Phloem transport of boron within avocado trees

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Abstract. A continuous supply of boron is vital for the formation of new cells during plant growth and development, and reproductive growth is particularly sensitive to any deficiency in boron supply. Insufficient transport of boron to flowers and developing fruitlets could be an important factor contributing to alternate bearing in avocado. In many plant species boron is not phloem mobile, as the mineral form of boron quickly leaks from the phloem into surrounding tissues, so distribution throughout the plant depends completely upon continuous xylem supply. Some plants have polyols (e.g. sorbitol, mannitol) as a component of their phloem sap, which forms a complex with boron ions, preventing its leakage from the phloem and making it phloem mobile. This has been well documented in many tree crops, such as apple and almond. Our hypothesis is that the presence of perseitol (a polyol) in the phloem sap of avocado results in boron being phloem mobile. While this has been previously suggested in the avocado literature, it has never been demonstrated. In this work we demonstrate that boron forms a complex with perseitol and that boron moves from mature to immature leaves and to flowers. This is consistent with boron being phloem mobile in avocado and confirms that mature leaves act as sources of boron to both vegetative and reproductive growth.

Key words. Avocado, boron, phloem, xylem

Introduction Boron (B) is a vital micro-nutrient for all plant growth. With hydroponic plants, where root growth is easily visualised, root growth is found to stop within minutes of replacing the root medium with a B-deficient medium. Such experiments show that plants require a continuous supply of B. Its primary role is in cell wall biosynthesis (Hu et al 1996). Plant cell walls consist of a matrix of cellulose and hemicelluloses fibres linked together by pectin. Intercellular attachment and plastic extensibility of cell walls are vital in the development of plant tissues, which is achieved by boron linkages between the pectin components. Under B-limiting conditions, cell wall B represents ~95% of cellular B. B deficiency results in the physical properties of the cell wall being markedly altered, correlating with the observed rapid inhibition of cell expansion. This role of B in the cell wall is consistent with the observation that B cannot be re-utilised and must be continuously supplied during plant growth

Higher plant requirement for B varies with species and tissue. For example, graminaceous monocot plants contain only a small amount of pectin, so have a low B requirement (3-10 µg B g^{-1} DW), while dicots have a relatively high pectin content and much higher B requirement (20-30 µg B g^{-1} DW) (Hu et al 1996).

B deficiency causes problems in the growth and development of higher plants, especially in reproductive growth. Inadequate B delivery to a developing flower has the potential to reduce fertilisation and fruit set. The gene responsible for borate cross-linking of pectin is strongly expressed in male and female tissues involved in fertilisation, and suppression of this gene causes defects in the development and function of the tissue of the pistil, pollen formation and pollen-tube germination and elongation (Iwai et al 2006).

Higher plants have two long-distance transport pathways. The xylem, responsible for water and mineral supply from the soil, uses a non-living structure for its conduits. Xylem flow is driven by transpiration from the aerial tissues, creating xylem tension that draws xylem sap up from the roots. The second long-distance pathway is the phloem. This consists of a living conduit structure, the sieve tubes, lined by a membrane. The driving force for phloem transport is generated by active loading of sugars within the mature leaves, generating a concomitant osmotic flow of water resulting in a high hydrostatic pressure, which drives flow of phloem sap from the mature leaves. Phloem transport is from the mature photosynthesising leaves to the sites of carbohydrate usage (e.g. immature tissues, reproductive organs) and storage in the stems and roots.

Within the soil environment and the xylem stream of a plant, B exits in the form of boric acid. In this chemical form, boron cannot be translocated out of a leaf within the phloem stream, as the phloem membrane is highly permeable to B. This results in it leaking out of the phloem stream into adjacent less concentrated xylem vessels and being transported back into the leaf. Hence B distribution within many plants is governed by the transpirational stream (xylem). There are many observations recorded over a long period of plants grown with adequate B supply having B concentrations that decrease from old to young leaves, and observations that B deficiency symptoms typically occur in meristematic tissues, while B toxicity symptoms occur first in the margins of the oldest leaves, which are at the end of the transpiration stream (Patrick & Hu 1996). When squash or tomato plants were transferred from adequate B supply to B-deficient conditions, the B content of the plant tissue established before the transfer did not decrease even though new apical growth was completely inhibited (Oertli 1993; Hu & Brown 1994). Such observations formed the basis of the historical classification of B as an immobile element (Zimmermann 1960).

