American Journal of Plant Sciences, **2012, 3, 1619-1624**
http://dx.doi.org/10.4236/ajps.2012.311196 Published Online November 2012 (http://www.SciRP.org/journal/ajps)

Scientific Research

Stimulation of Root Growth Induced by Aluminum in *Quercus serrata* Thunb. Is Related to Activity of Nitrate Reductase and Maintenance of IAA Concentration in Roots

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Received August 10th, 2012; revised September 24th, 2012; accepted October 16th, 2012

ABSTRACT

Aluminum (Al) is the most abundant metal in the earth's crust. Excess Al^{3+} released by soil acidification in soil solution is thought to be a growth limiting factor to many cultivated plant species, but it has been reported to stimulate plant growth in some crop and tree species in certain concentration of Al^{3+}. Previously, we had reported that Al treatment enhanced root development, NO_3^- uptake from growth media and *in vivo* nitrate reductase (NR) activity of roots. NR is one of the key enzymes in nitrogen metabolism and acts at the first step of nitrate assimilation in plants. In this study, we investigated the process of Al-induced root development in an early stage, focusing on the change in *in vitro* NR activity, and indole-3-acetic acid (IAA) and cytokinins concentration in roots of *Quercus serrata* seedlings, which were treated for 1 h with Al or Ca. In Al-treated roots, NR activity increased and IAA concentration was maintained at the same level as pretreatment, and indole-3-acetyl-L-aspartic acid (IA-Asp), which is a metabolic intermediate of IAA degradation, was not detected in roots. In Ca-treated roots, NR activity increased, but IAA concentration decreased as IA-Asp concentration increased. Thus, the maintenance of IAA concentration in Al-treated roots seems to result from suppression in the process of IAA decomposition. Al treatment increased the length and number of second lateral roots but Ca treatment did not. We concluded that root development induced by Al in the early stage was related to NR activity and maintenance of IAA concentration.

Keywords: Aluminum; Root; Development; NR; IAA; *Quercus serrata*

1. Introduction

Aluminum (Al), a component of primary and clay minerals, is the most abundant metal element on the planet, comprising about 7% by mass of the earth's crust. Al is released from the solid phase by soil acidification, and exchangeable Al increases when the pH of soil solution falls below 5.0. At a pH of less than 4.0, Al exists predominantly as Al^{3+}. Excess Al^{3+} in soil solution is considered to be toxic to cultivated plant species and is a limiting factor for plant growth in acidic soil. The pH of rhizospheric soil around the roots is lower than that of the bulk soil due to release of H^+ and organic anions from the roots, accompanied by the uptake of cations and respiration. Under these conditions, the concentration of Al^{3+} around the roots is thought to be higher than that of the average of the bulk soil [1-4]. Therefore, most plant roots are thought to be exposed to high concentrations of Al^{3+}. Although there are many reports about negative effects of Al on plant growth in both crop and tree species, enhancement of growth by Al treatment was reported much less than that and only a few in both crops and trees [5-9], and there seems to be an optimum concentration of Al for each plant species. Although much information on the mechanism of Al toxicity and Al tolerance in plants has been published [10,11], few reports have focused on the role of Al in plant growth.

Previously, we reported that Al enhanced root growth in *Quercus serrata* Thunb. seedlings in experiments with various Al treatments, and suggested that Al might act as a trigger to induce root elongation and rooting at 1.0 mM and 2.5 mM [7]. Then we showed that Al enhancement of root growth is related to stimulation of NO_3^- uptake and activation of *in vivo* nitrate reductase (NR) in roots

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[12]. Recently, lateral root development induced by NO has been reported to be related to auxin signaling and NR activity for *Lycopersicon esculentum* [13] and *Arabidopsis thaliana* [14]. Therefore, we considered that NR activity and auxin might be a key factor in Al-enhanced root growth in *Q. serrata*. In our previous study, stimulation of NO_3^- uptake and an increase in the number of lateral root primordia were observed in plants 3 days after Al treatment, but no increase in NR activity in roots measured by an *in vivo* system was detected after 3 days [12]. NR activity could not be detected possibly because in an *in vivo* system NR activity is measured according to the final amount of NO_2^- after some consumption through the metabolic processes in root cells.

