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Inhibition of Adventitious Root Growth in Boron-Deficient or Aluminum-Stressed Sunflower Cuttings

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The effect of boron and aluminum on the development of adventitious roots was studied in sunflower cuttings. Three-day-old seedlings were de-rooted and grown in nutrient solutions with or without boron and supplemented with different concentrations (from 50 to 700 μM) of aluminum. The number and length of the adventitious roots and proline content in adventitious roots in response to insufficient boron and aluminum stress were determined periodically. The micronutrient boron caused the development of numerous roots in the lower parts of the hypocotyl. A dose-response of boron-induced rooting yielded an optimum concentration of 0.1 mM boron. In the absence of boron, in the majority of the adventitious roots, a significant inhibition was observed with or without aluminum, indicating that the most apparent symptom of boron deficiency is the cessation of root growth. Increasing concentrations of aluminum caused progressive inhibition of growth and rooting of the hypocotyls, and a parallel increase in proline levels of adventitious roots. Supplemental boron ameliorated the inhibitory effect of aluminum, suggesting that aluminum could inhibit root growth by inducing boron deficiency. Ascorbate added to medium in the absence of boron improved root growth and induced a significant decrease in proline levels. These findings suggest that adventitious root growth inhibition resulting from either boron deficiency or aluminum toxicity may be a result of impaired ascorbate metabolism.

Key words : Adventitious roots, Aluminum, Ascorbate, Boron, Cuttings, Sunflower

1. Introduction

The first evidence for a boron requirement in plant growth and development was presented by Warington in 1923[1]. Since then, it is now well estabished that boron is an essential micronutrient for the normal growth of all higher plants and nitrogen-fixing cyanobacteria. However, in spite of decade of intensive research, the primary function of boron in the metabolism of higher plants is remains unknown[2]. Although the precise function of boron in plant metabolism is unclear, evidences suggest that it plays roles in cell elongation, nucleic acid synthesis, hormone response, membrane function and regulation of carbohydrate metabolism[3]. One of the reasons that primary function of boron in plants has not been elucidated is the diversity of symptoms produced by its deficiency. Boron-deficient plants may exhibit a wide variety of symptoms, depending on the species and the age of the plant. Boron deficiency causes many anatomical, physiological and biochemical changes. Rapid cessation of growth, followed by deterioration of meristems, is the earliest visible symptom of inadequate boron nutrition[4]. A characteristic symptom of young leaves, fruits, roots, and terminal buds is necrosis and abnomalities related to the breakdown of internal tissues. Boron deficiency disrupts cell division and cell elongation, but the mechanisms involved in these disruptions are not understood. This suggests that boron may be required for the maintenance of cell division, cell enlargement or both these processes.

In recent years, there is considerable information that boron plays essential roles in the structure and function of cell wall and cellular membranes including a direct effect of boron on

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ion uptake in developing plant tissues[5~7]. Boron influences cell wall expansion and plays an important role in cross-linking cell wall polysaccharides[8]. Boron interacts with polyhydroxy polymers such as pectins to form borate-ester cross-links and stabilize cell wall structure[9]. Variability in boron requirement between plant species is correlated with cell wall pectin content[10]. Recently, it has been shown in radish roots that boron, as boric acid, links two chains of rhamnogalacturonan II to form the boron-polysaccharide complex which seems to be ubiquitous in the cell walls of higher plants[11]. Boron caused a gradual hyperpolarization of the plasma membrane in sunflower root tips[12] and stimulated proton secretion and the activity of plasma membrane NADH oxidase in cultured carrot cells[13]. The plasma membrane NADH oxidase catalyzes the transfer of electrons to the ascorbate free radical in the transmembrane electron transfort reactions[14]. Through its effect on proton secretion and on the activity of plasma membrane NADH oxidase, boron could be directly associated with cell growth.

Numerous reports have shown that the plant hormone auxin has a central role in the initiation and growth of adventitious roots. Variations in peroxidase activity and the subsequent changes in the endogenous level of IAA have been frequently correlated with adventitious root formation in stem cuttings[15]. In several plant species, a supply of boron is essential for root development in stem cutting of light-grown seedlings[16]. Jarvis[17] proposed a model of adventitious root formation in which boron may have a role in the control of the level of endogenous auxin.

In our previous work we proposed that boron is required in the formation of adventitious roots in important crop species[18]. In this report we describe a connection between boron nutrition and ascorbate in adventitious roots and provide additional evidence linking ascorbate with root development to study the endogenous factors that control the formation of adventitious roots in sunflower seedlings. 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 growth.

