in a more basal part of the EZ (3.9 mm) in Quimbaya than in VAX-1 (3.3 mm). Elongation at the root axis is caused by the production and expansion of cells (Green, 1976). After cessation of cell division in the meristem, cells undergo a preparatory phase for rapid elongation resulting in an increase in width as well as in length (Baluška et al., 1990). Thereafter, cell elongation is characterized by extensive vacuolar expansion and an increase in the area of lateral cell walls (Pritchard, 1994). In common bean, differences in seed size among genotypes of different origin have been directly related to variation in cell volumes of different tissues (White and Gonzalez, 1990). Normally, genotypes of Mesoamerican origin (e.g., inter-specific genotype, VAX-1) are typically smaller seeded than those of Andean (i.e. Quimbaya) origin (Gepts et al., 1986; Singh, 1989). This is in agreement with our microscopic observations of root cross-sections at the TZ (A. Rangel and W.J. Horst, unpublished results), where higher numbers and larger cortical cells in Quimbaya than in VAX-1 could explain the greater size of the EZ in Quimbaya (8.8 mm) compared to VAX-1 (7.4 mm) (Fig. 1).

The division of the root apex into growth zones in common bean (Fig. 1) is arbitrary following the proposal by Ishikawa and Evans (1993) and it is in agreement with previous results obtained in mungbean (*Vigna radiata* L. (Blamey et al., 2004), maize (Baluška et al., 1990; Ishikawa and Evans, 1993 and 1995; Baluška et al., 1996). Using a computerized video system, Blamey et al. (2004) showed that in mungbean the zone of greatest expansion (CEZ) was located at 1.9 – 4.6 mm from the root tip. In maize, the EZ extended from 1.5 to 9.2 mm behind the tip, and the maximum relative elongation rate was 26 % h^{-1} at 4 mm in the CEZ extending from 3.2 to 6.5 mm behind the tip (Ishikawa and Evans, 1993). The TZ was located between 1.7 and 3.4 mm from the root tip in nutrient solution experiments (Ishikawa and Evans, 1993) or between 1 and 2 mm from the root tip in moist air experiments (Kollmeier et al., 2000). The localization of the distal transition zone (DTZ) in maize corresponded with the cytological studies conducted by Baluška et al. (1990).

The overall dynamics of root growth and Al accumulation in the present study confirmed our previous results on the characterization of the genotypes Quimbaya and VAX-1 as Al-resistant and Al-sensitive, respectively (Rangel et al., 2004 and 2005; Manrique et al., 2006). However, as observed previously by Cummings et al. (1992) the dynamics of the Al-stress perception and response varies on the spatial (Figs. 1-3) and temporal (Figs. 4-7) scale.

In both genotypes, accumulation of Al in the 5 mm root tips (Fig. 6) and even more in the 2 mm apical root zone (Fig. 7) during the first 4 h of Al treatment lead to enhanced inhibition of root elongation (Fig. 4). This initial inhibition of root elongation resulted from a generalized effect along the entire EZ (reduced maximal rate of relative elongation) without changing the shape or the length of the EZ. This observation is in agreement with Blamey et al. (2004) in mungbean and with Kollmeier et al. (2000) in maize who did not find any changes in the shape and length of the EZ due to Al treatment. In *Arabidopsis*, phosphorous deficiency shortened the EZ by reducing the production rate of epidermal cells but not cortical cells, and moderately decreased the maximal rate of relative elongation, while ethylene regulated the maximal rate of relative elongation rather than the length of the growth zone independently of the phosphorus status (Ma et al., 2003). Greater inhibition of root elongation by Al in the Al-sensitive VAX-1 cannot be related to a higher Al accumulation but appears to be related to its higher Al sensitivity of the TZ (Fig. 1). Localized application of Al to different zones of the root apex (1 mm or 20 mm agarose blocks) confirmed the previous results with maize obtained by Sivaguru and Horst (1998) and Sivaguru et al. (1999) and confirm that the TZ is the most Al-sensitive apical root zone not only in maize but also in common bean.

As well as in maize (Sivaguru and Horst, 1998; Kollmeier et al., 2000), localized application of Al to the MZ (0-1 mm zone) was significantly less inhibitory than when applied to more basal parts of the EZ in both common bean genotypes (Fig. 2). This effect has been normally explained by the strong capacity of the root-cap mucilage to bind Al, thus protecting the root tip from Al injury (Horst et al., 1982; Archambault et al., 1996; Li et al., 2000). Even more importantly, it is widely acknowledged that the DTZ is the root zone most responsive to a variety of hormonal and environmental stimuli (Ishikawa and Evans, 1993 and 1995; Borch et al., 1999; Kollmeier et al., 2000; Ma et al., 2003). The importance of the DTZ in Al perception and response has been demonstrated using different indicators (see Introduction). In our experiments application of Al to the TZ in both common bean genotypes resulted in root-growth inhibition to the same extent as if the whole root tip would have been treated (Fig. 2). Additionally, the pattern of Al accumulation along the 10 mm root tip of both genotypes suggested that enhanced Al injury during the first 4 h of Al treatment was particularly due to increasing Al contents in the MZ and TZ (Fig 7, 0-2 mm zone). This assumption is supported by a closer relationship ($R^2 = 0.61$ and 0.45 for Quimbaya and VAX-1, respectively) between root elongation rate and Al content of the 0-2 mm zone than with any other root zone. However, in contrast to maize (Kollmeier et al., 2000) and wheat (Ryan et al., 1993), application of Al to the EZ even up to 8 mm from the root tip (Figs. 2 and 3) also reduced root growth in both common bean genotypes, though to a lesser extent than when applied to the TZ. Dicotyledons and grasses (*Poales*) are well known to differ widely in the composition of their cell walls, particularly in their pectin content which is higher in dicotyledons (Carpita and Gibeaut, 1993), thus enhancing the capacity to accumulate Al. The important role of the cell-wall pectin-content for Al accumulation and Al sensitivity has been demonstrated by modifying the pectin contents of the maize root apex (NaCl treatment) and maize cell-cultures (Horst et al., 1999; Schmohl and Horst, 2000), and by the strong positive relationship between Al accumulation and the localization of pectin contents along the root apex (Schmohl and Horst, 2001; Eticha et al., 2005b). Comparing faba bean and maize, Schmohl and Horst (2001) showed that the four times greater Al accumulation in the first 5 mm of the root apex in faba bean corresponded to higher pectin contents. This higher Al-binding capacity of cell walls in faba bean could explain the differences in radial movement of Al in the DTZ. Marienfeld et al. (2000) showed that after short-term Al supply, Al was mostly restricted to the rhizodermis and outer cortical cells in faba bean while in maize Al was detected even in the inner cortex. The greater binding of Al to the cell walls in the EZ thus affecting the extensibility of the cell wall directly or indirectly by creating mechanical stress which is transferred to the cytoskeleton, leading to a disturbance of the processes that are necessary for cell elongation (Carpita and Gibeaut, 1993; Horst et al., 1999), might be responsible for the inhibitory effect of Al when applied to the EZ in common bean (Figs. 2 and 3).

The two common bean genotypes did not differ in the initial response of root elongation to Al exposure (Fig. 5), both were severely inhibited up to 4 h of Al treatment duration. After 4 h of Al treatment, both genotypes gradually recovered from the initial inhibition of root elongation (Fig. 4). Whereas this recovery continued in Quimbaya almost to the level of the control (without Al) after 24 h, in VAX-1 it was again increasingly inhibited by Al after 12 h of Al treatment. Similar patterns of recovery have been detected after 8 h of Al treatment in common bean (Cumming et al., 1992) and after 6 h of Al treatment in soybean (Yang et al., 2000 and 2001). This pattern of root elongation during medium-term Al exposure was reflected in decreasing Al contents of root apices (Fig. 6), particularly of the apical 2 mm (Fig. 7) in both genotypes and again increasing Al contents in the Al-sensitive VAX-1. However, only after 24 h of Al exposure the clear genotypic differences were observed in the recovery of root elongation. These differences were reflected in lower Al contents in the Al-resistant genotype Quimbaya which maintained the higher Al contents of the root tips for up to 16-20 h (Figs. 6 and 7). A similar or even smaller Al-induced inhibition of root elongation at higher Al contents in the root apices calls for an Al tolerance mechanism acting in the Al-resistant genotype Quimbaya in addition to an Al exclusion mechanism (see below).

The recovery of root growth after short-term (< 6 h) Al treatment is typical for plant species characterized by a Pattern II Al-induced release of organic acid anions (Ma et al., 2001; Ryan et al., 2001) such as soybean (Yang et al., 2000) and chakod (Ma et al., 1997). In both plant species the recovery of Al-resistant cultivars was directly related to an Al-induced citrate exudation. Our own unpublished work clearly shows that also in common bean recovery from short-term Al stress and medium-term established genotypic differences in Al resistance are related to Al-induced increase in release of citrate from root apices. This is in agreement with the studies of Shen et al. (2002), demonstrating that lower Al contents of root tips of the Al-resistant cultivar G 19842 compared to the Al-sensitive cultivar ZPV corresponded with its higher capacity to exude citrate that was assessed after 3 d of Al treatment (10 and 20 µM Al). Earlier, Miyasaka et al. (1991) had provided evidence that A1 resistance in common bean involves the efflux of citrate.

In conclusion, the results presented confirm that the expression of Al toxicity and Al resistance in common bean differs from maize. Although in both species the TZ appears to be the most Al-sensitive root zone, in common bean Al inhibits root elongation also when applied exclusively to the EZ. Aluminium resistance in common bean thus requires the protection of the entire EZ from Al injury. Genotypes differing in Al resistance in medium and long-term studies do not differ in their short-term sensitive response to Al. Aluminium resistance in common bean is building up during medium-term exposure to Al and is related to a reduced Al accumulation in the root tips, supporting the idea of a mechanism of Al resistance based on exclusion from the root apex mediated by citrate exudation. In addition to Al exclusion, it appears that the Al-resistant genotype Quimbaya possesses an Al tolerance mechanism detoxifying part of the Al accumulated in the root apices. Results on organic acid anion synthesis and exudation, and Al compartmentation and binding in root apices of common bean will be published elsewhere.

CHAPTER 2:

Aluminium fractionation in root apices of common bean (*Phaseolus vulgaris* L.) genotypes differing in aluminium resistance

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(To be submitted)

Abstract

The response of root elongation to aluminium (Al) in two common bean (*Phaseolus vulgaris* L.) genotypes differing in Al resistance characterized by a similar initial period (4 h) of Al sensitivity followed by a contrasting recovery period (8-24 h), which is typical for pattern II plant species, appeared to be particularly suitable to study the role of the cellular distribution of Al in Al toxicity and resistance. A higher initial Al accumulation of 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 negativity of the cell wall.

The binding stage 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 stabile-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. The comparative morin staining of Al in root cross-sections confirmed that morin staining does not reflect the quantitative cellular and tissue distribution of Al. However, it suggests that: (i) the initial inhibition of root elongation in both genotypes is related to accumulation of Al in the epidermis and outer cortical cells in the TZ as well as the EZ, (ii) Al moves quickly (< 4 h) across the cortex up to the endodermis, (iii) recovery from Al injury is accompanied with an internalization of Al into the symplast, and (iv) sustained inhibition of root elongation is characterized by a high mobility of Al across the cortex and into the central cylinder.

It is concluded that induced Al resistance in the Al-resistant genotype is mediated by reducing the stabile-bound Al into the apoplast thus allowing cell division and cell elongation to resume.

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). About 40 % of the common bean (*Phaseolus vulgaris* L.)-growing area in 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 responses is the root apex (Ryan et al., 1993), and particularly the distal part of the transition zone (DTZ, 1-2 mm), 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 (Chapter 1). 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, Chapter 1). 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., 2002a; 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 tolerance 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 and 1990). In the apoplast, the negativity 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. 2005b, Horst et al., 2007) through altering CW characteristics and functions, such as porosity and extensibility, hydraulic conductivity, displacement of ions from critical sites (Rengel, 1990; Blamey et al., 1993; Mimmo et al., 2003; Sivaguru et al., 2006) 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., 2005c). 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 tolerance rather than of Al toxicity.

The response to Al of common bean genotypes differing in Al resistance characterized by a corresponding initial period of Al sensitivity followed by a contrasting recovery period (Chapter 1) appeared to be particularly suitable for the study of the role of the cellular distribution of Al in Al toxicity and resistance.

Materials and methods

Plant material and growth conditions

Seeds of the Al-resistant common bean genotype (Quimbaya) and an Al-sensitive genotype (VAX-1) were germinated between filter-paper 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 into simplified nutrient solution (see above) 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 at a speed of 30/s 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 ultra-pure deionized water (dd 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 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 $\lambda = 405$ nm, emission $\lambda = 503$ 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 dd 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) 50 mM $BaCl_2$, for 15 min. Root tips were briefly washed in (500 µl) dd water and then transferred for 15 min to (500 µl) 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^oC 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 ^{o}C for 15 min. After collecting the WFSF, the root tips were frozen at -20 ^{o}C. The first symplastic-Al fraction (SYM-1) was recovered from the frozen-thawed samples by centrifugation (4000 g) at 4 ^{o}C for 15 min. The residue was then transferred to Eppendorf vials and homogenized in (500 µl) ethanol with a mixer mill (MM200; Retsch, Haan, Germany) at a speed of 30/s for 3 min. All further centrifugation steps were conducted at 23000 g (4 ^{o}C) for 5 min. After centrifugation, supernatant and pellet were separated and the pellet suspended again in (500 µl) ethanol. The complete process was repeated twice and both supernatants were 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) 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 dd water, centrifuged and the supernatant discarded. Thereafter, the pellet containing the stabile-CW Al fraction was dried in a centrifugal evaporator (RCT 10-22T; Jouan, Saint-Herblain, France) for later Al determination.

Aluminium localization

Free and loosely-bound Al in the root tissue was localized by staining with morin ($C_{15}H_{10}O_7$). Root tips were washed with dd water, excised, and thin cross-sections (Eticha et al., 2005c) were made at defined growth zones of the root apex (Chapter 1). The transition zone (TZ) was located between 1 to 2 mm and the central elongation zone (CEZ) between 4 to 5 mm from the root tip. Free-hand sectioning without fixation and embedding was employed in order to reduce artifacts related to cellular redistribution of Al. Root sections directly mounted in slides containing 100 µM aqueous solution of morin were examined with a microscope (Zeiss, Axioscope, Jena, Germany) equipped with epifluorescence illumination (Mercury lamp, HBO 50W), a band pass filter BP 395-440 nm (exciter), a beam splitter FT 510 nm, and a longwave pass filter LP 515 nm (emitter). Pictures were taken with a digital camera (AxioCam MRc, Carl Zeiss AG, Göttingen, Germany) mounted on the microscope.

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, WFSF, 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 an injection volume of 20 µl. When required, the samples were diluted with dd water.

Statistical analysis

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

Results

Effect of Al on overall root elongation

In presence of Al root elongation of both genotypes was severely (60-65 %) inhibited 4 h after the beginning of the Al treatment (Fig. 8A). 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.

The dynamics of Al accumulation in the 5 mm root tips during the Al-treatment duration (Fig. 8B) was inversely related to the Al-induced inhibition of root elongation. The severe inhibition of root growth after 4 h Al supply in both genotypes was related to high Al contents in the root tips. Recovery from root-growth inhibition after 8 h was accompanied by reduced Al contents. 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 supply. Whereas the Al contents after 4 and 8 h Al treatment were significantly higher in Quimbaya than in VAX-1, they were lower after 24 h which is reflected by the highly significant genotype x time interaction.

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 (Fig. 9). Constitutively, genotype Quimbaya had significantly higher CW pectin-contents than VAX-1. Aluminium treatment slightly increased the pectin contents in both genotypes, (Fig. 9A). The DM was also higher in Quimbaya than in VAX-1. The DM first decreased after 4 h Al treatment in both genotypes (Fig. 9B). 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. 9C) which is a measure of the negativity of the CW was consistently higher in Quimbaya than in VAX-1 (31 %). This 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 the content of unmethylated pectin decreased again in Quimbaya but not in VAX-1 corresponding to the observed recovery in DM in Quimbaya but not in VAX-1. This is reflected by the significant genotype x time interaction observed in DM and unmethylated pectin.

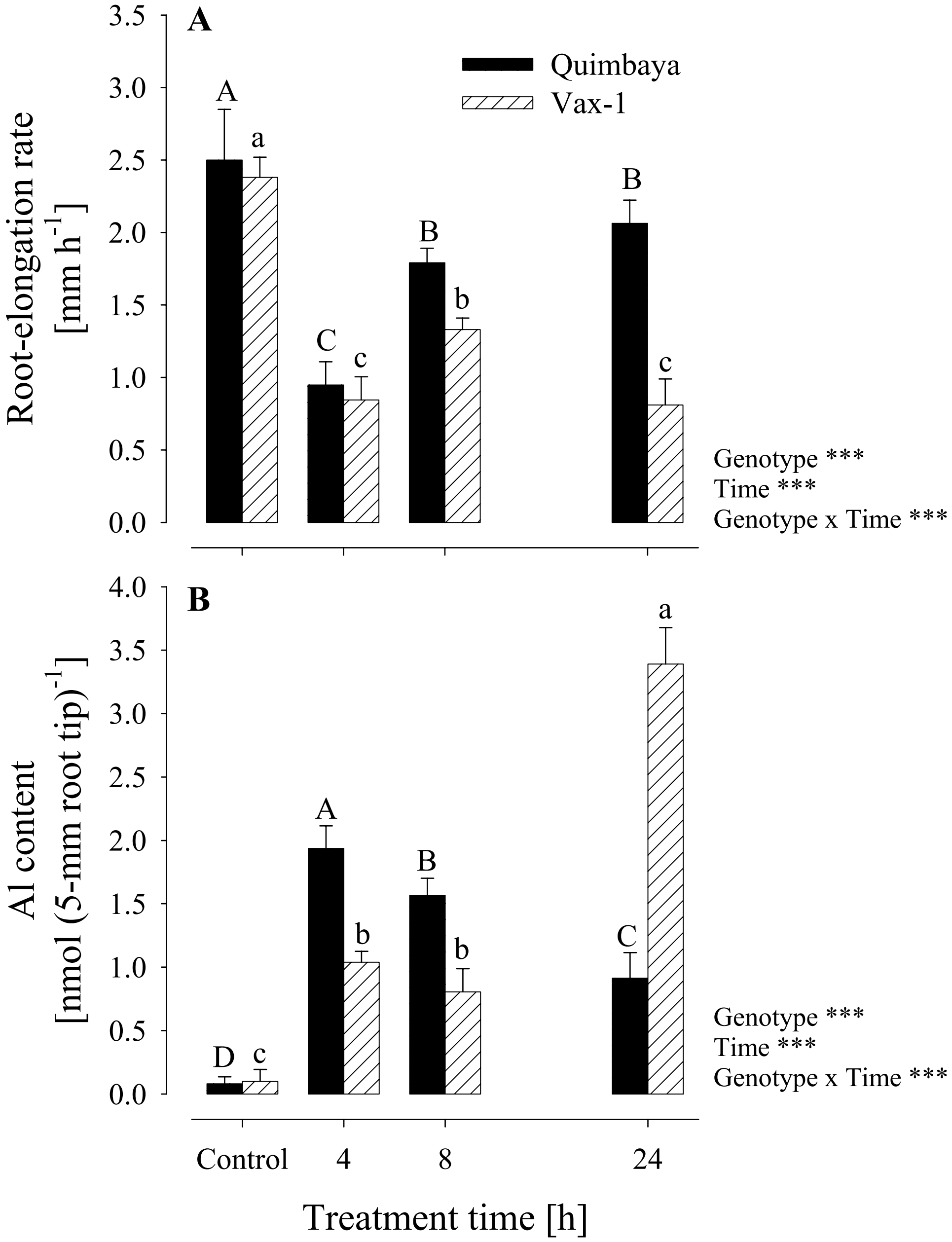

Figure 8. Effect of Al treatment on the root-elongation rate (A) and total Al contents (B) of the root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl and 8 µM H_3BO_3 with or without 20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

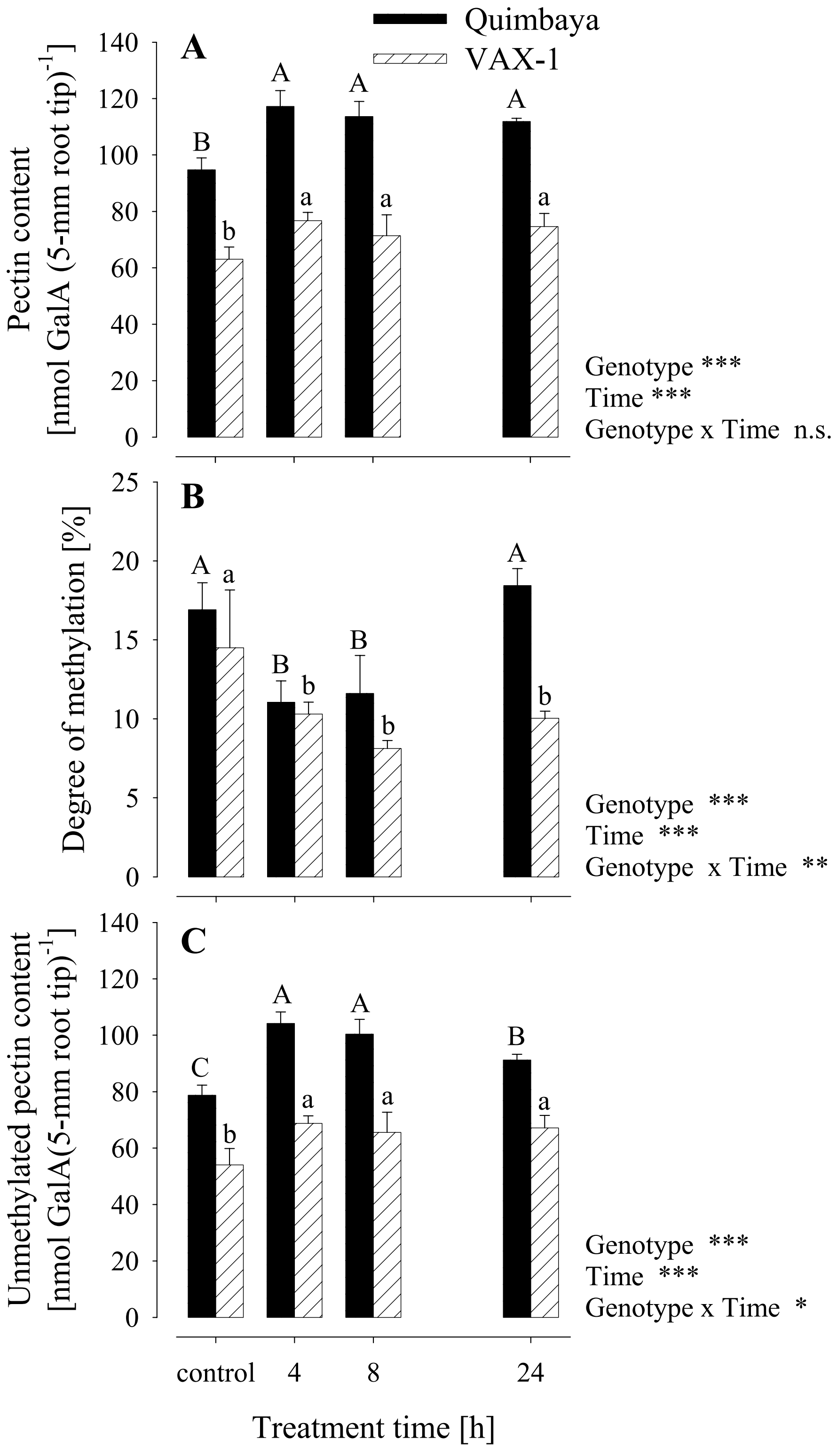

Figure 9. Total cell-wall pectin-content (A) its degree of methylation (B) and unmethylated pectin content (C) in 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 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 (Tukey test $p < 0.05$).

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 stage 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 not released from the root tips was termed 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. 10). During 4 h Al treatment, the time of maximum inhibition of root elongation (see Fig. 8A), Al accumulation was maximum in both Na_3citrate-exchangeable and non-exchangeable fractions in both genotypes. During the recovery from Al injury after 8 h, Al contents in both genotypes 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 particularly 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. 11). This was also true for VAX-1 for the recovery period 4-8 h 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. This is reflected by a significant correlation only between root-elongation rate and non-exchangeable (Fig. 11B) but not with Na_3citrate-exchangeable Al (Fig. 11A).

