Effect of Boron on the Development of Adventitious Roots in Sunflower Seedlings

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Abstract

Three-day-old sunflower(*Helianthus annuus* L.) seedlings were de-rooted and incubated in the nutrient solutions that contained either deficient or sufficient boron supply, and exposure to various pH and temperatures. In the absence of boron, no adventitious roots were formed in the majority of the seedlings. Boron caused the development of numerous adventitious roots in the lower part of the hypocotyl. The low pH damage was ameliorated by the simultaneous application of calcium, implying the involvement of calcium-requiring process in overcoming proton toxicity. Borate showed a strong ameliorative effect with Ca^{2+}. These results suggest that the primary target of proton toxicity may be linked to a disturbance of the stability in the pectic polysaccharide network, where calcium plays a key role in plant roots. Maximum temperature for the growth of adventitious roots was $25°C$. Although cessation of growth is the most apparent symptom of boron deficiency, exogenous ascorbate improved adventitious root growth in plants in the absence of boron. From the results it is suggested that the inhibition of adventitious root resulting from boron deficiency, low pH and temperature damage may be a consequence of disrupted ascorbate metabolism.

Key words − *Helianthus annuus*, adventitious roots, boron, low pH, calcium, ascorbate

Introduction

Boron(B) is an essential micronutrient for the normal growth of all higher plants and nitrogen fixing cyanobacteria. The first evidence for a boron requirement in plant growth and development was presented by Warington[30], indicating that a continual supply of boron is necessary in very low concentrations for growth of healthy broad bean plants. Since then, many roles for boron in plants have been proposed in decades of intensive research. However, the physiological and biochemical function of boron in growth processes of higher plants is still not known[21]. Boron is the least

well understood of all eight essential mineral micronutrients, despite the fact that boron deficiency symptoms have been described in detail. Boron deficiency causes many anatomical, physiological and biochemical changes; however, it is difficult to distinguish between primary and secondary effects[28]. Rapid cessation of growth, followed by deterioration of meristems, is the earlist visible symptom of inadequate boron nutrition[6]. This suggests that the micronutrient boron may be required for the maintenance of cell division, cell elongation or both of these processes. Boron deficient plants exhibit a shrunken aspect, with inhibition in root growth, short stems, affected youngest leaves and deformed fruits[5,21].

Substantial evidences have indicated a role for boron in cell wall organization that could be critical for proper

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cellular expansion[18], and may play a role as a structural component of the growing cell walls in developing plant tissues[14]. There is also considerable information that connects boron with membrane structure and function[8,22]. Evidence has also been reported that boron plays an important role in cross linkage cell wall polysaccharides and viability in boron requirement between plant species is correlated with cell wall pectin content[13,14]. Recently, Lukaszewski and Blevins[20] proposed that a close positive correlation between root growth and ascorbate concentration in response to insufficient boron. Low ascorbate concentration in the absence of boron could be attributed to accelerating catabolism or more likely to reducing ascorbate synthesis.

It is well known that Ca^{2+} ions play an important role in plant cell growth[7,27]. Ca-displacement by proton causes an inhibition of root elongation in wheat, and Ca^{2+} in the apoplast is one of the major targets of proton rhizotoxicity[17]. Proton toxicity is evident in culture solution consisting of calcium chloride[31]. Proton toxicity (low pH) inhibited root elongation in several plant species and proposed that Ca^{2+} displacement by proton is part of the toxic action of proton rhizotoxicity[17]. Pectic polysaccharides are one of the major constituents containing Ca^{2+}, which has a possible function to stabilize the cell wall network[9]. Although the biological role of pectic polysaccharides has not yet been clarified, another cross linkage of pectic polysaccharides is mediated by borate[18, 25]. Recently, it has been shown that tobacco cell death (lost of viability) was caused by borate deprivation[12]. On the other hand, the low pH of the apoplast has reported to stimulate pectin solubilization in tomato fruit during development[10]. These results raised a question as to whether the loss of viability in growing root tips from proton rhizotoxicity is due to the depletion of Ca^{2+} from the pectin network at low pH. Borate, which cross links pectin, also strongly ameliorated the effect of low pH damage, suggesting that the disturbance of the pectin network may be one of the primary targets of proton rhizotoxicity[25].