2. Materials and Methods

2.1. Plant materials

Seeds of sunflower(*Helianthus annuus* L.) were allowed to imbibe in aerated, deionized water overnight (ca.16 h) at 25 ℃ and imbibed seeds were sown in damp vermiculite in plastic trays. The seeds were germinated for 3 days at 25±1℃. Uniform 3-day-old seedlings were used in all the assays.

2.2. Plant growth conditions

Three-day-old seedlings were derooted by cutting the entire root at the base of hypocotyl and were placed in glass Petri dishes containing 50 ml of nutrient solution medium in the presence or absence of boron. The complete nutrient solution (+B) was composed of : 5 mM $Ca(NO_3)$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 22.8 μM FeNaEDTA(12-14%), 18 μM $MnCl_2 \cdot 4H_2O$, 1.6 μM $ZnCl_2$, 0.5 μM $CuCl_2 \cdot 2H_2O$, 0.2 μM $Na_2MoO_4 \cdot 2H_2O$ and 0.1 mM H_3BO_3. Derooted seedlings were kept for 1-7 days at 25 ℃, photon flux of about 160 μmol $m^{-2}s^{-1}$ and a relative humidity of about 70 % in the growth chamber with a 16 h light/8 h dark regime. During incubation, the hypocotyls were maintained by daily supply of medium to the dishes in order to compensate water loss. After 7 day of incubation, the cuttings were removed from the Petri dishes, washed with distilled water and blotted dry. The boron concentration required for optimal root growth was determined. Sunflower cuttings were transferred to nutrient solutions containing 0, 0.01, 0.1 and 1.0 mM boric acid and placed in the growth chamber as described above. Each treatment consisted of eight replications and each treatment was repeated at least twice.

2.3. Measurement of adventitious root growth

For the growth measurements, different parameters of rooting of hypocotyl, such as number and length of roots and the localization of roots along and around the hypocotyls were determined every 24 h. The number of roots per hypocotyl was counted and the mean value in rooted hypocotyl was calculated and the length of individnal root was measured.

2.4. Ascorbate supplementation

Treatment solutions contained 0.1 mM boric acid and the cuttings were supplemented with 100 μM ascorbate. Ascorbate stock solution was made fresh and added to the medium every 6 h. Adventitious root growth was measured after 24 h.

2.5. Aluminum treatment

Root length was measured and then seedlings were transferred to solutions supplemented with 50,400 or 700 μM $Al_2(SO_4)_3$ and 0, 0.01, 0.1 or 1.0 mM boric acid. 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. Aluminium treatment was continued over 7 days. After treatment derooted seedlings were rinsed and measured, and adventitious roots were collected for proline assay. In some experiments 100 μM ascorbate was added to the medium containing $Al_2(SO_4)_3$

2.6. Proline determination

Treated adventitious roots with distilled water and blotted on filter paper, were transferred into test tubes containing 10 ml of 3 % aqueous sulfosalicylic acid the homogenates were centrifuged at 1500 g for 10 min. Proline was determined by the method of Bates et al[19]. Two ml aliquot of the supernatant was treated with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100 ℃ and the reaction terminated in an ice bath. The reaction mixture was extracted with 5 ml toluene and mixed vigorously for 15-20 sec. The chromophore containing toluene was separated and the absorbance read at 520 nm. The proline content was then calculated on a fresh weight basis. Each value is the mean±SD of the results from three separate plants.

3. Results

3.1 Effect of boron on the development of adventitious roots

The experiments described in this study were carried out with 3-d-old sunflower seedlings that were grown in vermiculite moistened with distilled water. After removal of the roots at the base of the hypocotyl, stem cuttings were placed in the Petri dish that either contained nutrient solution with or without boron supply. In cuttings that were incubated for 4 days in the absence of boron, few adventitious roots were detected (Fig. 1). The cuttings ceased to grow and died within 2 weeks of the start of the treatment. However, in the presence of boron numerous adventitious roots were observed in the basel region of hypocotyl.

Fig. 1. Cuttings from 3-d-old sunflower seedlings incubated for 4 days in nutrient solution without boron (left) and with boron (right).

