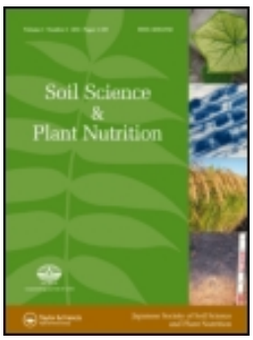

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Aluminium Tolerance Mechanisms in *Phaseolus vulgaris* L.: Citrate Synthase Activity and TTC Reduction Are Well Correlated with Citrate Secretion

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We characterised the physiological mechanisms of tolerance in two bean varieties (*Phaseolus vulgaris* L. viz. French Bean cv. Amy and Rosecoco (GLP2)) differing in Al tolerance of the varietal level. Root elongation at varying levels of Al over time clearly showed the Al tolerance superiority of Rosecoco over French bean. Aluminium uptake was much higher in French bean, in both root apex and 2 mm region of the root apex. The root cation exchange capacity of the Al-sensitive French bean was markedly higher than that of Rosecoco. Citric acid was the only organic acid whose secretion was stimulated by Al and was higher in Rosecoco than in French bean. The citrate synthase and $NADP^+$-isocitrate dehydrogenase activities were apparently higher in Rosecoco than in French bean but those of phosphoenolpyruvate carboxylase were not significantly different between the two varieties under Al stress. Triphenyl-tetrazolium chloride (TTC) reduction was greater in Rosecoco and was also well correlated with the citrate secretion. These results suggest the role of TTC reduction and citrate secretion as underlying factors in the Al tolerance mechanism of Rosecoco.

Key Words: **aluminium tolerance, CEC, citric acid, *Phaseolus vulgaris*, TTC reduction.**

Aluminium (Al) toxicity is a major constraint on crop production in acid soils which account for about 40% of the world's arable land (Foy et al. 1978; Taylor 1988). However the mechanism of Al toxicity has not yet been elucidated (Rengel 1992; Delhaize and Ryan 1995; Kochian 1995; Taylor 1995; Matsumoto 2000). Plant species and cultivars vary in Al tolerance and this aspect can be utilized in the selection of crops for acid soils (Foy et al. 1967). Plant breeders may introduce genes of Al-tolerant genotypes to the sensitive ones since Al tolerance is genetically controlled (Foy et al. 1978; Aniol and Gustafson 1984; Carver et al. 1988). However an understanding of the mechanisms of Al injury and tolerance is a prerequisite in the process of selection and breeding. There is however limited information on how Al affects the common bean (*Phaseolus vulgaris* L.), which is a major crop in East Africa.

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Al toxicity is primarily expressed through the inhibition of root growth (Foy et al. 1967, 1978). Al uptake has also been found to be higher in Al-sensitive varieties of wheat (Zhang and Taylor 1990; Rincón and Gonzales 1991). The capacity of plants to accumulate Al in roots, and subsequently lead to a higher Al sensitivity, has been associated with the capacity of roots to electrostatically attract Al and hold it in the Donnan free space in the apoplasm, i.e. cation exchange capacity (CEC) (Blamey et al. 1990, 1992). This area has not received much attention in the studies on Al tolerance.

Cumming et al. (1992) and de Lima and Copeland (1994) investigated the Al effect on respiration in roots. The results varied depending on the conditions and experimental design. Direct assessment of the mitochondrial activity might be better correlated with Al-tolerance.

One of the hypotheses for Al tolerance in plants is the chelation and subsequent detoxification of Al by organic acids secreted by roots into the rhizosphere (Foy 1988; Taylor 1988). Miyasaka et al. (1991) reported that *P. vulgaris* cultivars differed in citric acid exudation. The Al-tolerant Dade cultivar secreted ten times more citric acid than the Al-sensitive Romano cultivar.

Citric acid secreted by the roots is most likely derived from the tricarboxylic acid cycle (TCA). Therefore, measurements of the enzymes related to citric acid synthesis and degradation may be correlated with citric acid exudation by the roots and subsequently with the Al tolerance of plants. This area has not received much attention except for the work of Copeland and de Lima (1992) and Li et al. (2000) in wheat, and wheat and rye, respectively.

