Journal of Experimental Botany, Vol. 59, No. 3, pp. 575–583, 2008
doi:10.1093/jxb/erm336 Advance Access publication 4 February, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Evidence of phloem boron transport in response to interrupted boron supply in white lupin (*Lupinus albus* L. cv. Kiev Mutant) at the reproductive stage

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Received 9 October 2007; Revised 28 November 2007; Accepted 30 November 2007

Abstract

The present study investigates whether previously acquired boron (B) in mature leaves in white lupin can be retranslocated into the rapidly growing young reproductive organs, in response to short-term (3 d) interrupted B supply. In a preliminary experiment with white lupin in soil culture, B concentrations in phloem exudates remained at 300–500 μM, which were substantially higher than those in the xylem sap (10–30 μM). The high ratios of B concentrations in phloem exudates to those in the xylem sap were close to values published for potassium in lupin plants. To differentiate 'old' B in the shoot from 'new' B in the root, an experiment was carried out in which the plants were first supplied with 20 μM ^{11}B (99.34% by weight) in nutrient solution for 48 d after germination (DAG) until early flowering and then transferred into either 0.2 μM or 20 μM ^{10}B (99.47% by weight) for 3 d. Regardless of the ^{10}B treatments, significant levels of ^{11}B were found in the phloem exudates (200–300 μM in 20 μM ^{10}B and 430 μM in 0.2 μM ^{10}B treatment) and xylem sap over the three days even without ^{11}B supply to the root. In response to the 0.2 μM ^{10}B treatment, the translocation of previously acquired ^{11}B in the young (the uppermost three leaves), matured, and old leaves was enhanced, coinciding with the rise of ^{11}B in the xylem sap (to >15 μM) and phloem exudates (430 μM). The evidence supports the hypothesis that previously acquired B in the shoot was recirculated to the root via the phloem, transferred into the xylem in the root, and transported in the xylem to the shoot. In addition, some previously acquired ^{11}B in the leaves may have been translocated into the rapidly growing inflorescence. Phloem B transport resulted in the continued net increment of ^{11}B in the flowers over 3 d without ^{11}B supply. However, it is still uncertain whether the amount of B available for recirculation is adequate to support reproductive growth until seed maturation.

Key words: ^{10}B, ^{11}B, B recirculation, *Lupinus albus* L., phloem exudate, xylem sap.

Introduction

Boron mobility in higher plants lacking polyol-assisted phloem transport (Brown and Shelp, 1997) has been reported to vary from very low to moderate, across a range of studies, for example, in wheat (Huang *et al*., 2001), cotton (Oertli, 1994), canola (Stangoulis *et al*., 2001), broccoli, and lupin (Marentes *et al*., 1997; Shelp *et al*., 1998), and various trees (Lehto *et al*., 2004). In many crop species, boron (B) deposited in older leaves is not remobilized into new growth, even upon the interruption of external B supply to roots (Oertli, 1994). A very limited retranslocation of B into the young ear was reported in wheat, where the B that did enter the ear was predominantly dependent on long-distance transport in the xylem and concurrent B uptake from the roots (Huang *et al*., 2001). In a B-efficient canola cultivar, B was retranslocated out of mature leaves into young growing leaves when external B supply to the roots was interrupted for 5 d (Stangoulis *et al*., 2001).

Observations of high B concentrations in phloem exudates of lupin and broccoli have been attributed to an indirect mobilization of B through xylem to phloem

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transfer of the B concurrently taken up by the roots supplied with B (Marentes *et al*., 1997; Shelp *et al*., 1998). However, this xylem to phloem transfer of B acquired from concurrent root B uptake is unlikely to function in plants where the B supply is interrupted for days, such as in the canola study (Stangoulis *et al*., 2001). To maintain high levels of B in the phloem, and in the new growth when the external B supply in the rooting medium is interrupted, the B previously acquired in mature leaves would have to be retranslocated into the actively growing inflorescence through direct phloem loading or recirculation into roots via the phloem and transport in the xylem up into the growing shoot organs. Preliminary experiments revealed a high B concentration in phloem exudates collected from vegetative stems of lupin. However, there has been no direct evidence of B retranslocation out of mature leaves when the external B supply is severely limited, nor on the likely B retranslocation from shoot to root in species without the B–polyol complexes in their phloem to facilitate B mobility.

The objectives of the present study are to investigate if there are significant levels of B translocation out of leaves into the rapidly growing reproductive organs of lupin plants at the early stages of reproduction. To differentiate clearly 'old' from 'new' B uptake in the plants, the present study used the sequential treatments of the plants with enriched stable B isotopes, firstly ^{11}B (99.34% by weight) followed by ^{10}B (99.47% by weight). Solution culture was used in the present study as the actual B concentrations in solid rooting media (such as soil/sand) may be higher due to concentration effects from water loss, such as in the case of Marentes *et al*. (1997).