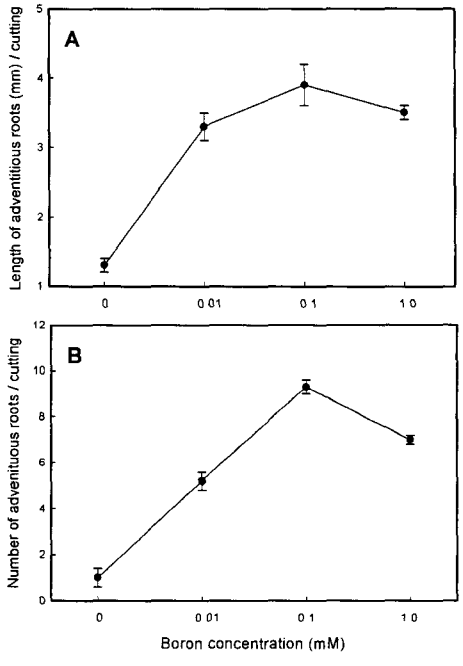

Fig. 2. The effect of boron nutrition on the length (A) and number (B) of the adventitious roots of 3-d-old sunflower cuttings incubated for 4 days in nutrient solution with or without boron supply.

Quantative data on the effect of boron on the initiation of adventitious roots in cuttings of sunflower seedlings are shown in Fig. 2. Adventitious root elongation of seedlings depended on boron concentrations of the medium. A maximum elongation rate was maintained across the range of 0.01 to 0.1 mM boron. 0.1 mM boron proved an optimal concentration for the initiation of organogenesis. In the absence of boron, root growth was limited to less than 67 % of the maximum elongation rate. A slight decline in adventitious root growth, presumably caused by boron toxicity, was observed with 1.0 mM boron. Unless stated otherwise, in subsequent experiments 0.1 mM boron was used as the boron-sufficient control and zero boron as the boron-deficient treatment.

3.2. Adventitious root development in aluminum-stressed plants grown in boron-deficient or boron-sufficient medium

The impact of boron nutrition on adventitious root formation was examined in aluminum-stressed roots. The number of adventitious roots was measured after various concentrations of aluminum were supplied to medium containing boron. The results showed that in the presence of 0.1 mM boron, increasing concentrations of aluminum in the medium resulted in a progressive inhibition of adventitious root growth (Fig. 3). In the presence of 700 μM $Al_2(SO_4)_3$, the number of adventitious roots was maintained at 25 % that of the aluminum-free control. In the absence of boron, in the majority of the adventitious roots, a significant inhibition in root development was observed with or without aluminum (Fig. 4). The data show that application of boron increased adventitious root development and the degree of stimulation of root growth was dependent on the boron supply.

3.3. Adventitious root development in boron-deficient medium supplemented with ascorbate

Higher concentrations of aluminum induced chlorosis of cotyledons and leaves, but application of ascorbate to the nutrient solution in the absence of boron greatly reduced chlorosis of leaves and maintained a greater length and number compared to the roots grown without boron and ascorbate (Fig. 5). Adventitious root elongation was measured after ascorbate was supplied to media with or without boron. Exogenous ascorbate, even in the absence of boron or aluminum stress conditions, resulted in a significant stimulation in root elongation (Table 1). The presence of ascorbate in boron-free medium increased root elongation to 35 % of the control. The results suggest that the effect of aluminum toxicity in the adventitious roots was ameliorated by exogenous ascorbate. The number of adventitious roots under aluminum stress in the absence of boron increased with application of exogenous ascorbate (Fig. 6), indicating that ascorbate can compensate for boron in root development. The results presented in this study suggest a connection between boron nutrition and ascorbate metabolism in root meristems and provide additional evidence linking ascorbate with adventitious root formation.

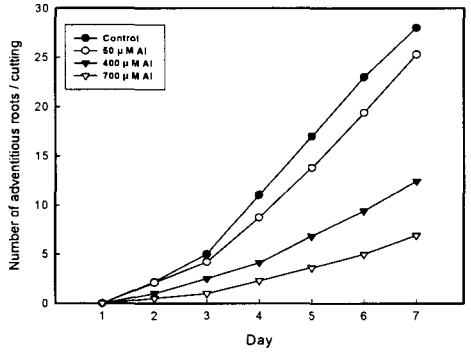

Fig. 3. Time course of adventitious root formation in sunflower seedlings incubated for 7 days with different concentrations of aluminum in the presence of boron.