These general patterns of B distribution and toxicity expression indicate that B is phloem immobile in most plant species, though this does not appear to be the case for species within *Prunus, Pyrus* and *Malus* genera, which include many horticulturally important fruit and nut trees (Brown & Hu 1996). When almond, peach or plum were exposed to high B in their growth medium, the predominant site of B accumulation was found to be the fruit and young tissues, and not the mature leaves. This is not consistent with B distribution within the xylem flow to sites of greatest water loss.

To be phloem mobile, boron must form a complex with a phloem mobile compound. It is now well documented that B forms a complex with sorbitol, and mannitol, both being polyols. Sorbitol is found in the phloem of the *Prunus, Pyrus* and *Malus* genera, and mannitol in some vegetables. Hence the existence of a polyol within the phloem sap is now believed to indicate that B will be phloem mobile.

The purpose of this work was to determine if B distribution within avocado trees is consistent with xylem distribution, or if there is phloem remobilisation from the mature leaves. Avocados are known to transport the polyol perseitol in the phloem sap. While this mode of phloem transport has been proposed, it has never been demonstrated in avocado.

Methods

Sample collection.

All samples were from 'Hass' avocado. New Zealand samples were collected from the Te Puke Research Centre in the Bay of Plenty (37˚ 49' S, 176˚ 19' E), and the Californian samples collected from the University of California South Coast Research and Extension Centre, Irvine (33˚ 31' N, 117˚ 43' W).

B-perseitol complex

The possibility of perseitol forming a complex with B was tested by mass spectroscopy of laboratory-prepared solutions. 10mM boric acid was mixed with 2mM ammonium acetate buffer (pH 8.0) and 1-10mM perseitol. This solution was then infused into a mass spectroscope.

Mineral analysis of leaves

Young avocado leaves were compared with mature leaves for both boron and calcium concentrations, calcium being an example that is not phloem mobile. Young avocado leaves are pinkish-red in colour, with this colour being gradually lost over several weeks as the leaf matures. This colouration was used as a marker of leaf age, with mature leaves defined as ones with no pink colouration, and a young leaf as one that was strongly coloured over its entire lamina and less that 40 mm in length. Samples of mature and young leaves were collected in New Zealand from the same vegetative shoots of four two-year-old 'Hass' trees that were still too young to flower. Another set of samples were taken from more mature trees.

These leaf samples were oven dried at ~70˚C, crushed and a sample ground then digested in nitric acid-perchloric acid (AOAC 1995), and the boron and calcium content determined by mass spectroscopy.

^{10}B labelling

About three weeks before flowers opened, the most apical mature leaf of three floral shoots of three on-flowering and three off-flowering trees were labelled with ^{10}B by immersing the leaf for five seconds into a solution of 1000 ppm ^{10}B in the form of boric acid in 0.05% Tween®20 (V/V) that had been pH adjusted to 6.5 with HCl. Samples for analysis were collected at mid-bloom and kept at -20˚C for analysis. Analysis involved dissolving 1 g of sample in 4 ml of HNO_3 (70% commercial conc., diluted 1 to 2) and 10 ml of HCl (36% commercial conc., diluted 1 to 5). These were refluxed at 75˚C for 2 hours in plastic 50-ml tubes, then left to settle over night and then diluted with water to 500 ml. An aliquot of this solution was then analysed for B using ICP/MS (PerkinElmer Elan DRCII/MS).

B concentrations in phloem sap

The currently accepted best method for collecting phloem exudate (sap collected from a severed sieve tube) is either by using an aphid stylet (van Helden et al 1994) or by cutting and collecting into EDTA (ethylenediaminetetraacetic acid),buffer solution (King & Zeevaart 1974). There is no suitable avocado phloem-feeding insect in New Zealand, so we had to use the latter method. The original method has been improved by solidifying the collection medium with agarose and including a bactericide (Lejeune et al 1993; Klages et al 1998).