Materials and methods

General

White lupin (*Lupinus albus* L. cv. Kiev Mutant) was used because of the ease of phloem exudate collection (Pate *et al*., 1974). Plants were germinated on paper towels moistened with 2 mM $CaSO_4$ at 25 °C in the dark, before being transplanted into soil or nutrient solution. The full-strength basal nutrient solution contained (μM): KNO_3, 2800; NH_4NO_3, 2000; $Ca(NO_3)_2$, 1600; $MgSO_4$, 1000; KH_2PO_4, 60; FeEDTA, 40; NaCl, 8; $ZnSO_4$, 2; $MnSO_4$, 2; $CuSO_4$, 0.5; $CoSO_4$, 0.2; and Na_2MoO_4, 0.08. Solution pH was maintained in the range of 5.0–6.0. Analytical grade chemicals were used to make up stock solutions of these nutrients, to which was added acid-cleaned B-specific resin (IRA-743, Sigma Chemical Co.) (5 g moist resin l^{-1}) to remove residual B present in the solution. Water used for making up all chemical and nutrient solutions was purified by passing through a column packed with B-specific resin. Boron isotopes were supplied as boric acid enriched with ^{10}B (99.47% weight) or ^{11}B (99.34% weight) (Eagle Picher, USA).

Experiment 1: B levels in xylem and phloem

The purpose of this experiment was to assess the levels of B in phloem exudate and xylem sap in B-adequate plants at early podding. Germinated seeds were planted in potting mix (composted pine bark and sand—3:1) fertilized with the complete nutrient solution described above which contained 10 μM B (boric acid) throughout the experiment. The plants were maintained under glasshouse conditions similar to those in Huang *et al*. (2001).

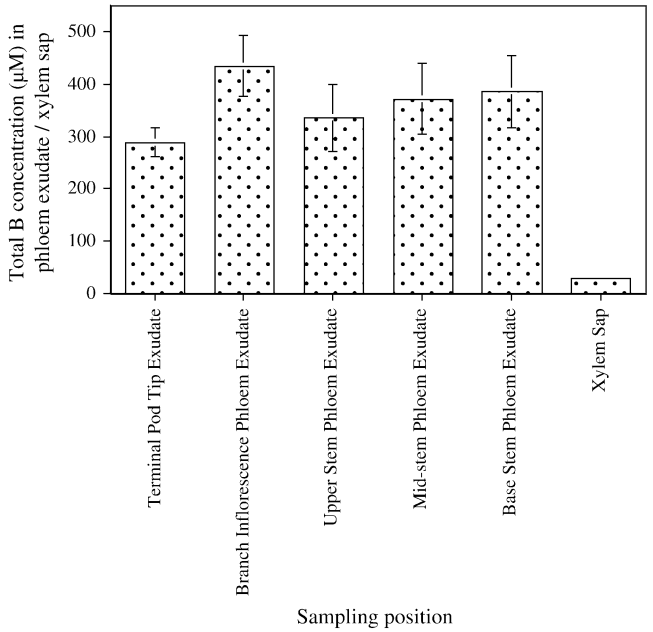

Fig. 1. Boron concentration in phloem exudate collected from different positions on the main stem and inflorescence and in the xylem sap from the base of the main stem of lupin plants grown in soil culture (Experiment 1). Values are means of four replicates and the bars where visible represent the SEM.

Experiment 2: B isotope labelling

To investigate the movement of B into the inflorescence, the plants were cultured in nutrient solution in the same manner as described in Huang *et al*. (2001), under glasshouse conditions: air temperature 18–34 °C, relative humidity 35–76%, photosynthetically active radiation (PAR) 425–1135 μmol m^{-2} s^{-1} (natural sunlight between 10.00 h and 16.00 h in the glasshouse). Briefly, after being acclimatized for 4 d in the 1/3 strength complete nutrient solution containing 20 μM ^{11}B, four plants were transplanted into each of the 5.0 l plastic pots lined with a polythene bag, containing 4.5 l of full-strength nutrient solutions. Plants were progressively thinned to one per pot by the date of commencement of the ^{10}B treatments. The plants were supplied with 20 μM ^{11}B for 48 d after germination (DAG)—until early flowering (14 d after budding) when the plants had ~16 leaflets on the main stem. During this period, aliquots of nutrients and ^{11}B were added to the solution of each pot based on a programmed nutrient addition as used in a previous study (Huang *et al*., 2001).

On 48 DAG (between 15.00 h and 17.00 h), the plants were transferred into fresh nutrient solutions containing full-strength basal nutrients and subjected to two levels of ^{10}B treatments: 0.2 μM or 20 μM ^{10}B. Before transfer into the treatments, the roots were rinsed with double-deionized water and then three changes of triple-deionized water (purified with acid-cleaned B-specific resin) containing 2 mM $CaSO_4$, to remove surface-adsorbed B. Each B treatment comprised 12 replicate pots, which allowed at least three consecutive harvests of three pots of plants at each time.

Phloem exudate and xylem sap collection and plant sampling

Experiment 1: At the early podding stage (61 DAG), phloem exudates were collected at shallow incisions according to Atkins

(1999), from the young (1–2 cm long) pod tips, the base of the inflorescence axis, the upper stem (between the second and fourth leaves of the main stem from the top), the mid stem (between the fifth and seventh leaves of the main stem from the top), and the basal stem (between the ninth and 12th leaves of the main stem from the top). Phloem exudate was collected into fine plastic tips over a 1 h period between, 9.00 h and 10.00 h in the glasshouse. The phloem exudate samples at each sampling position from plants of the same pot were pooled to form one sample and stored in a plastic microcentrifuge tube at –20 °C before analysis.

After phloem exudate collection, the shoot was cut at the hypocotyl, and root bleeding (xylem) sap was collected from the root stump. The droplets emerging from the root stump within the first 5 min were discarded (Jeschke *et al*., 1985). The collection of xylem sap lasted 1 h between 11.00 h and 12.00 h in the shade. The measured pH values in the phloem exudate and xylem sap were consistent with published values for these respective solutions (Pate, 1975), indicating minimal mixing of xylem and phloem fluids during collection.