Root play a primary role in supplying water and nutrients to plant tissues. Initiation and development of roots are critical to the growth and productivity of crop plants. Change in soil temperature, soil strength, composition of the soil atmosphere, and soil water content are among the environmental factors that can affect the growth and development of the root system[23]. An important environmental factor that impacts root growth and development is soil temperature. Root temperature also directly influences cell growth by accelerating the rate of cell division in root and shoot meristems.

Non-woody stem cuttings are suitable systems to study organogenetic processes such as the formation of adventitious roots. Numerous reports have shown that the plant hormone auxin has a significant role in the initiation and growth of these organs. The elongation of adventitious roots has been divided into various phases based on histological and biochemical observations. In several plant species a supply of boron is essential for root development in stem cuttings of light-grown seedlings[1]. Based on these findings, Josten and Kutschera[16] described a system to study the exogenous factors that control of adventitious roots in light-grown sunflower seedlings. In the presence of boron the cuttings formed numerous adventitious roots that entirely replaced the tap root system of intact seedlings. The purpose of this study is to investigate the effect of boron with Ca^{2+} and the viability of roots from rhizotoxicity due to Ca^{2+} at various pH and temperatures in growing adventitious roots of sunflower seedlings.

Materials and Methods

Plant materials

Seeds of sunflower(*Helianthus annuus* L.) were soaked in distilled water prior to sowing in vermiculite. The

seeds were germinated in the darkness for 3 days. Cuttings were made from 3-d-old seedlings of average size by severing the entire root at the base of hypocotyl. Cuttings were placed in glass Petri dishes which contained 50 mL nutrient solution medium in the presense or absence of boron. Seedlings were grown for 4 days at $25\pm1°C$, photon flux of about $160\,\mu mol\ m^{-2}s^{-1}$ and a relative humidity of about 70% in the growth chamber with a 16 h light/ 8 h dark regime.

Plant growth conditions

Cuttings were made from 3-or 4-day-old seedlings of average size by severing the entire root at the base of the hypocotyl. Stem cuttings were transferred to nutrient solutions containing 0, 0.01, 0.1, and 1.0 mM boric acid and were grown in a growth chamber for 1-7 d in conditions as described above. The boron concentration required for optimal root growth was determined. In some experiments the concentration of boric acid was 0.1 mM, in other experiment, no boric acid was added (nutrient solution -B). $CaCl_2$ was used as the source of Ca^{2+} and test solution was prepared by adding $CaCl_2$ to the nutrient solution (5, 20, 700 and $1000\,\mu M$ $CaCl_2$) at pH 3.5, 4.5, 5.5 or 7.2. The pH range from 3.5 to 7.2 in the nutrient solutions was adjusted by adding HCl or NaOH. For the temperature treatments, cuttings were exposed to various treatments of 10, 25 and 40°C. 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 $MnCl_2 \cdot$ 4 H_2O, 1.6 μM $ZnCl_2$, 0.5 μM $CuCl_2 \cdot 2H_2O$, $0.2\,\mu M$ $Na_2MoO_4 \cdot 2$ H_2O and 0.1 mM H_3BO_3. The solutions were renewed every day to prevent depletion and to avoid pH drift.

Ascorbate supplementation

Sunflower plants were grown as described above. Treatment solutions contained 0.1 mM boric acid and the cuttings were supplemented with $100\,\mu M$ ascorbate.

Ascorbate stock solution was made fresh and added to the medium every 6 h. Root growth was measured after 24 h.

Measurement of seedling growth

After 1-7 d of incubation, cuttings were removed from the Petri dishes, washed with distilled water and blotted dry. Thereafter, the length of adventitious roots were measured every 24 h by the scale having a marker at every 0.25 mm. The number of adventitious roots and root hairs was also counted. The fresh and dry weights of the cuttings were determined. The fresh weight was determined immediately after harvest and the dry mass production was determined by drying the seedlings at 80 °C for 48 h.

Proline assay

Proline contents were determined using the methods described by Bates et al.[3]. 0.5 g of plant tissue was homogenized in 10 mL of 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 1500 g for 10 min. Two mL aliquot of the supernatant was treated with 2 mL acid ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100°C. Tubes were cooled to ambient temperature (5 to 10 min in cool water bath) before 5 mL toluene was added, and solutions vigorously mixed. After separation of solution layers, 2 mL aliquot samples of the top chromophore (toluene) layers were placed in cuvettes and the absorbance read at 520 nm. Proline concentrations were determined from a standard curve and calculated on a fresh weight basis.