Samples of phloem sap were collected from a major peduncle of the inflorescences of both on- and off-flowering 'Hass' avocado trees, both in New Zealand and California, by bleeding into a vial containing 0.7% agarose (w/v) made without EDTA (water controls) or with 5 mM EDTA pH adjusted to 5.5 with NaOH, and both containing 20 µg ml^{-1} chloramphenicol and 0.1 mg adonitol as an internal standard. Samples were collected for 24 h, and kept for analysis at -80˚C. Boron measurements involved extracting in methanol and direct injection of 20 µl into the sample stream of a mass spectrometer (Brown et al 1992).

Statistics

ANOVA statistical analysis was carried out in R (xxx.r-project.org). A result is stated to be significant when $P < 0.05$.

Results

Laboratory solutions

With a perseitol-B mixture (1:10mM), data shown in Fig. 1 showing the presence of two major ions m/z 431 and 211 were found. The first of these was identified as the complex perseitol-B-perseitol (P-B-P') and the latter as de-protonated perseitol (P').

Leaf mineral ion concentrations

Samples of mature and young leaves were collected from the same shoots, so a paired comparison was possible, which is a much more powerful test than on simple means. The boron concentration in the young leaves was significantly higher than in the mature leaves, while the calcium concentrations were significantly higher in the mature leaves (Fig. 2).

^{10}B labelling

Three weeks after application of ^{10}B to the most apical mature leaf of a flowering shoot, the $^{10}B/^{11}B$ atom ratios of the flowers and young leaves were measured (Fig. 3). The natural atom ratio (Lederer et al 1968) is 0.244. Control values observed on unlabelled mature avocado leaves were significantly higher, demonstrating discrimination against the heavier isotope. Similar discrimination is seen between ^{12}C and ^{13}C (Bender 1968; Farquhar et al 1982). In both the on- and the off-flowering trees, the flowers and young leaves had $^{10}B/^{11}B$ ratios significantly above those of the controls, which implies that ^{10}B applied to the mature leaf must have been transported into the new tissues.

Phloem boron

Boron was found in all the phloem samples collected. There was no significant difference in total B collected in the phloem sap of on- and off-flowering trees in either New Zealand or California. The average amounts collected over 24 h in New Zealand from the on- and off flowering trees was 41 ng (±12ng, n=8) and in California was 27 ng (±5ng, n=9).

Discussion

Mass spectroscopy experiments confirmed that perseitol does form a complex with boron, similarly to the more common plant polyols investigated by other groups. Hence perseitol present in the phloem sap of avocado can be expected to form a complex with B, making B phloem mobile.

Calcium is well known to be delivered into all plant tissues within the xylem sap, and not to be transported within the phloem (Marschner 2005). So calcium is a good example of a xylem mobile mineral and if boron is also only xylem mobile, then the distribution of both of these would be similar. We expected that the mature leaves would have accumulated more calcium from their longer period of xylem sap import and resulting accumulation than younger leaves. This is what was observed (Fig. 2).

If boron was similarly accumulated in the transpiring leaves, and not exported in the phloem sap, then we would expect to have seen the same pattern as seen with calcium. This was not the case. With boron the younger leaves were found to have a higher concentration of boron than that found in the mature leaves. This is consistent with boron being exported from the mature leaves to the immature leaves concomitant with the younger leaves also accumulating boron from their xylem import. The export of boron from the mature leaves would be occurring along with export of carbohydrate, including perseitol, produced by photosynthesis within the mature leaf supplying the

immature growing leaf. Phloem perseitol would be a vital part of this process, providing the complexing agent to render leaf boron mobile within the phloem.

Boron occurs in nature as a mixture of the two isotopes ^{10}B and ^{11}B, with the ratio $^{10}B/^{11}B$ of naturally occurring boron being 0.244. While the two isotopes of boron are chemically identical, they do have small differences in mass, resulting in small differences in the rates of physical and chemical processes. This results in a small discrimination towards the lighter isotope (Fig. 3). The export of boron from a mature leaf was demonstrated by labelling a mature leaf with ^{10}B, and then finding this isotope of boron at above naturally occurring concentrations within the immature tissues. The only way that the ^{10}B concentrations could have increased above the naturally occurring concentrations was by import from the mature leaf labelled with pure ^{10}B.

