

## Short communication

### Brief look at sorbitol in 1-year-old shoots of apple (*Malus domestica*)

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**Abstract** The sugar alcohol sorbitol is a major translocatory product in most commercially important species in the Rosaceae family. Sugar alcohols are thought to play a variety of roles in plant tissue, including protecting metabolism during stress. Sorbitol has been found in high concentrations in 1-year-old apple (*Malus domestica*) shoots, however studies have shown that not all tissue throughout a tree has the ability to metabolise sorbitol. In this study, we looked at sorbitol metabolism in 1-year-old apple shoots using a variety of methods including carbohydrate depletion, metabolism of <sup>14</sup>C-sorbitol, and extraction of sorbitol dehydrogenase (SDH), the enzyme necessary to convert sorbitol to fructose. Although starch stores were able to be depleted in the shoot, sorbitol levels increased, corresponding to the decrease in starch, resulting in little net loss of carbohydrate. <sup>14</sup>C-sorbitol was not metabolised and no SDH was found in shoot tissue, suggesting that sorbitol has limited metabolism in 1-year-old apple shoots. Sorbitol may thus be ideal for

translocation in apple shoot tissue because of its ready movement into the transport pathway, but its restricted utilisation there.

**Keywords** apple; sorbitol; SDH; metabolism

## INTRODUCTION

Sorbitol is a sugar alcohol, present in a number of higher plants, but found most often in the Rosaceae (Loescher 1987). Sugar alcohols are thought to play a variety of roles in protecting metabolism during stress and thus there could be economic benefit in the production of sugar alcohols in plants that only produce sucrose (Sheveleva et al. 1998). However, it is vital to fully understand sugar alcohol metabolism in the plants in which it occurs naturally before we can successfully incorporate its synthesis into other plants. In this paper, an attempt was made to understand the metabolism of sorbitol in apple (*Malus domestica* Bork L.) stem tissue, something that has not been widely studied.

Apple leaves contain sorbitol as a major carbohydrate (60–70% of total sugar or 50–150 mM) with a lesser proportion of sucrose (16–24%) (Bielecki 1977). Sorbitol is a major translocation sugar (Bielecki 1969) and in apple phloem is found to comprise 65–70% of the sugars (Klages et al. 2001). Sorbitol is also a major component of the shoot carbohydrates (McQueen et al. 2004a).

The production of sorbitol in leaf tissue in Rosaceae and the conversion of sorbitol to other sugars in terminal sink tissue such as fruit have been well studied (Loescher & Everard 1996). The majority of degradation of sorbitol in Rosaceae sink tissue depends on the presence of the enzyme SDH in the reaction:

Sorbitol + NAD<sup>+</sup> → Fructose + NADH + H<sup>+</sup>  
(Loescher & Everard 1996).

Sorbitol can also be metabolised by sorbitol oxidase, which converts sorbitol to glucose in the absence of nicotinamide adenine dinucleotide

(NAD) and nicotinamide adenine dinucleotide phosphate (NADP). However, sorbitol oxidase activity has only been found in concentrations too low to explain *in vivo* sorbitol interconversions (Loescher & Everard 1996). In sink tissue, sorbitol is extensively converted to other carbohydrates (cellulose, starch, sucrose, fructose, glucose) (Bieleski 1982). In leaf tissue, which is only a net importer of photosynthate for a short time, the ability to metabolise sorbitol is lost at maturity, i.e., once leaves have fully expanded (Zhou et al. 2001). Surprisingly, very young leaves cannot metabolise sorbitol, and only expanding leaves have the ability to both use and make it (Bieleski & Redgwell 1985). Sorbitol is also not extensively metabolised by nectaries in a variety of Rosaceae, although Bieleski & Redgwell (1980) suggested the majority of sorbitol conversion occurred in the phloem.

In this study, we attempted to determine the role sorbitol plays in 1-year-old apple stem tissue. Sorbitol is present in stem tissue in very high concentrations (McQueen et al. 2004b), greater than can be accounted for simply if it is in transit from source to sink. It was thus important as part of understanding carbohydrate movement in apple stems to determine whether sorbitol could be metabolised for use in the stem. As not all tissue in Rosaceae has the ability to metabolise sorbitol, there is a possibility that sorbitol is present in the stem as a carbon store purely for use by higher priority sinks such as fruit, which have the ability to metabolise it.