B concentrations in the phloem exudate and xylem sap samples were determined by inductively coupled atomic emission spectrometry (ICP-AES, Varian).

Experiment 2: The collection of phloem exudate and xylem sap was carried out in the same manner as in Experiment 1 on 1, 2, and 3 d after the commencement (48 DAG) of ^{10}B treatments. Phloem exudates were collected from the base of the inflorescence axis and from the mid-stem position (between the fourth and sixth leaves from the top of the main stem). Xylem sap was collected from decapitated root stumps after phloem exudate collection (Jeschke *et al*., 1985).

All sap samples were stored at –20 °C for total B analysis by ICP-AES to determine initially the total B concentration. A known volume of each sap sample was then diluted with 4 ml of 1% nitric acid containing beryllium (Be) (2 μg l^{-1}) as an internal standard before inductively coupled plasma-mass spectrometry (ICP-MS; Perkin Elmer, Elan 6000, USA) analysis.

After phloem exudate collection, the shoots were divided into five strata: the inflorescence; young open leaves (YOL; the uppermost three leaves); recently matured leaves (RML; leaves 4, 5, and 6 from the top); matured leaves (ML; leaves 7, 8, and 9); and the rest of leaves (RL; below the 10th leaf of the main stem). The petioles were separated from the leaflets for plant analysis. The inflorescence was separated into flower buds and inflorescence stems (termed flower stalks).

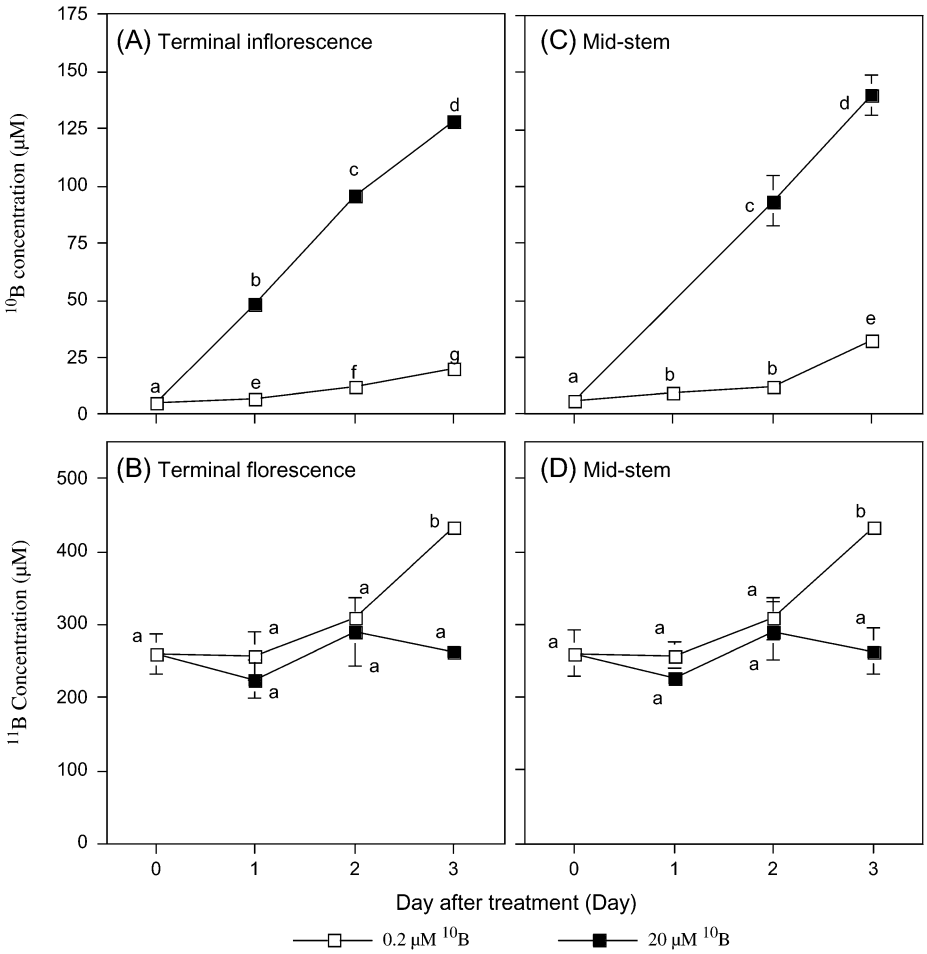

Fig. 2. Changes in ^{10}B (A, C) and ^{11}B (B, D) concentrations in phloem exudate collected from the base of the terminal inflorescence (A, B) and from the mid-stem (C, D) of lupin plants subject to B treatments: 0.2 μM or 20 μM ^{10}B in the nutrient solution for 3 d. Values are means of four replicates ±SEM. The means labelled with different letters represent a significant difference at $P \leq 0.05$.

Boron isotope analysis

Plant samples were dried at 70 °C to constant weight, finely ground, and then digested in concentrated nitric acid by means of a microwave digestion system (CEM Mars5, USA) (Huang *et al*., 2004). Sample digests were dissolved in B-free water. A known level of Be was added as an internal standard to all sap and digest samples and standard solutions for the analysis of ^{10}B and ^{11}B concentrations by ICP-MS.

