Effect of aluminum on metabolism of organic acids and chemical forms of aluminum in root tips of Eucalyptus camaldulensis Dehnh

Effect of aluminum on metabolism of organic acids and chemical forms of aluminum in root tips of *Eucalyptus camaldulensis* Dehnh.

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Abstract

Eucalyptus (*Eucalyptus camaldulensis*) has relatively high resistance to aluminum (Al) toxicity than the various herbaceous plants and model plant species. To investigate Al-tolerance mechanism, the metabolism of organic acids and the chemical forms of Al in the target site (root tips) in Eucalyptus was investigated. To do this, 2-year old rooted cuttings of *E. camaldulensis* were cultivated in half-strength Hoagland solution (pH 4.0) containing Al (0, 0.25, 0.5, 1.0, 2.5 and 5.0 mM) salts for 5 weeks; growth was not affected at concentrations up to 2.5 mM even with Al concentration reaching 6000 μg g^{-1} DW. In roots, the citrate content also increased with increasing Al application. Concurrently, the activities of aconitase and NADP$^+$-isocitrate dehydrogenase, which catalyze the decomposition of citrate, decreased. On the other hand, the activity of citrate synthase was not affected at concentrations up to 2.5 mM Al. ^{27}Al-NMR spectroscopic analyses were carried out where it was found that Al–citrate complexes were a major chemical form present in cell sap of root tips. These findings suggested that E. camaldulensis detoxifies Al by forming Al–citrate complexes, and that this is achieved through Al-induced citrate accumulation in root tips via suppression of the citrate decomposition pathway.

Keywords

1. Introduction

In acidic soils, which account for approximately 40% of the earth's arable land, Al^{3+} toxicity is a major factor limiting plant productivity (Taylor, 1991). The root growth of many agriculturally important crops, including wheat (*Triticum aestivum*) and maize (*Zea mays*), are suppressed by low (micromolar) levels of Al^{3+} within minutes or hours (Matsumoto, 2000). Therefore, it is important to understand the Al^{3+}-tolerance mechanisms of plants to establish strategies to increase crop productivity in acidic soils.

Many studies have examined Al^{3+}-tolerance mechanisms by using either tolerant cultivars or lines of Al-sensitive plant species. Until now, it was thought that Al^{3+} exclusion from the root apex, the so-called external detoxification mechanism, was the most important mechanism for Al^{3+} tolerance in these plant

species. In this process, plants can exclude Al^{3+} by actively transporting it out of the cytoplasm (Taylor, 1991 and Kochian, 1995), and when the concentration of Al^{3+} in the rhizosphere is reduced by the increase in root surface pH (Degenhardt et al. 1998) and by Al-chelating substances secreted from the roots (i.e. Kitagawa et al. 1986). Many plant species secrete low molecular weight organic acids as chelators from roots in response to Al^{3+}. For example, malate was released from the roots of Al^{3+}-tolerant cultivars of wheat (Delhaize et al. 1993), and citrate from Al^{3+}-tolerant cultivars of maize (Pellet et al. 1995). Al^{3+}-tolerant oat (*Avena sativa*) (Zheng et al. 1998) and rye (*Secale cereale*) (Li et al. 2000) also released both malate and citrate in response to Al^{3+}. Recently, genetic approaches showed that malate and citrate exudation were controlled by transporters, including an Al^{3+}-activated malate transporter (ALMT1) in wheat (Sasaki et al. 2004) and a multi-drug and toxic compound extrusion (MATE) process in sorghum (Magalhaes et al. 2007). Moreover, Al^{3+} treatments stimulated citrate synthesis in some plant species via increased citrate synthase (CS) activity and/or decreased isocitrate dehydrogenase (ICDH) activity in the tricarboxylic acid (TCA) cycle (Ryan and Delhaize, 2001 and Neumann and Martinoia, 2002).

