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Effects of Aluminum, Calcium, and Phosphate on Shoot Growth, Viability, and Aluminum Distribution in the Root Apices in Highbush Blueberry Cultured *in vitro*

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Summary

The viability, Al distribution in root apices, shoot growth, and excreted organic acid contents in blueberry cultured *in vitro* were examined to study the effects of aluminum (Al), calcium (Ca), and phosphate (P) ions on the plant's tolerance to Al.

1. Roots in the control, exposed to an Al-free acetate buffer were significantly damaged, but roots treated with 1mM Al or 1mM Al plus 1mM Ca retained their viability.

2. Al could not be detected in the root apex in the control, but the roots treated with 1mM Al and 1mM Al+Ca accumulated Al in the cortical area but not to the interior cortex as the treatment progressed. Thus, the accumulation of Al on the root surface seems to have protected the plant from the damage of low pH and acetate ions.

3. 1 mM Al in the medium hastened both the shoot and root growth for fifty days. The elimination of P from the medium inhibited root growth more than shoot growth.

4. The amount of organic acids excreted did not differ whether Al was present or not indicating that 1mM Al is not injurious.

Key Words: Al distribution, Al tolerance, FDA-PI staining, organic acids excretion, *vaccinium corymbosum* L..

Introduction

Recently the blueberry, an acidophilic plant, was found to tolerate low pH and the presence of Al (Suzuki et al., unpublished). Al is one of the most important factors influencing root growth inhibition in acidic soil. This inhibition, caused by Al, is lessened by the presence of a cation, particularly Ca (Kinraide and Parker, 1987; Matsumoto and Yamaya, 1986). However, root growth of the blueberry has not been affected by a high concentration of Ca, but, on the contrary, seems to decrease the fresh weight of the shoots (Korcak, 1989). It has, therefore, been suggested that the relationship between Ca and Al in the blueberry is different from that in other plants.

Delhaize et al. (1993a) reported that Al distribution in the roots of maize with Al tolerance is different from those of Al sensitive maize. Moreover, they explained that the maize with Al tolerance protects Al transport to the plant's upper parts because organic acids excreted from the root apex chelates with Al outside the root (Delhaize et al., 1993b). Although many hypotheses on the mechanisms of pH and Al tolerance have been reported for other plants, few reports have focused on the blueberry (Korcak, 1989; Peterson et al., 1987).

In this study, we examined the viability and Al distribution in root apex cells in highbush blueberry that have been treated with Al and Ca in vitro culture for fifty days. The effect on shoot growth and the amount of organic acids excreted from the root apex were also studied.

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Materials and Methods

Induction of root from subcultured shoot

An *in vitro* subcultured shoot of the highbush blueberry cultivar'Berkeley' was used. The shoot was cut off at a point 1-2 cm from its tip, dipped in 100 $mg \cdot l^{-1}$ indole butyric acid for one minute, then clipped to a silicon gum disk (10 mm diam.) with a gap along its radius. The disk was floated in distilled water in a test tube for 2 to 3 weeks until a root developed from the shoot. This root was used in the experiment.

Experiment 1: Effects of applying Al and Ca in the short term on the growth, viability, and Al distribution in the root apex

Treatment

The experimental media were made with $AlCl_3 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ in an acetate buffer solution (10 mM CH_3COOH-20 mM CH_3COONa) (pH 4.2). Three treatments were administered as follows: (1) control, acetate buffers only, (2) 1mM Al, and (3) 1mM Al and 1mM Ca (Al+Ca). The root before the treatment functioned normally. Fifteen ml of the medium was put

into a test tube (diameter 25 × 120 mm), and sterilized in an autoclave. After the medium was sterilized, the plantlet was floated with its silicon gum disk in the medium for one to ten days in the culture room (temp.: 25 ℃, illuminant: 38 μmol・m^{-2}・s^{-1}, continuous light). Each treatment consisted of 5 plantlets.

The growth of shoots and roots was measured with a caliper from the outside of the tube at 1, 5, and 10 days after the treatment. The viability of the root apex tissue was observed by double staining with fluorescein diacetate (FDA) and propidium iodide (PI) (Jones and Senft, 1985).

Determination of Al distribution in the root apex

Al distribution in the root apex of plantlets was determined by x-ray analysis. The root was immediately rinsed in distilled water after observation, frozen quickly in a freezer at -30 ℃, and then freeze-dried. The dried root was cut 35-70 μm from the root tip under a microscope, deposited on the carbon, and its Al distribution analyzed by using the scanning electron microscope (S-2300, HITACHI Co. Japan) and energy dispersion x-ray analyzer (EMAX-2770, HITACHI Co. Japan).

