Carbohydrate Storage and Remobilisation: The mechanisms involved in woody stem tissue of apple trees.

A thesis submitted in partial fulfilment of the requirements for the

Degree of

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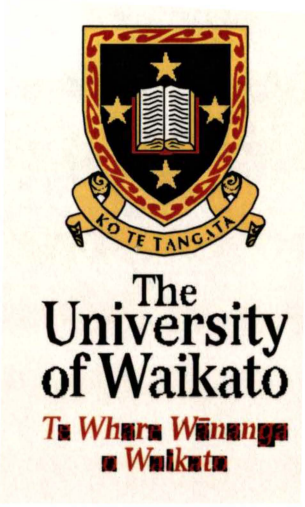

By

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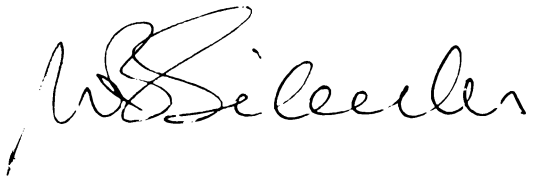
University of Waikato

2003

Certificate of Supervision

I certify that the experimental work contained in this thesis was planned, executed and written by Joanna McQueen while under the supervision of Prof. Warwick Silvester and Dr. Peter Minchin.



Prof. Warwick Silvester (Chief Supervisor)
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2003

Abstract

Carbohydrate storage and remobilisation: The mechanisms involved in woody tissue of apple trees.

Joanna C. McQueen

This thesis investigated the storage and remobilisation of carbohydrate reserves in perennial woody stem tissue, using one-year-old apple stems. In this study, the theories developed in herbaceous annual species were applied to woody perennial plants. Perennial plants are more complicated to study as they need reserves over a longer temporal scale and the woody tissue makes experimental manipulation difficult. In addition, apple is a multiple carbohydrate transporter and sorbitol (a sugar alcohol) is a major translocation product. These complications were overcome in this thesis, allowing hypotheses, developed from knowledge of carbohydrate movement in herbaceous plants, to be tested. The hypotheses were tested by manipulating sink conditions, ^{14}C and ^{11}C tracer experiments, use of inhibitors and enzyme extractions.

The findings allowed models of carbohydrate storage and remobilisation in stem tissue of apple to be made. The sieve elements of apple are leaky pipes and assimilates are lost through passive leakage or active unloading during their journey from source to terminal sink. During remobilisation of carbohydrate in spring, retrieval of this leaked assimilate back into the phloem stream is up-regulated. This retrieval is likely to be directly into the sieve elements and involves carriers similar to those found in herbaceous annuals. Buffering also occurs along the stem length to prevent interruption to flow from short-term changes in photoassimilate supply. Buffering, leakage and retrieval probably occur into the apoplast. Starch is initially remobilised from storage cells closest to the phloem region in the spring. Sorbitol in the stem probably cannot be metabolised, which allows sorbitol to exist in high concentrations in the stem where it could act as a temporary (days to weeks) storage pool and could play a buffering role, maintaining a constant flow of sorbitol to sink tissue.

During storage in autumn, when terminal sink tissue is either removed or saturated, assimilates continue to be lost from the leaky sieve tubes. However, retrieval is down-regulated so high concentrations of sugars build up in the stem apoplast. This results in storage of carbohydrate as starch, initially into the cells surrounding the phloem. Storage in the stem is given the lowest priority in terms of accumulating available carbohydrate and only occurs when there is an excess of photoassimilate, such as when higher priority sinks like fruit are removed or saturated. Eventually starch storage will saturate (at a concentration of 50mg g^{-1} in trees growing in the Hawkes Bay). Saturation of storage is likely to reduce concentration gradients between sieve elements and the apoplast, leading to a build up of sugars in the sieve elements and reduced assimilate flow. This is likely to result in a reduction or stoppage of photosynthesis, followed by leaf senescence.

The results of this thesis show that most of the processes of carbohydrate storage and remobilisation in herbaceous plants also occur in woody perennial plants and this has increased our knowledge of basic plant processes. In order to manipulate source-sink relationships in plants to increase crop yields, we need to understand the physiological processes involving the movement of carbohydrates. The retrieval and release of sugars along the transport phloem is important, as there is a balance between supplying terminal sinks with photosynthate and retention of photosynthate along the pathway. In addition, physiological studies into apple trees are sparse. It was important to go to the next stage of carbohydrate studies in apple trees, beyond orchard management, and understand the physiological processes involved in carbohydrate movement and remobilisation. Apple is a high yielding crop and knowledge of the carbohydrate processes in this plant could help to increase yields in other commercially important tree species.

Acknowledgements

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My supervisors, Professor Warwick Silvester and Doctor Peter Minchin deserve a big thank-you for seeing this project through. Warwick provided a level-headed perspective and got things sorted when they needed to be and Peter was always just down the corridor to ask a quick question and was always inspired by the science.

While it is hard not to sound clichéd about such things, a PhD is a long and difficult path to choose in the journey of life. It would not have been completed without the support of my husband, Mark, alongside me. There have been many stumbling blocks along the way, but at all times there was the support of a wonderful network of friends and family.

Many people have helped with this project and deserved to be thanked: HortResearch staff, especially, Michael Thorpe, Helen Boldingh, Carolyn Edwards, Karin Klages, Nick Gould, John Palmer, Jens Wunsche, Michael White, Iain Latter, Stuart Tustin, Armann Einerssan, Ian Hallett and Rod Bieleski.

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Chapter 1: Introduction

1.1. Introduction

This thesis aims to understand storage and remobilisation of carbohydrate reserves in perennial woody stem tissue. Apple was chosen as the experimental plant.

Carbohydrates are important because they function as energy storage molecules and as structural elements in the plant (cellulose). About three quarters of the dry weight of woody plants is made up of carbohydrates (Oliveira and Priestley, 1988), the majority of this being cellulose, leaving only a small fraction available for remobilisation and use. Carbohydrates play key roles in all aspects of plant life and when they are depleted, carbohydrate starvation has profound effects on a broad range of metabolic and developmental processes (Yu, 1999).

Carbohydrates (sucrose, glucose, fructose and in some plants, sugar alcohols) are synthesised in the leaves through the process of photosynthesis and transported many metres (e.g. in trees) or merely centimetres (e.g. in grass) to sites of growth and storage. In storage organs, such as tubers, starch accumulates where it is formed from sugars. Storage organs also consist of the roots and stem and these are the main sites of storage in plants without specialised storage structures. The leaves are temporary storage structures and starch builds up in the leaves during the day and is converted back to sugars at night. This sugar maintains a continuous supply of carbohydrate to sites of growth in the absence of photosynthesis. In the short-term, sugars in the leaves also maintain a carbohydrate supply when photosynthesis is temporarily interrupted. In the longer term, during times of carbohydrate deficiency, starch in storage organs is used to maintain a carbohydrate supply and is referred to as reserve carbohydrate. There are a number of occasions

throughout the year when carbohydrate depletion occurs in plants, such as during flowering and fruit set.

1.1.1. Reserve carbohydrate

Reserve carbohydrates can be defined as carbohydrates produced in excess of current requirements, which are laid down in stem and root tissue, and which may later be used in support of metabolism and growth as far ahead as the next season (Priestley, 1960).

Reserve carbohydrates may be used to ensure a continuous supply of carbohydrate to growing organs when there is a deficit of photosynthate in the short-term, such as on cloudy days. In the longer term, a major carbohydrate deficiency occurs in deciduous trees in the spring, when growth of new vegetative and fruiting buds occurs before any photosynthate is produced.

The reliance on reserves stored the previous season depends on the species; some species produce flowers before any leaves and thus rely entirely on reserves for the growth of those flowers e.g. sweet cherry, where flower and vegetative buds open simultaneously (Keller and Loescher, 1989). Apple leaves unfold shortly before flowers appear in the spring (Lakso, 1994), meaning that reserves are only drawn on until the leaves can begin to produce adequate amounts of photosynthate. At the other extreme, some deciduous plants do not produce flowers until leaves are fully formed and transporting photosynthate (e.g. kiwifruit).

Carbohydrate depletion is also faced in fruit trees when they become alternate bearing. During the non-fruiting 'off' years, trees store large amounts of carbohydrate and during the following heavy fruiting 'on' year, these carbohydrates are mobilised and used, leading to carbohydrate depletion in vegetative tree organs (Li *et al.*, 2003). In extreme cases, carbohydrate depletion causes collapse of trees at the end of an 'on' year (Smith, 1976).

In tree species, studies of carbohydrate storage and remobilisation have concentrated on measurements of the reserve pool over an annual cycle, with the research having an empirical focus. Some work has been carried out on the mechanisms involved in the storage of carbohydrates in wood (Lacointe *et al.*, 1995; Sauter, 2000; Sauter and Neumann, 1994), but little is known about utilisation of reserve carbohydrates in woody species. It is known that carbohydrate reserves play an essential role in trees, but very little is known about what specific roles they play in tree survival, growth and development (Loescher *et al.*, 1990). In contrast, there is extensive literature on the utilisation of reserve carbohydrates in cereals and grasses (Wardlaw, 1990) where carbohydrates in storage along the stem account for a significant amount of grain growth. There is some understanding of carbohydrate pathways (Hayes *et al.*, 1987; Patrick and Offler, 1996) and of enzyme activities associated with storage and remobilisation in stem tissues of these annual species (Wardlaw and Willenbrink, 1994). It is not known whether the same processes operate in woody perennial species. This study will attempt to determine whether the processes proposed in annual species apply to woody perennials.

