Journal of Plant Biology, June 1999, 42(2) : 151-158

Aluminum Uptake and Aluminum-Induced Rapid Root Growth Inhibition of Rice Seedlings

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Aluminum (Al) inhibits root growth in acidic soil, but the site of action of Al remains unclear. We investigated whether the rate of Al accumulation correlates to Al-indeced rapid root growth inhibition in rice seedlings (*Oryza sativa* L. cv. Youngnam). Growth of roots was significantly inhibited by 100 μM $AlCl_3$ as early as 1 h after the treatment. The inhibition of root growth was strongly dependent on Al concentration (I_{50} = 20 μM) and Al-exposure time (I_{50} = 23 min at 25 μM Al) in a solution of 10 mM KCl and 1 mM $CaCl_2$ buffered by 10 mM Mes/KOH (pH 4.5). Using ICPES, massive uptake of Al by roots was observed even at 15 min treatment of 25 μM Al. The kinetics of Al uptake by the roots closely corresponded to the inhibitory effects of Al on root growth. When the roots of seedlings were exposed to 50 μM Al for 1 h, then sectioned and stained with hematoxylin, all cell types of the roots showed the presence of Al in the cytoplasm. These results indicate that Al was rapidly taken up into the root cells and thereby reduced root growth.

Key words: Aluminum uptake, rice (*Oryza sativa* L. cv. Youngnam), root elongation, hematoxylin, cytoplasm

Aluminum (Al) is the third most abundant element in the crust of the earth. The solubilization of Al is enhanced in acidic environments. The soluble forms of Al are important in mineral phase formation and transitions, in the mobility of Al in soil and aquatic systems, and in the toxicity of Al to plants and aquatic organisms (Delhaize and Ryan, 1995). Al toxicity is one of the major factors limiting plant growth in acidic soils (Foy, 1983; Haug, 1984), and has thus received recognition as a potential problem for humans and animals (Ganrot, 1986).

Recently, intense research efforts have been directed to elucidate the cellular mechanisms of Al toxicity in higher plants. The initial symptom of Al toxicity is the inhibition of root growth (Kochian, 1995), which becomes apparent within hours of Al exposure (Wallace et al., 1982). Such a rapid inhibition of root growth by Al results in inhibition of nutrient and water uptake (Foy, 1983), and has been proposed to be caused by a number of different mechanisms, including Al interactions with cell wall, the plasma membrane, or the root symplasm (Kochian, 1995). Most importantly, it is still disputed whether the initial toxic effects of Al are manifested in the cell wall or within the plant cell. Al has been reported to cross the plasma membrane and thus cause the inhibition of root growth inside the cells (Rengel, 1996), although no information exists about which Al species or complexes take part in the transmembrane flux. On the other hand, Bennet and Breen (1991) have suggested that Al may act indirectly, via a signal-response pathway involving the root cap. Therefore, it is still not clear how the initial Al uptake is related to the root growth inhibition. One of the reasons for the discrepancies is the methodological problem owing to the difficulties of distinguishing between the Al residing in the apoplast and that in the symplast. Al may bind to cell surface, or form Al precipitates in the cell wall (Kinraide, 1991; Tice et al., 1992), and can also be taken up into the cytoplasm, and these are difficult to discriminate. A limiting resolution of microanalytical techniques, such as electron probe X-ray microanalysis (Lazof et al., 1994) and graphite furnace atomic absorption spectrometry (Vitorello and Haug, 1996) for the analysis of Al uptake is also one of the main factors contributing to the different Al content data in the tissues. Despite these research problems, some evidence supports the idea that Al is taken up into the root apical symplasm (Tice et al., 1992; Lazof et al., 1994; Kochian, 1995). It is probably taken up as a neutral Al ligand, by endocytosis, through membrane-bound proteins, or via stress-related lesions (Tice et al.,

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Abbreviations: Al=aluminum (total Al=Al, free Al=Al^{3+}); BSA=bovine serum albumin; ICPES=inductively coupled plasma emission spectrophotometry; PVP=polyvinylpyrrolidone.

1992). However, since these studies used fairly high concentrations of Al and long-term effects of Al, we still do not know whether Al is taken up into the root cells before the root growth is inhibited.