Experiment 2: Effects of Al application and P deficiency in vitro culture for fifty days.

Fresh Va3 medium made for the blueberry culture (Ishihara et al., 1992) was used as the basal medium. Four treatments to which roots were exposed were: (1) control, the basal medium, (2) +Al, 1 mM Al, (3) -P, phosphate-free basal medium, (4)-P+Al, phosphate-free, 1 mM Al solution. Each treatment consisted of 5 plantlets. The pH of each treatment medium was adjusted with NaOH solution before sterilization to be at pH 4.2 after sterilization. Thirty-five ml of each medium was pipetted into a test tube (25 × 200 mm) and sterilized in an autoclave. The rooting shoot was floated as above, and cultured for 50 days.

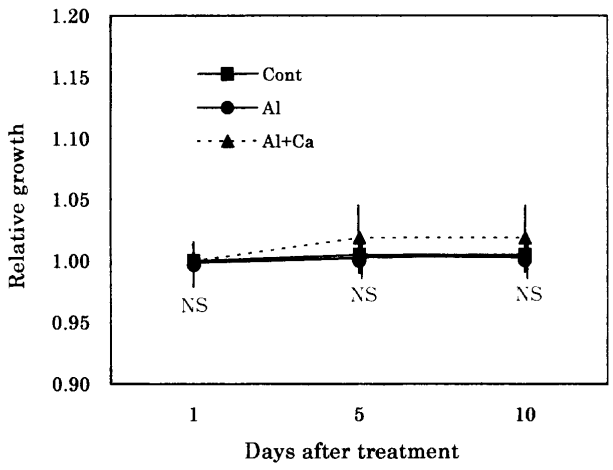

Fig. 1. Relative shoot growth (based on initial shoot length) of plantlets whose root were exposed to 1 mM Al and Ca for ten days. The initial shoot lengths are as follows: Cont.: 31 ± 8 mm, Al: 32 ± 7 mm, Al+Ca: 35 ± 7 mm. Vertical bars indicate SD.

The growth of the shoot and root was measured with a caliper from the outside of the tube at 1 and 5 days and at intervals of 10 days thereafter. The relative growth was calculated on the basis of the initial size.

The organic acids excreted into the medium 50 days after partial purification were identified using HPLC. The medium was filtered, the filtrate passed through an anion exchange columm (Dowex 1-X8, 200-400 mesh). The eluate was analyzed with HPLC (Shimadzu LC-10A, Japan); its components and characteristics: Detector: UV. 210 nm; column: Inertsil C8 250mm × 4.6mm I.D. (GL Science Co ltd. Japan); column temp: 40 ℃ ; Carrier solvent: 0.1 M $NH_4 4H_2 PO_4$ (pH2.5); flow rate: 1.0 ml・min^{-1}.

Results and Discussion

1. Effects of applying Al and Ca in the short term on the growth, viability, and Al distribution in the root apex

Shoots which were treated with 1 mM Al+Ca elongated about 2% during the culturing period, whereas those treated with Al and the control did not (Fig.1). Roots failed to elongate in all treatments (Data not shown). Control roots were significantly damaged by the buffer solution, whereas roots in 1mM Al and 1mM Al+Ca retained their initial viability (Fig. 2). This result indicates that Al application prevented potential root injury by the acetate buffer. The shoot in the control did not develop because of root injury; the roots in 1mM Al and 1mM Al+Ca treatment did not grow in spite of their healthy condition. This condition may have resulted because the acetate buffer contained no nutrients.

Generally, the injury caused by Al decreases with the coexistence of a cation (Matsumoto and Yamaya, 1986). Calcium (Ca), in particular, decreases the growth inhibition caused by Al (Kinraide and Parker, 1987). In our experiment, however, co-treatment of Ca with Al could not be associated with growth inhibition. Our results indicate that there are no injuries caused solely by high Al concentration in the blueberry, as evidenced by the high viability of roots treated with 1 mM Al. Korcak (1989) reported that the fresh weights of the aerial parts of plants decreased at a concentration of 500 μM Al in 1.3 mM Ca. In our experiment, plantlets were exposed to 1 mM Al and Ca but the roots remained viable. Because blueberry is recognized a calcihuges plant (Korcak, 1988b), we need to test at higher Ca levels.