Some plant species accumulate Al^{3+} at high concentrations in aerial parts without showing Al^{3+} toxicity. There is evidence that Al-tolerant plant species have not only external but also internal Al^{3+}-detoxification mechanisms, including chelation by organic acids. For example, the Al^{3+}-tolerant plant buckwheat (Fagopyrum esculentum Moench. cv. Jianxi) secretes oxalate from the roots in response to Al^{3+} (Ma et al. 1997a). Determination of chemical forms of Al^{3+} in buckwheat by ^{27}Al-nuclear magnetic resonance (^{27}Al-NMR) suggested that after Al^{3+} uptake, it was internally detoxified by formation of Al^{3+}-oxalate complexes in the roots (Ma et al. 1998), Al^{3+}-citrate complexes in the xylem sap, and Al^{3+}-oxalate complexes in the leaves (Ma and Hiradate, 2000), respectively. In the case of tea (*Camellia sinensis* L.), its growth was stimulated by Al^{3+} (Konishi et al. 1985), with Al^{3+} accumulating to more than 30,000 mg kg^{-1} in the leaves (Matsumoto et al. 1976). Tea also secreted oxalate from the roots in response to Al^{3+} (Morita et al. 2011), and Al^{3+} was also internally detoxified by the formation of Al^{3+}-oxalate complexes in roots (Morita et al. 2008) and Al^{3+}-citrate complexes in the xylem sap (Morita et al. 2004). Tea plants can also store Al^{3+} as Al^{3+}–catechin complexes in mature leaves (Nagata et al. 1992). As mentioned above, low molecular weight organic acids play a key role in Al^{3+} detoxification by forming Al^{3+}-organic acid complexes in the cytosol or at the root–soil interface.

Eucalyptus is grown worldwide as an ornamental tree and as a material for the paper industry. Previous reports have shown that *Eucalyptus gummifera* was able to use insoluble phosphate (aluminum phosphate and iron (III) phosphate) in acidic conditions (Mullette, 1973) and that its growth was stimulated in the presence of Al^{3+} (Mullette et al. 1974). Symonds et al. (2001) reported that Eucalyptus generally showed a higher growth rate under acidic conditions (pH 5.1-5.6) in all 35 species examined. Moreover, *Eucalyptus camaldulensis*, one of the most popular and valuable species in the genus Eucalyptus, can grow in acid soils (Osaki et al. 1998), i.e. where the soil pH is lower than 3.5 and the Al^{3+} concentration in the soil solution reaches the millimolar level (van Breemen and Pons, 1978). Indeed, in studies using these species under hydroponics (Osaki et al., 1997, Nguyen et al., 2003a and Tahara et al., 2005), *E. camaldulensis* was highly resistance to Al^{3+} than some of the crops mentioned above, and root elongation of this plant was not

inhibited even by 1 mM Al^{3+} for 20 days (Tahara et al. 2005). In addition, Nguyen et al. (2003a) and Tahara et al. (2008) suggested that *E. camaldulensis* showed increased citrate content in the roots in response to Al^{3+}. That study also reported that the secretion of citrate and oxalate from roots of *E. camaldulensis* increased in response to Al^{3+} (Nguyen et al., 2003b). These findings thus suggested that Eucalyptus has internal and external Al^{3+}-tolerance mechanisms based on chelation of Al^{3+} with organic acids, like buckwheat and tea. However, little is known about Al^{3+}-induced metabolic changes associated with the synthesis or degradation of organic acids and the chemical forms of Al^{3+} in Eucalyptus roots.

In this study, in order to clarify Al^{3+}-tolerance mechanisms of *Eucalyptus*, hydroponically grown *E. camaldulensis* was treated with Al^{3+} solutions (pH 4.0) for 5 weeks. To do this, the contents of Al^{3+} and low-molecular weight organic acids, the activities of enzymes in the TCA cycle, and the chemical forms of Al^{3+} in the roots were determined.

2. Results

E. camaldulensis grew normally in Al^{3+} treatments up to 2.5 mM Al^{3+}. Plants in those treatments showed similar fresh weights to that of the control (0 mM Al^{3+}) (Fig. 1). The growth of all plant parts was markedly decreased, however in the 5 mM Al^{3+} treatment, and the mean total fresh weight was approximately 60% of that of the control. Symptoms of Al^{3+} toxicity (leaf chlorosis or necrosis) were not visible in any treatment (data not shown).