In the current studies, we compared the Al tolerance mechanisms of two *P. vulgaris* varieties that differed in Al tolerance (unpublished): Rosecoco (GLP2), an Al-tolerant field bean, and an Al-sensitive French bean (snap bean), cv. Amy. Rosecoco had been selected from bean genotypes introduced into East Africa about 300 y ago and the French bean "Amy" had been bred in Holland and is currently grown in Kenya as a horticultural crop for export to the European markets. We found that the low CEC and high citrate secretion are the underlying factors in the higher Al tolerance mechanism of Rosecoco compared with French bean.

MATERIALS AND METHODS

Materials. Rosecoco bean (GLP2) seeds were supplied by the Kenya Seed Company and the "Amy" French bean seeds by the Royal Sluis Company (Holland) outlet in Nairobi, Kenya.

Growth of plants. Seeds were washed with tap water to remove the insecticide powder, rinsed with deionized water and then sterilized with 2% sodium hypochlorite for 10 min. The sodium hypochlorite was washed with running tap water and the seeds were rinsed with deionised water. The seeds were soaked in water for 6 h and then germinated in petri dishes under darkness at 25°C for 3 d. Seedlings with a uniform size were transferred to aerated solutions containing 0.5 mM $CaCl_2$ (pH 4.5) under a 14 h/25°C day and 10 h/20°C night regime at a light intensity of 40 W m^{-2}. On the third day, the seedlings were introduced to a new culture solution containing 100 μM $CaCl_2$ (pH 4.5) with the respective Al treatments for desired periods ranging from 0 to 24 h. The pH of the solutions was adjusted to pH 4.5 by either 1 N HCl or 1 N NaOH.

Seedlings for the collection of organic acid exudates and enzyme assay were transferred into 1-L plastic pots (4 seedlings per pot) containing aerated one-fifth strength Hoagland

nutrient solution, which consisted of 1.0 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$, 0.2 mM $(NH_4)H_2PO_4$, 20 μM NaFeEDTA, 3 μM H_3BO_3, 0.5 μM $MnCl_2$, 0.2 μM $CuSO_4$, 0.4 μM $ZnSO_4$, and 1.0 μM $(NH_4)_6Mo_7O_{24}$. The pH was adjusted to 4.5 using 1 N HCl or 1 N NaOH. The culture solution was renewed every 2 d for the first 4 d and every other day for the next 6 d. After 10 d the plants were subjected to the treatments described later.

Root elongation. Twenty roots from 3-d-old seedlings were marked with Indian ink at 1 cm from the root tip and placed in aerated 100 μM $CaCl_2$ solutions containing 0, 10, 20, and 50 μM Al (pH 4.5). The root length was then measured after 1, 3, 6, 12, and 24 h. The experiment was repeated three times.

Al uptake analysis. To examine the level of Al uptake in the two varieties, the roots of the Al-treated plants were excised, washed in distilled water (milli-Q water) and dried on a heating block at 100°C for 30 min. The dry materials were then digested in a mixture of concentrated 1 : 1 H_2SO_4 and HNO_3 (v/v) at 160°C for 3 h and Al content was determined using a graphite atomic absorption spectrophotometer (model Z-8270, Hitachi Co., Tokyo).

To identify the specific site of Al accumulation and the proportion of freely adsorbed and strongly bound Al, the content of Al before and after desorption was also determined. The method of Zhang and Taylor (1989) was used with minor modifications. Briefly, 2 mm root segments from 20 roots (0–2, 2–4, 4–6, 6–8, and 8–10 mm) were placed in 0.5 mM citric acid and maintained at 0°C on ice to prevent the loss of Al from the symplasm. After 30 min the root segments were rinsed with milli-Q water and prepared for Al determination as described above.

Root CEC. The CEC of the roots was determined according to the method of Crooke (1964) modified by Blamey et al. (1992). In short, roots of 3-d-old seedlings grown in a $CaCl_2$ solution for 3 d were excised, rinsed in distilled water and then dried at 70°C for 24 h. They were then milled to pass a 850 μm screen. Two hundred milliliters of 0.1 N HCl was added onto 100 mg of the milled root sample in a beaker. After 5 min of intermittent stirring, the sample was washed with milli-Q water to remove the HCl and then 200 mL of 1 M KCl (pH adjusted to 7.0 with KOH) was added. The pH of the KCl-milled root suspension was measured and the CEC of the roots was calculated from the decrease in pH.

