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INFLUENCE OF BORON DEFICIENCY ON PROTON RELEASE AND UPTAKE IN SUSPENSION CULTURED CELLS

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Boron (B) is known as an essential nutrient for plants since 1910 (1), but its role in plant metabolism is still far from being understood. A reduction in uptake and transport of other ions has been cited by different authors (4,6,9). According to Loughman's group (6,7) there is a reduced (Plasmalemma-?)ATPase activity after withholding B to plant tissues. The true mechanism of this interaction, however, remains obscure, as boric acid (or borate) does not form complexes with ATP (own unpublished observations), nor is there any indication, that it is bound directly to ATPases. In former experiments, though, we were able to observe effects of B-deficiency on the membrane potential of *Elodea* densa and root cells of *Helianthus annuus* (2).

In order to study further the effects of B on proton release in plants as a central mechanism for ion uptake and transport, we initiated a set of experiments with cell cultures of carrot and tomato, where an enhanced proton release was induced by the application of ferricyanide or by withholding Fe.

Material and Methods:

Plant cell cultures were grown as described earlier (4) in a modified Murashige and Skoog medium at 28°C on a gyratory shaker ($105 \cdot min^{-1}$). To obtain a faster response to B-deficiency, cell suspensions were precultured for at least four passages in a medium with the addition of only 10^{-5} M $B(OH)_3$. Cell growth was not affected for one week in this culture medium. For transfer to B-deficient conditions, the cells were separated from the spent medium by filtration over filter paper (S&S "white ribbon"), rinsed four times with an essentially B-free nutrient solution and transferred to the respective media (individual treatments as indicated in the figures and tables). The duration of the pretreatment was varied between 2 and 16 h. No significant effect on the growth of the cell cultures (as measured by fresh and dry weight increase) was observed within this period. 1 mM ferricyanide ($=K_3Fe(CN)_6$) was applied to some of the experiments as indicated. Proton release was measured by pH-decrease with a semi-micro electrode (Orion-Ross 8056). The medium

[illegible] the experiment contained 3% saccharose, 100 mg/l m-inositol $2 \cdot 10^{-4}$M $CaSO_4$, 0.1 times the concentration of trace elements and the complete vitamines and growth hormones of the full strength medium, with exceptions of auxins in the respective experiments. Auxins were added as 10^{-7}M 2,4-dichlorophenoxyacetic acid (2,4-D) alone (for tomato cells) or $2 \cdot 10^{-7}$M 2,4-D together with 10^{-9}M indole-3-acetic acid (carrot cell cultures). Vanadate was added as $4 \cdot 10^{-7}$M $NaVO_3$ to inhibit the activity of plasmalemma ATPases.
Proton uptake was followed by a pH-stat method using an automatic titrator with a FP/KF processor (Metrohm) connected to a pen chart recorder (BBC-Goertz Metrawatt). In these experiments, cells were kept after the respective pretreatment (with or without addition of B as 10^{-4}M $B(OH)_3$) in a simplified nutrient solution (3% saccharose, 100 mg/l m-inositol, trace elements at 0.1 times the normal concentration, growth regulators and vitamines as in the normal medium, and 10 mM $Ca(NO_3)_2$). The pH was kept constant by automatically adding 0.01 N HNO_3.
Statistical testing was performed either by variance analysis (factorial design) or t-test.

Results

The proton release of cell cultures, which were kept in a reduced medium (without Fe), was significantly lower under B-deficient conditions (Fig. 1, * denotes proton release *rates* to be significantly different). Depending on the preculture conditions, this effect was sometimes observed within less than one hour after transfer to B-deficient conditions (2). In different sets of experiments, the proton release was enhanced in the Fe-free medium between 0.5 to 1.5 h after transferring the cells. When Fe was supplied, the proton release was much lower, and the differences between the B-treatments could not be observed (unpublished results).
The proton release was enhanced by keeping the cell cultures in a medium with 1mM ferricyanide (Fig.2). Proton release rates were statistically significant one hour after transferring the cells. When continuing the experiments for several hours, the equilibrium pH which was reached by the B-deficient cells, was always higher than in the treatments with B supply (unpublished observations). When calculating on the basis of the cell dry weight, the final proton release was generally lowered by B-deficiency to about 40 to 50% of the B sufficient cell cultures. Addition of vanadate inhibited the net proton release in both

