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Root Growth Inhibition in Boron-Deficient or Aluminum-Stressed Squash May Be a Result of Impaired Ascorbate Metabolism[1]

Krystyna M. Lukaszewski and Dale G. Blevins*

Department of Agronomy, University of Missouri, 1–87 Agriculture Building, Columbia, Missouri 65211

Although cessation of growth is the most apparent symptom of boron deficiency, the biochemical function of boron in growth processes is not well understood. We propose that the action of boron in root meristems is associated with ascorbate metabolism. Total inhibition of root growth in squash (*Cucurbita pepo* L.) plants transferred to boron-free medium coincided with a major decrease (up to 98%) in the ascorbate concentration of root apices. Under low-boron conditions, in which root growth was partially inhibited, ascorbate concentration declined in proportion to growth rate. The decline in ascorbate concentration in boron-deficient root tips was not related to ascorbate oxidation. Ascorbate added to the medium improved root growth in plants supplied with insufficient boron. Increasing concentrations of aluminum in the nutrient medium caused progressive inhibition of root growth and a parallel reduction in ascorbate concentration of root apices. Elevated boron levels improved root growth under toxic aluminum conditions and produced root apices with higher ascorbate concentrations. To our knowledge, this is the first report of a correlation between boron nutrition, ascorbate concentration in root apices, and growth. These findings show that root growth inhibition resulting from either boron deficiency or aluminum toxicity may be a consequence of disrupted ascorbate metabolism.

The first evidence for a boron requirement in plant growth and development was presented in 1923 (Warington, 1923). Since then, many roles for boron in plants have been proposed, but its primary function remains unknown. Rapid cessation of growth, followed by deterioration of meristems, is the earliest visible symptom of inadequate boron nutrition (Bohnsack and Albert, 1977). Boron deficiency disrupts cell division and cell elongation, but the mechanisms involved in these disruptions are not understood. In recent years, substantial evidence has indicated a role for boron in cell-wall organization that could be critical for proper cellular expansion (Lewis, 1980; Loomis and Durst, 1992; Hu and Brown, 1994; Kobayashi et al., 1996). There is also considerable information that connects boron with membrane structure and function (Pollard et al., 1977; Tanada, 1983). A direct effect of boron on ion uptake was reported by Blaser-Grill et al. (1989) and Schon et al. (1990). Boron caused a gradual hyperpolarization of the plasma membrane in sunflower root tips (Schon et al., 1990) and stimulated proton secretion (Goldbach et al., 1990) and the activity of plasma membrane NADH oxidase (Barr et al., 1993) in cultured carrot cells. The plasma membrane NADH oxidase, also called AFR oxidoreductase (Arrigoni et al., 1981; Morré et al., 1986; Morré et al., 1987), catalyzes the transfer of electrons to the AFR in the transmembrane electron transport reactions. Through its effect on proton secretion and on the activity of plasma membrane NADH oxidase, boron could be directly associated with cell growth.

The link between boron and ascorbate metabolism and the subsequent impact on growth are the subject of this investigation. Ascorbate was examined in plants grown with sufficient and insufficient boron and under aluminum-toxic conditions in which supraoptimal boron was used to maintain root elongation (Blevins, 1987; LeNoble et al., 1996a, 1996b).

MATERIALS AND METHODS

Seeds of summer squash (*Cucurbita pepo* L. cv Sunbar, Stokes Seeds, Buffalo, NY) were allowed to imbibe in aerated, deionized water and placed between moistened sheets of germination paper (Anchor Paper, Hudson, WI) for 3 d at room temperature. Germinated seedlings with primary root lengths of 5 to 6 cm were placed in sponge support collars. Collars were then fitted into holes in the tops of 0.9-L plastic containers filled with a complete nutrient solution constituted of 1 mM KH_2PO_4, 2 mM $MgSO_4$, 5 mM $Ca(NO_3)_2$, 2 mM K_2SO_4, and 0.02 mM $FeSO_4$. The micronutrients consisted of 1.5 μM $ZnSO_4$, 2 μM $MnCl_2$, 0.16 μM $CuSO_4$, 0.12 μM Na_2MoO_4, and 10 μM H_3BO_3 (Bohnsack 1991). Plants were kept in a growth chamber under continuous illumination (400 μmol m^{-2} s^{-1}), constant temperature (28°C), and 70% RH. After 72 h roots were measured and rinsed consecutively in six aerated containers of deionized water (2 min each). Plants were then transferred to treatment solutions. Unless stated otherwise, each treatment consisted of eight replications and each experiment was repeated at least twice.