The Al^{3+} contents in roots and leaves are shown in Fig. 2. In young roots, the Al^{3+} content increased up to 2.5 mM Al^{3+}, reaching approximately 6000 µg g^{-1} DW in the 2.5 and 5 mM Al^{3+} treatments. In young and mature leaves, Al^{3+} contents also increased with higher levels of Al^{3+} treatments, and showed the highest values in the 5.0 mM Al^{3+} treatment. However, the Al^{3+} concentrations in leaves were much lower than those in young roots. In the 5.0 mM Al^{3+} treatment, approximately 4 µg Al g^{-1} DW accumulated in young leaves and 150 µg Al g^{-1} DW accumulated in mature leaves. These findings suggested that Al^{3+} mainly accumulated in roots of *E. camaldulensis*.

The contents of organic acids in young roots are shown in Fig. 3. The highest malate content (0.55 µmol g^{-1} FW) was in roots of plants in the 1.0 mM Al^{3+} treatment, but it did not respond to Al^{3+} supply. The citrate content increased with increasing Al^{3+} supply (citrate concentration of 0.79 µmol g^{-1} FW in the 2.5 mM Al treatment) and remained at a higher level in the 5 mM Al^{3+} treatment. Oxalate contents were more than 10-fold greater than those of malate and citrate, but this did not change in response to Al^{3+}. The oxalate content was approximately 14 µmol g^{-1} FW in the roots of all plants, regardless of Al^{3+} treatment.

The activities of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH), which catalyze the synthesis of malic acid, are shown in Fig. 4. The activity of PEPC was unchanged in treatments up to 2.5 mM Al^{3+} and then significantly decreased in the 5.0 mM Al^{3+} treatment. MDH activity gradually decreased with increasing levels of Al^{3+} in the treatments. By contrast, the activity of CS, which catalyzes citric acid synthesis, showed an increasing trend with higher levels of Al^{3+} application, but the increase was not statistically significant (Fig. 5). The activities of aconitase (ACO) and $NADP^+$-ICDH, which catalyze

the decomposition of citric acid, were lower in all of the Al^{3+} treatments than in the control.

Table 1 shows the contents of Al^{3+} and organic acids in cell sap extracted from Eucalyptus roots. The cell sap from root tips treated with 1.0 mM Al^{3+} was strongly acidic (pH 4.32) and contained 1.03 mM Al^{3+}. The contents of citrate (1.3 mM) and oxalate (1.49 mM) were similar to that of Al^{3+}, but higher than that of malate (0.26 mM). The ^{27}Al-NMR spectrum of the cell sap and Al-model solutions are shown in Fig. 6. In the spectrum of the cell sap, four signals were detected: a large, broad resonance at 11.3 ppm, two smaller broad signals at −1.4 and 3.8 ppm, and a small, sharp resonance at 15.9 ppm. In the spectrum of Al model solutions, broad peaks at 4.6 and 12.2 ppm were detected in the Al-malate (1:1) solution, and at 1.1 and 10.9 ppm in the Al-citrate (1:1) solution. Sharp peaks were detected at 1.4, 6.6, and 11.7 ppm in the Al^{3+}-oxalate (1:1) solution.

3. Discussion

E. camaldulensis was grown in half-strength Hoagland solution (pH 4.0) containing various concentrations of Al^{3+} (0–5.0 mM) for 5 weeks. The growth of E. camaldulensis was not affected by Al^{3+} up to 2.5 mM (Fig. 1). The Al^{3+} content in roots of plants in the 2.5 mM Al^{3+} treatment was approximately 6,000 mg g^{-1} DW (Fig. 2). These findings reconfirmed that *E. camaldulensis* was tolerant to Al^{3+}, as reported by Nguyen et al. (2003a).

The citrate content in the root increased with increasing Al^{3+} concentrations in the Al^{3+} treatments (Fig. 5). This was consistent with the results of Nguyen et al. (2003b) and Tahara et al. (2008), and suggested that Al^{3+} induced citrate accumulation in the roots of *E. camaldulensis*. Silva et al. (2004) reported that in seedlings of six species of *Eucalyptus*, *E. globulus* Labill., *E. urophylla* S.T. Blake, *E. dunnii* Maiden, *E. saligna* Sm., *E. cloeziana* F.J. Muell and *E. grandis* w. Hill ex Maiden, root exposure to Al^{3+} resulted in more than a 200% increase in malic acid concentration in the root tips, as well as a small increase in citric acid concentration. It was thus concluded that the Al^{3+} tolerance of these Eucalyptus species was mainly due to internal detoxification of Al^{3+} by complexation with malic acid. However, the content of malate in the roots of *E. camaldulensis* did not increase, or only slightly increased, with increasing Al^{3+} supply. These findings suggested that different Eucalyptus species might accumulate different types of organic acids in their roots in response to Al^{3+}.