Al distribution in the cross section of the root apex by x-ray analysis reveals that Al is undetectable in control roots, whereas those treated with 1mM Al and 1mM Al+Ca accumulated Al in the outer cortex, more than it did in the inner cortex as the treatment progressed (Fig.3). The control roots were damaged, whereas treated with Al were not, indicating that the accumulation of Al on the root surface may have protect the root

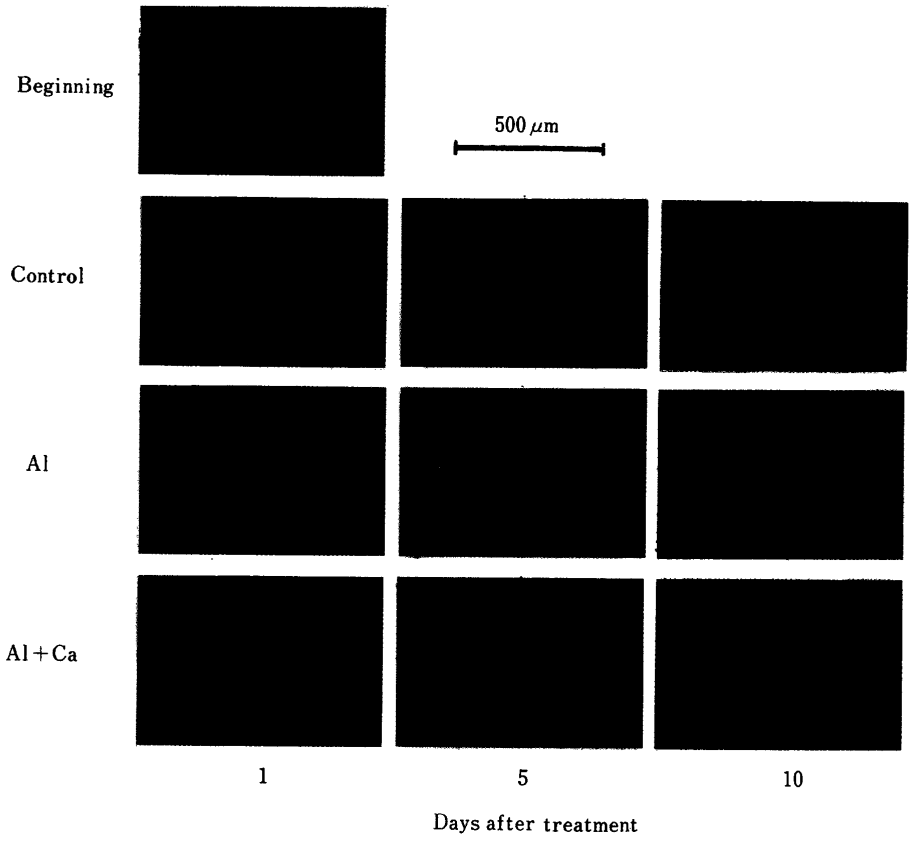

Fig. 2. Photomicrographs of root apices of blueberry plants treated with 1 mM Al and Ca, and dipped in fluorescein diacetate - propidium iodide solution. Green colored cells are viable, and red colored cells are damaged.