Organic acids. Roots of 10-d-old seedlings were washed to remove the salts by substituting 0.5 mM $CaCl_2$ (pH 4.5) for the nutrient solution for 12 h overnight. The washing solution was then replaced with 100 μM $CaCl_2$ (pH 4.5) containing 0, 10, and 20 μM Al for 24 h.

In order to detect the existence of a relationship between root triphenyl-tetrazolium chloride (TTC) reduction and citric acid secretion by the two bean varieties, roots of 10-d-old plants were excised and exposed to 20 μM Al in 100 μM $CaCl_2$ for 6 and 24 h. The amount of citric acid excreted for 0–6 and 6–24 h was analysed.

To determine whether the secretion of organic acids in the bean is specific to Al stress, both varieties were subjected to lanthanum (La) treatments consisting of 0, 10, and 20 μM in 100 μM $CaCl_2$ for 24 h.

Organic acids were determined as described by Ma et al. (1997). Briefly, the root exudates were passed through cation and anion exchange-resins. The organic acids retained in the anion-exchange resin were eluted by 2 N HCl. The eluate was then concentrated to dryness using a rotary vacuum evaporator at 40°C (EYELA Type N-N, Rikakikai Co., Tokyo, Japan). The residue was redissolved in 1 mL of milli-Q water adjusted to pH 2.1 with perchloric acid and the contents of organic acids were determined by high performance liquid chromatography (10A series, Shimadzu Co., Kyoto, Japan).

Enzyme assay. Plants were grown and treated with Al in the same way as those for the collection of organic acid exudates. After exposure to Al for 24 h, 10 root apices (1 cm) were rinsed with distilled water, blotted dry, and transferred immediately to Eppendorf tubes placed on ice. The roots were then homogenized with a micro-homogenizer (model NS-310E, Niti-on Co., Chiba, Japan) for 30 s in 50 mM HEPES-NaOH buffer (pH 7.5) containing 5 mM $MgCl_2$, 5 mM EDTA, 10% (v/v) glycerol and 0.1% (v/v) Triton X-100. The homogenate was centrifuged at $20,000 \times g$ for 5 min and the supernatant was used for the assay of citrate synthase (CS), phosphoenolpyruvate carboxylase (PEPCase), and $NADP^+$-isocitrate dehydrogenase (NADP-ICDH) according to the method of Johnson et al. (1994). The CS activity was monitored spectrophotometrically through the reduction of acetyl CoA with 5,5-dithio-bis-2-nitrobenzoic acid at 412 nm for 5 min. The reaction mixture was composed of 100 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 100 mM 5,5-dithio-bis-2-nitrobenzoic acid, 0.3 mM acetyl CoA and 0.5 mM oxalacetic acid. PEPCase was assayed spectrophotometrically by monitoring the reaction of NADH at 340 nm for 240 s in the coupled assay. The reaction mixture was similar to that above except for the substitution of 100 mM NaHCO, 30 mM phosphoenol pyruvate (PEP), and 2 mM NADP for acetyl CoA and oxalacetic acid. NADP-ICDH was assayed spectrophotometrically by monitoring the decrease of the amount of NADH at 340 nm for 240 s. The reaction mixture was similar to that used for the CS assay except for the substitution of 20 mM isocitrate and 2 mM NADP for acetyl CoA and oxalacetic acid.

TTC reduction. To examine the effect of Al on the TTC reduction in the roots of French bean and Rosecoco, roots of 3-d-old seedlings were tested for their capacity to reduce TTC. Ten excised roots of each variety were immersed in 10 mL of 1% TTC in 0.1 M phosphate buffer (pH 7.0) put in Thunberg tubes. The tubes containing the roots were incubated in darkness with shaking (60 rpm) at 30°C for 30 min. The reaction was stopped with the addition of 2 mL of 2 N H_2SO_4. The roots were rinsed with milli-Q water to eliminate the acid, cut into segments 0–2, 2–4, 4–6, 6–8, and 8–10 mm, blotted on tissue paper and transferred to pre-weighed Eppendorf tubes to measure the weight of roots. The root segments were then homogenised in acetyl acetate for 30 s with a micro-homogenizer. The homogenate was centrifuged at $13,500 \times g$ for 10 min and the optical density of the supernatant was then measured spectrophotometrically at 470 nm. The absorbance was compared with TTC standards previously reduced to formazan with $Na_2S_2O_4$.

