Plant and Soil (2005) 276:133–141
DOI 10.1007/s11104-005-3697-y

Effects of aluminum on the growth of tea plant and activation of antioxidant system

Faezeh Ghanati[1,3], Akio Morita[2] & Hiromi Yokota[2]
[1]*Faculty of Science, Department of Plant Science, Tarbiat Modarres University, POB 14155-111, Tehran, Iran.* [2]*Faculty of Agriculture, Department of Applied Biological Chemistry, Shizuoka University, 422-8529, Shizuoka, Japan.* [3]*Corresponding author**

Received 30 October 2004. Accepted in revised form 11 March 2005

Key words: aluminum tolerance, antioxidant system, *Camellia sinensis*, lignin, phenylalanine ammonia-lyase (PAL), reactive oxygen species (ROS)

Abstract

A possible connection between the effects of aluminum (Al) on the growth of tea plants and the active oxygen species scavenging system in root tips of intact tea plants and suspension-cultured tea cells was examined. Intact tea plants were treated with or without Al in a modified Hoagland solution, while suspension-cultured tea cells were treated with or without Al in a simple salt solution containing 3% sucrose and 0.2 m*M* calcium. Compared with the control treatments without Al, the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) increased by Al both in roots of intact plants and cultured cells. The level of peroxidation of membrane lipids, as well as the activity of wall-bound peroxidas, the content of lignin and wall-bound phenols, however, reduced by the treatment with Al either in tea roots or in cultured tea cells. The results indicated that Al-induced increase in the activities of antioxidant enzymes, resulting in increased membrane integrity and delayed lignification and aging, can be considered as a possible reason for the stimulatory effects of Al on the growth of the tea plants and this is irrespect of the presence of other micronutrients and their interaction with Al.

Abbreviations: APX – ascorbate peroxidase; CAT – catalase; CPO – covalently bound peroxidase; IPO – ionically bound peroxidase; MDA – malondialdehyde; PAL – phenylalanine ammonia-lyase; PO – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase; SPO – soluble peroxidase.

Introduction

The growth of many crop species is adversely affected by the occurrence of soluble species of aluminum (Al) in acid soils (pH below 5.0), although many plants exhibit genetic-based variability in Al sensitivity and Al resistance. The tea plant has been known as one of the most Al tolerant and Al accumulator plants species with markedly stimulated growth at high concentrations of Al supply (Konishi et al., 1985; Matsumoto et al., 1976). However, the mechanism of the stimulation of growth by Al has not been elucidated yet. Studies with high Al concentration in nutrient solutions are difficult to interpret in terms of physiological responses as a high proportion or nearly all of the Al added is presumably lost by precipitation, polymerization and complexion (Marschner, 1995). There is also a possibility that the observed beneficial effects of Al on growth of plants in nutrient solutions is a secondary effect brought about alleviation of toxicity caused by

*FAX No: 098-21-8009730. E-mail: ghangia@modares.ac.ir

H^+ at low pH or by other mineral elements e.g. excess phosphorus (Kinraide, 1993; Marschner, 1995). This hypothesis now is doubted by the results of the present study since the results obtained from experiments using suspension-cultured tea cells pre-treated with or without Al in simple salt solutions showed the same tendency as those of intact plants in nutrient solutions.