Phloem sap is notoriously difficult to collect. Use of a phloem feeding insect is the most reliable method, but the EDTA method much simpler and hence more frequently used. The EDTA method involves severing the tissue, so there is a huge potential for contamination of the phloem sap with cellular contents released when the tissue is damaged, and for mixing with apoplastic content during the entire collection time. A water blank is used to allow for potential contamination.

Boron was found in phloem sap of avocado, indicating that it is phloem mobile. The amount collected was very small - in the order of 30-40 ng being delivered through a major peduncle of the inflorescences over 24 h. With the EDTA collection method we have no measurement of the volume of phloem sap collected, so cannot calculate the B concentration.

In summary:

1) We have demonstrated that perseitol does form a complex with boron. This is directly comparable with other polyols found in the phloem sap of other plant species now believed to transport boron within their phloem sap.
2) Boron concentration in mature leaves has been shown to be much lower than that found in young leaf tissues, consistent with re-mobilisation of B within the mature leaves, with the phloem sap exported to young tissues, including developing leaves. This is exactly opposite to that found with calcium, which is well known not to be phloem mobile, and is also seen with B in plant species know not to transport B in their phloem sap.
3) Boron was found in the phloem sap of avocado.

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References

AOAC 1995, *Official methods of analysis: Plants.* In: Cunniff P (ed.). Official Methods of analysis. 16th Edition, Association of Official Analytical Chemists, Arlington, VA, USA. 3-4.

Bender, MM 1968, Mass spectrometric studies of carbon-13 variations in corn and other grasses, *Radiocarbon,* vol 10, pp. 468-472.

Brown & Hu 1996 Phloem mobility of boron is species dependent: Evidence for phloem mobility in sorbitol-rich species, *Annals of Botany,* vol 77, pp 497-505.

Brown, PH, Picchioni, G, Jenkin, M & Hu, H 1992, Use of ICP-MS and ^{10}B to trace the movement of boron in plants and soil, *Communications in Soil Science and Plant analysis,* vol 23, pp. 2781-2807.

Farquhar, GD, O'Leary, MH & Berry, JA 1982 On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology*, vol 9, pp 121-137.

Hu H & Brown PH 1994, Localization of boron in cell walls of squash and tobacco and its association with pectin, *Plant Physiology,* vol 105, pp. 681-689.

Hu H, Brown PH & Labavitch JM 1996, Species variability in boron requirement is correlated with cell wall pectin, *Journal of Experimental Botany*, vol 47, pp. 227-232.

Iwai, H, Hokura A, Oishi M, Ishii T, Sakai S & Satoh S 2006, The gene responsible for borate cross-linking of pectin rhamnogalacturonan-II is required for plant reproductive tissue development and fertilization, *Proceedings of the National Academy of Sciences,* vol 103, pp. 16592-16597.

King, RW & Zeevaart, JAD 1974, Enhancement of phloem exudation from cut petioles by chelating agents, *Plant Physiology*, vol 53, pp. 96-103.

Klages, K, Donnison, H, Boldingh, H & MacRae, E 1998, Myo-inositol is the major sugar in *Actinidia arguta* during early fruit development, *Australian Journal of Plant Physiology,* vol 25, pp. 61-67.

Lederer, CM, Hollander, JM & Perlman, I 1968, *Table of Isotopes*, 6th edition, Wiley.

Lejeune, P, Bernier, G, Requier, M -C and Kinet, L -M 1993, Sucrose increase during floral induction in the phloem sap collected at the apical part of the shoot of the long-day plant *Sinapis alba* L, *Planta*, vol 190, pp. 71-74.

Marschner 2005 *Mineral nutrition of higher plants*, 2nd edition, Academic Press.

Oertli, JJ 1993, The mobility of boron in plants, *Plant and Soil,* vol 155/156, pp. 301-304.

Patrick, H, Hu, H 1996, Phloem mobility of boron is species dependent: Evidence for phloem mobility in sorbitol-rich species, *Annals of Botany*, vol 77, pp. 497-505.

