J. For. Res. 5: 81–85 (2000)

Effect of Ca on Al-induced Activation of Antioxidant Enzymes in the Needles of Hinoki Cypress (*Chamaecyparis obtusa*)

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The effect of $Ca(NO_3)_2$ on the active oxygen scavenging system in hinoki cypress (*Chamaecyparis obtusa*) seedlings cultured in a nutrient solution containing aluminum was examined. The hinoki cypress seedlings were transferred to nutrient solutions containing 5 mM $AlCl_3$ together with various concentrations of $Ca(NO_3)_2$ in pots containing glass beads and Teflon tips. The growth in height and dry matter allocation to each organ was little influenced over a period of 12 weeks by either Al or the concentration of $Ca(NO_3)_2$. The activity of superoxide dismutase (SOD) in the needles was stimulated by Al, and the effect of Al was lowered significantly by simultaneous application of 25 mM $Ca(NO_3)_2$. At week 1, the activity of catalase (CAT) in the needles was increased by Al, but the effect was no longer observed at week 12. The Al concentration in the roots was increased by treatment with Al, whereas the Al concentration in needles was not. These results indicate that rhizospheric Al stress stimulates antioxidative enzyme activities in hinoki cypress needles and the activation of the enzymes is suppressed by addition of Ca. The transmission of Al stress to the needles, which induced a change in the enzyme activity, is not caused by the transfer of the Al ion itself from roots to needles.

Key words: calcium/aluminum (Ca/Al) ratio, catalase (CAT), hinoki cypress (*Chamaecyparis obtusa*), superoxide dismutase (SOD)

Soil acidification with pH below 4.0 can cause leaching of aluminum (Al) (Sato and Takahashi, 1996), which is toxic for many plants (Foy *et al.*, 1978; Rengel, 1992). Aluminum affects plant growth by causing an imbalance in the mineral nutrition of magnesium (Mg) and calcium (Ca) (Kelly *et al.*, 1990). On the other hand, it is well known that Al toxicity is ameliorated by cations, especially Ca and Mg (Kinraide and Parker, 1987). Ulrich (1983) concluded that Al stress in trees correlates with the molar ratio of the Ca/Al rather than the Al concentration in soil solution. Cronan and Grigal (1995) concluded in their review on Al stress in 18 species of trees that there is a 50% risk of an adverse impact on tree growth or nutrition when the Ca/Al ratio in soil solution reaches 1.0.

When plants are stressed, they produce large amounts of such active oxygen species as superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) that can result in photoinhibition and photooxidation. Most organisms have an enzymatic system associated with superoxide dismutase (SOD) and catalase (CAT) to scavenge such active oxygen species (Scandalios, 1993). The effects of environmental stresses, such as drought (Baisak *et al.*, 1994), ozone (Jung *et al.*, 1994; Inaba *et al.*, 1998), SO_2 (Tanaka and Sugahara, 1980), salt (Lechno *et al.*, 1997) and acidic mist (Tezuka *et al.*, 1998; Ogawa *et al.*, 1998), on plants have been estimated by determining their effects on the activities of SOD and CAT. Although many reports have been published on the effects of Al on plant growth and the morphological changes in roots caused by Al stress (Joslin and Wolfe, 1989; Kelly *et al.*, 1990; Zysset *et al.*, 1996; Hirano *et al.*, 1997), only a few have described the effect of Al stress on roots in detail (Cakmak and Horst, 1991; Severi, 1997; Richards *et al.*, 1998).

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Herein, we investigate the influence of $Ca(NO_3)_2$ given together with Al in the nutrient solution on the activities of SOD and CAT in needles of hinoki cypress (*Chamaecyparis obtusa*) seedlings.

Materials and Methods

1 Plant materials

Seedlings of hinoki cypress (*Chamaecyparis obtusa*) were grown in pots (plastic pot, 1/5,000 are [1 are = 100 m^2], 3.6 L; Iris Ohyama Inc., Sendai, Japan) containing soil composed of Kanuma soil (Otasangyo Co., Nagoya, Japan), Akadama soil (Otasangyo Co., Nagoya, Japan) and brown forest soil in a ratio of 2:3:5, v/v/v in the experimental field at Nagoya University in Aichi Prefecture, Japan.

