RESEARCH PAPER

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

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Running title: Citrate production and exudation and its relationship to Al resistance

Abstract

Two common bean (*Phaseolus vulgaris* L.) genotypes differing in aluminum (Al) resistance, Quimbaya (Al-resistant) and VAX-1 (Al-sensitive) were grown in hydroponics for up to 25 h with or without Al, and several parameters related to the exudation of organic acids anions from the root apex were investigated. Aluminum treatment enhanced the exudation of citrate from the root tips of both genotypes. However, its dynamic offers the most consistent relationship between Al-induced inhibition of root elongation and Al accumulation in and exclusion from the root apices. Initially, in both genotypes the short-term (4 h) Al injury period was characterized by the absence of citrate efflux independent of the citrate content of the root apices, and reduction of cytosolic turnover of citrate conferred by a reduced NADP-isocitrate dehydrogenase (EC 1.1.1.42) activity. Transient recovery from initial Al stress (4-12 h) was found to be dependent mainly on the capacity to utilize internal citrate pools (Al-resistant genotype Quimbaya) or enhanced citrate synthesis (increased activities of NAD-malate dehydrogenase (EC 1.1.1.37) and ATP-phosphofructokinase (EC 2.7.1.11) in Al-sensitive VAX-1). Sustained recovery from Al stress through citrate exudation in genotype Quimbaya after 24 h Al treatment relied on restoring the internal citrate pool and the constitutive high activity of citrate synthase (CS) (EC 4.1.3.7) fuelled by high phosphoenolpyruvate carboxylase (EC 4.1.1.31) activity. In the Al-sensitive genotype VAX-1 the citrate exudation and thus Al exclusion and root elongation could not be maintained coinciding with an exhaustion of the internal citrate pool and decreased CS activity.

Key words: aluminum toxicity, organic acids, exudation, synthesis, root apex

Abbreviations

ACO	Aconitase
CS	Citrate synthase
EZ	Elongation zone
GFAAS	Graphite furnace atomic absorption spectrometer
HPLC	High pressure liquid chromatography
ICDH	Isocitrate dehydogenase
MDH	Malate dehydrogenase
Al_{mono}	Monomeric aluminum
MATE	Multidrug and toxic compound extrusion
OA	Organic acid
PEPC	Phosphoenolpyruvate carboxylase
PFK	Phosphofructokinase
PCV	Pyrocatechol violet
TZ	Transition zone
TCA	Tricarboxylic acid
DTNB	5, 5´-dithio-bis-2-nitrobenzoic acid

Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important grain legume for direct human consumption (Broughton et al. 2003). It 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.) (Rao, 2001). 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 aluminum (Al) toxicity, the most important soil factor limiting crop yields on acid soils (Wortmann et al. 1998, Rao et al. 1993, 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 2001, Rangel et al. 2005, Manrique et al. 2006, Beaver and Osorno 2009). 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 remains a major challenge.

Aluminum 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). The analysis of spatial growth profiles in common bean revealed that the initial inhibition of root elongation by Al resulted from a generalized effect along the entire elongation zone (EZ) (Rangel et al. 2007). Additionally, localized application of Al to specific zones of the root apex in common bean showed that 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, confirming previous studies with maize, reporting that the TZ is the most Al-sensitive apical root zone (Kollmeier et al. 2000). However, in contrast to maize, application of Al to the EZ also reduced root growth in both common bean genotypes, though to a lesser extent than when applied to the TZ, indicating that not only the TZ but the entire EZ need to be protected from the Al injury (Rangel et al. 2007).

Plant species and genotypes within species vary widely in resistance to Al, suggesting that Al-resistant species or genotypes posses several mechanisms to avoid Al toxicity (Taylor 1991). Mechanisms 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. 1998*a*). 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. 2002, 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. 1993, 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 (Rangel et al. 2007) have shown that both Al-resistant and Al-sensitive common bean genotypes initially (at 4 h after Al treatment) were equally sensitive to Al. Thereafter, the root growth recovered in the Al-resistant genotype while it remains 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. 2002, 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. 2002). 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. 1993). Contrary to this observation, in soybean a typical pattern II-plant species, an Al-enhanced internal 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, 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 citrate synthase (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 have been 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, Barone et al. 2008), canola (*Brassica napus* L., Anoop et al. 2003), and *Nicotiana bethamiana* (Deng et al. 2009). Therefore, the main objective of the present study was to determine the effects of Al treatment (short- and medium-term) on the dynamics 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. Additionally, the effect of Al on some key enzymes of OA metabolism was also determined.

Materials and methods

Plant material and growth conditions

Seeds of two common bean genotypes with known differential Al resistance (Rangel et al., 2005) were used in this study. Seeds of the Al-resistant (Quimbaya) and Al-sensitive (VAX-1) genotypes kindly supplied by the Bean Program of CIAT (International Center for Tropical Agriculture, Cali, Colombia) 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). Plants 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 through 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 of ultra-pure deionized water and then digested in 500 ml of ultra-pure HNO_3 (65%) overnight on a rotary shaker. To complete the digestion, samples were incubated in a water bath at 80^{o}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 ultra-pure deionized water.

Collection of root exudates from intact root apices

To collect root exudates from intact root apices, twelve plants were bundled in filter paper soaked with nutrient solution. Approximately 1 cm of the main root apex of each plant was 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 (time dependent) 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 at 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 for 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 was immediately frozen at -20^{o} C for later determination. After each incubation period, the root tips (10 mm) were excised with a razor blade, washed with ultra-pure double deionized water, transferred to Eppendorf cups, and fixed immediately in liquid nitrogen to measure OA contents.

Determination of OA in root exudates and root apices

After OA collection, the incubation solution containing the OA was thawed and 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 and then poured into 20 ml poly-prep columns with a 200-400 mesh filter at the bottom of the column. The resulting solution containing the 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 filter tubes with a membrane pore size of 0.45 µm (GHP Nanosep® MF Centrifugal Device, Pall Life Sciences, Ann Arbor, USA), and centrifuged (10,000 g) 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). 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) at a speed of 20 oscillations per second for 3 min. Organic acids were extracted at 75^{o}C for 1 h with intermittent shaking in a vortex at every 15 minutes. Thereafter, the samples were centrifuged at 20,000 g for 10 min and the supernatant was transferred into a new Eppendorf cup. 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 into centrifugal filter tubes with a membrane pore size of 0.45 µm (GHP Nanosep® MF Centrifugal Device, Pall Life Sciences, Ann Arbor, USA), and centrifuged (10,000 g) 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 λ = 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 °C) cup holders of a micro-homogenizer (MM200 Retsch, Haan, Germany) at a speed of 20 oscillations per second for 3 min. 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 was centrifuged at 20,000 g for 5 min at 4°C, and the supernatant was used to determine 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 of malate dehydrogenase. The reaction was initiated by the addition of the enzyme extract, and the NADH oxidation was determined 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} of aldolase, 1 unit ml^{-1} of triose-P isomerase, 6 units ml^{-1} of α-glycerol-P dehydrogenase, and 0.5 mM ATP. The reaction was initiated with the addition of 180 µl of the enzyme extract. Reaction was monitored at 340 nm for 3 min, following the method of Carnal and Black (1983). All previously described enzymes were assayed using 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) according to 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 statistical program SAS 9.1 (SAS Institute, Cary, NC, USA) was used for analysis of variance. Means were compared using the Tukey test. *, **, ***, n.s. denote 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. 1A). 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. 1B) reflect 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 of Al contents of the root apices of Quimbaya while in VAX-1 the Al contents started to increase again after 8 h.