Data analysis

All experiments were done in triplate and repeated at least twice. Each value is the mean$\pm$SD of three replicates.

Results and Discussion

Three-d-old sunflower seedlings cultivated in vermic-

lite moistened with distilled water were used in the experiments. Under these growth conditions hypocotyl were 18-25 mm in length and elongated at an average rate of approximately 0.7 mm per h. After removal of the roots at the base of the hypocotyls, stem cuttings were placed in Petri dishes that contained nutrient solution with or without boric acid. After a period of 4 d in a growth chamber the cuttings were analyzed (Fig. 1). Under all conditions the hypocotyls were curved. The stems were significantly longer than at 3 d after sowing. In complete nutrient solution (+B) the cotyledons and primary leaves were much larger than in the nutrient solution (-B). Numerous adventitious roots were present in basal 5 mm-region of sturdy hypocotyl. In nutrient solution that lacked boron (-B), no or very few adventitious roots were detected. The results demonstrate that cuttings from de-etiolated seedings raised in the absence of B are a suitable system for responsive and sensitive plant species of B application.

Quantitative data on the effect of B on the initiation of adventitious roots in cuttings of sunflower seedlings are shown in Fig. 2. At a concentration of 0.01 mM boric acid a significant enhancement in the length and number of roots was observed compared to the control (-B). At a concentration of 0.1 mM boric acid proved an optimal concentration for the initiation of organogenesis. At higher concentrations above 1.0 mM boric acid inhibited adventitious root development.

The effects of low and high pH on the growth of adventitious roots of sunflower seedlings were determined. Cuttings from 3-d-old sunflower seedlings were cultured under different pH conditions for 7 days. The elongation of adventitious roots was influenced by pH (Fig.3). Elongation growth of the adventitious roots was reduced depending on the reduction of pH value in the range lower than 5.5 and significantly inhibited at pH 3.5. Length of adventitious roots grown at pH 3.5 was
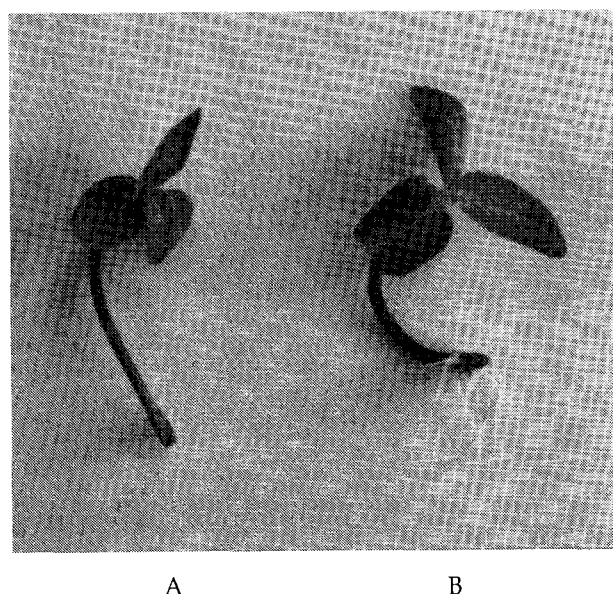

A   B

Fig. 1. Cuttings from 3-d-old sunflower seedlings that were incubated for 4 d in nutrient solution without boric acid (A) and with boric acid (B). Note that in the presence of boric acid (0.1mM) numerous adventitious roots are present (B).
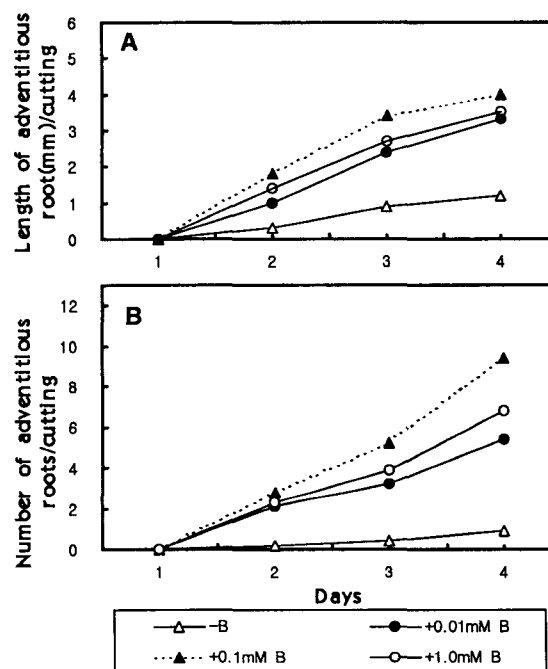