Phytohormones, especially indole-3-acetic acid (IAA) and cytokinins, are key factors in the regulation of plant growth, including root development [15,16]. We hypothesize that Al may act as an activator of NR in root cells and as a trigger to change the concentration of phytohormones such as auxin and cytokinin, which induce root development, in roots. Therefore to clarify the process/mechanism of Al-induced root development in an early stage, roots were treated with Al for 1 h to observe the change of *in vitro* NR activity, the concentrations of IAA and cytokinins, and root morphogenesis.

2. Materials and Methods

2.1. Plant Materials

Seeds of *Q. serrata* were collected from a secondary forest at Nagoya University, Japan. Seeds were germinated in siliceous sand. After root germination, seedlings were grown hydroponically in a modified version of the 1/10 Hoagland's No. 2 nutrient solution, containing 0.6 mM KNO_3, 0.4 mM $Ca(NO_3)_2·4H_2O$, 0.2 mM $MgSO_4·7H_2O$, 0.1 mM $NH_4H_2PO_4$, 45.5 µM $MnCl_2·4H_2O$, 8.95 µM $FeCl_3·6H_2O$, 0.4 µM $ZnSO_4·7H_2O$, 0.15 µM $CuSO_4·5H_2O$, 2.3 µM H_3BO_3, and 0.25 µM $NaMoO_4·2H_2O$ in a growth chamber at 23˚C with 65% relative humidity, in a photoperiod of 14 h/10 h (day/night) and irradiation of 150 $µmol·m^{-2}·s^{-1}$ for 8 weeks. The nutrient solution was changed once a week and the pH of the solution was adjusted to 4.0 ± 0.1 with 1 N HCl.

2.2. Al Treatment of Seedlings

Roots of 8-week-old seedlings were exposed to 2.5 mM $AlCl_3$ (pH 4.0) or 4.2 mM $CaCl_2$ (pH 4.0) solution for 1 h, which was then replaced with freshly prepared nutrient solution. Roots were collected from seedlings before treatment and 1, 2, and 3 days after the 1-h treatment. 15 seedlings were collected in each treatment at each sampling time, and 5 cm from the tip of the first lateral roots and the whole tissue of the second lateral roots were collected and used for further analyses.

2.3. Enzyme Extraction and Assay of NR Activity

For preparation of enzyme fractions, all subsequent steps were carried out at 0˚C to 4˚C. Roots were homogenized in a mortar with a grinding medium composed of 50 mM tris(hydroxymethyl)aminomethane (Tris-HCl, pH 7.5), 10 mM Ethylenediaminetetraacetic acid tetrasodium (Na_2-EDTA), 5 mM dithiothreitol (DTT), 1% bovine serum albumin (v/v), 20 µM p-amidinophenyl methanesulfonyl fluoride hydrochloride (p-APSMF), and 50% [wt/wt] polyvinylpolypyrorolidone (Polyclar AT). The homogenates were filtered with a layer of Miracloth (Calibiochem-Novabiochem, San Diego, CA, USA). The filtrate was centrifuged at 15,000 g for 10 min. The supernatant was applied to a Sephadex-G25 column (GE Healthcare Bio-Sciences, Uppsala, Sweden; gel volume, 5 ml) equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM Na_2-EDTA, 5 mM DTT, and 20 µM p-APSMF. The protein (enzyme)-rich flow through fractions was collected and used for the NR assay.

NR activity was measured by a modified version of the method of Hageman & Reed [17] as follows. The reaction mixture (1 ml) containing 23 mM K_3PO_4 (pH 7.5), 4.5 mM KNO_3, 9 µM flavin adenine dinucleotide (FAD), 69 µM nicotinamide adenine dinucleotide reduced (NADH), and enzyme fraction was incubated at 30˚C for 30 min in the dark. The reaction was stopped by adding 0.1 ml of the 0.5 M zinc acetate. Then the mixture was centrifuged at 5000 g for 5 min. An aliquot (0.75 ml) of the supernatant was mixed with 16 µl of 0.15 mM phenazine methosulfate (PMS) and incubated at 25˚C for 20 min. Then, 0.5 ml of 1% sulfanilamide (in 1.5 M HCl) and 0.5 ml of 0.02% N-1-naphthyl ethylenediamine dihydrochloride were added to the incubated solution. After incubation at 25˚C for 20 min, absorbance at 540 nm of the sample was measured using a spectrophotometer (model U-3310, Hitachi, Tokyo, Japan). NR activity on a fresh weight (fw) basis was calculated.

