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Influence of Boron on the Membrane Potential in *Elodea densa* and *Helianthus annuus* Roots and H^+ Extrusion of Suspension Cultured *Daucus carota* Cells[1]

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ABSTRACT

When following the membrane potential of *Elodea densa* leaf cells during a dark-light regime and analysing the different phases of the cycle, the pattern under boron deficiency resembled the one reported to occur after 3-(3,4-dichlorophenyl)-1,1-dimethylurea application. The potential in the dark slowly decreased when transferring *Elodea densa* leaflets and *Helianthus annuus* roots to a B-free medium and increased in the same way after B was added again. Addition of vanadate to inhibit plasmalemma ATPases in part mimicked the effects of B deficiency. It is suggested that B directly or indirectly affects the formation of a proton gradient. The effect of B on proton secretion was observed in various experiments with *Daucus carota* cell cultures. The results are discussed with respect to the possible involvement of B in membrane function and transport processes.

One of the very first observations of a reduced ion uptake under boron deficiency was made by Rehm (15) with *Impatiens balsamina.* Since that time, in several articles different authors have suggested a more or less direct involvement of B in plasmalemma-bound transport processes for higher plants and diatomaceous species (1, 12, 14, 17, 18, 20). It is still a matter of controversy whether these differences in net uptake are the consequence of a reduced active transport system such as H^+-pumping ATPases (14) or of a more or less general effect on membrane structure (13). The latter assumption is supported by the fact that in several cases of B deficiency lower flux rates for the ions K^+, Rb^+, and $H_2PO_4^-$ were found (4, 17, 18, 20), although a higher net efflux of K^+ (23) or no influence on ion fluxes (13) have been observed as well.

As early as 1945, Lundegårdh (10) stated that B "increases the acidity of the surface of the cytoplasma." Tanada (21) found an effect of B on the electric potential of red-light irradiated *Vigna* hypocotyls and observed B-induced differences with respect to the adhesion of *Vigna* root tips on negatively charged surfaces (22). In view of these observations, we started experiments to study the effects of B on the membrane potential E_m, proton secretion, and ferricyanide reduction. This might help in separating primary and secondary events of B deficiency and lead to a better understanding of boron's role in plant metabolism.

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MATERIALS AND METHODS

Plant Cultivation and Nutrient Solutions

Elodea densa was grown in a 0.1 strength nutrient solution according to Hoagland and Arnon (7). The B supply as $B(OH)_3$ was modified as stated in the tables and figures. *Helianthus annus* was raised hydroponically in a 0.2 N Hoagland solution (see above) for about 6 to 8 weeks after germination. Suspension-cultured cells of *Daucus carota* were raised as described earlier (4). The B-deficiency treatments were started either by changing the solution during the potential measurements or by preincubating plant parts (*Elodea*), whole plants (*Helianthus*), or thoroughly washed cells (*Daucus*) in an essentially B-free solution. The nutrient solution employed for the experiments was either a 0.2 strength Hoagland solution (*Elodea, Helianthus*) or a modified Murashige and Skoog medium (without Fe for ferricyanide reduction to avoid the formation of blue-colored cyanide-complexes). For continuously monitoring H^+-release of *Daucus* cells, a simplified solution, containing only 3% (w/v) saccharose, 0.1 mM $CaSO_4$, and 10 mM K_2SO_4 was used. To minimize B contamination, highest purity grade chemicals and ultrapure water (Seradest Up) were used, and the storage of solution in glassware and low quality plastics was avoided throughout. If required, solutions were treated additionally with the borate-specific Amberlite IRA 743 ion exchanger (analytical grade, prewashed in ultrapure water).

K was determined by flame photometry (Elex 6361, Eppendorf) after wet ashing in $HNO_3/HClO_4/H_2SO_4$ (20:2.5:1, v/v).