1.2. Current Knowledge of Carbohydrate Movement

1.2.1. Phloem anatomy

Central to a discussion of carbohydrate movement is knowledge of the phloem tissue, through which carbohydrates move from sources to sinks. Phloem is a perplexing tissue to study because it is deeply embedded in other tissues and, because it is under extremely high pressure, immediately explodes following any type of cutting or impalement, necessary for experimental manipulation (van Bel, 2003). Therefore, phloem must be studied *in situ* using non-invasive techniques such as short-lived radioisotopes, nuclear magnetic resonance imaging and confocal laser scanning microscopy (van Bel, 2003).

The phloem of angiosperms consists of several cell types; the phloem sap conducting sieve elements, the companion cells and the phloem parenchyma cells. Sieve elements are connected to one another by sieve pores at each end to form a pipe network. Sieve elements and companion cells are produced by a common mother cell, after which the sieve element loses most of its organelles including the nucleus (van Bel *et al.*, 2002). Thus the companion cell provides genetic information and metabolites to the sieve elements via plasmodesmata (van Bel *et al.*, 2002).

Throughout the plant, phloem has slightly different characteristics depending on its location, such as the size of companion cells and plasmodesmal frequencies between companion and parenchyma cells. The relative size of the companion cell in different tissue types suggests a strong involvement in sugar accumulation processes (van Bel, 1996), with a decreasing volume ratio between companion cells and sieve elements along the length of the phloem; large companion cells are found in the collection phloem (in the leaves) (van Bel, 1996).

Because of these different characteristics, in a discussion of phloem it is important to differentiate between different phloem types. In this study, the term 'collection phloem' will be used to describe phloem in the leaves (van Bel, 2003). 'Transport phloem' will be used to describe phloem in the pathway between source and sink and 'sink phloem' will be used to describe phloem in sink tissue. Sink phloem has been labelled 'release phloem' by van Bel (2003) but this term is avoided in this study to prevent confusion with other release processes.

Throughout the plant, different terminology is used to describe various processes. Phloem loading has mainly been studied in leaf tissue and thus the term 'phloem loading' will refer to uptake into the collection phloem of mature leaves (van Bel, 2003) (Fig. 1.1). Phloem unloading has been studied in terminal sinks, so the term 'phloem unloading' will refer to unloading at terminal sinks. Loading and unloading of phloem also occur

along the length of the pathway between sources and sinks (transport phloem) and will be termed release/retrieval (van Bel, 2003) (Fig. 1.1), although it is possible that these mechanisms are the same as those found in sources and sinks.

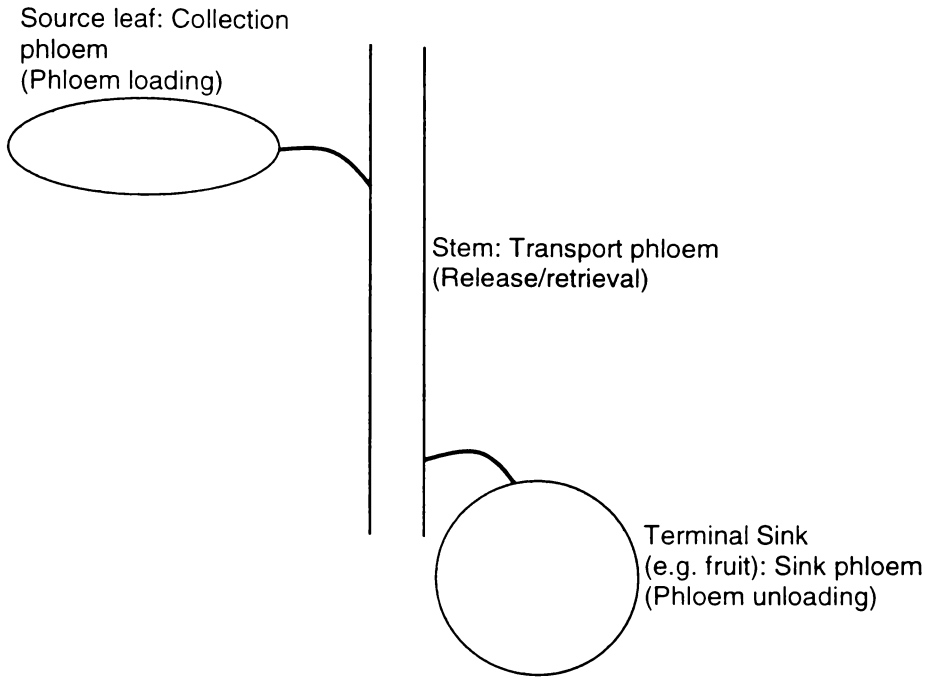

Figure 1.1. The terminology used to describe the various types of phloem found in a plant and the processes that occur here (after van Bel 2003). Sink phloem has been called release phloem by van Bel (2003), but this term is not used here to avoid confusion with the release (unloading) process occurring in stems.

1.2.2. Phloem loading in source leaves

Carbohydrates are produced in the leaves by photosynthesis and then transported around the plant to where they are required for growth or storage. Sugars are loaded into the collection phloem for transport either by an apoplastic or symplastic route. In the apoplastic route, sucrose is unloaded into the apoplast from parenchyma cells and taken up into the sieve element/companion cell complex by an energy dependent transport system (Fig. 1.2) (van Bel, 1992). In the symplastic route, sugars move from the mesophyll cells into the conducting elements of the phloem via

plasmodesmata (Kuhn *et al.*, 1999; Turgeon and Beebe, 1991) (Fig. 1.2). Species have been classified as apoplastic or symplastic loaders according to the plasmodesmata density between the leaf parenchyma and companion cell interface (Gamalei, 1991). Evidence suggests that phloem loading and unloading can occur either apoplastically or symplastically, depending on the species and may even depend on the tissue type studied (Komor *et al.*, 1996; van Bel, 1989). Different solutes (e.g. sorbitol versus sucrose) may have different modes of loading within the same tissue type.

Apoplastic phloem loading involves a set of sucrose/H^+ symporters and sugar transport across membranes is facilitated by these energy-dependent, cargo-specific transporters (Shakya and Sturm, 1998). Four sucrose transporters have been identified in Solanaceous species (SUT1, SUT2, SUT3 and SUT4) (Barker *et al.*, 2000) and an excess of 20 genes have been identified in different plant species, including monocots and dicots (Patrick *et al.*, 2001). SUT1 and SUT4 are found in source tissue, while SUT2 is found on the sieve element plasma membrane of transport and sink phloem (Weise *et al.*, 2000). Whether these transporters are ubiquitous remains to be determined.

Sugar transporters are thought to be important control points for the allocation of carbohydrate between competing sink organs (Bush, 1999). Changing the expression of these transporters may allow modification of the flow of sugar to a particular sink (Lemoine, 2000). Sugars also act as regulatory signals that affect gene expression and hence plant development (Williams *et al.*, 2000). Again, signalling properties of sugars have not been studied in woody species.

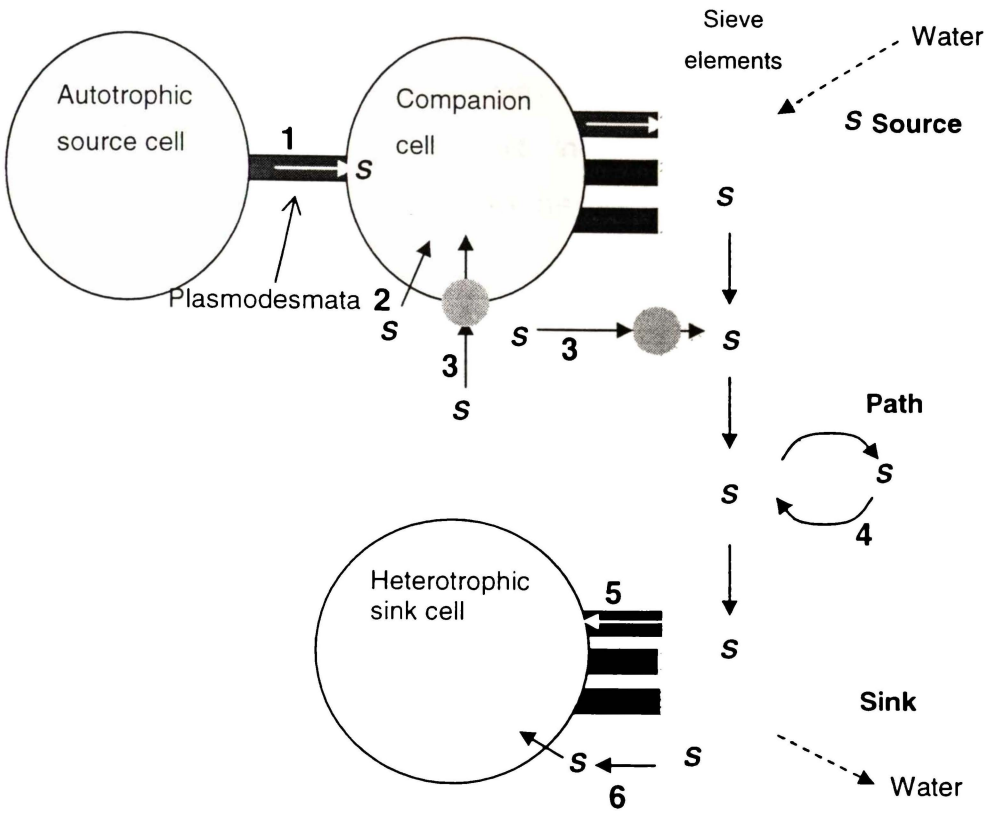

Figure 1.2. The phloem showing the source-path-sink continuum of sugar (***S***) transport (modified from Williams et al. 2000). Sugar is loaded into the sieve elements either symplastically (**1**) or apoplastically (**2**). Loading of sucrose is facilitated by sucrose transporters (**3**) either bound to the sieve elements or companion cell membranes. Along the pathway, sugar is released or leaked from the sieve elements and retrieved (**4**). The release (unloading) and retrieval (reloading) processes here are probably similar to those at the source and sink, but comparatively little study into these processes has been carried out on stems. At the sink, sugar is unloaded from the sieve elements either symplastically (**5**) or apoplastically (**6**).