Five-year-old seedlings (35 samples, about 70 cm height) of hinoki cypress were rinsed with streaming water and each transplanted to a plastic pot (Wagner pot, 1/5,000 are, 3.6 L; Fujimoto Kagaku Kogyo Co., Tokyo, Japan) filled with glass beads 2.0–2.8 mm in diameter and Teflon tips on May 30, 1997. Glass beads were used for precise control of the root environment in the growth medium, and the Teflon tips were used to avoid excessive compression of the root system. A five-fold-diluted solution of Hoagland No. 2 (Hoagland and Arnon, 1950) was used as the basic nutrient solution. The concentration of each element in the nutrient solution is given in Table 1. During the experiment, all pots were placed in an open frame (1.9 × 5.3 × 1.8 m), this was covered with plastic film to shelter the seedlings from rain.

2 Experimental treatment

The seedlings were divided into five groups, of seven pots per group, and supplied with the nutrient solution containing 5 mM $AlCl_3$ and $Ca(NO_3)_2$ at various concentrations. Table 2 shows the concentrations of Ca and Al and the Ca/Al ratio for each group. Seedlings in Group 1 (control) were supplied with the basal nutrient solution containing 0.8 mM $Ca(NO_3)_2$,

Table 1 Composition of fivefold-diluted Hoagland solution No.2.

Element	Compound	Concentration (mM)
K	KNO_3	1.2
Ca	$Ca(NO_3)_2 \cdot 4H_2O$	0.8
Mg	$MgSO_4 \cdot 7H_2O$	0.4
P	$NH_4H_2PO_4$	0.2
Mn	$MnCl_2 \cdot 4H_2O$	0.0091
Fe	Fe EDTA	0.0179
Zn	$ZnSO_4 \cdot 7H_2O$	0.0008
Cu	$CuSO_4 \cdot 5H_2O$	0.0003
B	H_3BO_3	0.0046
Mo	$Na_2MoO_4 \cdot 2H_2O$	0.0005

Table 2 Concentrations of Ca and Al in the nutrient solutions.

Group	$Ca(NO_3)_2$ (mM)	$AlCl_3$ (mM)	Ca/Al ratio
1	0.8	0	–
2	25.0	5.0	5.00
3	12.5	5.0	2.50
4	5.0	5.0	1.00
5	0.8	5.0	0.16

and those in Groups 2–5 were supplied with 5 mM $AlCl_3$ together with various concentrations of $Ca(NO_3)_2$. The 5 mM Al treatment is known to cause growth inhibition of hinoki cypress significantly (Kohno *et al.*, 1995). On the other hand, it is known that Al toxicity is ameliorated by Ca (Kinraide and Parker, 1987), and there is a 50% risk of an adverse impact on tree growth when the Ca/Al ratio in soil solution reaches 1.0 (Cronan and Grigal, 1995). The treatment group conditions were decided according to those previous researches. All nutrient solutions were adjusted to pH 3.5–4.0 with HCl. About 200 mL of each solution was applied to each pot two or three times a week in the evening. The experimental treatments began on August 18, 1997.

3 Measurement of tree height and dry matter allocation to each organ

The height of the seedlings (7 samples for each group) was measured at 0, 8 and 12 weeks after the start of the treatments. At week 12, fresh needles, dead needles, stems, white roots and other roots were separated and rinsed with deionized water (3 samples for each group). The half bulk of the fresh needles was used for the enzymatic assays. The remaining needles and other organs were oven-dried at 85°C for 48 h, placed in a desiccator overnight, and weighed to determine dry matter allocation to each organ (% of dry weight of each organ to that of the whole plant).

4 Assays of enzyme activities

At 0, 1, 7, and 12 weeks after the start of the treatments, a branch was cut from each seedling. Current-year needles (1.0 g f. wt) from the cut branch were homogenized in 3 mL of grinding medium composed of 0.1 M MOPS (3-morpholinopropanesulfonic acid)-KOH (pH 7.5), 1 mM Na_2EDTA, DTT (dithothreitol) (1 mM) and 0.1 g polyvinylpolypyrrolidone (Polyclar AT, Sigma Chemical Co.). The homogenate was centrifuged at 20,000 *g* for 5 min. The supernatant was dialyzed for 4 h and the resulting solution was used for assays of enzyme activities.