RESULTS

Al effect on root growth

The addition of Al led to a measurable root growth inhibition starting in the first hour. The reduction of root growth increased with increasing concentrations of Al up to 50 μM for both French bean and Rosecoco (Fig. 1A). The inhibition of root elongation by Al was greater in French bean than in Rosecoco and increased with time (Fig. 1B).

Al uptake

The content of Al in the root apex increased with increasing Al concentration except for French bean at 50 μM Al (Fig. 2A). At 50 μM Al, the increase in the rate of cell death which was revealed by Evans blue staining showing a 200% higher rate of cell death in French bean than in Rosecoco (data not presented), and the severe inhibition of root apex elongation resulted in a lower Al uptake in French bean. The Al content was higher in

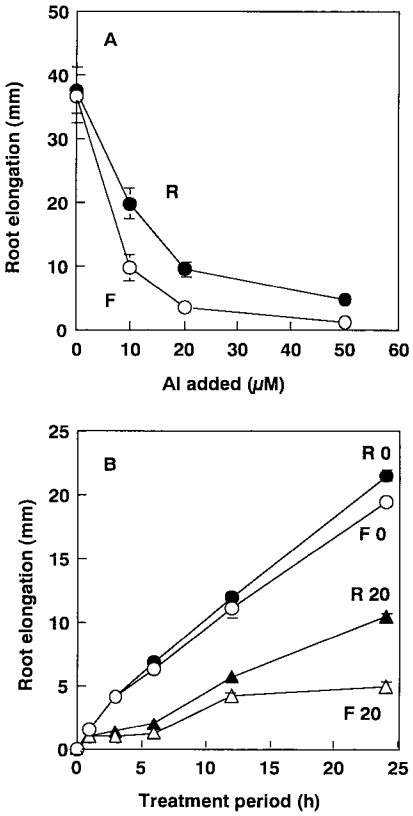

Fig. 1. Root elongation of French bean (F) and Rosecoco bean varieties (R) after 24 h treatment with 0, 10, 20, and 50 μM Al (A), time course (1, 3, 6, 12, and 24 h) with 20 μM Al treatment (B). After germination, the seedlings were pre-cultured in 0.5 mM $CaCl_2$ (pH 4.5) for 2 d before exposure to Al in a 100 μM $CaCl_2$ (pH 4.5) solution containing relevant Al concentrations. Values denote means$\pm$SE ($n=3$).

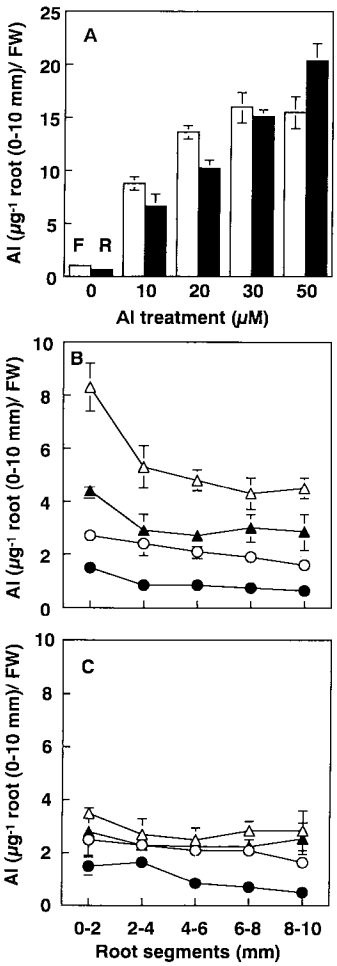

Fig. 2. Al uptake by French bean (F) and Rosecoco (R) in 10 mm root after 24 h Al treatments with 0, 10, 20, 30, and 50 μM Al (A), and in root segments after treatment for 24 h with either 0 (▲, F; ●, R) or 10 μM Al (△, F; ○, R) before (B) and after desorption with 0.5 mM citric acid (C). After germination the plants were pre-cultured in 0.5 mM $CaCl_2$ (pH 4.5) for 2 d before exposure to a 100 μM $CaCl_2$ (pH 4.5) solution containing relevant Al concentrations. Values denote means$\pm$SE ($n=3$).

