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Structural and functional damage caused by boron deficiency in sunflower leaves

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Abstract

Boron deficiency induced a dramatic inhibition in sunflower plant growth, shown by a reduction in dry mass of roots and shoots of plants grown for 10 d in nutrient solution supplied with 0.02 μM B. This low B supply facilitated the appearance of brown purple pigmentation on the plant leaves over the entire growth period. Compared to B-sufficient (BS) leaves, leakage from B-deficient (BD) leaves was 20 fold higher for potassium, 38 fold for sucrose, and 6 fold for phenolic compounds. High level of membrane peroxidation was detected by measuring peroxidase activities as well as peroxidative products in BD sunflower plants. Soluble and bound peroxidase activities measured in BD thylakoid membranes were accelerated two fold compared to those detected in BS-membranes. No detectable change in soluble peroxidase activity in roots whereas a 4 fold stimulation in bound peroxidase activity was detected. Thylakoid membranes subjected to low B supply showed enhancement in lipoxygenase activity and malondialdehyde (MDA) content in parallel with 40 and 30 % decrease of linoleic and linolenic acid contents (related to total unsaturated fatty acids). A slower rate of Hill reaction activity (40 %) and a suppressed flow of electron transfer of the whole chain (30 %) were detected in BD thylakoid membranes. This reduction was accompanied with a decline in the activity of photosystem 2 shown by a diminished rate of oxygen evolution (42 %) coupled with a quenching (27.5 %) in chlorophyll *a* fluorescence emission spectra at 685 nm (F_{685}). Thus B is an important element for membrane maintenance, protection, and function by minimizing or limiting production of free oxygen radicals in thylakoid membranes of sunflower leaves.

Additional key words: chlorophyll; dry mass of roots and shoots; fluorescence; Hill reaction; lipoxygenase; malondialdehyde; peroxidase; photosystem 2; potassium; saccharose.

Introduction

Optimization of mineral nutrition is one of the environmental factors that limit plant growth and production. Exposure of plants to mineral deficiency results in changes in

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most physiological and biochemical processes leading to a disturbance in plant growth and yield (Wenzel and Melhorn 1995, Yu and Bell 1998). Role of B in plant growth and development is rarely reported. Many physiological functions could be affected by B deficiency. These include cell division and elongation (Kouchi 1977), nucleic acid metabolism, protein synthesis, lignification of cell wall, and xylem differentiation (Lewis 1980). A diminished rate of photosynthetic oxygen evolution and a declined quantum yield and efficiency of photosystem 2 (PS2) were recorded in BD sunflower leaves (Kastori *et al.* 1995). Boron is required for membrane integrity and function because it stimulates proton pumping and potassium uptake in sunflower roots (Schon *et al.* 1990). Deficiency of B resulted in a decrease in H^+-ATPase activity and an increase in sunflower membrane ability for passive conductance of protons (Roldan *et al.* 1992, Ferrol *et al.* 1993). Involvement of oxygen free radicals leading to peroxidative damage of the plant tissues is one of various postulates concerned with the injury signals induced by many macronutrient deficiencies (Yu *et al.* 1998). Thus the activity of many scavenger enzymes such as peroxidases may be altered (Cakmak *et al.* 1995, Hodges *et al.* 1997). The hydroperoxidation of unsaturated fatty acids by lipoxygenase (Axelrod *et al.* 1981) is involved in the peroxidative damage of plants under stress (Lynch *et al.* 1985, Borrell *et al.* 1997). Lipoxygenase functions to incorporate molecular oxygen into both linoleic and linolenic acids forming different lipid peroxides which decompose to produce free oxygen radicals, malondialdehyde (MDA) and jasmonic acid (Vick and Zimmerman 1984) that cause lipid peroxidation. However, only little information is available on the correlation between the effect of B nutrition and thylakoid membrane characteristics and functions.

In the present investigation we analysed thylakoid membranes of sunflower leaves excised from plants grown in nutrient solution supplied with various B concentrations. The effects of B on the membrane characteristics were determined by measuring their permeability to potassium, sucrose, and phenolics, by detecting the contents of peroxidation products, MDA, and linoleic and linolenic acids, and by determining the activities of both peroxidase and lipoxygenase. The effects of B on the membrane functions were detected by measuring various photosynthetic parameters.