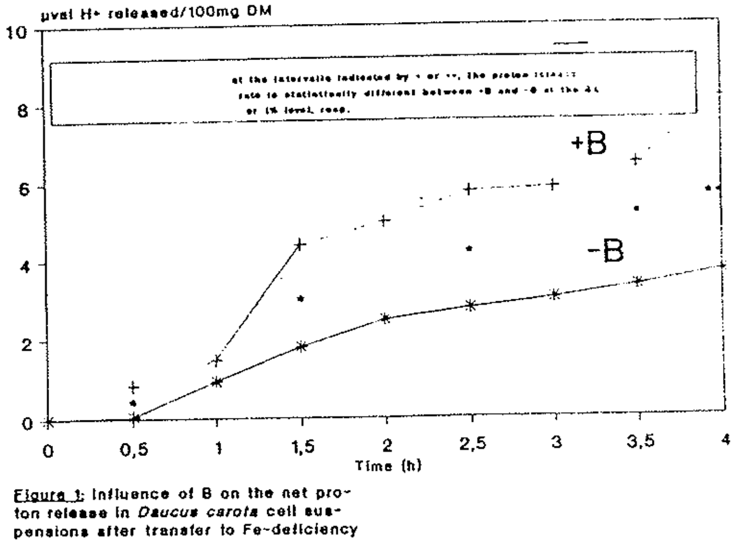

Figure 1: Influence of B on the net proton release in *Daucus carota* cell suspensions after transfer to Fe-deficiency

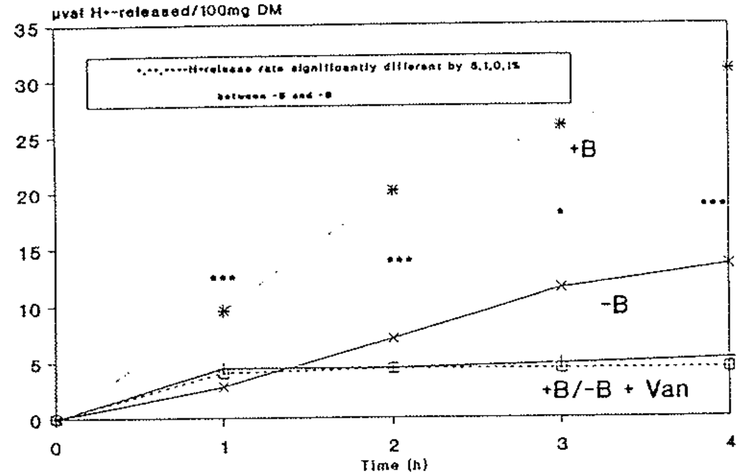

Figure 2: Influence of 18 h B-deficiency on the ferricyanide-induced proton secretion and its inhibition by 400µMvanadate

treatments completely within one hour, indicating the participation of (a) plasmalemma-bound ATPase(s) (Fig.2).
In those experiments without additions of auxins, especially when they have been withheld also during the pretreatment period, the net proton

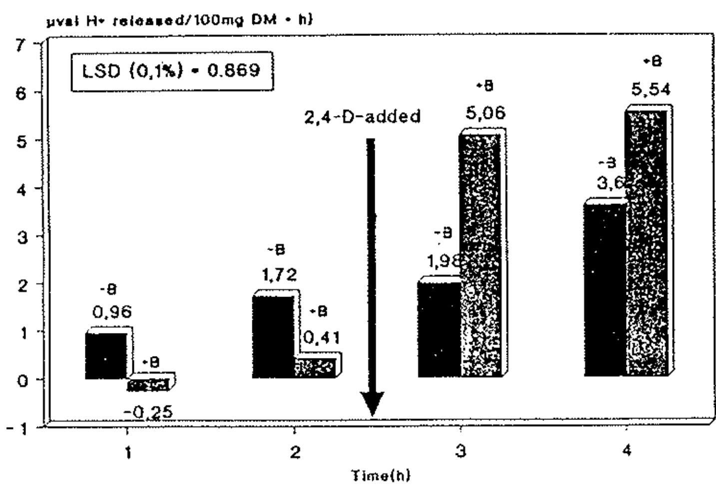

Figure 3: Effect of added 2,4-D on net proton release of tomato cell cultures, pretreated 16 h +/-B and without 2,4-D

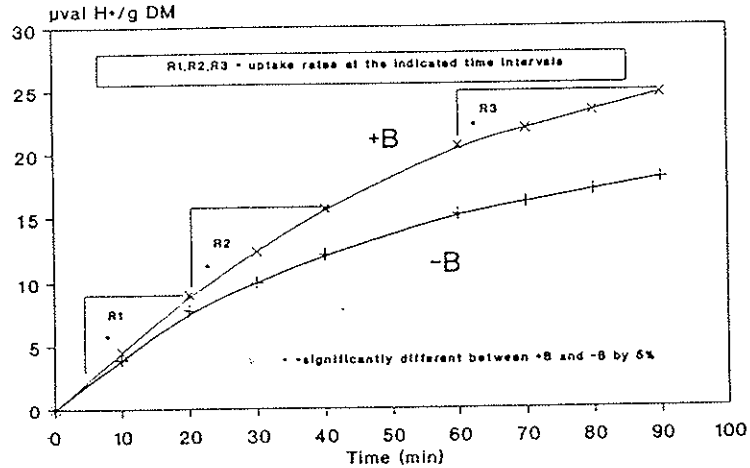

Figure 4: H+-uptake from 10mM Ca(NO3)2 by *Daucus carota* cells after 2h +/- B as revealed by a pH-stat method

release was much lower and no further differences between the B-treatments could be observed (data not shown here). There was even a slight tendency towards some higher proton secretion of the B-deficient cells. After the auxins have been added, however, the proton secretion

was enhanced within 0.5 h, and the cells, which have been supplied with B, showed a significantly higher net proton release (Fig.3).