Materials and methods

Plant materials and treatments

Two year-old rooted cuttings of *Camellia sinensis* L. cv. Yabukita were grown for 8–10 months in an aerated, modified nutrient solution containing (in m*M*): $(NH_4)_2SO_4$, 0.713; NH_4NO_3, 073: KH_2PO_4, 0.1; K_2SO_4, 0.46; $CaCl_2$, 0.5; $MgSO_4$, 0.41; Fe-EDTA, 0.032; H_3BO_3, 0.046; $CuSO_4$, 0.002; MnSO4, 0.09; Na_2MoO_4, 0.0026; $ZnSO_4$, 0.0091. pH was adjusted to 4.2 and the nutrient solution was exchanged every week. In Al treatments, Al was added as $AlCl_3$ to a final concentration of 400 μ*M* and the treatment was continued for 12 weeks. The duration for the treatment and the applied concentration of Al were decided according to the previous reports by Konishi et al. (1985) and Konishi (1992). Suspension cultures were prepared from the calli of the same variety of tea. The calli were derived from anthers (Kuboi and Kaji, 1994) and were generous gifts from professor Kuboi, Shizuoka University. The cell line was fast growing, a property which allowed us to detect the effects of Al on growth, at shorter periods of time. The cells were grown in B5 medium in darkness at 25 °C on rotary shakers (100 rpm) with weekly exchange. Exponentially growing cells at 7 days were harvested by suction filtration, suspended and rinsed three times with a simple salt solution containing 3% sucrose, 0.2 m*M* $CaCl_2$, pH 4.2, buffered with 2 m*M* Homo-pipes (Research Organics, Cleveland, USA). A stock solution of $AlCl_3$ was prepared just before using, sterilized by passing through a filter (pore size 0.45 μm), and added to the aforementioned simple salt solution to final concentrations of 0, 50 or 500 μ*M*. Preliminary experiments on suspension-cultured tea cells revealed that the concentrations of 50 μ*M* of Al was optimal, whereas the concentration of 500 μ*M* was relatively high but still had stimulatory effect (unpublished observation). After 24 h they were harvested, a batch of them were washed thoroughly with deionized water, frozen in liquid N_2 and kept at −80 °C for further analytical experiments. The second batch was washed thoroughly with B5 medium and equal amounts of them returned to B5 medium in order to determine the effect of Al on their further growth for 1 week (Yamamoto et al., 1994). In one experiment a group of flasks containing tea cells in simple salt solutions with or without Al were harvested just after 24 h, while the others were kept for an additional 5 days and the viability and the growth of their cells were evaluated. Cell viability was checked by Evans Blue (0.1% water solution, 5 min) and growth was measured by determination of fresh weight. All observations and measurements were carried out with three independent repetitions.

Determination of the level of lipid peroxidation

Level of damage of membranes was determined by measuring malonyldealdehyde (MDA) as the end product of peroxidation of membrane lipids (De Vos et al., 1991). In brief, samples were homogenized in an aquatic solution of TCA (10% w/v) and aliquots of the filtrates were heated in 0.25% TBA. The amount of MDA was determined from the absorbance at 532 nm followed by correction for the nonspecific absorbance at 600 nm.

Extraction and assay of the enzymes involved in scavenging ROS

Frozen samples (200 mg fresh weight) were homogenized in 3 mL HEPES--KOH buffer (pH 7.8) containing 0.1 m*M* EDTA. The homogenate was centrifuged at $15{,}000 \times g$ for 15 min. All operations were performed at 4 °C. In the supernatant, superoxide dismutase (SOD) was assayed by a photochemical method (Giannopolitis and Reis, 1977). Reaction mixture (3 mL) consisted of 50 m*M* HEPES-KOH buffer (pH 7.8), 0.1 m*M* EDTA, 50 m*M* Na_2CO_3 (pH 10.2), 12 m*M* L-methionine, 75 μ*M* NBT, 300 μL enzyme extract and 1 μ*M* riboflavin. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of

the rate of NBT reduction measured at 560 nm. Activity of catalase (CAT) was measured in a reaction mixture which consisted of 25 m*M* Na-phosphate buffer (pH 6.8), 10 m*M* H_2O_2 and diluted enzyme extract in a total valium of 1 mL. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm (Cakmak and Horst, 1991). Activity of ascorbate peroxidase (APX) was measured according to the method of Nakano and Asada (1981). In brief, samples were homogenized in 1 mL of 50 m*M* Na-phosphate buffer (pH 7.8) containing 5 m*M* ascorbate, 5 m*M* DTT, 5 m*M* EDTA, 100 m*M* NaCl and 2% (w/v) PVP. The homogenate was centrifuged as 15000 × *g* for 15 min at 4 °C. The reaction was initiated by adding H_2O_2 to a final concentration of 44 μ*M*. The reaction rate was monitored by the decrease in absorbance at 290 nm. The rate constant was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and corrected for the rate obtained prior to the addition of H_2O_2. Protein contents were determined by the method of Bradford (1976), using BSA as standard.