Materials and methods

Plant growth: Sunflower seeds (*Helianthus annuus* L. cv. Romsun 90) were thoroughly washed with water and surface sterilized with 1 % sodium hypochlorite solution, germinated in the dark at 25 °C in sterilized quartz sand, and watered daily with distilled water. After 3 to 4 d, the seedlings were transferred to plastic pots with sand saturated with nutrient solution containing different B concentrations (0.02, 0.2, 2.0, 20.0, and 50.0 μM H_3BO_3). The nutrient solution consisted of: 0.75 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.1 mM $CuSO_4$, 0.01 M $(NH_4)_6Mo_7O_{24}$. Nutrient solution was renewed every 3 d. Plants were harvested after 4 to 10 d growth in nutrient solution.

Roots, aboveground parts, and leaves with various treatments were separated and frozen dried for the different measurements. An automatic Area Meter (*LI-3000, Licor*, USA) was used for measuring the leaf area. Different plant organs were dried at 70 °C for the determination of dry mass. The content of B in samples ashed at 450 °C) was determined spectrophotometerically.

Measurement of membrane leakage: Leaves with a fresh mass of 0.7 g were excised from petiole, gently rinsed in distilled water, and transferred to plastic vessels (100 cm^3) containing aerated distilled water. After incubation for 2-4 h in leakage solution, leaves were removed and a sample of 2 cm^3 of the leakage solution was taken to determine the concentrations of potassium, sucrose, and phenolics. Potassium concentration in the leakage solution was measured by flame photometry. Using the P-hydroxybenzoic acid hydrazide reaction, reducing sugars were measured (Blakeney and Mutton 1980). The difference between reducing sugar before and after invertase treatment gave sucrose concentration. Phenolic compounds were determined by colorimetry using the Folin reagent (Swain and Hillis 1959).

Peroxidase activity: Different plant parts (roots or leaves) were frozen and ground in a mortar after mixing with extraction buffer (50 mM K phosphate, pH 5.8) to extract soluble peroxidase or the same plus 800 mM KCl to extract total peroxidase (both soluble and ionically bound). The homogenates were filtered through 2 layers of *Miracloth* and the filtrates were centrifuged at 10 000×*g* for 35 min at 20 °C. The supernatant was used as the enzyme source. The assay medium contained 50 mM K-phosphate buffer (pH 5.8), 7.2 mM guaiacol, 11.8 mM H_2O_2, and 0.1 cm^3 enzyme extracted in a final assay volume of 3.0 cm^3. The reaction was initiated by the addition of H_2O_2 and the change in absorbance at 470 nm was measured. One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM tetraguaiacol per min (Mac-Adam *et al.* 1992).

Lipoxygenase activity: Frozen leaves were mixed with 50 mM phosphate buffer (pH 7.6) and homogenized in a mortar. The homogenates were filtered and the filtrates were centrifuged as described above. The assay medium contained 1.4 cm^3 of tetraborate buffer (pH 10), 10 cm^3 of linoleic acid, and 0.1 cm^3 enzyme extract. The change in absorbance was monitored at 234 nm. One unit of lipoxygenase activity causes an increase in 0.001 per min at 234 nm (Sekhar and Reddy 1982).

Malondialdehyde content: The level of lipid peroxidation was measured in terms of MDA content using the thiobarbituric acid reaction. Leaf samples (0.5 g) were homogenized in 0.1 % (m/v) trichloroacetic acid and centrifuged for 5 min at 10 000 ×*g*. 4 cm^3 of 20 % (m/v) trichloroacetic acid and 0.5 % (m/v) thiobarbituric acid were added to 1 cm^3 of the supernatant. The mixture was heated at 95 °C for 30 min and rapidly cooled in an ice-bath. Following a 15 min centrifugation at 2 300×*g*, the absorbance of the supernatant was read at 532 nm and the value for the non-specific absorption at 600 nm was subtracted. The concentration of thiobarbituric acid was calculated using a molar extinction coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as nmol MDA (Du and Bramlage 1992).

Fatty acid content: Lipids were extracted from chloroplast leaves and fatty acids were identified and analyzed using gas chromatography (El-Shintinawy and Selim 1995).