Fig. 1

Since an Al-induced release of OA-anions has been implicated in Al resistance of common bean and other plant species (see introduction), the cumulative OA-anion exudation from 10 mm root tips treated with Al for 10 h was characterized in a first approach (Fig. 2). 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 cumulative 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. The kinetics of the exudation of these OA-anions showed 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 (Fig. 3). The exudation rates of malate and oxalate were stimulated by Al 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 of Al treatment.

Fig. 2

Fig. 3

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. 4). In agreement with results from Fig. 2, citrate exudation (Fig. 4A) 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, $P < 0.001$). Partially in agreement with the observation in Fig. 3, succinate exudation was enhanced after a 3-5 h lag period (Fig. 4B) not only in Quimbaya, but also in VAX-1, however, to a lesser extent, and decreased to a lower level thereafter.

Fig. 4

Aluminum-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. 5). 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 first 4 h of Al treatment and thereafter in both genotypes reaching a minimum after 9 h of Al treatment. Then, the OA contents recovered reaching the original levels (no Al treatment) in Quimbaya after 25 h. In genotype VAX-1 this recovery was only transient and less marked before the contents reached very low levels after 25 h of Al treatment. This general pattern of response of OA levels to Al treatment was particularly expressed for citrate, malate, oxalate and fumarate.

Fig. 5

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. 6). 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.

Fig. 6

Since the exudation of OA anions (Figs. 3 and 4) and the internal OA contents (Fig. 5) clearly showed differences between the genotypes and the Al treatment duration, we determined activities related to OA synthesis and degradation (Fig. 7). 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. Aluminum 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 and NAD-MDH were also reduced by Al treatment, but they recovered after longer Al treatment. In genotype VAX-1, CS activity remained constant at a low level and was further reduced after 8 h Al treatment. In contrast, Al treatment consistently enhanced the activities of NAD-MDH and ATP-PFK while PEPC was only enhanced after exposure to Al for longer duration (> 8 h).

Fig. 7

Discussion

In this study, the effect of Al treatment on the dynamics of root elongation and Al accumulation in root apices of the two genotypes differing in Al-resistance (Fig. 1) was in agreement with our previous results (Rangel et al. 2007, 2009) and with an Al-induced Al resistance mechanism in common bean (Cumming et al. 1992). According to these results 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 (Rangel et al. 2009); (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). Collectively, these results suggest an Al-exclusion mechanism acting in common bean after the initial inhibition of root elongation.

Constitutively, higher total internal OA-contents, particularly succinate, citrate, aconitate and pyruvate were observed in genotype Quimbaya compared to Al-sensitive VAX-1 (Fig. 5). This could be a result of differences in the gene pools (Quimbaya is Andean and VAX-1 is Mesoamerican) of different origin (Gepts et al. 1986, Singh 1989) leading to higher constitutively expressed biosynthetic capacity of organic acids through greater CS and PEPC activities (Fig. 7). White and Gonzalez (1990) found that large-seeded Andean genotypes (such as Quimbaya) possess larger cell volumes in different tissues compared to the small-seeded Mesoamerican (such as VAX-1). Constitutively higher OA-contents of the Al-resistant common bean genotype Dade compared to the Al-sensitive Romano have been suggested to contribute to a higher potential for Al chelation and detoxification (Lee and Foy 1986, Miyasaka et al. 1991).

However, in spite of the higher OA contents of the root apices of Quimbaya than of VAX-1 (Fig. 5), both genotypes were equally Al-sensitive during the first 4 h of Al treatment (Fig. 1A). This can be attributed to low exudation of OAs particularly citrate and succinate which did not differ between the genotypes (Fig. 3). The lack of enhanced OA anion exudation after short-term exposure of the root apices to Al in spite of high OA contents in the root tips could be due to either an inhibition of anion permeases in the plasma membrane that control OA anion transport from the cytoplasm to the apoplast, or due to a low citrate concentration in the cytosol owing to sequestration of the OAs in other symplastic compartments such as the mitochondria and the vacuoles.

Thus, recovery from the initial Al injury depends on the capacity to release organic acid anions (Figs. 3 and 4A) mediated by anion permeases. The importance of anion permeases facilitating the efflux of OA in Al resistance has been well documented (reviewed by Delhaize et al. 2007). However, the response differs with plant species, with regard to OA species, secretion patterns, temperature sensitivity and dose response (Ma and Furukawa 2003). Shen et al. (2004) provided circumstantial evidence for the existence of a plasma-membrane anion-channel mediating Al-enhanced citrate

exudation in common bean which was inhibited by the action of the anion-channel inhibitor anthracene-9-carboxylic acid and K-252a, a broad range inhibitor of protein kinases.

Aluminium treatment increased the cumulative total OA anions secreted to the same extent in both genotypes (Fig. 2). 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 particularly for succinate, malate and oxalate. The observed Al-induced secretion of malate and oxalate in VAX-1 (Fig. 3) was in agreement with the reduction of oxalate and malate contents after 5 h of Al treatment (Fig. 5). 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. 1A) and was not sustained over time. Similarly in soybean, Al treatment also stimulated a transient secretion of malate during the first 6 h of root exposure, and sharply decreased thereafter without any contribution to Al resistance (Silva et al. 2001).

Succinate was exuded following the same exudation pattern as citrate (Figs. 3 and 4). Aluminium-induced succinate exudation in addition to malate or citrate in Al-resistant genotypes of different plant species has also been reported. However, considering the Al-detoxifying capacity of different OA anions, it can be assumed that succinate will not contribute much to Al resistance (Hue et al. 1986); hence citrate exudation offers the most consistent explanation for the effects observed in root elongation of both genotypes during the Al treatment (Fig. 1A).