Extraction and assay of the enzymes involved in the metabolism of phenolics

In order to determine the activity of phenylalanine ammonia-lyase (PAL), the frozen cells were homogenized in ice-cold K-borate buffer (0.1 *M*, pH 8.8) containing 2 m*M* *β* -mercaptoethanol with a mortar and pestle and centrifuged at 16,000 × *g*, 10 min at 2 °C. The supernatant was used as a crude enzyme solution. The reaction mixture (total of 2 mL) was composed of 0.5 mL of crude enzyme and 1 mL of extraction buffer (without *β*-mercaptoethanol). The reaction started with the addition of 0.5 mL of 4 m*M* Phenylalanine and after 1 h incubation at 37 °C was stopped with 100 μL of 5 *M* HCl. The mixture was extracted three times with EtOAc. The EtOAc extract was air-dried, redissolved in 50% MeOH and analyzed by HPLC on an ODS-80 Ts column (4.6 × 250 mm, Tosoh, Japan). Cinnamic acid (CA), as the products of PAL reaction was eluted at a flow rate of 0.5 mL min_{-1} with a linear gradient of 30–80% MeOH containing 0.1% HOAc and was monitored at 273 nm (Wakabayashi et al., 1997). The enzyme activity was expressed as amounts of CA produced for 1 h per mg of protein in the reaction mixture.

Peroxidase (PO) was extracted and determined in three fractions; the soluble (SPO), ionically (IPO) and covalently (CPO) wall bound fractions. The first one, using guaiacol as an electron donor is involved in the stress response and the two latter, using syringaldazine as an electron donor, are supposed to be more related to the lignification and suberization of the cells (Fukuda and Komamine, 1982; Pandolfini et al., 1992). Samples were homogenized in 50 m*M* Tris-maleate buffer (pH 6.0) and centrifuged at 1000 × *g* for 10 min at 2 °C. The supernatant was re-centrifuged at 18,000 × *g* for 20 min at 2 °C. This second supernatant was used to assay soluble PO. Pellets of the first and the second centrifugations were pooled, incubated with 0.2 *M* $CaCl_2$ for 2 h at room temperature, and then centrifuged at 18,000 × *g* for 20 min at 2 °C. The supernatant was used to measure the activity of IPO. The pellet was used directly for assay of CPO (Pandolfini et al., 1992). Activity of SPO fraction was assayed in 60 m*M* K-phosphate buffer (pH 6.1) containing 28 m*M* guaiacol and 5 m*M* H_2O_2. The increase in the absorbance was recorded at 470 nm. For IPO and CPO assay, the final reaction mixture (3 mL) contained 41.6 nM syringaldazine, 40 m*M* Tris-maleate buffer (pH 6.0) and 16 m*M* H_2O_2. These anionic types of PO utilize syringaldazine as a specific substrate and have been supposed to be more related to the lignification of the cell walls and polymerization of phenolic domains of suberin (Goldberg et al., 1983; Pandolfini et al., 1992). Activity of IPO was expressed as the increase in absorbance at 530 nm per min per mg protein and activity of CPO was expressed as the increase in absorbance at 530 nm against cell wall dry weight.

Determination of lignin and wall-bound phenolics

Cell wall preparations were obtained by homogenization of frozen samples in water with a mortar and pestle followed by centrifugation at 1000 *g* and sequential washing of the pellet with EtOH, $CHCl_3$–MeOH (2:1) and acetone and then drying in air. Lignin content of wall preparations was measured via a modified acetyl bromide procedure (Iiyama and Wallis, 1990). The lignin content was determined by measuring of absorbance at 280 nm using specific absorption coefficient

value 20.0 g^{-1} L cm^{-1}. Phenolics were liberated from the walls with ammonium oxalate (20 m*M*, 70 °C) and then with 0.1 *M* NaOH, under N_2 for 24 h. After acidification of both fractions to approximately pH 3.0 with HCl, phenolics were extracted three times with EtOAc, air-dried and re-dissolved in 50% MeOH before determination by HPLC on an ODS-80 Ts column (4.6 × 250 mm, Tosoh, Japan) (Wakabayashi et al., 1997). Monophenols were monitored at 280 nm and the sum of them in the ammonium oxalate and NaOH fractions were calculated.