In the present work, we used a rice plant cultivar highly sensitive to Al for investigating the cellular mechanisms of Al toxicity in root elongation. We have especially focused on whether Al is taken up into the root cells within the short term before Al induces root growth inhibition. Al distribution in several types of root cells was visualized by hematoxylin staining, and the results showed that the root cells can rapidly take up Al from the surroundings, within the time frame of the Al-inhibition of root growth in rice seedlings.

MATERIALS AND METHODS

Plant Material

Seeds of rice (*Oryza sativa* L. cv. Youngnam) were imbibed in distilled water for 2 days at room temperature. The seeds were surface-sterilized for 30 min by soaking in a freshly prepared, filtered solution of 1.5% (w/v) calcium-hypochlorite, thoroughly washed with tap water and rinsed with distilled water. Seeds were grown on a nylon net in plastic boxes holding 3 L of deionized water in a growth chamber (Conviron. model no. E15, Controlled Environments. Pembina, ND) for 3 days. The culture for seed germination was aerated and carried out at 30/25°C, 16-/8-h and 80% RH in the dark. The axial roots from 3-day-old seedlings were used for all experiments.

Measurement of Root Elongation

The measurement of root elongation was done through a video image (x70) equipped with a light microscope. Six seedlings were selected for uniform initial root length. The mean length of the whole roots was 3.2 ± 0.3 cm. Each primary root from the seedlings was marked with India ink at 1.5 mm intervals from the tip and transferred to a 250 mL solution containing 10 mM Mes, 10 mM KCl, and 1 mM $CaCl_2$, pH 4.5. The solution was prepared just before use. The pH of the solution was found to be quite stable during the experiments. The Al stock solution was 10 mM $AlCl_3$ in 0.1 N HCl. For comparison, a stock solution of Fe^{3+} was made as 10 mM $FeCl_3$ in 0.1 N HCl. A stream of air was bubbled throughout the measurements. All experiments were done under dim light at room temperature.

Analysis of Ion Uptake by Inductively Coupled Plasma Emission Spectrophotometry (ICPES)

The seedlings were treated for 1 h in a solution containing 10 mM Mes, 10 mM KCl, and 1 mM $CaCl_2$, pH 4.5 with and without $AlCl_3$. In other experiments, we tested the possibility of competitive uptake between Al^{3+} and Fe^{3+} into the roots. The seedlings were treated for 1 h with a combination of Al and Fe at 200 μM each in the solution above. The seedlings after 1 h exposure to Al were transferred to 0.5 mM citrate (pH 4.5) at 0°C for 30 min to desorb cell wall-bound Al, and then thoroughly rinsed with cold deionized water (Zhang and Taylor, 1990). Al-treated roots and their corresponding controls were harvested and dried at 60°C for 24 h. Collected tissues (about 20 mg) were suspended overnight in a mixture of 1 mL of HNO_3 plus 0.5 mL of H_2O_2. They were then digested by the addition of a few drops of HCl at 150°C for 6 h. A colorless transparent solution was obtained by the addition of 1% (v/v) HNO_3. Concentrations of ions were determined by ICPES (model IV ICPES, Shimadzu, Kyoto, Japan).

Hematoxylin Stain

The intact roots and the root cells of 3 day-old seedlings were stained using hematoxylin described by Delhaize et al. (1993). The roots of seedlings were exposed to 50 μM $AlCl_3$ for 1 h in a solution (pH 4.5) of 10 mM Mes, 10 mM KCl, and 1 mM $CaCl_2$. After Al treatment, they were thoroughly rinsed in deionized water for 10 min, and then photographed. To identify whether Al localizes within cells of Al-treated roots, Al-treated seedling roots were sectioned, plasmolyzed and stained with hematoxylin. Each part of the primary roots from Al (at 50 μM for 1 h, pH 4.5)-treated seedlings was excized and chopped with a razor blade in a solution containing 0.4 M mannitol, 10 mM Mes, 1 mM $CaCl_2$, 0.5% (w/v) polyvinylpyrrolidone (PVP, MW 40,000; Sigma), and 0.1% (w/v) BSA (fraction V; Sigma, St. Louis, MO, USA), pH 5.6. They were then thoroughly washed with a solution of 0.4 M mannitol and 1 mM $CaCl_2$ containing 0.5 mM citrate (pH 4.5) to remove cell wall-bound Al. To obtain single cell layers with living cells from Al-treated roots, the root segments were digested in an enzyme solution which contained 2% (w/v) cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.2% (w/v) pectolyase Y-23