Aluminum-enhanced citrate exudation has been previously implicated in Al resistance of common bean (Miyasaka et al. 1991, Mugai et al. 2000, Shen et al. 2002, Rangel and Horst 2006, Stass et al. 2007). The citrate exudation pattern observed in both common bean genotypes is characteristic for Pattern II plants with a 4-5 h lag phase corresponding to the maximum period of root-growth inhibition (Fig. 1A) before the onset of enhanced exudation of citrate in both genotypes during the recovery period (Figs. 3 and 4). Thereafter, citrate exudation was enhanced in the Al-resistant genotype Quimbaya, while it was reduced in the Al-sensitive genotype VAX-1. Similar patterns of exudation have been observed with soybean (Yang et al. 2001, Silva et al. 2001), rye (Li et al. 2000), *Cassia tora* (Ma et al. 1997), sorghum (*Sorghum bicolor*, Magalhaes et al. 2007), Arabidopsis (Lui et al. 2009) and *Citrus junos* (Deng et al. 2009).

In common bean, there is overall agreement that citrate exudation follows a Pattern II response (Mugai et al. 2000, Shen et al. 2002) which is consistent with the Al-induced expression of Al resistance genes (Kochian et al. 2004). In contrast to Pattern I plant species, in Pattern II plant species the genes coding for the anion permeases are just being identified. Recently, three genes (*HvMATE, SbMATE, and AtMATE*) belonging to the multidrug and toxic compound extrusion family (MATE) have been shown to be associated with Al-activated citrate secretion conferring Al resistance in barley (*Hordeum vulgare*, Furukawa et al. 2007), sorghum (Magalhaes et al. 2007) and Arabidopsis (Liu et al. 2009). Fontecha and coworkers (2007) detected an Aluminum-Activated-Malate-Transporter (*ALMT*) homologue of the *TaALMT1* that was characterized in wheat (Sasaki et al. 2004) and showed in

common bean at least 72% similarity to the wheat sequence, however, its role in Al resistance in common bean remains unknown.

In Pattern I plant species the Al-induced release of OA anions neither depends on internal OA-contents nor on gene induction (Delhaize et al. 1993, Yang et al. 2006), but on the activation of a plasma membrane anion-channel (Delhaize et al. 2007). In Pattern-II species, the exudation of OA anions was related to internal OA contents (Lee and Foy 1986, Silva et al. 2001), induction of genes related to OA synthesis and catalysis (Li et al. 2000, Yang et al. 2004), activation of anion channels in plasma (Ma et al. 2000, Magalhaes et al. 2007, Liu et al. 2009) or mitochondrial (Yang et al. 2006) membranes. In our study with common bean, Al 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. 5). 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 in 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 reduced 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). However, in triticale, also classified as Pattern-II species (Ma et al. 2000) the levels of citrate in root tips increased during the exposure to Al in both Al-resistant and Al-sensitive genotypes (Hayes and Ma 2003).

In this study, after the initial lag period (4h), Al-induced citrate exudation was switched on earlier in genotype VAX-1 than in Quimbaya (Fig. 3, 4A) indicating a more rapid expression of anion permeases or increase in cytosolic citrate concentration driving the citrate release. Increased accumulation of citrate in the cytosol can be mediated by reduced activity of the enzymes aconitase (ACO) and isocitrate dehydrogenase (NADP-ICDH) that are involved in citrate turnover (Massonneau et al. 2001, Anoop et al. 2003, Fig. 8). Aluminium treatment rapidly reduced the activity of NADP-ICDH (Fig. 7) in both genotypes, suggesting a rapid response leading to citrate accumulation in the cytosol. Phosphorus deficiency-induced citrate exudation was related to reduce NADP-ICDH activity in lupin (*Lupinus albus* L., Kania et al., 2003, Kihara et al., 2003*a*) and carrot cells (*Daucus carota* L., Takita et al. 1999, Koyama et al. 1999, Kihara et al. 2003*b*). Transformed yeast (*Saccharomyces cerevisiae*) defective in ACO and NADP-ICDH action showed more accumulation and exudation of citrate and were less Al-sensitive than the wild type (Anoop et al. 2003). However, in tobacco the down-regulation of NADP-ICDH and up-regulation of citrate synthase enhanced citrate accumulation but not citrate exudation (Delhaize et al. 2003) indicating that citrate accumulation in the cytosol and citrate exudation are independently controlled processes with the latter being the decisive step for Al resistance.

Fig. 8

Both genotypes were able to recover from the initial Al injury (Fig. 1A) coinciding with an enhanced exudation of citrate (Fig. 4A). A quantitative comparison per root tip of the change in citrate content (Fig. 5) with the enhanced citrate exudation during the same period (Fig. 4A) revealed that in Quimbaya the enhanced citrate exudation between 4 and 8 h of Al treatment could be maintained for at least two hours through the constitutive large internal pool of citrate. Therefore, in Quimbaya there seems to be less need to up-regulate citrate synthesis through CS (Fig. 7) particularly at the constitutively higher activity level than in VAX-1. However, in VAX-1 with a much lower citrate internal pool size (Fig. 5) the initially enhanced citrate exudation (Fig. 4A) can only be maintained by this pool for less than one hour. Thus the enhanced citrate exudation between 4-16 h Al treatment can only be maintained by VAX-1 by up-regulating citrate synthesis through maintaining CS activity and enhancing malate dehydrogenase (NAD-MDH) and phosphofructokinase (ATP-PFK, Fig. 7) which is the key enzyme channeling assimilates from glycolysis to the TCA cycle (Carnal and Black 1983, Stryer 1988, Fig. 8). After 24 h Al treatment the release of citrate can no longer be sustained in VAX-1 (Fig. 4A) owing to reduced CS activity (Fig. 7). In contrast Quimbaya is capable of resuming CS (Fig. 7) and increasing NAD-MDH activities thus maintaining a high citrate release necessary to prevent Al from accumulation in the root apex (Fig. 1B).

A key role of CS in citrate synthesis in roots and citrate exudation 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 (Takita et al. 1999, Koyama et al. 1999, Kihara et al. 2003*a*). Enhanced CS activity by Al treatment has been observed in soybean (Yang et al. 2001) and rye (Li et al. 2000), where the increased CS activity coincided with the beginning of citrate exudation. Aluminum-enhanced citrate exudation related to an apparently higher CS activity has been previously reported in common bean (Mugai et al. 2000). Aluminum-induced citrate exudation driven by Al-inducible expression of mitochondrial CS enhancing Al resistance in *Paraserianthes falcaria* (L.) Neilson, a leguminous tree, was reported: Al treatment increased the accumulation of mitochondrial CS transcripts, its activity and gene expression (Osawa and Kojima 2006). Recently, the over-expression of the mitochondrial CS gene from *Citrus junos* conferred Al resistance in *Nicotiana bethaminana* (Deng et al. 2009).