Fig. 2. Time course of length (A) and number (B) of the adventitious roots of 3-d-old sunflower cuttings that were incubated for 4 d in nutrient solutions (±boric acid).
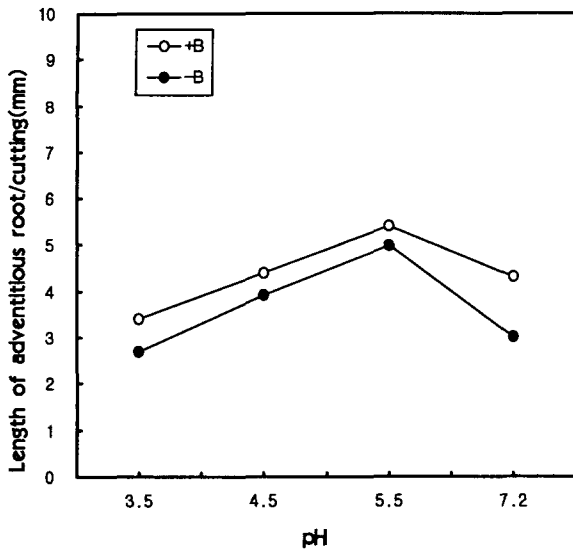
Fig. 3. Effects of various pH on the adventitious root growth of sunflower seedlings.

The adventitious roots of seedling cultured for 7 d in the nutrient solutions were exposed at pH 3.5, 4.5, 5.5 and 7.2 in the presence or absence of boric acid.

Fig. 4. Time course of adventitious root elongation of sunflower seedling grown for 7 d without boron in the medium (A), or with 100 μM ascorbate in the absence of boron (B).

only 20 % of the value of the root length grown at pH 5.5. The elongation of roots showed a similar tendency regardless of presence of boric acid. Time course of various pH induced adventitious root formation was determined (Fig. 4). The number of roots was gradually increased regardless of pH values. Root hair formation is controlled by several environmental factors such as calcium[4], nitrate and pH[11], as well as phytohormones [26]. The elongation growth of roots was highly suppressed depending on the lowering of the media pH. Inoue et al.[15] reported that root hair formation was induced by lowering the pH of the growing medium for lettuce seedlings grown under continuous white light. Vigorous root hair formation was observed in the seedlings cultured under low pH condition. The amount of root hairs formed in the pH 4.5 was about 7 times greater than that at pH 5.5[15]. Those results supported that the acidic condition was favorable for the formation of root hairs. This observation suggests that the primary target of proton in rhizotoxicity is the disturbance of biological functions involved in root
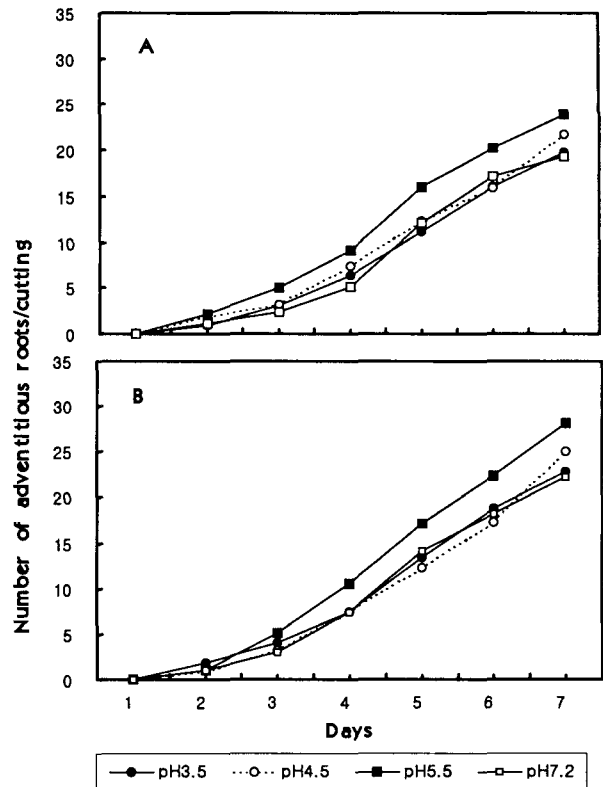
elongation. It has been demonstrated that Arabidopsis thaliana displays high sensitivity to short-term proton rhizotoxicity[19]. A visual low-pH damage was detectable within a short term, and the result are comparable to those obtained using inhibition of root elongation as an indicator for rhizotoxicity, which requires a long term. Exogenous ascorbate promoted adventitious root formation in the absence of boron. Vigorous root hair formation was observed at pH 5.5. The number of adventitious roots grown in the nutrient solution without boron was less than in the roots grown in the nutrient solution in the presence of ascorbate. Borate increased ascorbate synthesis[20]. Ascorbate, an antioxidant, is a major metabolite in plants and in association with plants against oxidative system protects plants against oxidative damage resulting from aerobic