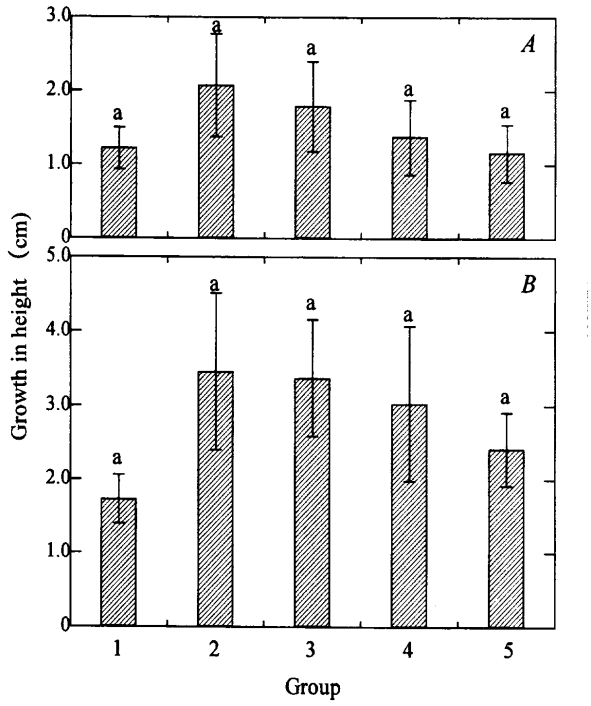

Fig. 1 Effects of Ca supplied with Al on height growth of hinoki cypress seedlings. The seedlings in groups 1–5 (Table 2) were measured after 8 (*A*) and 12 weeks (*B*) of measurement. Values shown are the means ± SE ($n = 7$). Signs with different letters indicate significant differences at $p < 0.05$ according to Fisher's LSD-test.

The enzymes were assayed at 25°C with a spectrophotometer (model U-3210; Hitachi, Tokyo). SOD activity was estimated by the method of Asada *et al.* (1973). The assay mixture (1 mL) contained 50 mM K-PO_4 (mixture solution of K_2HPO_4 and KH_2PO_4) (pH 7.8), 0.1 mM disodium-EDTA, 0.01 mM cytochrome *c*, 0.1 mM xanthine, xanthine oxidase (3.5 to 4.0 mU) and an enzyme fraction. CAT was assayed in a reaction mixture (1 mL) that contained 90 mM K-PO_4 (pH 7.0), 15 mM H_2O_2 and an enzyme fraction by the method of Beers and Sizer (1952). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. All analytical assays were repeated three times.

5 Measurement of Al concentration in roots and needles

At 12 weeks after the start of the treatment, white roots and needles were rinsed with deionized water, oven-dried and ground for the measurement of Al concentration (7 samples for each group). The Al content in 0.5-g samples was measured by an inductively coupled plasma-optical emission spectroscopy after wet ashing (Bernas, 1968).

Results

1 Tree height and dry matter allocation to each organ

The heights of hinoki cypress seedlings at weeks 8 and 12 are shown in Fig. 1*A* and 1*B*, respectively. At both stages, plants in group 2 [25 mM $Ca(NO_3)_2$] were the highest and seedling height decreased with decreasing concentration of $Ca(NO_3)_2$, although the difference was not statistically significant ($p > 0.05$, Fisher's LSD-test). Aluminum had no

Table 3 Dry matter allocation to each organ in hinoki cypress.

Group	Fresh needles (%)	Dead needles (%)	Stems (%)	White roots (%)	Other roots (%)	Above-ground part* (%)	Subterranean part** (%)
1	37.2 ± 4.4^{a}	4.3 ± 2.4^{ab}	31.3 ± 2.9^{a}	5.9 ± 1.3^{a}	21.4 ± 4.7^{a}	72.7 ± 4.7^{a}	27.3 ± 4.7^{a}
2	40.0 ± 2.8^{a}	2.6 ± 1.2^{ab}	34.9 ± 1.3^{ab}	2.7 ± 0.2^{b}	19.9 ± 2.9^{a}	77.4 ± 3.2^{a}	22.6 ± 3.2^{a}
3	40.4 ± 3.6^{a}	1.9 ± 0.5^{a}	31.5 ± 2.9^{a}	2.7 ± 0.1^{b}	23.5 ± 2.7^{a}	73.8 ± 2.5^{a}	26.2 ± 2.5^{a}
4	37.0 ± 0.6^{a}	4.0 ± 1.3^{ab}	33.7 ± 2.1^{ab}	4.3 ± 0.3^{ab}	21.1 ± 1.6^{a}	74.6 ± 1.4^{a}	25.4 ± 1.4^{a}
5	39.5 ± 2.7^{a}	2.1 ± 0.6^{ab}	39.7 ± 2.9^{b}	4.3 ± 0.3^{ab}	15.0 ± 2.6^{a}	81.2 ± 2.3^{a}	18.8 ± 2.3^{a}

* Above-ground part = Fresh needles + Dead needles + Stems, ** Subterranean part = White roots + Other roots. Values shown are the means ± SE ($n = 3$). Signs with different letters indicate significant difference at $p < 0.05$ according to Fisher's LSD-test.