(Seishin Pharmaceutical Co., Ltd., Tokyo, Japan), 0.4 M mannitol, 1 mM $CaCl_2$, 0.5% (w/v) PVP, 0.1% (w/v) BSA, pH 5.6. They were agitated by shaking in the dark at 28°C for 30 min. From the digested root segments, single cell layers were harvested on a nylon mesh of 200 μm and washed again with a solution of 0.4 M mannitol and 1 mM $CaCl_2$ containing 0.5 mM citrate (pH 4.5). This procedure allowed separation of a fair number of tissue pieces in single cell layer, clearly showing plasmolyzed cells. Three cell types, root hairs, elongated cells, and root meristematic cells, differing in the cell size, and morphology, were observed under a light microscope. The cells were all viable as determined by using fluorescein diacetate (FDA) (Goh et al., 1995). They were stained with hematoxylin in 0.4 M mannitol and 1 mM $CaCl_2$, and then photographed.

Free Al^{3+} Activity

Table 1 shows the free Al^{3+} activities and ionic strengths of the solution of pH 4.5 at total Al concentrations used in this study as calculated by the computer program GEOCHEM-PC version 2.0 (Parker et al., 1995).

RESULTS

Effects of Al on the Root Elongation

Under control conditions, the elongation of the primary root axes proceeded about 1.1 ± 0.2 cm per day. When the roots were exposed to various concentrations of Al, the root elongation was rapidly inhibited at 1 h after the treatment and showed about 90% inhibition at 100 μM Al (pH 4.5) (Fig. 1). The concentration required to achieve 50% of the maximum rate was at 20 μM Al when the rates of root elongation were normalized to percentage of untreated controls. At further investigation, the inhibitory effect occurred as early as 30 min when the roots were placed in the different concentrations of Al (Fig. 2). However, a complete inhibition of root elongation was not observed even after 3 days of exposure to 200 μM Al in this system (elongation rate was 13% of maximum). These results showed that Al toxicity in root elongation was strongly dependent upon Al concentration and became significant within 30 min.

Table 1. Total Al concentration, free Al^{3+} activity, and ionic strength in uptake solutions of 10 mM Mes (pH 4.5) containing 10 mM KCl and 1 mM $CaCl_2$.

Total Al (μM)	Al^{3+} activity (μM)	Ionic strength (mM)
0	0.00	13.08
10	1.78	13.12
20	3.55	13.18
50	8.92	13.30
100	17.73	13.50
200	35.10	13.92

The free activity of Al^{3+} and the ionic strength in the uptake solutions (pH 4.5) were estimated using the computer software program GEOCHEM-PC version 2.0. The log K values of thermodynamic constants used were −5.01 $(AlOH)^{2+}$, −8.7 $(Al(OH)_2)^{+1}$, −15.2 $(Al(OH)_3)$, and −23.3 $(Al(OH)_4)^{-1}$.

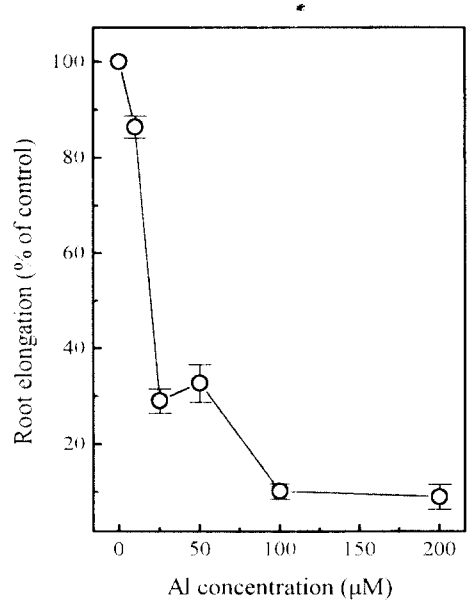

Figure 1. Root elongation of rice seedlings treated with various concentrations of Al. Seedlings exposed to Al for 1 h in the solution containing 10 mM Mes (pH 4.5), 10 mM KCl, and 1 mM $CaCl_2$. The root elongations were normalized by percentage of the controls without treatment of Al (mean ± SE, $12 \leq n \leq 18$). The absence of an error bar indicates that the size of the error does not exceed the size of the symbol.

