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Effects of Boron on Leaf Expansion and Intercellular Airspaces in Mung Bean in Solution Culture

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ABSTRACT

Mung bean (*Phaseolus aureus* Roxb.) was grown in solution culture to study the effect of boron (B) deficiency on leaf expansion and intercellular airspaces. Different B concentrations were supplied just after initiation of the second trifoliate leaf because this is the first leaf whose growth depends on the exogenous B supply. The central leaflet of the second trifoliate was employed in this study. When plants were grown in 0.2 μmol L^{-1} B, leaf expansion was limited compared with that of plants supplied with 50 μmol L^{-1} B. During the progress of leaf development, plants grown in 0.2 μmol L^{-1} B were transferred to medium with 50 μmol L^{-1} B medium at days 2, 4, 6, and 8. The final leaf area of plants transferred on day 2 was half that of plants raised in 50 μmol L^{-1} B solution. Plants transferred on days 6 and 8 produced a leaf area similar to that of plants grown in 0.2 μmol L^{-1} B. Uptake experiments with ^{10}B-enriched boric acid showed that there was enough B present at the sites of cell expansion after 50 μmol L^{-1} B was supplied for two days. The failure of deficient leaves to resume elongation may therefore have been due either to a lack of incorporation of B into the cell wall or boron or to a requirement for B early in leaf development to enable future cell wall expansion. In addition to the reduced leaf expansion resulting from B deficiency, the formation of intercellular airspaces was also depressed. The proportion of the spongy mesophyll region comprising airspaces in

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B-deficient leaves was less than 50% of that observed in leaves of plants supplied with sufficient B.

Keywords: B deficiency, B sufficiency, intercellular airspaces, leaf expansion, *Phaseolus aureus*

INTRODUCTION

Boron deficiency is the most widespread micronutrient shortage in many plant species throughout the world (Shorrocks, 1997; Goldberg, 1993). The deficiency causes adverse effects on plant morphological features (Dell and Huang, 1997; Goldbach, 1997), including restricted root elongation (Hirsch and Torrey, 1980; Cohen and Lepper, 1977) and diminished leaf expansion (Jiao et al., 2003; Noppakoonwong et al., 1993). Boron deficiency influences root elongation by affecting cell expansion and cell division (Hirsch and Torrey, 1980; Cohen and Lepper, 1977) while limited leaf development is primarily a result of reduced cell elongation (Hu and Brown, 1994). Previous research has demonstrated that the inhibition of root elongation caused by B deficiency can be corrected if B is re-supplied before the meristematic region of root tips is permanently damaged (Bohnsack and Albert, 1977). However, it is still not known whether or not the inhibition of leaf expansion resulting from B deficiency can be reversed by supplying sufficient B to growing plants.

The intercellular airspaces between the spongy mesophyll cells in leaves facilitate internal CO_2 diffusion for photosynthesis (Evans et al., 1994; Evans and von Caemmerer, 1996) and are also an important component of leaves (Dale, 1988) providing leaf flexibility. Thus, leaves deficient in B are brittle and fragile (Goldbach, 1997). The *BOR*1-1 mutant of *Arabidopsis thaliana* forms fewer intercellular airspaces in leaves than the wild type (Takano et al., 2001) and it has, therefore, been postulated that limited leaf expansion under conditions of B deficiency are related to the inhibition of intercellular airspaces. The objectives of the present study were to characterize recovery of leaf expansion in B-deficient mung bean plants after sufficient B was supplied and to investigate the effects of B deficiency on the formation of intercellular airspaces.

MATERIALS AND METHODS

Seeds of mung bean (*Phaseolus aureus* Roxb.) were surface-sterilized and rinsed thoroughly with distilled water. Seedlings were then placed in fine granular vermiculite in seed trays that were placed in distilled water maintained at a depth of 2.5 cm. Prior to the start of all experiments, seedlings were raised for five days under a continuous light source from warm white fluorescent tubes (PAR 100 μmol $m^{-2}s^{-1}$) and at a temperature of 22°C. Only distilled water was supplied during these five days.