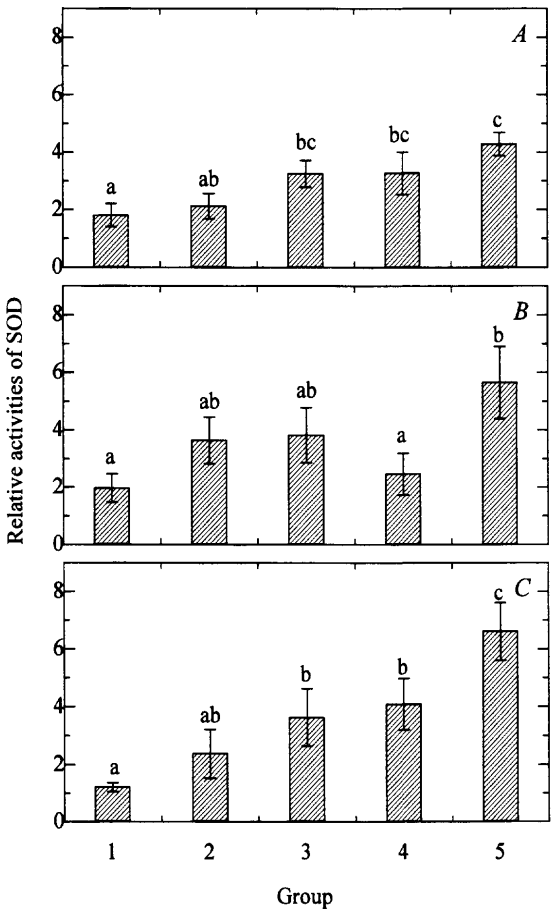

Fig. 2 Effects of Ca supplied with Al on superoxide dismutase (SOD) activity in needles of hinoki cypress seedlings assayed after 1 (*A*), 7 (*B*) and 12 weeks (*C*) of measurement. Relative activity of SOD just before the treatment was expressed as 1. The specific activity of needles just before the treatment was initiated was approximately 5.32 U mg^{-1} protein. Values shown are means ± SE ($n = 7$). Signs with different letters indicate significant differences at $p < 0.05$ according to Fisher's LSD-test.

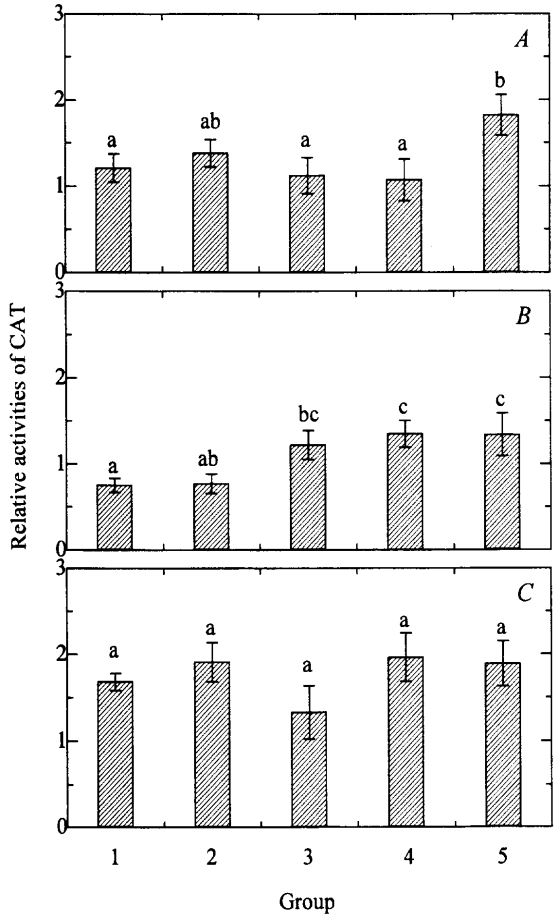

Fig. 3 Effects of Ca supplied with Al on catalase (CAT) activity in needles of hinoki cypress seedlings assay after 1 (*A*), 7 (*B*), and 12 weeks (*C*) of measurement. Relative activity of CAT just before the treatment was expressed as 1. The specific activity of needles just before the treatment was initiated was approximately 29.68 μM mg^{-1} protein min^{-1}. Values shown are the means ± SE ($n = 7$). Signs with different letters indicate significant differences at $p < 0.05$ according to Fisher's LSD-test.

effect on seedling height (compare groups 1 and 5).