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* Corresponding author; e-mail dale_blevins@muccmail.missouri.edu; fax 1–573–882–1469.

Abbreviations: AFR, ascorbate free radical; DHA, dehydroascorbate.

The boron concentration required for optimal root growth was determined. Squash plants were transferred to nutrient solutions containing 0, 1, 2.5, 5, 10, 20, 40, or 100 μM boric acid and placed in the growth chamber in conditions as described above. Root length increase was measured after 24 h.

Ascorbate Supplementation

Squash plants were grown as described above. Treatment solutions contained 0, 10, or 30 μM boric acid. Half of the plants at each boron concentration were supplemented with 100 μM ascorbate. Ascorbate stock solution was made fresh and added to the medium every 6 h. Root growth was measured after 24 h. To eliminate the possibility that boron was added as an impurity with the ascorbate supplement, ascorbate stock solution (200 mM) was analyzed for boron contamination. No measurable boron was detected in the solution by inductively coupled plasma spectrometry (detection limit of 0.03 parts per million).

Aluminum Treatment

Germinated squash seedlings were grown as described above. Root length was measured and then seedlings were transferred to solutions supplemented with up to 0.7 mM $Al_2(SO_4)_3$ and 0, 10, or 40 μM boric acid. The relatively high concentration of aluminum needed to inhibit root growth in hydroponically grown squash plants can be explained by the chemical composition of the nutrient solution used in these experiments. In our complete medium, 98% of the Al^{3+} ion was bound by SO_4^{2-}; therefore, 1 mM $Al_2(SO_4)_3$ corresponds to 40 μM free Al^{3+} (as determined by the GEOCHEM-PC program, D.R. Parker, University of California, Riverside). The pH of all media was adjusted to 4.0 with 0.4 M H_2SO_4 or 4 M KOH, and maintained throughout the treatment period by additional adjustments every 6 h as required. Aluminum treatment was continued over a period of 24 h. After treatment roots were rinsed and measured, and root tips (5 mm) were collected for enzymatic analysis or for determination of dry weight. Root tip samples were dried for 12 h at 60°C. In some experiments 100 μM ascorbate was added to the medium containing $Al_2(SO_4)_3$.

Ascorbate, AFR, and DHA Determinations

The ascorbate concentration of squash root tips was estimated as described by Liso et al. (1984). Apical root sections (5 mm) of squash plants were excised and weighed, and 125 mg fresh weight of root tips was homogenized in 1.25 mL of 5% (w/v) *m*-phosphoric acid. Extracts were centrifuged at 10,000*g* for 4 min. Samples (100–300 μL) of supernatant were placed in quartz cuvettes with 0.1 M citrate-0.2 M phosphate buffer, pH 6.2, to make a final volume of 3 mL. The initial A_{265} was determined, and ascorbate concentration was estimated by monitoring the decrease in absorbance after the addition of 2 units of commercial ascorbic acid oxidase (Sigma). After the oxidation of ascorbate was complete, ascorbate oxidase was inhibited with 10 mM sodium azide and DTT was added to the cuvettes to a final concentration of 2.5 mM. Following reduction with DTT (3–4 min at room temperature), the A_{265} was recorded again. DHA and AFR were determined from the difference between the final reading and the initial absorbance (Takahama and Oniki, 1994). Ascorbate concentration was expressed as micrograms of ascorbate per 100 milligrams fresh weight.

Ascorbic Acid Oxidase

Ascorbic acid oxidase activity was determined spectrophotometrically as described by Esaka et al. (1988) with minor modifications. Excised apical and subapical root segments (5 mm in length, 20 each) were placed in 2 mL of incubation solution consisting of 10 mM citrate-20 mM phosphate buffer, pH 6.2, 0.002% (w/v) *m*-phosphoric acid, and variable concentrations of EDTA (0–5 mM) or boric acid (0–1 mM). Following a 10-min preincubation in a shaking water bath (60 rpm) at 30°C, ascorbate was added to the test solutions (100 μM final concentration) and incubation was continued for 30 min. At the end of this period, 500-μL samples of the incubation solution were mixed 1:1 (v/v) with 0.1% (w/v) *m*-phosphoric acid, centrifuged at 14,000*g* for 4 min, and the A_{265} of the supernatant was measured. The amount of ascorbic acid oxidized in the reaction was quantified based on a standard curve, and the activity of ascorbate oxidase was expressed as micromoles of ascorbic acid oxidized per minute.