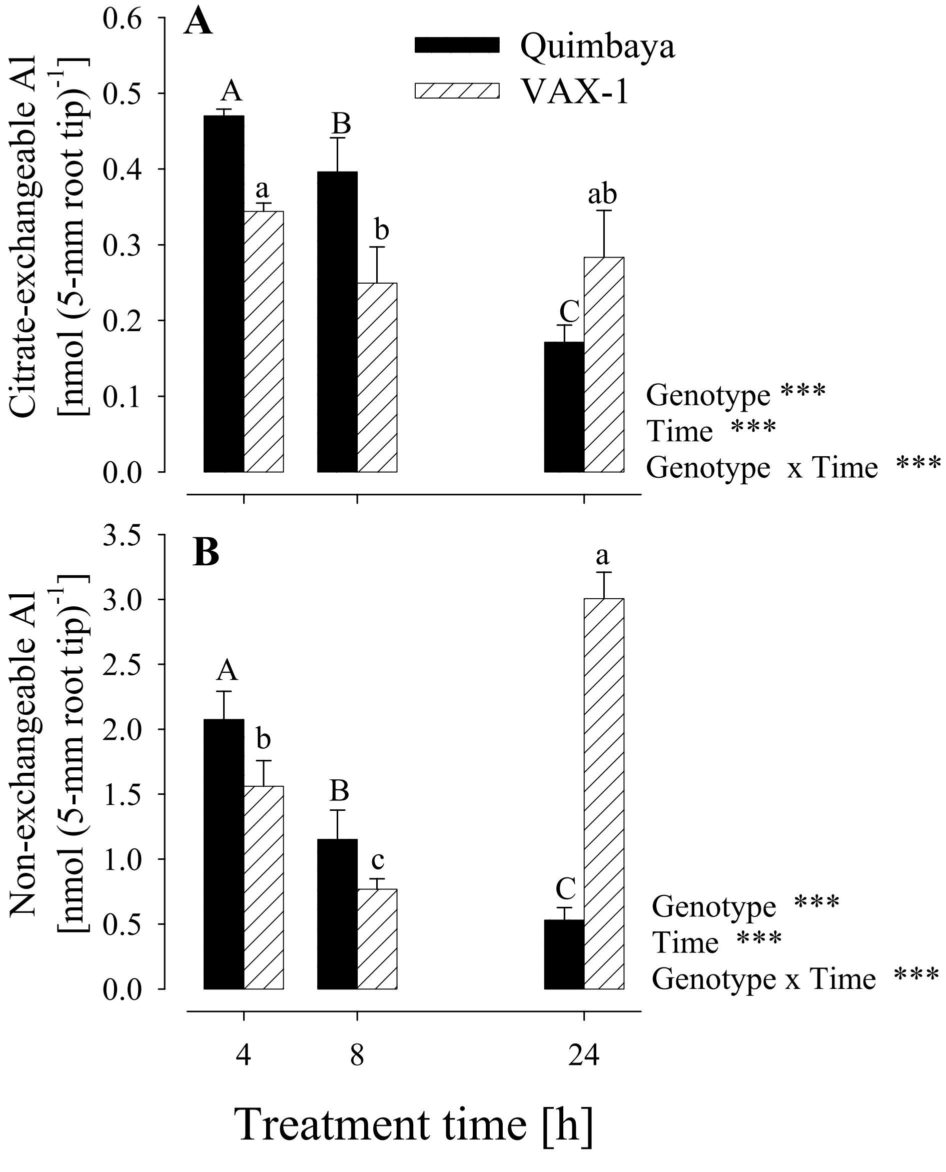

Figure 10. Citrate-exchangeable (A) and non-exchangeable (B) Al contents in 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 20 µM Al for up to 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 (Tukey test $p < 0.05$).

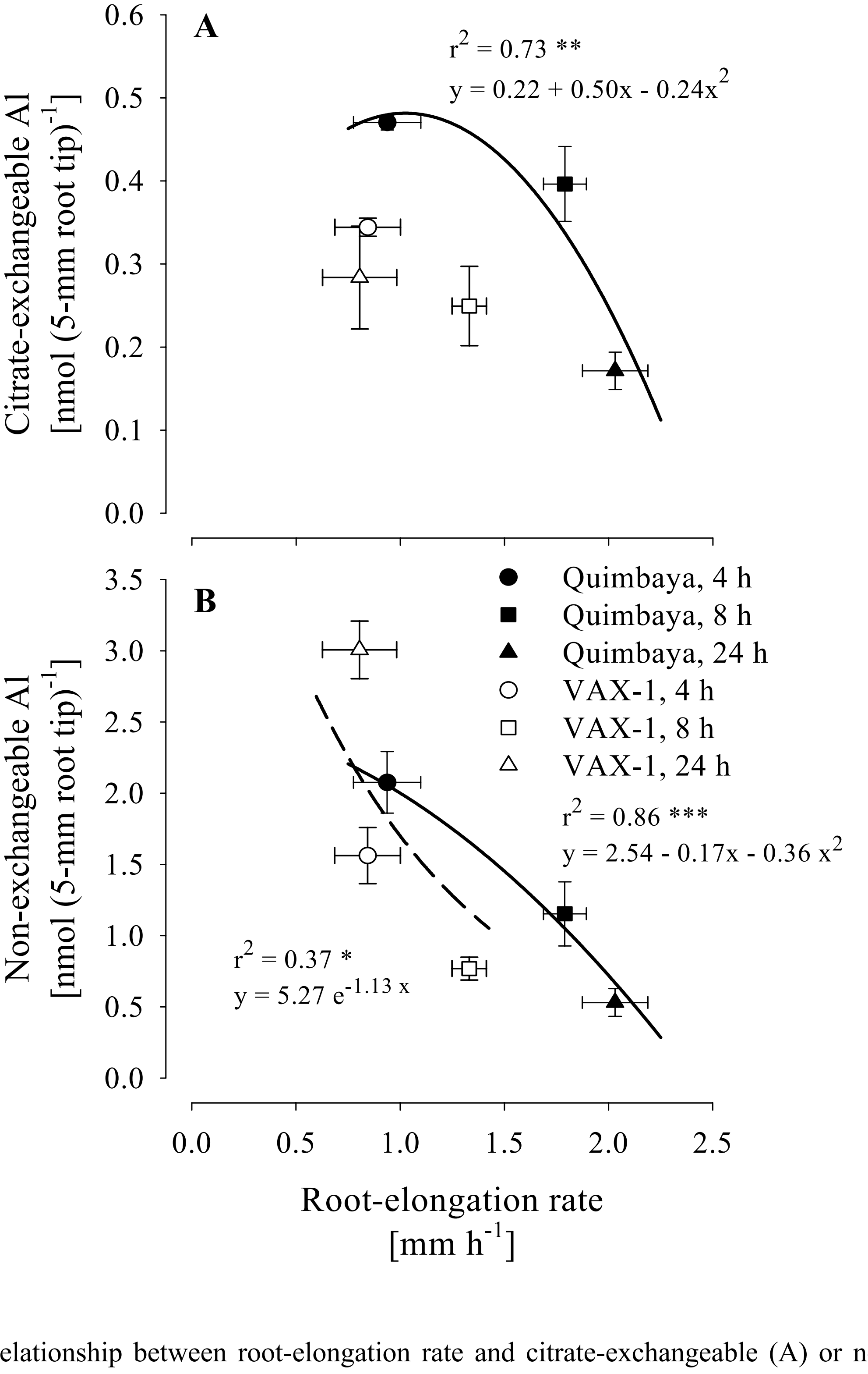

Figure 11. Relationship between root-elongation rate and citrate-exchangeable (A) or non-exchangeable (B) Al contents of root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 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.

In order to better characterize the cellular distribution of Al in the root apices in addition to the binding stage, the root tips were subjected to a more differentiated fractionation procedure (Fig. 12). The Al contents in the WFSF fraction were initially two times higher in Quimbaya than in VAX-1 but decreased over time particularly in Quimbaya, whereas the level remained rather constant in VAX-1 leading to a higher Al content 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 much 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 higher in Quimbaya, too. It increased up to 8 h 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 stabile-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 first slightly decreased after 8 h and then increased after 24 h Al treatment.

The stabile-bound CW Al fraction represented about 80 % of the total Al content (Fig. 13). Whereas the relative importance of this fraction was enhanced in Quimbaya with time, in VAX-1 this fraction first decreased (8 h) but then increased again after 24 h Al treatment. The quantitatively second important Al fraction was the symplastic fraction (combining the two symplastic Al fractions). This fraction became increasingly important 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 importance of the WFSF (smallest fraction) and the labile-bound CW Al fractions did not vary much over time. However, the latter was more important in VAX-1 (8 %) than in Quimbaya (4.5 %).

As in the Al-exchange experiments (see Fig. 11), 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. 12). In both genotypes, root-elongation rate was negatively related to the Al content of the apoplast (Fig. 14A). 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.

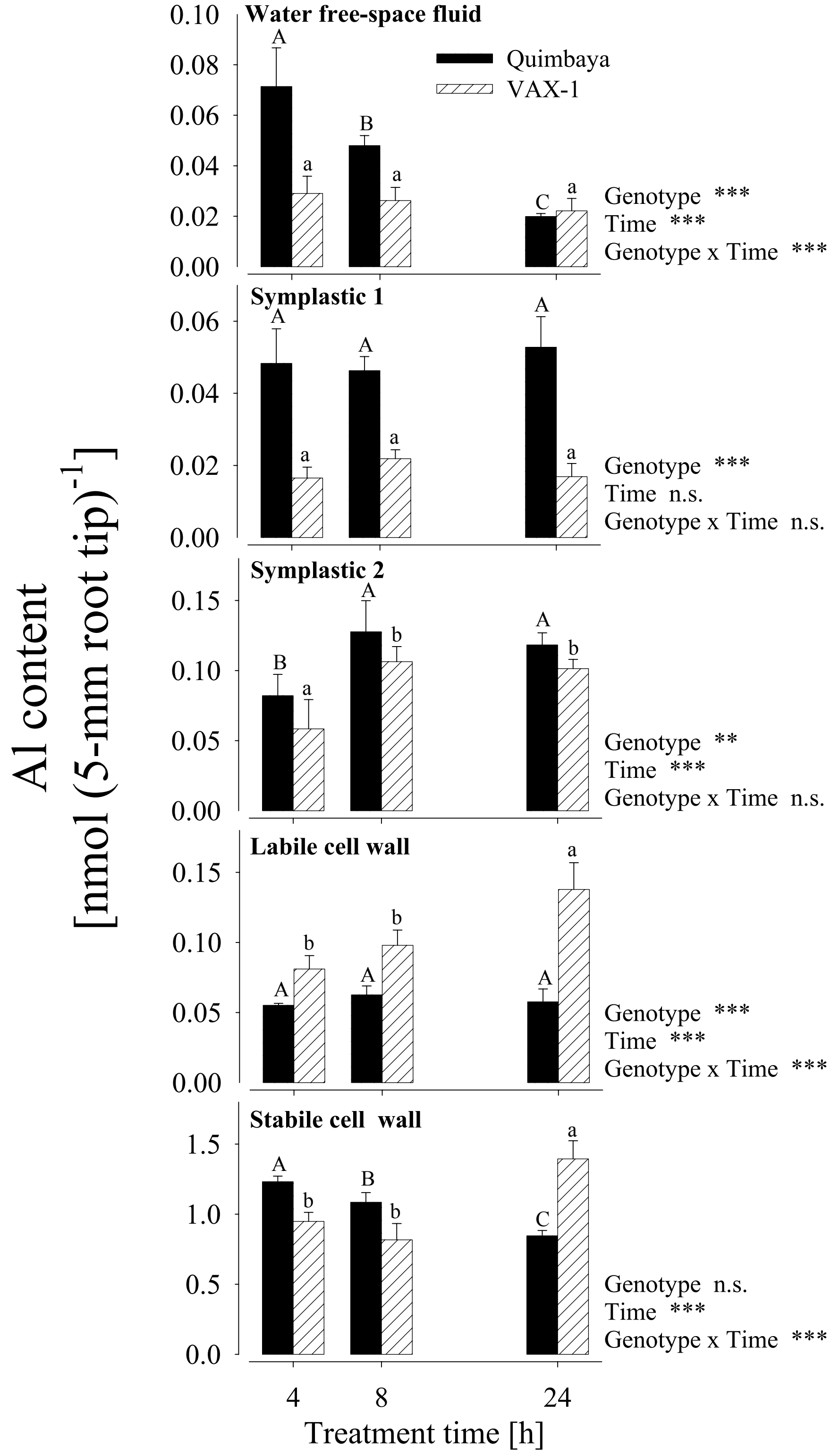

Figure 12. Aluminium contents of different cell compartments in 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 20 µM Al for up to 24 h, pH 4.5. 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 (Tukey test $p < 0.05$).

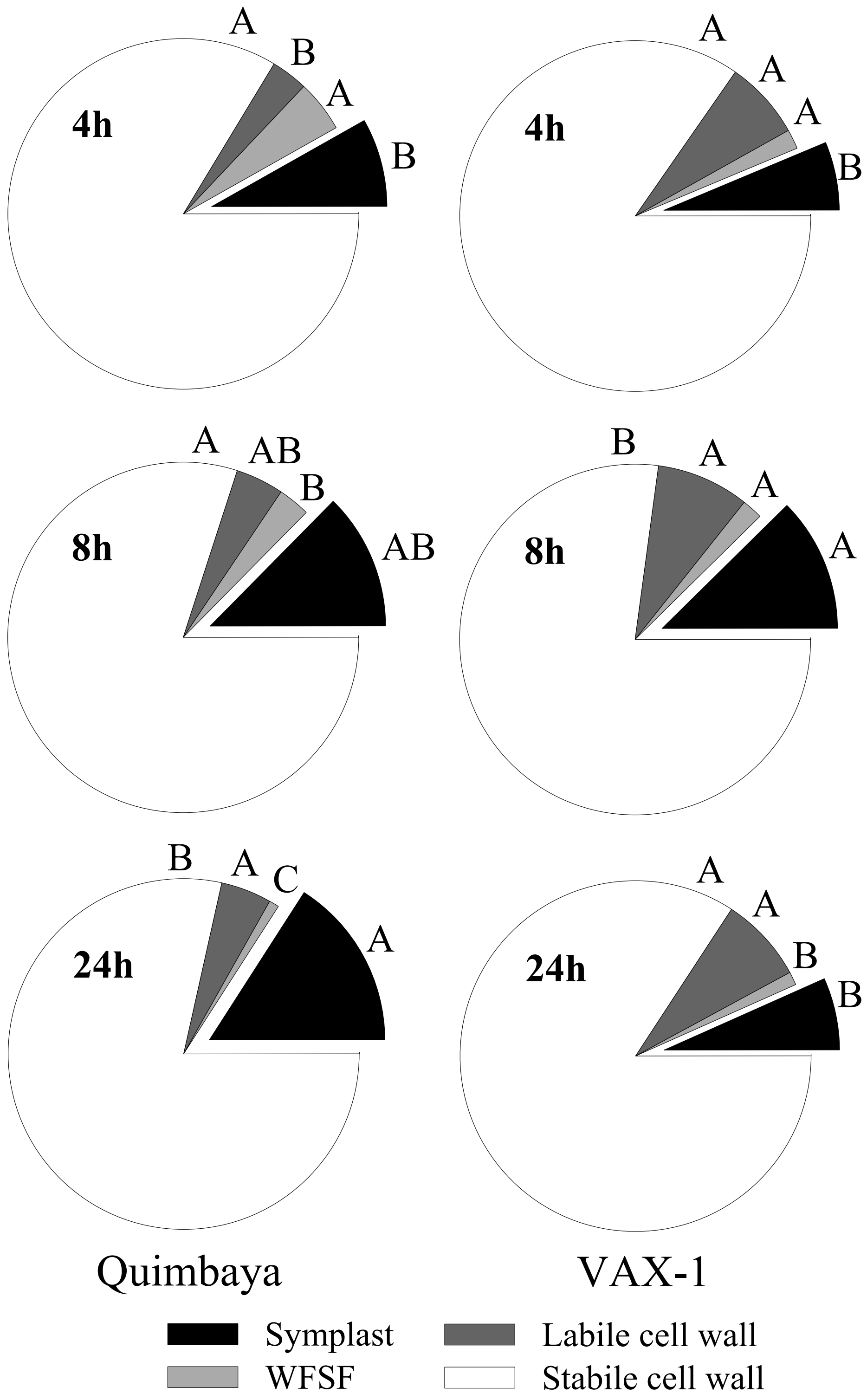

Figure 13. Relative distribution of Al contents of different cell compartments in the 5 mm root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 20 µM Al for up to 24 h, pH 4.5. Means with the same letter are not significantly different between times in each fraction (Tukey test $p < 0.05$).

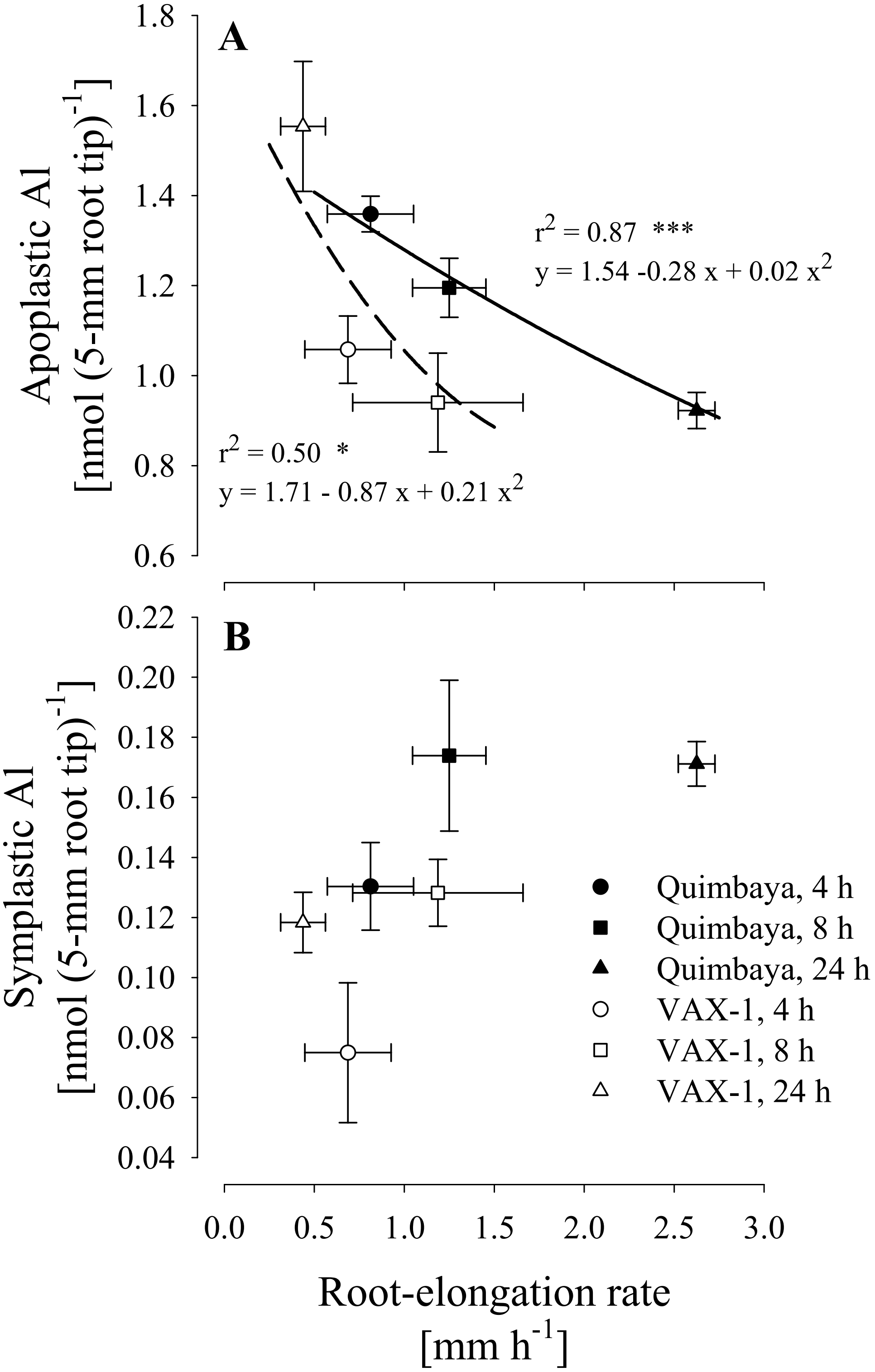

Figure 14. Relationship between root-elongation rate and apoplastic (A) or symplastic(B) Al contents of root tips of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 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.

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 fraction, correlations were calculated (Fig. 15). The WFSF and the stabile-bound CW, but not the labile-bound CW Al fractions showed a highly-significantly negative relationship with the enhanced root-elongation rate during the Al treatment.

Localization of Al in the root tips

Staining techniques are relatively simple and rapid tools for examining Al distribution in plant roots. The observed differences in the WFSF Al fraction between genotypes and throughout the Al treatment duration, and the importance of this Al fraction in the expression of Al injury and recovery suggested an apparent difference of Al localization and distribution in the root apices. Therefore, the radial distribution of Al during up to 24 h of Al treatment in the TZ and CEZ was studied using morin which most sensitively stains free and loosely bound Al in the apoplast and the symplast (Eticha et al., 2005c).

In the control roots (no Al treatment) no Al-morin fluorescence could be detected at the standardized settings of the fluorescence microscope. Therefore, no pictures are presented. Constitutively, genotype Quimbaya has a higher number (7 to 8 lines of cells) and larger cortical cells compared to VAX-1 (5 to 6 lines of cells) (Fig. 16). This was especially evident in the CEZ. In presence of Al, all root cross-sections were characterized by bright green fluorescence of the Al-morin complex. After 4 h of Al treatment, the time of maximum inhibition of root-elongation, in the TZ a zone of intense staining was observed up to the third row of outer cortical cells in Quimbaya (Fig. 16A) while it was confined primarily to the subepidermal cortical cell layer in VAX-1 (Fig. 16E). Both apoplast and symplast were stained more intensively in Quimbaya than in VAX-1. At a lower intensity Al-morin fluorescence was also observed along the entire cross-section up to the endodermis in both genotypes, especially in VAX-1(Fig. 16E)

In the CEZ after 4 h of Al treatment, the Al-morin complex was mainly located at the subepidermal cortical cell layer in both genotypes (Figs. 16B, 16F). In contrast to the TZ, the fluorescence was more intense in genotype VAX-1 than in Quimbaya. Although less intense, Al-morin staining of the apoplast could also be observed in the entire cortex especially in VAX-1 (Fig. 16F). Evidence of cell destruction (spots of high fluorescence intensity) of some epidermal cells was observed in both genotypes, but more clearly in VAX-1 than in Quimbaya.

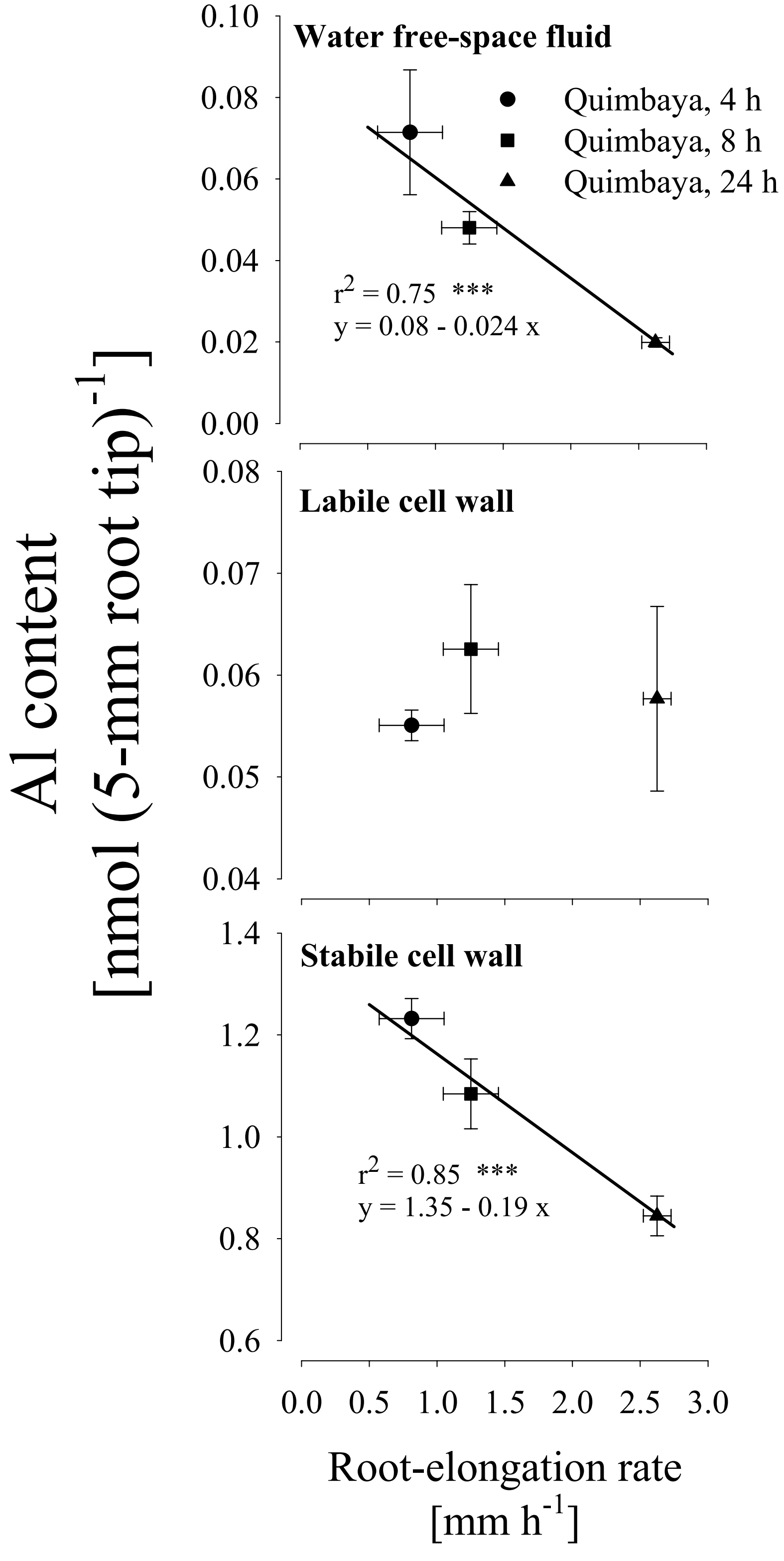

Figure 15. Relationship between root-elongation rate and the Al contents of three different apoplastic fractions in 5 mm root tips of genotype Quimbaya (Al-resistant) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 with or without 20 µM Al for up to 24 h, pH 4.5. For the ANOVA *** denote levels of significance at $p < 0.001$.