Five-day-old seedlings were transplanted into acid-washed black plastic pots (8.2 × 8.2 × 11.6 cm deep) filled with Rorison's solution according to Hewitt (1966), but with different amounts of B. The nutrient solution had the following composition: 2 mmol L^{-1} $Ca(NO_3)_2$, 1 mmol L^{-1} $MgSO_4 \cdot H_2O$, 1 mmol L^{-1} K_2HPO_4, 68 μmol L^{-1} Fe-EDTA, 9 μmol $L^{-1}MnSO_4 \cdot 4H_2O$, 0.15 μmol L^{-1} $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.5 μmol $L^{-1}ZnSO_4 \cdot 7H_2O$, 1.5 μmol $L^{-1}CuSO_4.5H_2O$. Boron was supplied as H_3BO_3. Seedlings were raised hydroponically in a growth cabinet under a 16h/8h light/dark cycle with a day temperature of 25°C, a night temperature of 18°C, and an irradiance level of 350 ± 20 μmol $m^{-2}s^{-1}$ (PAR) from metal-halide lamps. The nutrient solution was replenished every two days for the duration of all experiments.

Since the growth of the second trifoliate leaf depends on the exogenous B supply (Jiao, 2001), investigations were carried out on the central leaflet of this leaf. In all experiments, 0.2 μmol L^{-1}B was supplied until the second trifoliate leaf emerged; this process usually took 9 days. One day later, half of the plants were transferred to medium containing 50 μmol L^{-1}B, the optimum B concentration, and the remainder maintained at 0.2 μmol L^{-1}B. This time point was taken as day 0 in leaf development. There were two seedlings per pot for the first 12 days of this development and one plant per pot remained until the end of the experiment.

A second experiment was designed to investigate whether or not leaf expansion could recover after having been limited by B deficiency for various time periods. Plants were transferred from 0.2 to 50 μmol L^{-1}B after 0, 2, 4, 6, and 8 days. In a further treatment, plants were maintained on 0.2 μmol L^{-1}B throughout the experiment.

Leaf area was determined using a leaf area meter (Delta-T Devices Ltd, Cambridge, UK). All data were subjected to 2-sample t-test using Minitab 11 for Windows. The Tukey multiple comparison test was employed following ANOVA to compare differences among mean values.

Application and Analysis of ^{10}B

Plants were raised in 50 or 0.2 μmol L^{-1} B for six days. The (natural) isotopic abundance of the solutions was: $^{11}B/^{10}B = 4.26$. The roots were washed with distilled water and the plants were then transferred to 0.2 or 50 μmol L^{-1}B enriched with ^{10}B so that the isotopic abundance was: $^{11}B/^{10}B < 0.04$ (Eagle Pitcher Industries, Quapaw, OK, USA). Two days later the central leaflets of the second trifoliate leaf were harvested and immediately frozen in liquid N_2. They were stored at −20°C until required for further testing.

Isotope ratios for ^{11}B and ^{10}B were determined by ICP-MS following laser ablation, which employed an LSX-200 series device (CETAC Technologies Inc, Omaha, NE, USA). The ICP-MS employed was an Agilent 4500 (Hewlett-Packard Co., USA). ^{13}C was used as an internal standard to which data were

normalized. The leaflet was scanned at a speed of 40 μm s^{-1} at 10 Hz with a 200 μm diameter laser. The sample cell employed to hold the tissue was too small to hold the entire leaflet when plants had been raised in 50 μmol L^{-1} B. The most apical 40 mm of the leaflet was scanned for plants supplied with 50 μmol L^{-1} B. Leaflets were scanned parallel to, but about 0.5 mm away from, the midrib.

Measurement of Intercellular Airspaces in Leaves

Four, 7, and 20 days after plants were subjected to different B concentrations, 1 cm^2 segments of leaf tissue were excised from the middle leaflet of three individual plants from sufficient (50 μmol L^{-1}B) and deficient (0.2 μmol L^{-1}B) plants. The segments were carefully dissected from the area halfway along the leaf and 0.2 cm away from the midrib and were immediately fixed in a solution consisting of formaldehyde (20 mg cm^{-3}) and sucrose (7.5 mg cm^{-3}) in NaH_2PO_4(100 mmol L^{-1}, pH 7.2) and kept under a mild vacuum until they were penetrated by the fixative. The samples were then dehydrated through a series of ethanol concentrations and transferred to embedding solvent (Histoclear, BDH, UK), and finally saturated with paraffin (Paraplast Xtra, Sigma, USA). Sections (15 μm) were cut with a microtome (Reichert, Austria). They were attached to adhesive-treated microscope slides (Polysine slides, SLS, Germany) in a water bath and dried at 37°C. After the paraffin was removed, the sections were re-dehydrated through a series of ethanol concentrations. Sections were covered with a few drops of Histoclear and the slides mounted in DePeX (Serva Feinbiochemica, Heidelberg, Germany). Intercellular airspaces were determined by means of a SEESCAN computer image analysis system employing a CCD video camera to view embedded leaf sections. The area of the spongy mesophyll layer, including airspaces, was recorded over a 350 μm section of each leaf segment. Given that the thickness of the spongy mesophyll layer was also recorded, the percentage area contributed by airspaces could be calculated. Six observations were made for each leaf with each treatment, providing three leaf replicates.