Since the TCA cycle requires stoichiometric parity between acetyl Co-A and oxaloacetate to proceed, removal of oxaloacetate or any of its precursors disrupts the cycle (Hill 1997, Fig. 8). 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,

Fig.8). Root isoforms 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). Aluminum 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. 7). However, the constitutively higher PEPC activity in Quimbaya might suggest a more efficient anaplerotic function than in VAX-1. This assumption is supported by the fact that in VAX-1 the enhanced citrate exudation triggers the activities of particularly PFK (see above). A role of PEPC in Al resistance of Al-induced citrate-exuding Al-resistant soybean genotypes has been demonstrated by Ermolayev et al. (2003).

In conclusion, the kinetics of citrate exudation from root tips offers the most consistent explanation for the response in root elongation and Al uptake of both genotypes of common bean to Al treatment. The Al-induced citrate exudation pattern is characteristic for a Pattern-II response, with a 4-5 h lag phase, which corresponds to the period of maximum Al accumulation in the root apices and root-growth inhibition. Initial Al-dependent efflux of citrate from the root tips in both genotypes is not regulated by the internal citrate levels in the roots but requires the expression of an organic anion permease in the plasma membrane. Transient recovery from initial Al stress was found to be dependent on the capacity to utilize internal citrate pools (Al-resistant genotype Quimbaya) or enhance citrate synthesis (Al-sensitive VAX-1) associated with a reduced cytosolic turnover of citrate. However, sustained recovery from Al stress in the Al-resistant genotype relied on the synthesis of citrate particularly via PEPC. The results observed and the conclusions drawn from this study need to be substantiated through the analysis of the transcription of those genes coding for the proteins/enzymes involved.

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Figure Legends

Fig. 1. 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. Average root-elongation rates under control conditions were 2.5 ± 0.3 and 2.38 ± 0.1 for Quimbaya and VAX-1, respectively. 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); * on top of data points denote significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Fig. 2. 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.

Fig. 3. 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.

Fig. 4. 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. 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. Fof the ANOVA **, *** denote probability levels at $P < 0.01$ and 0.001. Means with the same letter are not significantly different between exudation periods for Quimbaya (capital) and VAX-1 (small); * on top of data points denote significant differences between genotypes within each treatment time (Tukey test $P < 0.05$)

Fig. 5. 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); * on top of columns denote significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Fig. 6. 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. For the ANOVA *** denotes 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); * on top of columns denote significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Fig. 7. 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. Fot the ANOVA *, *** 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); * on top of data points denote significant differences between genotypes within each treatment time (Tukey test $P < 0.05$).

Fig. 8. Schematic presentation of key enzymes involved in citrate biosynthesis (CS, PEPC, MDH) or degradation (ICDH) and the supply of C skeletons to the TCA cycle (PFK, PEPC, NAD malic enzyme) that might contribute to accumulation in and secretion of citrate from the roots of common bean (*Phaseolus vulgaris* L.). NAD malic enzyme is shown in the scheme although it was not assayed in our experiments. Dashed lines show substrates (ATP and citrate) that negatively control the activity of PFK. Dots at the membranes represent transporters, mostly antiporters that transport two substrates. However, to simplify the scheme, only one substrate is shown according to their importance in the discussion of this paper. Cytoplasm (c) or mitochondrial (m) isoenzymes of ICDH and MDH are shown according to their location. Bold letters represent enzymes and substrates directly involved in Al responses.