## MATERIALS AND METHODS

In these experiments, 'Braeburn' trees, grown on MM106 rootstock in a commercial orchard (Ruakura Road, Hamilton, New Zealand) were used. Trees were planted at a spacing of 4.5 × 2.75 m in east-west oriented rows. Dormant pruning, fertilisers, pesticides, and herbicides were applied as required according to standard commercial practices. To determine whether all the carbohydrates (including sorbitol) in a 1-year-old shoot were available for stem growth, seven shoots were girdled to obtain isolated experimental shoots preventing carbohydrate flow while maintaining xylem continuity, on one tree. A 2-cm-wide strip of phloem and cambial tissue was removed on 6 November 2000 and an aluminium splint was taped across the girdled area for support; the girdle was left uncovered. Leaves were removed immediately after girdling and

subsequently at 1/3 their final size before they could become carbon exporters (Hansen 1971), and thereafter checked and removed if necessary every 3 days. Leaves were also removed from seven intact (non-girdled) shoots on a different tree.

The experiment was terminated on 11 December 2000 when no new leaves grew from the girdled shoots. Shoots were then removed at the girdle and were immediately cut into small pieces and frozen in liquid nitrogen. Frozen samples were then stored at -80°C until freeze-drying. Once freeze-dried, the samples were stored at -20°C until carbohydrate extractions (via enzymatic assay) were carried out.

Shoot samples were ground into a fine powder (Wiley Mill Grinder, General Electric Motors, United States). Samples were then well mixed and re-freeze-dried before weighing. A 100 mg sample of powered tissue was subsequently extracted for carbohydrates by heating in 80% ethanol for 60 min. at 60°C. After mixing well and centrifuging, sugars were assayed (in duplicate) from the supernatant as described by Jones et al. (1977) and starch was assayed from the pellet as described by Jones (1979). Glucose and fructose concentrations were not separated but were measured together for a total value. Blank samples and standards were run for each carbohydrate extracted. Each extraction and assay also included a spiked sample for each of the carbohydrates to ensure that all the carbohydrate was extracted and that there were no enzyme inhibitors in the sample. The carbohydrate concentrations found in untreated shoots were used for comparison.

To further determine if all carbohydrates in the stem were available and could be used for metabolism, three replicate whole seedling plants (Baujade × A151R4T128; 30 cm high) were placed in full natural light (glasshouse) or in darkness (cupboard of similar temperature) and harvested 10 weeks later. Extraction of starch, sorbitol, sucrose, and glucose + fructose was carried out as described above.

To determine whether <sup>14</sup>C-labelled sorbitol or sucrose were metabolised when taken into the apple stem via a bathing solution, internodal pieces of 1-year-old shoots were cut into 1–2 mm lengths. To cut pieces of such size, the stem was split in half lengthwise to avoid the splitting of bark from wood that occurred when whole stems were used. Immediately after cutting, 10 half-round discs were placed into 10 ml of continuously aerated medium containing 20 mM 2(N-morpholino)ethanesulphonic acid (MES) buffer (pH 6.5), 1 mM CaCl<sub>2</sub> and mannitol and sorbitol or sucrose at concentrations of

0.1 and 7 mM. The osmolarity of the incubation medium was maintained at 125 OsM by addition of appropriate amounts of mannitol. The discs were equilibrated at room temperature for 30 min, followed by a change into fresh medium to remove any potential inhibition to uptake resulting from the contents of damaged cells at the tissue ends.

Uptake studies commenced with the addition of  $^{14}\text{C}$ -sucrose (7.4 MBq ml<sup>-1</sup>) or  $^{14}\text{C}$ -sorbitol (7.4 MBq ml<sup>-1</sup>). Incubation was carried out at room temperature for 2 h and was terminated by removal of the  $^{14}\text{C}$  incubation solution. The stem discs were immediately washed (3 times for 10 min per wash, with 10 ml of the initial equilibrium) to remove any radioactivity within the apparent free space of the tissue. Discs were kept on ice during washing to prevent further metabolic uptake.

