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BRIEF COMMUNICATION

Effect of boron supply on nitrate concentration and its reduction in roots and leaves of tobacco plants

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Abstract

Shoot and root mass of tobacco plants treated with only 0.05 μM boron was decreased by 25 and 50 %, respectively, when compared to plants sufficiently supplied with B (2 and 5 μM). Leaf B content of 0.05 μM B-treated plants decreased (about 80 - 90 %) when compared to 2 μM B treated plants; this drop of B content were not as marked (about 25 - 45 %) in roots. Leaf and root nitrate contents in B-deficient plants were 45 - 60 % and 35 - 45 % lower, respectively, than those from 2 and 5 μM B treated plants. It is suggested that B deficiency might decrease nitrate uptake rather than nitrate reductase activity in tobacco plants.

Additional key words: boron deficiency, hydroponic culture, *Nicotiana tabacum*, nitrate assimilation.

Although in recent years attention is being paid to the toxic effects of boron (Gunes *et al.* 2007), especially when combined to salinity (Edelstein *et al.* 2005, Bishnoi *et al.* 2006, Sotiropoulos *et al.* 2006b), B deficiency is also a significant nutritional disorder since it causes impairments in several metabolic and physiological processes (Brown *et al.* 2002, Bolanos *et al.* 2004, Camacho-Cristóbal *et al.* 2008). Due to the difficulty to ascertain which of these responses are of primary or secondary nature (González-Fontes *et al.* 2008), direct physiological effects of B in plant nutrition remain still undiscovered, although there is evidence for a direct role of B in cross-linking of cell wall rhamnogalacturonan II (RG-II) and pectin assembly, which shows that B is essential for both cell wall structure and function (O'Neill *et al*. 2004).

In our laboratory it was shown that tobacco plants grown without B since the sowing had leaves with significantly decreased magnesium, calcium, potassium, and especially nitrate concentrations when compared to control leaves. Moreover, B deficiency caused a drop in leaf nitrate reductase (NR) activity throughout the day (Camacho-Cristóbal and González-Fontes 1999). This is a very interesting result because it points out a possible relationship between B and N metabolism in vascular plants. Plants require large amounts of N for the biosynthesis of amino acids and secondary metabolites and, hence, nitrate availability in the soil and its tissue concentration can limit plant growth. Nitrate reduction, catalysed by NR, is the primary step in the nitrate assimilation process, and it is essential for the production of ammonium to be incorporated into carbon skeletons for amino acid biosynthesis (Campbell 1999). Although the reduction of nitrate takes place in both roots and leaves in many herbaceous plants (*e.g.*, tobacco) this reduction occurs mainly in leaves (Gojon *et al.* 1998). Thus, nitrate accumulation in leaves depends mostly on the balance between the rate of nitrate uptake and its assimilation. The aim of this work has been to evaluate the effect of different B supplies on nitrate accumulation and its reduction in both roots and leaves of tobacco plants.

Seeds of tobacco (*Nicotiana tabacum* L. cv. Gatersleben) were sown in seedbeds filled with a mixture of *Perlite* and *Vermiculite* (1/1, v/v) in a growth chamber with a 12-h photoperiod, day/night temperature of 25/20 ºC, relative humidity of 70/80 %, and irradiance of 350 μmol m^{-2} s^{-1} (photosynthetically active radiation).

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Abbreviations: d.m. - dry mass; f.m. - fresh mass; *Nia* - nitrate reductase structural gene; NR - nitrate reductase.

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During a 4-week period three sets of plants were watered every two days with the complete nutrient solution described by Camacho-Cristóbal *et al*. (2005) except that contained 0.2, 10, or 20 µM H_3BO_3, respectively. Afterwards, each set of plants was transferred to separate 30-dm^{-3} plastic containers with 0.2× complete nutrient solution, the final B concentration being adjusted in the hydroponic cultures to 0.05, 2.0, or 5.0 µM, respectively. Ultra-purified water with an electric conductivity lower than 5.5 µS m^{-1} and analytical-grade salts were always used to prepare nutrient solutions and hydroponic cultures. Nutrient solutions were continuously aerated and completely renewed twice a week (pH 5.6 - 6.3). Plants were grown hydroponically for two weeks and then harvested. Whole plants were removed from the hydroponic cultures at three different times of the day (30 min before the beginning of the light period, after 4 h from the beginning of the light period, and 30 min before the beginning of the dark period). Shoots and roots were quickly separated, dried with paper towels, and weighed. Roots and the two highest leaves were rapidly frozen in liquid N_2, and stored at -80 °C.