RESULTS AND DISCUSSION

Boron and Leaf Expansion

Comparison between the leaf area of plants supplied with 50 μmol L^{-1}B and the expansion of the leaves of plants raised in 0.2 μmol L^{-1}B medium indicated a significant level of restriction at day 6, which was only 54% that of control plants ($p < 0.05$) (Fig. 1). A significant difference between these two treatments

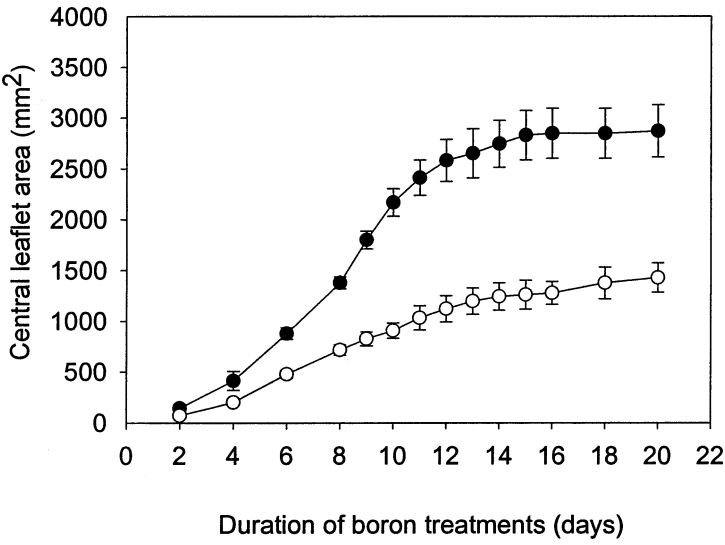

Figure 1. Effects of boron on expansion of the central leaflet of the second trifoliate leaf of plants grown in 50 μmol L^{-1} (●) or 0.2 μmol L^{-1} (○) B from day 0. Data are means ± 1 SD of six replicates.

was maintained until the leaves had stopped expanding in both treatments ($p < 0.05$).

When plants were transferred from 0.2 μmol L^{-1}B to 50 μmol L^{-1}B within two days, the normal leaf area was achieved. Those plants transferred after 4 days of growth in B-deficient growth media indicated a significant enhancement in expansion relative to that in plants maintained under deficient conditions. Despite this, effective leaf area was only about two thirds of that attained by leaves of plants supplied with 50 μmol L^{-1}B throughout the experiment, which lasted 20 days. When the deficient treatment extended beyond six days, transfer to 50 μmol L^{-1}B failed to induce any significant expansion beyond that seen under permanent deficiency (Fig. 2). It is evident from the data that unless the supply of B is restored to an adequate level within 2 days, the second trifoliate leaf lost its ability to expand.

This is the first demonstration that transfer of plants from B-deficient to B-sufficient medium does not lead to a recovery of leaf expansion even when applied prior to the cessation of its expansion, as shown in Figure 2. It has been demonstrated previously that the cell number of the second trifoliate leaf is not influenced by B deficiency (Jiao, 2001). Thus, the lack of an appropriate concentration of B during the first week of growth seems to inhibit cell wall expansion. Boron is predominantly localized in the cell wall (Hu and Brown, 1994; Hu et al., 1996; Matoh et al., 1993) and cross links with rhamnogalacturonan II