Van Helden, M, Tjallingii, WF & van Beek, TA 1994, Phloem sap collection from lettuce (*Lactuca sativa* L.): Chemical comparison among collection methods, *Journal of Chemical Ecology,* vol 20, pp. 3191-3206.

Zimmermann, MH 1960, Transport in the phloem, *Annual Review of Plant Physiology*, vol 11, pp. 167-190.

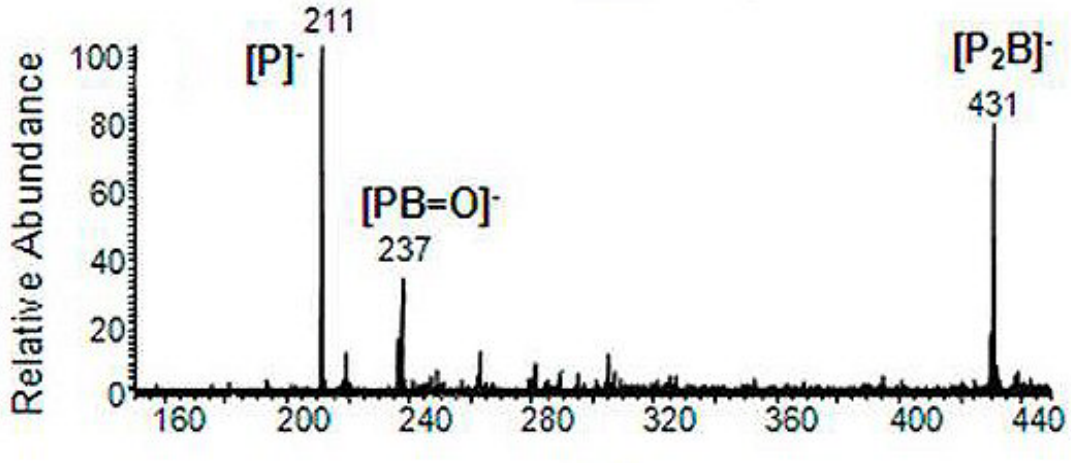

Figure 1. Mass spectrometer scan of perseitol boric acid solutions (1:10mM). Peaks for ionised perseitol (P-), perseitol-boron and perseitol-boron-perseitol are labelled.