Al Accumulation by Rice Root

On the basis of the results from Figures 1 and 2, we investigated Al content in roots by using the inductively coupled plasma emission spectrophotometer (ICPES) method. Al uptake by roots was found to be critically dependent upon the exposure time (Fig. 3A). The time-course showed a rapid increase up to 30 min, then a steady state of Al content in the tissue. During such an active Al uptake period, there were no significant changes in K^+ content (Fig. 3B). Comparison of these results with that of the time course of

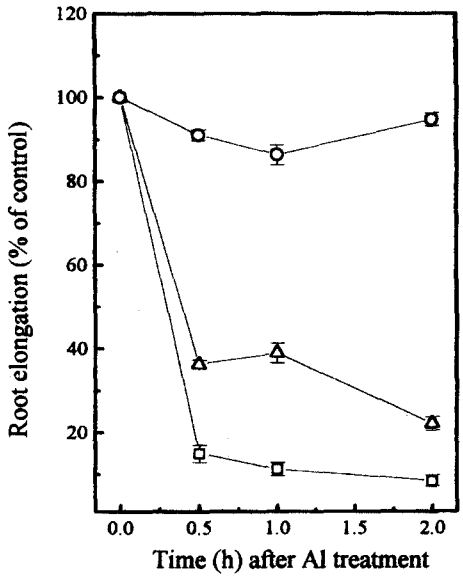

Figure 2. Time course of root elongation of rice seedlings in a solution containing 10 (○), 25 (△), and 100 (□) μM Al. The experimental conditions were the same as in Figure 1. The mean ± SE is shown ($14 \leq n \leq 18$).

root elongation inhibition suggests that there may be a close correspondence between Al content and the inhibitory rates of root elongation during the first hour of Al exposure.

Distribution of Al in Root Cells

To identify whether Al is taken up into the cells of the roots within the time frame of Al-induced root growth inhibition, we observed cells from Al-treated seedling roots (50 μM Al for 1 h) as shown in Figure 4A and B. These materials were distinctly stained by hematoxylin, which has been widely used to locate binding sites of aluminum in plant and animal cells (Havas, 1986; Ono et al., 1995). Our pictures clearly show the binding of hematein (oxidized hematoxylin, purplish blue color) to constituents of cells and explicitly indicate the localization of Al in the cytoplasm (Fig. 4, D, F, and H) when compared to untreated controls (Fig. 4, C, E, and G). Especially, it was much more densely stained in the root meristematic cells (Fig. 4H). The results strongly suggest that Al could readily enter the root cells of rice seedlings.

Fe^{3+}/Al^{3+} Interaction for Al Uptake

In order to understand the physiological mechanism for Al uptake by roots, we investigated a possible interaction between Al^{3+} and Fe^{3+}, which are solubilized in acidic soil. Since rice (a strategy II plant) releases Fe^{3+}-binding compounds called "phytosid-