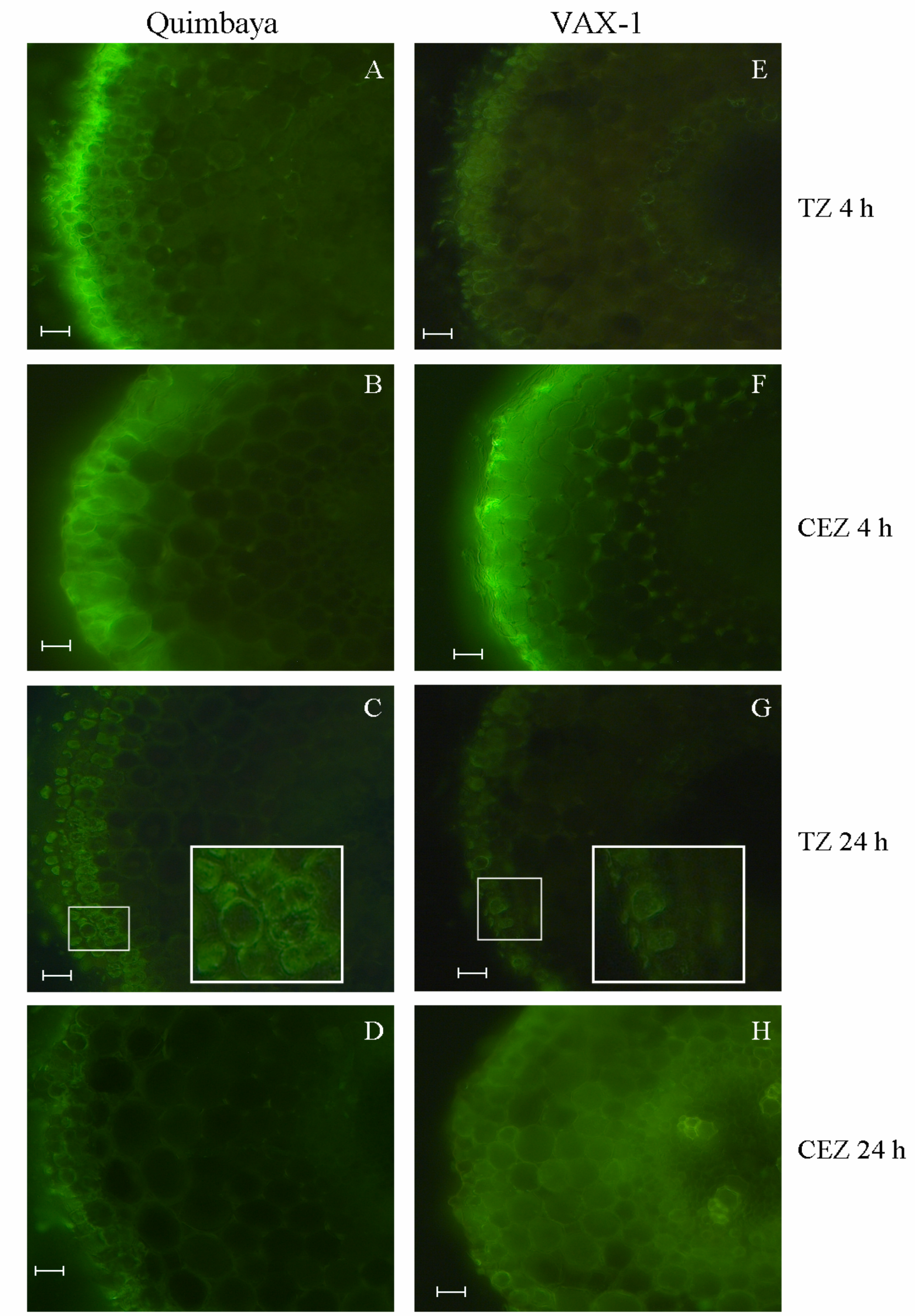

Figure 16. Aluminium distribution localized by morin staining in root-cross sections of Quimbaya (Al-resistant, left) and VAX-1 (Al-sensitive, right) common bean genotypes. (A, E) Cross-section from the TZ after 4 h of Al treatment. (B, F) Cross-sections from the CEZ after 4 h of Al treatment. (C, G) Cross-section from the TZ after 24 h of Al treatment. (D, H) Cross-sections from the CEZ after 24 h of Al treatment. Plants were grown in a simplified nutrient solution with or without 20 µM Al for up to 24 h, pH 4.5. In order to compare the fluorescence intensities, all pictures were taken at the same magnification (200X), excitation filter 395 to 440 nm, barrier filter 470 nm and 140 ms light exposure. A picture summarizing thirty-two sections taken from 8 plants (four consecutive cross-sections per plant). Scale bars = 50 µm.

After 24 h of Al treatment, when roots of genotype Quimbaya had nearly completely recovered from Al injury and VAX-1 got severely damaged by Al, cross sections in the TZ (Figs. 16C, 16G) did not show major differences in the Al-morin staining intensity and distribution between the genotypes. The fluorescence mostly represented symplastic Al accumulation in subepidermal cortical cells particularly in Quimbaya (inserts in Figs. 16C and 16G). There was hardly any fluorescence of Al-morin beyond the outer cortical cell layers.

Clear differences between the genotypes in the staining intensity and radial Al distribution were observed in the CEZ after 24 h of Al treatment (Fig. 16D, 16H). In Quimbaya, the fluorescence was restricted mainly to the symplast of epidermal cells (Fig. 16D). In VAX-1, Al was evenly distributed across the cortex and clearly reached the central cylinder with the xylem vessels particularly well stained (Fig. 16H).

Discussion

The results clearly corroborate previous information that in contrast to many other plant species, Al resistance in common bean is an Al-inducible trait involving a lag phase of 4-6 hours (Cumming et al., 1992; Chapter 1; Fig. 8A). Therefore, unraveling the physiological and molecular basis of Al toxicity and resistance requires a systematic kinetic study over 24 h of the dynamics of the Al response in the root apex which is missing so far.

As hypothesized by Taylor (1995), *Phaseolus vulgaris* proved to be an ideal experimental system to elucidate the relationship between Al response and recovery from initial Al injury and the dynamics of Al uptake, distribution and compartmentation in the root apex.

Aluminium treatment resulted in rapid Al accumulation (after 4 h, Fig. 8B) more in the Al-resistant genotype Quimbaya than in the Al-sensitive genotype VAX-1 leading to severe inhibition of root elongation in both genotypes (Fig. 8A). 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 cereals and dicotyledons (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., 2005b), potato

(*Solanum tuberosum* L., Schmohl et al, 2000, Horst et al., 2007) and common bean (Stass et al., 2007) using different experimental approaches. Therefore, it appears reasonable to assume that the higher initial Al accumulation of the Al-resistant common bean genotype Quimbaya in the root apex compared to VAX-1 (Fig. 8) is due to its higher content of unmethylated pectin and thus higher negativity of the CW (Fig. 9).

Aluminium treatment initially enhanced the root-tip pectin-content in both genotypes (Fig. 9A). This is in agreement with previous studies conducted in maize (Eticha et al., 2005b), cultured tobacco cells (*Nicotiana tabacum* L., Chang et al., 1999), squash roots (*Cucurbita maxima* Duch., Le Van et al., 1994), and wheat (*Triticum aestivum* L., Hossain et al., 2006). In all cases, Al stress increased not only the pectin contents but also cellulose and hemicellulose contents indicating that root elongation is more inhibited by Al than CW synthesis leading to a higher specific weight of the root apex (Tabuchi and Matsumoto, 2001). In addition, Al treatment decreased the DM of the pectin (Fig. 9B) thus enhancing the overall negativity of the CW (Fig. 9C). Several studies have shown that the Al-induced changes in the CW structure might enhance the activity of PME by releasing enzymes molecules that were initially bound to block of carboxylic groups rather than a direct effect on the enzyme (Moustacas et al., 1991). This release might facilitate the contact of the enzyme and its substrate (highly methylated pectin) leading to lower DM (Nari et al., 1991; Goldberg et al., 1992; Schmohl et al., 2000). However, at high concentrations metal ions will inhibit the enzyme reaction (Nari et al., 1991). With Al treatment duration up to 24 h, the pectin content and the DM fell back to the initial values in Quimbaya but remained lower in VAX-1. However, the negativity of the CW consistently remained higher in genotype Quimbaya than in VAX-1. Therefore, it appears that the changes in pectin content and its DM are not the cause but rather the consequence of the resumption of root elongation after the initial Al injury and thus build up of Al resistance in Quimbaya.

It has been argued that the strong binding of Al in the cell wall represents a detoxification mechanism in squash (Le Van et al., 1994). However, the recovery from initial Al injury in the Al-resistant genotype Quimbaya after 24 h Al treatment was negatively related to the citrate non-exchangeable Al fraction of the root apices (Fig. 11) and the stabile-bound Al cell-wall fraction (Fig. 15). This suggests that the strong binding of Al to the pectic matrix of the cell wall is a main factor of Al toxicity and not a tolerance mechanism in common bean. In contrast to the stabile-bound cell-wall Al fraction, there was no indication that the labile-bound (citrate-exchangeable) Al fraction was related to Al-induced inhibition of root elongation (Fig. 15). 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 principle difference between grasses and dicotyledons in Al binding to cell walls, which is not surprising given the difference in cell–wall composition (see above). This is well illustrated by the fact that treatment of cell walls with 50 mM $BaCl_2$ removed about 20 % of the cell wall-bound Al in maize (Wang et al., 2004), and nearly all Al adsorbed on wheat cell-walls 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. 11A) might be explained by the contribution of free apoplastic Al to this fraction (Fig. 15).

The fractionated extraction procedure allowed to separate operationally defined apoplastic and symplastic Al fractions (Fig. 12). Among the 5 fractions the WFSF Al and the stabile-bound CW Al fractions are expected to best represent in vivo compartmentation of Al, the former 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. 15). 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 this 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 (Klug et al, personal information) compared to 6-15 % in common bean (Fig. 13).

The symplastic Al fraction neither reflected 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. 13). 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. 12 and 16) seems to indicate that higher symplastic Al contents are characteristic for enhanced/acquired Al resistance which is in line with the observations by Vázquez et al.

(1999) and Illes et al. (2006) who ascribed internalization of Al into the symplast as an Al tolerance mechanism in maize and *Arabidopsis*, respectively. Greater accumulation of Al in the symplastic fraction in genotype Quimbaya might by explained by the greater cell volume and thus vacuoles (root cross sections in Fig. 16) typical for genotypes of Andean origin in comparison to the Mesoamerican origin of VAX-1 (Gepts et al., 1986; Singh, 1989; White and Gonzalez, 1990). However, it is rather unlikely that this can explain enhanced Al resistance because of the quantitatively small Al fraction in the symplast (Fig. 13).

The transitory (VAX-1) or sustained (Quimbaya) recovery from initial Al-induced inhibition of root elongation (Fig. 8A) typical for pattern II plant species (see introduction) is related to a decrease in Al contents of the root tip (Fig. 8B), particularly the apical 2 mm (Chapter 1). The close negative correlation of root-elongation rate and Al contents of the WFSF and the stabile-bound cell-wall fraction (Fig. 15) 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., 2002a; Chapter 3). 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. However, the decrease of the Al content of the stabile-bound cell-wall Al fraction with increasing Al treatment duration (Fig. 15) by root-released citrate appears to be implausible because this fraction is defined as citrate non-exchangeable. It thus appears unlikely that once stabile bound Al is 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 protecting the root for further Al accumulation (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.

As shown by Eticha et al. (2005c) in maize, morin is unable to stain pectin and CW-bound Al. However, we cannot exclude that labile-bound CW (citrate-exchangeable) Al will at least partly react with morin considering that citrate has an Al binding affinity constant (log K = 7.8) close to that of morin (log K = 4.2-6.5). The assumption is supported by Vitorello and Haug (1997) who observed that citrate quenched the Al-morin complex only by 37%. Similarly, Klug and Horst (2006) demonstrated that up to 40% of the Al-citrate complex could be detected by using morin or lumogallion staining protocols. Thus, the observed Al-morin complex (Fig. 16) is expected to represent in addition to the WFSF and symplastic Al also part of the labile-bound (citrate-exchangeable) CW Al. Consequently, morin staining did not allow any statement about quantitative distribution of Al between cell compartments. Our results show that Al-morin fluorescence was mainly and primarily observed in the epidermal and outer cortical cells, independently of the genotype, the observed root zone, or the Al treatment time (Fig. 16). This is in agreement with previous studies conducted in *Avena sativa* (Marienfeld and Stelzer, 1993), wheat (Delhaize et al., 1993a) and *Picea abies* (Godbold et al., 1995; Godbold and Jentschke, 1998) using energy-dispersive X-ray analysis (EDXMA), in soybean (Glycine max) using secondary ion mass spectrometry (SIMS, Lazof et al., 1994, 1996) or lumogallion staining (Kataoka et al., 1997), or in maize (Wang et al., 2004) using morin staining. During the maximum inhibition of root growth (4 h) the intensity of the Al-morin complex was similar in the TZ and the CEZ in Quimbaya, in agreement with its higher WFSF and symplastic Al contents. On the other hand, it was specifically intense at the CEZ in VAX-1, possibly explaining the labile-bound Al to the CW, the only fraction consistently higher in VAX-1 compared to Quimbaya. Although less intense, it appears that Al was able to penetrate up to the endodermis in both genotypes in both zones. In maize and *Vicia faba*, the transport from the rhizodermis to the endodermis was shown to be time-dependent (1 to 3 h, respectively) without any transport of Al into the central cylinder through the fully differentiated endodermis within 3 h of Al treatment (Marienfeld et al. 2000). Similarly, in soybean Al was able to penetrate up to the endodermis after 4 h of Al treatment, along the entire elongation zone (Lazof et al., 1996). Although in these two studies different experimental approaches were used, both agreed that Al entered the symplast. In agreement with these studies also in common bean, Al accumulated in the symplast of both genotypes, more in Quimbaya than in VAX-1, and particularly during the recovery of root growth especially at the TZ (insert in Fig. 16C). After 24 h of exposure to Al, the clearly observed genotypic differences in Al resistance (Fig. 8A) were characterized by a restriction of the Al-morin fluorescence to the symplast of epidermal cells (Fig. 16D) in the Al-resistant genotype Quimbaya, while it was homogeneously distributed across the cortex into the central cylinder in the Al-sensitive genotype VAX-1 (Fig. 16H) in agreement with the enhanced Al accumulation in the labile-bound CW fraction (Fig. 12). In agreement with these results, Lazof et al. (1994) reported similar Al distribution in soybean, a typical pattern II plant type (Yang et al., 2001), after 18 h of Al treatment.

In conclusion, these results support the idea that induced Al resistance in the Al-resistant genotype mediated by the release of citrate into the apoplast which will be the subject of the subsequent chapter. A high citrate concentration in the WFSF will reduce the binding of Al in the stabile-bound Al fraction of the apoplast thus allowing resumption of cell division and cell elongation and leading to a reduction of the Al contents in the root apex.

CHAPTER 3:

Aluminium resistance in common bean (*Phaseolus vulgaris* L.) involves induction and maintenance of citrate exudation from root apices

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(To be submitted)

Abstract

Citrate exudation has been reported to contribute to aluminium (Al) resistance in common bean (*Phaseolus vulgaris* L.). Nevertheless, a clear characterization of the exudation pattern or its association with internal organic acid contents and enzyme activities involved in citrate synthesis or degradation has not been conducted so far. Therefore, in the current study, two common bean genotypes differing in Al resistance, Quimbaya (Al-resistant) and VAX-1 (Al-sensitive), were grown for up to 25 h with or without Al supply, and several parameters related to the exudation of organic acids anions from the first centimeter of the root tip were investigated. Among the organic acid anions determined Al treatment enhanced particularly the exudation of citrate from the root tips of both genotypes and its dynamics offers the most consistent relationship with the kinetics of Al accumulation and Al-induced inhibition of root elongation of both genotypes. The genotype-independent short-term (4 h) Al injury period was characterized by the accumulation of Al in the root tips, absence of citrate efflux in spite of appreciable root citrate-contents (Quimbaya >> VAX-1). Thereafter (5 – 9 h), in both genotypes root elongation recovered (VAX-1 faster than Quimbaya) by an Al-enhanced exudation of citrate from the internal organic acid pool, leading to reduced citrate and Al contents. In VAX-1 citrate efflux could be sustained during this period by a down-regulation of the activity of NADP-isocitrate dehydrogenase (NADP-ICDH) thus reducing the cytosolic turnover of citrate a low but constant citrate synthase (CS) activity. In Quimbaya, the citrate efflux was sustained by both a lower NADP-ICDH activity and the greater internal citrate pool in spite of a decreased CS activity. The recovery of the CS activity after 25 h Al treatment allowed sustaining an enhanced capacity to exude citrate and to restore the internal organic acid contents leading to decreasing Al contents and thus recovery of root growth in Quimbaya. In VAX-1, a decreased CS activity coupled with an exhausted internal citrate pool, resulted in lower rates of citrate secretion, resumption of Al accumulation and, consequently, to severe inhibition of root elongation. It is concluded that Al resistance in common bean requires the expression/activation of a citrate permease and the maintenance of the cytosolic citrate concentration through up-regulated synthesis and down-regulated degradation.

Introduction

Common bean (*Phaseolus vulgaris* L.) is the second most important source of protein in eastern and southern Africa and the fourth in tropical America. It is also the third most important caloric source after cassava (*Manihot esculenta* Crantz) and maize (*Zea mays* L.) (CIAT, 1999). Common bean is mainly produced on small-scale farms (80 % of the world's dry bean production) where about 40 % and 30 to 50 % of the bean-growing area in Latin America and central, eastern and southern Africa, respectively are affected by Al toxicity, the most important soil factor limiting crop yields on acid soils (von Uexküll and Mutert, 1995), leading to 30 to 60 % yield reduction (CIAT, 1992). First attempts to characterize genotypic differences in Al resistance in beans date from the mid 70´s (Foy et al., 1972; Howeler, 1991). Since then, considerable progress has been attained in identifying and improving germplasm better adapted to acid soils (Rao et al., 1993; Rangel et al., 2005; Manrique et al., 2006). However, common bean is still among the plant species, least adapted to soil acidity, generally, and Al toxicity, specifically. Therefore, improving Al resistance of common bean to reduce the dependence of small farmers on lime and nutrient inputs (CIAT, 1999) remains a major challenge.

Aluminium rapidly inhibits root growth (Foy, 1988; Llugany et al., 1995) by injuring the most Al-susceptible part of the plant, the root apex (Ryan et al., 1993; Sivaguru and Horst, 1998; Kollmeier et al., 2000). Plant species and genotypes within species vary widely in the resistance to Al, suggesting that Al-resistant species or genotypes posses several mechanisms to avoid Al toxicity (Taylor, 1991). Mechanism of Al resistance have been broadly classified as those which prevent the entrance of Al into the plant (Al exclusion) and those that detoxify or sequester Al internally (Al tolerance) (Rengel, 1996; Delhaize et al., 2007). Several possibilities have been proposed for each type of mechanism (Taylor, 1991), but most of them remain speculative (Kochian et al., 2004). However, since 1991 accumulating evidence has shown that organic acids (OA) play an important role in detoxifying Al internally and externally (Ma, 2000; Ma et al., 2001; Ryan et al., 2001; Ma and Furukawa, 2003). Both, the kind and the amount of exuded OA-anions contribute to the Al-detoxification capacity (Zheng et al., 1998a). Consequently, the OAs have been classified as strongly (citrate, oxalate and tartrate), moderately (malate, malonate and salicylate) and weakly (succinate, lactate, formate and acetate) Al-detoxifying compounds according to the stability of the Al complex (Hue et al., 1986). The enhanced exudation of citrate in response to Al stress has been reported in common bean (Miyasaka et al., 1991; Mugai et al., 2000; Shen et al., 2002a, Rangel and Horst, 2006; Stass et al., 2007), maize (Pellet et al., 1995, Kollmeier et al., 2001) and soybean

(*Glycine max* L., Yang et al., 2000; 2001; Silva et al., 2001), the exudation of malate in wheat (*Triticum aestivum* L., Delhaize et al., 1993b; Pellet et al., 1997), a combination of both (citrate and malate) in rye (*Secale cereale* L., Li et al., 2002) and triticale (*x Triticosecale* Wittmark., Ma et al., 2000; Hayes and Ma, 2003), and oxalate in buckwheat (*Fagopyrum esculentum* Moench., Zheng et al., 1998b) and taro (*Colocasia esculenta* L. Schott., Ma and Miyasaka, 1998). These OAs are thought to complex Al within the apoplast of the root apex (Kinraide et al., 2005).

Two patterns of OA exudation can be recognized, based on the timing and amount of secretion (Ma et al., 2001). In Pattern-I plants species, exudation is switched on without a delay after exposure of plants to Al and kept constant over time. In Pattern-II plants species, OA secretion is induced by the addition of Al after a lag phase of several hours and the rates of release increase over time. This characterization suggests a constitutive mechanism of OA release in the Pattern-I, whereas in Pattern-II the induction of genes and synthesis of proteins involved in the OA synthesis and/or membrane transport is required (Ma et al., 2001; Ryan et al., 2001). Cumming and coworkers (1992) proposed that Al resistance appears to be an inducible trait in common bean. In agreement with their studies, our previous results (Chapter 1) have shown that both Al-resistant and Al-sensitive common bean genotypes initially (4 h) react equally sensitive to Al. Thereafter, the root growth recovered in the Al-resistant genotype while it remained inhibited after a transient recovery in the Al-sensitive genotype. This dynamic of root elongation in response to Al was closely related to the Al content in the root tip and suggested the presence of an Al-exclusion mechanism. Al-induced citrate exudation has been reported in common bean (Miyasaka et al., 1991; Mugai et al., 2000; Shen et al., 2002a, 2004; Rangel and Horst, 2006; Stass et al., 2007): However, the pattern of OA exudation in common bean remains a matter of debate (Ma and Furukawa, 2003). In some experiments, a rapid Al-induced exudation of citrate could be observed after 2 h of Al treatment (Shen et al., 2004), while in others, the citrate exudation seems to be delayed for a period of 3-5 h (Mugai et al., 2000; Shen et al., 2002a). Therefore, a better characterization of the pattern of Al-induced citrate exudation in relation to the observed changes in the dynamic of root elongation is necessary to generate physiological markers to quantify differences in Al resistance in common bean.

Contrary to the better characterized process of Al-induced exudation of OA-anions by plant roots, the role of the metabolism and accumulation of OA in Al resistance is still elusive (Ryan et al., 2001, Horst et al., 2007). In many species, where OA-anion release is activated by Al, no correlations are apparent between internal OA concentrations and efflux. For instance, Al-sensitive and Al-resistant wheat genotypes did not differ in root concentrations of malate, although the Al-resistant genotypes excreted 5- to 10-fold more malate than the Al sensitive genotypes (Delhaize et al., 1993b). Contrary, in soybean, a typical patter II-plant type, an Al-enhanced accumulation of malate (Yang et al., 2001) and citrate (Silva et al., 2001) contributed to the enhanced citrate secretion. A similar controversy exists about the role of enzymes involved in OA synthesis/decomposition in Al-induced OA-anion efflux. In wheat, the high Al-induced malate secretion occurred without significant changes to the activities of phosphoenolpyruvate carboxylase (PEPC) or malate dehydrogenase (NAD-MDH). Moreover, the activities of these enzymes were not significantly different between genotypes (Ryan et al., 1995). An enhanced CS activity has been reported in soybean (Yang et al., 2001) and common bean (Mugai et al., 2000) after 12 and 24 h of Al treatment, respectively. In addition, strategies to over-express enzymes involved in OA metabolism has proven to be effective in enhanced OA exudation leading to Al resistance in transgenic plants of *Arabidopsis thaliana* (Koyama et al., 2000), alfalfa (*Medicago sativa* L., Tesfaye et al., 2001) and canola (*Brassica napus* L., Anoop et al., 2003; Ligaba et al., 2004). Therefore, in the present study the effects of Al treatment (short- and medium-term Al treatment) in the dynamic of OA accumulation and their exudation from the root apex (1 cm) of an Al-sensitive (VAX-1) and Al-resistant (Quimbaya) common bean genotypes were analyzed. Additionally, the effect of Al in the dynamic of some metabolic key enzymes of the citric acid cycle was studied.