Generally, citrate accumulates in higher plants via suppression of citrate decomposition in the cytosol, since the activity of CS in mitochondria is not stimulated by Al^{3+} (Neumann and Martinoia, 2002). Meanwhile, the Al^{3+}-tolerant carrot suspension culture cell line selected by Koyama et al. (1988) secreted citrate. In these cells, there was an increase in CS mRNA transcripts (Takita et al. 1999) and a decrease in ICDH mRNA transcripts (Kihara et al. 2003a). CS activity increased in response to the Al^{3+} has been reported in various plant species such as soybean (Yang et al. 2001), common bean (Mugai et al. 2000) and *Cassia tora* L. (Yang et al. 2004). These results suggested that Al^{3+} regulates CS activity and citrate accumulation in roots. On the other hand, accumulation of citrate in the cytosol is achieved by reduced activity of the ACO and NADP-ICDH that are involved in citrate turnover (Massonneau et al., 2001, Anoop

et al., 2003 and Rangel et al., 2010). In white lupin, which showed high citrate exudation activity, the citrate metabolic system (activities of ICDH and ACO) in the cytosol was blocked under phosphate deficiency (Kania et al., 2003 and Kihara et al., 2003b). In lemon, ACO activity was suppressed at the transcriptional level at the stage when citrate accumulated in the fruit (Sadka et al. 2000). In this study, the activities of ACO and NADP$^+$-ICDH in *E. camaldulensis* roots decreased in the Al^{3+} treatments, while CS activity remained unchanged up to the 2.5 mM Al^{3+} treatment (Fig. 6). Therefore, in roots of *E. camaldulensis*, Al^{3+} resulted in suppressed citrate decomposition via the reduction of ACO and NADP$^+$-ICDH activities, resulting in increased citrate content.

In the ^{27}Al-NMR spectrum of the cell sap from root tips treated with 1.0 mM Al^{3+}, there were four signals: at 1.4, 3.8, 11.3, and 15.9 ppm. A resonance at around 0 ppm suggests the presence of Al^{3+} monomeric species (Kerven et al. 1995) and another around 3 ppm indicates Al^{3+} bound to a carboxyl group of an organic acid, such as malic acid (Hiradate, 2004). Therefore, the two signals at −1.4 and 3.8 ppm suggested the presence of Al^{3+} monomeric species and Al^{3+} binding to malic acid, respectively. Two resonances were detected at around 11.3 ppm: one large broad signal at 10.9-12.9 ppm in the Al-citrate solution and another sharper resonance at 11.7 ppm in the Al-oxalate solution. In addition, the peak pattern when the Al-oxalate complexes was observed as a main peak at 6.6 ppm, the concentration of oxalate in the cell sap appears to be lower than ratio of the peak area at 11.7 ppm (Table 1). Therefore, the large broad signal at 11.3 ppm in the cell sap indicated the presence of Al-citrate complexes in the roots of *E. camaldulensis*. In a previous report (Morita et al. 2008), it was demonstrated showed that the peak at 16.1 ppm in the tea cell sap at pH 5.0 was due to Al^{3+}-oxalate (1:3) complex, although the pH value in eucalyptus cell sap solutions was 4.3 in this study. Consequently, the resonance at 15.9 ppm in cell sap did not derive from the Al^{3+}-oxalate (1:3) complex. Tahara et al. (2008) reported that the unknown low-molecular-weight (LMW) Al^{3+}-binding ligand contributes to Al^{3+} resistance in *E. camaldulensis*. It is thus possible that the spectrum of the cell sap might represent not only the A1-citrate complex but also the unknown Al-LMW complex.

4. Conclusions

In conclusion, the results of this study demonstrate that the roots of *E. camaldulensis* display internal tolerance to Al^{3+} by forming Al^{3+}–citrate complexes through increasing citrate contents and suppressing citrate decomposition. These mechanisms resulted in accumulation of Al^{3+} up to 6000 μg g^{-1} DW in the roots.