Membrane potential was measured by standard electrophysiological methods with 3 M KCl-filled glass microelectrodes (tip diameter approximately 0.8–1 μm), with the reference electrode submersed in the surrounding medium. To minimize noise signal, part of the experiments were performed without continuous circulation of the nutrient solution. Later experiments with a slightly modified setup did not reveal any differences when compared to experiments with flowing solutions. The potential was shunted to a high impedance voltmeter (Keithley 610, $>10^{14}\Omega$) connected to a single pen chart recorder. Light was supplied by a microscope lamp with an intensity of 44 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400–700 nm) during the

'light' cycles; during the 'dark' phase, light intensity was <0.01 $\mu E \cdot m^{-2} \cdot s^{-1}$. Temperature fluctuations between dark and light phases were ≤1°C under our experimental conditions. Vanadate was supplied as Na_3VO_3 in the concentrations indicated in the tables.

Ferricyanide Reduction

The initial ferricyanide concentration was adjusted to 1 mM $K_3Fe(CN)_6$. An aliquot of about 1.5 to 2.0 g of cells (fresh weight) was suspended in a 100 mL solution to start the experiments. Aliquots were taken from the gently stirred solutions at 30 min intervals and the cells were removed by filtration through purified cotton wool or several layers of filter paper (Schleicher & Schüll, White Ribbon). Ferricyanide reduction was monitored photometrically at 420 nm and the pH was measured by an Orion-Ross semimicro electrode.

Proton Release

In a further set of experiments, proton release was followed by automatically registering the pH every 5 min in a Solartron Datalogger. The temperature in these experiments was controlled to about 22 to 24°C, and the cell suspensions were stirred by bubbling CO_2-free air through the solutions. Cell cultures which were used in these experiments have been kept for 3 to 4 weeks in solutions containing 10 μM $B(OH)_3$. The experiments started about 30 to 40 min after separating the cells from their preculture medium (this time was needed for rinsing the cells and adjusting the whole device). The experiments were carried out in quadruplicate.

Boron Determination

Where necessary, boron was determined by ICP-plasma emission spectrometry either directly in the solutions (sensitivity better than 0.1 μg/mL) or after concentration (plus addition of mannitol to avoid losses caused by evaporation of boric acid) and wet ashing in covered PTFE crucibles.

Statistics

The influence of B on the membrane potential in different phases of the dark/light cycles was analyzed statistically for the parameters indicated in Figure 1 by variance analysis and Duncan's multiple range test. For all other experiments, treatments were compared by the *t*-test.

RESULTS

The resting potential in the dark was lowered in the cells of *Helianthus* roots (Table I) and *Elodea* leaflets (Table IIA, column a) when transferred from 10 μM $B(OH)_3$ to essentially B-free media for 20 h. As far as could be recognized, the potential decreased steadily and recovered slowly to approximately the same level after adding B again (Table I, data of *Elodea* not shown). We were unable to detect major breaks or rapid changes of the membrane potential as a consequence of the changes in B supply. The observed potentials of *Helianthus* roots were relatively low, which might be attributed to the comparatively low concentration of the nutrient solution (less than one-fifth-concentrated Hoagland solution) at the time of sampling for the experiment.

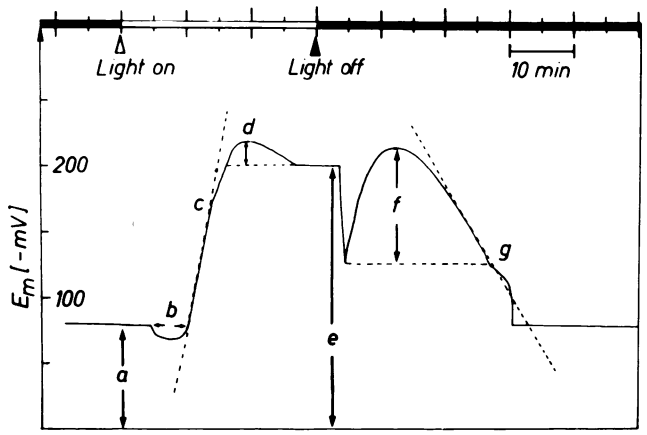

Figure 1. Scheme showing the different phases of the pattern of membrane potential in *E. densa* under a dark/light regime as used in the statistical analysis for Table II. a, 'Resting' potential (dark phase) (mV); b, lag phase for raising the potential at the beginning of the light phase (min); c, rate of potential increase in the light (−mV/min); d, transient hyperpolarization before reaching the 'equilibrium potential' in the light (−mV); e, equilibrium potential in the light (−mV); f, transient hyperpolarization at the beginning of the dark phase (−mV); g, rate of potential decrease in the dark (−mV/min).