To further elucidate this behaviour, we applied a pH_{stat}-technique to suspension-cultured carrot cells. As long as there is no increased proton secretion (cell cultures with an adequate supply of Fe and without ferricyanide), the equilibrium pH always reached a value around 5.5 - 5.8. When adding physiologically alkaline salts such as $Ca(NO_3)_2$ or $CaCl_2$ and starting with solutions adjusted to a pH lower than the equilibrium pH, the cells usually responded by an enhanced proton uptake. By automatic titration the pH could be kept constant, and the amount of acid, required to maintain the pH, gave a direct measure for the protons taken up by the cells. As only one treatment at a time could be tested without *direct* comparison to the other treatments, the results were somewhat more variable. It was to be seen, however, that the proton uptake rate was generally lower under B-deficient conditions. This effect could be noticed as early as 2 h after transfer to the B-deficient medium (Figure 4). Application of DCCD tended to eliminate the differences between the B-treatments, especially at R2 and R3 (data not shown). An increased proton uptake under B-deficiency, as described earlier (2), seemed to be only a transitory effect after changing from B-sufficient to B-deficient conditions.

Discussion

The above mentioned results clearly indicate, that B is involved in the regulation of plasmalemma-bound processes, and that it modifies the movement of protons through this membrane. According to results from Loughman's group, B-deficiency causes an inhibition of the ATPase activity (6,7), presumably of a plasmalemma-bound type. Although we were able to suppress the proton secretion in plant cell cultures by the addition of ATPase inhibitors such as vanadate, DES and DCCD (Fig.2 and still unpublished results), this does not necessarily indicate a *direct* influence of B on the ATPase activity. It only shows, that the proton transport, which is influenced by the supply of B, involves the activity of an ATPase, and that there is apparently no major contribution of a plasmalemma-bound reductase (NAD(P)H-consuming). If B supply would in fact directly modify the activity of ATPases, than there should be major differences in the plasmalemma-bound ATPases of monocotyledonous and dicotyledonous species, as the former ones posses only a low B-requirement (10) which was also reflected by the behaviour of *Zea mays* cell cultures (own unpublished results). Although boric acid

tends to act as an electron acceptor due to its unpaired electron at the p-orbital and thus might also participate as an intermediate in redox processes (such as described by Crane et al. 3), we were unable to find a significant effect of B on the ferricyanide reduction (2).
In order to directly influence a given metabolic process, B should be able to complex as boric acid or borate anion with the respective (mostly polyhydroxy-)compounds. There is practically no complexation with ATP, however, and it is unlikely, that B would modify the ATPase activity by complexing to the protein moiety of the molecule. It is obvious from our results (and has been verified in numerous experiments), that B influences the auxin-controlled part of the proton release activity in our (auxin-heterotrophic) cell cultures. This indicates, that B rather affects the action of auxins, possibly by modifying the plasmalemma in a way that auxins may bind to their receptors, or by changing the uptake rate of auxins. It has been observed by different authors that the uptake of anions is reduced under B-deficiency (4,6,8), which also might be relevant to the uptake of auxins. It should be noted, however, that this could have been caused by a reduced proton permeability of the plasmalemma as well (we have found a higher membrane resistance under B-deficiency; own, still unpublished results). Determination of the cytoplasmic pH as related to the supply of B would be helpful in further elucidating the primary role of B in membrane-bound processes, especially with respect to the interactions with auxin controlled mechanisms. Earlier, we have found a higher rate of auxin degradation under conditions of B-deficiency, caused by an increased peroxidase-/IAA-oxidase activity (5). But this is a rather late occurring alteration (under our experimental conditions between 20 and 30 h after transferring the cells to the respective medium), and should not be responsible for the rapid effect of added 2,4-D (less than 30 minutes, Fig.3), as 2,4-D is not metabolised by IAA-oxidases to a significant extend.
The most likely explanation for the effect of B is thus, that it might alter the structure of the plasmalemma and therefore allow auxin to bind to receptors, which in turn would stimulate the net proton secretion under inducing conditions such as Fe-deficiency or ferricyanide-application. Indications for a complex interaction between B and auxin (transport) have been described recently (11,12).

Summary

It was shown, that B deficy conditions reduces the proton release of cell cultures of *Lycopersicon esculentum* and *Daucus carota* under conditions, which stimulate the net proton secretion (Fe-deficiency and ferricyanide application). The effect of B was only observed, when auxin was added to the cultures. It is assumed, that B modifies the membrane of Dicots by binding to hydroxylic groups, so that will bind (more) auxins, and/or that it affects the uptake of anions in general.

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