5. Experimental

5.1. Plant materials and treatment conditions

Rooted cuttings of *E. camaldulensis* (2-years old) were transferred into Wagner pots containing 12 L tap H$_2$O adjusted to pH 6.0 with either 1 N H$_2$SO$_4$ or 1 N NaOH. After 1 week, in order to adapt the

hydroponic culture system, the H_2O was replaced and the plants were grown in 1/8 strength Hoagland's solution (Hoagland and Snyder, 1933) for 1 week, in 1/4 strength Hoagland's solution for 1 week, and then in half-strength Hoagland's solution until the start of the treatment. After 1 week in half-strength solution, plants were transferred to the treatment solutions; half-strength Hoagland's solution containing 0, 0.25, 0.5, 1.0, 2.5, or 5.0 mM Al as $Al_2(SO_4)_3 \cdot 14$-$18H_2O$. The pH was adjusted to 4.0 with 1 N H_2SO_4. At the start of the treatments, the fresh weight of *E. camaldulensis* was 20 ± 2 g plant−1. The treatment solutions were renewed every week and continuously aerated. The pH of the treatment solution was adjusted every day during the treatment. Each experiment was carried out in triplicate. After 5 weeks from the start of treatment, three rooted cuttings from each treatment were immersed in dilute H_2SO_4 solution (pH 3) for 3 min to remove Al^{3+} compounds from the root surface, and then washed with deionized H_2O. After wiping with a paper towel, the plants were separated into leaves, stems, and roots, and each part was weighed. Then, leaves and roots were separated into young parts (which developed after the start of Al treatment) and mature parts. Young root samples were immediately stored at −80 °C until analyses of organic acid contents and enzyme activities. At the same time, samples of young roots treated with 1.0 mM Al were cut with scissors, and root tips (0–5 mm) were used for ^{27}Al-NMR analysis. The rest of the young roots and mature and young leaves were freeze-dried and ground into a fine powder to measure Al contents.

5.2. Measurement of Al content

Aliquots of powdered plant samples (approx. 50 mg) were digested with condensed HNO_3:$HClO_4$ (1:1 v/v) and completed to 25 ml in a volumetric flask. This solution was used to measure Al using an atomic absorption spectrometer with a graphite furnace (Spectra AA with Zeeman correction, Varian, Australia).

5.3. Measurement of organic acid contents

Frozen young root samples (approx. 1.5 g) were macerated with 3 M $HClO_4$ (7.5 ml) and 1 mM EDTA·2Na (3 ml) with a cold mortar and pestle. After centrifugation at 5000g for 10 min at 4 °C, the supernatant was adjusted to pH 5.0 with 0.5 N KOH and kept on ice for 30 min before filtering through filter paper (No. 6, Advantec, Tokyo, Japan). The filtrate was used to analyze the contents of malate and citrate using enzymatic methods (Mollering, 1985). Water-soluble oxalate in frozen samples was extracted with deionized H_2O for 1 h. After filtering the solution, oxalate was analyzed using an ion-chromatograph (IC 500S, Yokogawa-Hokushin Electric, Tokyo, Japan) equipped with a guard column (IonPac AG4A-SC, 4 × 50 mm, Dionex, Sunnyvale, CA, USA) and an analytical column (IonPac AS4-SC, 4 × 250 mm, Dionex).

5.4. Measurement of enzyme activities

Young roots stored at −80 °C were homogenized with a mortar and pestle in cold 100 mM HEPES-KOH buffer (pH 8.0) containing 30 mM sorbitol, 1% (v/v) bovine serum albumin (BSA) and 5% (v/v) polyvinylpolypyrrolidone. The homogenate was filtered through nylon mesh (120 mesh) and centrifuged at 10,000g for 15 min, with the supernatant then used for enzyme assays. Activities of PEPC, MDH, CS, and

NADP^{+}-ICDH were measured according to the method of López-Millán et al. (2000). The activity of ACO was measured by the method of Cooper and Beevers (1969). Briefly, the activities of PEPC and MDH were assayed spectrophotometrically by monitoring disappearance of NADH at 340 nm. CS activity was assayed spectrophotometrically by following reduction of acetyl coenzyme A with 5,5′-dithio-bis-2-nitro benzoic acid at 412 nm for 3 min. NADP^{+}-ICDH activity was assayed spectrophotometrically by monitoring disappearance of NADPH at 340 nm. ACO activity was measured spectrophotometrically by monitoring reduction of NADPH at 340 nm.