metabolism, photosynthesis and a range of pollutants.

To determine whether lowering of solution pH increases the Ca^{2+} requirement for root growth, cuttings were soaked in solutions containing Ca^{2+} at various concentrations at pH 3.5, 4.5, 5.5 and 7.2, and root elongation was measured 6 h after exposure. Root elongation as a function of Ca^{2+} concentration at pH 5.5 had sharp peaks at $20\,\mu M$, and then slightly decreased at higher Ca^{2+} concentrations (Fig. 5). Thus, the Ca^{2+} requirement of growing roots strongly increased by lowering the pH. The addition of $CaCl_2$ known to bind with pectin strongly ameliorated low pH damage. Borate strongly ameliorated low pH stress in the presence of Ca^{2+}. High concentrations of Ca^{2+} were required to maintain root tip viability and root elongation of Arabidopsis thaliana at low pH. Borate strongly ameliorated low pH stress in the presence of Ca^{2+}[19]. However, borate provided no amelioration of

low pH stress when applied without Ca^{2+}. This Ca^{2+} requirement of borate amelioration fits the mechanism of cross linkage of pectic polysaccharides with borate, which requires Ca^{2+}[25]. Although the internal effects of metals have been reported, these results indicate that short-term proton rhizotoxicity is caused by weakening of Ca-mediated cross-linkage of pectin due to low pH. Further analysis is needed to test this hypothesis in relation to the vital importance of the development of the pectin network for root growth.

Maximum growth for adventitious root was shown at $25\,°C$, while very little root growth occurred at $10\,°C$ and $40\,°C$ (Fig. 6). Under temperature stress condition, the development of adventitious roots of sunflower seedlings was found to be enhanced by borate. In addition, exogenous ascorbate promoted adventitious root elongation in the absence of boron (Fig. 7). The number of adventitious roots reached its maximum at $25\,°C$. Very little adventitious root growth occurred at $10\,°C$ and $40\,°C$. In general the growth of roots tends to increase with increases in soil temperature[23]. In cultivated
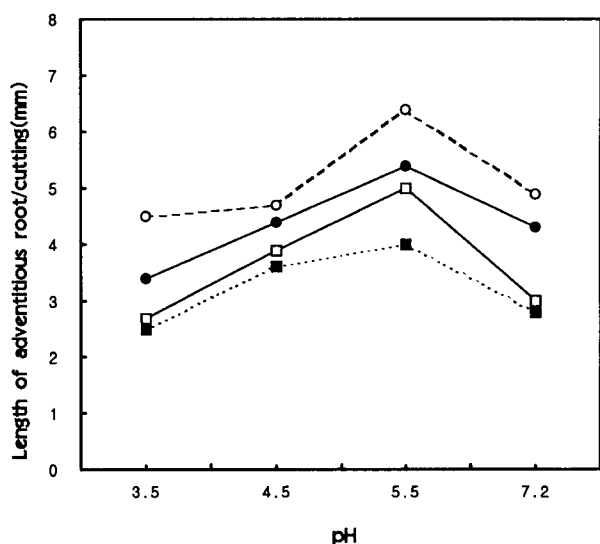

Fig. 5. Ameliorative effect of Ca^{2+} on the development of adventitious roots in sunflower seedlings.