French bean than in Rosecoco for the 0 μM Al treatment (control) in both citric acid-desorbed and undesorbed roots. The Al desorption treatment with citric acid markedly reduced the Al content especially in French bean (Fig. 2C). The difference in Al content between French bean and Rosecoco was negligible in the Al-desorbed roots (Fig. 2C) but substantial in the non-desorbed roots (Fig. 2B). A higher Al uptake in the roots of both French bean and Rosecoco without desorption was observed in the 0–2 mm root segments (Fig. 2B).

The high Al content in the non-desorbed roots of French bean can be ascribed partly to the high CEC value of French bean (Blamey et al. 1992). The CEC values of French bean and Rosecoco were 5.01 ± 0.3 and 3.4 ± 0.2 cmol(+) kg^{-1} dry matter, respectively. The CEC value of French bean was 55% higher than that of Rosecoco. The Al bound to the apoplast could be excluded by the desorption with citrate and the amount of Al was related to the CEC.

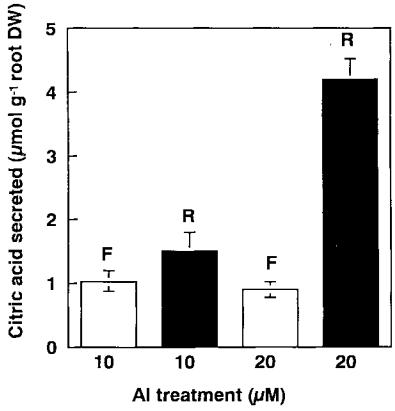

Fig. 3. Exclusion of citric acid in French bean (F) and Rosecoco (R). The roots of 10-d-old seedlings were exposed to 0, 10, and 20 μM Al (pH 4.5) in 100 μM $CaCl_2$ for 24 h. The root exudates were collected and analyzed as described in MATERIALS AND METHODS. The exclusion of citric acid was not detected at 0 μM Al. Values denote means $\pm$ SE ($n=3$).

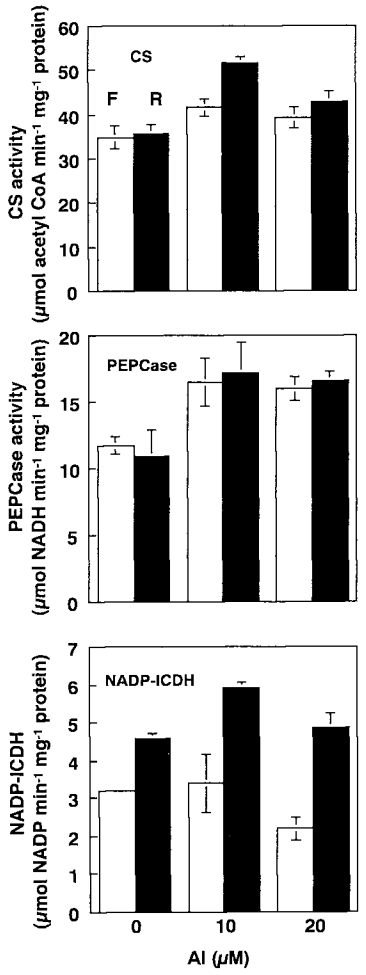

Fig. 4. Effect of Al on the activities of enzymes related to citric acid synthesis. The roots of 10-d-old French bean (F) and Rosecoco (R) were exposed to 100 μM $CaCl_2$ (pH 4.5) containing 0, 10, and 20 μM Al for 24 h. The root apices (10 mm) were excised and assayed for the activity of citrate synthase (CS), NADP-specific isocitrate dehydrogenase (NADP-ICDH) and phosphoenolpyruvate carboxylase (PEPCase). Results indicate means $\pm$ SE ($n=3$).