Data analysis

The contents and concentrations of $^{10/11}B$ in plant parts, phloem exudate, and xylem sap were compared using the analysis of variance for treatment effects. Differences among mean values were determined by Duncan's multiple range test ($P \leq 0.05$) with the SPSS statistical package (SPSS, version 6.0, SPSS Inc., 1993).

Results

Boron in phloem exudate and xylem sap

Boron concentrations in the phloem exudates were substantially higher than those in the xylem sap (Figs 1–3). In Experiment 1, using soil culture containing adequate B supply, B concentrations were the highest (400–500 μM) in the phloem exudates collected from the stem segment immediately adjacent to the inflorescence (the oldest first order branch), and 25% lower (300–420 μM) in the upper, mid, and basal stem segments. In contrast, B concentrations in the xylem sap were only 25–35 μM (Fig. 1).

In Experiment 2, after the interruption of ^{11}B supply in the nutrient solution, the level of ^{11}B concentrations in the phloem exudates remained relatively stable at 200–300 μM over the first 2 d, but significantly increased up to 430 μM in the 0.2 μM ^{10}B treatment at day 3 (Fig. 2). The ^{10}B concentration in the phloem exudates from the terminal inflorescence and mid-stem linearly increased with time in the 20 μM ^{10}B treatment, up to 120–130 μM ^{10}B while, surprisingly, the concentration of previously acquired ^{11}B remained at 200–300 μM in the phloem exudates, even 3 d after removal of ^{11}B from the nutrient solution (Fig. 2).

The level of ^{10}B in the xylem sap rapidly increased up to ~15 μM in the 20 μM ^{10}B treatment at day 1 after commencing the treatment and remained relatively stable for the rest of the experimental period (Fig. 3). Regardless of the ^{10}B treatments, significant levels of ^{11}B in the xylem sap were detected over the 3 d (Fig. 3). However, the previously acquired ^{11}B concentration in the xylem sap remained as high as 10–15 μM in the xylem sap for 2 d, and increased to >15 μM at day 3 in the 0.2 μM ^{10}B treatment. In contrast, the ^{11}B concentration in the xylem sap significantly declined to 7–8 μM at day 3 in the 20 μM ^{10}B treatment (Fig. 3).

Boron partitioning in reproductive organs and leaves

Despite the biomass increment, ^{11}B concentrations in the inflorescence components (flower buds and stalks) remained relatively stable at 37–50 mg kg^{-1} dry weight, in 0.2 μM ^{10}B over the 3 d (Fig. 4). In contrast, there was a linear increase in ^{10}B concentration in the flower buds and stalks over the 3 d, in response to 20 μM ^{10}B supply. Coincidently, ^{11}B concentrations declined significantly over the 3 d in both ^{10}B treatments in the old leaves (ML), but decreased by day 2 and remained stable at day 3 in the oldest group of leaves (RL) in which early senescence was observed (Table 1). Comparatively, the decrease in ^{11}B concentrations in the old leaves (ML and RL) was larger at 0.2 μM ^{10}B than that at 20 μM ^{10}B treatment.

Even though the supply of ^{11}B to the roots was interrupted, there was a continual increase in ^{11}B content over the 3 d in both the flower buds and stalks at 0.2 μM ^{10}B (Fig. 5). The net increment of ^{11}B in the inflorescence at 20 μM ^{10}B was smaller than that at 0.2 μM ^{10}B (Fig. 5). The ^{10}B contents in the flower buds and stalks of the inflorescence increased linearly with time in the 20 μM ^{10}B plants, but only a small change occurred in the 0.2 μM ^{10}B treatment (Fig. 5).

The effects of the ^{10}B treatments on leaf B content were observed mostly in the leaf blades (Fig. 6a, b). At both

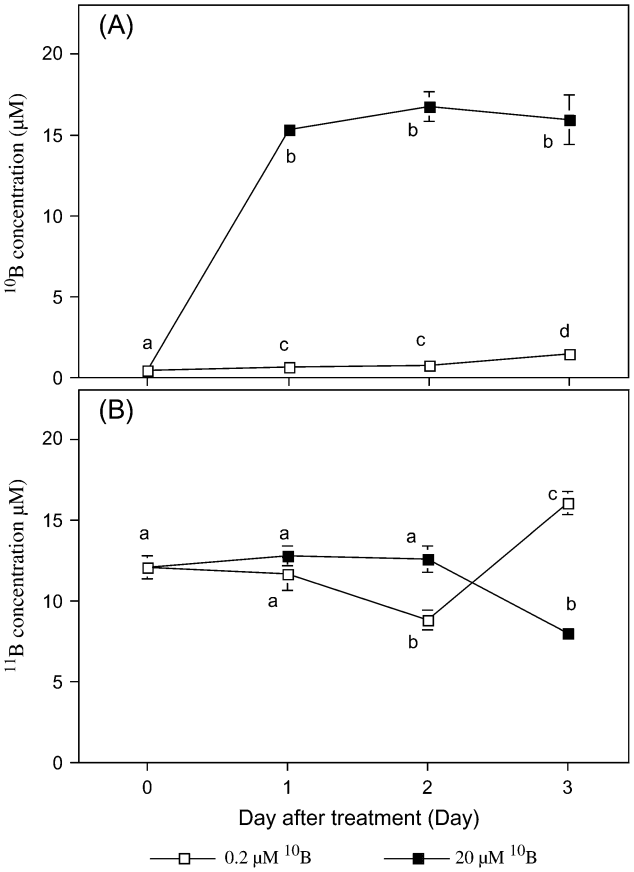

Fig. 3. Changes in B isotope concentrations in the xylem sap at the stem base, in response to the B supply treatments: 0.2 μM or 20 μM ^{10}B in the nutrient solution for 3 d. The means labelled with different letters represent a significant difference at $P \leq 0.05$.