In addition, a longer-term bathing period of 5 h was carried out using the method described above. Samples were analysed by HPLC (Perkin Elmer Isocratic LC Pump, Perkin Elmer PE Nelson interface, TotalChrom 6.2 software) to determine the radioactivity of products after bathing. Once extracted in 6 ml 80% ethanol (60 min at 60°C), samples were dried down and redissolved in 200 µL H<sub>2</sub>O. Samples were passed through a membrane filter before HPLC analysis using an ion-exchange lead column (Shodex SP 0810) with water as eluent (0.6 ml min<sup>-1</sup>) at 70°C. Sugars and associated radioactivity were detected using a refractive index (RI) detector (RID-6A Shimadzu) and in-line radioactivity detector with a solid glass cell (Ray Test Ramona 2000). For two of the bathed samples, fractions were collected from the HPLC at 1 min intervals. The radioactivity of these samples was determined by liquid scintillation (counted for 60 min per sample or to a precision of 2%, whichever came first).

Sorbitol dehydrogenase (SDH) was extracted from 1-year-old shoot tissue in April and December 2002, using the method of Lo Bianco & Rieger (1998). The sensitivity of the method was increased by reading the production of NADH per min at 25°C over a 5-min period on a fluorometer (Farrand Optical Co, Inc. United States) within the range of 0.1–5 × 10<sup>-9</sup> moles. In addition to 1-year-old shoot tissue, tissue known to contain SDH such as roots and growing shoot tips (including expanding leaves) was extracted. Samples were also spiked with known quantities of SDH at the assay stage to measure any SDH inhibition induced by the plant extract. Enzyme activity (nmol min<sup>-1</sup> g<sup>-1</sup> fresh weight) was calculated from the net change in fluorescence per min.

The data were analysed statistically using Analysis of Variance (ANOVA) (Minitab). The assumption that the data were independent and normally distributed was tested before an analysis was performed.

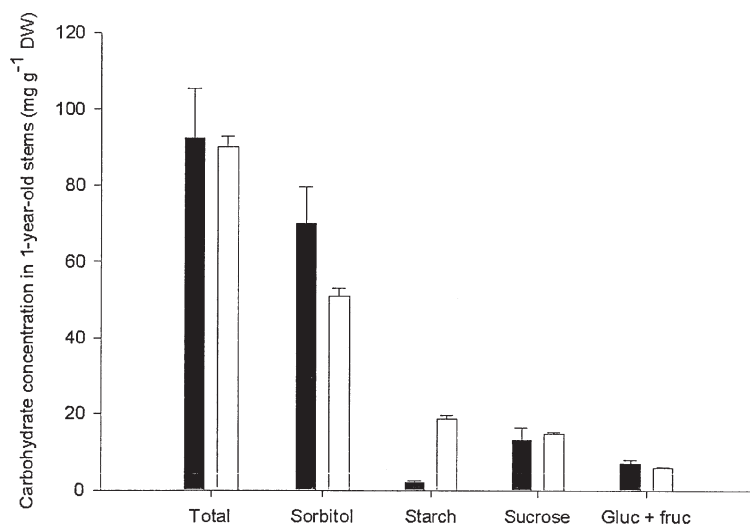
## RESULTS

The starch concentration remaining in the defoliated girdled stems was significantly lower ( $P < 0.05$ ) than the control (ungirdled, non-defoliated) shoots (Fig. 1). Concentrations of sucrose and glucose + fructose in the defoliated girdled stems corresponded to the concentrations normally found in 1-year-old shoots at that time of year. Sorbitol was present in high concentrations in defoliated girdled shoots and was significantly increased by the defoliated girdling treatment. Total carbohydrates in the defoliated girdled shoots were not significantly different from control stems. The loss of starch in the defoliated girdled shoots was paralleled by a gain in sorbitol concentration. Removing leaves on an intact shoot showed that eventually no new leaves formed on the branch.

Starch concentrations were significantly lower ( $P < 0.05$ ) in whole seedling plants kept in the dark for 10 weeks than whole seedlings kept in the light (Table 1). The concentration of sorbitol was significantly higher in plants kept in the dark than in light. Glucose + fructose was also significantly higher ( $P < 0.05$ ) in dark treated plants than those in light, whereas the sucrose concentration was not significantly different. Plants kept in the dark lost their leaves within 2 weeks.