1.2.3. Phloem unloading at terminal sinks

A sink is defined as a net importer of photosynthate (Minchin and Thorpe, 1993), for example, the roots, growing shoots, flowers, fruit and storage. A terminal sink can be defined as a sink where carbohydrate is irreversibly metabolised or stored, such as fruit, in contrast to a sink where the carbohydrate can be remobilised, such as storage.

At the terminal sink, sugars are unloaded from the sieve elements either via an apoplastic or symplastic route (Fig. 1.2), although a symplastic route has been found most often in the plants studied to date (mainly pea and maize) (Fisher and Oparka, 1996; Patrick, 1997; Patrick and Offler, 1996; Schmalstig and Cosgrove, 1990; van Bel, 2003). Study of unloading is extremely difficult because the vascular bundles are buried deep within the sink tissue where they form intimate anatomical associations (Patrick, 1997) and as such very few species have been examined. However, loading and unloading of phloem are important to study as they are often considered to be the rate limiting steps to crop production (Patrick, 1997; van Bel, 1993).

1.2.4. Transport phloem

Solutes move along a source-path-sink continuum through the sieve tubes (Fig. 1.2). A hydrostatic pressure gradient is created from the source to the sink and solutes move down this. The pressure gradient is created by the active uptake of solutes and other osmotica into the sieve tubes at the source, thus creating an osmotic pressure difference across the membrane (Patrick *et al.*, 2001). Water will then move passively into the sieve tubes, increasing the turgor pressure in the sieve tube at the source, creating a concentration gradient between source and sink and resulting in bulk flow of solutes and water through the phloem to the sink tissue (Patrick *et al.*, 2001).

The stem tissue includes the pathway of phloem transport, but it is more than just a pipe network for the flow of photosynthate en route to sink tissue. Sieve tubes are not hermetically sealed pipes (Minchin and Thorpe, 1987; Vreugdenhil, 1985; Vreugdenhil and Koot-Gronsveld, 1989), but actually lose appreciable amounts of photosynthate, part of which is retrieved (Hayes *et al.*, 1987; Minchin and Thorpe, 1987). In bean, sieve tubes lose 6% of photosynthate per centimetre of stem, of which, two thirds is retrieved (Minchin and Thorpe, 1987). Retention of photosynthate to supply terminal sinks coincides with release along the transport phloem to supply sinks such as storage along the pathway (van Bel, 1996).

Long-term storage of sugars and starch occurs in the stem and can be a major part of the carbohydrate reserves in a plant, particularly in trees. Studies into long-term stem storage in trees have mostly involved examination of the carbohydrate flux. No studies have been carried out on the mechanisms involved in long-term storage in woody species. Is storage of carbohydrate reserves in stems of woody species dependent on carbohydrate supply and sink demand, or is there a seasonal component to it? In wheat and barley, carbohydrate storage in the stem is not competitive with grain filling (Schnyder, 1993). However, storage does occur when there are excess photosynthates caused by less demand from other sinks and carbohydrate stored in the stem has a buffering action, providing carbohydrate during deficits in photosynthate production (Schnyder, 1993).

Short-term storage of sugars in the stem also plays a major role in the carbohydrate status of a plant. Sugars can accumulate in phloem parenchyma cells for temporary or long-term storage (as starch) and then be distributed back to the sieve element/companion cell complex when there are fluctuations in photoassimilate supply. Storage of carbohydrate along the length of the pathway in the stem tissue has a buffering action on the fluctuations in the supply of photosynthate available for growth of e.g. fruit (Minchin *et al.*, 1984). Short-term (several hours) changes in

photosynthate supply are buffered, resulting in no change to photosynthate supply at the sink. In bean, this buffering pool is found in the stem apoplast (Minchin *et al.*, 1984). Does this buffering pool, present in annuals, exist in perennial woody species?

Retention of photosynthate to supply terminal sinks and release along the transport phloem to supply sinks such as storage along the pathway is achieved by a rigorously regulated release/retrieval balance in the sieve element/companion cell complexes of the transport phloem (van Bel, 2003). The strict control seems to require a degree of symplastic isolation of sieve element/companion cell complexes in transport phloem. Few plasmodesmata connections have been found between sieve element/companion cell complexes and phloem parenchyma cells in stems of *Phaseolus* (Hayes *et al.*, 1985), *Lythrum, Cucurbita, Vicia* and *Zinnia* (Kempers *et al.*, 1998). Further evidence for symplastic isolation was found by the containment of flurorchromes in the sieve element/companion cell complex of transport phloem after injection into sieve tubes of various plants including *Ricinus, Solanum,* and *Lupinus* (van Bel and Kempers, 1990; van Bel and van Rijen, 1994). Electrical conductance and membrane potentials provide further evidence for isolation (van Bel, 2003). However, in some cases contradictory data has been obtained and this is probably due to differences in sink conditions. This indicates that sieve element/companion cell complexes are able to shift between symplastic and apoplastic routes (Patrick and Offler, 1996) by gating their plasmodesmal connections towards the phloem parenchyma cells (van Bel, 2003). What route is found in woody stems remains to be determined.

In the stems of some plants, retrieval is apoplastic because the phloem is symplastically isolated (Goggin *et al.*, 2001). Release (unloading) in stem tissue has mostly been studied in sugar cane (Glasziou and Gayler, 1972) and bean (Hayes *et al.*, 1987; Minchin *et al.*, 1984) and has been found to be apoplastic, however in pea, there is evidence that release is symplastic (Schmalstig and Cosgrove, 1990) (Fig. 1.3). The mode of retrieval and

release in woody stems has not been studied. It can be hypothesised that the same mechanisms found in annual plants will apply to woody species; this hypothesis will be addressed in this thesis.

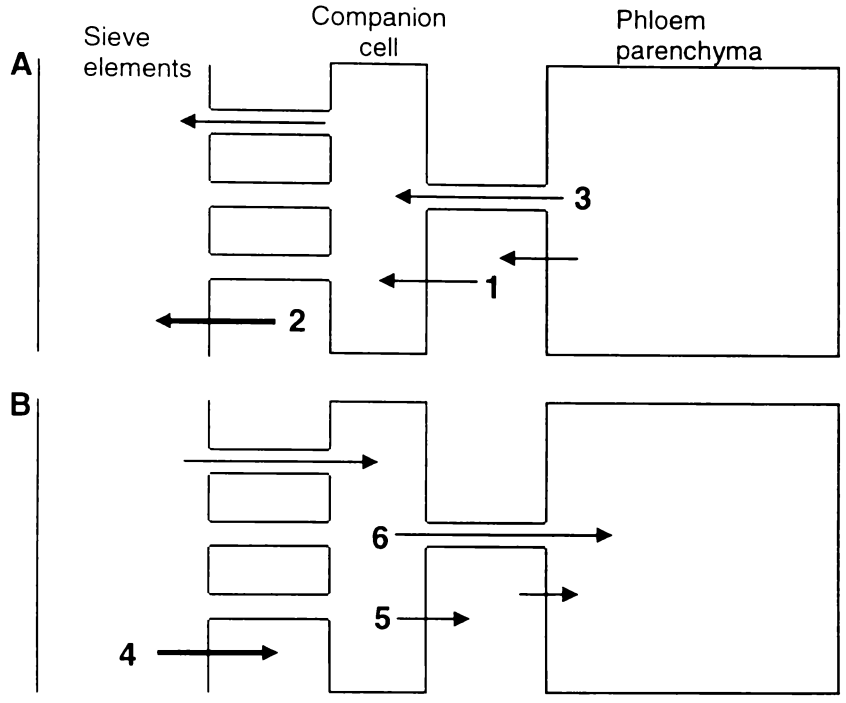

Figure 1.3. Retrieval (A) and release (B) of sugars in the transport phloem. Sieve element/companion cell ratios indicate that transport phloem is a functional hybrid between collection and sink phloem, which is consistent with balanced release/retrieval processes along the transport path (van Bel, 2003). Few symplastic connections exist between the sieve element/companion cell complex and phloem parenchyma in stems. Retrieval may be apoplastic, either into the companion cells (1) (from where sugar is transferred symplastically into the sieve element) or more likely directly into the sieve elements (2). Retrieval could be symplastic (3), but this is less likely in herbaceous plants; the route in woody plants has not been studied. Release of sugar is most likely to be directly from the sieve elements (4). However if the companion cell is involved, release could be apoplastic from the companion cell (5) or symplastic (6) depending on sink conditions (Patrick and Offler, 1996). Routes can be changed by the gating of plasmodesmal connections towards the phloem parenchyma cells (van Bel, 2003). What route is found in woody stems remains to be determined.

Release/retrieval events are controlled by carrier systems, energised by proton-motive force (van Bel, 1995). Sucrose has been found to be mainly retrieved by companion cells (Stadler *et al.*, 1995). However in transport phloem, sucrose carriers dominate on the sieve element plasma membrane (van Bel, 2003). This favours an energy supply by companion cells and photoassimilate retrieval by sieve elements (van Bel, 2003) (Fig. 1.3). Only 25% of the sieve element surface in transport phloem of advanced herbs has an interface with a companion cell, so sugars could easily escape from sieve element areas uncovered by companion cells (van Bel, 1996) (Fig. 1.3).