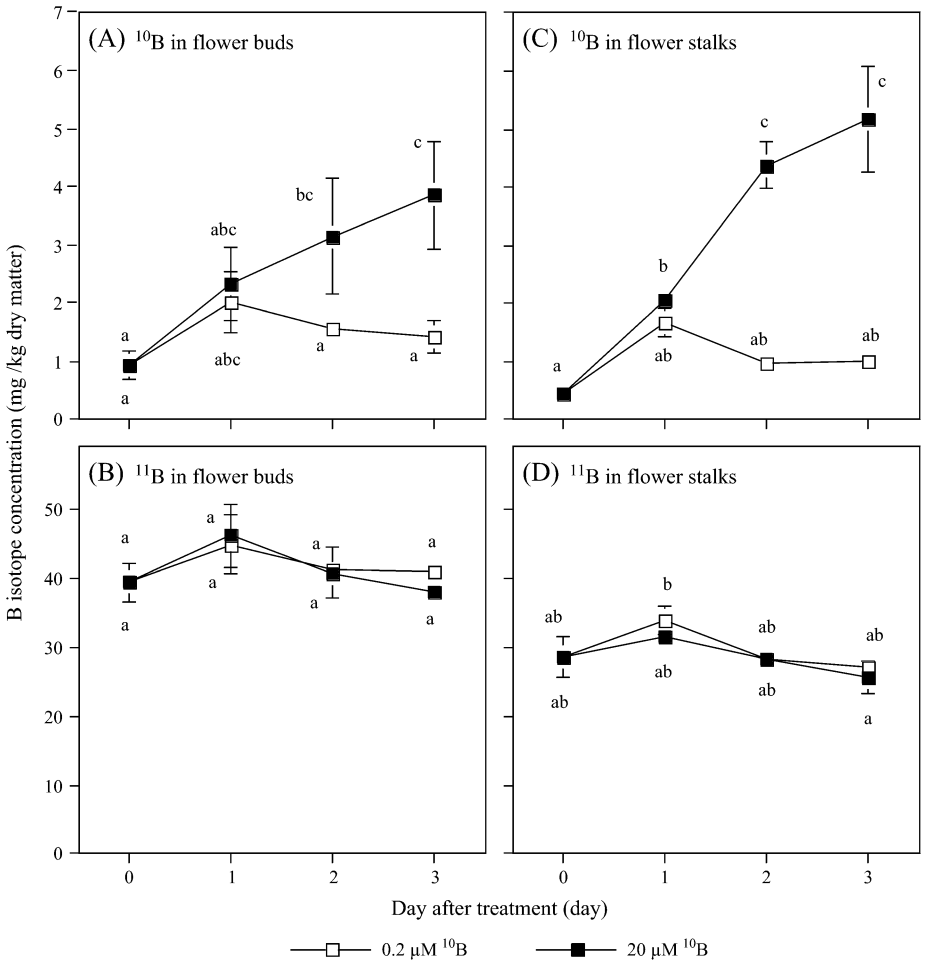

Fig. 4. Boron isotope concentrations in the flower parts of lupin plants subjected to B supply treatments of 0.2 μM or 20 μM ^{10}B in the nutrient solutions for 3 d. The means labelled with different letters represent a significant difference at $P \leqslant 0.05$.

0.2 μM and 20 μM ^{10}B, the ^{11}B content in YOL and RML blades rapidly declined over the 3 d. Regardless of the B treatments, the ^{11}B content in the petiole of YOL remained relatively stable (Fig. 6a), but decreased in the RML petioles (Fig. 6b). The ^{10}B content at 20 μM ^{10}B increased linearly with time in the YOL and RML blades and petioles (Fig. 6a. b).

Discussion

The present study is the first to demonstrate the retranslocation of B from the shoot to the root in crop species without a polyol transport mechanism, and B retranslocation out of mature leaves into actively growing reproductive organs via phloem and xylem. This is supported by several pieces of evidence, notably: (i) the presence of significant levels of ^{11}B in the xylem sap 3 d after the removal of ^{11}B supply in the nutrient solution. Indeed the ^{11}B increased at day 3 in the 0.2 μM ^{10}B treatment, but decreased in the 20 μM ^{10}B treatment. (ii) The presence

Table 1. *The concentrations (mg B kg^{-1} dry matter) of ^{10}B and ^{11}B in old leaves (ML, RL) of lupin plants, in response to the sequential supply treatments of ^{11}B and ^{10}B*

At the end of the ^{11}B supply period, the plants were subject to 0.2 μM and 20 μM ^{10}B supply in the nutrient solution for 3 d. The values are averages of three replicates with standard errors in parentheses.