Cuttings were grown in nutrient solutions containing $CaCl_2$ of various concentrations at pH 3.5 (1000 μM), 4.5 (700 μM), 5.5 (20 μM), 7.2 (5 μM) for 7 d in optimal boron condition (●), with $CaCl_2$ in the presence of boron(○), in the absence boron (□), with $CaCl_2$ in the absence of boron (■).
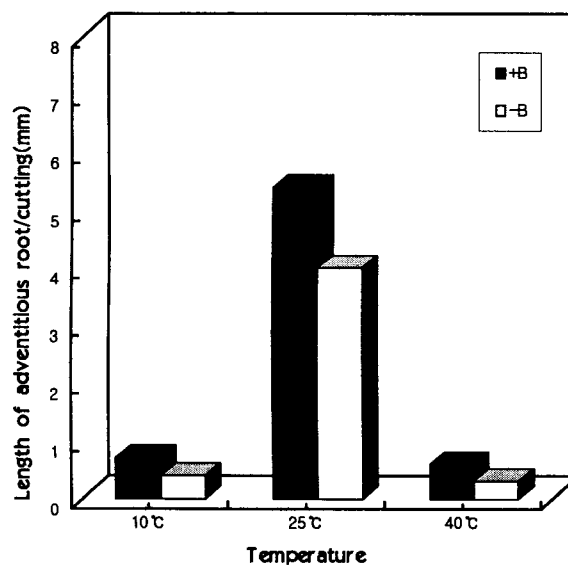

Fig. 6. Effect of temperature on the development of adventitious roots in sunflower seedlings grown for 7 d in the presence or in the absence of boron.
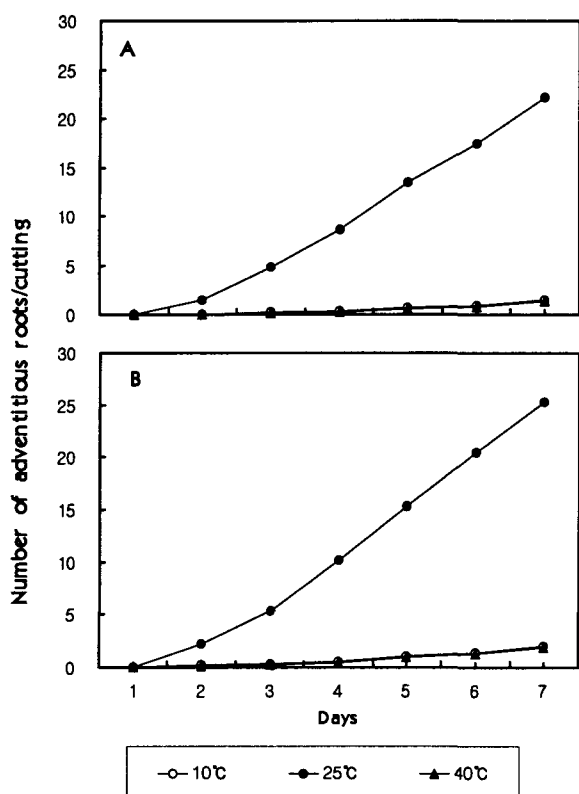
Fig. 7. Time course of adventitious root formation in sunflower seedlings grown for 7 d without boron in the medium (A), or with 100 μM ascorbate in the absence of boron (B).
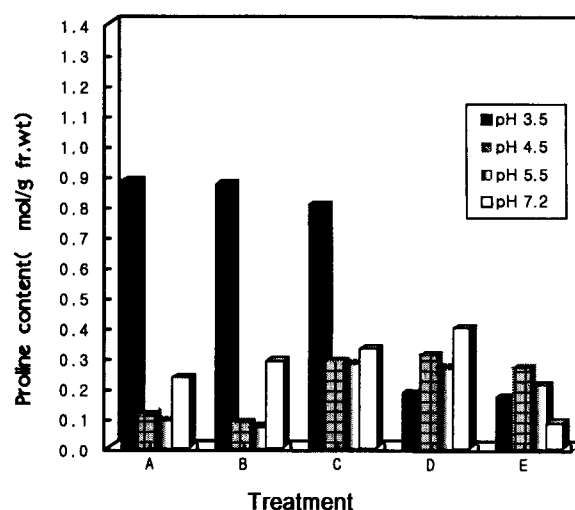

Fig. 8. Proline content in the adventitious roots of sunflower seedlings after 7-days of exposure to various pH.