1.3. Carbohydrate Partitioning

Sink tissues (including storage) compete against one another for limited carbohydrate resources. The carbohydrate available for movement around the plant depends on how much is produced by photosynthesis, how much is transferred to the phloem and how much is unloaded from the phloem at various sinks (Giaquinta, 1983). Because there is competition between sinks for resources, the concept of sink strength was developed. Sink strength can be defined as the flow of photosynthate per unit of time into a sink (Warren Wilson, 1972). However, the concept of sink strength is not that useful because it is difficult to measure; photosynthate flow into a sink is not determined by the sink alone, so this flow is not a measure of sink strength (Minchin and Thorpe, 1993). A better concept is sink priority where certain sinks can capture photosynthate at some times at the expense of others. A hierarchy of sink priority exists: growth of seeds > growth of fleshy fruit parts = growth of shoot apices and leaves > growth of cambium > growth of roots > storage (Wardlaw, 1990). Thus fruit and seed growth have dominance over vegetative organs (Wardlaw, 1990).

Carbohydrate partitioning is the process of balancing the maintenance of vegetative tissue with growth of fruit and vegetation (Geiger and Fondy, 1991; Proctor *et al.*, 1976). A range of factors may influence carbohydrate partitioning, such as changes in photosynthate supply, hormonal and nutritional controls or vascular constraints (Wardlaw, 1990) and as such there is still much to learn about this process. Carbohydrate partitioning is a key determinant of harvest index (Daie, 1985; Giaquinta, 1983; Patrick and Offler, 1996) so it must be understood to enable increases in harvest index to be made. The increased grain yield of wheat and barley in modern crops can be entirely accounted for by increases in harvest indexes (Ho, 1988). The higher photosynthetic capacity of modern crops has generally been achieved by increasing the light–intercepting area of leaves (increase in leaf number, more erect leaf posture, larger individual leaf area) or by increasing the size or number of individual grains (Ho, 1988). Manipulation of source-sink relationships is therefore necessary to continue to improve crop yield. Understanding of the physiological processes involving the movement of photoassimilate is necessary before this can take place.

1.4. Application to Woody Stems

So far, the theory developed for carbohydrate resource allocation and the mechanisms behind carbohydrate movement have been developed in herbaceous, mainly annual species. Theories on carbohydrate accumulation and remobilisation have been tested in few woody perennial plants (*c.f.* Sauter, 2000; Sauter and Neumann, 1994; Sauter and van Cleve, 1991; Sauter and van Cleve, 1993 in poplar). In annual species, there is a short time sequence of storage and reserves can be depleted completely at fruit development. In a perennial species, carbohydrate reserves are needed on a longer temporal scale. Seeds and fruit cannot completely deplete carbohydrate reserves, as vegetative parts need to be maintained. This complicates the perennial system, as alternate sinks are

available to carbohydrates. In addition, in deciduous perennial species, reserves are needed to ensure the species survival after winter.

Woody species present additional complications to study. A major problem in the study of woody species is their distinguishing feature, the wood! Many of the techniques used to develop theories on carbohydrate transport rely on the ease of getting substances into soft tissue with minimal damage. Woody tissue is extremely hard and therefore difficult to cut, especially as it increases in size with age. Getting substances into the tissue is challenging. In addition, woody species generally are slow growing and can take years to produce sink tissue, in the form of fruit, necessary for experimental manipulations. These challenges have thus far limited studies on woody tissue. In this thesis, an attempt was made to overcome these challenges to broaden our understanding of the mechanisms involved in carbohydrate movement and remobilisation in woody tissue. The aim was to determine whether the mechanisms proposed in herbaceous annual species can be applied to perennial woody tissue.

Apple, a species of economic importance was chosen to carry out this study. This study focussed on the stem tissue of the apple tree. Specifically, one-year-old stems (last season's extension shoots) were used. This meant that tissue could be destructively harvested from the tree for analysis without adversely affecting the health of the tree. Samples could be taken throughout the year, as this wood was always present.

1.4.1. Apple

This study was carried out on *Malus sylvestris* (L.) Mill. var *domestica* Borkh. Mansf. (apple), a member of the Rosaceae family, which includes other commercially important fruit trees such as *Prunus* (apricot, peach, plum and cherry) and *Pyrus* (pear). Apple is a deciduous temperate fruit crop species that is very adaptable to different climates, growing

commercially from the tropics to the high latitudes in Norway (Lakso, 1994). Apple is essentially a wild-type plant that has had few fundamental changes through modern breeding (Lakso, 1994). Little selection for physiological responses has occurred in commercial cultivars (Lakso, 1994) and few physiological differences in environmental responses can be found between a 200yr old cultivar and a newly released cultivar (Lakso, 1994). This could explain the variability of fruit set and yield that is a bane of horticultural practitioners (Lakso, 1994). Progress in increasing productivity has been made in the past by overcoming nutrient limitations by fertilisation, water deficits and pests, improved thinning, pruning and orchard design (Lakso, 1994). In spite of the lack of breeding improvements, photosynthetic rates in young, fully-expanded apple leaves are substantially higher than what is thought typical of trees or many C_3 plants (Loescher *et al.*, 1985).

Fruit yields in apple can be extremely high, averaging 100 t fruit ha^{-1} yr^{-1} in New Zealand (Bieleski, 2000), which translates to a yield of 14 t sugar equivalents ha^{-1} yr^{-1} in fruit (Bieleski, 2000). This is substantially higher than production in parts of the Northern Hemisphere; New Zealand apple yields are almost twice as high as New York State, USA (Lakso, 1994) but unfortunately, little is known of the physiological basis for this. The long photosynthetic period after harvest in New Zealand trees may be responsible (Tustin *et al.*, 1997) allowing plenty of carbohydrate reserves to be laid down in autumn for use the following spring. Understanding of the physiology of the apple tree is now necessary for further yield improvements to be made and to apply the high yields achievable in apple to other commercially important crop species.

Woody species are difficult to experimentally manipulate for the reasons described earlier. But as an added complication, apple trees are multiple carbohydrate transporters. In the majority of species, sucrose is the only translocation product. The selection of sucrose as a major transport sugar has been related to its non-reducing nature and relative insensitivity to

metabolism (Lalonde *et al.*, 2003). However, a smaller number of species translocate other carbohydrates as a major product with sucrose being a minor component. In apple trees, the major translocation carbohydrate is sorbitol, which is not that well known as a transport carbohydrate.

1.4.2. Sorbitol

Sorbitol is a sugar alcohol and these are sugars with an extra alcohol grouping, also called polyols, polyalcohols or polyhydric alcohols (Bieleski, 1982). Sorbitol is derived from glucose (Fig. 1.4) and in some literature is referred to as glucitol because of its derivation from glucose e.g. Lewis (1984). Chemically, physically and biologically, the sugar alcohols closely resemble the sugars from which they are derived and it is usually convenient to treat them as special kinds of sugars or carbohydrates (Loescher, 1987).

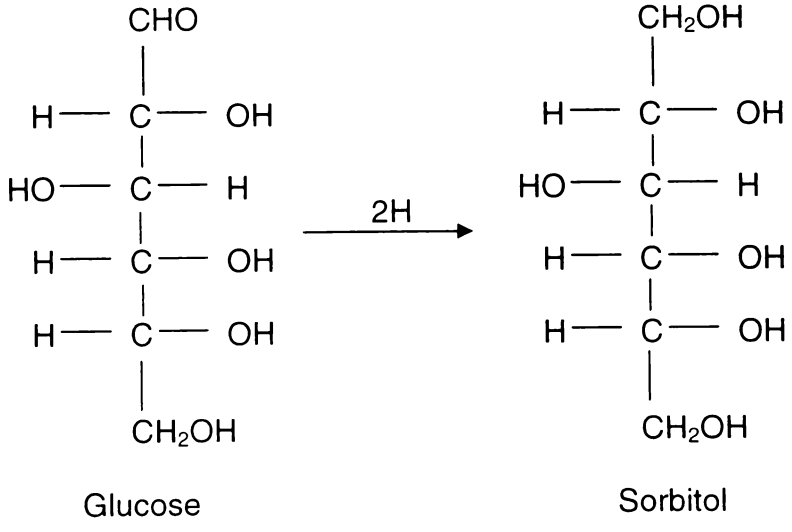

Figure 1.4. Chemical relationship between glucose and sorbitol (Lewis, 1984)

Sorbitol is produced in apple leaves by the following reaction, catalysed by aldose 6-phosphate reductase:

Glucose 6-phosphate + NADPH + $H^+ \rightleftharpoons$ sorbitol 6-phosphate + $NADP^+$
Sorbitol 6-phosphate + $NADP^+ \rightarrow$ Sorbitol + Pi
(Loescher and Everard, 1996)

Sixty to 90% of the carbon exported from the apple leaf is in the form of sorbitol (Loescher, 1987), with sucrose making up most of the balance (Loescher and Everard, 1996). Sorbitol appears to be a temporary carbon store in the leaf, accumulating in the light and being translocated in the dark once sucrose pools, which also form a temporary storage role, have been depleted (Loescher and Everard, 1996). Sorbitol comprises 65-70% of the sugars in the transport phloem (Klages *et al.*, 2001), yet little is known about its transport.

The mode of phloem loading and unloading in the apple tree stem has yet to be characterised. Some studies have been carried out on sorbitol producing species e.g. peach (Moing *et al.*, 1997), where apoplastic loading of sorbitol was found in leaves, but little study has been carried out in stem tissue of any species. The mode of phloem unloading and loading in apple stems will be discussed in more detail in chapter 6. It is necessary to determine the mode of retrieval so as to manipulate these complex interactions for horticultural benefit. To do this, it is vital to understand the underlying transport processes.