^{10}B treatment (μM)	Days after treatment		
	1	2	3
Matured leaves (ML)			
	^{10}B concentration		
20	3.16 (0.240)	6.65 (0.597)	8.53 (0.443)
0.2	1.22 (0.282)	1.70 (0.307)	1.06 (0.157)
	^{11}B concentration		
20	44.6 (2.64)	37.5 (1.55)	35.7 (1.75)
0.2	41.2 (2.05)	37.0 (1.43)	31.6 (0.86)
Rest of leaves (RL)			
	^{10}B concentration		
20	2.33 (0.245)	4.12 (0.856)	4.88 (0.279)
0.2	0.792 (0.142)	0.782 (0.117)	0.699 (0.013)
	^{11}B concentration		
20	46.7 (4.33)	37.6 (1.94)	39.4 (2.37)
0.2	42.9 (1.23)	34.7 (2.98)	35.7 (0.51)

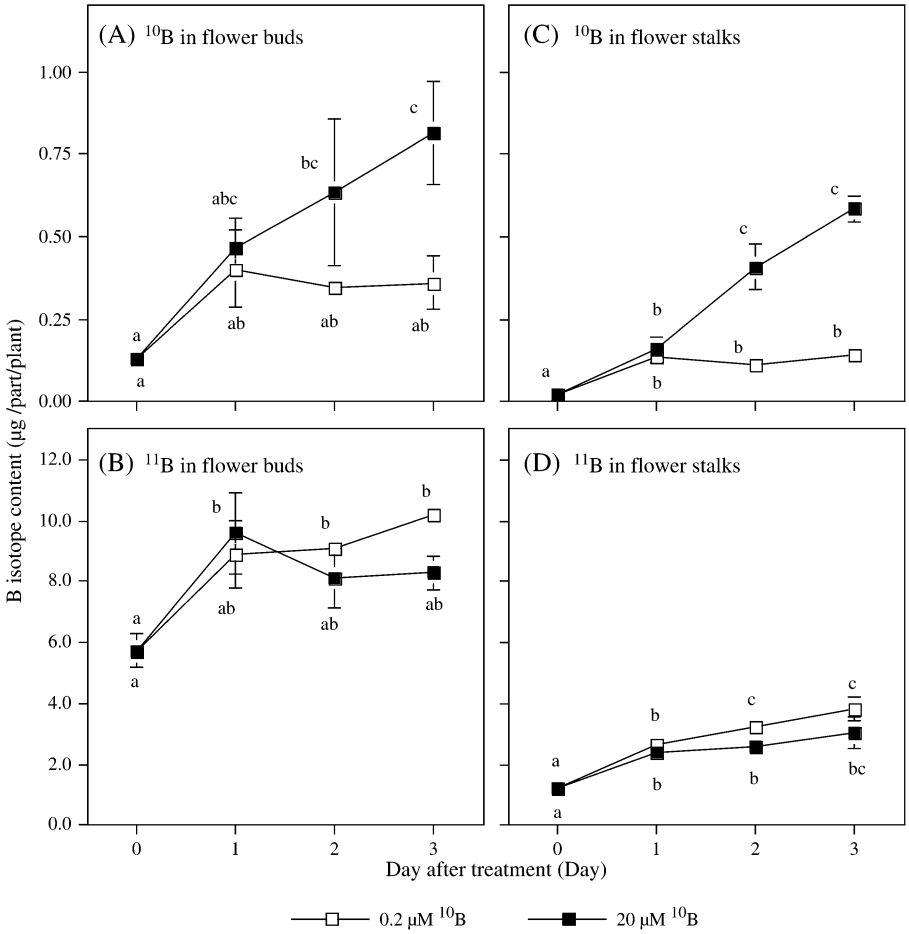

Fig. 5. Boron isotope contents in the flower parts of lupin plants subject to B supply treatments of 0.2 μM or 20 μM ^{10}B in the nutrient solutions for 3 d. The means labelled with different letters represent a significant difference at $P \leqslant 0.05$.

of high levels of ^{11}B in the phloem exudates collected from the base of the terminal inflorescence and the mid-stem positions, which increased up to 430 μM in the 0.2 μM ^{10}B treatment; and (iii) continued net transport of ^{11}B into the flowers (particularly at 0.2 μM ^{10}B) coinciding with the rapid loss of ^{11}B out of the leaf blades of mature and old leaves, which resulted in stable ^{11}B concentrations in the flowers despite their increase in dry matter and the termination of external ^{11}B supply for 3 d.

It is highly likely that B in the shoot was recirculated into the root via the phloem, transferred into the xylem, and carried upwards in the xylem sap, because there were 7–17 μM ^{11}B present in the xylem sap over the 3 d after withdrawing ^{11}B supply in the external nutrient solution. The source of this ^{11}B in the xylem sap cannot be quantitatively accounted for by the residual effects of water-soluble B stored in root cells (Dannel *et al*., 2000). A substantial proportion of B was detected in the symplastic pool of root cells of sunflower plants, when provided with 50 μM B, but not in those with 1 μM B (Dannel *et al*., 2000). In sunflower, the newly absorbed B accounted for >80% of the B in xylem sap when supplied with 10 μM B (Matoh and Ochiai, 2005). However, the 'old' B (^{11}B) in the xylem sap still accounted for almost 40% of the xylem B in the present study. If only root uptake of external B occurred without recirculation of ^{11}B from the shoot to the root, the high levels of ^{11}B would not have been detected in the xylem sap of the lupin plants. The recycled ^{11}B in the phloem may have been efficiently loaded into the xylem in the roots by the B transporters in the root pericycle cells (Takano *et al*., 2002).