5.5. ^{27}Al-nuclear magnetic resonance (NMR) analysis

Cell sap was extracted from root tips (0–5 mm) according to the method of Ma et al. (1997b) and collected into an NMR tube (5 mm diameter). ^{27}Al-NMR spectra were obtained at 156.3 MHz (JNM-α600 spectrometer, JEOL, Tokyo, Japan) according to Morita et al. (2008). $AlCl_3$ (0.2 mM in 0.1 N HCl) was used as an external reference to calibrate the chemical shift (0 ppm). The ^{27}Al-NMR spectrum of the Al–citrate, Al–malate, and Al–oxalate complexes, prepared by mixing $AlCl_3$ with citric acid (1:1), malic acid (1:1), and oxalate (1:1), respectively, were recorded for comparison. The pH of the mixture was adjusted to that of the cell sap (pH 4.3 ± 0.2) with 0.1 N HCl. In addition, the contents of Al, malate, citrate, and oxalate in root cell sap were determined in young root samples as described above.

5.6. Statistic analysis

Data were analyzed statistically using Tukey test to determine significant difference in the data among the groups. *P* values less than 0.05 were considered significant. The values are represented as mean ± S. D.

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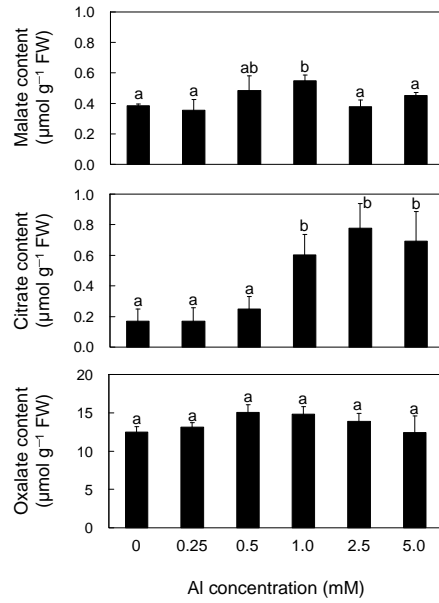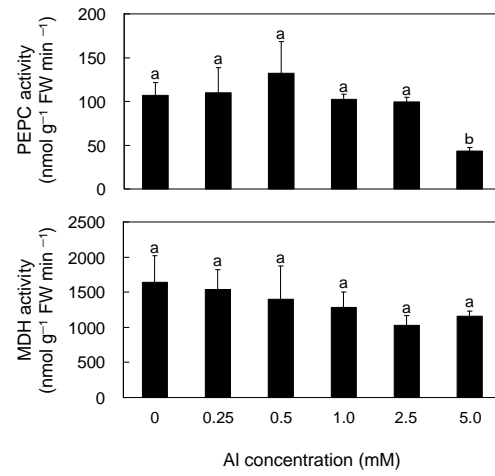**Figure**
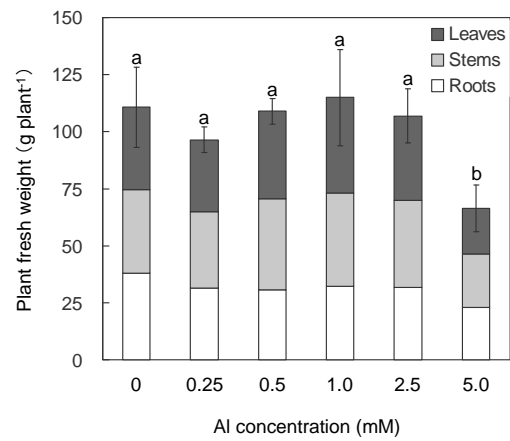
Fig. 1 Effect of Al on growth of *Eucalyptus camaldulensis*.
Values are mean ± SD (*n*=3). Means followed by different
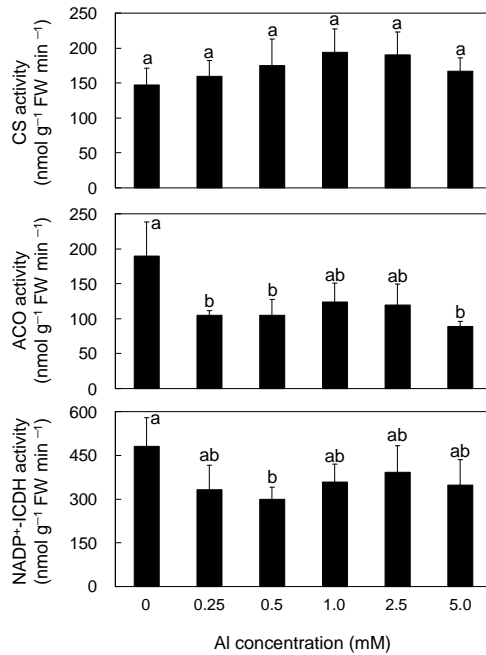letters are significantly different at *P*<0.05.
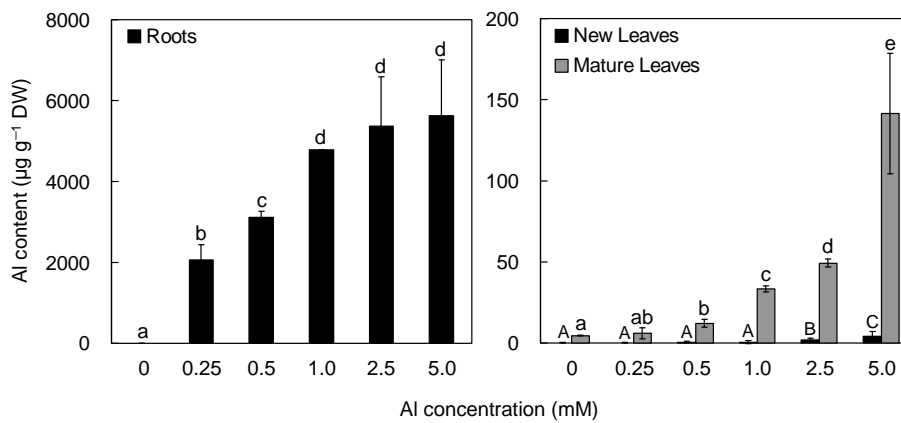
Fig. 2 Effect of Al on Al content in young and old leaves and young roots of *Eucalyptus
camaldulensis*. Values are mean ± SD (*n*=3). Means followed by different letters are
significantly different at *P*<0.05.