Table I. *Membrane Potential Difference (−mV) in H. annuus Root Cells from the Elongation Zone (≈10–20 mm behind the root tip) as Influenced by Boron (+B = $3 \cdot 10^{-5}$ M H_3BO_3, −B = without added B)*

Pretreatment 3 d	E_m	Treatment 20 h	E_m	ΔE_m
	−mV		−mV	
+B	74.5	+B	73.2	15.7
		−B	57.5*	
−B	56.9***	+B	65.5	13.9
		−B	51.6*	

*, ***, Statistically significant between the B treatments at the 5% and 0.1% level, respectively, according to the *t*-test.

The typical pattern of the membrane potential under a dark-light regime and the respective changes after transfer to B deficient conditions are depicted in Figure 2A. In as few as 20 min after removing B, the following changes were observed (see also Fig. 1): (a) an increased lag phase for the rise of the potential after turning the light on; (b) a reduced rate of potential increase after illuminating, especially during the second part of this phase; (c) a loss of the transient hyperpolarization before reaching the equilibrium potential in the light; (d) a lower depolarization rate toward the resting potential in the dark. These differences persisted as long as B was withheld from the nutrient solution. When adding B again, it took about 18 to 24 h until the shape of the potential curve equaled the original one (Fig. 2B). Especially, the second phase of the light-induced increase in E_m (Fig. 1, b) recovered only slowly.

After the addition of Na_3VO_3 to inhibit plasmalemma-

Table II. *A. Membrane Potential Difference E_m (−mV) and Its Changes (δ mV) in E. densa during Different Phases of a Dark/Light Regime, as Influenced by B and Vanadate Concentration*

Vanadate	(a) Dark E_m (−mV)		(b) Lag phase (min)		(c) Light δE_m		(d) light Hyperpolarization		(e) light E_m		(f) dark Hyperpolarization		(g) dark E_m	
	+B	−B	+B	−B	+B	−B	+B	−B	+B	−B	+B	−B	+B	−B
μM					$mV \cdot min^{-1}$		−mV		−mV		−mV		−mV/min	
0	109.2ef	87.2**h	6.8b	8.6*a	38.2a	11.0***d	25.5a	2.7***c	216.8ef	201.5fg	62.1c	58.8cd	10.7a	7.8***bc
100	125.2cd	90.8***gh	4.0d	8.8**a	29.1b	15.4***c	26.1a	0.0***c	231.2cde	194.8***g	83.0a	73.3*b	6.8cd	8.5**b
400	137.2bc	104.3***def	6.3bc	3.8**d	16.0c	7.8***def	0.0c	0.0c	237.0bcd	225.7de	34.4f	48.3**e	5.0efg	4.5efgh
800	156.2a	125.0***cde	8.5a	5.1**cd	5.2f	5.4f	0.0c	0.0c	248.0ab	229.3cde	31.8f	51.0***de	4.2fgh	3.3hi

B. Influence of Elevated B Concentrations on the Membrane Potential Difference and Its Changes in E. densa

Boron Concentration	(a) Dark E_m	(b) Lag Phase	(c) Light ΔE_m	(d) Light Hyperpolarization	(e) Light E_m	(f) Dark Hyperpolarization	(g) Dark ΔE_m
	−mV	min	$mV \cdot min^{-1}$		−mV		$-mV \cdot min^{-1}$
10 μM B	109.2	6.8	38.2	25.5	216.8	62.1	10.3
1000 μM B	102.8	5.5	30.6	29.2	220.2	51.5	11.7

*, **, *** Shows statistical differences between the B treatments of the same $NaVO_3$ concentrations at the 5, 1, and 0.1% level, respectively, according to the *F* test; values followed by different letters are significantly different at the 5% level according to Duncan's multiple range test (data for the treatments with 200 μM and 1600 μM vanadate are not given in the table, but have been considered for statistical analysis); for identification of the different phases see Figure 1; B supplied as $B(OH)_3$ in the indicated concentrations.

bound ATPases, the light-induced increase of E_m was delayed in the +B treatment, but was accelerated under −B conditions (Table IIA, column b) when compared to the respective behavior of the control (+B/without vanadate). The rate of increase of E_m was significantly lower without B up to 400 μM Na_3VO_3 (Table IIA, column c). The transient hyperpolarization preceding the stationary phase of E_m in the light was almost absent in all −B treatments, and could be completely suppressed by the addition of vanadate at a concentration of ≥400 μM (Table IIA, column d).