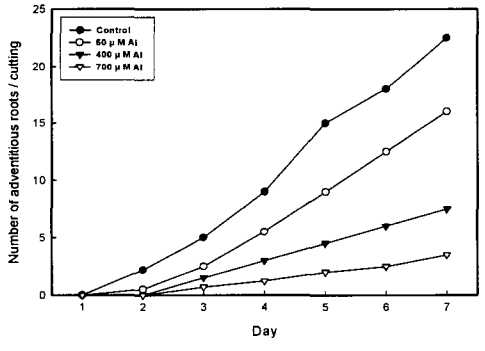

Fig. 4. Adventitious root formation in aluminum-stressed sunflower seedlings incubated for 7 days in the medium in the absence of boron.

Fig. 5. Cuttings from 3-d-old sunflower seedlings exposed to 50 μM Al for 24 h in the nutrient solution without boron (left), with 100 μM ascorbate in the absence of boron (middle), or optimal boron conditions (right).

Table 1. Length of adventitious roots of 3-d-old seedlings following treatment with different concentrations of aluminum and ascorbic acid. The de-rooted seedlings were incubated for 7 days in nutirent solution with or without boron

Treatment	Adventitious root length (mm)	
	- Ascorbate	+ Ascorbate
Control (-B)	4.8±0.3	6.5±0.5
Control (+B)	5.4±0.4	—
50 μM Al	4.4±0.4	5.6±0.4
50 μM Al + B	5.8±0.6	—
400 μM Al	4.2±0.2	4.7±0.3
400 μM Al + B	5.6±0.4	—
700 μM Al	4.0±0.2	4.3±0.3
700 μM Al + B	5.2±0.4	—

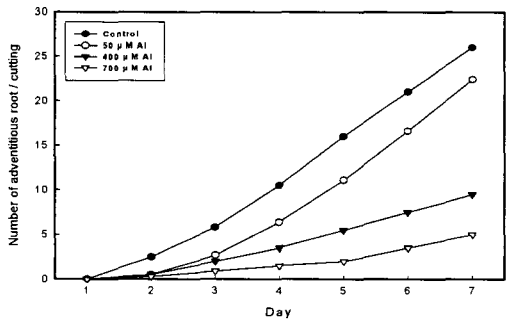

Fig. 6. Adventitious root formation in aluminum-stressed sunflower seedlings incubated for 7 days in the medium with 100 μM ascorbate in the absence of boron.

3.4. Proline content in aluminum-stressed adventitious roots grown in boron-deficient medium

Changes in the proline content in 7-d-old adventitious roots of sunflower seedlings exposed to aluminum-stress were monitored (Fig. 7). The proline accumulation in the adventitious roots gradually increased both with increasing concentration and duration of exposure to aluminum in the medium in the absence of boron. A significant rise in proline content was observed at 700 μM $Al_2(SO_4)_3$ at the end of the experiment, regardless of absence of boron.

Under insufficient boron conditions that produced

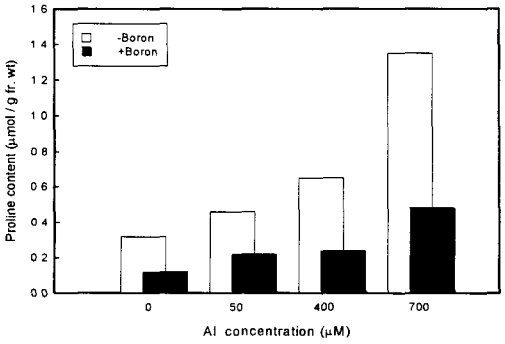

Fig. 7. Proline content in the adventitious roots of sunflower seedlings after 7-days of exposure to different concentrations of aluminum in the presence or in the absence of boron.

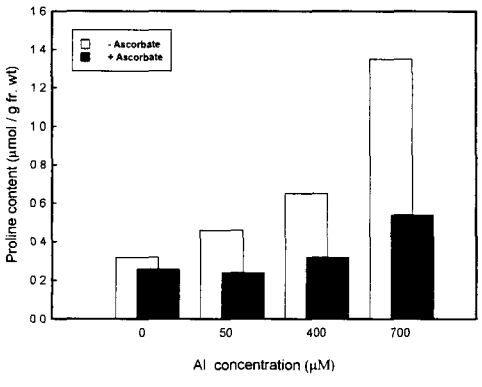

Fig. 8. Proline content in the adventitious roots of sunflower seedlings after 7-days of exposure to different concentrations of aluminum with 100 μM ascorbate in the absence of boron.

approximately 2.0 to 2.5-fold rise in proline content exogenous ascorbate reduced to nearly 2.0-fold of that in aluminum-stressed plants (Fig. 8). These results indicate that proline accumulation under aluminum stress conditions is assumed to be involved in stress tolerance and ascorbate ameliorated the inhibitory effect of aluminum toxicity.