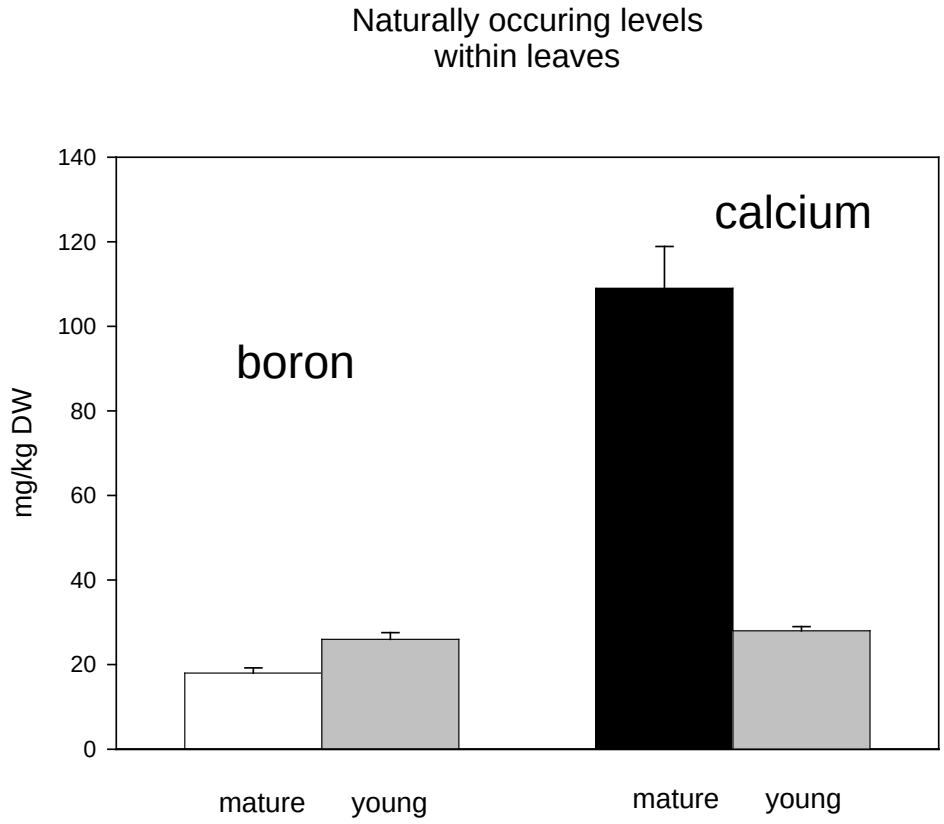

Figure 2. Boron and calcium (Ca) concentrations in mature and young avocado leaves. With blocking by tree, ANOVA showed no significant difference between trees. Both boron and calcium concentrations were significantly different according to the paired Student t-test, which is appropriate test as all samples were paired at the shoot level.

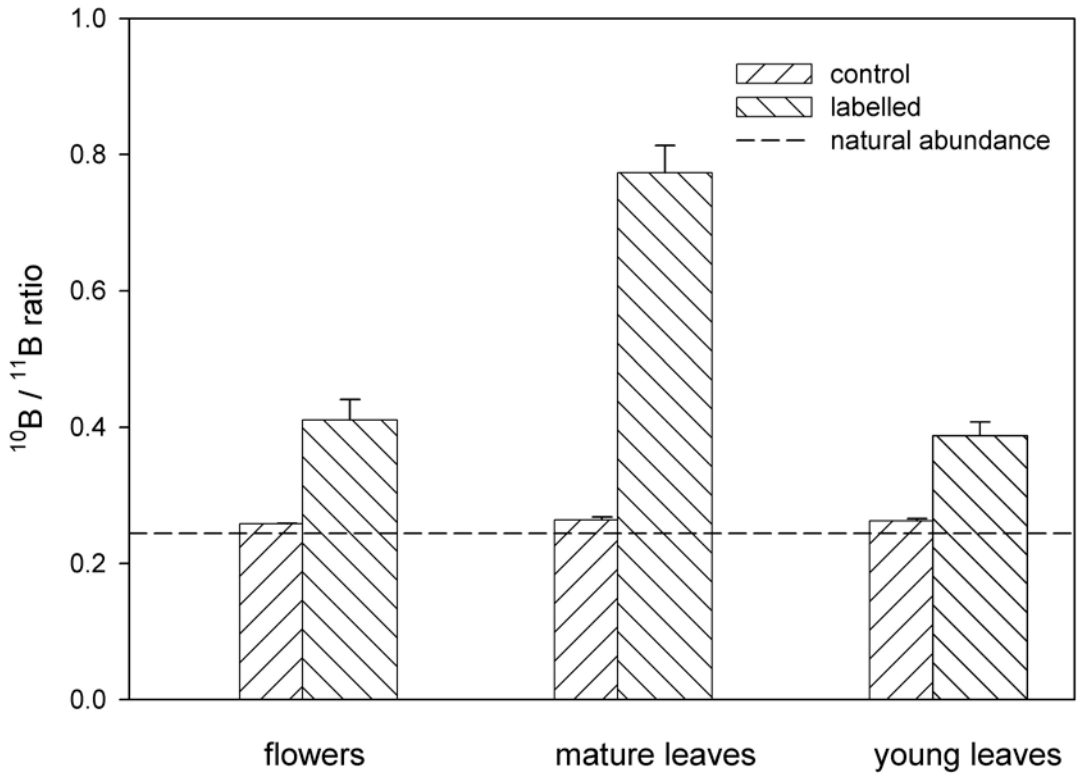

Figure 3. Boron ratio $^{10}B/^{11}B$ in avocado tissues supplied with photosynthate from the ^{10}B labelled mature leaf compared with an unlabelled shoot from the same tree. Samples were taken 3 weeks after labelling. Data are the means and standard errors of equal numbers of on- and off-flowering shoots: control n=8, labelled n=20.