For determination of nitrate reductase (NR) activity, frozen leaves and roots were ground to a fine powder in a mortar pre-cooled with liquid N_2. Then NR (EC 1.6.6.1) activity in the absence of Mg^{2+} was measured immediately according to Camacho-Cristóbal and González-Fontes (1999). Boron, nitrate, amino acids, and proteins were measured as previously described by Camacho-Cristóbal and González-Fontes (1999). All analytical determinations and enzyme assays were carried out on roots and leaves from three separate plants harvested randomly, and measured in duplicate. The data shown are mean values ± SD.

When plants are grown in soil, nutrients are added in higher concentrations in order to compensate for those amounts lost by leaching as well as to replace ions in the soil solution. Thus, B concentration in nutrient solution usually ranges between 20 and 100 µM, 25 or 50 µM being values very common (Hoagland´s solution and modifications). Nevertheless, in hydroponic cultures there is no leaching process and ions are much more available to roots provided that pH is controlled. This makes easier to decrease nutrient concentrations without affecting plant growth as long as the nutrient solution is renewed frequently. In this work we have used 5 µM B concentration in the hydroponic cultures for the control treatment. This value has been reported to be enough to grow herbaceous plants in soil (tobacco, Ruiz *et al*. 1998) and in hydroponic cultures (barley, Roessner *et al*. 2006).

In a previous work we showed that the lack of B in the culture medium for six weeks caused a drastic decrease in the growth of shoots (about 80 %) and roots (about 90 %) of tobacco plants (Camacho-Cristóbal and González-Fontes 1999). Therefore, to avoid this important alteration in the plant growth, it has been used a B supply as low as 0.05 µM for two weeks to get B-deficient plants in the present work. There were no differences in the shoot and root mass of tobacco plants when treated with 2 or 5 µM B. However, these plants had shoots and roots bigger than tobacco plants treated with 0.05 µM B (Fig. 1*A*,*B*). It is well known that B deficiency inhibits plant growth (Dugger 1983), this effect being more marked in roots, which resulted in a higher shoot/root ratio in B-deficient plants when compared to those sufficiently supplied with B (cf. Figs. 1*A* and *B*; Camacho-Cristóbal *et al*. 2005).

Tobacco plants treated with 0.05 µM B had drastically decreased (about 80 - 90 %) leaf B content when compared to plants treated with 2 or 5 µM B (Fig. 1*C*). Ruiz *et al.* (1998) considered leaf B contents about 10 mg(B) kg^{-1}(d.m.) in tobacco plants treated with 0.5 µM B to be deficient concentration, whereas those resulting from a treatment with 5 µM B as normal contents [about 40 mg(B) kg^{-1}(d.m.)]. Concentrations of leaf B consistent with this last value have been also reported by our group in tobacco plants treated with 10 µM B [about 40 mg(B) kg^{-1}(d.m.); Camacho-Cristóbal and González-Fontes 1999, Camacho-Cristóbal *et al*. 2005]. Accordingly, these data, together with those from plant growth, indicate that tobacco plants treated with 0.05 µM B are B-deficient plants while those treated with 2 or 5 µM B are plants sufficiently supplied with B. This conclusion is also supported by the fact that these plants had similar shoot and root mass as those treated with 10 µM B (Fig. 1*A*,*B*; Camacho-Cristóbal and González-Fontes 1999, Camacho-Cristóbal *et al*. 2005). Interestingly, there was a decline in leaf B content at the end of the light period irrespective of the B treatment (Fig. 1*C*), which might be due to a lower B uptake rate in evening hours or increased amount of photosynthates and so d.m.

Differences of root B content among the three treatments were not as marked (about 25 - 45 %) as in leaves, although B content in B-deficient plants were always lower than in plants supplied with 2 or 5 µM B (Fig. 1*D*). These results support that B concentration is less affected in roots than in leaves under B deficiency (Dannel *et al*. 1998, Camacho-Cristóbal *et al*. 2002), which could be explained in terms of growth (Camacho-Cristóbal *et al*. 2005). Thus, when the B supply is very low (0.05 µM), a tissue dilution of this micronutrient occurs as the plant biomass increases. Nevertheless, because of root growth is more sensitive to B deficiency than shoot growth (Fig. 1*A*,*B*; Huang *et al*. 1996, Camacho-Cristóbal and González-Fontes 1999), the effects of this mineral deficiency on the B concentration would be more marked in leaves than in roots. Moreover, another explanation may be given in terms of B uptake and translocation from root to shoot. In *Arabidopsis* plants, Takano *et al*. (2002) showed that B uptake into roots occurs mainly by passive transport, which would