The effect of boron and EDTA on commercial ascorbate oxidase (Sigma) was also tested. The preincubation without ascorbate was shortened to 5 min and the assay of 0.1 unit of ascorbate oxidase was performed in the presence of 0, 2, and 5 mM EDTA or 0, 50, and 100 μM boric acid. Change in A_{265} was recorded every 30 s over a period of 12 min and the activity of ascorbate oxidase was determined as previously described.

RESULTS

Boron Nutrition, Ascorbate Concentration, and Ascorbate Oxidase Activity in Squash Root Tips

Root elongation of squash seedlings depended on boron concentration of the medium (Fig. 1). A maximum elongation rate of 2.5 mm h^{-1} was maintained across the range of 5 to 40 μM boron. A 45% inhibition was observed when boron concentration was lowered to 1 μM. In the absence of boron, root growth was limited to less than 10% of the maximum elongation rate. A slight decline in root growth, presumably caused by boron toxicity, was observed with 100 μM boron. Unless stated otherwise, in subsequent experiments 10 μM boron was used as the boron-sufficient control and zero boron as the boron-deficient treatment.

Inhibition of root elongation in treatments with inadequate boron was accompanied by a decline in ascorbate concentration in root apices (Table I). In plants supplied with sufficient boron, the concentration of ascorbate in root tips ranged from 70 to 115 μg 100 mg^{-1} fresh weight. In plants transferred to boron-deficient conditions, ascorbate concentration dropped to less than 1.9 μg 100 mg^{-1} fresh weight. Under low boron conditions (1 μM boron) that

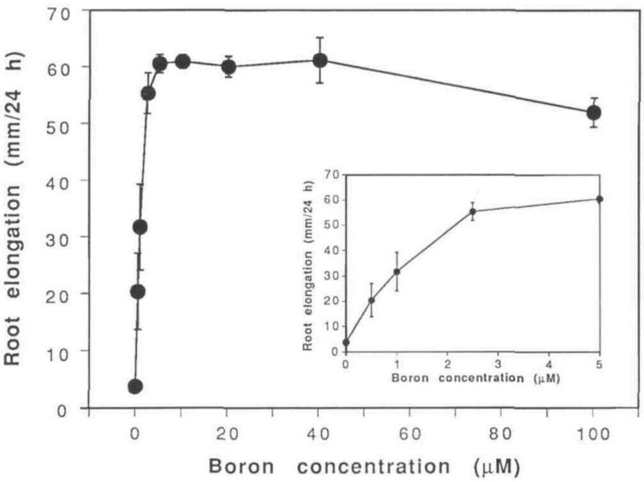

Figure 1. The effect of boron nutrition on squash root elongation rate. Germinated squash seedlings were grown for 3 d in boron-sufficient hydroponic medium, then for 24 h with variable concentrations of boron. Bars represent SE.

produced partial (approximately 45%) inhibition of root growth, ascorbate concentration was reduced to nearly 50% of that in boron-sufficient controls.

The oxidized forms of ascorbate (AFR and DHA) showed no apparent response to boron treatment. The concentrations of AFR and DHA in root apices excised from plants treated with 0, 10, and 40 μM boron were 12.0 ± 0.5, 9.6 ± 2.1, and 10.0 ± 1.1 μg 100 mg^{-1} fresh weight, respectively.

Ascorbate oxidase activity in excised squash root tips changed very little with the addition of boron (up to 1 mM) to the incubation mixture (data not shown). These results were supported by in vitro assays of commercial ascorbate oxidase isolated from *Cucurbita* species (Sigma), in which no change in activity was observed in the presence of up to 100 μM boron (data not shown). The activity of ascorbate oxidase (commercial) also was not affected by EDTA (data not shown).