Materials and methods

Plant material and growth conditions

Seeds of two common bean genotypes with known differential Al resistance were used in this study. Seeds of the Al-resistant (Quimbaya) and Al-sensitive (VAX-1) genotypes were germinated between filter-paper styrofoam-sandwiches soaked with tap water, in an upright position. Uniform seedlings were transferred to 18 l pots with constantly aerated simplified nutrient solution containing 5 mM $CaCl_2$, 0.5 mM KCl, and 8 µM H_3BO_3 (Rangel et al., 2005). Plans were cultured in a growth chamber under 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. Unless indicated, the 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 aluminon or pyrocatechol violet method (PCV) according to Kerven et al. (1989). Nominal 40 or 20 µM Al treatments resulted in 32 ± 3 or 16 ± 2 µM Al_{mono} after 24 h, respectively.

Effect of Al on root growth and Al contents of 5 mm root apices

Root growth was measured at 4, 8 and 24 h of Al treatment by marking the tap root 3 cm behind the root tip 2 h before the beginning of the Al treatment with a blue permanent marker (Sharpie- Fine Point, Sanford, FL). For the determination of the Al content, 5-mm root tips were washed with 1 ml ultra-pure deionized water (dd water) and then digested in 500 ml ultra-pure HNO_3 (65 %) overnight on a rotary shaker. To complete the digestion, samples were incubated in a water bath at 80°C for 20 min. Aluminium was measured by GFAAS (Unicam 939 QZ; Analytical Technologies Inc., Cambridge, UK), at a wavelength of 308.2 nm. When required, the samples were diluted with dd water.

Collection of root exudates and determination of organic acids in exudates and root apices

To collect root exudates from root apices, twelve plants were bundled in filter paper soaked with nutrient solution. Approximately 1 cm of the main root apex of each plant were immersed into 18 ml of a constantly aerated collection solution containing 5 mM $CaCl_2$, 8 µM H_3BO_3 and 0 or 40 µM $AlCl_3$ (nominal concentration), pH 4.5, in 20 ml poly-prep filtration columns (BioRad Laboratories, Richmond, California, USA). Two different approaches were used for the collection the root exudates. (i) For the study of short-term Al effects, root apices of non-pretreated plants were incubated in presence of Al for a period of up to 10 h, changing the poly-prep columns with the incubation medium every 2 h. (ii) For the study of short to medium-term Al effects, root apices of plants pretreated with Al (20 µM) for 0, 3, 7, 15 and 23 h at pH 4.5 were subsequently incubated in an identical solution with 0 or 40 µM Al for 2 h. The Al concentration in the incubation medium was doubled in order to compensate the small volume and thus low total Al supply. In both approaches, the basal part of the root system was constantly moistened with collection solution (see above) to prevent dryness but avoiding dripping into the columns. After 2 h the incubation solution containing the root exudates were immediately frozen at -20° C. After thawing, the incubation solution was passed through 5 g of a cation-exchange resin (AG50W-X8 with a 75-150µm mesh) at a flow rate of 1 ml min^{-1}. The resin had been charged with 20 ml (3 M) HCl and washed four times with ultra-pure deionized water (dd water), and then mounted in 20 ml poly-prep columns with a 200-400 mesh filter at the bottom of the column. The resulting solution containing the organic acids (OA) was concentrated to dryness in a rotary vacuum evaporator (RCT 10-22T, Jouan, Saint-Herblain, France). The residue from each sample was re-dissolved in 500 µl (10 mM) perchloric acid, sonicated for 15 min, filled into centrifugal Eppendorf filter tubes with a membrane pore size of 0.45 µm (GHP Nanosep® MF Centrifugal Device, Pall Life Sciences, Ann Arbor, USA), and centrifuged at a speed of 13000 rpm for 25 sec. The centrifuged samples were immediately measured or frozen.

The OA content of root tips was determined by the modified method of de la Fuente et al. (1997). Briefly, after each incubation period, the root tips (10-mm) were excised with a razor blade, washed with dd water, transferred to Eppendorf cups, and fixed immediately in liquid nitrogen. Before thawing, 400 µl of cold 70 % (v/v) ethanol was added to the samples which were then homogenized in a micro-homogenizer (MM200 Retsch, Haan, Germany) for 3 min at 20 shake sec^{-1}. Organic acids were extracted at 75 °C for 1 h with intermittent shaking in a vortex every 15 minutes. Thereafter, the samples were centrifuged at 15000 rpm for 10 min and the supernatant was transferred into a new Eppendorf tube. The supernatant was concentrated to dryness in a rotary vacuum evaporator (RCT 10-22T, Jouan, Saint-Herblain, France). The concentrated residue from each sample was re-dissolved in 200 µl (10 mM) perchloric acid, sonicated for 15 min, transferred to centrifugal Eppendorf filter tubes with a membrane pore size of 0.45 µm, and centrifuged at a speed of 13,000 rpm for 25 sec. The samples were immediately measured or frozen.

The OAs concentrations in the root exudates as well as in the extracts of root tissue were measured by isocratic High Pressure Liquid Chromatography (HPLC, Kroma System 3000, Kontron Instruments, Munich, Germany). The OAs were detected through a 20 µl loop-injector (Auto-sampler 360) of the HPLC, separating different OAs on an Animex HPX-87H (300 x 7.8 mm) column (BioRad, Laboratories, Richmond, California, USA), supplemented with a cation H^+ micro-guard cartridge, using 10 mM perchloric acid as eluant at a flow rate of 0.5 ml per minute, constant temperature of 35 °C (Oven 480), and 74 hPa of atmospheric pressure. Measurements were performed at a wavelength $\lambda = 214$ nm (UV Detector 320). The peak of each OA was identified by the retention time (time window 20 %) of the same OA in a standard solution in 10 mM perchloric acid containing [mM]: 0.08 KNO_3, 0.10 oxalate, 0.05 cis-aconitate, 2 citrate, 0.5 tartrate, 0.10 pyruvate, 5 malate, 0.1 trans-aconitate, 4 succinate, 2 formate and 0.1 fumarate. Organic acid-anion exudation was expressed as nmol per root tip per hour and OA contents of root tissue as µmol per gram fresh weight of root tip.

Enzyme activities in root apices

After pH adaptation (see above), plants were treated with Al (20 µM, pH 4.5) for 0, 4, 8 and 24 h and activities of several enzymes relevant for the OA metabolism were quantified in root apices. Roots of 8 plants per replicate were rinsed with distilled water and 5 mm root tips (primary plus the 4 longest basal roots per plant) were excised using a razor blade, transferred to Eppendorf cups and fixed immediately in liquid nitrogen. Before thawing, 500 µl cold 100 mM Hepes-NaOH buffer (pH 7.5) was added to the samples which were then homogenized in pre-cooled (-18 ^{o}C) cup holders of a micro-homogenizer (MM200 Retsch, Haan, Germany) for 3 min at 20 cycle sec^{-1}. The procedure was repeated after cooling down of the Eppendorf cup containing the homogenate. Thereafter, a second 500 µl Hepes-NaOH was added, the homogenate centrifuged at 20,000 g for 5 min at 4^{o}C, and the supernatant was used to assay the enzyme activities.

Citrate synthase (CS, EC 4.1.3.7) was measured by incubation of 150 µl extract in 1 ml of solution containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.1 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 0.3 mM acetyl CoA, and 0.5 mM oxaloacetate. The CS reaction was initiated by addition of the enzyme extract and measured by following the reduction of acetyl CoA in the presence of DTNB during 3 min at 412 nm according to Srere (1969) and Johnson et al. (1994). For the determination of NADP-isocitrate dehydrogenase (NADP-ICDH, EC 1.1.1.42) 250 µl of extract was incubated in 1 ml of assay solution, composed of 84 mM triethanolaminhydrochloric (pH 7,5), 42 mM NaCl, 4 mM DL-isocitrate, 4 mM $MnSO_4$ and 0.4 mM NADP. The NADP-ICDH reaction started with the addition of the enzyme extract and the activity recorded as the rate of reduction of NADP, monitored at 340 nm for 3 min according to Bernt and Bergmeyer (1974). NAD-malate dehydrogenase (NAD-MDH, EC 1.1.1.37) activities were measured by monitoring the oxidation of NADH at 340 nm for 2 min, following the method of Macnicol and Jacobsen (1992). The reaction started by adding 100 µl of a ten-fold diluted extract in 1 ml of assay solution containing 50 mM Hepes-NaOH (pH 7.5), 0.5 mM EDTA, 0.2 mM NADH, and 1 mM oxaloacetate. The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was measured by incubating 200 µl extract in 1 ml of assay solution containing 100 mM bicine (pH 8.5), 0.2 mM NADH, 5 mM $MgCl_2$, 1 mM glucose-6-phosphate, 2 mM phosphoenolpyruvate (PEP), 10 mM $NaHCO_3$ and 2 U malate dehydrogenase. The reaction was initiated by the addition of the enzyme extract, and the NADH oxidation was followed by monitoring the change rate of the absorbance at 340 nm for 2 min, following the method of Hatch and Oliver (1978) and Macnicol and Jacobsen (1992). ATP-phosphofructokinase (ATP-PFK, EC 2.7.1.11) was assayed in 1 ml reaction mixture containing 100 mM Hepes-NaOH, pH 8.0, 2.5 mM $MgCl_2$, 0.10 mM NADH, 10 mM Fru-6-P, 6 units ml^{-1} aldolase, 1 unit ml^{-1} triose-P isomerase, 6 units ml^{-1} α-glycerol-P dehydrogenase, and 0.5 mM ATP. The reaction was initiated with the addition of 180 µl of the enzyme extract. Reaction progress was monitored at 340 nm for 3 min, following the method of Carnal and Black (1983). All previously described enzymes were assayed in a spectrophotometer (UVICOM 943, Kontron Instruments, Munich, Germany) at room temperature (24 ^{o}C).

The total buffer (Hepes-NaOH)-extractable protein content of the root apices was determined in a Microplate Spectrophotometer (µQuant, Bio-Tek Instruments Inc, Winooski, Vermont, USA) after Bradford (1976). The specific enzyme activities were expressed in nmol of the monitored reaction product per min per mg protein.

Statistical Analysis

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

Results

The inhibition of root elongation by Al reached about 70 % in both genotypes after 4 h (Fig. 17A). Whereas Al-resistant genotype Quimbaya completely recovered after 24 h, the Al-sensitive genotype VAX-1 recovered only to 40 % after 8 h and then got increasingly damaged up to 60 % again after 24 h Al treatment. The Al contents of the 5 mm root apex (Fig. 17B) reflected the inhibition of root elongation induced by Al. Enhanced inhibition of root elongation up to 4 h of Al treatment was related to increasing Al contents in the root tips even more in genotype Quimbaya than in VAX-1. Recovery from Al stress was accompanied by continued decreasing Al contents of the root apices of Quimbaya while in VAX-1 the Al contents started to increase again after 8 h.

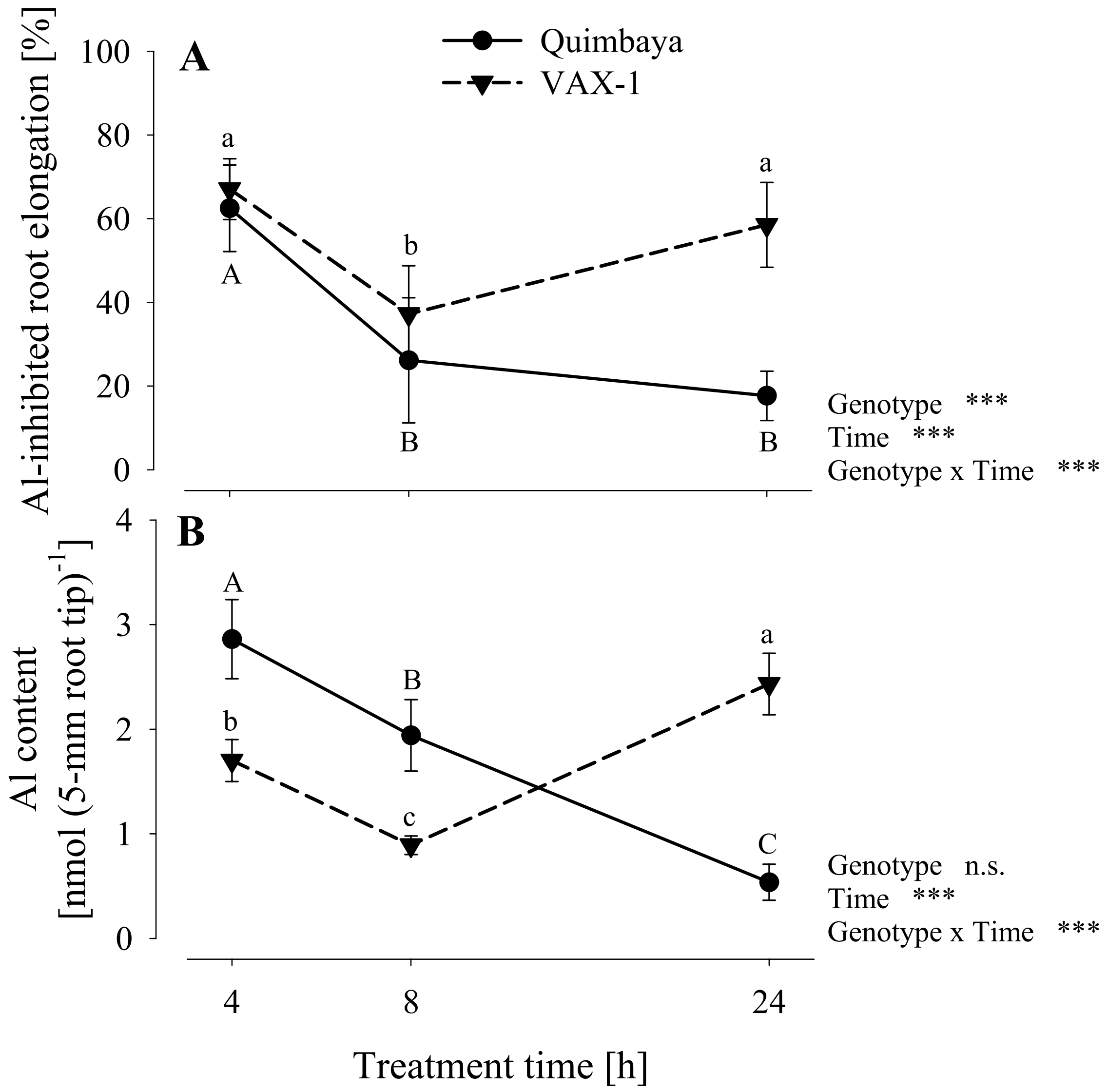
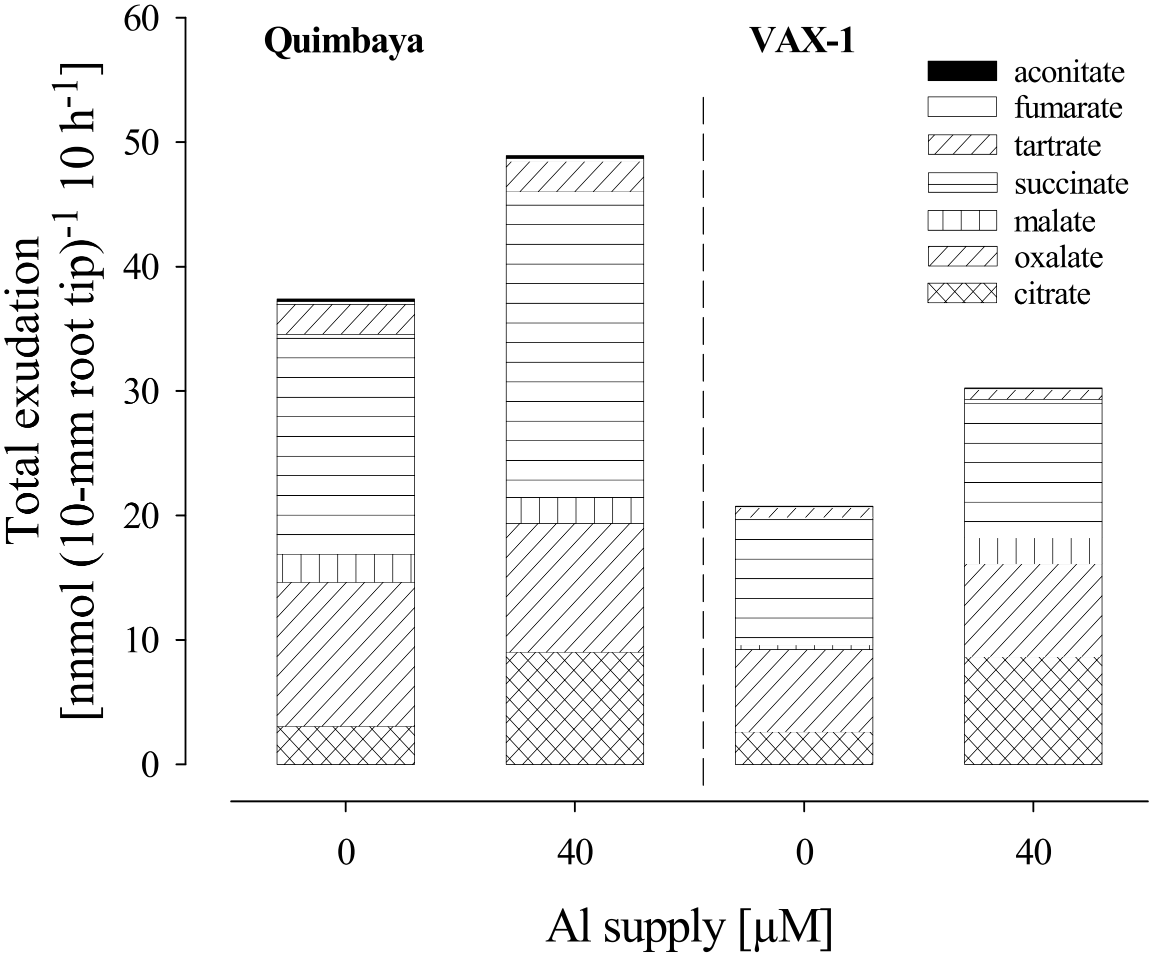


Figure 17. Effect of Al supply (20 µM) on root elongation (A) and Al contents in the root tips (B) of the common bean genotypes Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) grown in a simplified nutrient solution containing 0.5 mM $CaCl_2$, 0.5 mM KCl and 8 µM H_3BO_3 for up to 24 h, pH 4.5. Symbols represent means ± SD of eight replicates (root growth) or four replicates (Al content). For the ANOVA *** denotes a level of significance at $p < 0.001$; n.s. = not significant. Means with the same letter are not significantly different between treatment times for Quimbaya (capital) and VAX-1 (small), Tukey test $p < 0.05$.

Since an Al-induced release of OA-anions has been implicated in Al resistance of common bean and other plant species (see introduction), the cumulated OA-anion exudation from 10 mm root tips treated with Al for 10 h was characterized in a first approach (Fig. 18). The identified OA-anions in the root exudates and their relative abundance independently of genotype and Al treatment were succinate > citrate ≈ oxalate > malate ≈ tartrate > fumarate > aconitate. The total OA-anion exudation was greater in Quimbaya than in VAX-1 with and without Al supply. Aluminium treatment did not modify the exudation of tartrate, fumarate or acconitate, but enhanced the exudation of citrate in both genotypes, oxalate and malate in VAX-1, and succinate in Quimbaya, and reduced oxalate exudation in Quimbaya.



Source ANOVA	citrate	oxalate	malate	succinate	tartrate	fumarate	aconitate	total
Genotype	*	***	***	***	***	***	***	***
Treatment	***	n.s	***	***	n.s	n.s	n.s	***
Genotype x Treatment	n.s	***	***	***	n.s	n.s	n.s	n.s

Figure 18. Cumulated organic acid-anion exudation from 10 mm root tips of two common bean genotypes grown for 10 h in a simplified collection solution containing 5 mM $CaCl_2$ and 8 µM H_3BO_3 at 0 or 40 µM Al supply, pH 4.5. Values for every organic acid-anion represent means of four replicates. For the ANOVA *, *** denote levels of significance at $p < 0.05$ and 0.001. n.s. = not significant.

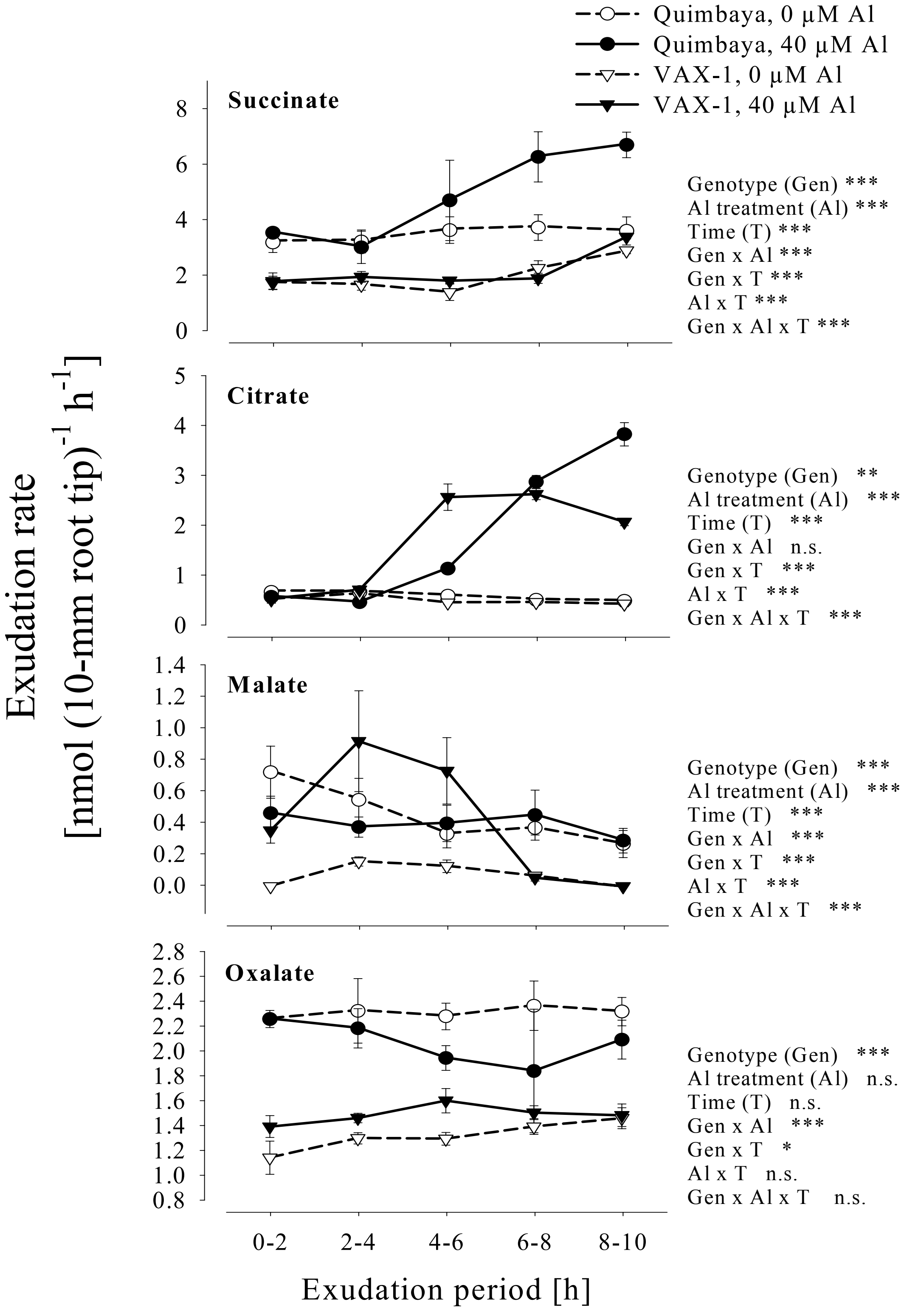


Figure 19. Kinetics of the exudation of succinate, citrate, malate and oxalate from the 10 mm root tips of two common bean genotypes grown over a 10 h period in simplified nutrient solution containing 5 mM $CaCl_2$ and 8 µM H_3BO_3 at 0 (—) or 40 µM Al (----), pH 4.5. Root exudates were collected over 2 h periods. Bars represent ± SD of four replicates. For the ANOVA *, **, *** denote levels of significance at $p < 0.05$, 0.01 and 0.001. n.s. = not significant.

The kinetics of the exudation of these OA-anions showed (Fig. 19) that the Al-enhanced release of succinate (Quimbaya only) and citrate (Quimbaya and VAX-1) was characterized by a lag phase of 4-6 h. The exudation rates of malate and oxalate were stimulated by Al already during the 0-2 h Al treatment in genotype VAX-1 whereas in genotype Quimbaya they were even decreased. However, genotype VAX-1 could not maintain this Al-enhanced exudation of OA-anions beyond 6-8 h Al treatment.