In addition, the retranslocation of B from the shoot to the root seemed to be regulated by the concurrent B supply to the root, as the concentration of the previously absorbed ^{11}B significantly increased in the xylem sap at day 3 in the 0.2 μM ^{10}B treatment, while it declined in the xylem sap at 20 μM ^{10}B. This suggests that increased recirculation of ^{11}B from shoot to root, coinciding with increased translocation of ^{11}B out of mature and old leaves into the phloem at 0.2 μM ^{10}B, was a response to B demand in the inflorescence that was the most actively growing shoot organ at the time. In contrast, a decreased recirculation of ^{11}B from shoot to root may have occurred

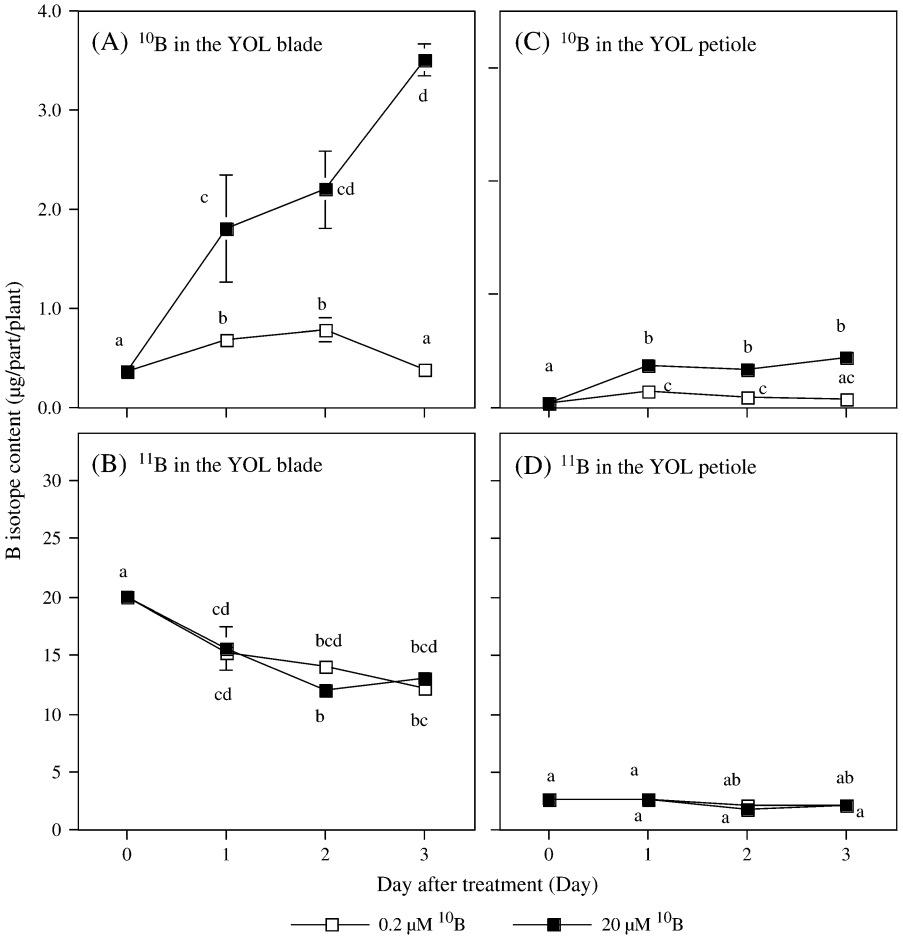

Fig. 6. (a) Boron isotope contents in the youngest open leaf (YOL) blade and petiole leaf parts of lupin plants subject to B supply treatments of 0.2 μM or 20 μM ^{10}B in the nutrient solutions for 3 d. The means labelled with different letters represent a significant difference at $P \leqslant 0.05$. (b) Boron isotope contents in the recently matured leaves (RML) of lupin plants subject to B supply treatments of 0.2 μM and 20 μM ^{10}B in the nutrient solutions for 3 d. The means labelled with different letters represent a significant difference at $P \leqslant 0.05$.

when supplied with adequate B in the nutrient solution (20 μM ^{10}B) due to the direct transport of concurrent ^{10}B uptake into the inflorescence.

The possibility of B retranslocation in lupin plants is further supported by the substantial levels of ^{11}B in the phloem exudates collected from both the mid-stem and the base of the terminal inflorescence. In particular, there was a trend for an increase in ^{11}B concentration over the first 2 d (from 250 μM to 300 μM) and a significant increase at day 3 (~430 μM) in the 0.2 μM ^{10}B treatment. This coincided with the rise of ^{11}B in the xylem sap at day 3 in the plants treated with 0.2 μM ^{10}B. Increased ^{11}B recirculation into the roots and transport upwards to the shoot would be needed to meet the demand of the rapidly growing sink—the inflorescence—when the roots were not supplied with adequate B. The circulation of K^+ and Mg^{2+} has been reported previously in white lupin: 76% or 87% of the phloem-borne K^+ and Mg^{2+} were circulated into the root and re-entered the xylem (Jeschke *et al.*, 1985), and then were preferentially transported into the inflorescence (Jeschke *et al.*, 1987). The preferential partitioning of recirculated B into growing tissues may have occurred as net ^{11}B content actually declined in the top leaves (YOL group) while it increased in the rapidly growing inflorescence.