The dry matter allocation to each organ of hinoki cypress after a 12-week culture in the test solutions are shown in Table 3. Except for white roots, the value for each organ was not significantly influenced by the concentration of $Ca(NO_3)_2$ (Ca/Al ratio). Although there were significant differences between the rates of white roots in group 1 (control) and groups 2 and 3 [25.0 and 12.5 mM $Ca(NO_3)_2$] ($p < 0.05$, Fisher's LSD test), there was no clear tendency between dry matter allocation to white roots and the concentration of $Ca(NO_3)_2$.

2 Enzyme activities in the needles

The enzyme activities in the needles were measured at weeks 0, 1, 7, and 12. Figures 2 and 3 show the enzyme activities of SOD and CAT as a relative value to that before the treatment (Figs. 2, 3). The comparison of the result of group 1 with that of 5 shows the effect of Al addition, because their Ca concentration in the nutrient solution were the same. The SOD activities at weeks 7 and 12 were significantly stimulated by $AlCl_3$ (Fig. 2). In the other hand, the results of groups 2–5 show the effect of Ca addition to the nutrient solutions containing Al in the same concentration, that is the

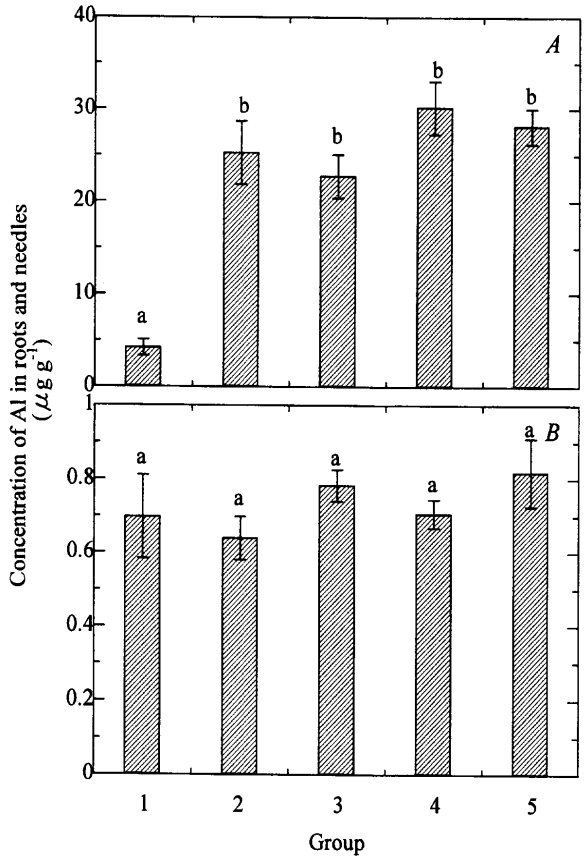

Fig. 4 Effect of Ca supplied with Al on the Al concentration of roots (*A*) and needles (*B*) of hinoki cypress seedlings measured after 12 weeks of measurement. Values shown are means ± SE ($n = 7$). Signs with different letters indicate significant differences at $p < 0.05$ according to Fisher's LSD-test.

effects of Ca/Al ratio. The effect of $AlCl_3$ was lowered by simultaneous application of $Ca(NO_3)_2$ (groups 2–4). Namely, the SOD activities depended on Ca/Al ratios in the nutrient solutions.

At weeks 1 and 7, the CAT activity was stimulated by the addition of $AlCl_3$ to the medium (compare group 1 with 5) (Fig. 3). The effect of $AlCl_3$ was significantly decreased by simultaneous application of $Ca(NO_3)_2$ at 12.5 and 5.0 mM (groups 3 and 4; Ca/Al = 2.5 and 1.0) when examined at week 1 and at 25 mM (group 2; Ca/Al = 5.0) when examined at week 7. That is to say, the activities tended to increase with the decreasing Ca/Al ratios. At week 12, no significant difference in CAT activity was observed between measurements.