Fig. 3 Effect of Al concentration on contents of malate, citrate and oxalate in young roots of *Eucalyptus camaldulensis*. Values are mean ± SD (*n*=3). Means followed by different letters are significantly different at *P*<0.05.

Fig. 4 Effect of Al concentration on activities of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) in young roots of *Eucalyptus camaldulensis*. Values are mean ± SD (*n*=3). Means followed by different letters are significantly different at *P*<0.05.

Fig. 5 Effect of Al concentration on activities of citrate synthase (CS), aconitase (ACO), and NADP+-isocitrate dehydrogenase (NADP+-ICDH) in young roots of *Eucalyptus camaldulensis*. Values are mean ± SD (*n*=3). Means followed by different letters are significantly different at *P*<0.05.
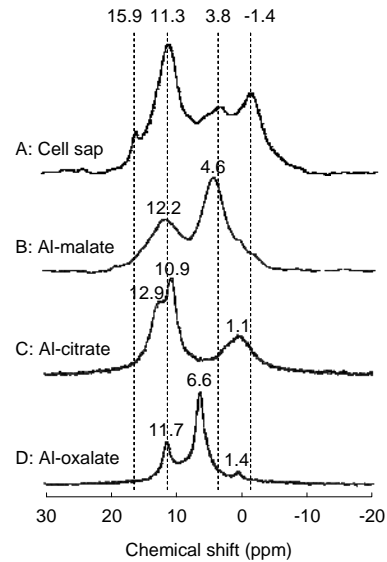
Fig. 6 ^{27}Al-NM Spectrum of cell sap from root tips (A) of *Eucalyptus camaldulensis* grown in nutrient solution containing Al 1.0 mM for 5 weeks and Al model solutions (pH 4.3 ± 0.2); Al-malate (B), Al-citrate (C), and Al-oxalate (D) solutions. Numerical values show chemical shifts (ppm) referenced to $AlCl_3$ (0 ppm).

Table 1 pH values and contents of Al and organic acids in cell sap of root tips treated with 1.0 mM Al.

Measurement	Value
pH	4.32
Al (mM)	1.032
Malate (mM)	1.489
Citrate (mM)	1.300
Oxalate (mM)	0.258