Statistical analysis

All of the experiments were carried out with at least three independent repetitions. All values are shown as the mean ± SD. Statistical analysis was performed using Student's *t*-Test and the differences between treatments were expressed as significant at level of $P < 0.05$.

Results

Growth of the root tips of tea plants was markedly stimulated in the presence of Al compared to those grown in the absence of Al (Figure 1a). The rate of the elongation of Al-treated roots (8.3 ± 1.0 mm/10 days) was also significantly higher than that the one treated without Al (3.1 ± 0.4 mm/10 days). In addition, in treatments without Al, the abundance of brown roots and the emergence of second or third order lateral roots were noticeable (Figure 1B). Pretreatment of suspension-cultured tea cells with simple salt solutions containing 50 or 500 μ*M* Al for 24 h stimulated their subsequent growth in B5 medium, compared to those control cells pretreated in simple salt solutions without Al (Figure 2). The viability of Al-pretreated cells in B5 medium was almost similar to those in B5 without pretreatment with Al (96 and 94%, respectively). In the cells that were kept in simple salt solution, a reduction in the fresh weight was observed after 24 h. This phenomenon is usually observed in cell culture, apparently due to the rinsing procedure and exposing the cells to a new medium. However, a 33% reduction was observed in the fresh weight of the cells treated without Al, while 20 and 22% reductions were observed in 50 and 500 μ*M* Al-treated cells, respectively. Further increase in the fresh weight of the cells during 24 h to 5 days after treatment was also higher in 50 μ*M* Al-treated cells, compared to those treated without Al or with 500 μ*M* (Figure 2). After 5 days, the viability of the cells cultured in simple salt solutions containing 0, 50 and 500 μ*M* of Al

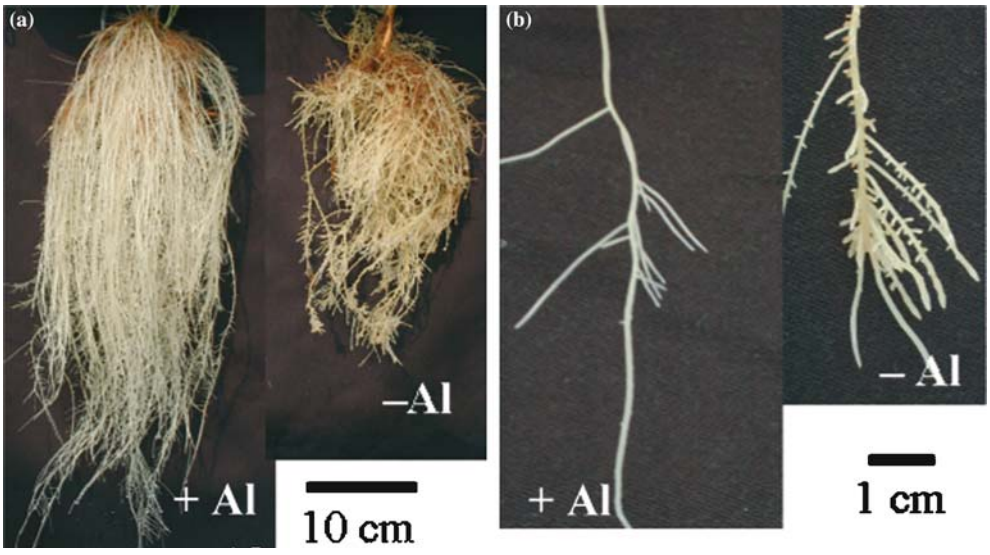

Figure 1. Effect of Al on the growth of root tips of intact tea plants in nutrient solution. The plants treated with 0 or 400 μ*M* Al in nutrient solution for 12 weeks.