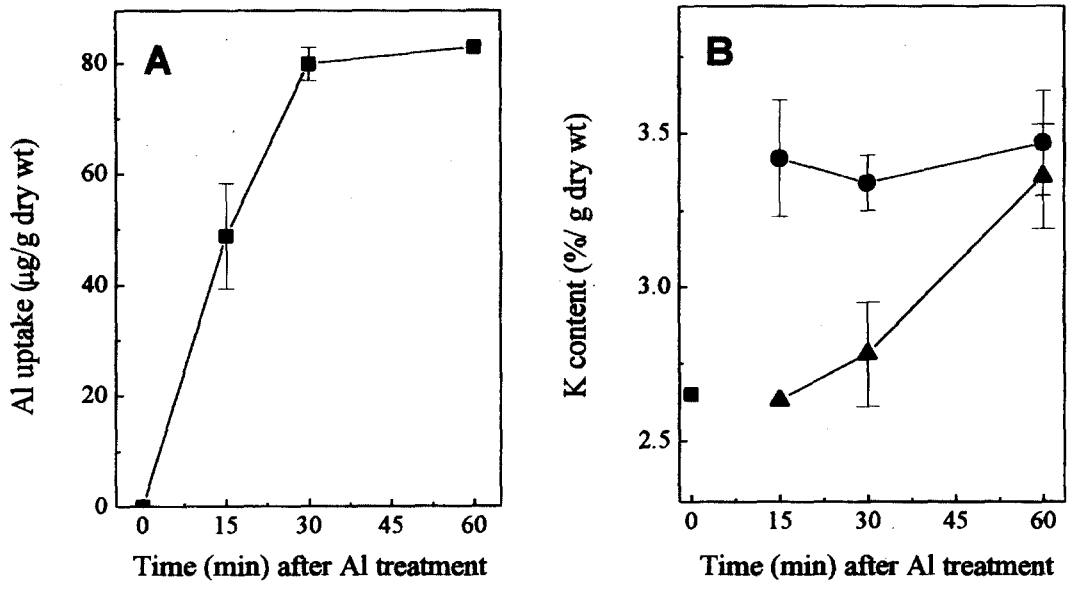

Figure 3. Time course of total Al (A) and K (B) accumulated by roots exposed to 20 μM Al, assayed using ICPES. The experimental conditions were the same as in Figure 1. The mean ± SE of three independent experiments (n = 225 seedlings) is shown, and the absence of error bars indicates that the size of the error does not exceed the size of the symbol. (A), Al amount was below 0.1 μg Al/g dry wt during 1 h exposure to 20 μM Al at 4°C. (B), K amount was analyzed from the samples used in (A). The filled square (■) indicates K content in control samples without treatment with Al at T = 0. Accumulated K amount during 1 h exposure to 20 μM Al at 4°C was 2.47 ± 0.07%/g dry wt.Both Al-treated (●) and untreated control (▲) samples were analyzed.

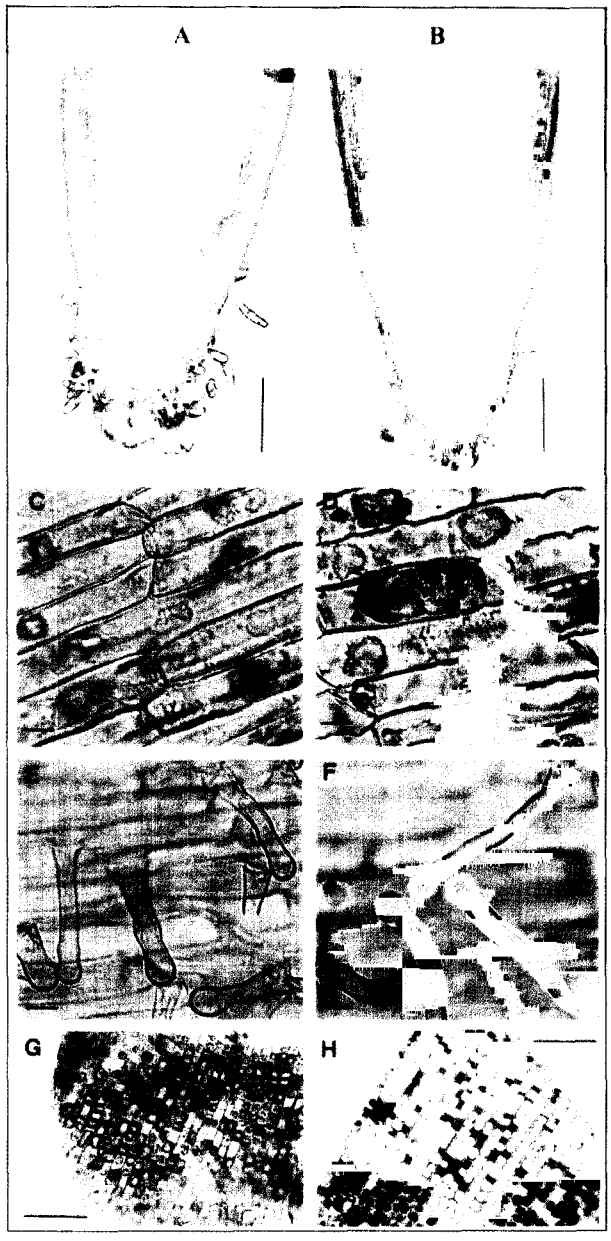

Figure 4. Localization of Al in intact roots and root cells of rice seedlings. (A) and (B), Localization of Al in an intact root. Seedlings were exposed to 50 μM Al for 1 h in a solution of 10 mM Mes (pH 4.5), 10 mM KCl, and 1 mM $CaCl_2$. A, control; B, Al-treated root. The bar is 30 μm. The other conditions were the same as in Figure 1. (C-H), Localization of Al in the root cells separated from Al-treated and control roots. Al-treated roots were obtained from the seedlings exposed to 50 μM Al for 1 h in a solution of 10 mM Mes (pH 4.5), 10 mM KCl, and 1 mM $CaCl_2$. C (control) and D (Al-treated), mature elongated cells; E (control) and F (Al-treated), root hairs; G (control) and H (Al-treated), root meristematic cells. Hematoxylin staining was done for 30 min (See "Materials and Methods"). The bars are 16 μm for C-F and 30 μm for G and H.