A, optimal boron conditions; B, with Ca^{2+} in the presence of boron; C, without boron in the medium; D, with Ca^{2+} in the absence of boron; E, with 100 μM ascorbate in the absence of boron.
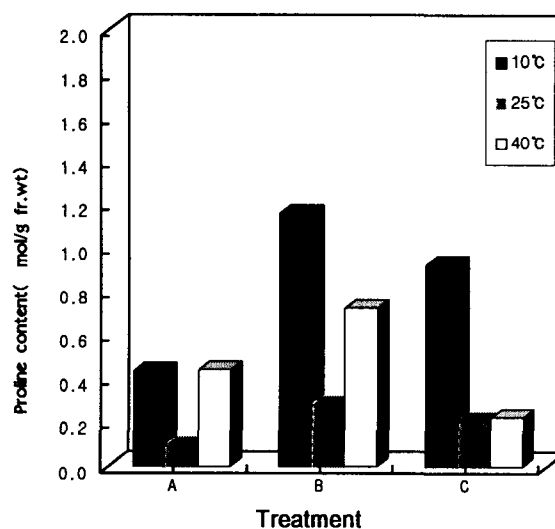

Fig. 9. Proline content in the adventitious roots of sunflower seedlings after 7-days of exposure to various temperatures.

A, optimal boron conditions; B, without boron in the medium; C, with 100 μM ascorbate in the absence of boron.

sunflower the optimum temperature for primary and lateral root growth was reported to be in the range of 23-25°C[24]. The exposure of sunflower roots to higher or lower than optimum temperature can have an adverse effect on the growth and development of the root system. It appears that temperature is directly reducing root growth and thus indirectly decreasing water and nutrient uptake.

Changes in the proline content in 7-d-old adventitious roots of sunflower in response to various pH were monitored (Fig. 8). The proline accumulation in the adventitious roots, as a function of pH values, increased progressively in response to the low pH. A significant rise in the proline content was observed at pH 3.5, regardless of presence of boron. The pattern of proline

accumulation according to temperatures was different. The increase in the proline level at 10°C was prominent

n the absence of boron (Fig. 9), suggesting that chilling s related to the increase of proline content[2]. Proline ías been shown to accumulate in tissue and organs of ɔlant subjected to water deficit, high salinity, chilling, ow and high temperature stress, heat and heavy metal ɛxposure[29]. However, proline content in roots ʝecreased when plant was grown in combination with ɔorate and Ca^{2+}. The present observations showing a ɔositive relationship between stress agents and proline ıccumulation suggest a protective role of this amino acid ıgainst stress and some mechanism which compensate ɔr the reduction of root function induced inhibition of ʝhe adventitious root elongation.

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초록 : 해바라기 유식물의 부정근 발달에 미치는 붕소의 효과

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암하에서 3일간 발아시킨 해바라기 유식물의 유근을 절단한 후 붕소 첨가구와 비첨가구에서 온도와 pH 처리에 의한 부정근 발생을 조사하였다. 붕소 미첨가구에서는 부정근이 발생하지 않았으나, 붕소 첨가구에서는 배축의 하위부에서 부정근 발생이 유도되었다. 낮은 pH 처리에 의해 부정근이 손상되었으나 Ca^{2+}을 함께 처리한 붕소 첨가구에서는 현저하게 회복되었는데, 이것은 proton 독성을 극복하는데 calcium이 필요하며 proton 독성이 뿌리에서 중요한 기능을 하는 펙틴 다당류의 안정성을 방해하는 현상과 관련이 있음을 시사해 준다. 부정근 생장에 최적온도는 25℃이었다. 붕소 결핍의 가장 뚜렷한 현상은 부정근 생장의 정지이었으나 붕소 첨가구에서 부정근 생장은 향상되었다. 붕소 비첨가구에 ascorbate를 처리하였을 때 부정근의 생장은 촉진되었다. 이러한 사실들로 보아 붕소 결핍, 낮은 pH 및 온도저해로부터 야기된 부정근 생장의 억제는 ascorbate대사가 교란된 결과에 기인되는 것으로 사료된다.