Chloroplasts were isolated (Osman and El-Shentinawy 1988) from leaves excised from plants grown with different B levels. Hill reaction activity was measured spectrophotometerically (Biswal and Mohanty 1976). Rates of oxygen evolution were measured with a Clark type oxygen electrode (*Yellow Springs Instruments*, USA) and a slide projector providing an irradiance of 300 W m^{-2} at the cuvette surface and temperature of 25 °C. The basic reaction mixture (pH 7.8) used for all electron transport assays contained 40 mM sorbitol, 50 mM K_2PO_4, and 4 mM $MgCl_2 \times 6\ H_2O$. Steady state of electron transport measurements from water to 2,5-dimethyl-*p*-benzoquinone (BQ; 1 mM) in presence of 0.5 mM $K_3Fe(CN)_6$ showed PS2 activity, and from water to methyl viologen (MV; 0.5 mM) showed whole chain electron transport activity. Fluorescence emission spectra at room temperature were measured using a *Perkin-Elmer LS50B* fluorimeter. The emitted radiation was measured using a slit width of 2 mm. Samples were dark adapted for 15 min.

Results and discussion

After 6-10 d growth in nutrient solution containing low B concentrations both shoot and root growth were inhibited (Fig. 1). At day 10 of growth, both shoot and root dry masses of plants grown at 0.02 μM B were reduced by 67.0 and 62.5 % in

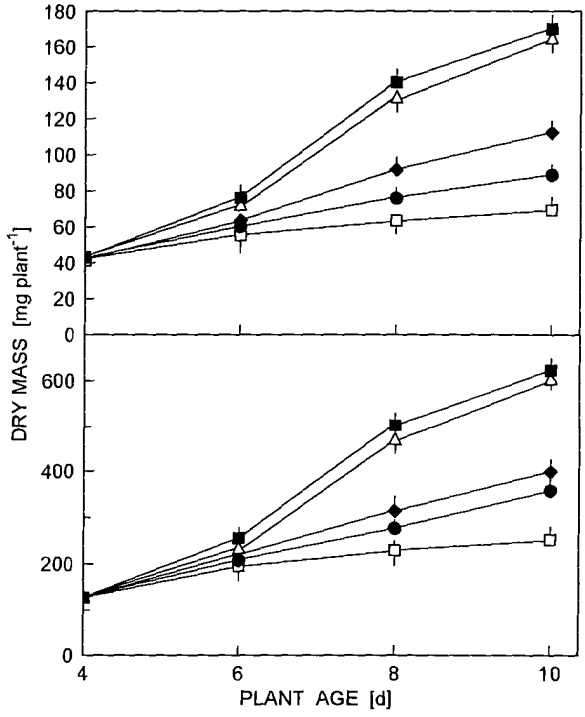

Fig. 1. Effect of different B concentrations in nutrient solution (μM: □ 0.02; ● 0.2; ◆2.0; Δ 20.0; ■ 50.0) on dry mass of sunflower plants of different age. Vertical bars indicate ±SD of 3 replications.

comparison to those grown at 20.0 or 50.0 μM B, respectively. Based on these values, plants grown at 0.02 and 20.0 μM B are described as BD and BS plants, respectively. With increasing content of B in nutrient solution, concentration of B in dry mass of roots and shoots increased (Table 1), the increase being much larger in shoots than roots. The BD symptoms increased also with the plant age. Dark green leaf pigmentation changed to brown purple at 0.02 μM B, particularly in the leaf base; this was accompanied by a change in root color into grayish. Plant species differ greatly for B requirement and sunflower has the highest requirement among the field crops (Blamey 1976). Thus dry mass reduction under BD shown in this work reflected primarily the injury of the overall processes of growth which is in agreement with that observed in rice plants (Yu and Bell 1998).

Table 1. Effect of different boron (B) concentrations in nutrient solution on B content, and soluble (SPOX) and bound (BPOX) peroxidase activities in sunflower roots and shoots. Values are means ± SD of 5 (dry mass) or 3 (enzymes) replications.