2.4. Extraction and Quantification of Phyto-Hormones

The roots were collected from 15 seedlings before treatment and 1, 2, and 3 days after the 1-h treatment, as mentioned above. Plant hormones such as IAA and cytokinins in root tissues were extracted and quantified using a liquid chromatography-tandem mass chromatography system (Waters; AQUITY UPLC System/Quattro Ultima Pt), as described previously [18]. Also, the amount of indole-3-acetyl-L-alanine (IA-Ala), indole-3-acetyl-L-

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aspartic acid (IA-Asp), indole-3-acetyl-L-iso-leucine (IA-Ile), indole-3-acetyl-L-leucine (IA-Leu) and indole-3-acetyl-L-phenylalanine (IA-Phe), which are a metabolic intermediate of degradation of IAA, was determined to assess turnover of IAA.

2.5. Root Morphogenesis

The lengths of the first and second lateral roots were measured before, 3 and 7 days after the 1-h treatment and the numbers of second lateral roots were counted 7 days after the 1-h treatment by LIA 32 software [19].

3. Results

The lengths of first and second lateral roots did not change 3 days after 1-h treatment with Al or Ca (data not shown). At 7 days after treatment, the length and number of Al-treated second lateral roots were significantly increased compared with those of pretreatment and Ca-treated roots (**Table 1**).

The *in vitro* NR activity gradually increased to 175% on day 3 after 1-h treatment with both Al and Ca compared with pretreatment levels. However, there was no obvious difference between treatments (**Figure 1**).

IAA concentration (87.3 pmol/g fw) in *Q. serrata* roots was comparable with that of rice (82.1 pmol/g fw) [18]. IAA concentration in *in vitro* cultured shoots of *Q. robur* was about 300 to 700 nmol/g dry weight (dw) in basal area and about 10 to 70 nmol/g dw in apical section [20]. IAA concentration in roots of *Q. serrata* was about 82 to 88 pmol/g fw in the present study, so the IAA concentration in *Q. serrata* roots was very low compared with that of *Q. robur* shoots. The IAA level in roots treated with Al remained at the same level as that of the pretreatment sample until 2 days after 1-h treatment (**Figure 2(a)**). In this study, the amount of metabolic intermediate of degradation of IAA, was also determined to assess turnover of IAA. IA-Asp was not detected in the Al-treated roots even after 2 days (**Figure 2(b)**). IAA concentration in Ca-treated roots decreased by approximately 50% 2 days after treatment. As IAA level decreased, IA-Asp concentration increased in Ca-treated roots (**Figure 2(b)**). Other metabolic intermediate of degradation of IAA, such as IA-Ala, IA-Ile, IA-Leu, IA-Trp and IA-Phe, were not detected in both treatments.

We detected 17 cytokinin species (data was not shown), including active cytkinins such as N6-(⊿2-isopentenyl)-adenine (iP) and trans-zeatin (tZ) (**Figures 2(c)** and **(d)**). There were not clear difference between Al and Ca treatment in both active cytokinins, but the total amount of cytokinins in Ca-treated roots gradually increased to approximately 1.79 pmol/g fw at 3 days after treatment. The total level of active cytokinins in Al-treated roots was lower than that of Ca-treated roots, especially on day 3 (**Figures 2(c)** and **(d)**).

4. Discussion

To elucidate the stimulatory effect of Al on lateral root development in an early stage, in this study we focused on *in vitro* NR activity and the phytohormones such as auxin and cytokinins.