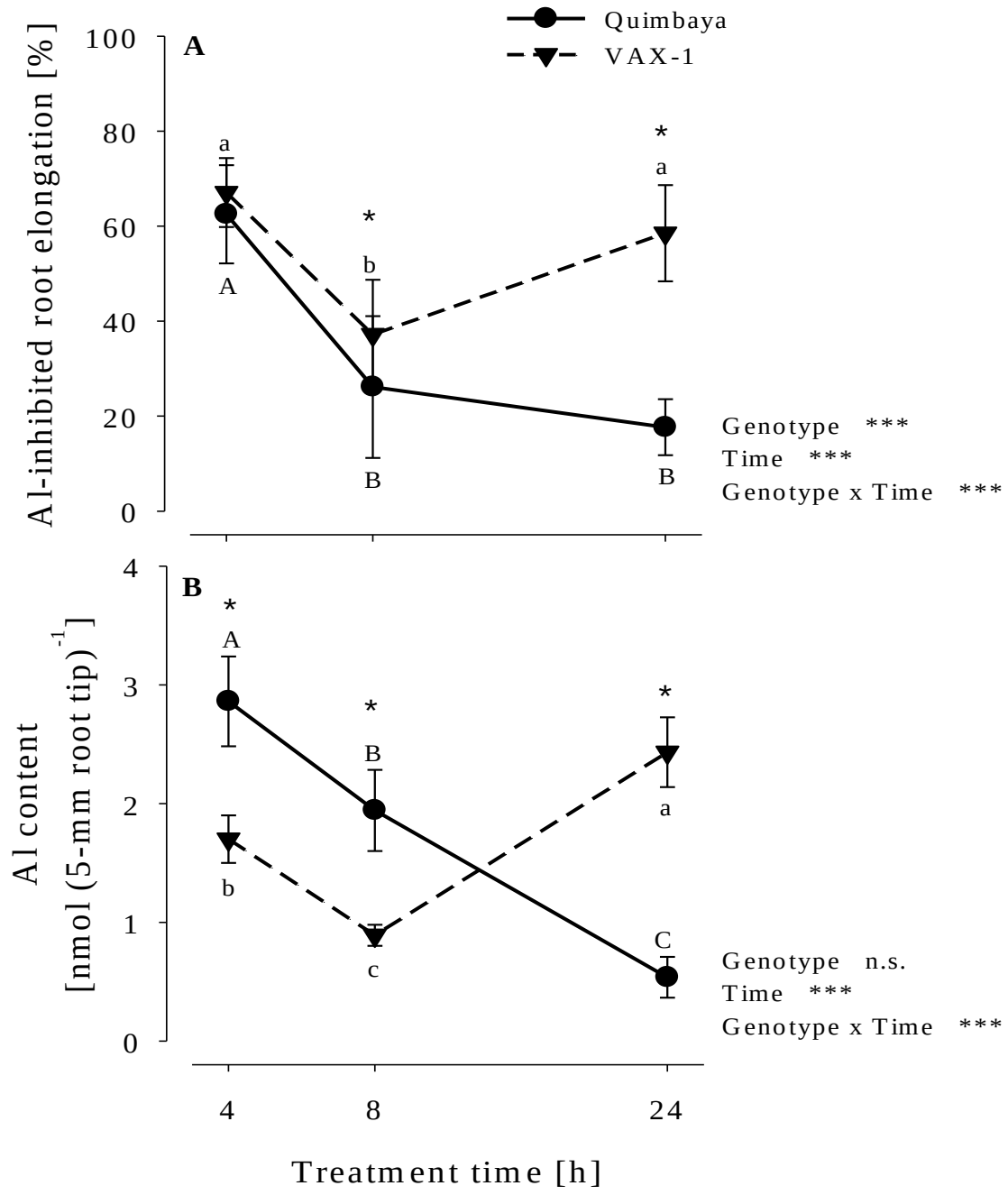
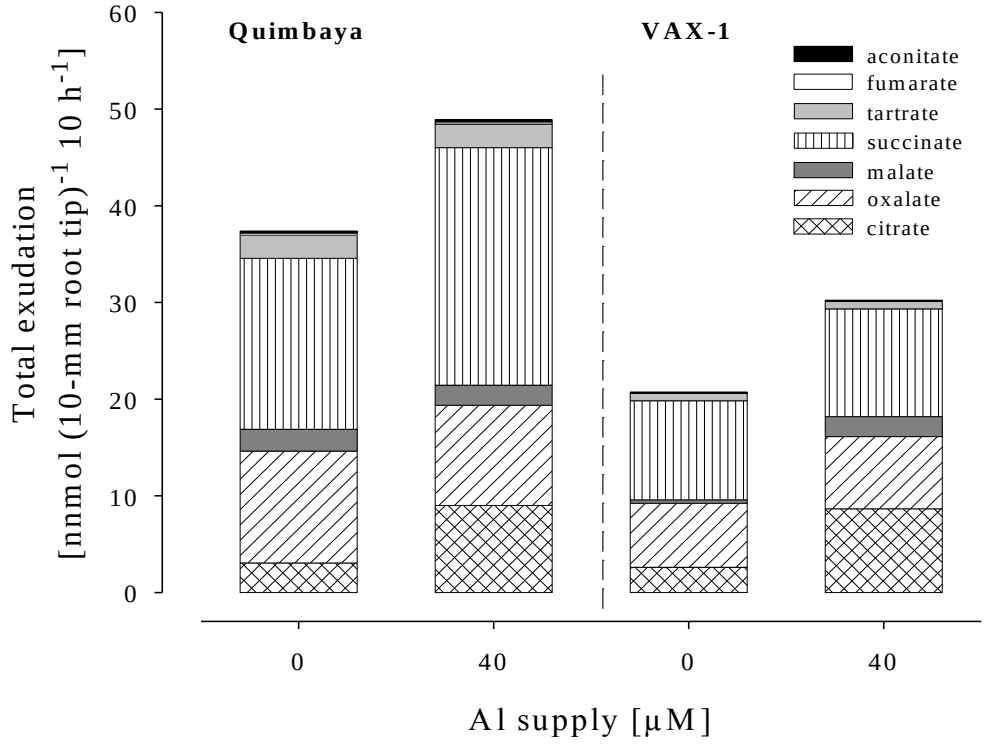
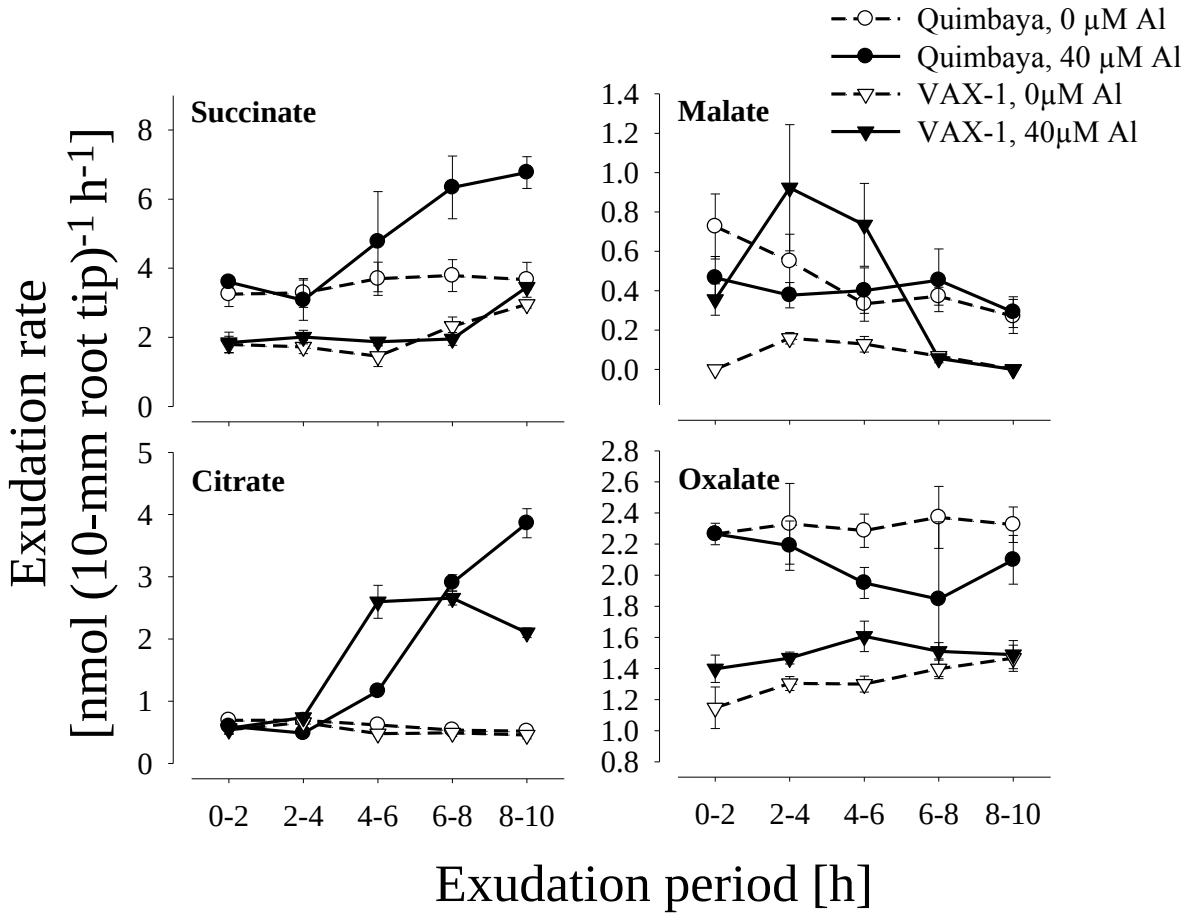

Quimbaya
VAX-1
A
Al-inhibited root elongation [%]
100
80
60
40
20
0
a
b
a
A
B
B
*
*
*
Genotype ***
Time ***
Genotype x Time ***
B
Al content
[nmol (5-mm root tip)-1]
4
3
2
1
0
A
B
C
a
b
c
*
*
*
Genotype n.s.
Time ***
Genotype x Time ***
4
8
24
Treatment time [h]

Fig. 1



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

Fig. 2



Source ANOVA	Succinate	Citrate	Malate	Oxalate
Genotype (Gen)	***	**	***	***
Al treatment (Treat)	***	***	***	n.s.
Exudation period (Time)	***	***	***	n.s.
Gen x Treat	***	n.s.	***	***
Gen x Time	***	***	***	*
Treat x Time	***	***	***	n.s.
Gen x Treat x Time	***	***	***	n.s.