No consistent changes between the B treatments have been observed for 'light potential' (Fig. 1, e) and transitory hyperpolarization after turning the light off (Fig. 1, f). Comparatively low levels of vanadate (0.1–0.2 mM) sufficed to equalize the depolarization rates at the onset of the dark phase (Fig. 1, g) for plants grown with or without B (Table IIA, column d).

In a series of similar experiments we also tested the influence of an elevated B concentration. There were no differences at all between a normal (10μM) and the 100-fold (1mM) B concentration (Table IIB; Fig. 2B).

When analyzing the K^+ content of *Elodea densa* incubated for 20 h in a one-fifth concentrated Hoagland solution, the K^+ concentration decreased only slightly, statistically not significant, in the B deficient plants (39.0 mM *versus* 38.2 mM). In *Daucus* cells, too, the K^+ content was only slightly affected by B deficiency (data not shown).

Pretreatment in B-free solutions for 16 to 17 h decreased the proton release of suspension-cultured *Daucus* cells by over 50%, whereas it did not affect the rate of ferricyanide reduction (Fig. 3). The first differences between the B treatments were to be seen after about 6 h under our experimental conditions (these data are not presented here). When using cells precultivated in a solution with lower B concentrations (5 μM, with a maximum of 9–10 g cells per 70 mL nutrient solution, changed weekly), cells responded more rapidly (Fig. 4), although they did not show any signs of a beginning B deficiency. Here, significant differences between the B treatments became apparent in as few as 50 minutes after the start of the experiment (*i.e.* about 1.5 h after transfer to B-deficient or -sufficient conditions). The external B concentration in the +B treatments did not vary noticeably during the time course of the experiment; the data are thus omitted.

DISCUSSION

B deficiency lowers the resting potential during the dark phase, in both *Helianthus* roots and *Elodea* leaf cells. It is not clear whether this is directly related to an influence of B on proton-pumping plasmalemma ATPase activities, as relatively high levels of vanadate were needed to supress this effect. As our nutrient solutions contained phosphate, the uptake of vanadate might have been somewhat restricted. In a crude membrane vesicle preparation, Pollard *et al.* (14) observed a stimulation of ATPase activity by B. It should be taken into consideration, however, that the effect of B might also be an indirect one (see below).

From experiments with *Elodea* (9) or *Riccia fluitans* (3), it was concluded that the dark potential is probably due mainly to an unequal distribution of K^+ between cell (vacuole) and external medium. Although a flux equilibrium can be as-