Previously, we reported that rhizospheric Al enhanced NR activity measured using an *in vivo* system and increased lateral root primordia in 2-year-old *Q. serrata* seedlings [12]. In the present study, we also confirmed

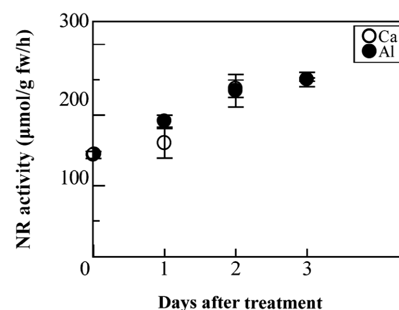

Figure 1. The activity of NR in roots measured by an *in vitro* system at 0 (Pre), 1, 2, and 3 days after treatment with 2.5 mM $AlCl_3$ (pH 4.0) (Al) or 4.2 mM $CaCl_2$ (pH 4.0) (Ca) for 1 h. Vertical bars represent the means ± SE (n = 3).

Table 1. Length of first and second lateral roots and number of second lateral roots at 0 (Pre) and 7 days after treatment. Roots of 8-week-old seedlings were treated with 2.5 mM $AlCl_3$ (pH 4.0) or 4.2 mM $CaCl_2$ (pH 4.0) solution for 1 h and then replaced with freshly prepared nutrient solution.

Treatment	First lateral roots		Second lateral roots		
	Length		Length		Number
	Pre	After 7 days	Pre	After 7 days	After 7 days
	(cm)	(cm)	(cm)	(cm)	(number/cm)
Al	25.5 ± 2.1	28.4 ± 3.0	2.62 ± 0.23	4.45 ± 0.33**	2.5 ± 0.3**
Ca	25.4 ± 2.4	27.5 ± 3.0	2.29 ± 0.12	2.58 ± 0.31	1.7 ± 0.2

Values are the means ± SE (n = 20). Asterisks indicate significant differences between Al treatment and Ca treatment ($p < 0.01$) according to the *t*-test.

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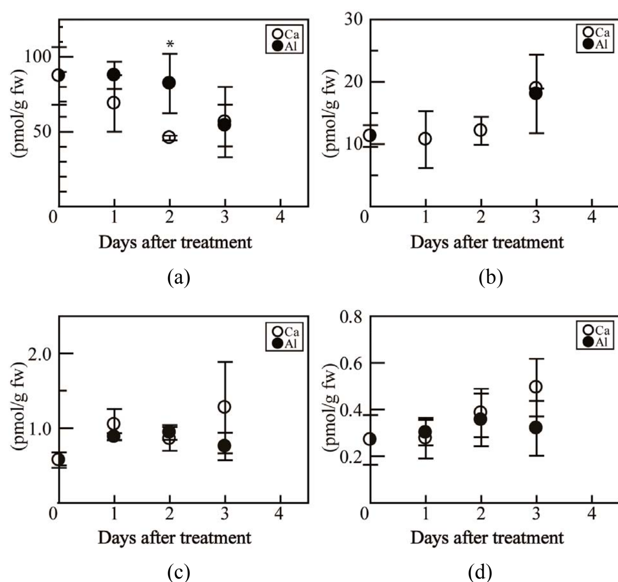

Figure 2. Concentration of phytohormones: IAA (a), IA-Asp(b), iP(c), and tZ (d) in roots at 0 (Pre), 1, 2, and 3 days after treatment. Roots of 8-week-old seedlings were treated with 2.5 mM $AlCl_3$ (pH 4.0) or 4.2 mM $CaCl_2$ (pH 4.0) solution for 1 h and then replaced with freshly pre- pared nutrient solution. Vertical bars represent the means ± SE (n = 3). Asterisks indicate significant differences between Al treatment and Ca treatment ($p < 0.05$) according to the *t*-test.

that Al treatment enhanced NR activity in roots measured using an *in vitro* system and was detected 1 day after 1-h 2.5 mM Al treatment (**Figure 1**). The activity of NR in roots treated with 2.5 mM Al was slightly higher than that of root treated 4.2 mM Ca after a day from 1-h treatment, however, it did not show clear differences between Al and Ca treatments after 2 and 3 days (**Figure 1**). Both Al and Ca are able to reduce the surface potential and neutralize negative surface charge by binding to membrane phospholipids [21,22]. In our previous study, the uptake of NO_3^- by roots from the cultivation medium was enhanced at 3 days after Al treatment, which was considered to be a result of an increase in positive charge on root cell membrane [12]. Thus, it is considered that enhancement of NR activity induced by 1 h treatment with Al or Ca may be involved in an increase in NO_3^- influx, caused by an increase in positive charge on the cell membrane due to absorption of Al^{3+} or Ca^{2+}. Moreover, these observations support our previous hypothesis that enhancement of NR activity in Al-treated roots may be induced by promotion of NO_3^- uptake due to a change in the surface polarity of the cell membrane.