B supply [μM]	B content [mg(B) kg^{-1}(d.m.)]		SPOX [unit kg^{-1}(d.m.)]		BPOX [unit kg^{-1}(d.m.)]	
	roots	shoots	roots	shoots	roots	shoots
0.02	11.4±0.5	8.2±0.1	29 400±300	11 900±400	28 400±300	10 500±200
0.20	16.2±0.4	34.6±0.5	28 200±400	4 200±800	18 400±1400	8 700±600
20.00	27.3±0.9	135.3±0.8	31 300±1200	6 200±900	7 200±700	5 200±600

Plants possess scavenging enzyme systems that overcome the injury induced in plant tissues by various environmental stresses (Hodges *et al.* 1997, Yu *et al.* 1998). In order to investigate whether an oxidative damage is involved in the injury caused by B deficiency, peroxidase activities were measured in roots and leaves of sunflower plants (Table 1). B deficiency did not affect the soluble peroxidase activity in roots, but the activity of ionically bound peroxidase activity was much lower in BS than BD roots. Both soluble and bound peroxidase activities in BD leaves were twice those detected in BS leaves. The production of active oxygen species may be a general alarm signal that notifies possible modification of metabolism (Rao and Ormrod 1995). The altered activity of the antioxidative peroxidase enzyme that functions in the removal of H_2O_2 from biological systems (Gosset *et al.* 1994) reflects the response of sunflower plants to increased free radicals production under BD. The action of ionically bound peroxidase is located in the cell wall, induces its rigidity, and prevents later expansion involved in the growth process (Fry 1986, Mac-Adam *et al.* 1992). Thus, BD-induced inhibition in sunflower seedlings growth may also result from cell wall tightening due to the formation of cross linkages among cell wall polymers. A similar reduction in peroxidase activities induced under cadmium stress was observed in rice seedlings (Chen and Kao 1995). This shows that an oxidative damage was created in BD sunflower plant tissues. Free radicals of oxygen are highly toxic to various cellular compounds, particularly membrane lipids and proteins, which results in a disorganization of membrane structure and facilitated membrane leakage (Thompson *et al.* 1987, Van Ginkel and Sevanian 1994).

Increasing B supply from 0.02 to 20.0 μM enhanced B contents in sunflower leaves which was accompanied by a significant reduction in leakage of potassium, sucrose, and phenolics (Table 2). In BD leaves, leakage was higher by 20 fold for potassium, 38 fold for sucrose, and 6 fold for phenolics compared with BS leaves. This shows a high net efflux of the examined solutes across the plasma membrane of BD leaves. These effects indicated a special role of B in the maintenance of plasma membrane integrity of sunflower leaves. A similar effect of B deficiency on solute efflux was observed in sunflower hypocotyl segments (Tang and De La Fuente 1986) and leaves (Cakmak *et al.* 1995). The oxidation of the accumulated phenolic compounds induced by B deficiency (Loomis and Durst 1991) resulted in a generation of free radicals of oxygen and reflected the modification and injury of the membrane structure (Appel 1993). Boron is a structural factor required for plasma membrane integrity due to its ability to form complexes with the membrane constituents such as glycoprotein and glycolipids (Parr and Loughman 1983, Shelp 1993).

Table 2. Effect of different B concentrations in the nutrient solution on B content in dry mass and leakage [mg kg^{-1}(f.m.) h^{-1}] of potassium, sucrose [glucose equivalent], and phenolic compounds [caffeic acid equivalent] and on contents of linoleic (18:2) and linenic (18:3) acids [% of total], lipoxygenase activity and malonylaldehyde (MDA) content [related to lowest B concentration] in sunflower leaves. Values represent means ± SD of 3 replications (leakage) or 3 independent experiments.

B supply [μM]	B content [mg kg^{-1}]	Leakage K	sucrose	phenolics	(18.2)	(18:3)	Lipoxygen.	MDA
0.02	9.1±0.5	202.0±3.4	494.0±3.2	37±8	12.5±1.5	48.7±0.8	-	-
0.20	42.2±0.9	160.0±2.5	245.0±1.7	34±12	16.7±2.3	58.5±1.7	22.5±0.6	15.5±1.1
20.00	90.6±2.7	10.0±0.8	13.0±0.5	6±2	20.5±0.9	70.2±1.1	29.2±0.9	19.8±2.3

To ascertain the observed membrane damage under B deficiency, various peroxidative products were detected in thylakoid membranes isolated from sunflower leaves. BD chloroplasts had declined linoleic (18:2, by 40 %) and linolenic (18:3, by 30 %) acid contents with regard to total unsaturated fatty acids, and also lipoxygenase activity and MDA content in thylakoid membranes were declined (Table 2). Hence the most abundant unsaturated fatty acids constituting thylakoid membranes were affected by B deficiency. The altered composition and degradation of fatty acids in stressed photosynthetic organism or in organized membrane system were found by Goes *et al.* (1994) and DeLong and Steffen (1998). This could be achieved *via* the cleavage of covalent bonds through the activation of toxic oxygen molecules that can attack fatty acid chains (Chamberlain and Moss 1987). The enhancement of lipoxygenase activity and the high accumulation of MDA observed in BD chloroplasts are important signals for membrane modifications due to high production of free radicals in stressed plant tissues (Borrell *et al.* 1997, DeLong and Steffen 1998).