Fig. 3

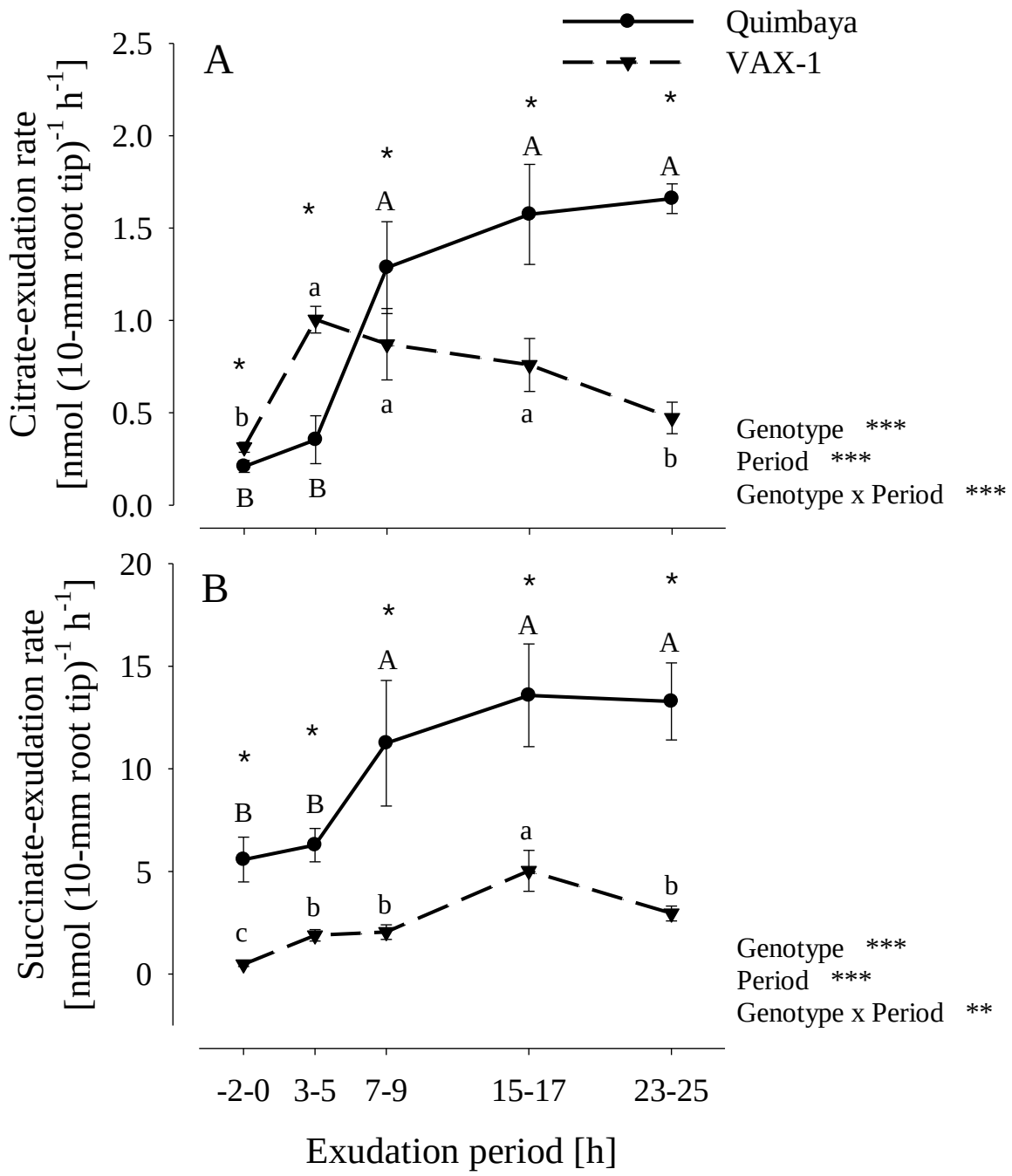

Quimbaya
VAX-1
A
B
Citrate-exudation rate [nmol (10-mm root tip)-1 h-1]
Succinate-exudation rate [nmol (10-mm root tip)-1 h-1]
Genotype ***
Period ***
Genotype x Period ***
Genotype ***
Period ***
Genotype x Period **
-2-0
3-5
7-9
15-17
23-25
Exudation period [h]