Organic acid exudation

The two varieties secreted only citric acid based on the HPLC analysis. There was no measurable citric acid in the exudates from the control and lanthanum-treated roots (results not presented). The French bean secreted less citric acid than Rosecoco under the 10 and 20 μM Al treatment. In the 10 to 20 μM Al treatments citric acid secretion by Rosecoco increased 3-fold while that of French bean decreased (Fig. 3).

Enzymes

Figure 4 shows the activity of CS, PEPCase, and NADP-ICDH. The stimulation of the enzyme activity by Al was relatively greater in Rosecoco than in French bean. CS and PEPCase activity in Rosecoco increased with increasing Al concentration by 160 and 140%,

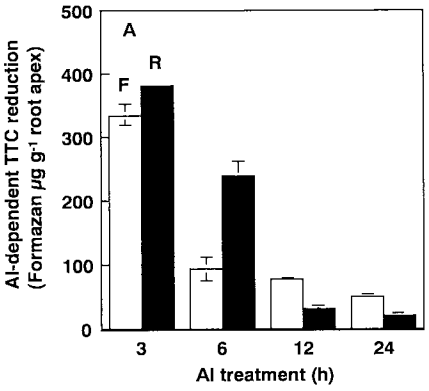

Fig. 5. Effect of exposure to 20 μM Al on triphenyl-tetrazolium chloride (TTC) reduction in roots of French bean (F) and Rosecoco (R); TTC reduction from root apex (10 mm) treated with 20 μM Al for 3, 6, 12, and 24 h (A) and in root segments after 6 h (B). After germination, the plants were pre-cultured in 0.5 mM $CaCl_2$ (pH 4.5) for 2 d before exposure to a 100 μM $CaCl_2$ (pH 4.5) solution containing 20 μM Al. TTC reduction in roots was determined as described in MATERIALS AND METHODS. Values denote means$\pm$SE ($n=3$).

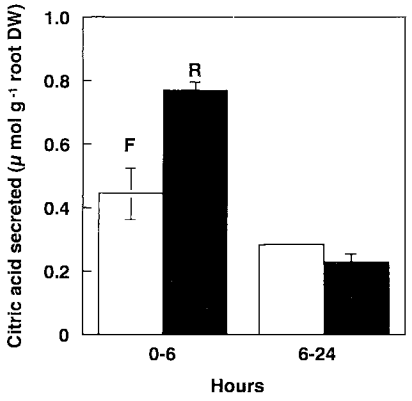

Fig. 6. Effect of Al on the secretion of citric acid by excised roots of French bean (F) and Rosecoco (R). The roots were exposed to a 100 μM $CaCl_2$ (pH 4.5) solution containing 20 μM Al. Root exudates were collected during 0-6 h and 6-24 h and the citric acid content was determined as described in MATERIALS AND METHODS. Values denote means$\pm$SE ($n=3$).

respectively but declined at 20 μM Al in French bean. These findings were well correlated with the citric acid secretion trend (Fig. 3). The NADP-ICDPH activity was clearly stimulated by 10 μM Al in Rosecoco but inhibited by 20 μM Al in both varieties. This enzyme converts isocitrate to 2-oxoglutarate, resulting in the decrease of the amount of citrate which is the precursor of isocitrate. The activity of NADP-ICDH was higher in Rosecoco than in French bean, indicating that the secretion of citrate might largely depend on the synthesis of citrate with prior activation of CS under Al stress in Rosecoco.

TTC reduction

TTC reduction in roots was used to assess the level of mitochondrial activity. TTC reduction increased with Al treatment in both varieties (Fig. 5A) and the Al effect was more pronounced in Rosecoco than in French bean at least until 6 h. The TTC reduction under Al stress in the root apex (0–10 mm) decreased with time in both varieties but the decrease was much greater in French bean after 6 h (Fig. 5A). After 6 h the Al effect on TTC reduction in Rosecoco was less conspicuous than in French bean. This was probably due to some Al-injury recovery mechanism in Rosecoco and was closely related to citrate exudation from the excised roots. The Al effect was pronounced in the 0–4 mm region and less conspicuous along the root profile (Fig. 5B). There was a clear difference in the TTC reduction activity of the root segments between French bean and Rosecoco. The TTC reduction activity in the 0–2 and 2–4 mm segments of Rosecoco was extremely high and two times as high as that of French bean. The TTC reduction activity tended to decrease from the root tip to mature zones, indicating that the mitochondrial activity was strongest at the root tip (0–4 mm region). This finding is noteworthy because organic acids are secreted from this region by plants under Al stress (Zheng et al. 1998). These results suggested that the increased synthesis of citric acid may depend on the mitochondrial activity, based on the TTC reduction activity under Al stress. This observation was further supported by the fact that the total excretion of citrate in the 0 to 6 h treatment with Al was higher than that in the 6 to 24 h treatment (Fig. 6). Furthermore this time-dependent excretion of citrate was much higher in Rosecoco.