3 Concentration of Al in roots and needles

The concentrations of Al in the roots and needles of hinoki cypress seedlings cultured in the nutrient solution containing 5 mM Al together with various concentrations of $Ca(NO_3)_2$ for 12 weeks are shown in Fig. 4. In the roots, the concentration of Al in group 1 (control) was significantly lower than in the other measurements ($p < 0.05$, Fisher's LSD-test) (Fig. 4A). In the needles, however, the concentration of Al was not influenced by the treatments (Fig. 4B).

Discussion

Kohno *et al.* (1995) grew hinoki cypress and Japanese cedar (*Cryptomeria japonica*) plants in nutrient solutions containing Al at various concentrations (Ca concentration was fixed to 0.8 mM) for 4 months and found that growth was significantly decreased by Al at concentrations higher than 5 mM or a Ca/Al ratio smaller than 0.16. Zysset *et al.* (1996) observed a decrease in the biomass of needles and fine root ratio after a 31-week culture in a medium containing Ca and Al at a ratio of 0.1, in the European chestnut (*Castanea sativa*). In our experiment, height growth and dry matter allocation to each organ in hinoki cypress seedlings were not significantly affected by the presence of Al in a growth medium (Table 3). The culture period of 12 weeks may not have been long enough to cause the morphological changes.

The CAT activity in the needles was significantly increased by the application of Al within 1 week and SOD activity within 7 weeks (Figs. 2 and 3). In other words, the effect of Al stress appears in the needles within 1 or 7 weeks after the start of Al treatment in contrast to the likely immediate response in the roots (Cakmak and Horst, 1991; Richards *et al.*, 1998). Many researchers have reported the effects of Al on the morphology of tree roots (Joslin and Wolfe, 1989; Kelly *et al.*, 1990; Zysset *et al.*, 1996; Hirano *et al.*, 1997), but none have reported an effect on needles as a result of Al stress in roots.

In our experiments, the SOD activity in needles was increased by $AlCl_3$ and the effect of $AlCl_3$ was lowered by simultaneous application of $Ca(NO_3)_2$. CAT activity in the needles was also increased by $AlCl_3$ within one week, but the effect of $AlCl_3$ was no longer observed at week 12 for all Ca/Al ratios (Figs. 2 and 3). The effect of $Ca(NO_3)_2$ on the CAT activity was slight and complicated (Fig. 3). Ascorbate peroxidase, as well as CAT, is a scavenger of hydrogen peroxide (H_2O_2), which is a kind of active oxygen species, and plants have specific characteristics in H_2O_2 scavenging systems (Asada, 1992). Studies on the dose-response of Al-induced activation of each antioxidant enzyme are in progress.

As shown in Fig. 4, the concentration of Al in the roots was increased by the treatment with $AlCl_3$ whereas the Al concentration in needles was not, indicating that Al stress to the needles, which induced a change in the enzyme activity, is not caused by the transfer of Al ion itself from roots to needles. Godbold *et al.* (1988) reported that the endodermis is a barrier for Al in Al-treated roots of Norway spruce (*Picea abies*). Larsen *et al.* (1996) suggested callose depositions are responsible for the blockage of root growth by Al in Al-sensitive mutants of *Arabidopsis thaliana*. However, how Al stress in the root system is conducted to needles is not clear.

In summary, height growth and dry matter allocation to each organ were negligibly affected by both the application of $AlCl_3$ and the concentration of $Ca(NO_3)_2$ in hinoki cypress seedlings. However, the activity of SOD and CAT in the needles was rapidly stimulated by rhizospheric Al treatment. That is, Al stress increased activities of these antioxidative enzymes. In addition, the activities of SOD and CAT

decreased with increasing Ca concentration, indicating that Al stress might be smaller. These results show that Ca might mitigate the phytotoxicity of Al. On the other hand, the Al concentration in the roots was increased by all Al treatments, whereas the Al concentration in needles was not effected by any treatment. The results also show that Al stress to the needles is not caused by the transfer of Al ion itself from roots to needles. To clarify the mechanism of stem transmission from roots to needles, further study will be required.

This work was supported in part by funding from the Japan Science and Technology Corporation, the CREST program 1996–2001, and the Center for Forest Decline Studies.

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(Accepted March 28, 2000)