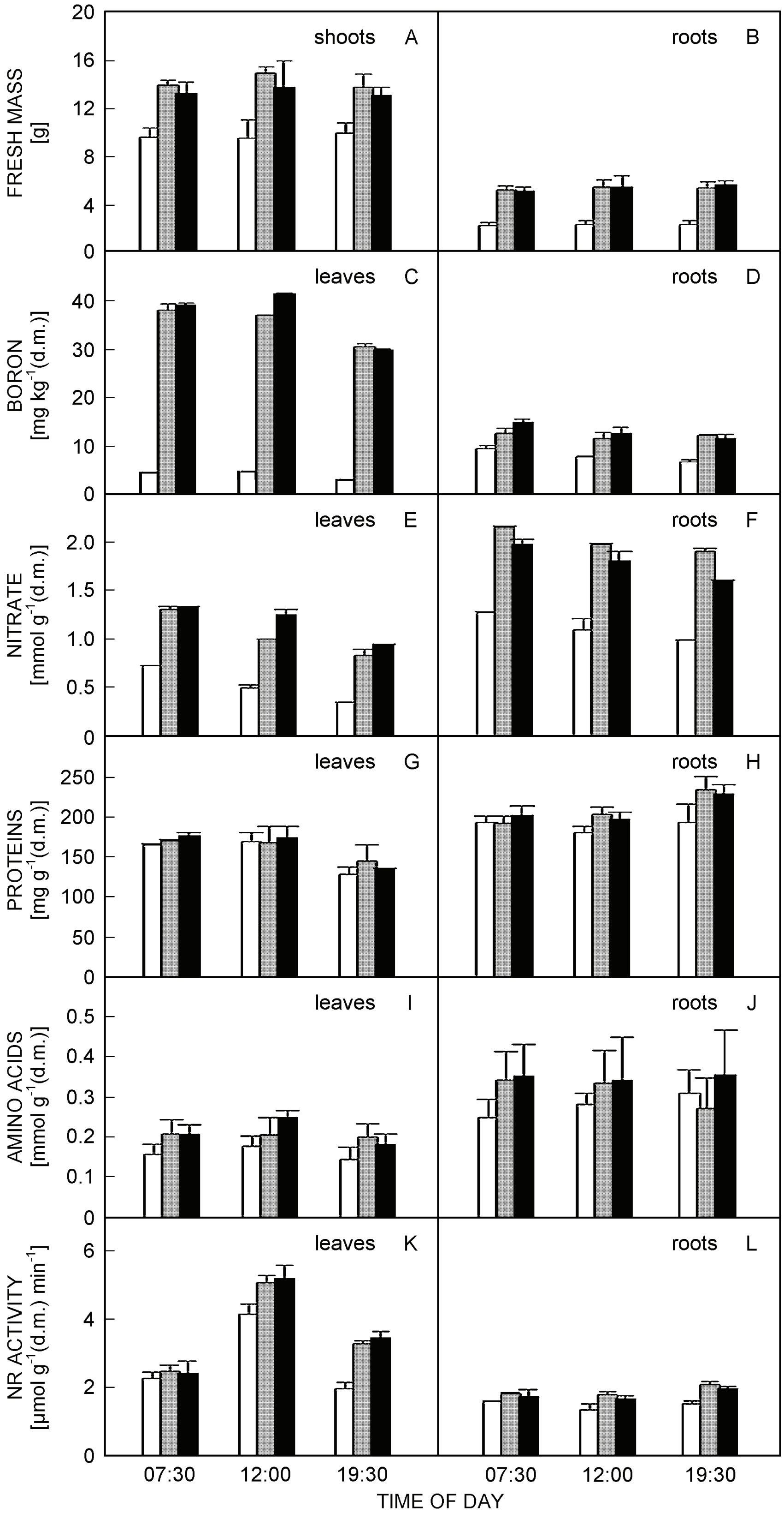

Fig. 1. Effects of B supply on fresh mass (*A*, *B*), contents of boron (*C*, *D*), nitrate (*E*, *F*), proteins (*G*, *H*), and amino acids (*I*, *J*), as well as NR activity (*K*, *L*). Tobacco plants were grown hydroponically for two weeks in a nutrient solution containing 0.05 (*white columns*), 2.0 (*grey columns*), or 5.0 (*black columns*) µM B. Plants were harvested at the indicated time. Means ± SD, *n* = 3.

explain the lower B content in B-deficient roots (Fig. 1*C*,*D*). However, these authors concluded that B translocation from root to shoot at low external B supply is mediated by BOR1, a B transporter for xylem loading that works against a concentration gradient of B. Taking this last finding into account, our data suggest that B translocation from root to shoot was very affected after two weeks of plant growth under a limiting B concentration of 0.05 μM, which led to lower leaf B contents (Fig. 1*C*,*D*).