Root Elongation in Boron-Deficient Medium Supplemented with Ascorbate

The decline in ascorbate concentration of boron-deficient squash root apices could be either a cause or a result of root growth inhibition. To address this question, root elongation was measured after ascorbate was supplied to media containing a range of boron concentrations. Exogenous ascorbate promoted root elongation in the absence of boron and under low-boron conditions (Fig. 2). The presence of ascorbate in boron-free medium increased root elongation from 4 to 33% of the boron-sufficient control, whereas with 0.5 μM boron, elongation increased from 43 to 82%, and with 1 μM boron elongation increased from 55 to 105% of the control (Fig. 3).

Based on the inductively coupled plasma analysis the highest possible concentration of boron added as an impurity with ascorbate could account for at most 20 pM boron in the medium, a quantity too small to explain the observed enhancement of growth. Stimulation of growth by exogenous ascorbate in the absence of boron suggests that ascorbate can compensate for boron in root elongation. Therefore, the change in ascorbate concentration is responsible for the observed growth effects and is not a result of root growth inhibition.

Table I. *Root elongation and ascorbate concentration in squash root apices following 24 h of treatment with different levels of boron*

Germinated squash seedlings were grown for 3 d in a complete nutrient solution with 10 μM boron, then transferred to treatment solutions containing 0, 1, or 10 μM boron. Values represent means of 12 replications ± SE.

Boron Concentration in the Medium	Root Elongation Rate	Ascorbate Concentration in the Root Tips
µM	*mm/24 h*	*µg ascorbate 100 mg^{-1} fresh weight*
10	60 ± 1.9	93.5 ± 4.1
1	37 ± 1.0	55.6 ± 1.4
0	3 ± 0.2	[illegible]

Root Growth and Ascorbate Concentration in Root Tips of Aluminum-Stressed Plants Grown with Supplemental Boron

The impact of boron nutrition on ascorbate concentration was also examined in aluminum-stressed roots. This ap-

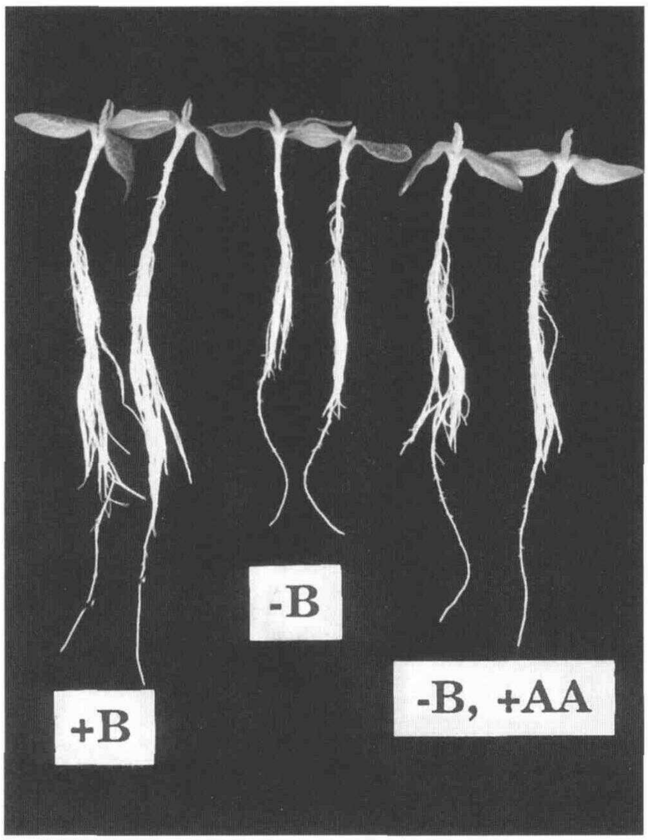

Figure 2. Squash plants grown for 3 d in boron-sufficient hydroponic medium, then for 24 h in optimal boron conditions (left), without boron in the medium (middle), or with 100 μM ascorbate in the absence of boron (right).