The growth hormone auxin has many roles in plant development [15]. It plays a central role in cell growth, gravitropism, apical dominance, lateral root initiation, leaf abscission, vascular differentiation, flower bud formation, and fruit development [15,23]. In this study, we succeeded in determining the concentrations of IAA and a metabolic intermediate of degradation of IAA (IA-Asp). IAA concentration in Al-treated roots was kept at the same level as in pretreated roots, and IA-Asp was not detected 1 and 2 days after 1-h Al treatment, whereas IAA concentration in roots treated with Ca for 1 h decreased and a relatively high amount of IA-Asp was detected at all stages (**Figure 2(b)**). This result indicates that IAA concentration is maintained at more than 82 pmol/g fw in Al-treated roots because Al may limit irreversible inactivation or metabolic degradation of IAA. The concentration of each active cytokinin species did not show an obvious tendency to vary between treatments (**Figures 2(c)** and **(d)**). The mechanisms of nitrogen assimilation and nitrogen acquisition which involves plant hormones have been shown, and it has reported that endogenous or exogenous cytokinin took part in process of NR activation [24]. In this study, there was not clear relationship between iP or tZ and NR activity in root after 1-h Al or Ca treatment. At present, the physiological role of individual cytokinin species involving NR activity and root development in an early stage cannot be explained in *Q. serrata*.

In general, cytokinins and auxin act antagonistically in controlling meristem activities [25]. Moreover, the level of IAA concentration required to promote growth in each tissue is different [23]. As mentioned above, the IAA in Al-treated roots was kept at about 80 pmol/g fw for at least 2 days, in contrast to the decrease in Ca-treated roots. The ration of IAA to sum of iP and tZ tended to decrease with time after both 1h-treatments, and the ratio was higher in Al treatment than in Ca treatment (**Figure 3**). Enhanced root elongation and increased number of

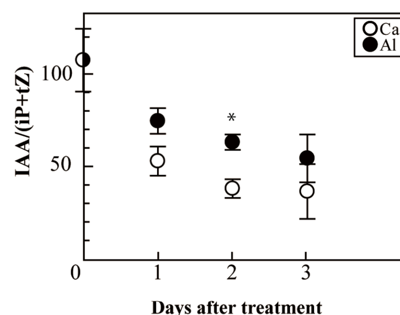

Figure 3. The ration of IAA to sum of iP and tZ in roots at 0 (Pre), 1, 2, and 3 days after treatment. Roots of 8-week-old seedlings were treated with 2.5 mM $AlCl_3$ (pH 4.0) or 4.2 mM $CaCl_2$ (pH 4.0) solution for 1 h and then replaced with freshly prepared nutrient solution. Vertical bars represent the means ± SE (n = 3). Asterisks indicate significant differences between Al treatment and Ca treatment ($p < 0.05$) according to the *t*-test.

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second lateral roots induced by Al in the present study (**Table 1**) may result in Al maintaining IAA concentration, and the hormone balance (auxin/cytokinin ratio) may be essential for efficient lateral root development.

A close relationship between auxin-induced lateral root formation and NO-induced lateral root development has been reported recently. Initiation of lateral roots is accompanied by an increase in NO level, an increase that is involved with auxin signaling [13]. Furthermore, Kolbert *et al*. [14] reported that lateral root development by auxin-induced NO production was associated with NR activity. In the present study, the 1-h Al treatment enhanced NR activity in roots and the elongation and development of second lateral roots, but 1-h Ca treatment did not enhance second lateral root growth even though NR activity increased (**Figure 1** and **Table 1**).

Al-induced enhancement of root growth in *Q. serrata* may be caused by stimulation of NR activity in roots and IAA-induced NO synthesis. Furthermore, these data, including those on phytohormones, might be useful for further investigation of tree development.

5. Acknowledgements

This work was supported by Grants-in-Aid for Young Scientists (B) (20780114) from the Ministry of Education, Sports, Culture, Science and Technology of Japan, and The Salt Science Research Foundation.

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