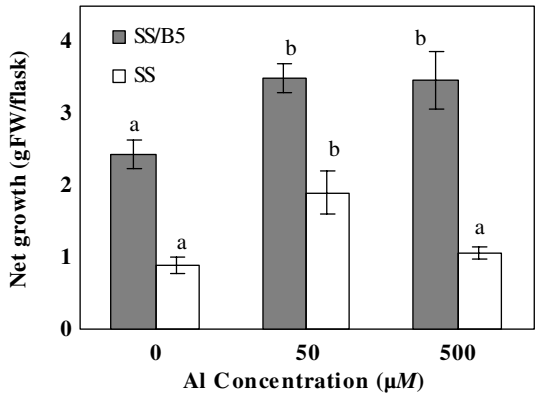

Figure 2. Effect of Al on the growth of suspension-cultured tea cells. A batch of cells were pretreated with 0, 50 or 500 μ*M* Al in simple salt solutions for 24 h and then returned to B5 medium and their capability for growth was compared after 1 week (SS/B5). The second group kept in simple salt solution for 5 days with or without Al (SS). Data are means obtained from at least three different experiments in triplicate ±SD. Signs with different letters in each group indicate significant differences at $P < 0.05$ according to Student's *t*-Test.

was 93, 86, and 85%, respectively. In comparison with the treatments without Al, the level of peroxidation of lipids of Al-treated roots and Al-treated cultured cells was significantly low (Figure 3). The activity of SOD of tea plant roots was improved by the treatment with Al, although the level of increase was not significant. The activity of SOD in cultured cells, however, was significantly increased with Al treatment, compared to that of control cells (Figure 4a). Similarly, treatment of tea roots and tea cells with Al increased the activity of CAT, compared to that of treatments without Al. The level of increase of CAT

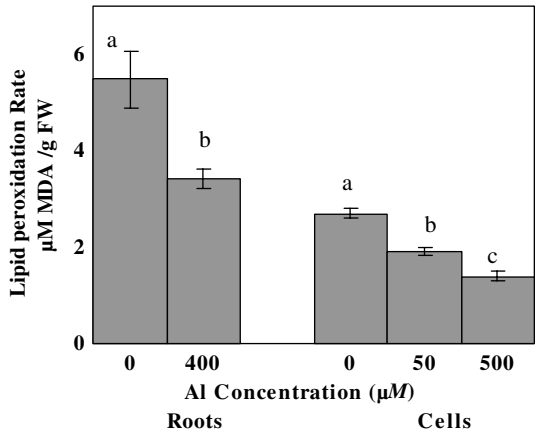

Figure 3. Effect of Al on the rate of lipid peroxidation in root tips of tea plants and suspension-cultured tea cells. The plants were treated with 0 or 400 μ*M* Al in nutrient solution for 12 weeks and the cells were treated with 0, 50, and 500 μ*M* Al in simple salt solutions for 24 h. Data are mean ± SD, $n = 3$. Signs with different letters in each group indicate significant differences at $P < 0.05$ according to Student's *t*-Test.