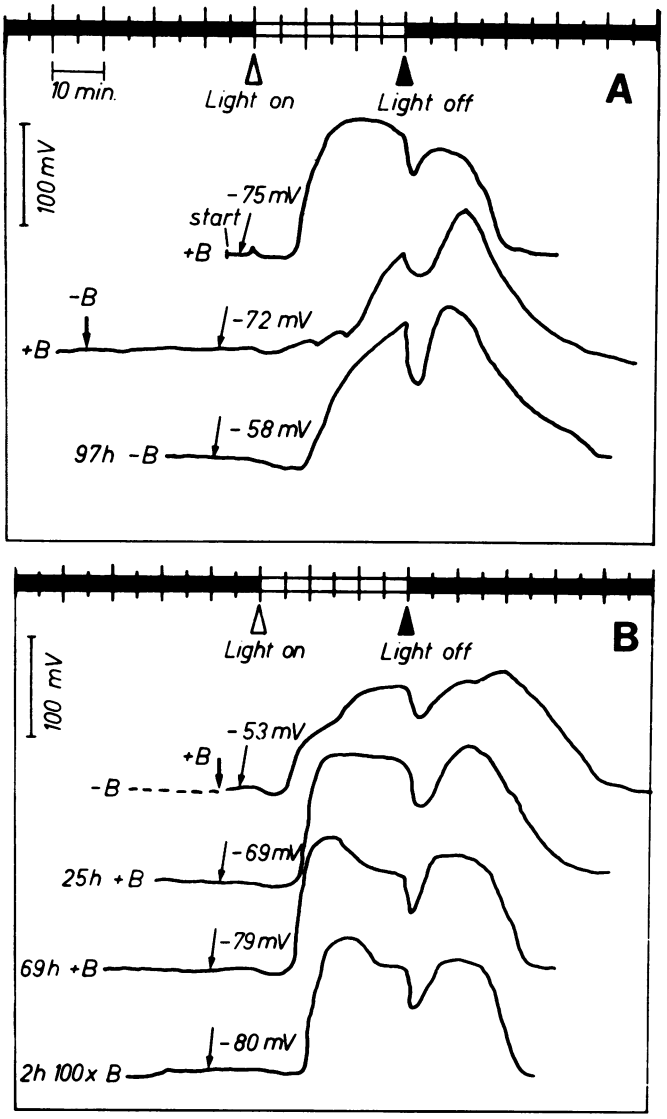

Figure 2. Pattern of membrane potential in *E. densa* under dark/light cycles as influenced by the supply of boron (−B = supplying a B-free solution, +B = 10 μM B; 100 × +B = 1 mM B); the beginning of the respective treatments is indicated by the boldface arrows in each panel; the 'dark potential' of the different curves is written next to the lightface arrows. A, E_m at the change from +B (top curve) to −B (center curve) and after 97 h of −B treatment (bottom curve). B, E_m when changing from −B to +B (after 143 h −B treatment, top curve) and recovery of the E_m pattern (center two curves, after 25 and 69 h +B, respectively). Bottom curve, E_m at 1 mM B as $B(OH)_3$.

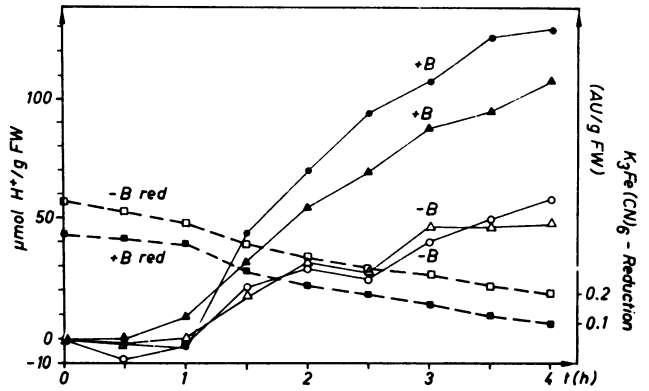

Figure 3. Ferricyanide reduction (red, □, ■) and proton secretion (○, ●, △, ▲), by *D. carota* cell suspensions as influenced by B nutrition; the figure shows the average net proton release in two independent experiments (△, ▲, ○, ●). Open symbols (△, ○, □) = −B treatment, without added B; solid symbols (▲, ●, ■) = +B treatment, 100 μM $B(OH)_3$.

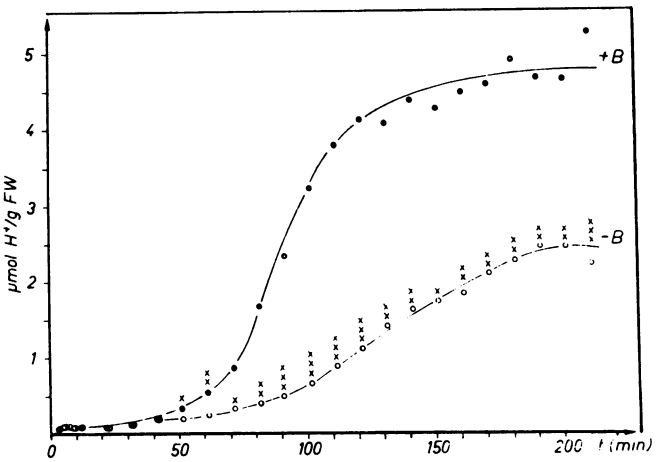

Figure 4. Proton release by suspension-cultured carrot cells per g of fresh weight as influenced by the B supply (as in Fig. 3); *, **, *** show the differences to be significant according to the *t*-test at the 5, 1, and 0.1% level, respectively.