4. Discussion

Boron deficiency is a widespread nutritional disorder under natural conditions. If the availability of boron is insufficient, a rapid cessation or complete inhibition in root growth, short stems and deformed fruits occurs[2,20]. Among the postulated roles of boron in higher plants, recent evidence favors boron involvement in cell wall organization and / or membrane structure and function[6,7], which could be critical for cell growth. In the present study, as pointed out by Shorrocks[21], the seedlings of sunflower which is one of the most responsive and sensitive plant species of boron application were used.

Aluminum toxicity is a growth-limiting factor for plants grown on acid soil. One of the most obvious symptoms of aluminum toxicity is the rapid inhibition of root growth[22,23], which results in poor nutrient acquisition and consequently leads to nutrient deficiencies and decreased crop yields[24]. The growth inhibition of roots associated with aluminum exposure suggests that the cytoskeleton may be a target of aluminum phytotoxicity[25]. In the present results aluminum inhibited adventitious root growth regardless of presence of boron and supraoptimal concentrations of boron in aluminum- toxic medium greatly reduced adventitious root growth inhibition. The toxic effect of aluminum was greatly diminished by supplemental boron, suggesting that aluminum could inhibit root growth by inducing boron deficiency. The results are consistent with other reports regarding the action of boron in root growth inhibition[26]. Supplemental boron ameliorated symptoms of aluminum toxicity in squash and alfalfa roots[27,28].

Depending on time and length of application, exogenous additions of ascorbate are known to lead to growth stimulation in normal, aerobically-grown plant tissues, including roots[29]. Ascorbate also stimulates cell proliferation in roots and shows a positive, long-lasting effect on root growth. It is, therefore, important to note that in this present study adding ascorbate to the plants had an effect on promoting growth of adventitious roots. The effect of aluminum toxicity in the adventitious root development was ameliorated by exogenous ascorbate in the absence of boron, suggesting that ascorbate can compensate for boron in root growth. Recently, Lukaszewski and Blevins[26] proposed a close correlation between root growth and ascorbate concentration in squash root apices. Ascorbate concentration was reduced in response to insufficient boron, indicating that boron may involve in maintaining ascorbate levels in root meristems. The mechanisms for boron-ascorbate interaction could be related to boron association with the ascorbate redox cycle and plasma membrane electron transport. Barr et al.[9] demonstrated an inhibition of plasma membrane NADH oxidase in the absence of boron. Substantial evidences have shown that inhibition of NADH oxidase in the absence of boron could alter the redox state of ascorbate and, therefore, growth. The decline in ascorbate concentration induced by boron deficiency represents a decrease in the total pool of ascorbate in root apices, and could be attributed to accelerated catabolism, or, more likely, to reduced ascorbate synthesis[26]. An explanation for the improved growth and survival of the ascorbate-treated seedlings may, therefore, be due to the role of ascorbic acid in the detoxification of oxygen radicals. The application of reductant compounds presumed to play a protective role against oxidative stress have altered the redox balance with concomitant disturbance of plant morphogenesis[30]. 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 interaction between boron and ascorbate.

There are well established metabolic traits leading to proline accumulation under stress conditions that are assumed to be involved in

stress tolerance[31]. Under some environmental stress conditions, proline was shown to provide an important contribution to osmotic adjustment in the growing regions of the primary root of maize[32]. A recent paper could conciliate these apparently conflicting results[33]. The authors showed that, in stressed maize plants, proline accumulated in the growing one of the primary root was presumably imported and not synthesized in these tissues.

There was an evidence that proline itself had a protective effect against the consequences of aluminum stress. Adventitious roots from sunflower seedlings grown with aluminum had higher proline than roots grown without aluminum. Other result have also shown that greater proline accumulation may occur in plants grown with excess aluminum[34]. However, sorghum grown with aluminum did not have higer proline than plants grown without aluminum[35]. A significant decline in proline levels in response to ascorbate in the absence of boron indicates that ascorbate ameliorates the symptoms of aluminum toxicity in sunflower roots. Thus, it is questionable that there is any adaptative value for proline synthesis and /or its accumulation in aluminum- stressed tissues.

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