DISCUSSION

Differential root elongation of plants after exposure to Al has been used as a parameter by many researchers to screen for Al tolerance (Foy et al. 1974; Rincón and Gonzales 1992; Sasaki et al. 1994). Based on this parameter, it was confirmed that the Rosecoco variety showed a higher Al tolerance than French bean.

A higher Al uptake in the roots of Al-sensitive varieties has been reported in wheat (Zhang and Taylor 1990; Rincón and Gonzales 1991). In this study, the results of Al analysis in the root apex showed that Rosecoco accumulated less Al than French bean. However at 50 μM Al, Rosecoco accumulated more Al, presumably due to the very severe root growth inhibition in French bean which resulted in the reduced apex volume of root for Al absorption. This observation underscores the importance of selecting appropriate levels of Al treatments in root Al uptake studies for use as a tool in screening plants for Al tolerance.

The first 2 mm part of the root apex has been shown to be the most active region for Al uptake in wheat (Rincón and Gonzales 1991) and maize (Sivaguru and Horst 1998; Kollmeier et al. 2000). In this study, both varieties accumulated more Al in the 0–2 mm

region than in the rest of the root apex. French bean showed a sensitivity to Al based on the accumulation of much higher amounts of Al than Rosecoco. Therefore Rosecoco had developed better Al-exclusion mechanisms. These Al-exclusion mechanisms have been identified in this study based on the lower CEC value and higher citric acid secretion.

The Al remained in the root apex after desorption with citric acid which corresponds to the tightly bound Al in either the apoplasm or symplasm confers a sensitivity to Al because it corresponds to the metabolic Al (Zhang and Taylor 1989). The results (Fig. 2B, C) revealed a significant difference in Al content between French bean and Rosecoco, especially in the undesorbed 0–2 mm region, indicating that this region of the root apex is the most active in Al absorption.

It was interesting to note that the Al content of undesorbed control roots (without Al treatment) was 2 fold higher in French bean than in Rosecoco and was almost 3 fold higher in desorbed control roots (Fig. 2B, C). This observation suggests that Al in the control roots which corresponded to the originally stored Al in the seeds is strongly bound in either the apoplasm or symplasm. Based on these findings, we determined the Al content of the seeds of French bean and Rosecoco. The Al content of the French bean seeds was 0.201 μmol Al g^{-1} seed D.W., a value 3 times higher than that of Rosecoco. It is suggested that Al stored in seed, might be translocated to the growing roots upon germination.

The high CEC value of roots has been associated with a higher Al uptake and consequently higher sensitivity to Al (Wagatsuma 1983; Blamey et al. 1990). We, therefore, measured the CEC to determine whether it was related to the difference in Al content. The Al-sensitive French bean showed a 55% higher CEC value than Rosecoco, thus confirming some role of root CEC in Al tolerance and sensitivity in the two varieties of *P. vulgaris*.

Among the organic acids commonly secreted in large amounts, citric acid exhibits the highest Al-detoxifying capacity. *P. vulgaris* genotypes have been reported for the first time to secrete citric acid upon exposure to Al (Miyasaka et al. 1991). In this study, only citric acid was detected under Al stress. In the exudates from roots treated with La or in the control (no aluminium treatment), no measurable citric acid was detected, suggesting that the secretion of citric acid in both French bean and Rosecoco was Al-specific (data not presented).

The secretion of citric acid by French bean decreased at 20 μM Al, presumably due to the reduced activity of CS (Fig. 4).