1.4.3. Role of sorbitol

Sorbitol plays a central role in metabolism and physiology in Rosaceae species, however, in spite of the important commercial nature of these species, the role of sorbitol in these higher plants is not fully understood, possibly because sorbitol was only discovered in these plants 35 years ago (Bieleski, 1982). Many functions for polyols (including sorbitol) have been proposed and these include:

1. Osmoregulation – an increased concentration of sorbitol is seen in response to osmotic stress (Bieleski, 1982). Half the weight of sorbitol is needed to generate the same osmotic potential as that generated by sucrose; therefore sorbitol ties up less carbon per unit of osmotic potential than an equimolar concentration of sucrose (Lo Bianco and Rieger, 2002).
2. Cryoprotection role - sorbitol can lower the freezing point of the tissue because it is present in such high concentrations and/or protect the enzyme system if freezing occurs (Bieleski, 1982). Sorbitol dehydrogenase is the major enzyme known to degrade sorbitol in sink tissues. Sorbitol oxidase also degrades sorbitol but has never been found in high concentrations in sink tissue (Yamaki, 1980a; Yamaki, 1980b). Because so few enzymes degrade sorbitol, it is possible to accumulate sorbitol in high concentrations.
3. Osmoprotectants because water-like hydroxyl groups in polyols allow them to form an artificial sphere of hydration around macromolecules (e.g. proteins) thus preventing metabolic inactivation when there is low osmotic potential (Williamson *et al.*, 2002).
4. Protective – polyols can scavenge hydroxyl radicals and so protect enzymes from denaturation during stress (Loescher and Everard, 1996). Stresses may include salt stress, cold hardiness, cold tolerance, freezing resistance, or drought.
5. Use of NADPH in production of the polyol – in plants that only synthesise sucrose, NADPH is used up by photorespiration. Plants that synthesise polyols have substantially higher photosynthetic rates than other C_3 plants; this might be due to a reduction in photorespiration as NADPH is used up in the production of the polyol (Loescher *et al.*, 1985).
6. High mobility of boron in polyol synthesising plants (Noiraud *et al.*, 2001b). Boron deficiency symptoms can occur even when boron is in ample supply in some plants, however in plants that synthesise polyols, boron is highly mobile. Transgenic tobacco and rice plants

genetically engineered to produce sorbitol exhibited less symptoms of boron deficiency (Bellaloui *et al.*, 2003; Noiraud *et al.*, 2001b).

Despite the numerous functions attributed to polyols, very little is known about the mechanisms of their transport inside the plant (Noiraud *et al.*, 2001b) or their importance in carbon partitioning (Lo Bianco *et al.*, 1999).

1.5. Carbohydrate Reserves in Apple

Some studies into the carbohydrate reserves in apple have been carried out although these generally have had an empirical focus. Few studies have investigated the physiological basis behind the storage and movement of these reserves.

Carbohydrate reserves (in apple trees) may constitute 20 – 30 % of the total dry matter (Oliveira and Priestley, 1988). Reserves are important for respiration during winter and the growth of flowers in spring. Starch generally accumulates during periods of photosynthetic activity and is then used for spring growth (Dowler and King, 1966). There are times of the year when trees contain high levels of carbohydrate, in the form of starch and times when levels are depleted (Dowler and King, 1966). In fruit trees, starch declines from bud break in spring until late summer when replenishment occurs and a maximum is reached in autumn, followed by a decline over winter (Corelli-Grappadelli *et al.*, 1994; Dowler and King, 1966; Gaudillere *et al.*, 1992; Hagidimitriou and Roper, 1994; Mochizuki and Hanada, 1957; Oliveira and Priestley, 1988; Priestley, 1960; Smith *et al.*, 1992; Tromp, 1983; Yoshioka *et al.*, 1988). A second minimum sometimes occurs in late summer when growth of shoots is most vigorous. Sorbitol measurements have been excluded from many earlier studies into carbohydrate reserves because the importance of it was not known. It is not known whether sorbitol plays a storage role in apple trees or under what conditions it is stored.

Reserves are formed in the autumn, particularly in the roots (Hansen and Grauslund, 1973) and the early stages of growth in the spring depend on these reserves (Oliveira and Priestley, 1988). Roots are probably the most important storage organ (Loescher *et al.*, 1990) and may play a critical role in the management of carbohydrate reserves. The stem is also an important storage organ and the reserves stored here may play an important role in buffering changes in photosynthate supply. While the fluctuations in carbohydrate supply have been characterised in various woody tissue including the stem, few studies have probed the underlying mechanisms of carbohydrate storage and remobilisation. We do not know if storage of carbohydrate is under seasonal control or what carbohydrates are long-term reserves. Does sorbitol play a role in long-term storage?

Leaf and early fruit development originate from reserves within the tree (Hansen, 1971; Oliveira and Priestley, 1988; Wardlaw, 1990). At this time, starch stored in the ray parenchyma cells of the stem or in the root cortex is converted back into sugars that are then transported to the growing buds. Thus the storage of adequate supplies of carbohydrate is critical for both fruit yield and quality. However, the importance of carbohydrate reserves in deciduous trees may be overemphasised as a tree loses only 1/3 of the extractable carbohydrate classified as reserves at budbreak (Oliveira and Priestley, 1988). Only 20% of the carbohydrate used in the growth of new shoots has to be supplied by reserves, with the remaining 80% coming from the early production of photosynthates (Hansen and Grauslund, 1973; Johnson and Lakso, 1986). What happens to the rest of the carbohydrates stored in a tree? Do we need to redefine our definition of reserves?

The mechanisms involved in carbohydrate movement in apple trees are not well understood. Sorbitol transporters have recently been identified in sour cherry fruit and leaf sink tissue (Gao *et al.*, 2003) and a transporter for mannitol (also a sugar alcohol) has also recently been discovered in the transport phloem of celery (AgMaT1) (Noiraud *et al.*, 2001a). It is not known if the mannitol carrier is responsible for retrieval along the pathway

or for storage by phloem parenchyma (van Bel, 2003). Sugar alcohols can be apoplastically phloem loading (Lalonde *et al.*, 2003); is this true of sorbitol retrieval in apple stems?

In summary, although a literature search reveals many references to research carried out on apple trees, physiological studies are sparse. Much work into the seasonality of reserves has been carried out over forty years ago, failing to recognise the important role that sorbitol plays in apple trees. Much of this early work was designed with the problem of alternate bearing in mind, although today, through orchard management practices, this problem has largely been overcome. It is now important to go to the next stage of carbohydrate studies in apple trees, beyond orchard management, and understand the physiological processes involved in carbohydrate storage and remobilisation.

1.6. Hypotheses

Our current beliefs on the mechanisms of carbohydrate storage and remobilisation are based on studies of herbaceous, annual plants. The aim of this thesis is to understand the mechanisms of storage and remobilisation of carbohydrates in woody stem tissue, using apple as the experimental plant. In carrying out this aim, this thesis addresses the following hypotheses:

Storage of carbohydrates in herbaceous plants occurs when there is less demand by other sinks. In trees, there is little fundamental understanding of the storage process and there is an interaction between sink strength and season making it hard to distinguish one from the other. The hypothesis that storage of carbohydrate in the stem is determined by alternate sink demand in apple trees is tested in chapter 3.

Sugar transporters have been characterised in herbaceous annuals and exhibit specific uptake behaviours and responses to inhibitors. These

transporters may be ubiquitous and this hypothesis is tested in chapter 4. Similarities to known transporters are tested by examining uptake kinetics and responses to inhibitors. In addition, some aspects of the sorbitol transporter(s) operating in the stem tissue are also characterised.

In herbaceous plants, there is leakage and retrieval of sugars along the length of the transport phloem, which can buffer short-term changes in photosynthate supply. The hypothesis that the transport phloem in apple stems involves leakage and retrieval into apoplastic space is tested in chapter 5.

The role of sorbitol as a storage carbohydrate is ambiguous. It is possible that sorbitol is present in the stem purely for use by higher priority sinks such as fruit that have the ability to metabolise it. In chapter 6, the hypothesis that sorbitol cannot be metabolised in apple stem tissue is tested.

In addition to these hypotheses, chapter 2 lays the framework on which to base other studies by an examination of the reserve fluxes present in apple stems throughout the entire year. This information of an undisturbed system was needed to provide a basis for further experimentation.

Finally, in chapter 7, a model is proposed for carbohydrate storage and remobilisation in apple stems. Avenues for future research are also presented.

All cited references in this thesis are listed together following chapter 7.

2.1. Introduction

In carrying out a study involving perennial plants, it is necessary to consider the reserve fluxes present throughout an entire year or more. Most studies into carbohydrate reserves have been carried out on annual plants, which do not have this temporal aspect to reserve storage. In this chapter, the information on reserve fluxes in a perennial plant was gathered to provide a basis for further experiments.

In apple trees, leaf and early fruit development in the spring originate from reserves within the tree (Hansen, 1971; Oliveira and Priestley, 1988; Wardlaw, 1990) and thus the storage of adequate supplies of carbohydrate is critical for both fruit yield and quality. Reserve carbohydrates are used until about six weeks after dormancy when the production of photosynthate equals the rate of utilisation (Hennerty and Forshey, 1971). Many tree responses such as flower bud initiation and fruit set have been explained on the basis of stored levels of carbohydrate (Hennerty and Forshey, 1971). Therefore, knowledge of the nature of these carbohydrate reserves is important for experiments involving manipulation of these reserves.