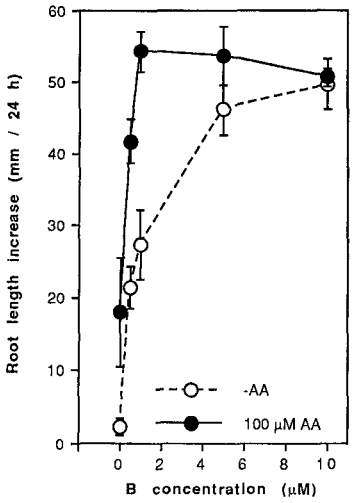

Figure 3. Root elongation rates of squash plants grown for 24 h with various concentrations of boron in the presence or absence of ascorbate. Plants were prepared as described in Figure 1. Exogenous ascorbate (100 μM) was supplied to the rooting medium during the 24-h treatment and was replaced every 6 h. Bars represent SE.

proach originated from our previous work in which supplemental boron ameliorated symptoms of aluminum toxicity in squash and alfalfa roots (LeNoble et al., 1996a, 1996b). Without boron, total cessation of growth and up to a 99% reduction in ascorbate concentration in root apices were observed with and without aluminum (data not shown). In the presence of 10 μM boron, increasing concentrations of aluminum in the medium resulted in a progressive inhibition of root elongation (Fig. 4A). The decrease in root elongation was highly correlated with declining ascorbate concentration in the root tips ($r = 0.99$). When boron concentration was elevated to 40 μM, inhibition of root growth by aluminum was less severe, and in the presence of 500 μM $Al_2(SO_4)_3$, root elongation was maintained at 75% that of the aluminum-free control (Fig. 4B). As in the 10-μM boron treatment, ascorbate concentration in root apices was closely correlated with root elongation rate ($r = 0.96$).

Changes in root tip dry weight were similar to changes in root elongation (Fig. 5, A and B). With 500 μM $Al_2(SO_4)_3$, dry weight and ascorbate concentration increased progressively as boron was increased from 0 to 10 to 40 μM ($r_{0-40\ \mu M\ B} = 0.99$). With 700 μM $Al_2(SO_4)_3$, toxicity was severe, but the decline in ascorbate concentration was again correlated with a decrease in dry weight ($r_{0-40\ \mu M\ B} = 0.96$; $r_{0-100\ \mu M\ B} = 0.95$).

DISCUSSION

One of the reasons that the primary function of boron in plants has not been elucidated is the diversity of symptoms produced by its deficiency. Among the postulated roles, recent evidence favors boron involvement in cell wall and/or membrane structure and function (Loomis and Durst, 1992; Shelp, 1993; Marschner, 1995), which could be critical for cell growth. In this study, we modified boron nutrition to alter squash root elongation and found a close positive correlation between root growth and ascorbate concentration in root apices. The reduction in ascorbate concentration in response to insufficient boron indicates that boron may be involved in maintaining ascorbate levels in root meristems.

The mechanism for a boron-ascorbate interaction could be related to boron association with the ascorbate redox cycle and plasma membrane electron transport. Barr et al. (1993) demonstrated an inhibition of plasma membrane NADH oxidase (AFR oxidoreductase) in the absence of boron. The biological role of NADH oxidase is not clearly understood, but some relationship to growth has been postulated. AFR is generated by wall-bound ascorbate oxidase, and Lin and Varner (1991) reported high ascorbate oxidase activity in fast-growing regions of squash fruits and leaves. Ascorbate oxidase catalyzes the oxidation of ascorbate to DHA via an AFR intermediate. Both DHA and AFR were postulated to affect growth. Lin and Varner (1991) suggested that DHA might be involved in cell-wall loosening. AFR was shown to enhance cell-wall acidification and proton extrusion, as well as to stimulate cell elongation in onion root tips (Morre et al., 1987; Hidalgo et

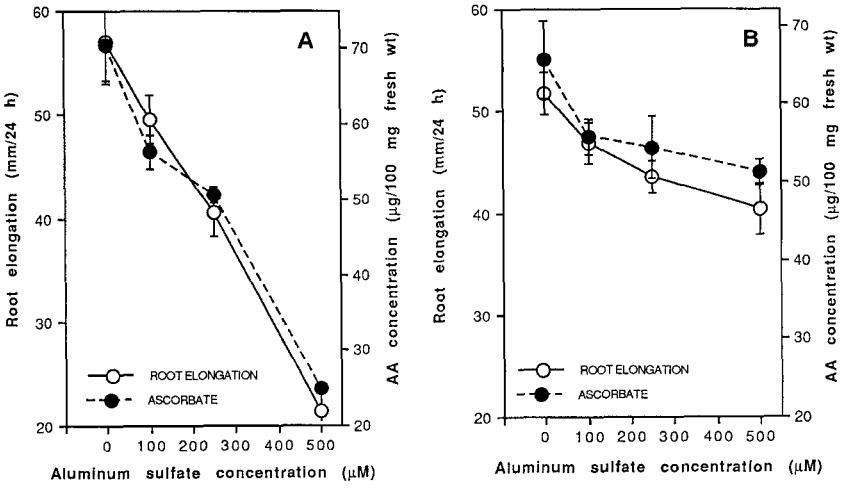

Figure 4. Root elongation and ascorbate concentration in root tips of aluminum-stressed squash plants treated with supplemental boron. Germinated squash seedlings were grown for 3 d in a complete nutrient solution, then for 24 h with increasing concentrations of aluminum and 10 μM boron (A) or 40 μM boron (B). Bars represent SE.