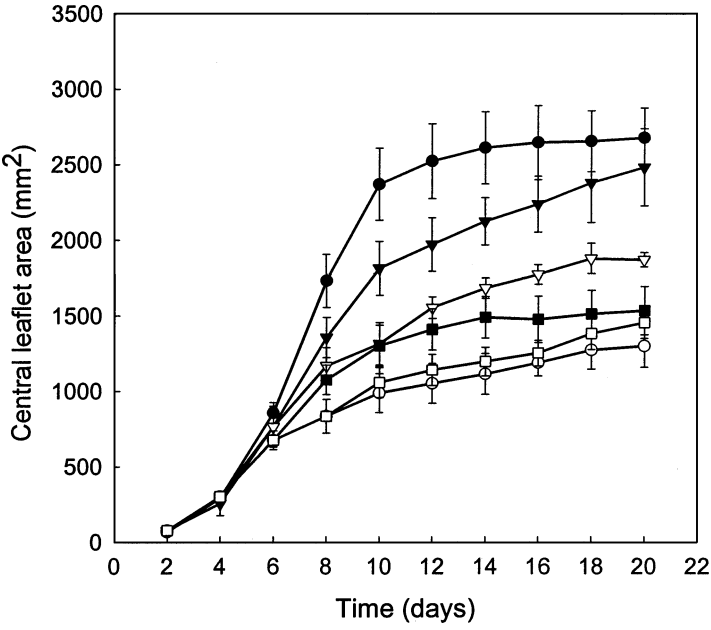

Figure 2. Recovery of the expansion of the leaf in response to enhanced B supply. Seedlings were supplied with 0.2 μmol L^{-1}B for 9 days before the second trifoliate emerged. This time point is referred to as day 0. Plants were then transferred to 50 μmol L^{-1}B. The transfers were made after 0 (●), 2 (▼), 4, 6 (■) and 8 (□) days. One treatment involved no transfer (○). Data are means ± 1 SD of six replicates.

(Kobayashi et al., 1996; O'Neill et al., 2001), which determines cell wall extensibility (Hu and Brown, 1994; Findeklee and Goldbach, 1996). Boron is distributed unevenly in the leaf, thereby creating gradients that occur from the leaf apex to base (Brown and Shelp, 1997). Cell maturation also proceeds from apex to base (Donnelly et al., 1999; Poethig and Sussex, 1985). It was found previously that epidermal cells of the second trifoliate leaf expand until day 11 (Jiao, 2001). Therefore, the failure of leaf expansion to respond to the reintroduction of a sufficient supply of B (Fig. 2) may be explained in one of two ways: (1) there is not enough B present at the sites where cell expansion occurs vigorously; or (2) the ability of cell walls to expand is determined before cell wall expansion ceases, perhaps even earlier than day 4 of leaf development.

Distribution of ^{10}B in Leaves

^{10}B was used to trace the distribution of B within the leaf after plants were transferred from B-deficient to B-sufficient medium. Preliminary investigations

established that when plants were raised on B at 0.2 or 50 μmol L^{-1}, the natural abundance of ^{11}B and ^{10}B was evidenced along the length of the leaf (data not shown). Transfer of plants into enriched ^{10}B resulted in a reduction in the $^{11}B/^{10}B$ ratio (Fig. 3A). This occurred irrespective of whether plants had previously been raised under conditions of B deficiency (0.2 μmol L^{-1}) or sufficiency (50 μmol L^{-1}). This reduction was more pronounced when the transfer had been into a higher concentration of B. The important point from

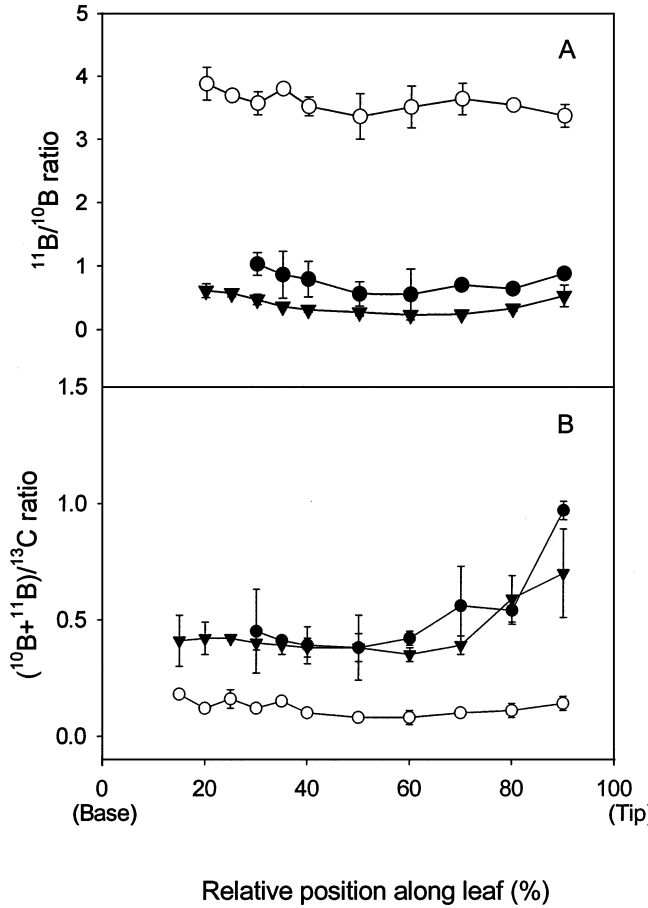

Figure 3. Boron distribution along the blade of the central leaflet of the second trifoliate leaf. $^{11}B/^{10}B$ (A) and $(^{11}B + ^{10}B)/^{13}C$ (B). (●) 50 μmol L^{-1}B then plants transferred to 50 μmol L^{-1}B enriched in ^{10}B; (▼) 0.2 μmol L^{-1}B then plants transferred to 50 μmol L^{-1}B enriched in ^{10}B; (○) 0.2 μmol L^{-1}B then plants transferred to 0.2 μmol L^{-1}B enriched in ^{10}B. Relative position along leaf is expressed as the percentage distance from the base. Data are means ± 1 SD of three replicates.