Although seasonal carbohydrate partitioning has been studied in fruit trees in detail (Hansen and Grauslund, 1973; Kandiah, 1979b; Murneek, 1933; Priestley, 1960; Priestley, 1962a), most studies have not measured the full range of non-structural carbohydrates found in apple trees (starch, sorbitol, sucrose, glucose, fructose). Sorbitol is a major photosynthetic product and component of the non-structural carbohydrates present in apple trees (Bieleski, 1969), but was not known to exist in these plants before 1968 (Bieleski, 1982). Reducing sugar assays were used in many earlier studies and these were not sensitive to the sorbitol component. Past studies have also often used small, non-bearing trees (Keller and

Loescher, 1989) and in most cases, samples have not been collected over the entire year.

New Zealand has a long mild autumn period resulting in a long photosynthetic period after fruit harvest. After harvest, apple trees translocate carbon and nitrogen to the woody perennial parts of the tree where carbon is stored as starch. The rate and magnitude of this translocation in autumn depends on available carbohydrate in the leaves and on the photosynthetic rate (Tartachnyk and Blanke, 2001). It is thought that the long photosynthetic period after harvest could be responsible for the very high yields of New Zealand apple trees compared to most of the Northern Hemisphere (e.g. the mean fruit weight over three years of young 'Jonagold' apple trees growing in New Zealand was nearly twice that of trees growing in New York) (Tustin *et al.*, 1997). Because previous studies into carbohydrate partitioning have been carried out in the Northern Hemisphere (Hansen and Grauslund, 1973; Kandiah, 1979b; Murneek, 1933; Priestley, 1960; Priestley, 1962a), it was important to repeat these experiments using apple trees grown in New Zealand in which the carbohydrate profile might be expected to be quite different due to the long photosynthetic period after harvest.

In this study, an examination into the seasonal non-structural carbohydrate partitioning in 'braeburn' apple stems growing in New Zealand was carried out over the entire year. The full range of major carbohydrates was examined (starch, sorbitol, sucrose, glucose and fructose).

2.2. Method

The change in non-structural carbohydrates in one-year-old stems (last seasons extension shoots) of seven-year-old 'Braeburn' apple trees, grown on MM106 rootstock in a commercial orchard (Gowanlea Orchard, Ruakura Road, Hamilton, New Zealand: NZMS 260 S14 775 163) was examined from July 2000 to July 2002. Trees were planted at a spacing of 4.5 x 2.75m in east - west oriented rows. Dormant pruning, fertilisers, pesticides and herbicides were applied as required according to standard commercial practices. Harvested fruit volumes were (t ha^{-1}) 1999/00 74.9, 2000/01 60.2, 2001/02 67.4. Alternate bearing was evident in individual trees within the block, with the 2001/02 season being an 'off' year for individual trees (no/few fruit produced). 'Off' trees were excluded from analysis.

In the first year of collection (July 2000 – July 2001) four trees were randomly selected within the fruiting trees in the block, excluding the first tree in each row. Three one-year-old stems were collected from each tree each month. Bark and wood were not separated.

In the second year of collection (July 2001 – July 2002) three different trees were randomly selected within the block, excluding the first tree in each row. Two one-year-old stems were collected from each tree at monthly or bimonthly intervals. Over the summer months, bark and wood were able to be separated from one another easily and thus analysed separately. For the rest of the year, when the bark and wood did not separate easily, bark and wood were analysed together.

One-year-old stems were removed from the tree just above the lowest bud, leaves removed and any dead wood excluded. Branches were cut into small pieces and frozen in liquid nitrogen on site. Frozen samples were then stored at $-80^{0}C$ until freeze-drying. Once freeze-dried, the

samples were stored at -20^0C until carbohydrate extractions were carried out.

Stem samples were ground into a fine powder (Wiley Mill Grinder, General Electric Motors). Samples were then well mixed and re-freeze-dried before weighing. 100mg of powered sample was subsequently extracted for carbohydrates by heating in 80% ethanol for 60mins at 60^0C. After mixing well and centrifuging (two repetitive washings were carried out), sugars were assayed (in duplicate) from the supernatent as described by Jones et al. (1977) and starch was assayed from the pellet. 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 sucrose assay involved the hydrolysis of sucrose and reduction of $NADP^+$ by a series of coupled enzyme reactions:

$$\text{Sucrose} \xrightarrow{\text{invertase}} \text{glucose + fructose}$$

$$\text{Glucose + fructose + 2ATP} \xrightarrow{\text{hexokinase}} \text{glucose 6-P + fructose-6-P + 2ADP}$$

$$\text{Fructose-6P} \xrightarrow{\text{P-glucoisomerase}} \text{glucose-6-P}$$

$$\text{2 glucose-6-P + 2 } NADP^+ \xrightarrow{\text{Glucose-6-P-dehydrogenase}} \text{2 6-P-gluconate + 2 NADPH}$$

All enzymes used in the reactions except invertase were from Boehringer Mannheim, Germany. Invertase used was from Sigma Chemical Company, USA. The experimental conditions under which enzymes were applied are detailed in Jones (1977) and Jones et al. (1979). A concentrated stock buffer solution was kept in the freezer as multiple freeze thawing did not affect its stability (Jones et al. 1977). Enzymes were added to this solution immediately before use.

The fluorescence of NADPH was measured by a fluorometer (Farrand Optical Co., Inc. USA) within the range of 0.1 – 5 x 10^{-9} moles. Because any pre-existing glucose and fructose would also have been hydrolysed, glucose and fructose (not separated) were also measured by omitting the first stage of enzyme reactions above. This value was then subtracted from the sucrose value.

The enzymatic reaction for the assay of sorbitol was reversible, thus it was necessary to remove free fructose by heating in NaOH before carrying out the assay. Sorbitol underwent the following enzymatic reaction:

$$\text{Sorbitol} + \text{NAD} \xleftrightarrow{\text{Sorbitol dehydrogenase}} \text{fructose} + \text{NADH} + \text{H}^+$$

NADH, which has an identical fluorescence to NADPH (Lowry and Passonneau, 1972) was also measured on the fluorometer.

The pellet leftover after sugar extraction was used to determine the starch concentration. Starch was measured as glucose equivalents after digestion with amyloglucosidase (Sigma-Aldrich Co. International) as described by Jones (1979). Heating with amyloglucosidase for sixty minutes gave maximal glucose equivalents yields when the temperature was set at 55^0C. The glucose released by amyloglucosidase was measured by reduction of $NADP^+$ by the coupled enzymatic reactions described above. The efficiency of starch hydrolysis was compared with acid hydrolysis using starch standards. This showed that enzyme hydrolysis was 80% efficient and results were corrected appropriately.

The data was analysed statistically using Analysis of Variance (ANOVA). The assumption that parametric parameters were filled (i.e. the data has equal variance, is independent and normally distributed) was tested before an analysis was performed.

Visualisation of starch in the stem was made at monthly intervals. One-year-old stems were removed from the tree and cut into cross-sections

using a microtome. Cross-sections were immersed in an iodine stain (1:1 (w.w) potassium iodide: iodine flakes) for 2 minutes to stain starch black. Sections were then visualised (Leica DMRE binocular system, Germany) and photographed using a digital camera (Carlzeiss Axio-Cam HRc, Germany).

2.3. Results

The seasonal trends for all soluble carbohydrates from July 2000 to July 2001 for one-year-old stems of apple trees grown in the Waikato were investigated (Fig. 2.1a). Total non-structural carbohydrates were found to be significantly higher in February compared to November, December and May ($P<0.001$). Sorbitol made up the majority of the total carbohydrates and consequently determined the shape of the total carbohydrate curve. Sorbitol concentrations increased steadily until October, and then declined slightly until February (mid-summer) when a 2-fold increase in concentration was observed. Sorbitol concentrations then fell below that of starch by May (late autumn) and ended up having a similar value in July 2001 as was found in July 2000 (midwinter). The starch concentration declined before full bloom (25th October) until February when it began to increase again. The starch concentration found in July 2001 was similar to that found in July 2000 (35 vs. 47 mg g^{-1} dry weight). Starch and sorbitol were weakly negatively correlated ($r^2 = 0.48$); an increase in starch coincided with a decrease in sorbitol. Sucrose concentrations were constant until November, and then dropped significantly in December ($P < 0.001$) where they remained constant until a significant increase in July. Glucose + fructose (not measured separately) was present in very low concentrations and therefore no further analysis was carried out.