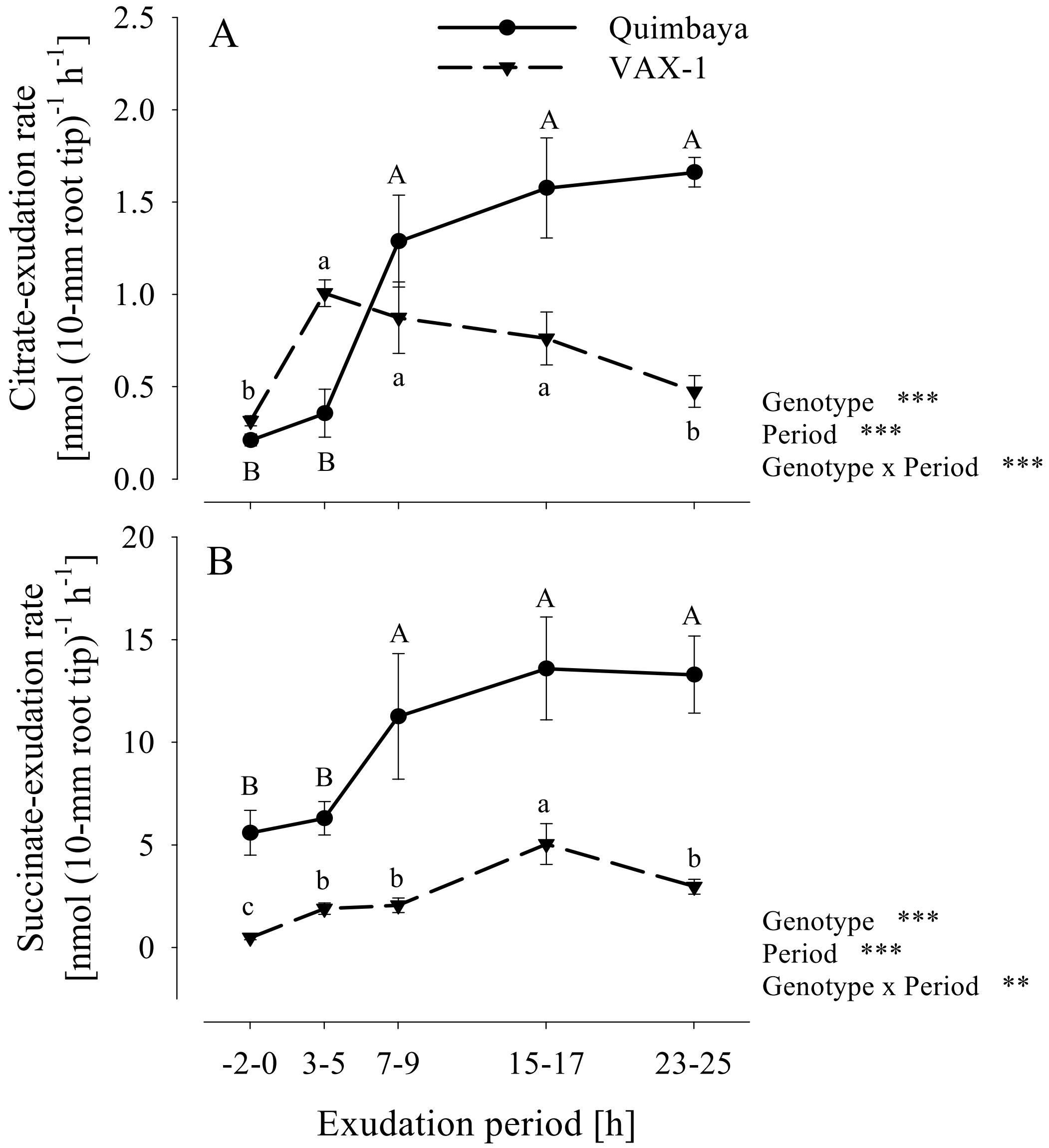


Figure 20. Citrate (A) and succinate (B) exudation-rate of 10 mm root tips of two common bean genotypes precultured in simplified nutrient solution for 0 or 3, 7, 15 or 23 h at 20 µM Al, pH 4.5. Thereafter root exudates were collected for 2 h in simplified collection solution at 0 (-2-0) or 40 µM Al supply, pH 4.5. Bars represent ± SD of four replicates. Means with the same letter are not significantly different between exudation periods for Quimbaya (capital) and VAX-1 (small), Tukey test p <0.05. **, *** denote probability levels at p < 0.01 and 0.001.

In order to be able to monitor the exudation of OA-anions from growing root tips for up to 24 h, the experimental system had to be changed. Therefore, intact plants were treated with Al for 0, 3, 7, 15 or 23 h prior to the collection of the root exudates for a 2 h period (Fig. 20). In agreement with figure 19, citrate exudation (Fig. 20A) was induced by Al treatment after a lag phase of 3-5 h in genotype VAX-1, whereas in genotype Quimbaya the lag phase lasted 7-9 h. By then the exudation rate already declined in genotype VAX-1. In contrast, in genotype Quimbaya the citrate exudation continued to increase up to 23-25 h of Al treatment (highly significant genotype x time interaction, $p > 0.0001$). Partially in agreement with the observation in figure 19, succinate exudation was enhanced after a 3-5 h lag period (Fig. 20B) not only in Quimbaya, but also in VAX-1. However, this appeared to a lesser extend and decreased to a lower level thereafter.

Aluminium-stimulated exudation of OA-anions could be related to the OA contents of the root tips. Therefore, the OA contents of the 10 mm root tips were determined after the collection of root tip exudates for 2 h in order to characterize the influence of Al treatment duration on the dynamics of the OA content (Fig. 21). Eight OAs could be quantified with succinate >> malate > tartrate > citrate ≈ oxalate > pyruvate > aconitate ≈ fumarate. With the exception of malate, tartrate and oxalate genotype Quimbaya had constitutively higher OA contents than VAX-1. Aluminium treatment generally reduced the OA contents in the Al-sensitive genotype (VAX-1) during the period of maximum root growth inhibition (4 h) and thereafter in both genotypes, reaching a minimum after 9 h of Al treatment. Then, the OA contents recovered reaching the original contents (no Al treatment) in Quimbaya after 25 h. In genotype VAX-1 this recovery was only transient and less obvious before the contents reached very low levels after 25 h Al treatment. This general pattern of response of OAs contents to Al treatment was particularly expressed for citrate, malate, oxalate and fumarate.

Differences between the genotypes in the constitutive contents of OAs and their changes induced by Al treatment could be due to differences in the activities of enzymes involved in their synthesis and degradation. Since the enzyme activities per unit of root weight depends on the protein content of the sample, the root-tip protein-concentrations were determined (Fig. 22). The protein contents were consistently up to 2 times higher in genotype Quimbaya than in VAX-1. Aluminium-treatment for 4 h enhanced the protein contents particularly in genotype VAX-1. Subsequently, total protein contents returned to the control levels without Al in both genotypes.

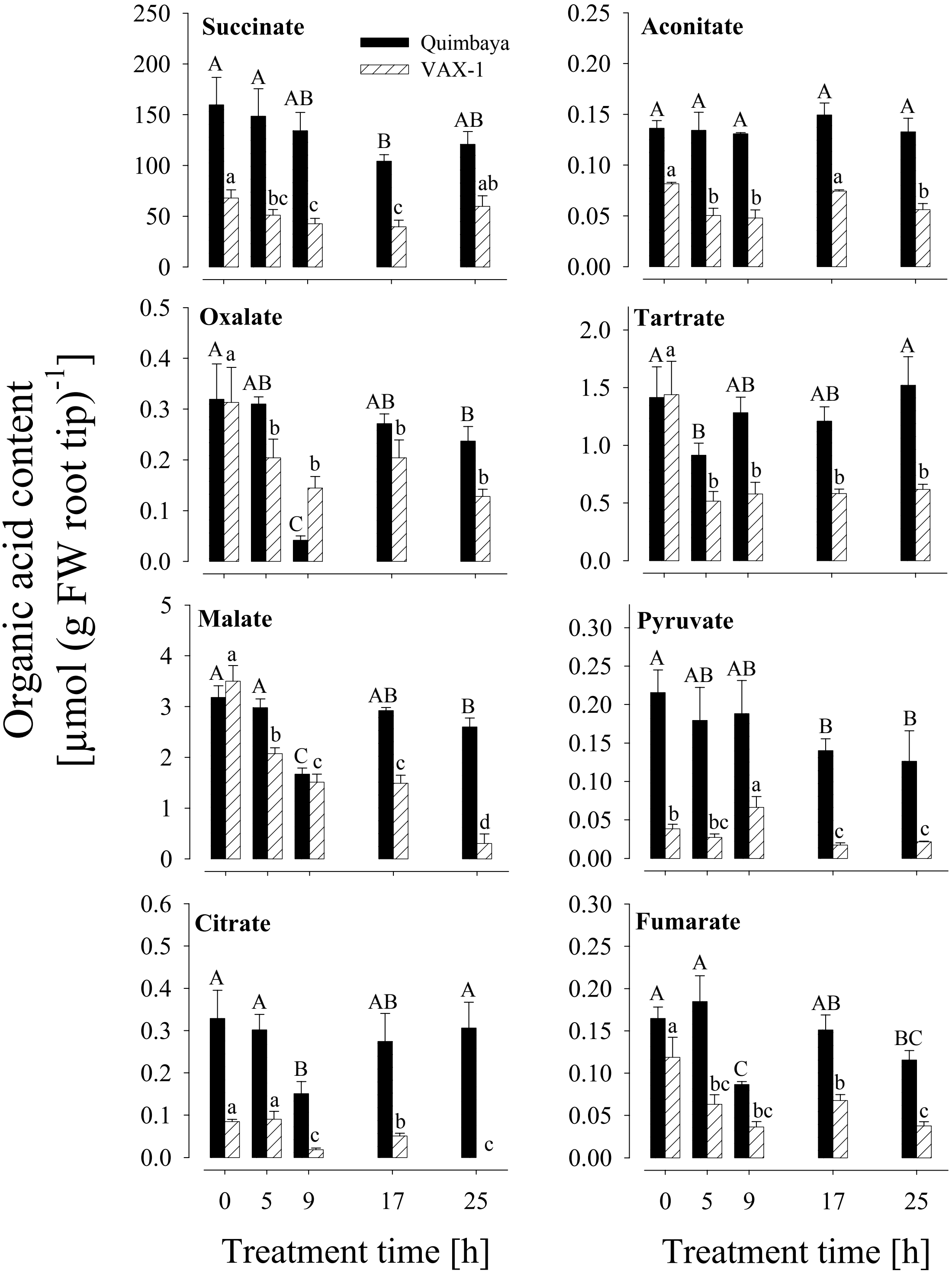


Figure 21. Dynamics of the contents of succinate, oxalate, malate, citrate, aconitate, tartrate, pyruvate and fumarate in 10 mm root tips of two common bean genotypes precultured in simplified nutrient solution for 0 or 3, 7, 15 or 23 h at 20µM Al, pH 4.5. Organic acid contents were measured after subsequent collection of the root exudates for 2 h in simplified collection solution at 0 or 40 µM Al supply, pH 4.5. Bars represent means ± SD of four replicates. Means with the same letter are not significantly different between treatment times for Quimbaya (capital) and VAX-1 (small), Tukey test $p < 0.05$.

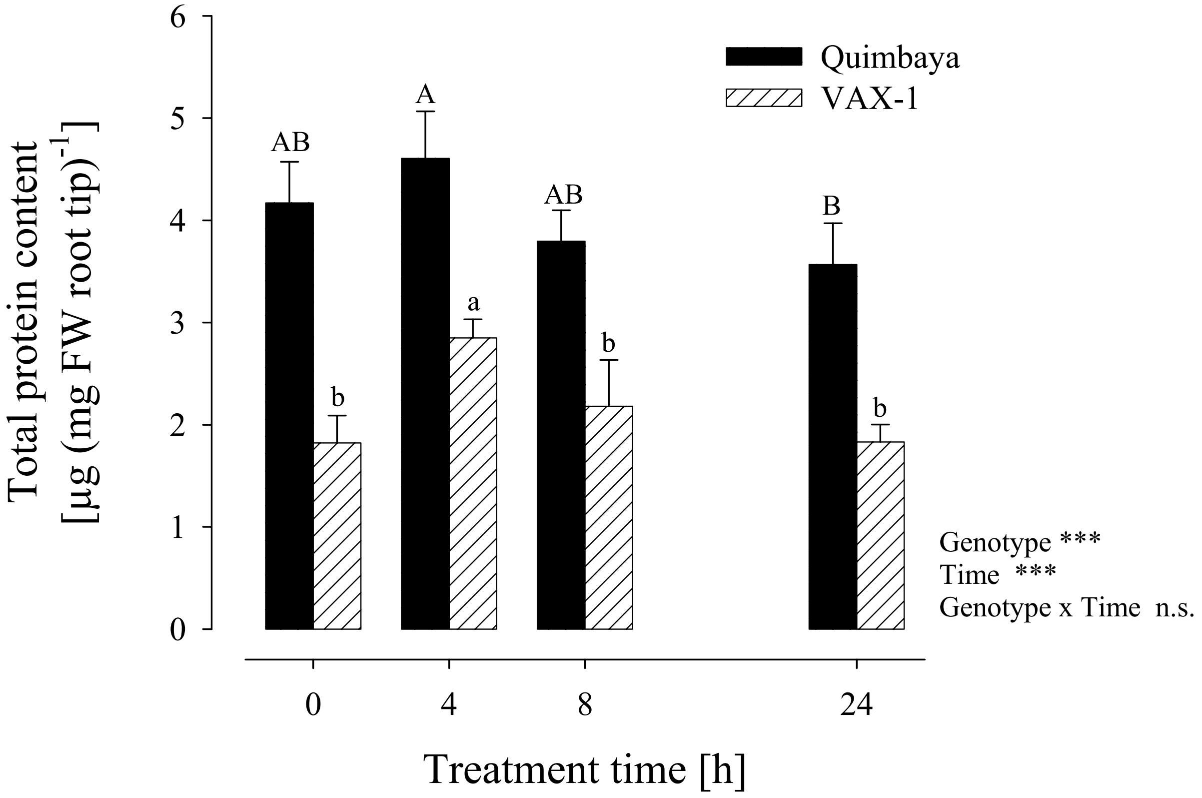


Figure 22. Effect of Al on buffer (Hepes-NaOH)-extractable protein content from 5-mm root tips of two common bean genotypes grown up to 24 h in simplified nutrient solution at 20 µM Al, pH 4.5. Bars represent means ± SD of four replicates. *** denote probability level at $p <$ 0.001; n.s = not significant. Means with the same letter are not significantly different between treatment times for Quimbaya (capital) and VAX-1 (small), Tukey test $p < 0.05$.

Since the exudation of OA anions (Figs. 19 and 20) and the OA contents (Fig. 21) clearly showed differences between the genotypes and the Al treatment duration, we determined activities related to AO synthesis and degradation (Fig. 23). In root apices not treated with Al, genotype Quimbaya had significantly higher specific activities of CS (4-fold) and PEPC (1.6-fold) than VAX-1. The activities of NADP-ICDH, NAD-MDH or ATP-PFK did not differ between the genotypes. Aluminium treatment for 4 h drastically reduced the activity of NADP-ICDH in both genotypes and remained at the low level up to 24 h Al treatment. Only in genotype Quimbaya, the activities of CS, NAD-MDH, PEPC and ATP-PFK were also reduced by Al treatment, but they recovered after a longer Al treatment. In genotype VAX-1, CS activity remained constant at a low level and was even further reduced after 8 h Al treatment. In contrast, NAD-MDH, ATP-PFK activities were consistently and PEPC after a short depression (< 4 h) enhanced by Al treatment.

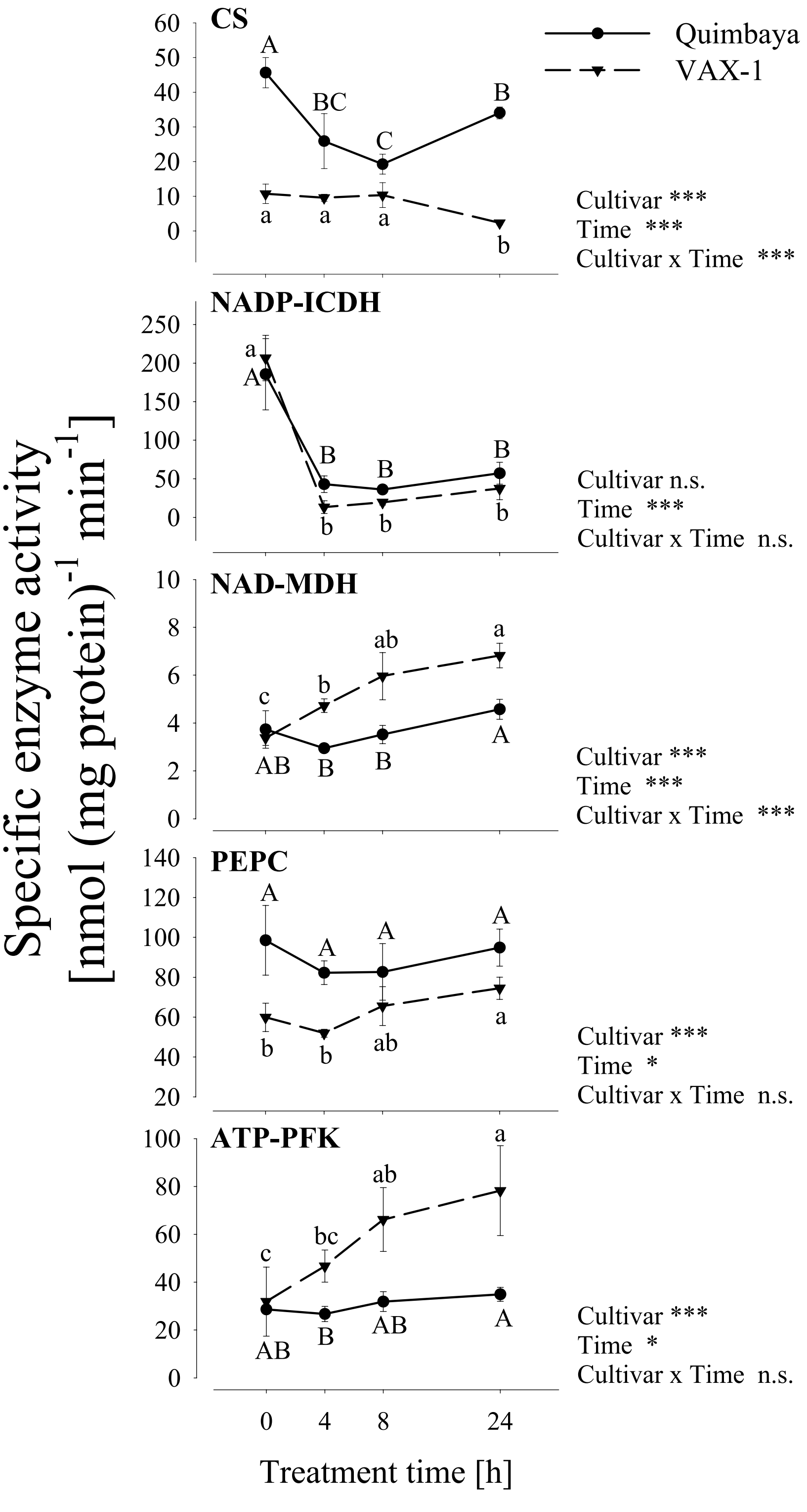


Figure 23. Effect of Al on the specific activities of citrate synthase (CS), NADP-isocitrate dehydrogenase (NADP-ICDH), NAD-malate dehydrogenase (NAD-MDH), Phosphoenolpyruvate carboxylase (PEPC) and ATP-phosphofructokinase (ATP-PFK) from 5 mm root tips of two common bean genotypes grown up to 24 h in simplified nutrient solution at 20 µM Al, pH 4.5. Bars represent ± SD of four replicates. *, *** denote probability level at $p < 0.05$ and 0.001; n.s = not significant. Means with the same letter are not significantly different between treatment times for Quimbaya (capital) and VAX-1 (small), Tukey test $p < 0.05$.

Discussion

Aluminium binds readily to binding sites in the apoplast and the plasma membrane (Blamey et al., 1990; Horst, 1995) especially in the most Al-sensitive sites of the root apex leading to enhanced transport of Al to the symplast (Taylor et al., 2000) and/or to impairment of root growth and functions (for recent reviews see, Kochian et al., 2004, 2005; Horst et al., 2007). Therefore, it has been hypothesized that reduced binding of Al in the apoplast, specifically to the stabile-bound cell-wall Al fraction in common bean (Chapter 2), is a prerequisite for Al resistance (Horst, 1995; Rengel, 1996; Blamey, 2001). Screening techniques for Al resistance have been largely developed and applied based on measurements of Al-induced inhibition of root elongation. The effect of Al treatment in the dynamic of root elongation in both genotypes (Fig. 17A) was in agreement with our previous results (Chapter 1 and 2) and with an Al-induced Al resistance in common bean (Cumming et al., 1992). Also, the results (Fig. 17B) corroborated results showing that changes in root elongation are closely related with modifications in the Al contents of the 5 mm root tip (Chapter 1 and 2). According to these results, with two genotypes differing in Al resistance, three clear phases of the Al response could be observed: (i) a genotype-independent short-term (<4 h) Al injury period, characterized by an enhanced accumulation of Al in the root tip mainly determined by cell-wall characteristics such as pectin content and its degree of methylation (Chapter 2); (ii) a genotypic-independent recovery of root elongation with a concomitant reduction of Al accumulation in the root tip; and (iii) either a constant decrease of Al contents leading to a steady recovery of root growth in the Al-resistant genotype (Quimbaya) or a resumption of Al accumulation leading to severe inhibition of root growth in the Al-sensitive genotype (VAX-1). These three phases can also be observed when Al-inhibited root elongation is calculated from figure 1B in Mugai et al. (2000) and in the study of Stass et al. (2007) working with the medium Al-resistant common bean genotype (Rangel et al., 2005). Collectively, these results suggest an Al-exclusion mechanism acting in common bean after the initial inhibition of root elongation. Constitutively, higher total OA-anions exudation (Fig. 18) and OA-contents (Fig. 21) were observed in Quimbaya (Al-resistant) compared to VAX-1 (Al-sensitive). This is possibly a result of differences in the gene pools of different origin (Gepts et al., 1986; Singh, 1989), as well as better constitutively biosynthetic machinery triggered by potentially greater CS and PEPC activities (Fig. 23). Accordingly, White and Gonzales (1990) found that large-seeded Andean genotypes (i.e. Quimbaya) possess larger cell volumes in different tissues compared to the small-seeded Mesoamerican (i.e. VAX-1). In agreement to this, the better capacity to mobilize P from Al- and Fe-bound phosphates of large-seeded Andean genotypes

(G19833) than small-seeded Mesoamerican genotypes (DOR364) has been related to its greater capability to exude OA-anions, especially citrate and tartrate (Shen et al., 2002b). Constitutively higher OA-contents of the Al-resistant Dade compared to the Al-sensitive Romano common bean cultivars appeared to contribute to a higher potential for Al-chelation and detoxification (Lee and Foy, 1986; Miyasaka et al., 1991). Aluminium treatment increased the cumulative total OA-anions secreted to the same extends in both genotypes (Fig. 18). However, with the exception of citrate, the highly significant genotype x Al treatment interaction indicated that the pattern of exudation in both genotypes was differentially expressed especially for succinate, malate and oxalate. Many plant species and cultivars respond to Al stress by secreting specific OA-anions from the roots (Ma et al., 2001). However, the response differs with plant species, as well as OA species, secretion patterns, temperature sensitivity and dose response (Ma and Furukawa, 2003). The observed Al-induced secretion of malate and oxalate in VAX-1 (Fig. 19) was in agreement with the reduction of oxalate and malate contents after 5 h of Al treatment (Fig. 21). However, this exudation did not measurably contribute to Al resistance because it was expressed during the time of maximum inhibition of root elongation (< 4 h, Fig. 17A) and was not sustained over time. In soybean, Al treatment also stimulated a transient secretion of malate during the first 6 h of root exposure to 1.4 µM Al, and sharply decreased thereafter without any contribution to Al-resistance (Silva et al., 2001). In the Al-sensitive ruzigrass (*Brachiaria ruziziensis* Germain and Evrard cv Common) 13 days of Al treatment enhanced the exudation of oxalate, citrate and malate to a greater extend than in the Al-resistant signalgrass (*Brachiaria decumbens* Stapf cv Basilik) also without any contribution to Al resistance (Wenzl et al., 2001).

Mainly in Quimbaya succinate was exuded in parallel, following the same exudation pattern as citrate (Figs. 19 and 20). Succinate exudation in Al-resistant cultivars in response to Al has been also reported in wheat (Delhaize et al., 1993b; Christiansen-Weniger et al., 1992), barley (Guo et al., 2007), maize (Gaume et al., 2001) and alfalfa (Tesfaye et al., 2001). In all cases, succinate was exuded in parallel to malate (wheat) or malate and citrate (barley, maize and alfalfa). Similarly, succinate exudation in parallel to citrate has been reported in radish (*Raghanus sativus* L.), rape (*Brassica napus* L.), potato (*Solanum tuberosum* L.) and cabbage (*Brassica oleraceae* L.) under low P conditions (Zhang et al., 1997; Dechassa and Schenk, 2004). Additionally, succinate reducing translocation of Mn or Zn to the shoots has been involved indirectly in Mn resistance (Doncheva et al., 2005) and directly in Zn resistance in pea (*Pisum sativum* L., Doncheva et al., 2001). Aluminium toxicity is associated with peroxidative damage of membrane lipids due to the stress-related increase in the production of ROS in soybean (Cakmak and Horst, 1991) and sorghum (*Sorghum bicolor* Moench., Peixoto et al., 2001). Although, Al-induced lipid peroxidation does not occur rapid enough to be an initial mechanism of Al toxicity (Horst et al., 1992; Yamamoto et al., 2001), Al-induced ROS generation and associated mitochondrial dysfunction could still play a more general role in Al inhibition of root growth (Yamamoto et al., 2002). Succinate has been proven to reduce organic hydroperoxides in mitochondria inhibiting the formation of lipid hydroperoxides. Similarly it could act by preventing or removing some lipid hydroperoxides once they are formed, involving the electron transport-linked reduction of endogenous ubiquinone (Bindoli et al., 1982; Pai and Gaur, 1988).