Table 3. Effect of different B concentrations on photosynthetic activities measured as leaf area, chlorophyll (Chl) *a* and *b* contents, Hill reaction activity, rates of electron transport of the whole chain ($H_2O \rightarrow MV$) and photosystem 2 (PS2) ($H_2O \rightarrow BQ$), and maximum fluorescence spectra at 685 nm (F_{685}). Values represent means ± SD of 3 replications.

Parameter	B supply [μM]		
	0.02	0.20	20.00
Leaf area [cm^2]	27.3±0.5	32.4±0.9	46.4±0.8
Chl *a* [g kg^{-1}(d.m.)]	8.6±0.9	9.1±1.1	10.3±1.4
Chl *b* [g kg^{-1}(d.m.)]	3.1±0.8	3.5±0.5	4.0±0.3
Hill activity [μmol(DCPIP red.) kg^{-1}(Chl) s^{-1}]	43.3±1.7	53.8±2.9	75.5±1.4
Whole chain, $H_2O \rightarrow MV$ [μmol(O_2) m^{-2} s^{-1}]	66.7±1.5	74.2±2.4	96.8±1.1
PS2, $H_2O \rightarrow BQ$ [μmol(O_2) m^{-2} s^{-1}]	41.3±1.4	55.2±1.4	71.3±2.4
F_{685} [relative]	72.5±2.5	95.0±2.1	100.0±0.5

To determine whether peroxidative damage of the thylakoid membranes facilitated by B deficiency influenced its function, various photosynthetic activity parameters were measured (Table 3). B deficiency induced a significant reduction in leaf area (41 %) as well as chlorophyll (Chl) contents (Chl *a* of 16.5 % and Chl *b* of 22.5 %) with regard to BS leaves. The decrease in Chl content suggests that the Chl synthesizing system and/or chlorophyllase activity were affected by B deficiency. The change in Chl content, common in plants under stress, alters the composition and disorganizes the structure of the light-harvesting protein complexes of thylakoid membranes (Kastori *et al.* 1995) which leads to an altered chloroplast architecture (Khavari-Nejad and Mostofi 1998). Hill reaction activity expressed as DCPIP photoreduction declined by 43 % in BD chloroplasts compared to that detected in BS chloroplasts (Table 3). A maximum rate [96.8 μmol(O_2) m^{-2} s^{-1}] of the whole chain ($H_2O \rightarrow MV$) electron transfer was detected in BS chloroplasts. B deficiency induced a pronounced inhibition (31 %) of the whole chain electron transport rate compared to BS chloroplasts. Moreover, the activity of PS2 ($H_2O \rightarrow BQ$) declined in BD chloroplasts by 42 % with regard to that in BS chloroplasts. The fluorescence emission at 685 nm (F_{685}) of PS2 Chl molecules was by 27.5 % lower in BD than BS chloroplasts. The impairment of electron transfer of PS2 and F_{685} indicated the diminished photochemical efficiency of the PS2 protein complex (Cao and Govindjee 1990). Similar damage at or near the reaction centre of PS2 was observed in chloroplasts isolated from plant leaves under stress (Velitchkova and Fedina 1998). Hence the B deficiency may affect directly or indirectly the primary photosynthetic process and/or the overall process of photosynthesis.

Our results support the possible role of B in thylakoid membrane maintenance, protection, and function in sunflower leaves. This role was confirmed by the increased activities of peroxidases and lipoxygenase associated with the decrease in the percentage of both linoleic and linolenic acid contents in BD chloroplasts. The altered enzyme activities were accompanied with increasing MDA accumulation and with facilitating leakage of phenolic compounds, sucrose, and potassium. These modifications in the thylakoid membranes were coupled with an impairment of

photosynthetic efficiency judged by the decline in the electron transport rate of PS2 and the quenching in the F_{685}. Thus B deficiency is one of the environmental stresses that facilitate the production of free oxygen radicals and result in structural and functional damage of thylakoid membranes of sunflower leaves.

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