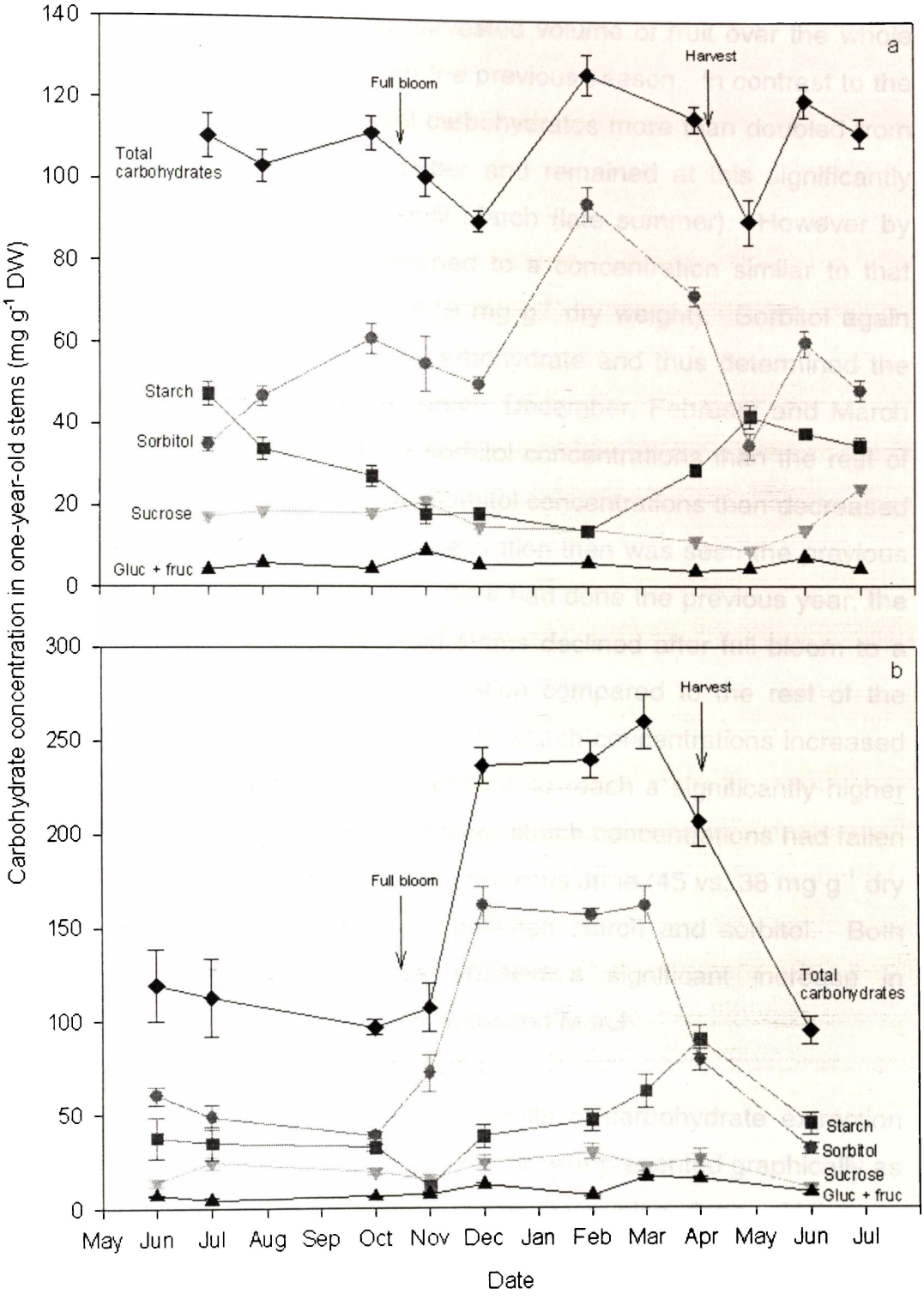

Figure 2.1. Carbohydrate concentration of one-year-old stems (mg g^{-1} dry weight) from fruiting trees grown in the Waikato. Sampling dates July 2000 to July 2001 (a) and sampling dates June 2001 to June 2002 (b). Error bars are standard errors (n = 6 - 12). Note different y-axes. 'Off' trees were excluded from analysis.

The seasonal trends for all carbohydrates from June 2001 to June 2002 are shown in Figure 2.1b. The harvested volume of fruit over the whole block was higher this season than the previous season. In contrast to the previous year, total non-structural carbohydrates more than doubled from November (late spring) to December and remained at this significantly higher concentration ($P<0.001$) until March (late summer). However by June 2002 (winter) they had returned to a concentration similar to that found the previous June (94 vs. 119 mg g^{-1} dry weight). Sorbitol again made up the majority of the total carbohydrate and thus determined the shape of the total carbohydrate curve. December, February and March had significantly higher ($P<0.001$) sorbitol concentrations than the rest of the year (up to a 3-fold increase). Sorbitol concentrations then decreased by June to a significantly lower concentration than was seen the previous June (31 vs. 60 mg g^{-1} dry weight). As it had done the previous year, the starch concentration of one-year-old stems declined after full bloom to a significantly ($P<0.001$) lower concentration compared to the rest of the year. However, unlike the previous year, starch concentrations increased from November and continued to increase to reach a significantly higher concentration in April. By June, however, starch concentrations had fallen to a similar concentration as found the previous June (45 vs. 38 mg g^{-1} dry weight). No correlation was found between starch and sorbitol. Both sucrose and glucose + fructose showed a significant increase in concentration during December, February and March.

There is some contention about how results of carbohydrate extraction should be presented. In this case, the results are presented graphically as mg g^{-1} dry weight as many other authors have also done. However, Priestley (1973) suggests that expression on the basis of residue dry weight is more suitable when looking at reserves. In this study, the dry weight of the residue left after extraction was measured and was found to be similar to the dry weight of sample initially used (results not shown). Expressing results in terms of fresh weight may give the best measure of metabolic activity, but can be considered inappropriate due to fluctuations in water content throughout the day. Units of mmoles g^{-1} is a more

meaningful expression when looking at the physiology of the carbohydrate reserves and results are presented in this way in Table 1. Presenting the results as mmoles g^{-1} does not alter the trends described above. Presenting results as absolute amounts per shoot also did not alter the trends described above, however the variation between limbs increased, resulting in larger standard errors.

Table 2.1. Results from Figure 2.1 expressed as mmoles g^{-1} dry weight.

Date	Carbohydrate concentration (mmoles g^{-1})			
	Starch	**Sorbitol**	**Sucrose**	**Gluc + fruc**
July-00	0.267	0.193	0.039	0.024
August-00	0.172	0.243	0.040	0.032
October-00	0.115	0.344	0.019	0.025
November-00	0.046	0.281	0.018	0.050
December-00	0.115	0.280	0.028	0.033
February-01	0.082	0.422	0.027	0.033
April-01	0.174	0.395	0.012	0.022
May-01	0.260	0.191	0.015	0.024
June-01	0.233	0.331	0.022	0.040
July-01	0.163	0.251	0.059	0.026
October-01	0.165	0.213	0.039	0.036
November-01	0.171	0.397	0.026	0.041
December-01	0.215	0.887	0.036	0.071
February-02	0.278	0.578	0.045	0.039
March-02	0.152	0.690	0.003	0.091
April-02	0.422	0.425	0.027	0.085
June-02	0.188	0.157	0.007	0.045

When the one-year-stems were separated into bark (cambium, phloem, periderm) and wood (xylem and pith) all carbohydrates were found in the highest concentrations in the bark (Fig. 2.2). The concentration of sorbitol found in the bark was four times higher than what was extracted from the wood. In contrast, the concentration of sucrose found in the bark was only 2.5 times higher than in the wood. Twice as much starch was found in the bark than in the wood. Glucose + fructose was found in similar concentrations in both the bark and wood.

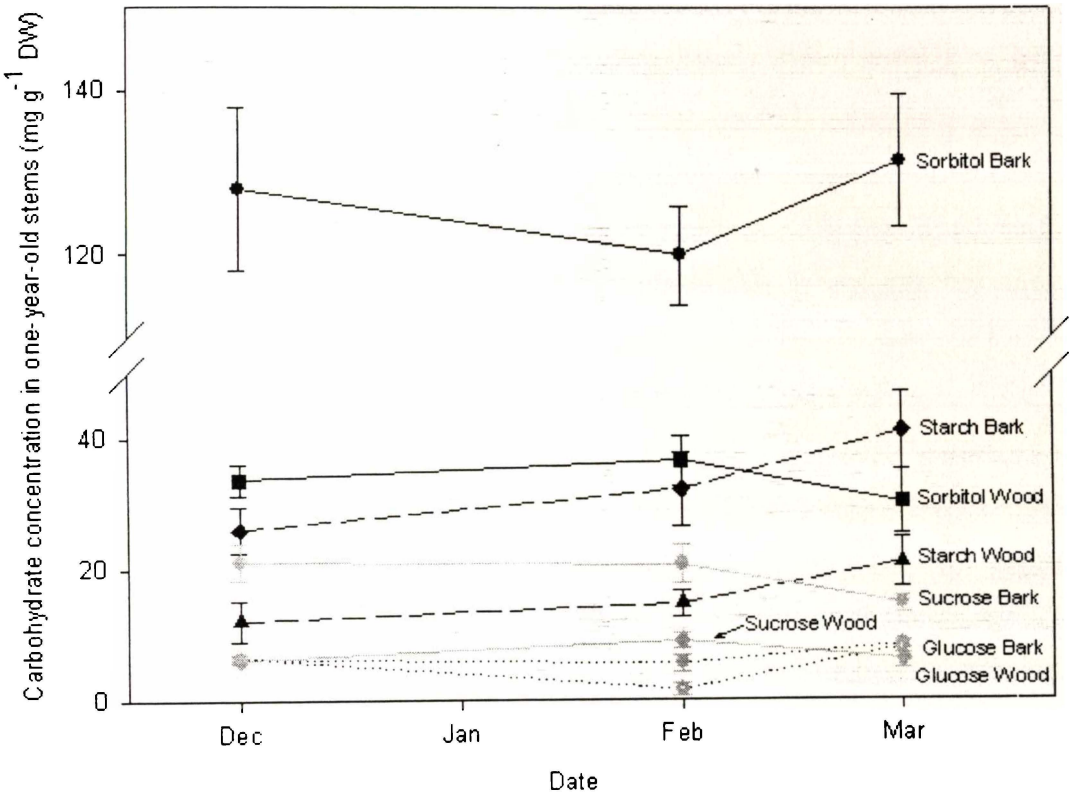

Figure 2.2. Carbohydrate concentrations (mg g^{-1} dry weight) of one-year-old stems separated into bark and wood components from trees grown in the Waikato (December 2001 to March 2002). Error bars are standard errors (n=6).