To determine whether sorbitol and sucrose could be metabolised by the stem, stem pieces were bathed in labelled sugars and the products of metabolism measured by both an in-line solid cell radioactivity detector and scintillation counting. In stems that had been bathed in  $^{14}\text{C}$ -sorbitol, no metabolism took place as only radioactive sorbitol was found. In stems bathed in  $^{14}\text{C}$ -sucrose, metabolism of sucrose was confirmed by the presence of labelled glucose and fructose (data not shown).

SDH activity was found in growing tips and expanding leaves from trees of various ages and varieties growing at the Ruakura Research orchard (Table 2). SDH was also detected in the roots of potted plants, but never found in 1-year-old shoots at any time of the year (Table 2).



**Fig. 1** Carbohydrate concentration of girdled 1-year-old shoots when new leaves were removed before becoming net exporters of photosynthate (dark bars). Carbohydrate concentrations of control stems (not girdled) are also shown (light bars). Error bars are standard errors ( $n = 7$ ). (DW, dry weight.)

**Table 1** Carbohydrate concentration of whole plants kept in either the dark or light for 10 weeks. Letters denote significant differences ( $P < 0.05$ ).

	Carbohydrate concentration (mg g <sup>-1</sup> )			
	Starch	Sorbitol	Sucrose	Gluc + fruc
Light	67.2 <sup>a</sup>	25.1 <sup>a</sup>	8.9 <sup>a</sup>	4.8 <sup>a</sup>
Dark	41.8 <sup>b</sup>	38.3 <sup>b</sup>	10.5 <sup>a</sup>	8.6 <sup>b</sup>

**Table 2** Sorbitol dehydrogenase (SDH) activity (fresh mass basis) in various organs of apple (*Malus domestica*) at two different times of the year. Estimates of error are based upon the measurement uncertainty of SDH levels. (FW, fresh weight.)

Month	Organ	Average SDH activity (nmol min <sup>-1</sup> g <sup>-1</sup> FW)
Apr	Root	49 ± 3% ( $n = 2$ )
Apr	Stem	0 ± 0.1% ( $n = 4$ )
Dec	Growing tip	30 ± 2.4% ( $n = 4$ )
Dec	Stem	0 ± 0.1% ( $n = 5$ )

## DISCUSSION

Depletion of carbohydrates by defoliation did not occur to the extent expected. Total carbohydrates were similar in both defoliated girdled and control branches, suggesting that there is a degree of internal control of carbohydrate use within apple trees (Priestley 1970). This also suggests that there were limited sinks available to cause carbohydrate

depletion. Removing leaves on ungirdled branches showed that despite a continuous supply of carbohydrates, new leaves eventually stopped being formed. This suggests that exhaustion of leaf initiation sites prevented the growth of new leaves.

In an attempt to fully deplete the carbohydrate reserves in apple stem tissue, whole plants were kept in the dark for 10 weeks. Plants kept in the dark did show a reduction in total carbohydrates compared with their light equivalents, however, even in the dark, starch levels were not depleted completely. As in the defoliation experiment, sorbitol concentrations rose in conjunction with the decline in starch, suggesting that at least some of the starch in the stem was simply converted to sorbitol rather than being utilised for metabolism.

The sugar alcohol is often the last carbohydrate to be utilised in other sugar alcohol containing plants. When *Protea eximia*, which contains polygalatol, was put into the dark, starch levels dropped to near zero after 5 days, whereas levels of polygalatol remained the same as plants in the light (Bialeski et al. 1992). This shows that the polyol was unavailable

for remetabolism (Bieleski et al. 1992). In celery, mannitol pools began to decrease only when other stored sugars fell below 1% of dry weight during flowering (Williamson et al. 2002). Priestley (1962) found that carbohydrate reserves in a healthy apple tree are unlikely to be exhausted in normal circumstances or even by many days in darkness. Indeed, after 10 weeks in darkness, the plants in this study only lost 6% of the available carbohydrate. Priestley (1970) reports that plants grown in complete darkness lost more than a third of their extractable carbohydrates and then died. Unfortunately we are unable to find any more information about this particular experiment, and sorbitol levels were not discussed.