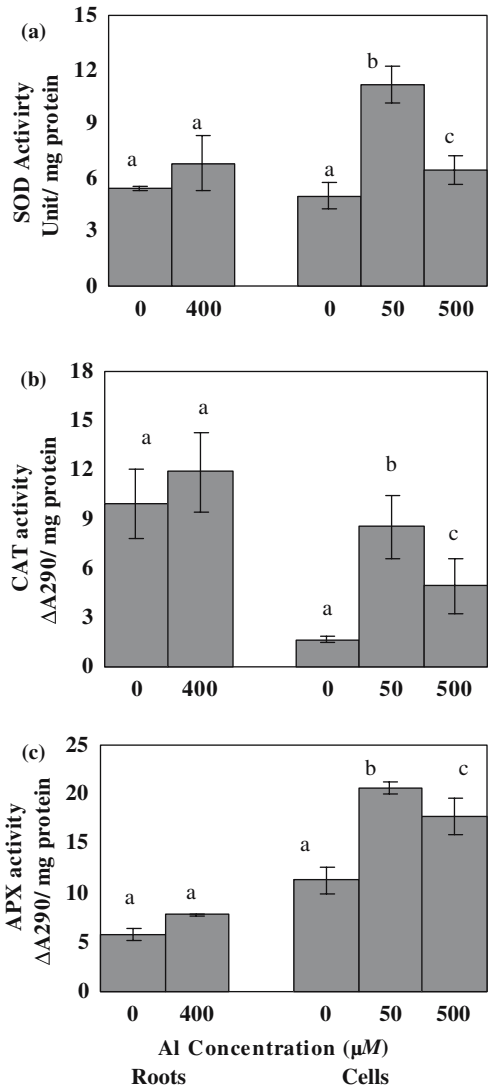

Figure 4. Effect of Al on the activities of SOD (a), Catalase (b), and APX (c) in root tips of tea plants and suspension-cultured tea cells. The plants were treated with 0 or 400 μ*M* Al in nutrient solution for 12 weeks and the cells were treated with 0, 50, and 500 μ*M* Al in simple salt solutions for 24 h. Data are mean ± SD, $n = 3$. Signs with different letters in each group indicate significant differences at $P < 0.05$ according to Student's *t*-Test.

activity induced by Al treatment was more significant in cultured cells than in intact plants (Figure 4B). As shown in Figure 4C, treatment with Al enhanced the activity of APX. However, the effect was statistically significant only in cultured cells.

Enhancement of the activities of the ROS scavenging enzymes should result in reducing level of H_2O_2, a major substance for lignification and biosynthesis and accumulation of phenoilc compounds in plants. The activity of PAL, a key enzyme involved in the metabolism of phenolics and lignification of cell walls, was lowered by treatments of tea plants and suspension-cultured tea cells with Al, compared to that of those corresponding treatments without Al (Table 1). The activity of soluble fraction of peroxidase (SPO), that is a cationic type of PO and is of relevance for stress conditions, was repressed in Al-treated roots, but was not significantly changed in Al-treated cultured cells (Figure 5). The activities of ionically and covalently bound fractions of peroxidase (IPO and CPO, respectively) in roots tips of tea plants and in cultured tea cells reduced by Al treatment (Figure 5). In this context and as shown in Table 1, the content of lignin and sum of the monophenols esterified to the cell wall polysaccharides of root tips and suspension-cultured cells treated without Al were higher than those corresponding treatments with Al.

Discussion

The growth of roots of intact tea plants in the presence of Al and further growth of Al-pretreated suspension-cultured cells of tea (Figures 1 and 2 implied that the response of this plant to Al is not only expressed at the level of intact plants but also at the cellular level. The fast growing tea cell line used in the present study was initially derived from anther (Kuboi and Kaji, 1994) and has maintained for several years as calli. The calli were then introduced to suspension media with several subcultures. Thus, they provided a homogenous pool of developmentally identical and undifferentiated cells capable of showing the mechanism of physiological response of tea plant to Al.

Evaluation of the effects of Al on the growth of tea cells in nutrient solution was out of the goal of the present study. On the other hand, growing of tea plants in a simple salt solution for a long period of time seems to be impractical. Nevertheless, based on the results presented here the growth responses of tea roots to Al in the presence of other micronutrients was similar to that of the cells in simple salt solutions without other micronutrients. These results may cast in doubt the previous hypotheses that explained the beneficial effect of Al exclusively based on the interaction of Al with other micronutrients and alleviation of their toxicity (Konishi et al., 1985; Ogawa et al., 2000). The possible interaction of Al at high concentrations with other micronutrients however, cannot be excluded and is currently under investigation.

Promotion of the growth of tea plant roots and tea cells by Al treatment was accompanied by the maintenance of cell viability and decrease of the level of lipid peroxidation (Figure 3) and the increase of the activities of antioxidant enzymes (Figure 4). The initial rates of lipid peroxidation and the activities of antioxidant enzymes were different in the suspension-cultured

Table 1. Lignin content, total content of wall-bound phenols, and PAL activity of root tips of tea and suspension-cultured tea cells treated with different concentrations of Al