Considering the Al-detoxifying capacity of different OA anions, it is known that succinate will not contribute directly to Al resistance (Hue et al., 1986); hence citrate exudation offers the most consistent relationship to the effects observed in the root elongation of both genotypes during the Al treatment (Fig. 17A). Aluminium-enhanced citrate exudation has been previously implicated in Al resistance of common bean (Miyasaka et al., 1991; Mugai et al., 2000; Shen et al., 2002a, Rangel and Horst, 2006; Stass et al., 2007). The citrate exudation pattern observed in both common bean genotypes was classified into Pattern II with a 4-5 h lag phase, which corresponds to the maximum period of root-growth inhibition (Fig. 17A), prior to the increased exudation of citrate in both genotypes during the recovery period (Figs. 19 and 20). Thereafter, citrate exudation was enhanced in the Al-resistant genotype Quimbaya, while it was constantly reduced in the Al-sensitive genotype VAX-1. Similar patterns of exudation have been reported in soybean, where citrate secretion in an Al-resistant genotype begins after 6 h of Al treatment (Yang et al., 2001); in rye, malate and citrate exudation were delayed for 6 and 10 h, respectively (Li et al., 2000); and in *Cassia tora*, the secretion of citrate in response to Al was increased after a 4 h lag phase (Ma et al., 1997). Ambiguous data has been reported regarding the pattern of citrate exudation in common bean. Even though not described, the results presented from Mugai et al. (2000) and Shen et al. (2002) also suggested a Pattern II response of citrate secretion with 6 h and 3-6 h lag phases, respectively. However, Al induced rapid exudation of citrate from excised tap and/or basal 5 mm root tips, suggesting a Pattern-I type, have also been demonstrated (Shen et al., 2004). Independently, all above mentioned groups have agreed that citrate exudation in response to Al treatment increases over the exposure time, a common characteristic of Pattern-II plants (Ma, 2000; Ma et al., 2001), which is consistent with the induction of Al tolerance genes (transport and/or OA synthesis) contributing to this increased capacity (Kochian et al., 2004).

Recently, Fontecha and coworkers (2007) detected the putative Aluminium-Activated-Malate-Transporter (*ALMT1*) sequence from wheat (Sasaki et al., 2004) in eight grass genomes and *Phaseolus vulgaris*, showing at least 72 % similarity to the wheat sequences. Similarly, a gene (*HvAACT1*) responsible for the Al-activated citrate secretion in barley (*Hordeum vulgare* L.) belonging to the multidrug and toxic compound extrusion family (MATE) was identified (Furukawa et al., 2007). The importance of anion channels facilitating the efflux of OA in Al resistance has been well documented (Ryan et al., 1995; Ryan et al., 1997; Piñeros and Kochian, 2001; Kollmeier et al., 2001; Delhaize et al., 2004; Sasaki et al., 2004; Delhaize et al., 2007). Independently of the internal citrate contents, which are constitutively higher in Quimbaya than in VAX-1 (Fig. 21), both genotypes were equally sensitive during the first 4 h of Al treatment (Fig. 17A). Thus, recovery from the initial Al injury depends on the capacity to release citrate (Figs. 19 and 20A) after a lag phase mediated by anion channels. Shen et al. (2004) demonstrated the existence of a plasma membrane anion channel mediating Al-enhanced citrate exudation that was inhibited by the action of anthracene-9-carboxylic acid and K-252a, a broad range inhibitor of protein kinases. However, the dynamics of citrate exudation after the transient root recovery period (> 9 h) differs significantly between genotypes suggesting that internal OA-contents, which in turn are determined by OA biosynthesis, might also influence citrate secretion (Fig. 20A).

Opposite to the Pattern I of secretion where it has been demonstrated that the release of OA-anions (i.e. malate in wheat or oxalate in buckwheat) neither depends on internal OA-contents nor gene induction (Delhaize et al., 1993b; Yang et al., 2006), but on the activation of the plasma membrane anion-channel *ALMT1* (Delhaize et al., 2007). In Pattern-II plants, the influence of internal OA-contents (Lee and Foy, 1986; Silva et al., 2001), gene induction related to metabolism (biosynthesis and catalysis) of OA (Li et al., 2000; Yang et al., 2004), activation of anion channels (plasma membrane and/or tonoplast) or transport of OAs from the mitochondria in the release of OA-anions has been observed (Ma et al., 2000; Ma and Furukawa, 2003; Anoop et al., 2003; Kochian et al., 2004; Yang et al., 2006; Fontecha et al., 2007). Aluminium treatment generally reduced the OA-contents (especially citrate, malate, oxalate and fumarate) in both genotypes, more in VAX-1 than in Quimbaya, reaching a minimum after 9 h of Al treatment (Fig. 21). Thereafter, the OA-contents in Quimbaya recovered nearly to the control levels without Al, while it remained low in VAX-1. These results are in agreement with earlier work on the effect of Al on common bean, in which long-term Al treatment (> 3 d) generally reduced the OA-contents (especially citrate and malate), more in the Al-sensitive than in the Al-resistant genotype (Lee and Foy, 1986). In soybean, short-term Al treatment (3 h) rapidly dropped the malate but not the citrate contents of the root apex (Yang et al. 2001), while long-term Al treatment (72 h) enhanced the accumulation of both (citrate and malate) in the root tips of Al-resistant genotypes (Silva et al., 2001). By contrast in triticale, a Pattern-II plant characterized by a 6 to 12 h lag phase prior to the exudation of malate and citrate, respectively (Ma et al., 2000), the levels of citrate (root tips and mature root segments) or malate (mature segments only) in roots increased during the exposure to Al in both Al-resistant and Al-sensitive genotypes (Hayes and Ma, 2003).

After the lag period (4h), citrate exudation was observed to be high in VAX-1 but low in Quimbaya (Fig. 20A), whereas the concentration of citrate in the root tips were unmodified at that time (Fig. 21). Both genotypes were able to overcome the initial Al injury (Fig. 17A). However, it appears that in Quimbaya, high internal OA-contents, especially those of citrate (constitutively higher), can be maintained by lowering citrate metabolism (accumulation of OA), while VAX-1 is enhancing its citrate production at expenses of internal OA-contents (constitutively low). Later on, the drastic reduction of OA-contents (30 and 50 % of citrate pool in VAX-1 and Quimbaya, respectively), which corresponded with the period of transient recovery of root growth (Fig. 17A), indicated that the enhanced or steady citrate efflux in Quimbaya and VAX-1, respectively, was influenced not only by their internal citrate contents but by their citrate-synthesis capacity, leading to exhausted internal citrate pools, especially in VAX-1. Complete recovery of root growth in Quimbaya was related to restoring OA-contents resulting in an enhanced citrate exudation. Conversely, the enhanced root-growth inhibition in VAX-1 was related to depletion of internal OA contents in general, leading to a reduced capacity to exude citrate (Fig. 20A).

Evidence of internal complexation and detoxification of Al has come from research focusing of Al includers plants (Ma et al., 2001; Kochian et al., 2004). Aluminium in the leaves of hortensia (*Hydrangea macrophylla* L.) exists primarily as Al-citrate complex (Ma et al., 1997). In buckwheat, Al is complexed in both roots and leaves in the form of Al oxalate. Additionally, buckwheat is able to immobilize and detoxified Al by accumulating P in the roots (Zheng et al., 2005). Aluminium resistance in common sorrel (*Rumex acetosa* L.), a Pattern-II plant specie, implies both exclusion of Al and tolerance of high Al concentration in the roots mediated by citrate exudation and increased accumulation of citrate and anthraquinone (Tolra et al., 2005). Our previous results have shown that Quimbaya accumulated higher amounts Al in the symplast than VAX-1 (Chapter 2). Moreover, in both genotypes the absolute and relative Al contents in the symplastic fraction increased during the recovery period. Thus higher OA-contents might contribute to detoxify this Al. Internal detoxification of Al was shown to involve Al chelation in the cytosol and subsequent storage of the Al-carboxylate complex in the vacuoles (Kochian et al., 2004). Internalization of Al into vacuolar compartments during recovery of root growth has been observed in maize (Vázquez et al., 1999) and *Arabidopsis thaliana* (Illes et al., 2006).

In the citric acid cycle (TCA), succinate is oxidized to fumarate by succinate dehydrogenase, an enzyme located to the inner mitochondrial membrane. It is the only enzyme that participates in both the TCA and the mitochondrial electron-transport chain. Therefore, inhibition of this enzyme or preventing the formation of succinate or the oxidation to fumarate may directly represent a reduced cell respiration (Stare and Bauman, 1936; Stryer, 1988). Aluminium treatment reduced the contents of succinate and fumarate in both genotypes, more in VAX-1 than in Quimbaya (Fig. 21). While both OA remained at low concentrations in VAX-1, they were increased nearly up to the control levels in Quimbaya. Therefore, it appears that Al treatment reduced root respiration in both genotypes, remaining lower in VAX-1 and recovered in Quimbaya thereafter.

Reduced respiration rates are normal during stress periods, although this might not imply a reduction in the concentration of ATP suggesting a lower ATP demand under conditions of root inhibition (Yan et al., 1992). Recently, Ligaba et al. (2004) observed that Al treatment reduced the rates of respiration in canola favoring the internal accumulation of citrate and malate. Similarly, Cumming and coworkers (1992) observed that the Al-induced resistance in common bean was related to increased rates of respiration after 72 h of Al treatment. After partitioning into growth and maintenance expenditures, they observed that a large portion of the respiration was allocated to maintenance processes in the Al-resistant cultivar (Dade), potentially reflecting diversion of energy to metabolic pathways. Contrary, Al injury was reflected in reduced rates of both growth and maintenance of respiration in the Al-sensitive cultivar (Romano). Interestingly, after 8 h of Al treatment the rates of growth respiration were significantly reduced in both cultivars, while the maintenance respiration remained unaffected (Cumming et al., 1992). Using Triphenyl-tetrazolium-chloride (TTC), an indirect indicator of respiration and cell viability, Mugai and coworkers (2000) suggested that the secretion of citrate upon Al stress in common bean was related to increased mitochondrial respiratory activity in the Al-resistant genotype.

Organic acids play an important role in cell metabolism (energy production, cation charge balance or osmotic potential regulators), hence, their synthesis and concentrations tends to be strictly regulated (Ryan et al., 2001). Therefore, the activities of several enzymes involved in synthesis and turnover of citrate were examined. The evidence that increased CS activity is associated with an enhanced capacity to accumulate citrate leading also to a greater efflux of citrate from roots is supported by a range of results reported in the literature. Enhanced CS activity under low or moderate P supply has been demonstrated in lupin (Johnson et al., 1994, 1996) and mutant carrot cells (*Daucus carota* L., Takita et al., 1999; Koyama et al., 1999; Kihara et al., 2003a). Citrate synthase activity enhanced by Al treatment has been observed in soybean (Yang et al., 2001) and rye (Li et al., 2000), where the improved CS activity coincides with the beginning of citrate efflux. In contrast, in triticale the observed citrate efflux was not related to *in vitro* enzyme activities in general, but to the transport of citrate out of the cells (Hayes and Ma, 2003). Contrasting responses have been described in *Cassia tora* related to CS activity under Al toxicity. Yang et al. (2004) reported an increase in the CS activity within 3 h of Al treatment prior to the beginning of citrate exudation (6 h). On the other hand, recently Yang et al. (2006) indicated that Al-regulated citrate efflux was not related to CS activity. Aluminium-enhanced citrate exudation related to an apparently higher CS activity has been previous reported in common bean (Mugai et al., 2000). Our results show that Al treatment differentially affected the activity of CS in both genotypes (Fig. 23). In Quimbaya, the constitutively higher activity of CS was significantly decreased during the first 8 h of Al treatment and, thereafter, recovered nearly to the initial levels prior to Al treatment, while in VAX-1 at a lower level the activity of CS was initially unaffected but decreased beyond 8 h of Al treatment. These results suggest that the enhanced capacity to exude citrate observed in VAX -1 after 3-5 h Al treatment lead to exhausted internal citrate pools because the CS activity was insufficient to meet the citrate loss through exudation. In Quimbaya, the increased citrate efflux during the recovery period (7-9 h) could be met by the much greater internal citrate pool (50 % of the citrate pool) even at a decreasing citrate synthesis-rate. The capacity to maintain or even enhance citrate exudation (Quimbaya) (Fig. 20A) required the resumption of CS activity after longer Al treatment duration (Fig. 23).

Since the citric acid cycle (TCA) requires stoichiometric parity between acetyl Co-A and oxaloacetate to proceed, removal of oxaloacetate or any of its precursors disrupts the cycle (Hill, 1997). Anaplerotic “fill up” synthesis of intermediates (i.e. malate) redresses imbalances that may occur as a result of consumption of TCA-cycle intermediates for amino-acid synthesis or other processes (Miller and Cramer, 2005). Oxaloacetate can be replenished via NAD-malic enzyme, glutamate oxidation catalyzed by glutamate dehydrogenase, glyoxylate cycle or via PEPC (Palmieri et al., 1997; Held, 2005). Root forms of PEPC are known to have various anaplerotic functions that include providing the C skeletons for N assimilation, cytoplasmic pH maintenance, and osmolarity regulation (Nisi and Zocchi, 2000).

Aluminium treatment equally affected the activity of PEPC in both genotypes implying a common pathway to replenish C skeletons after the beginning of citrate exudation (Fig. 23). However, the constitutively higher PEPC activity in Quimbaya might suggest a better anaplerotic function than in VAX-1. The activity of NAD-MDH, an enzyme that catalyses the reversible conversion of oxaloacetate to malate was significantly enhanced in VAX-1 in presence of Al, and after a transient reduction was also enhanced in Quimbaya. This suggests that in VAX-1, the metabolic pathway were directed to produce more citrate, while in Quimbaya this takes place after a lag phase.

Carbohydrates are fuel for the energy metabolism and also a C source for the synthesis of cell structures. The glycolysis pathway has a fundamental role in the utilization of carbohydrates (Held, 2005). Phosphofructokinase is the most important control element in the glycolytic pathway, since this enzyme is a direct indicator of the energy charge in the cell (Carnal and Black, 1983; Stryer, 1988). Aluminium treatment increased the activity of ATP-PFK in VAX-1 in a similar pattern as the activity of NAD-MDH (Fig. 23). In Quimbaya the ATP-PFK activity which was initially at the same level as in VAX-1, remained unaffected by the Al treatment. Phosphofructokinase is inhibited by high levels of ATP or by high levels of citrate, which actually enhanced the inhibitory action of ATP (Held, 2005). A higher ATP-PFK activity under Al stress in VAX-1 may reflect an enhanced need of C skeletons through glycolysis in order to cope with the release of OA-anions: malate and oxalate initially (Fig. 19) and citrate thereafter. The lack of response of ATP-PFK activity in Quimbaya in spite of increasing release of citrate may indicate that this genotype is capable to meet the additional C need through its higher PEPC activity.

Increased accumulation of citrate contents in the cytosol can also be mediated by reduced activity of enzymes involved in citrate turnover (Massonneau et al., 2001). Degradation of citrate by aconitase (ACO) and NAD(P)-ICDH which are mainly located in the cytosol may directly influence the cytosolic citrate concentration (Kihara et al., 2003b) and thus release through anion channels in the plasma membrane. Aluminium treatment rapidly reduced the activity of NADP-ICDH (Fig. 23) in both genotypes, suggesting a rapid and constant response to accumulate citrate in the root tips. This is corroborated by the invariability of citrate contents in both genotypes (Fig. 21) just after the lag phase (4-5 h) of citrate efflux. Similar results have been reported in lupin (*Lupinus albus* L., Kania et al., 2003), mutant carrot cells (Takita et al., 1999; Koyama et al., 1999; Kihara et al., 2003a), transformed yeast (*Saccharomyces cerevisiae*; MMYO11) defective in ACO and NADP-ICDH action (Anoop et al., 2003) and transgenic tobacco plants defective in NADP-ICDH activities (Delhaize et al., 2003). With the exception of the last group, the increased internal citrate contents contributed to the observed citrate efflux. Molecular characterization of NADP-ICDH in lupin indicated that the cytosolic isoenzymes functions as a hetero-dimer, and that the activity is apparently regulated by the lower transcript level for this enzyme (Kihara et al., 2003b). In *Cassia tora* Al treatment caused a reduction of ACO instead of NADP-ICDH, also leading to citrate accumulation and efflux (Yang et al., 2004). In maize, reduced levels of aconitate were related to increment of citrate acid, suggesting that Al reduced the activity of ACO (Piñeros et al., 2002). Similarly, Al treatment reduced the aconitate contents in VAX-1 (Fig. 21), implying that the activity of ACO was also reduced by Al, favoring citrate accumulation (Fig. 20A). The unmodified internal aconitate acid contents suggest that the activity of ACO was also unchanged and part of the citrate could be isomerized to isocitrate. Isocitrate is a key branching point between the TCA and glyoxylate cycle in plants. When energy is needed, it is oxydatively decarboxylated to 2-oxoglutarate. At high levels of ATP or possibly low consumption of ATP like those under stress condition (Yan et al., 1992), isocitrate is channeled into the glyoxylate pathway for the formation of biosynthetic intermediates (i.e. succinate, glyoxylate and citrate) and bypassing the oxidative phase of the TCA cycle (Stryer, 1988; Palmieri et al., 1997). In addition to the role of glyoxylate cycle in gluconeogenesis an anaplerotic role (anaplerotic pathway) has also been suggested (Olsen and Harada, 1995; Ismail et al., 1997; Eastmond and Graham, 2001). The anaplerotic pathway involves the combined action of microbodies (i.e. peroxisome or glyoxysome), mitochondria and cytosol (Tolbert, 1981). In a simplified form, isocitrate is transformed to succinate by the action of isocitrate lyase (ICL) and then succinate passes from the microbody into the mitochondria and enters the TCA cycle, where it is converted to malate. This malate is either exported to the cytosol in exchange for succinate to fuel the synthesis of soluble carbohydrates via PEP carboxykinase (gluconeogenesis pathway) or is directly converted to oxaloacetate and, thereafter, to citrate thus completing the cycle (Eastmond and Graham, 2001). Exposure to Al increases the production of glyoxylate-cycle intermediates like tartrate, glycolate, formate and oxalate in *Pisolithus tinctorius*, an Al-resistant ectomycorrhizal fungi, possibly conferring Al resistance to their host trees (Cumming et al., 2001). Apart from the Al-enhanced exudation of succinate, in parallel to citrate (Fig. 20B) and the rapid recovery of the tartrate and oxalate acid contents, after the initial Al injury period (Fig. 21) observed in Quimbaya, our data do not allow us to corroborate the existence of an anaplerotic glyoxylate-pathway. Thus, further research dealing with the presence of ICL and malate synthase in root tips, as those developed by Ismail et al. (1997) might contribute to confirm this assumption.

In conclusion, the Al-induced citrate exudation pattern observed in both common bean genotypes belongs to a Pattern-II type, with a 4-5 h lag phase, which corresponds to the maximum period of root growth inhibition. Initial Al-dependent efflux of citrate from the root tips in both genotypes is not regulated by the internal levels in the roots but requires the activation or expression of an organic anion permease in the plasmalemma and/or the tonoplast. Transient recovery from initial Al stress depends on the capacity to utilize internal citrate pools associated with a reduced cytosolic turnover of citrate. However, sustained recovery depends additionally on the capability to enhance synthesis of OAs thus maintaining the cytosolic OA concentration.

GENERAL DISCUSSION

Knowledge on the mechanisms of Al toxicity can contribute to the development of more accurate, rapid, and simple screening procedures through the characterization of the physiological/molecular basis of Al toxicity and Al resistance. Therefore, the present study focused on three areas, which are considered to particularly contribute to quantify genotypic differences in Al resistance and to develop quick screening techniques for Al resistance in common bean: (i) the temporal and spatial effects of Al on root growth and accumulation of Al in the root apex, (ii) the role of cellular distribution of Al in Al toxicity and resistance, and (iii) the characterization of the role of Al exclusion mediated by the efflux of organic acid anions in the Al resistance of common bean.

The most widely recognized symptom of Al toxicity is inhibition of root elongation (Foy, 1988). Detailed studies on the kinetics of the response of root-growth rates to exposure to Al offer the possibility to differentiate between constitutive or inducible Al-resistance mechanism and to verify whether this is consistent across genotypes (Parker, 1995). In agreement with Cumming and coworkers (1992), our results clearly corroborated that Al resistance in common bean is an Al-inducible trait (chapter 1, 2 and 3). Root elongation of both genotypes was severely inhibited during the first 4 h of Al treatment. Thereafter, both genotypes showed gradual recovery. However, this recovery continued in genotype Quimbaya (Al-resistant) until the root-elongation rate reached the level of the control (without Al), while the genotype VAX-1 (Al-sensitive) was increasingly damaged by Al after 12 h of Al treatment (Figs. 4, 5, 8A and 17A). This dynamic of root elongation in both genotypes was closely reflected by the Al contents of the root apex (Figs. 6, 7, 8B and 17B) and corresponded with the presence of an Al-exclusion mechanism mediated by citrate exudation (Figs. 19 and 20A).

A common Al injury period, “the lag phase”

During the first 4 h of Al treatment, both common bean genotypes did not differ significantly in the initial response of root elongation and were severely inhibited (Figs. 4, 5, 8A and 17A). It is widely acknowledged that root growth in plants is confined to the apical regions along which diverse spatial patterns of growth intensity exist as a result of different gradients of cellular activities (Erickson and Sax, 1956; Gandar, 1983; Pritchard, 1994). In presence of Al, the analysis of spatial growth profiles revealed that the initial inhibition of root elongation resulted from a generalized effect along the entire elongation zone (EZ). This was reflected by a reduced maximal rate of relative elongation, without changing the shape or the length of the entire EZ (Fig. 1). Regarding to Al, similar effects have been observed in other plants such as mungbean (Blamey et al., 2004), or maize (Kollmeier et al., 2000), whereas other factors like P deficiency are known to modify the shape of the EZ (Ma et al., 2003). Localized application of Al to specific zones of the root apex showed that in both genotypes application of Al to the transition zone (TZ) resulted in root-growth inhibition to the same extent as if the whole root tip would have been treated with Al (Figs. 2 and 3). These results confirmed studies with maize, reporting that the TZ is the most Al-sensitive apical root zone (Sivaguru and Horst, 1998; Sivaguru et al., 1999). However, in contrast to maize (Kollmeier et al., 2000) and wheat (Ryan et al., 1993), application of Al to the EZ even up to 8 mm from the root tip (Figs. 2 and 3) also reduced root growth in both common bean genotypes, though to a lesser extent than when applied to the TZ. This discrepancy between plant species suggested that cell wall (CW) characteristics, known to differ between grasses and dicotyledons (Carpita and Gibeaut, 1993), might influence the capacity to accumulate Al (Schmohl and Horst, 2001) resulting in greater binding of Al to the CW in the EZ, thus affecting directly the extensibility of the CW or indirectly by creating mechanical stress which is transferred to the cytoskeleton, leading to a disturbance of the processes that are necessary for cell elongation (Carpita and Gibeaut, 1993; Horst et al., 1999).

The observed Al-induced root inhibition corresponded with an enhanced capacity to accumulate Al in the root tip, determined by CW characteristics (i.e. pectin content) of each genotype, but especially between the 0-2 mm zones of the root apex (Fig. 7), which is in agreement with the high sensitivity of the TZ (see above). Zhang and Taylor (1989, 1990) observed that Al accumulates in roots with a rapid initial phase (accumulation of easily exchangeable Al in the apoplast) and a lower rate thereafter (metabolism-dependent binding of Al into the apoplast and transport of Al into the symplast). In the apoplast, the pectin matrix of the CW and its negative charges, determined by its degree of methylation (DM), represents the primary binding site of Al (Blamey et al., 1990; Schmohl and Horst, 2000) and Al injury (Schmohl et al., 2000, Eticha et al. 2005b, Horst et al., 2007). The pectin content per millimeter root tip generally declined from the tip to the base of the root tip in the first 5 mm of the root (Eticha et al., 2005b; Stass et al., 2005). Therefore, the high Al accumulation in the 0-2 mm root zone coupled with the enhanced Al sensitivity of the TZ could be related to the higher pectin contents of this zone. Additionally, the higher initial Al accumulation of Quimbaya in the 5 mm root apex compared to VAX-1 could be related to its higher content of unmethylated pectin and thus higher negativity of the CW (Fig. 9). Aluminium treatment enhanced the root-tip pectin-content in both genotypes and decreased its DM (Fig. 9), thus enhancing the overall negativity of the CW, an effect commonly observed in other plants under Al stress (Le Van et al., 1994; Chang et al., 1999; Eticha et al., 2005b; Hossain et al., 2006; Stass et al., 2007).