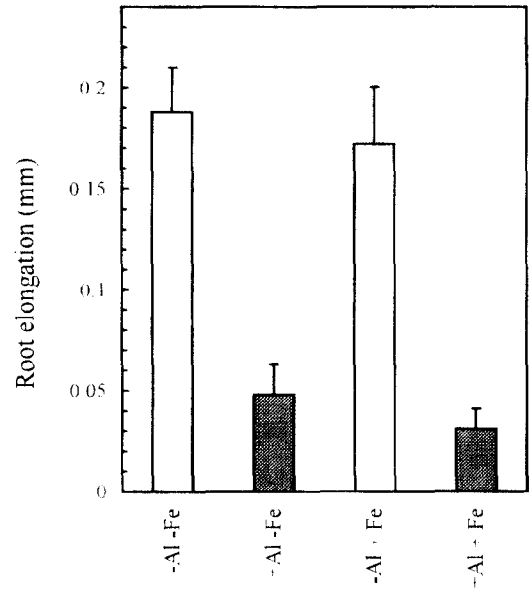

Figure 5. Effect of Fe^{3+} in Al-induced inhibition of root elongation of rice seedlings. Total Al and Fe concentrations in the solution were 200 μM each at pH 4.5, respectively. The treatment was 1 h. The other experimental conditions were the same as in Table 2. The error bars denote the SE values of the mean of 15 seedlings.

Table 2. Effects of $AlCl_3$ and $FeCl_3$ on levels of aluminum, iron, and potassium in rice root.

Treatment	Al	Fe	K
	(μg/g dry wt)	(μg/g dry wt)	%/g dry wt
−Al−Fe	N.D.	82.5 ± 1.7	2.47 ± 0.07
+Al−Fe	245.3 ± 3.8	99.1 ± 35.4	2.35 ± 0.09
−Al+Fe	21.7 ± 1.2	1506.7 ± 20.3	2.21 ± 0.03
+Al+Fe	382.0 ± 6.3	1890.0 ± 11.6	2.53 ± 0.05

Roots of seedlings (n = 75) were treated with either $AlCl_3$ (200 mM) or $FeCl_3$ (200 mM) or both for 1 h in the dark at room temperature. Solutions used for root uptake were buffered by 10 mM Mes (pH 4.5; See Materials and Methods). The values are the mean (±SE) concentrations of three experiments. N.D. = not detected.

erophores" into the surrounding soil, which bind and take up Fe^{3+} and possibly other cations into the roots (Fide et al., 1996), we expected that the number of Al binding sites would decrease if there was a competitive interaction between Al and Fe uptake into rice seedlings. However, contrary to our expectation, Fe^{3+} did not ameliorate the inhibition of root elongation by Al; in the presence of Fe^{3+}, the elongation of Al-treated root was not significantly different from controls that were treated with only Al (Fig. 5). Addition of Fe alone into solution had a negligible effect

on root elongation. The uptake analysis also showed a 55.7% increase of Al contents in the presence of Fe^{3+} and a 25.4% increase of Fe^{3+} content in the presence of Al (Table 2). A synergistic effect in Al and Fe uptake by roots is not consistent with the hypothesis of a common binding site of Fe and Al in the plasma membrane of root cells. When the seedlings were incubated for 1 h at 20 μM Al in the presence of $FeCl_3$ ranging from 0 to 40 μM, we could neither detect a significant effect of Fe on Al accumulation in roots nor on the Al-inhibition of root elongation (data not shown). The results indicate that Al uptake by roots is not related to a specific uptake system for iron in acidic soil.