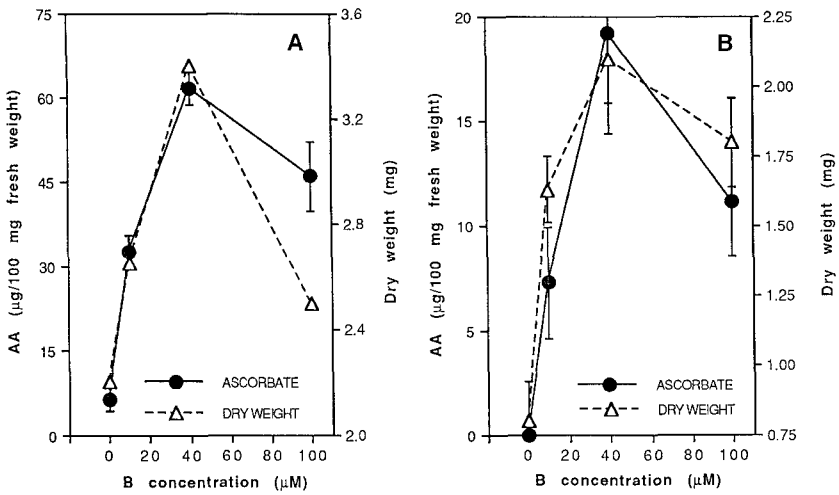

Figure 5. Root tip dry weight and ascorbate content in squash root apices in aluminum-toxic medium supplied with different concentrations of boron. Germinated squash seedlings were grown for 3 d in a complete nutrient solution, then for 24 h treated with 500 µM $Al_2(SO_4)_3$ (A) or 700 µM $Al_2(SO_4)_3$ (B) in the presence of increasing concentrations of boron. Bars represent SE.

al., 1989, 1991; Gonzáles-Reyes et al., 1992, 1994; De Cabo et al., 1993). Based on these findings, it is reasonable to hypothesize that inhibition of NADH oxidase in the absence of boron could alter the redox state of ascorbate and, therefore, growth.

In squash root apices, little variation was detected in the concentration of the oxidized forms of ascorbate (DHA and AFR), and the small changes did not correlate with the boron levels used in experiments or with the inhibition of growth. This indicates that the decline in ascorbate concentration induced by boron deficiency represents a decrease in the total pool of ascorbate in root apices, and cannot be ascribed to an interference of boron with ascorbate oxidation. Low ascorbate concentration in the absence of boron could be attributed to accelerated catabolism, or, more likely, to reduced ascorbate synthesis. Although no studies have shown that boron can stimulate ascorbate synthesis, this could explain our data, as well as the results of Gum et al. (1945), Govindan (1950), and Mondy and Munshi (1993), who reported an increase in ascorbate content following boron treatment of beets, tomatoes, and potatoes, respectively.

In our previous work we proposed that aluminum could inhibit root growth by inducing boron deficiency (Blevins, 1987), and demonstrated that supraoptimal concentrations of boron in aluminum-toxic medium greatly reduced root growth inhibition (LeNoble et al., 1996a, 1996b). The present results show that with increasing concentrations of aluminum in the medium, ascorbate concentration of root apices declines in a manner highly correlated with root elongation. In agreement with earlier findings, the toxic effect of aluminum was greatly diminished by supplemental boron, and in each case enhanced root elongation was accompanied by increased ascorbate concentration in root apices.

Results presented in this study suggest a connection between boron nutrition and ascorbate concentration in root meristems, and provide additional evidence linking ascorbate with root elongation. Despite the essential role of ascorbate as an antioxidant, little is known about the pathway of ascorbate biosynthesis or the control of the ascorbate level in plants. Further research is needed to define the biological importance of this ascorbate/boron interaction.

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