Plant material	Al supply (μM)	Wall-bound phenols (μg g dry weight^{-1})	Lignin	PAL activity (μg CA/mg protein/h)
Roots	0	72.0 ± 6.0[a]	81.1 ± 6.1[a]	10.15 ± 1.3[a]
	400	56.0 ± 1.0[b]	54.6 ± 1.7[b]	2.43 ± 0.4[b]
Cultured cells	0	40.1 ± 1.5[a]	70.5 ± 9.0[a]	14.04 ± 0.5[a]
	50	36.4 ± 1.0[b]	57.9 ± 9.9[b]	12.23 ± 1.6[b]
	500	35.4 ± 1.0[b]	58.6 ± 9.9[b]	12.80 ± 0.2[b]

Data show mean ± SD, $n = 3$. In each group signs with different letters indicate significant differences at $P < 0.05$ according to Student's *t*-Test.

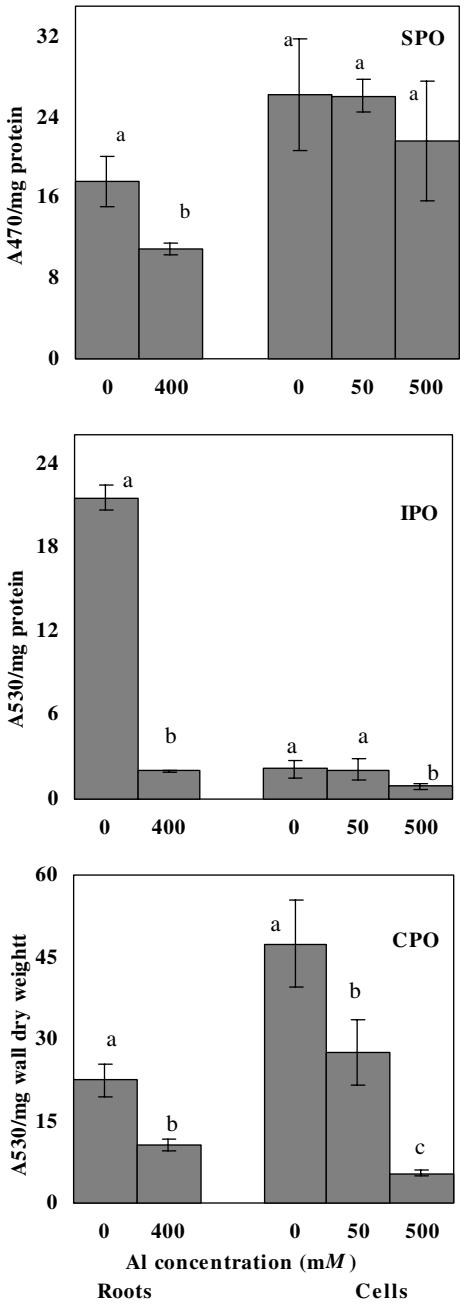

Figure 5. Peroxidase activity of root tips of tea plants and suspension-cultured tea cells. The plants were treated with 0 or 400 μM Al in nutrient solution for 12 weeks and the cells were treated with 0, 50, and 500 μM Al in simple salt solutions for 24 h. Activity was determined in three fractions; SPO, soluble fraction using guaiacol as an electron donor; IPO and CPO, ionically and covalntly wall bound PO, respectively, using syringaldazine as an electron donor. Data are mean ± SD, $n = 3$. Signs with different letters in each group indicate significant differences at $P < 0.05$ according to Student's t-Test.

tea cells and the roots of the intact plants. The extent to which the activities of the enzymes were changed after Al treatment was also different between the two groups. Although the real reason for such differences needs to be clarified by further experiments, it might be linked to the different media as well as different durations of the treatments of intact plants and cultured cells.