DISCUSSION

Plants in acidic soil grow and develop in an environment with the constant presence of Al, experiencing the cumulative toxic effect of Al. The most easily recognized symptom of Al toxicity is the inhibition of root growth. A number of reports have shown that the initial symptom of Al toxicity can occur within 1-2 h after exposure to Al (Wallace and Anderson, 1984; Ownby and Popham, 1989; Kim and Lee, 1998). The inhibitory effect of Al in squash roots was found mainly in the elongation zone, indicating that the inhibition is primarily caused by a decline in cell elongation (Van et al., 1994). In this paper we showed that Al inhibits elongation of rice seedling roots in concentration-dependent (Fig. 1) and time-dependent (Fig. 2) manners. Especially striking was that the root elongation was strongly inhibited by Al as early as 30 min of treatment time. Such a rapid inhibition by Al agrees well to the results from wheat (Wallace and Anderson, 1984; Ryan et al., 1992) and squash seedlings (Van et al., 1994).

However, since it is still unclear whether the inhibition of root elongation by Al is directly related to the degree of Al uptake in roots, we conducted the analysis of Al accumulation by roots for ≤1 h incubation at 20 μM Al (pH 4.5). Figure 3A showed the uptake kinetics with a rapid phase up to 30 min followed by a phase of steady accumulation of Al in roots. Such uptake kinetics of Al well corresponded with that of root elongation by Al in Figure 2. Therefore, our results are consistent with the hypothesis that the amount of Al absorbed by the cells was a determining factor in the inhibition of growth by Al (Yamamoto et al., 1994).

The inhibition of root elongation by Al seems to be largely due to Al^{3+}, a dominant form in the acidic condition of pH 4.5 (Table 1). Many trivalent cations are toxic to plants and, because Al toxicity is largely restricted to acid conditions, it is generally assumed that Al^{3+} is the major phytotoxic species (Martin, 1988; Kinraide, 1991; Delhaize and Ryan, 1995; McDonald-Stephens and Taylor, 1995). We therefore investigated the effect of a possible competing metal for Al accumulation in roots, Fe^{3+}. However, contrary to our expectation, we could not detect any competitive relationship between Al/Fe for their accumulation and the inhibition of elongation in rice roots (Fig. 5 and Table 2). Al accumulation in the roots was rather slightly accelerated by Fe^{3+} supply while the inhibition of root elongation did not change significantly. These results indicate that at least during the short term incubation, the uptake of two metals are not via the same transporter.

Leakage of K^+ ions from cells has often been used as a sensitive measure of membrane dysfunction (Taylor, 1988). Ono et al. (1995) thus showed a significant decrease of K^+ content in the cells after 18 h treatment with Al which resulted in dysfunction of membrane permeability barrier in the presence of both Al and Fe. In contrast, our result did not show any significant change in the K^+ content of Al-treated cells (Table 2), and this difference may be due to the short time frame of the experiment. K^+ channels have been reported to have sensitivities to Al; recent patch clamp studies have shown that Al^{3+} blocks inward-rectifying K^+ channels in the mature region of roots (Gassmann and Schroeder, 1994) and inhibits K^+ uptake (Taylor and Foy, 1985; Miyasaka et al., 1989). In addition, Al was found to be an effective antagonist of the outward-rectifying K^+ channels (Ryan et al., 1995).

To investigate whether Al enters the root cells within 1 h, we used the cells separated from Al-treated roots, which clearly showed Al distribution in the cytoplasm (Fig. 4). This result is in contrast to the result of Yamamoto et al. (1994), who showed no uptake of Al by tobacco suspension cells within 10 h exposure time. This discrepancy may be due to the difference in plant species. Our pictures show quite remarkably that the cells in the meristematic region were much more densely stained than those in the other areas of the root, suggesting a high concentration of Al binding molecules and/or Al transporters in meristematic cells.

In conclusion, we propose that all root cells can rapidly take up Al, and the cells in elongation zone are inhibited in elongation, which ultimately results in retardation of the root growth. However, the toxic

mechanisms of Al in meristematic and differentiated zones are still not clearly understood and they merit further studies.

ACKNOWLEDGEMENT

This work was supported by grants from the Korea Research Foundation made in the program year of 1998 and from the Korea Institute of Science and Technology Evaluation and Planning awarded to Y. Lee and a postdoctoral fellowship from Korea Research Foundation (300-599) to C-H. Goh. We wish to thank Dr. Magaly Rincon-Zachary, Department of Biology, Midwestern State University, U.S.A. for the generous gift of GEOCHEM-PC program used in this study. In addition, we thank Korea Basic Science Institute for the tissue mineral analysis.

Received June 10, 1999; accepted June 19, 1999.

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