sumed only in *Elodea* and not in roots, their vacuolar K^+ concentration may be calculated according to the Nernst equation, provided K^+ is the main ionic species responsible for the equilibrium potential. The K^+ concentration would thus be 37.8 mM with B and 15.8 mM without B. In contrast, in most of our experiments the actual K^+ concentration in the plants was only marginally affected, at least during the earlier phases of B deficiency. Thus, the observed potential differences must have been caused by other ions. As our medium contained only traces of Na^+ and Cl^- in the *Elodea* medium, the most likely candidate for this effect is H^+, besides the possible effect of B on anion uptake (4, 18). This assumption is supported by the fact that B-deficient cells show a significantly lower net proton release (Figs. 3 and 4). The difference is about in the same order of magnitude as the one observed in E_m. Lack of response of the ferricyanide reduction to B deficiency for up to 1 d stands against a participation of plasmalemma-bound redoxpumps in this B-influenced proton excretion (Fig. 3)., although the B effect was more pronounced in the presence of ferricyanide (Fig. 3) or when Fe was omitted from the nutrient solution (Fig. 4). Results of preliminary experiments with the pH stat method revealed that B deficiency increased the proton uptake independently from the accompanying anion within 2 h after starting the deficiency treatment (our unpublished results). It is also noteworthy, that the equilibrium-pH (*i.e.* where proton release equals proton uptake) in our experiments was higher for B-deficient cells (see *e.g.* Fig. 4). This was independent from the

experimental conditions, *i.e.* even when there was no stimulation of net proton release (our unpublished results).

$B(OH)_3$ may in part exert effects comparable to other easily permeating weak (organic) acids (11, 16), presumably by lowering the cytoplasmatic pH. As boric acid has a high ether partition coefficient, it passes lipophilic layers quite easily. At the higher internal pH it may react either with OH^- or hydroxylic groups, forming a borate anion or a negatively charged borate ester, thus lowering the cytosolic pH, which in turn may affect proton uptake and release, and transport processes linked to H^+-movement. Other than with the supply of weak organic acids, the effects of B deprivation and resupply are relatively slow, which may be due to the binding characteristics of boric acid/borate to the various ligands in cell wall, plasmalemma, and probably other cellular compartments. It can be excluded that the observed difference in proton release is based on a *direct* stoichiometric reaction of boric acid in the cells with a concomitant release of protons liberated by borate formation, as the difference between the treatments with respect to the protons released was 5 to $6 \cdot 10^{-5}$ mol $H^+ g^{-1}$ (fresh weight), whereas the total supply of B amounted to about $5 \cdot 10^{-6}$ mol $B(OH)_3 \cdot g^{-1}$ (fresh weight), and the B concentration of the nutrient solution did not vary significantly during the time course of the experiment (data not shown). It is noteworthy that in our experiments the effect of B on the proton extrusion was especially pronounced in Fe-free solutions, *i.e.* conditions where H^+ extrusion is known to be highly stimulated, at least in Fe-efficient species (19).

The mechanism(s) by which B may influence membrane potential and proton movement through membranes are still subject to speculations. It was hypothesized that $B(OH)_3$ increases the electron transport rate in phytochrome-mediated reactions (22). In agreement with these assumptions, there is a striking similarity in the pattern of the membrane potential under dark/light regimes between B-deficient *Elodea* and the ones that have been treated with DCMU, an inhibitor of electron transport (2).

In actually running pH stat experiments with auxin-heterotrophic cell cultures, we found that the effect of B on net proton release as well as proton uptake was strictly dependent on the presence of auxins (our unpublished results). Besides boron's influence on peroxidase/IAA-oxidase activities (5, 6), a similar interaction between auxins and B has been found for the rooting of mung bean cuttings (8). B may either affect the cellular distribution of auxins (6) or even somehow regulate their action. The effects of B deficiency on E_m and proton release are in agreement with earlier observations of a reduced phosphate efflux (4). It is very likely, that these differences will influence the distribution of substances like acidic plant growth regulators (e.g. auxins) as well.

Experiments are under way to further examine boron's influence on mechanisms related to proton movement in membranes such as membrane resistance, cytosolic pH, anion uptake, and auxin metabolism.

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