Fig. 4

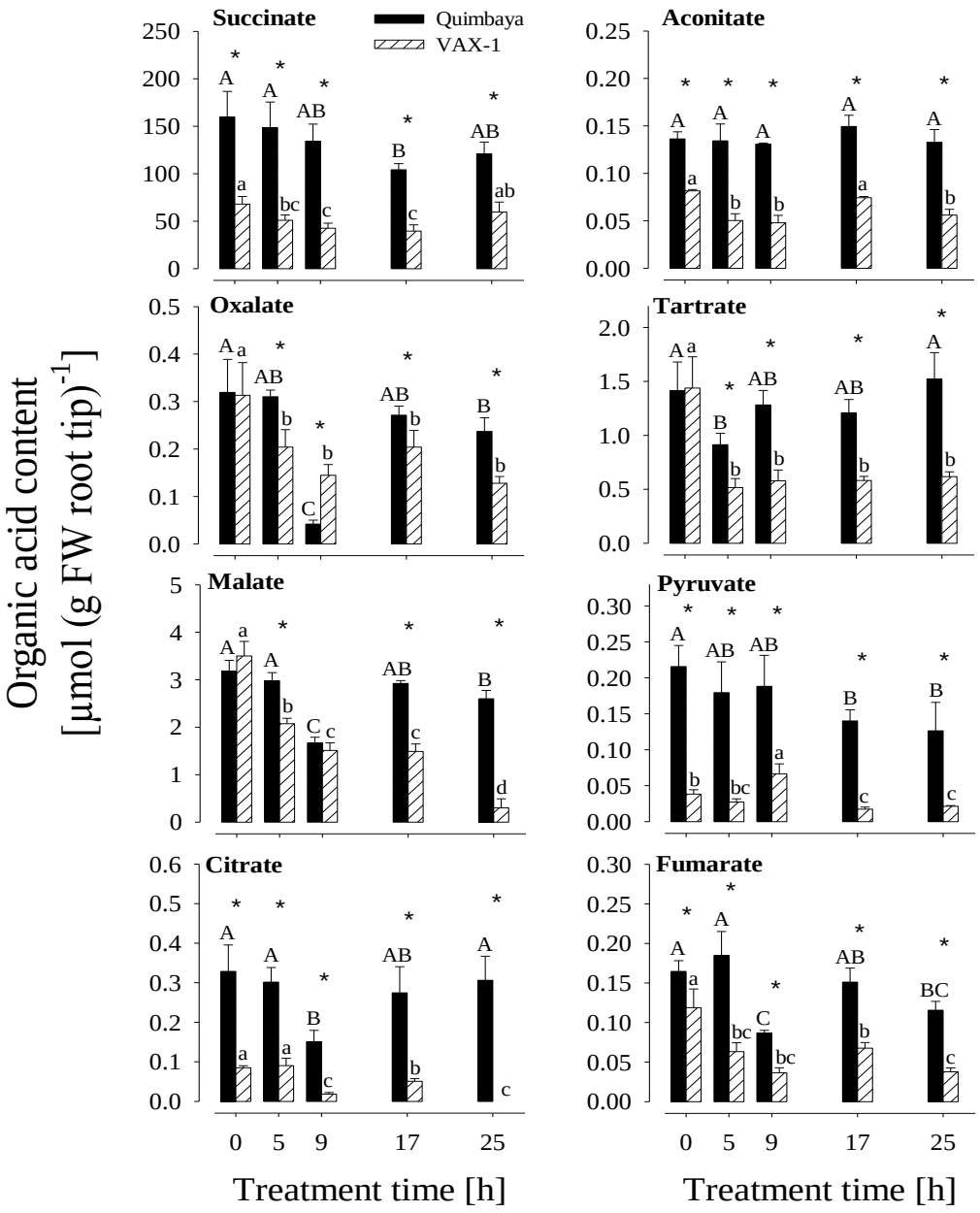
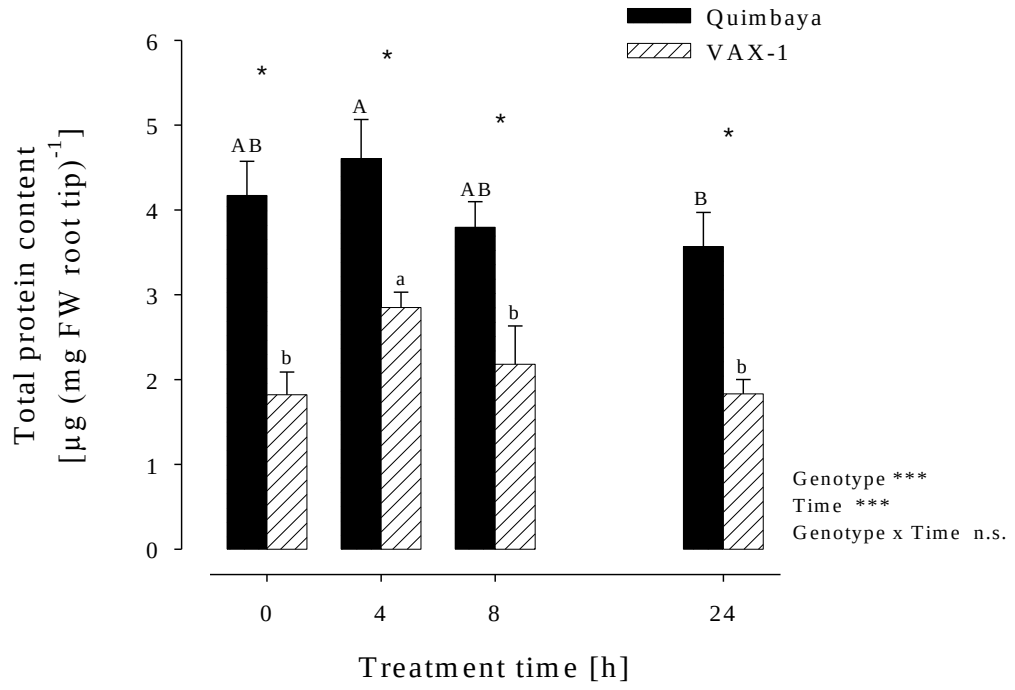

Succinate
Quimbaya
VAX-1
Aconitate
Oxalate
Tartrate
Malate
Pyruvate
Citrate
Fumarate
Organic acid content
[µmol (g FW root tip)-1]
Treatment time [h]
Treatment time [h]