Irrespective of B treatment, leaf nitrate contents showed maximal values at the end of the dark period and decreased during the photoperiod (Fig. 1*E*). Although it is well known that nitrate uptake in tobacco plants is higher during the light period (Matt *et al.* 2001), the nitrate content increased during the dark period as consequence of a lower leaf nitrate reductase (NR) activity (Fig. 1*K*) and, hence, a lower nitrate reduction rate.

Plants treated with 2 or 5 μM B had higher leaf and root nitrate contents than B-deficient ones (Fig. 1*E*,*F*). The lower nitrate content under B deficiency could be the consequence of an increase in NR activity and/or a decrease in nitrate uptake by roots. NR activity was not higher in B-deficient plants when compared to plants treated with 2 or 5 μM B (Fig. 1*K*,*L*); therefore, the lower nitrate content in B-deficient plants could be caused by decreased nitrate uptake. It is widely known that active transport of nitrate across the plasma membrane is powered by the proton gradient generated by plasma-membrane H^+-ATPases (McClure *et al.* 1990a,b), whose activity (Ferrol *et al.* 1993) and transcript level (Camacho-Cristóbal and González-Fontes 2007) decrease under B deficiency. Moreover, the putative inhibition of nitrate uptake in B-deficient plants may be mediated by several regulation mechanisms, including the indirect pathways (*e.g.*, the inhibition of some intermediates of carbon metabolism or growth processes under B deficiency may cause a higher concentration of some products of nitrogen metabolism that might down-regulate nitrate uptake).

Under different B treatments, there were no significant differences among protein and amino acid contents in both leaves and roots (Fig. 1*G*-*J*).

A key step in nitrate assimilation is the reduction of this anion to nitrite in the reaction catalyzed by NR, enzyme that is highly regulated at transcriptional and post-transcriptional levels (Campbell 1999, Kaiser and Huber 2001). Irrespective of B treatment, leaf NR activity had maximal values 4 h after the beginning of the light period (Fig. 1*K*). This fact has been also reported by other authors; thus, in tobacco plants grown with high nitrate and with a photoperiod of 12 h, both NR activity and *Nia* transcripts showed fluctuations throughout the day (Scheible *et al.* 1997a,b). It is also interesting to point out that leaf nitrate content decreased from the onset of the photoperiod (Fig. 1*E*), even though NR activity had its maximal value 4 h after the beginning of the photoperiod (Fig. 1*K*). These results show that there is no relationship between the daily fluctuations of nitrate content and the post-translational regulation of NR in leaves (Ferrario *et al.* 1996, Scheible *et al.* 1997b). In addition, B supply affected leaf NR activity during the photoperiod since leaf NR activity in tobacco plants treated with 0.05 μM B was lower than that in plants treated with 2 and 5 μM B (Fig. 1*K*). These results support the idea that light combined with B deficiency represent conditions particularly negative for NR activity, probably due to a higher degradation of *Nia* transcript and NR protein (Camacho-Cristóbal and González-Fontes 1999). Nevertheless, in spite of a decrease in leaf nitrate content and NR activity, B-deficient plants were actually not limited by nitrogen since their leaf amino acid and protein contents were similar to plants sufficiently supplied with B (Fig. 1*G*-*J*). This is in agreement with the results found for kiwifruit shoots cultured *in vitro* where low B concentrations in the medium did not change significantly organic N content of shoots (Sotiropoulos *et al.* 2006a).

Root NR activity did not follow the diurnal pattern of NR activity in leaves, and was kept almost constant during the day (Fig. 1*L*). In addition, B-deficiency did not affect root NR activity as notably as it did in leaves, NR activity being only slightly lower at end of the light period when compared to roots of plants treated with 2 or 5 μM B (Fig. 1*L*). It is also interesting that NR activity was clearly lower in roots than in leaves of tobacco plants, which supports the idea that the reduction of nitrate in herbaceous plants is carried out mainly in leaves (Gojon *et al.* 1998).

In conclusion, this work shows that a B-deficient supply (0.05 μM) decreased the nitrate concentration in both leaves and roots of tobacco plants, probably due to a decreased nitrate uptake by roots since NR activity was not notably affected by B deficiency. Nevertheless, a more precise explanation of B effect on nitrate uptake and reduction may be complicated by existence of several types of nitrate transport proteins and different forms of NR.

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