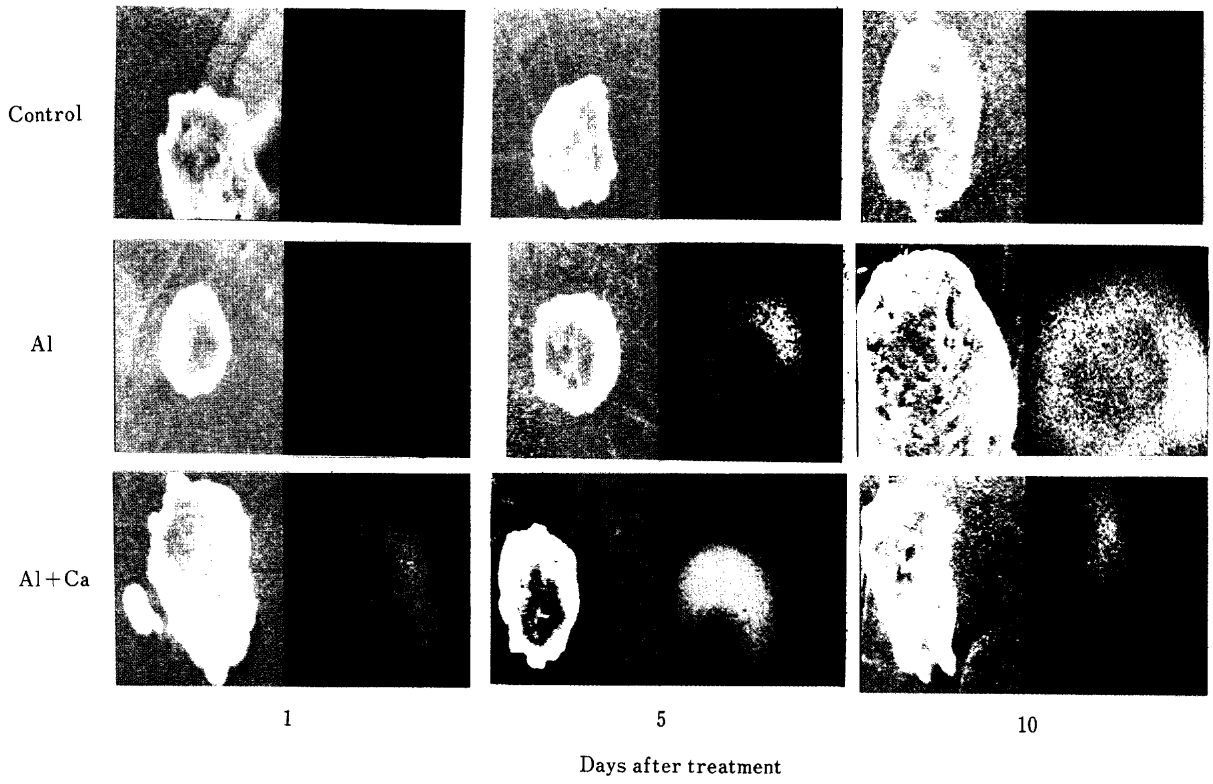

Fig. 3. Photomicrographs of cross sections of root apices (left) and distribution of aluminum (right). The region with Al accumulation is bright.

from low pH and acetate ion.

That Al tolerance was activated after the root reacted with Al in the rhizosphere was found in *Phaseolus vulgaris* (Cumming et al., 1992; Olivetti et al., 1995), and *Triticum aestivum* L. (Delhaize et al., 1993a, 1993b; Basu et al., 1994). That the blueverry roots remained healthy in 1 mM Al supports that idea. The Al content was higher in roots than in leaves (Korcak, 1988a; Suzuki et al., 1995), whereas *in vitro* cultured shoot without roots absorbed Al readily and growth inhibition was proportionate to the concentration of Al in the medium (Suzuki et al., 1995). Hence the high concentration of Al in the upper parts of the blueberry is the basis of growth inhibition. Thus, we believe that the accumulation of Al on the root protects the blueberry from being damaged by Al at a lower pH.

2. *Effects of Al application and P deficiency in vitro culture for fifty days.*

The shoots on the control and the +Al-treated plantlets gradually elongated for the first 40 days of the treatment and then stopped (Fig.4). Control shoots and those on +Al-P plantlets stopped elongating 20 days after treatment. Their shoot growth was significantly smaller than those on separate +Al and -P treatment. These data indicate that phosphate deficiency was accentuated by Al.

Roots in the control and -P treatment did not elongate during the experimental period, whereas, those in +Al grew 10 % during the same period, which was significantly superior over that of the control (Fig.5). Although the root growth was inhibited in the Al and -P treatment, shoots in all treatment elongated, indicating that the roots were able to absorb water and nutrient.

The amount of individual organic acids excreted into the medium by root were not significantly different among treatment (Table 1). Excretions, such as organic acids, which result in Al-chelation in the rhizosphere, are factors affecting Al tolerance (Miyasaka et. al., 1991; Samuels et al., 1997). In this experiment, the amount of excreted organic acids did not differ whether Al was present or not, indicating that at 1mM, Al does not induce Al injury.

In summary, 1 mM Al in the medium hastens both the shoot and root growth, and the lack of P in the medium inhibits root growth more than it does shoot growth. Furthermore, 1 mM Al in a P-free medium inhibits shoot growth unlike P deficiency. It is necessary to examine those effects in the field condition.