Analysis of the binding stage, compartmentation and localization of Al in the root apices have shown that the strong citrate non-exchangeable binding of Al to the CW which represented quantitatively (80 %) the most important Al fraction (Fig. 6) best reflected the observed Al-induced inhibition of root elongation (Figs. 4, 5, 8A and 17A). In agreement with these results, Taylor et al. (2000) using the model giant algae *Chara corallina* showed that after surgical removal of the CW, more than 99 % of the total Al taken up accumulates in the CW, but it can be also transferred into the symplast. With the exception of the labile-bound CW Al fraction, Quimbaya accumulated significantly more Al in the water free space fluid (WFSF) and symplastic fractions compared to VAX-1 (Fig. 12). Morin, a fluorochrome that forms a fluorescent complex with Al (Browne et al., 1990), is known to most sensitively stain free and loosely bound Al in the apoplast and the symplast, but not the stabile bound Al to the CW (Eticha et al., 2005c). Our Al localization experiments using thin hand-cut sections from the TZ and central elongation zone (CEZ) showed that the Al corresponding to the WFSF, labile-bound and symplastic fractions were mainly and primarily accumulated in the epidermal and outer cortical cells and in consequence, the Al-morin complex was more intense in Quimbaya than in VAX-1 (Fig. 16). Similar results related to radial mobilization of Al and symplastic Al accumulation have been reported in soybean (Lazof et al., 1996) and maize (Marienfeld et al., 2000). Greater accumulation of Al in the symplastic fraction in genotype Quimbaya might by explained by the greater cell volume and thus vacuoles (root cross sections in Fig. 16) typical for genotypes of Andean origin in comparison to the Mesoamerican origin of VAX-1 (Gepts et al., 1986; Singh, 1989; White and Gonzalez, 1990).

Two patterns of OA exudation can be recognized based on the timing and amount of secretion. In Pattern-I plants, exudation is switched on without a delay after exposure of plants to Al and kept constant over time. In Pattern-II plants, OA secretion is induced by the addition of Al after a lag phase of several hours and the rates of release increase over time (Ma et al., 2001). This characterization suggests a constitutive mechanism of OA release in the Pattern-I plants, whereas in Pattern-II plants, the induction of genes and synthesis of proteins involved in the OA synthesis and/or membrane transport is required (Ma et al., 2001; Ryan et al., 2001). With the exception of malate and oxalate in VAX-1 only (Fig. 19), Al treatment during the initial period of root inhibition did not induce the exudation of any other organic acid (OA)-anion in both genotypes. The exudation of oxalate and malate corresponded with the reduction of internal contents of each OA observed after 5 h of Al treatment (Fig. 21). However, this exudation did not measurably contribute to Al resistance because it was expressed during this period of strong inhibition of root growth and was not consistent during the Al treatment duration. Similar transient efflux of OA-anions has been observed in soybean (Silva et al., 2001) and ruzigrass (Wenzl et al., 2001) without contributing to Al resistance. Al-induced citrate exudation has been reported in common bean (Miyasaka et al., 1991; Mugai et al., 2000; Shen et al., 2002a, 2004; Rangel and Horst, 2006; Stass et al., 2007) and, as discussed later, citrate exudation offers the most consistent relationship to the effects observed in the root elongation of both genotypes during the Al treatment (Figs. 4, 5, 8A and 17A). Although, the efflux (Figs. 19 and 20A) and internal contents of citrate (Fig. 21) were not altered during the initial Al injury in both genotypes, Al treatment significantly modified the activities of some enzymes related to citrate synthesis (Fig. 23). In VAX-1, Al treatment reduced the activity of phosphoenolpyruvate carboxylase (PEPC) and NADP-ICDH, while Al enhanced the activity of NAD-malate dehydrogenase (NAD-MDH) and ATP-phosphofructokinase (ATP-PFK), and did not alter the activity of citrate synthase (CS). In Quimbaya, the activities of all 5 analyzed enzymes were reduced. In both genotypes, especially the activity of NADP-ICDH were drastically reduced and remained at the low level up to 24 h of Al treatment. This suggested a rapid and constant response in both genotypes to maintain the internal citrate levels in the root tip a prerequisite for maintaining the further exudation. This enzyme in combination with aconitase (ACO) is involved in citrate turnover (Massonneau et al., 2001). Additionally, both are know to act mainly in the cytosolic pathway, thus enhancing the accumulation/maintenance of cytoplasmic citrate contents influencing directly the citrate secretion in comparison to the mitochondrial cycle (Kihara et al., 2003b).

A common gradual recovery from Al injury, “the activation of citrate efflux”

While the initial period of root inhibition (4 h) was characterized by the absence of OA-anion exudation, the transient recovery of root elongation rates observed in both genotypes (Figs. 4, 5, 8A and 17A) was characterized by the beginning of an Al exclusion mechanism based on exudation of citrate and succinate (the latter especially in Quimbaya). Similar patterns of root growth recovery have been observed in common bean (Cumming et al., 1992; Stass et al., 2007), soybean (Yang et al., 2000 and 2001) and chakod (Ma et al., 1997), also classified as Pattern-II plant species. After a lag period (4h), citrate exudation was observed to be high in

VAX-1 but low in Quimbaya (Figs. 19 and 20A). This contrasting capacity to initially exude citrate in both genotypes is partially explained by the continuous capacity to produce citrate (unmodified CS activity coupled with reduction of OA contents), and a reduced citrate turnover in VAX-1, whereas in Quimbaya the capability to produce citrate is drastically reduced (reduced CS activity coupled with maintenance of OA contents) resulting in low rates of exudation. The observed citrate exudation pattern characterized common bean as Pattern-II plant. It is thought that the lag phase in Pattern-II plants is consistent with the induction of Al resistance genes (transport and/or OA synthesis) contributing to the increased capacity to exude OA-anions (Ma et al., 2001; Ryan et al., 2001;Kochian et al., 2004). The importance of anion channels facilitating the efflux of OA-anions in Al resistance has been well documented (Ryan et al., 1995; Ryan et al., 1997; Piñeros and Kochian, 2001; Kollmeier et al., 2001; Delhaize et al., 2004; Sasaki et al., 2004; Delhaize et al., 2007), also mediating citrate exudation in common bean (Shen et al., 2004). Considering that the initial internal citrate contents were not modified during the lag phase in both genotypes, the observed Al-enhanced citrate exudation is consistent with the activation of a plasma membrane anion channel. However, it is not clear: (i) if the higher rates of citrate exudation in VAX-1 corresponded to a rapid activation of anion channels compared to Quimbaya, (ii) why both genotypes equally overcome the initial Al injury, although the rates of citrate exudation differed among them, and (iii) if the exudation of succinate, which runs in parallel to citrate in both genotypes, but especially in Quimbaya might contribute to the observed initial recovery.

Exudation of succinate in parallel to malate or citrate in Al-resistant cultivars has been reported in wheat (Delhaize et al., 1993b; Christiansen-Weniger et al., 1992), barley (Guo et al., 2007), maize (Gaume et al., 2001) and alfalfa (Tesfaye et al., 2001). Succinate has been involved in the resistance of pea to Mn and Zn toxicities (Doncheva et al., 2001, 2005) and in the control of oxidative stress, especially in mitochondria (Bindoli et al., 1982; Pai and Gaur, 1988). However, a specific contribution to Al resistance has not been reported and remains speculative. Additionally, it is known that succinate will not contribute directly to Al resistance, due to the low stability of the Al complex (Hue et al., 1986), hence citrate exudation offers the most consistent relationship to the effects observed in root elongation of both genotypes during Al treatment (Figs. 4, 5, 8A and 17A). During this transient root-growth improvement, the initially higher amounts of citrate excreted by VAX-1 remained fairly constant or even decreased, whereas citrate release from Quimbaya was enhanced up to the same levels of those from VAX-1 (Figs. 19 and 20A). Although, in both genotypes the observed citrate efflux corresponded to a reduction of the internal OA contents (Fig. 21) and the activities of enzymes involved in citrate synthesis (Fig. 23) seems to indicate two contrasting strategies to sustain the observed citrate exudation. In VAX-1, citrate exudation was related to the reduction of citrate contents (30 % of the constitutively low internal pools), coupled with an apparently better capacity to produce citrate (normal CS activity), which results in a generalized consumption of OA (exhaust approach). In Quimbaya, a reduced metabolic activity (conservative approach) leading to better conservation of internal OA-contents, indicated that the incrementally citrate exudation is mainly related to the use of internal citrate contents (50 % of the constitutively higher internal pools).

Recovery from Al stress was reflected by reduced Al contents, especially in the TZ but also in the EZ (Fig. 7). It has been argued that the strong binding of Al in the cell wall represents a detoxification mechanism in squash (Le Van et al., 1994). However, the negative relationship between stabile-bound Al to the CW and root growth during the recovery period in both genotypes (Figs. 11, 14 and 15), indicated that the strong binding of Al to the pectic matrix of the CW is a main factor of Al toxicity and not a tolerance mechanism in common bean. Additionally, the decrease of the Al contents in the WFSF fraction, especially in Quimbaya, suggested that citrate exudation acts mainly by keeping the Al concentration in the WFSF low and thus reducing the amount of Al which could potentially bind to the CW. A further investigation dealing with the interaction of citrate and Al accumulation in each cell compartment is needed to better understand the mode of action of citrate exudation in each cell compartment and to clarify the low or the enhanced Al accumulation in the labile-bound (citrate-exchangeable) Al fraction in Quimbaya and VAX-1, respectively.

The symplastic Al fraction did not reflect the recovery from initial Al stress and was even increased in both genotypes during up to 24 h of Al treatment (Figs. 12 and 14). Internalization of Al in the symplast has been suggested to contribute to Al tolerance rather than to Al toxicity in maize (Vázquez et al., 1999) and *Arabidopsis* (Illes et al., 2006). Hence, this Al internalization may contribute to Al tolerance in common bean, too, although the small quantity of this fraction questions the relative quantitative contribution of this mechanism to Al resistance.

A contrasting steady recovery or severe root inhibition period, "enhanced or reduced citrate efflux"

Between 9 and 25 h of Al treatment, the Al-resistant genotype Quimbaya enhanced the exudation of citrate, while it constantly declined in the Al-sensitive genotype VAX-1 (Fig. 20A), leading to a strong reduction in Quimbaya or an increase in VAX-1 of Al contents along the entire root apex (Figs. 6, 7, 8B and 17B). This explains the gradual recovery of root growth rates up to the control levels without Al in Quimbaya and the increasing inhibition in VAX-1 (Figs. 4, 5, 8A and 17A). This result is in agreement with the studies of Shen et al. (2002a), who demonstrated that lower Al contents of root tips of the Al-resistant common bean cultivar G 19842 compared to the Al-sensitive cultivar ZPV corresponded with its higher capacity to exude citrate after 3 d of Al treatment (10 and 20 µM Al).

During this period, the activities of all enzymes studied, especially CS, gradually increased in Quimbaya (Fig. 23), leading to a replenishment of all internal OA, especially citrate (Fig. 21), thus sustaining the enhanced citrate exudation. On the other hand, in VAX-1 the lower citrate exudation corresponded to a decreased CS activity (Fig. 23) and the complete depletion of internal citrate pool (Fig. 21). It is important to notice that ATP-PFK activity which is an important indicator of the internal OA levels and the energy charge in the cell (Carnal and Black, 1983, Stryer, 1988) was particularly enhanced in VAX-1, showing that citrate level was constantly depleted. Citrate synthase activity enhanced by Al treatment has been observed in soybean (Yang et al., 2001) and rye (Li et al., 2000), where the higher CS activity coincided with the beginning of citrate efflux. In contrast, in triticale the observed citrate efflux was not related to *in vitro* enzyme activities in general (Hayes and Ma, 2003).

Since the citric acid cycle (TCA) requires stoichiometric parity between acetyl Co-A and oxaloacetate to proceed, removal of oxaloacetate or any of its precursors disrupts the cycle (Hill, 1997). Anaplerotic "fill up" synthesis of intermediates (i.e. malate) redresses imbalances that may occur as a result of consumption of TCA cycle intermediates for amino-acid synthesis or other processes (Miller and Cramer, 2004). Oxaloacetate can be replenished via NAD-malic enzyme, glutamate oxidation catalyzed by glutamate dehydrogenase, glyoxylate cycle or via PEPC (Palmieri et al., 1997; Held, 2005). The activity of PEPC was equally affected by Al treatment in both genotypes (Fig. 23), suggesting a similar capacity to refill intermediates of the TCA. However, the constitutively higher PEPC activity in Quimbaya might suggest a better anaplerotic function than in VAX-1. The enhanced capacity to exude succinate in parallel to citrate, especially in Quimbaya (Figs. 19 and 20), suggests the presence of a glyoxylate pathway. This pathway is known to replenish TCA intermediates and bypassing the oxidative phase (controlled by ACO and NADH-ICDH) of the TCA cycle, thus refilling succinate for the normal development of the TCA cycle and favoring the production of citrate for further exudation. Evidence of the glyoxylate pathway as anaplerotic function has been suggested (Olsen and Harada, 1995; Ismail et al., 1997; Eastmond and Graham, 2001). Recently, the expression of isocitrate lyase (ICL) and malate synthase (MS), both enzymes characterizing the presence of active peroxisomes in the glyoxylate cycle, was demonstrated *in vivo* in cucumber roots in response to shading or defoliation (Ismail et al., 1997). Thus, further research dealing with the presence of ICL and MS in root tips of common bean on the lines developed by Ismail et al. (1997) might contribute to confirm this assumption.

The Al contents of the root tips in the Al-resistant genotype Quimbaya were consistently higher for up to 16-20 h compared to VAX-1, and deceased only thereafter (Figs. 6, 7, 8B and 17B). This might be associated with the constitutively higher negativity of the CW observed in Quimbaya, and suggests that cell wall characteristics (i.e. pectin content and its DM) are a principal factor of Al injury but not Al resistance in common bean. In genotype Quimbaya the increase of citrate exudation was accompanied by a reduction of Al contents in the WFSF, leading to lower accumulations of Al in the stabile-bound CW fraction. In genotype VAX-1, the slowdown of citrate exudation was reflected in a strong accumulation of stabile and labile-bound Al to the CW. It is interesting to note that during the entire Al treatment, the labile-bound Al CW fraction was the only fraction consistently higher in the Al-sensitive genotype VAX-1. Localization of free and symplastic Al by morin showed that the observed increase in the symplastic Al fraction corresponded mainly to Al internalized in epidermal cells in the TZ (inserts in Figs. 16C and 16G), more in Quimbaya than in VAX-1. However, Al-morin staining in the CEZ clearly reflected the observed genotypic differences in Al resistance. In this zone, Al-morin fluorescence was restricted to the epidermal cells (Fig. 16D) in genotype Quimbaya while it was homogeneously distributed across the cortex into the central cylinder in the Al-sensitive genotype VAX-1 (Fig. 16H). Lazof et al. (1994) reported similar Al distribution in soybean after 18 h of Al treatment.

In conclusion, the study shows that in common bean the TZ is the most Al-sensitive apical root zone, however, Al applied to the entire EZ is also root growth-inhibiting. Thus the entire 10 mm root apex requires protection against Al injury in the Al-resistant genotype Quimbaya. Common bean proved to be a typical Pattern II plant species characterized by a most Al-sensitive response to short-term Al supply and then recovery from Al injury through the release of particularly citrate. Since citrate could only be released in response to Al after a lag phase of 4 hours in spite of high citrate concentrations in the root apex, this suggest that Al resistance in common bean requires the expression/activation of a citrate permease. Thereafter, the continuous release of citrate and maintenance of the cytosolic citrate concentration depends on the up-regulation of citrate synthesis and down-regulation of its degradation.

OUTLOOK

The present work clearly indicated that Al-resistance in common bean is an induced trait, where the effect of citrate exudation is expressed during the medium-term exposure of roots to Al. Therefore, development and application of screening techniques, based on root elongation, Al accumulation and citrate exudation as sensitive parameters, should focus in the genotypic differences in Al-resistance between 16 and 24 h after Al treatment. Considering the impossibility to use Al-induced callose formation as a sensitive parameter to select Al resistant genotypes in common bean, inhibition of root elongation remains the principal, most simple and fastest observable morphological symptom of Al injury, thus the main parameter to be used in Al-resistance screenings in common bean. The acknowledged importance of the root apex and particularly the DTZ as the most Al-sensitive root zone particularly in maize was corroborated in common bean. However, Al inhibited root growth also when Al was supplied to more basal zones along the EZ, indicating that not only the first 0-2 mm root zone but also the entire root tip (10 mm) needs to be protected from Al injury and, therefore, should be considered in further studies.

Common bean proved to be an ideal experimental plant to elucidate the relationship between Al response and recovery from initial Al injury and the dynamics of Al uptake, distribution and compartmentation in the root apex. Further experiments targeting the cell compartmentation of citrate in relation to Al, coupled with the pattern of citrate exudation along the entire root tip needs to be conduct in order to clarify: (i) the similar capacity of both genotypes to overcome the initial Al injury (transient recovery period), (ii) the higher initial Al accumulation and lower citrate exudation in Quimbaya compared to VAX-1 at comparable inhibition of root elongation (higher Al tolerance of Quimbaya), and (iii) the contrasting recovery response between the two genotypes during the medium-term exposure of roots to Al.

Since the early 90´s, cumulative evidence has shown that organic acids play an important role in detoxifying Al internally and externally. As demonstrated here, it also represents a mayor role in the Al resistance in common bean. However, evaluation of more genotypes is required in order to validate the importance of organic acids in the Al resistance in common bean.

The role of succinate in Al resistance has been normally neglected due to its low Al-detoxifying capacity. The direct involvement of succinate in the respiration status of the cell, its protecting role controlling lipid peroxidation in mitochondria and the parallel exudation to malate or citrate in wheat, maize, soybean and common bean by Al-resistant cultivars suggest that succinate might be an additional important factor in the expression of Al tolerance.

Additionally, the possible contribution of a glyoxylate pathway as an anaplerotic function to replace citrate and other intermediates of the citric acid cycle, including succinate, requires further research addressing the roles of isocitrate lyase (ICL) and malate synthase (MS). Both enzymes are specific to the glyoxylate cycle and known to be induced in the roots during limitation of assimilates a possible explanation of the breakdown of the citrate synthesis and efflux in VAX-1 after 24 h Al treatment.

The characterization of common bean as a pattern-II plant of organic acid exudation and the implementation of new molecular/physiological methods might contribute to elucidate the importance of gene induction and/or synthesis of proteins involved in the organic acid synthesis and/or transport out of the cells. Furthermore, such tools will be helpful in bean improvement programs through molecular-assisted breeding. Advanced screening efforts using our previously developed nutrient solution technique revealed that Andean genotypes were generally superior to Mesoamerican genotypes in Al resistance based on Al-inhibited root elongation. In the field, however, Mesoamerican genotypes proved to be well adapted to acid soils due to abundant shallow roots that escape Al toxicity in the subsoil layers. Due to out-breeding depression in Andean X Mesoamerican crosses, obtaining high-yielding genotypes with high Al resistance is difficult using this approach. Therefore, presently interspecific crosses between *Phaseolus vulgaris* and *Phaseolus acutifolius* or *Phaseolus coccineus* and backcrossing to selected common bean genotypes is being exploited as a promising breeding strategy aiming at combining genes from both gene pools and progenies to improve common bean for adaptation to acid Al-toxic soils.

SUMMARY

Common bean needs significant improvement in aluminium (Al) resistance to reduce the dependence of small farmers on lime and fertilizer inputs on acid soils. Variation exists for adaptation to acid soils and Al resistance among common bean genotypes. Knowledge on the mechanisms of Al toxicity can contribute to the development rapid and reliable screening procedures needed for accelerated breeding for Al resistance.

The initial response of plants to Al is an inhibition of root elongation. Detailed studies on the kinetics of the response of root-growth rates to Al supply offer the possibility to differentiate between constitutive or inducible Al-resistance mechanism and to verify whether this is consistent across genotypes. Therefore, the present study focused on the temporal and spatial effects of Al on root growth, Al accumulation and cellular localization, and Al exclusion mediated by the release of organic acid anions.

Root elongation of both, the Al-resistant (Quimbaya) and the Al-sensitive (VAX-1) genotype were severely inhibited during the first 4 h of Al treatment. Whereas the transition zone (TZ, 1-2 mm) was the most Al-sensitive apical root zone, Al applied to the elongation zone (EZ, 2-10 mm) also induced inhibition of root elongation. After the initial Al injury, both genotypes showed gradual recovery. However, this recovery continued in genotype Quimbaya until the root-elongation rate reached the level of the control (without Al), while the genotype VAX-1 was increasingly damaged by Al after 12 h of Al treatment. These observed effects on root elongation in both genotypes were reflected by the dynamics of Al contents in the root apex. Enhanced inhibition of root elongation during the initial 4 h of Al treatment was related to high Al accumulation in root apices in both genotypes (Quimbaya > VAX-1). Recovery from Al stress was not only reflected by decreasing Al contents especially in the TZ, but also in the EZ. After 24 h of Al treatment the high Al resistance of Quimbaya was reflected by much lower Al contents in the entire root apex. The results presented in chapter 1 suggested the activation and maintenance of an Al exclusion mechanism acting along the entire EZ but especially in the TZ.

A higher initial Al accumulation of Quimbaya in the 5 mm root apex compared to VAX-1 could be related to its higher content of unmethylated pectin and thus higher negativity of the cell wall (CW). The binding stage and cellular distribution of Al in the root apices characterized using a fractionated Al extraction from intact roots and isolated cell walls revealed that the observed changes in the root elongation-rate of both genotypes was significantly negatively correlated with the free apoplastic and the stabile-bound, non citrate-exchangeable CW-Al representing the most important Al fraction in the root apex (80 %), but

not with the symplastic and the labile-bound, citrate-exchangeable CW-Al. The increase of Al in the symplastic fraction during recovery form initial Al injury particularly in Quimbaya indicated that internalization of Al into symplast might contribute to Al resistance rather than Al injury. The radial distribution studies using morin as stain for Al indicated that Al in the free apoplastic, labile-bound CW and symplastic Al fractions mainly accumulated in the epidermis and outer cortical cells of the EZ, but particularly in the TZ. Sustained Al injury in genotype VAX-1 after 24 h Al treatment was related to the penetration of Al across the cortex and the endodermis up to the central cylinder.

The initial genotype-independent Al injury was due to the absence of citrate exudation from the root tips in both genotypes. Thereafter (5–9 h), in both genotypes root elongation recovered by an Al-enhanced exudation of citrate from the internal organic acid pool, leading to reduced citrate and Al contents. In VAX-1 citrate efflux could be sustained during this period by a down-regulation of the activity of NADP-isocitrate dehydrogenase (NADP-ICDH) thus reducing the cytosolic turnover of citrate and a low but constant citrate synthase (CS) activity. In Quimbaya, the citrate efflux was sustained by both a lower NADP-ICDH activity and a greater internal citrate pool in spite of a decreased CS activity. The recovery of the CS activity after 25 h Al treatment allowed sustaining an enhanced capacity to exude citrate and to restore the internal organic acid pool leading to decreasing Al contents and thus recovery of root growth in Quimbaya. In VAX-1, a decreased CS activity coupled with an exhausted internal citrate pool resulted in lower rates of citrate secretion, resumption of Al accumulation and, consequently, to severe inhibition of root elongation.

In conclusion, the study shows that in common bean the TZ is the most Al-sensitive apical root zone, however, Al applied to the entire EZ is also root growth-inhibiting. Thus the entire 10 mm root apex requires protection against Al injury in the Al-resistant genotype Quimbaya. Common bean proved to be a typical Pattern II plant species characterized by a most Al-sensitive response to short-term Al supply and then recovery from Al injury through the release of particularly citrate. Since citrate could only be released in response to Al after a lag phase of 4 hours in spite of high citrate concentrations in the root apex, this suggest that Al resistance in common bean requires the expression/activation of a citrate permease. Thereafter, the continuous release of citrate and maintenance of the cytosolic citrate concentration depends on the up-regulation of citrate synthesis and down-regulation of its degradation.

ZUSAMMENFASSUNG

Die Verbesserung der Aluminium (Al) Resistenz in Bohnen kann einen wichtigen Beitrag dazu leisten, die Abhängigkeit von Kleinbauern von Kalk- und anderen Düngern auf sauren Böden zu verringern. Schwankungen zwischen Bohnenarten bestehen bei der Anpassung an saure Böden und in der Al Resistenz. Das Wissen um die Mechanismen der Al Toxizität kann dazu beitragen, schnelle und zuverlässige Selektionsmethoden zu entwickeln, die benötigt werden um den Züchtungserfolg im Hinblick auf Al Resistenz zu beschleunigen.