The ^{11}B in the phloem exudates was most likely to have come from that translocated out of young, mature, and old leaves (YOL, RML, ML, and RL), but the proportion was not quantitatively determined. There may be some initial water-soluble ^{11}B stored in stem tissues (Matoh and Ochiai, 2005), but this pool of B would have been depleted quickly without continual supply of ^{11}B. Decreased B concentrations in mature/old leaves were observed in canola plants with interrupted B supply, particularly in B-efficient cultivars (Stangoulis *et al.*, 2001). The retranslocation of ^{11}B out of the mature leaves was enhanced when external ^{10}B supply was very low (0.2 μM), coinciding with the rise of ^{11}B concentration in the phloem sap at day 3. In contrast, the newly absorbed ^{10}B in 20 μM ^{10}B plants contributed <30% towards the total B in the phloem exudates.

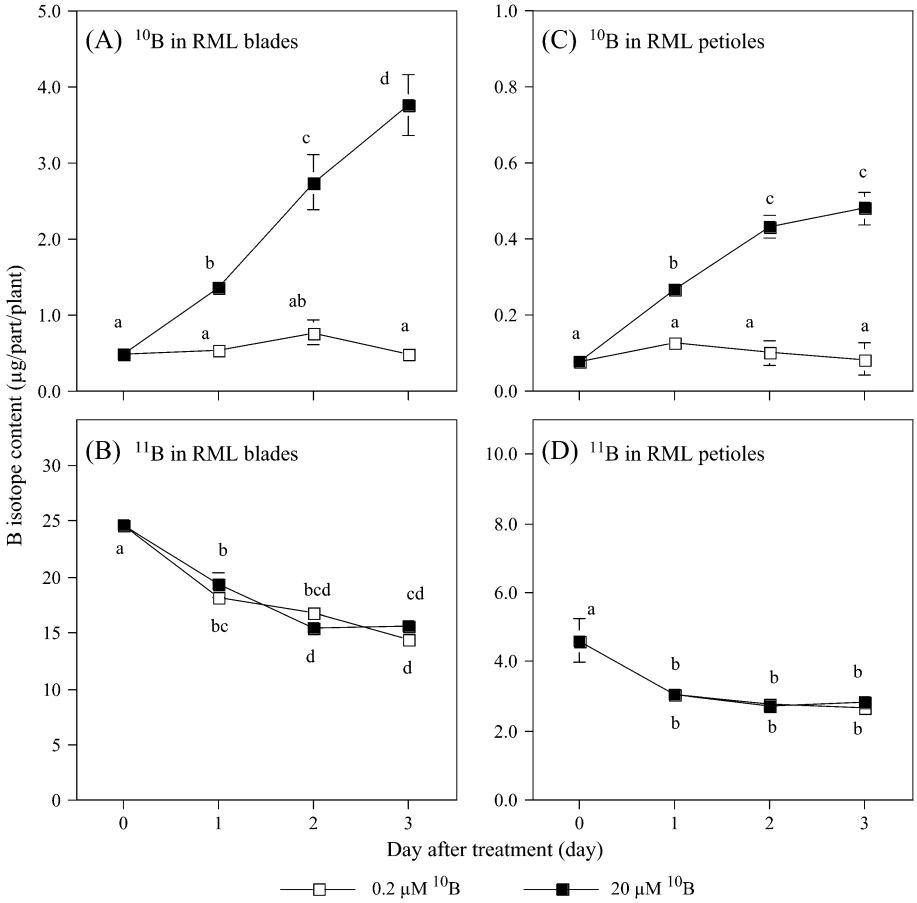

Fig. 6. (b) (*Continued*).

B concentrations (300–500 μM) in the phloem exudates were substantially higher than those in the xylem sap (10–30 μM) within the 3 d period, regardless of B supply to the roots. By calculation, the ratios of total B concentrations in the phloem exudates to those in the xylem sap were ~13 and 18 in 20 μM and 0.2 μM ^{10}B treatments, respectively. In comparison, the ratios of K (phloem-mobile) and Ca (phloem-immobile) concentrations in the phloem exudates from lupin (*L. albus*) fruit tips to those in the xylem sap are ~17 and 1.2, respectively (Pate, 1975). Marentes *et al*. (1997) found that the ratios of B concentrations in the phloem exudates (380–30 μM) to those in the xylem sap ranged from 4 to 23 in lupin plants.

Collectively, the evidence presented here strongly suggests that the previously acquired ^{11}B in the shoot can be recirculated to the root via the phloem within the short term. This is supported by the significant levels of ^{11}B in the xylem sap over the 3 d following interruption of ^{11}B supply to roots, coinciding with the pattern and levels of ^{11}B changes in the phloem exudates collected from the upper stem (the base of the terminal inflorescence) and the mid-stem. The results support the argument that previously acquired B can be translocated out of mature/old leaves of the lupin plant into the rapidly growing inflorescence via recirculation in the phloem to the root, followed by xylem transport to the shoot. Nevertheless, uncertainty remains regarding the amount of B available for recirculation and whether it would be adequate to support reproductive growth during a prolonged period of low B uptake by roots, since a significant proportion of B in vegetative tissues is bound in cell walls and not available for retranslocation, especially in low B plants (Matoh and Ochiai, 2005). Further investigation using techniques such as split-root studies with B isotopes are necessary to quantify the amount of B in the shoot that is recirculated to the root and from there to rapidly growing vegetative and reproductive tissues.

Acknowledgements

We thank Dr Brenda Rohl and Mr Michael Smirk at the University of Western Australia for their invaluable help in the analysis of B isotopes by the ICP-MS (PE, Elan 6000). Ms Jinjuan Zhang provided assistance in the glasshouse experiments. The present study was funded by an Australian Research Council small grant at Murdoch University.

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