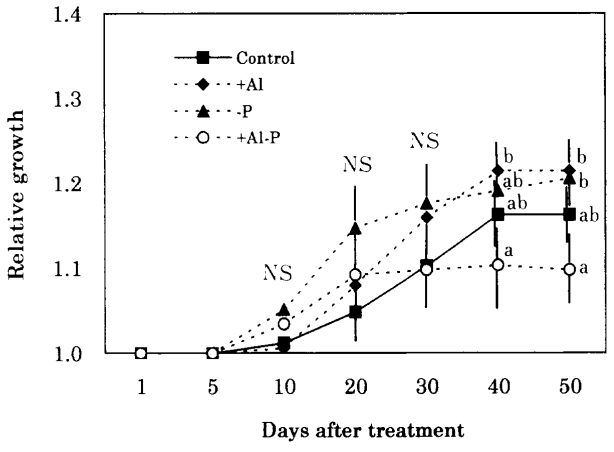

Fig. 4. Relative shoot growth (based on initial shoot length) of plantlets whose root were exposed to 1 mM Al and P for over 50 days. The initial shoot lengths are as follows : Cont.: 33 ± 6 mm, +Al: 33 ± 7 mm, -P: 31 ± 10 mm, +Al-P: 34 ± 10 mm. Vertical bars indicate SD; means were significant at P=0.05 by F-test.

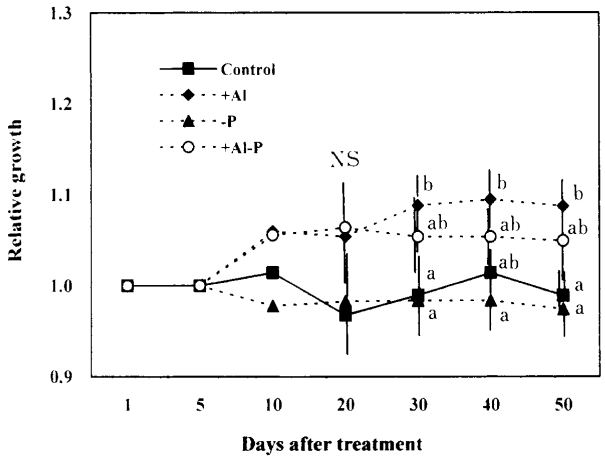

Fig. 5. Relative root growth (based on initial root length) of root exposed to 1 mM Al and P for over 50 days. The initial root lengths were : Cont.: 26 ± 2mm, +Al: 23 ± 6 mm, -P: 33 ± 8 mm, +Al-P: 30 ± 7 mm. Vertical bars indicate SD; means were significant at P=0.05 by F-test.

Table 1. Effect of Al and P on concentrations of organic acids in the medium.

Treatment	Organic acids (μ mol · l^{-1} · $root^{-1}$)		
	Citric acid	Maric acid	Oxalic acid
Control	0.46 ± 0.38[z]	0.54 ± 0.25	6.69 ± 3.13
+Al	0.35 ± 0.16	0.58 ± 0.32	4.22 ± 1.53
-P	0.45 ± 0.56	0.43 ± 0.07	5.59 ± 6.64
+Al-P	0.47 ± 0.31	0.49 ± 0.22	2.46 ± 1.40

[z] Mean ± SD

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ハイブッシュブルーベリーのシュート生長および根端組織の細胞活性，Al分布に対するAl, CaおよびPの影響

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摘　要

ハイブッシュブルーベリーのAl耐性に対するAl，Caおよびリン酸の影響を明らかにするため，in vitro培養で発根させたブルーベリーにAlおよびCa処理し，根端細胞活性と根端部のAl分布を調査した．また，AlおよびPの有無培地で50日間培養し，生長および根からの有機酸放出量を調査した．得られた結果は以下の通りである．

1. 対照区(Al無添加，酢酸緩衝液のみ)の根は，根端部に著しく障害を受けたが，1mM Al処理および1mM Al+Ca処理では処理前の根と同程度の活性を維持した．

2. X線分析で，根端組織のAl分布を測定した結果，対照区の根にはAlの集積はみられなかったが，1mM Al処理および1mM Al+Ca処理では，根横断面の皮層部に処理日数とともにAlが集積していくことが認められた．根表面へのAl集積が低pHや酢酸イオンの影響を防いでいることが示唆された．

3. 1 mM Alを添加した培地はシュートおよび根の生長を対照区よりも促進した．またリン酸を欠如した培地ではシュートよりも根の生育が抑制されたが，リン酸欠如培地に1 mM Alを添加した処理はリン酸欠如培地とは逆にシュートの生長が抑制された．

4. 処理終了時における根から培地中への有機酸放出量は，いずれの有機酸とも処理間に有意差がなく，Alストレスによる増加は認められなかった．これらの結果は，50日間の培養においても1mM濃度のAlは障害を誘導しないことを示した．