The amount of excretion of citric acid by the excised roots in the subsequent 18-h-period from 6 to 24 h was much smaller than that in the first 6-h-period, suggesting that the energy and carbon skeleton for citrate synthesis are supplied from the shoots in both French bean and Rosecoco (Fig. 6). The results indicate that these factors may be related to the regulation of citric acid excretion under Al stress.

The activity of CS and PEPCase increased at 10 μM Al in both French bean and Rosecoco but at 20 μM Al only Rosecoco showed an increase in the activity of these enzymes (Fig. 4). PEPCase catalyses the dark fixation of CO_2 and supplies the carbon skeleton for the synthesis of citrate. Andrade et al. (1997) reported the possible involvement of PEPCase in the Al tolerance mechanism but the exposure of Al-tolerant wheat root to 160 μM Al hardly affected the PEPCase activity within 24 h. The difference in the PEPCase activity between Rosecoco and French bean was negligible (Fig. 4). Thus, the role of PEPCase in *Phaseolus* seems to be less significant in terms of Al tolerance mechanism.

The role of CS in the exclusion of citric acid upon Al stress was confirmed by the fact that Al-tolerant rye could induce a CS activity in the roots. The fluctuations of the induced

activity of CS paralleled the excretion of citric acid (Li et al. 2000). Furthermore, Koyama et al. (1999) reported that the mitochondrial CS gene improved the growth of carrot cells in Al-phosphate medium due to the increased synthesis of citric acid which led to the release of phosphate from the Al-phosphate complex by Al-citrate chelation. In this regard, Li et al. (2000) have recently reported that pyridoxal 5′-P and phenylisothiocyanate which are inhibitors of citrate transport in the mitochondrial membrane suppressed the exclusion of citrate upon Al stress, resulting in a decrease of Al tolerance. Transgenic tobacco and papaya into which the bacterial CS gene had been introduced became Al-tolerant due to the increase of the synthesis and exclusion of citrate (de la Fuente et al. 1997). These observation were well correlated with the citrate secretion pattern of the two bean varieties, implying that CS has a direct influence on citrate synthesis.

Takita et al. (1999) reported the role of NADP-ICDH located in the cytoplasm of carrot cells in citrate excretion upon Al stress. They indicated that the enzyme activity was related to the decrease of the citrate content because this enzyme degrades isocitrate to 2-oxoglutarate, since isocitrate is metabolised from citrate. However, the NADP-ICDH activity was higher in Rosecoco than in French bean in this study. Thus NADP-ICDH in *Phaseolus* may not be involved in the mechanism of Al tolerance, although the compartmentation of NADP-ICDH was not investigated in this study.

Based on these considerations, the activation of CS by Al may play a role in the Al-resistance mechanism of Rosecoco.

TTC reduction has been used to characterize cell viability in the case by freezing (Stattin and Lindström 1999) and this reaction is considered to reflect the activity of succinate dehydrogenase in the TCA cycle. We suggest that the secretion of citrate upon Al stress is related to the mitochondrial respiratory activity of excised roots based on the TTC reduction. In both Rosecoco and French bean, a higher TTC reduction, especially in Rosecoco, was observed up to 6 h after the Al treatment and thereafter it declined. These finding are in agreement with the demonstration of citrate secretion in the excised roots (Fig. 6).

Furthermore, TTC reduction was compared in different root segments. The results clearly showed that the Al-treated 0–2 mm root segments exhibited a higher TTC reduction than other more mature regions. Again the distinctly high TTC reduction of the root segments treated with 20 μM Al was observed in Rosecoco. Secretion of organic acids is confined to the root apex (0–4 mm) (Zheng et al. 1998) and our results are consistent with this fact. Thus it is considered that the secretion of citrate in *P. vulgaris* is related to the activation of the mitochondrial activity based on the TTC reduction as well as to the increased activity of CS in mitochondria.

In conclusion our study revealed the differential mechanisms of Al tolerance in *P. vulgaris* in terms of root elongation, Al uptake, CEC, TTC reduction, and organic acid exudation. We have also shown that there was a clear relationship between the activity of CS and citric acid secretion in two varieties differing in Al tolerance. The TTC reduction has for the first time been found to be stimulated more by Al in a Al-tolerant variety than in the Al-sensitive one.

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