these data is that there was no significant difference in $^{11}B/^{10}B$ ($p > 0.05$) along the leaf blade, irrespective of whether or not seedlings were deficient in B at the time of transfer.

The ratio of $(^{11}B + ^{10}B)/^{13}C$ provides a reference for comparing the amounts of B along leaves of plants raised under different B regimes. Leaves of plants raised in 50 μmol L^{-1}B but transferred into a solution enriched in ^{10}B at the same concentration produced a similar ratio along most of its length, as did those raised initially in 0.2 μmol L^{-1}B, from 0.4 to 0.6 ($p > 0.05$). The enhanced ratio towards the leaf tip to a value of 0.8 reflects normal B distribution in leaves (Brown and Shelp, 1997). The $(^{11}B + ^{10}B)/^{13}C$ ratio was reduced to 0.12 when B-deficient plants were transferred into solution with enriched ^{10}B but at lower (0.2 μmol L^{-1}) concentration (Fig. 3B). This limited supply of B, shown already to induce classic deficiency symptoms, did not result in an increase in the ratio of $(^{11}B + ^{10}B)/^{13}C$ at or near the leaf tip. Nevertheless, it is clear that impaired transport of B into and along the leaf is not the reason why expansion fails after a previous period of B deficiency. However, we do not know if the B has been incorporated into the cell wall.

Boron and Intercellular Airspaces

Intercellular airspaces in spongy mesophyll layer increased during the process of leaf expansion, regardless of B concentration. However, leaves of plants supplied with 50 μmol L^{-1}B developed more intercellular airspaces than did deficient leaves. Differences in intercellular airspaces between the two treatments were significant on days 7 and 20, with 50% fewer intercellular airspaces in the leaves of B-deficient plants (Table 1).

Previous studies have focused on the effects of B deficiency on leaf development in terms of cell elongation and cell division (Hu and Brown, 1994). Little information is available on the effect of B on intercellular airspaces in leaves. The diminished intercellular airspaces found in this study are consistent

Table 1
Effects of boron on the percentage of spongy mesophyll region constituted by intercellular airspaces in mung bean leaves

	Duration of boron treatment (days)		
Boron concentration	Day 4	Day 7	Day 20
50 μmol L^{-1}	6.80 ± 0.91	17.43 ± 0.94	25.74 ± 1.00
0.2 μmol L^{-1}	2.90 ± 1.40	9.23 ± 1.30*	10.79 ± 1.00*

Data presented are means ±1 SD of 3 replicates. *denotes significant difference ($p < 0.05$) between two boron treatments.

with the observation on leaves of the wild type subjected to B deficiency and *BOR*1-1 *Arabidopsis thaliana* supplied with sufficient B (Takano et al., 2001). The formation of intercellular airspaces is determined by cell turgor (Jarvis, 1998). Cell turgor is affected by membrane integrity (Cakmak and Römheld, 1997). Moreover, expansion of epidermal cells coincides with the formation of intercellular airspaces in the mesophyll (Dale, 1988). It was found previously that B deficiency inhibits epidermal expansion to a greater extent than other mesophyll or palisade cell expansion (Jiao, 2001). Therefore, it was concluded that the reduced intercellular airspaces in leaves caused by B deficiency is subject to the role of B in membrane and cell wall structure.

CONCLUSIONS

Boron deficiency severely limited leaf expansion. While the effects of B deficiency on leaf expansion were not apparent on day 4, transferring B-deficient plants to B-sufficient growth medium did not fully restore leaf expansion of the B-deficient plants, which was only half that of leaves supplied with sufficient B from day 0. Transfer on day 6 or 8 showed similar leaf area compared with plants raised exclusively in deficient solution. Perhaps the failure of leaf expansion was not due to B availability in the region where growth normally occurs. This implies that B is required at early stages of leaf development to ensure full leaf expansion. In addition, diminished leaf expansion caused by B deficiency was coupled with a decline in intercellular airspaces in the leaves. The percentage of the spongy mesophyll region comprising intercellular airspaces in B-deficient leaves was less than half of that found in B-sufficient leaves.

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