Fig. 5

1

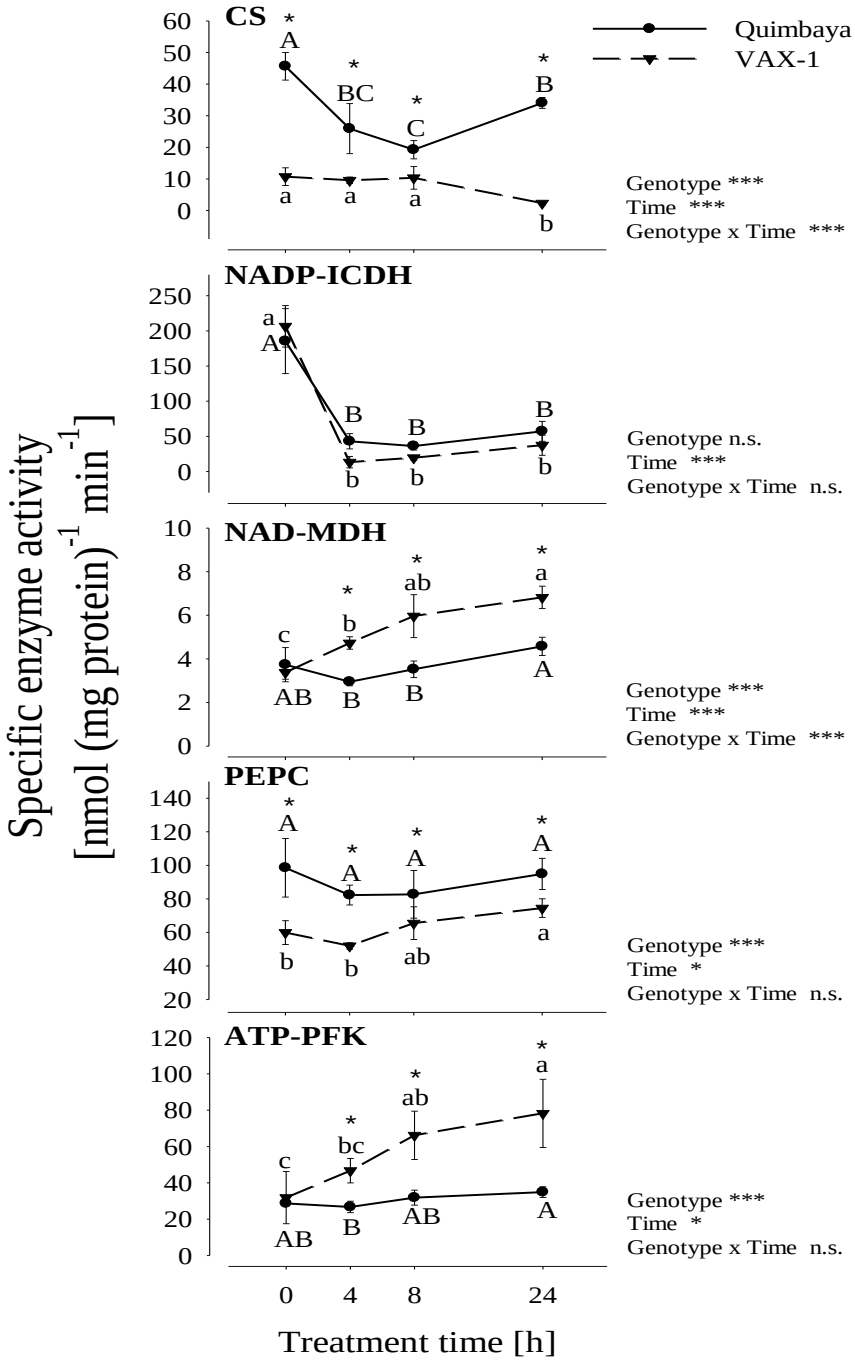

Quimbaya
VAX-1
Total protein content
[µg (mg FW root tip)-1]
6
5
4
3
2
1
0
AB
b
A
a
AB
b
B
b
*
*
*
*
Genotype ***
Time ***
Genotype x Time n.s.
0
4
8
24
Treatment time [h]

2

3

4 Fig. 6

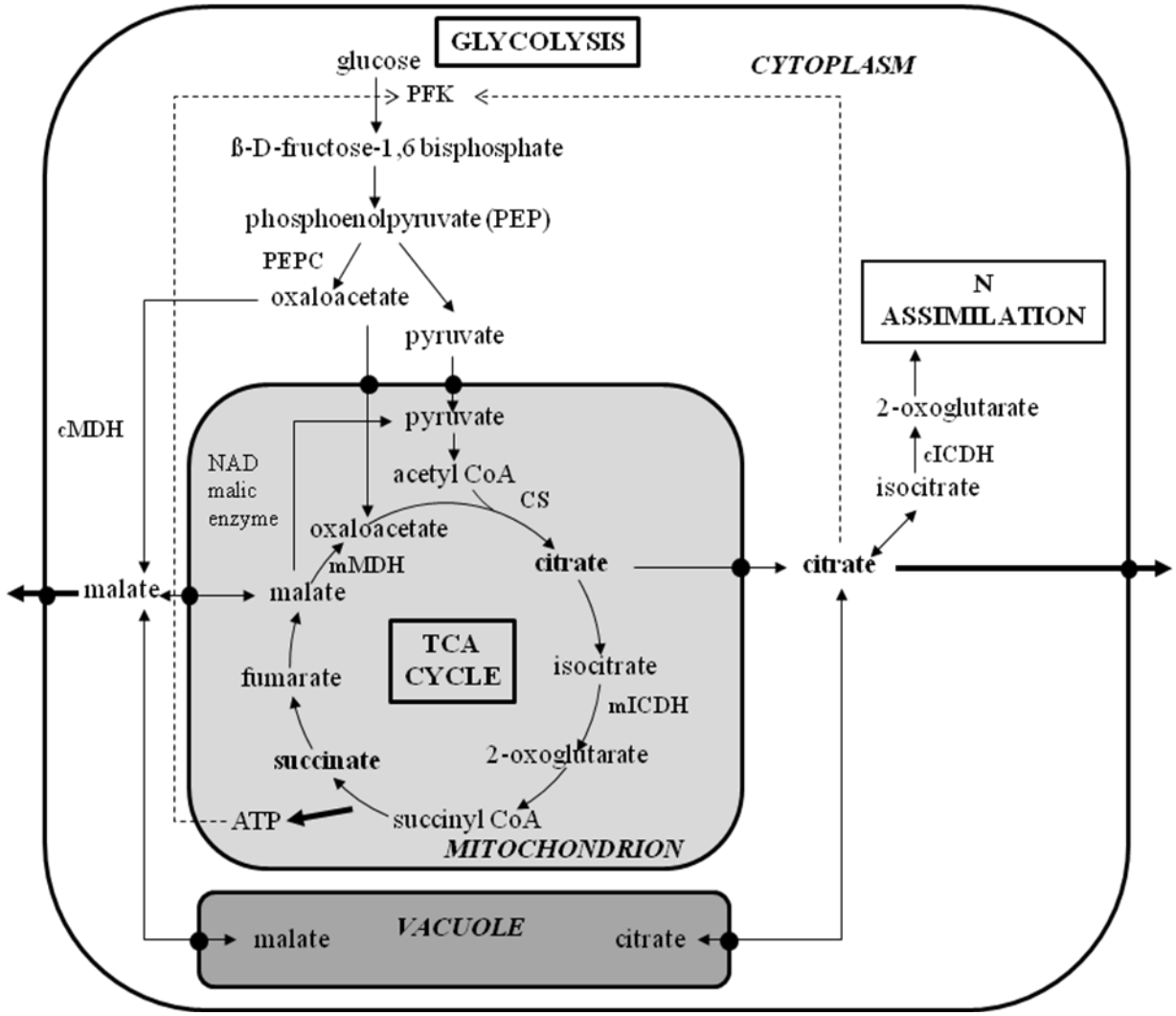
5


CS
NADP-ICDH
NAD-MDH
PEPC
ATP-PFK
Quimbaya
VAX-1
Genotype ***
Time ***
Genotype x Time ***
Genotype n.s.
Time ***
Genotype x Time n.s.
Genotype ***
Time ***
Genotype x Time ***
Genotype ***
Time *
Genotype x Time n.s.
Genotype ***
Time *
Genotype x Time n.s.
Specific enzyme activity
[nmol (mg protein)-1 min-1]
Treatment time [h]
0
4
8
24

1

2 Fig. 7

3


GLYCOLYSIS
glucose
CYTOPLASM
PFK
ß-D-fructose-1,6 bisphosphate
phosphoenolpyruvate (PEP)
PEPC
oxaloacetate
pyruvate
N
ASSIMILATION
2-oxoglutarate
cICDH
isocitrate
cMDH
pyruvate
NAD
malic
enzyme
acetyl CoA
CS
oxaloacetate
mMDH
citrate
malate
malate
citrate
TCA
CYCLE
isocitrate
mICDH
fumarate
succinate
2-oxoglutarate
ATP
succinyl CoA
MITOCHONDRION
malate
VACUOLE
citrate

1

2 Fig. 8