Die erste Antwort der Pflanzen auf Al ist die Hemmung des Wurzelwachstums. Ausführliche Untersuchungen über die Kinetik der Wurzelwachstumsraten als Antwort auf Al Zugabe ermöglichen es, zwischen konstitutiven oder induzierbaren Al Resistenzmechanismen zu unterscheiden, um herauszufinden, ob diese einheitlich zwischen Genotypen sind. Die vorliegende Arbeit beschäftigt sich mit den zeitlichen und räumlichen Effekten von Al auf Wurzeln, der Akkumulation von Al und der Lokalisierung im Gewebe, sowie mit Ausschlussmechanismen, die durch die Ausschüttung von organischen Säuren vermittelt werden.

Das Wurzelwachstum sowohl des Al resistenten Genotypen Quimbaya als auch des Al sensitiven Genotypen VAX-1 wurde in den ersten 4h der Al Behandlung stark eingeschränkt. Die Zugabe von Al zur Elongationszone (EZ, 2-10mm) hatte eine Hemmung des Wurzelwachstums zur Folge; die am meisten sensitive Zone der Wurzelspitze war jedoch die Transitionszone (TZ, 1-2mm). Nach der anfänglichen Verwundung der Wurzelspitze durch Al zeigten beiden Genotypen eine allmähliche Erholung von dieser Behandlung. Diese Erholung hielt in Quimbaya an, bis die Wurzelwachstumsraten wieder der Höhe der Kontrollen (ohne Al) entsprachen. VAX-1 dagegen war nach 12h durch die Al Zugabe nachhaltig im Wachstum gehindert. Diese Effekte auf das Wurzelwachstum konnten auch bei der Dynamik der Al Gehalte in den Wurzelspitzen beobachtet werden. Eine gesteigerte Hemmung des Wurzelwachstums während der ersten 4h der Al Behandlung war verbunden mit einer hohen Al Akkumulation in den Wurzelspitzen beider Genotypen (Quimbaya > VAX-1). Eine Erholung des Al Stresses wurde nicht nur durch absinkende Al Gehalte besonders in der TZ angezeigt, sondern auch durch stark verringerte Gehalte in der gesamten Wurzelspitze. Die in Kapitel I vorgestellten Ergebnisse sprechen daher für eine Aktivierung und Aufrechterhaltung eines Mechanismus zum Ausschluss von Al, der entlang der gesamten EZ, aber in speziellem Maße in der TZ wirksam wird.

Eine anfänglich höhere Al Akkumulation in der 5mm Wurzelspitze von Quimbaya als in VAX-1 konnte mit dessen höherem Gehalt an unmethyliertem Pektin, und damit höherer

Negativität der Zellwand (CW) in Verbindung gebracht werden. Der Bindungsstatus und die zelluläre Verteilung von Al in den Wurzelspitzen konnten durch eine fraktionierte Al Extraktion von intakten Wurzeln und isolierten Zellwänden charakterisiert werden. Die beobachteten Veränderungen in der Wurzelwachstumsrate beider Genotypen waren signifikant negativ mit dem freien apoplastischen und dem stabil gebundenen, nicht durch Citrat austauschbaren Zellwand-Al korreliert. Der Anstieg der Al Gehalte in der symplastischen Fraktion während der Erholung der Wurzelspitzen von der anfänglichen Verwundung durch Al, besonders in Quimbaya, wies darauf hin, dass die Internalisierung von Al in den Symplasten eher zur Al Resistenz als zur Verletzung der Wurzelspitze beitrug. Die Untersuchung der radialen Verteilung, bei der Morin als Färbemittel für Al eingesetzt wurde, zeigte, dass Al zwar im freien Apoplasten und in der labil gebundenen Zellwandfraktion hauptsächlich in der Epidermis und den äußeren kortikalen Zellschichten der EZ akkumuliert, sich besonders jedoch in der TZ wieder finden ließ. Anhaltender Schaden durch Al im Genotypen VAX-1 nach 24h Al Behandlung konnte mit dem Eindringen des Al durch den Kortex und die Endodermis bis hinein in den Zentralzylinder erklärt werden.

Der anfängliche, vom Genotypen unabhängige Schaden durch Al entstand durch die Abwesenheit der Citrat Exudation in den Wurzelspitzen beider Genotypen. Danach (5-9h) erholte sich das Wurzelwachstum in beiden Genotypen, ausgelöst durch eine durch Al angeregte Ausschüttung von Citrat aus den internen Vorräten an organischen Säuren, was zu reduzierten Citrat und Al Gehalten führte. In VAX-1 konnte der Citratefflux während dieser Zeit durch ein Herunterschrauben der NADP-Isocitratdehydrogenaseaktivität erreicht werden, wodurch der cytosolische Gesamtumsatz von Citrat reduziert, und eine niedrige, aber konstante Aktivität der Citratsynthase erreicht wurde. In Quimbaya wurde die Citratausschüttung durch eine niedrigere NADP-ICDH-Aktivität und einen größeren internen Citratvorrat trotz einer verringerten CS Aktivität aufrechterhalten. Die Wiedereinsetzen der CS Aktivität nach 25h Al Behandlung erlaubte es, eine erhöhte Kapazität beizubehalten, um Citrat auszuschütten, und um die internen Vorräte an organischen Säuren zu erneuern, was zu niedrigeren Al Gehalten und somit zu einer Erholung des Wurzelwachstums in Quimbaya führte. In VAX-1 resultierte eine verringerte CS Aktivität, verbunden mit erschöpften internen Citratvorräten, zu einer Wiederaufnahme der Al Akkumulation und einer nachfolgenden schweren Hemmung des Wurzelwachstums.

Die Ergebnisse dieser Untersuchungen zeigen, dass in Bohnen die EZ die am meisten sensitive apikale Wurzelzone ist; jedoch führt Al auch in der gesamten EZ zu einer Hemmung des Wurzelwachstums. Somit benötigt die gesamte 10-mm Wurzelspitze einen Schutz vor

Verwundung durch Al im Al resistenten Genotypen Quimbaya. Bohnen sind bewiesenermaßen typische Pattern II Pflanzen, charakterisiert durch eine sehr sensitive Antwort auf Kurzzeit Applikation von Al und anschließender Erholung der Verletzung durch die Ausschüttung insbesondere von Citrat, aber auch anderen organischen Säuren. Citrat wurde trotz hoher Konzentrationen in der Wurzelspitze erst nach einer Verzögerung von 4h als Antwort auf Al entlassen, was darauf schließen lässt, dass Al Resistenz in Bohnen eine Aktivierung der Citratpermease verlangt. Demnach hängen die kontinuierliche Ausschüttung von Citrat und die Beibehaltung der cytosolischen Citratkonzentrationen von der Hochregulierung der Citratsynthese und der Herabregulierung deren Degradation ab.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. Dr. Walter J. Horst for giving me the opportunity to continue the work begun six years ago on this interesting area and for his support, supervision, critical guidance along the entire work and to sharper the axe when the work turned dull and blunt. I also want to extend my sincere thankfulness to Mrs. Horst for all the support during difficult moments and keeping Germany close to the best French stile.

To Dr. Idupulapati M. Rao for his trust, support, always enthusiastic messages and contributing to a successful accomplishment of a dream.

I am grateful also to Prof. Dr. H. P. Braun for his willingness to be my co-referee, always having time for discussion and guidance; to Prof. Dr. Scherer and Prof. Dr. H. Stützel for kindly accepting the request to be my examiners.

Special thanks go to Dr. Angelika Stass for her critical comments, contributions, time to answer "I have one question with a, b, c and d", and the help during the preparation of this manuscript.

I would like to express my heart felt gratitude to Caroline Maria Quignon for all the help during the experiments, preparation of this manuscript and now sharing more than one life.

Many thanks to my office mates Zorica Cevetic and Dr. Dejene Eticha for all the work, discussions, sharing ideas and good time in the office. To Mrs. Dusy who has taken care of actually every social or administrative aspect of this wonderful time in Germany. To all friends and colleagues in the institute for sharing six years of my life.

My special gratitude goes to all "Rancheros": Peter, Sara Maria, Nathalie, Daniel, Fredy Andrés, Natalia, Alejandro, Freddy Rodrigo, Diana, David, Magdalena, los Saucedos, Lina, Marcela, Claudia, Thomas, Tanja, Caroline and Junior for being my family in Germany.

I also want to thanks my family including my "Gastmutter" Mrs. Ute Brüning for all the love and attention.

This research was supported by two restricted core projects from BMZ/GTZ (2000.7860.0-001.0 entitled "An Integrated Approach for Genetic Improvement of Aluminium Resistance on Crops of Low-fertility Soils" and No. 05.7860.9-001.00 entitled "Fighting drought and aluminum toxicity: Integrating functional genomics, phenotypic screening and participatory evaluation with women and small-scale farmers to develop stress-resistant common bean and *Brachiaria* for the tropics" granted to the International Centre for Tropical Agriculture (CIAT).

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Soil biology and soil fertility

INTERNATIONAL RESEARCH EXPERIENCE

Plant nutrition institute, Leibniz University of Hanover, Hanover, Germany (2005-2007) GTZ/CIAT/UNI-HANNOVER/PABRA. Fighting drought and aluminum toxicity: Integrating functional genomics, phenotypic screening and participatory evaluation with women and small-scale farmers to develop stress-resistant common bean and *Brachiaria* for the tropics. PhD, student. "*Short- and Medium-Term Effects of Aluminium Toxicity and Resistance in Common Bean (Phaseolus vulgaris L.)*"

Plant nutrition institute, University of Hanover, Hanover, Germany (2003-2005) GTZ/CIAT/UNI-HANNOVER/PABRA/TROPILECHE/SWNM An integrated approach for genetic improvement of Aluminium resistance of crops on low-fertility acid soils.

PhD, student. "*Short- and Medium-Term Effects of Aluminium Toxicity and Resistance in Common Bean (Phaseolus vulgaris L.)*

Plant nutrition institute, University of Hanover, Hanover, Germany (2001-2003)
BMZ/CIAT/UNI-HANNOVER/PABRA/TROPILECHE/SWNM An integrated approach for genetic improvement of Aluminium resistance of crops on low-fertility acid soils.

MSc, student. "*Differences in Aluminium resistance among common bean Phaseolus vulgaris* L. *cultivars*"

Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia (1995-2001)
CIAT/ICRISAT/NARS Latin American Sorghum and Pearl millet Project
Responsible for the organization and conduction of field experiments, data analysis, reporting and budget administration of the Latin American sorghum and pearl millet project at CIAT station. Identification of sorghum and pearl millet genotypes adapted to acid soil conditions, generation of new breed population, inbred lines and their progenies. Coordination of the Latin American Sorghum and Pearl Millet Network.

International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh India (Dec1998- Feb1999)
Visiting Scientist, Sorghum and Pearl Millet Breeding
Methodologies and techniques in sorghum and pearl millet breeding

Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia (1994-1995)
Soils and Plant Nutrition Unit
B.S thesis "Effect of the earthworm Martiodrilus carimaguensis sp.nov. on nitrogen dynamic and zero point of net charge (ZPC)in a Colombian savanna Oxisol".

SUPERVISORY EXPERIENCE

Supervision of (1) BSc student registered in the Leibniz University of Hanover: Aluminium resistance in Brachiaria species (2005-2006)

Supervision of (10) technicians and workers, CIAT-Colombia (1995-2001).

Supervision of (1) student registered in the Tolima University-Colombia: Emasculation and cross-pollination in sorghum and pearl millet (2000).

EDUCATION

PhD., Dr. rer. hort. -*Plant nutrition* (2003-2007), Gottfried Wilhelm Leibniz University of Hanover, Faculty of Natural Science, Institute of Plant Nutrition, Hanover, Germany.

MSc., International Horticulture-*Plant nutrition* (2003), University of Hanover, Department of Horticulture, Hanover, Germany.

Specialization on sorghum and pearl millet breeding (1999), International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh India.

B.S., Agronomy engineer (1997), National University of Colombia, Palmira-Valle del Cauca, Colombia.

SCIENTIFIC SOCIETY MEMBERSHIP

Soil Science Society of Colombia

GRANTS

2007-2007. Fellowship International Research Assistant CIAT/BMZ
2006-2007. Research and teaching assistantship program- STIBET-DAAD
2001-2003. Fellowship from the German Academic Exchange Service (DAAD)

AWARDS

Rangel AF., Mobin M., Rao I and Horst WJ. "Aluminium-induced callose formation is not a suitable parameter for accessing genotypic differences in aluminium resistance in *Phaseolus vulgaris* L." **3. Preis** Posterauszeichnung auf der Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung, 01.-03. September 2004, Göttingen, Deutschland.

PUBLICATIONS

Refereed Journal Articles

Rangel AF, Rao IM, Horst WJ. Spatial aluminium sensitivity of root apices of two common bean (*Phaseolus vulgaris* L.) genotypes with contrasting aluminium resistance. (To be submitted)

Rangel AF, Rao IM, Horst WJ. Aluminium fractionation in root apices of common bean (*Phaseolus vulgaris* L.) genotypes differing in aluminium resistance. (To be submitted)

Rangel AF, Rao IM, Dubey RS, Stass A, Horst WJ. Aluminium resistance in common bean (*Phaseolus vulgaris* L.) involves induction and maintenance of citrate exudation from root apices. (To be submitted)

Rangel AF, Mobin M, Rao I and Horst WJ 2005. Proton toxicity interferes with the screening of common bean (*Phaseolus vulgaris* L.) genotypes for aluminium resistance in nutrient solution. *J. Plant Nutr. Soil Sci.* 168, 607-616.

Rangel AF, Thomas RJ., Jiménez JJ and Decaens T. 1999. Nitrogen dynamics associated with earthworm casts of *Martiodrilus carimaguensis* Jiménez and Moreno in a Colombian savanna Oxisol. Pedobiologia 43, 557-560.

Rangel AF, Madero E, Thomas RJ, Friesen D, and Decaens T. 1999. Ion exchange properties of casts of the anecic earthworm (*Martiodrilus carimaguensis* Jiménez and Moreno) in a Colombian savanna Oxisol. Pedobiologia 43, 795-801.

Decaens T., **Rangel A.F**., Asakawa N and Thomas R.J. 1999. Carbon and nitrogen dynamic in ageing earthworm casts in grassland of the eastern plains of Columbia. Biol. Fertil. Soils 30, 20-28.

Rangel AF, Thomas RJ, Madero E, Lavelle P, Friesen DK, Jiménez JJ, Decaens T. y Amezquita E. 1997. Efecto de la lombriz de tierra *Martiodrilus carimaguensis sp. nov* en la dinámica del nitrógeno de un Oxisol de los Llanos Orientales de Colombia. Revista Suelos Ecuatoriales 27, 235-241.

Rangel AF, Madero E Thomas RJ, Lavelle P, Friesen DF, Jiménez JJ. Decaens T. y Amezquita E. 1997 . Efecto de la lombriz de tierra *Martiodrilus carimaguensis sp. nov* en el punto de carga cero (ZPC) de un Oxisol de los Llanos Orientales de Colombia. Revista Suelos Ecuatoriales 27, 231-247.

Invited Contributions to Books

Reddy, BVS, **Rangel, AF,** Ramaiah, B and Ortiz, R. 2004. "A Research and Network Strategy for Sustainable Sorghum Production Systems for Latin America". In Sorghum Genetic Enhancement: Research Process, Dissemination and Impacts. MCS Bantilan, UK Deb, CLL Gowda, BVS Reddy, AB Obilana and RE Evenson (Eds.). ICRISAT, International Crops Research Institute for the Semi-Arid Tropics Patancheru 502 324, Andhra Pradesh, India.

Rangel AF, Thomas RJ, Jiménez JJ and Decaens T. 2000. Nitrogen dynamics associated with earthworm casts of *Martiodrilus carimaguensis* Jiménez and Moreno in a Colombian savanna Oxisol. *in* Nature's plow: Soil Macroinvertebrate Communities in the Neotropical Savannas of Colombia. J. J. Jiménez and R. J. Thomas (Eds.). CIAT, A.A 6713, Cali, Colombia

Decaëns T, Jiménez JJ, **Rangel AF**, Cepeda A y Lavelle P. 2000. La macrofauna del suelo de la sabana bien drenada de los Llanos Orientales de Colombia. En: G. Rippstein, F. Motta y G. Escobar (Eds.). *Los Pastos Nativos de los Llanos Orientales de Colombia. Diversidad-Dinámica-Productividad*. CIAT-CIRAD (bajo prensa)

Rangel AF and Reddy BVS 2000. Network trials: Prospects and problems. p 57-65. *In* The proceedings of the workshop "A research and network strategy for sustainable sorghum and pearl millet production systems for Latin America, La Libertad, Villavicencio Meta, Colombia, 24-26 Noviembre 1998.

Reddy BVS and **Rangel AF**. 2000. Genotype (G) and G x environment (E) interactions in sorghum in acid soil of the Oriental Llanos of Colombia. p 46-53. *In* The proceedings of the workshop "A research and network strategy for sustainable sorghum and pearl millet production systems for Latin America, La Libertad, Villavicencio Meta, Colombia, 24-26 Noviembre 1998.

Reddy BVS, **Rangel AF**, Iglesias C and Bernal J. 2000. Evaluation of sorghum and pearl millet for acid soil tolerance in the Oriental Llanos of Colombia. p 37-45. *In* The proceedings of the workshop "A research and network strategy for sustainable sorghum and pearl millet production systems for Latin America, La Libertad, Villavicencio Meta, Colombia, 24-26 Noviembre 1998.

Reddy BVS, **Rangel AF** and Iglesias C. 1998. Latin American sorghum project: some results. P. 70-79 *in the* proceedings of the First International Symposium on Sorghum, Rio Bravo, Tamaulipas, Mexico, 27-30 May 1998.

Papers published in conference proceedings

Rangel AF, Rao IM, Horst WJ (2007) Spatial aluminium sensitivity of root apices of two common bean (*Phaseolus vulgaris L.*) genotypes with contrasting aluminium resistance. In: Jahrestagung der Deutschen Gesellschaft für Planzenernährung. Berlin, Humbolt-Univerität zu Berlin. 57.

Rangel AF and Horst WJ (2006) "Short and medium term root-growth responses to aluminium in common bean (*Phaseolus vulgaris* L.)". First join conference of The German Society of Plant Nutrition-DGP and The Research Centre Biotechnology and Plant Breeding Uni-Hohenheim-FSP, "Plant Nutrition Meets Plant Breeding". September 26-28, 2006, Stuttgart, Germany.

Rangel AF, Mobin M, Rao I and Horst WJ (2004) "Aluminium-induced callose formation is not a suitable parameter for accessing genotypic differences in aluminium resistance in *Phaseolus vulgaris*". The 6th International Symposium on Plant-Soil Interactions at Low pH. Aug 1-5, 2004 Sendai, Japan.

Rao I, Wenzel P, Chavez A, Arango A, Manrique G, Buitrago M, Miles J, Beebe S, Kimani P, Argel P, Schmidt A, Peters M, Thome J, Ishitani M, **Rangel A**, Mobin M and Horst W (2004) " Advances in improving acid soil adaptation of tropical crops and forages: The case of common bean and brachiaria". 20th RIB Symposium, International Symposium on Frontier Research to Improve Crop Productivity in Acid Soils. Research Institute for Bioresources, Okayama University, Kurakashi, Japan, January 2004.

Rangel AF, Reddy BVS, Bernal J y Ceballos H (2000). "Sorgo y Millo Perla Alternativas para el Manejo Sostenible de los Suelos en los Llanos Orientales de Colombia". II Segundo Seminario Regional "Agrociencia y Tecnología", Siglo XXI, Orinoquia Colombiana Villaviencio, Meta Colombia, Agosto 23-25 del 2000.

Rangel AF, Reddy BVS (1998) "Network trials, Prospects and Problems". A Research and Network Strategy for Sustainable Sorghum and Pearl Millet Production Systems for Latin America. Workshop Centro experimental "La Libertad", Corpoica, November 24-26 of 1998.

Rangel AF, Madero E, Decaëns T, Thomas RJ, Lavelle P, Friesen DK, Jiménez JJ and Amézquita E (1998) "Effect of the earthworm Martiodrilus carimaguensis on the zero point charge (ZPC) of an oxisol from the Colombian savannas" VI International Symposium on Earthworm Ecology Vigo - España 31 August 4 September 1998

Rangel AF, Madero E, Decaëns T, Thomas RJ, Lavelle P, Friesen DK, Jiménez JJ and Amézquita E (1998) "Effect of the earthworm Martiodrilus carimaguensis on the Nitrogen dynamics in an oxisol from the Colombian savannas" VI International Symposium on Earthworm Ecology Vigo - España 31 August - 4 September 1998

Rangel AF, Thomas RJ, Madero E, Lavelle P, Friesen DK, Jiménez JJ, Decaëns T y E. Amézquita (1996) "Efecto de la lombriz de tierra Martiodrilus carimaguensis sp. nov. en la dinámica del nitrógeno de un oxisol de los Llanos Orientales de Colombia" VIII Congreso Colombiano de la Ciencia del Suelo. "Manejo de Suelos del Trópico Semiárido" Santa Marta - COLOMBIA 2-5 Octubre 1996

Rangel AF, Thomas RJ, Madero E, Lavelle P, Friesen DK, Jiménez JJ, Decaëns T y E. Amézquita (1996) "Efecto de la lombriz de tierra Martiodrilus carimaguensis sp. nov. sobre el punto de carga cero (ZPC) de un oxisol de los Llanos Orientales de Colombia" VIII Congreso Colombiano de la Ciencia del Suelo. "Manejo de Suelos del Trópico Semiárido" Santa Marta – COLOMBIA. 2-5 Octubre 1996

Rangel AF, Thomas RJ, Madero E, Jiménez JJ y Decaens T (1996) "Influencia de las lombrices de tierra en la dinámica de los nutrientes del suelo, un caso específico en los Llanos Orientales". Universidad Nacional de Colombia, Sede Palmira. Simposio "Relaciones de la Macrofauna con la Fertilidad y con el Efecto de la Perturbación de los Suelos".1996

Rangel AF, Thomas RJ, Madero E, Lavelle P, Friesen DK, Jiménez JJ, Decaëns T y Amézquita E (1996) Efecto de la lombriz de tierra Martiodrilus carimaguensis sp. nov en la dinámica del nitrógeno de un Oxisol de los Llanos Orientales de Colombia". Taller Regional "Agrociencia y Tecnología Siglo XXI Orinoquia Colombiana Villavicencio Meta Agosto 1996

Rangel AF, Thomas RJ, Madero E, Lavelle P, Friesen DK, Jiménez JJ, Decaëns T y Amézquita E (1996) "Efecto de la lombriz de tierra Martiodrilus carimaguensis sp. nov en la dinámica del nitrógeno de un Oxisol de los Llanos Orientales de Colombia". XII Congreso Nacional de Egresados Universidad Nacional de Santander, "Seminario Internacional del Medio Ambiente y Desarrollo Sostenible", Bucaramanga 1996

Rangel AF y Madero E (1994) "Determinación del punto de carga cero (ZPC) de dos suelos de carga variable y su retención de cargas al lavado después del encalamiento". Universidad Nacional de Colombia, Sede Palmira. Realización problema especial en el área de química de suelos. Diciembre 1994

Reports

GTZ/CIAT/UNI-HANNOVER/PABRA/TROPILECHE/SWNM. 2002. An integrated approach for genetic improvement of Aluminium resistance of crops on low-fertility acid soils. Annual report 2002. Centro Internacional de Agricultura Tropical CIAT. A.A 6713, Cali, Colombia

GTZ/CIAT/UNI-HANNOVER/PABRA/TROPILECHE/SWNM. 2001. An integrated approach for genetic improvement of Aluminium resistance of crops on low-fertility acid soils. Annual report 2001. Centro Internacional de Agricultura Tropical CIAT. A.A 6713, Cali, Colombia

ICRISAT, NARS and CIAT. 2000a. A research and network strategy for sustainable sorghum production systems for Latin America. Ninth season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

ICRISAT, NARS and CIAT. 1999b. A research and network strategy for sustainable sorghum production systems for Latin America. Eighth season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

ICRISAT, NARS and CIAT. 1999a. A research and network strategy for sustainable sorghum production systems for Latin America. Seventh season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

ICRISAT, NARS and CIAT. 1998b. A research and network strategy for sustainable sorghum production systems for Latin America. Sixth season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

ICRISAT, NARS and CIAT. 1998a. A research and network strategy for sustainable sorghum production systems for Latin America. Fifth season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

ICRISAT, NARS and CIAT. 1997b. A research and network strategy for sustainable sorghum production systems for Latin America. Fourth season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

ICRISAT, NARS and CIAT. 1997a. A research and network strategy for sustainable sorghum production systems for Latin America. Third season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

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Rangel AF, Thomas RJ, Decaens T., Jiménez JJ, Lavelle P and Madero E. 1996. Effect of Earthworms on Soil Fertility, in Soils and plant Nutrition Unit, Project # 10. Productive and regenerative agricultural systems for marginal and degraded soils of tropical Latin America. Centro Internacional de Agricultura Tropical (CIAT).

ICRISAT, NARS and CIAT. 1996a. A research and network strategy for sustainable sorghum production systems for Latin America. First season report. Patancheru 502 324, Andhra Pradesh, India: ICRISAT. (Limited distribution)

Rangel AF y Madero E. 1994. Determinación del punto de carga cero (ZPC) de dos suelos de carga variable. Problema especial. Universidad Nacional de Colombia, Facultad de Ciencias Agropecuarias, Palmira.