Treatment of cultured tea cells and tea plants caused the activation of antioxidant system. It suggests that the fundamental role of Al in promotion of growth of tea plant is connected with and scavenging of ROS. The latter is formed in normal cell metabolism and its regulation is a common cellular event. Oxidative damage is often associated with plant stress. A number of studies indicated that the degree of oxidative cellular damage in plants exposed to abiotic stress is controlled by the capacity of the antioxidant system (Sreenivasulu et al., 2000). Genetics-based studies in plants suggested the involvement of oxidative stress in Al toxicity (Yamamoto et al., 2002). Most of Al-induced genes are generally stress-inducible genes (Ezaki et al., 2001). Enhancement of antioxidant capacity may protect plants under specific conditions but it will also interfere with the signaling cascade involved in plant adaptation (Dat et al., 2001). A main protective role against ROS is attributed to SOD in catalyzing and dismutation of superoxide anions to O_2 and H_2O_2 (Sreenivasulu et al., 2000). The great difference between tea plant (and probably other Al-tolerant species, (Ogawa et al., 2000; Ezaki et al., 2001), and sensitive species (Cakmak and Horst, 1991), can be explained upon the upregulation of SOD followed by other ROS scavenging enzymes, in particular CAT and APX. In Al-sensitive plants, SOD activity is mainly triggered by Al that in turn results in dismutation of $O_2^{\cdot -}$ and more production of another toxic species, H_2O_2. In Al-treated tea plants, however, the produced H_2O_2 is subsequently detoxified by APX and CAT. Peroxidase also eliminates H_2O_2, but due to its role in the stiffening of the walls (Fry, 1986), a lower activity of it will be more beneficial for the growth. It has been already shown that CAT removes the bulk of H_2O_2 whereas APX can scavenge H_2O_2 that is not removed by CAT. Also, due to the higher affinities for H_2O_2, APX can remove

lower concentrations of H_2O_2 at different subcellular locations (Dat et al., 2001). In the present study we measured cytoplasm-located anionic PO. This isoenzyme of APX utilizes ascorbic acid as a specific electron donor (Shigeoka et al., 2002) and probably plays a key role in the defense system, through detoxification of H_2O_2 as well as prevention of ascorbic acid peroxidation (Zheng and Huystee, 1992). Cytosolic APX was significantly upregulated by H_2O_2 and/or paraquat treatment in cell suspension culture of rice (Morita et al., 1999). Addition of diethyldithiocarbamat (a SOD inhibitor) lowered the induction of APX, whereas inhibition of CAT or APX (resulting in H_2O_2 accumulation) upregulated APX expression, suggesting that H_2O_2 is part of signaling cascade that induces cytosolic APX (Morita et al., 1999). Thus, although all H_2O_2 scavenger enzymes act in a cooperative or synergistic way for the survival of the cell even under normal conditions (Michiels et al., 1994), it is more likely that among ROS scavenger enzymes, CAT is the key enzyme that effectively eliminates H_2O_2, thereby regulates the activity of APX and PO. This may explain the fact that in Al-treated tea cells and tea plants the level of the increase in the activity of APX was not as remarkable as the activity of CAT. Also, it suggests that the lowered activity of PO following the treatment of the plants and cells with Al was potentially related to the higher activity of CAT.

In tea plants treated without Al, accumulation of one of ROS, e.g., H_2O_2, in normal cell metabolism, can stimulate accumulation of other ROS (Dat et al., 2001). H_2O_2 is a signaling intermediate in programed cell death (Alvarez and Lamb, 1997). It is mostly consumed in peroxoidation of membrane lipids (Cakmak and Horst, 1991), and increases the mechanical strength and lowers the extensibility of plant cell walls (Schopfer, 1996). Hydrogen peroxide as an electron acceptor for wall-bound PO, plays a major role in polymerization of phenolic monomers in the synthesis of lignin and also establishment of covalent bonds between lignin and carbohydrate in the cell walls (Fry, 1986). Consist with this, the higher activities of PAL (Table 1) and wall bound fractions of PO (Figure 5), the more lignin content and the bigger pool of monophenols bound to the cell wall polysaccharides (Table 1) of tea plant materials in the treatments without Al, can be explained upon the more available ROS, in particular H_2O_2. Such a H_2O_2-mediated rigidity of cell walls would explain the faster abolishment of growth occurring in those plants treated without Al. Therefore, the beneficence of Al for the growth of tea plants is likely, at least in part, mediated by scavenging of ROS, resulting in the increase of cell membrane and cell wall stability and postponement of aging and death of tea plant cells.

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Section editor: T.B. Kinraide