No metabolism of sorbitol occurred when apple stem pieces were bathed in  $^{14}\text{C}$ -sorbitol. This is consistent with the findings of Bieleski (1969) who found that  $^{14}\text{C}$ -sorbitol applied to excised apple phloem remained in the aqueous phase rather than being converted to lipids or proteins. In contrast, however, the findings of Wang & Quebedeaux (1997) concluded that sorbitol can be converted into other carbohydrates in apple stems. They found that when  $^{14}\text{C}$ -sorbitol was applied to stems, 76% remained as sorbitol in the stems and 18% was converted to glucose and sucrose. However, this was achieved by placing cut ends of shoots (with leaves) into solution containing  $^{14}\text{C}$ -sorbitol and allowing transpiration to take the solution up. This means that the glucose and sucrose component that was found in the stems could in fact have been converted elsewhere in the plant (e.g., in the leaves or buds) and retransported back to the stem.

$^{14}\text{C}$ -sucrose was metabolised into glucose, galactose, fructose, and a small quantity of sorbitol, showing that this method was sensitive enough to show any metabolism present. Bieleski (1966) found that c. 70% of the  $^{14}\text{C}$ -sucrose taken into apple phloem tissues stayed in the form of sucrose and <0.5% was recovered as sorbitol. Apple root tips, bark and wood of stems, and root tissues have been found to only synthesise a little sorbitol from fructose (Loescher & Everard 1996). Using the cut stems in solution described above, Wang & Quebedeaux (1997) found that 7% of  $^{14}\text{C}$ -glucose was converted to sorbitol in the stem (although this also could have been converted in the leaves and retransported).

SDH was recovered from growing tips and roots of apple trees and ranged from 25 to 50  $\text{nmol min}^{-1} \text{g}^{-1}$ . This is consistent with the range that Lo Bianco & Rieger (1998) found for growing tips of different *Prunus* species (10–311  $\text{nmol min}^{-1} \text{g}^{-1}$ ),

but is lower than reported values for root tips (164–244  $\text{nmol min}^{-1} \text{g}^{-1}$ ). Lo Bianco et al. (1999) found SDH only in the first 13 mm of root tip of 15-day-old peach seedlings and activity decreased from 300–25  $\text{nmol min}^{-1} \text{g}^{-1}$  in a distance of only 5 mm. Because root tissue was taken from more than 13 mm of root tip in this study, our results were possibly “diluted” to some extent by tissue that did not contain SDH.

No SDH was recovered from 1-year-old apple shoots at either early or late periods of the growing season. This is consistent with the shoot not being able to metabolise sorbitol into other products if SDH is not present. This appears to be the first time that attempts have been made to extract SDH from 1-year-old shoot tissue of apple trees. Further research is required to determine that sorbitol oxidase, which has only been found in very low concentrations in some tissue to date (Loescher & Everard 1996), is not present in higher amounts in apple shoot tissue where it could be responsible for cleaving sorbitol to glucose.

Sucrose cleavage enzyme activity in sink tissues was correlated to sink growth rate in plants where sucrose was the only translocated form of carbon (Lo Bianco et al. 1999). SDH activity also may be an indicator of sink strength (Bantog et al. 2000) and there is a correlation between sink growth and SDH activity for vegetative parts of peach (Lo Bianco et al. 1999). Stress in plants has been found to reduce SDH activity and result in the accumulation of sorbitol, leading to osmotic adjustment (Lo Bianco et al. 2000). Sucrose metabolism is only marginally reduced and may therefore support maintenance activities and some growth during drought (Lo Bianco et al. 2000). Thus SDH activity could be used as a measure of sink strength or stress levels in vegetative sinks of sorbitol transporting species. That no SDH was found could suggest that the 1-year-old shoot is a very low priority sink.

From these experiments, it appears that sorbitol has limited metabolism in 1-year-old apple shoots. Sorbitol may act entirely as a translocation product in the apple stem, where it is blocked from utilisation by the absence of the appropriate metabolic pathway. Bieleski & Redgwell's (1985) finding that sorbitol acts only as a translocation product in developing apricot leaves lends further support to this hypothesis. If sorbitol is able to move readily into the phloem, but has restricted utilisation along the transport pathway, sorbitol may be the ideal translocation substance (Bieleski 1969). The lack of metabolism in the stem could allow sorbitol to play

a buffering role in the carbohydrate balance of the tree, maintaining a constant flow of sorbitol to sink tissue.

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