The iodine stained cross sections of the one-year-old stems showed that in September 2000 (a time when starch concentration was high), starch was easily visible (as black or dark blue grains) in the parenchyma around the vessel cells near the cambium (Fig. 2.3a). Starch was also present in the ray cells. By December 2000 (a time when starch concentration was reduced), no starch was visible near the cambium, however starch could still be seen in the xylem parenchyma and ray cells near the pith and as well as in the pith (Fig. 2.3b).

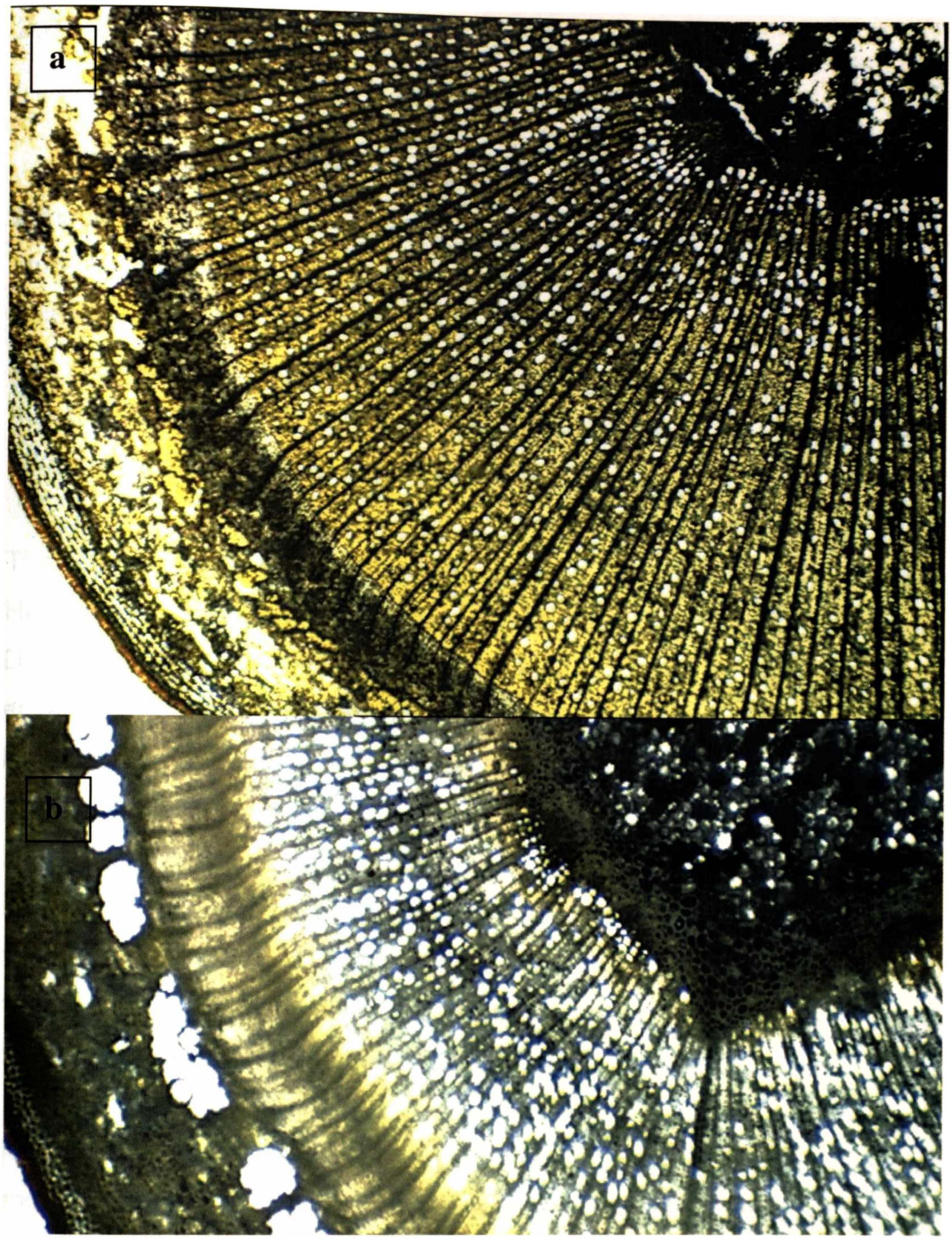

Figure 2.3. Cross sections of one-year-old apple stems stained with iodine stain. Black or navy blue areas represent starch grains. a) September 2000 b) December 2000. Higher resolution pictures were unable to be obtained due to the difficulty in cutting very thin sections of woody tissue.

2.4. Discussion

2.4.1. Seasonal carbohydrates

Although carbohydrate interactions in apple trees have been the subject of study for many years, no data has been obtained for trees growing in New Zealand. Knowledge of the carbohydrate reserves in New Zealand trees was necessary for experiments involving manipulation of these reserves. It was important to know when carbohydrates were at maximum and minimum levels for interpretation of further experiments.

The reported carbohydrate fluctuations in fruit trees in variety of Northern Hemisphere locations (Mochizuki and Hanada 1957, Priestley 1960, Dowler and King 1966, Tromp 1983, Oliveira and Priestley 1988, Yoshioka et al. 1988, Gaudillere et al. 1992, Corelli-Grappadelli et al. 1994, Hagidimitriou and Roper 1994) were similar to those seen in trees grown in New Zealand. In summary, starch declined from bud break in spring until late summer when replenishment occurred. A maximum was reached in autumn, followed by a decline over winter. A second minimum has been reported in late summer when growth of shoots is most vigorous, although this was not seen in these results. Sorbitol and sugar concentrations have been shown to increase as starch decreases.

This study was carried out on one-year-old stems due to ease of sampling and the ability to remove this wood over time without adversely affecting the health of the tree. Oliveira and Priestley (1988) state that all parts of a plant (shoots, stems, roots) have the same pattern of seasonal variations, only the magnitude varies depending on the nature of the tissue, its position to sites of photosynthesis and its growth. Thus it is a reasonable assumption that the one-year-old stems are a reflection of the carbohydrate status of the rest of the tree.

Interestingly, in both years of this study, the carbohydrate concentration of the one-year-old stems returned to similar levels over winter, regardless of what happened to the carbohydrates earlier in the season. In 2001/2002, the 2-fold increase in total carbohydrates over the summer still resulted in lower carbohydrate levels in winter. The cause of this 2-fold increase is uncertain, but one suggestion is differences in climate at critical times of fruit development. Alternatively, the trends found could be a consequence of the previous years fruit load. The return to similar concentrations of carbohydrates over winter, regardless of concentrations early in the season, raises questions about the importance of reserves in one-year-old stems.

Reserves are probably only important in the very earliest stages of growth in spring (Hansen, 1971; Kandiah, 1979a) and there is evidence that carbohydrate reserves do not always influence growth the following season (Avery *et al.*, 1979; Hennerty and Forshey, 1971; Priestley, 1981; Scholefield *et al.*, 1978). Priestley (1963) found that initial reserves did not determine the amount of new growth in the spring and that shading at this time only influenced the rate of reserve replenishment.

Priestley (1970) suggests that there is a degree of internal control of carbohydrate use within apple trees. This suggests that extra carbohydrate laid down in the summer was moved to other parts of the tree to maintain this internal control. Carbohydrate levels in one-year-old stems may be kept at a minimum over winter, as these branches are likely to be prone to loss (e.g. through storms, snowfall).

Walton et al. (1999) have estimated the cost of apple fruit growth of an average 'Braeburn' apple to be 33.3g glucose. In October, immediately before budbreak, one-year-old stems over the entire tree contain approximately 43g glucose equivalents[1]; enough reserves for the growth

[1] Value obtained by converting moles of carbohydrates to glucose equivalents and estimating the fresh weight of one-year-old stems by averaging stem weights harvested in October. The total number of one-year-old stems was estimated from counts of the number of stems per tree.

of less than two fruit. Thus carbohydrate from other parts of the tree and from photosynthate production early in the season must have a much greater importance to the growth of fruit than the reserves available in one-year-old stems.

A tree with an adequate supply of carbohydrates will support flowering and setting of large numbers of fruit in the spring (Tustin *et al.*, 1997). However there is a lack of information in the literature of what constitutes an adequate supply of carbohydrate. Total carbohydrates increased 2-fold over the summer of the second year during this study. This suggests that at no time were carbohydrates limiting growth, as the apple tree was able to maintain or even increase its level of carbohydrate reserves during periods of strong sink demand from fruit.

New Zealand has very high apple yields compared to the Northern Hemisphere (Tustin *et al.*, 1997), and it is thought that this is due to the long photosynthetic period after harvest resulting in a lot of carbohydrate storage. Certainly when trees are prematurely defoliated immediately after harvest there is delayed bud break the following year with less fruit set and lower yields (Loescher et al. 1990, Lakso 1994, Tustin et al. 1997), however the reduced growth of defoliated trees in spring does not distinguish between low reserve carbohydrate and low reserve nitrogen (under normal senescence, N is removed from leaves before leaf fall). Artificial defoliation will remove N reserves, which may play an equal or greater role than carbohydrate reserves in spring growth (Cheng and Fuchigami, 2002; Sauter and Neumann, 1994). A recent study by Cheng and Fuchigami (2002) has found that carbohydrate reserves may not be as important as nitrogen reserves in spring growth of young apple, thus research on amino acids as the limiting factor in fruit growth may prove valuable.

Tartachnyk and Blanke (2001) found that apple leaves enhance their photosynthesis in autumn, possibly resulting in a lot of carbohydrate being laid down over the long New Zealand autumn. However an increase in

carbohydrate concentration was not seen after harvest in this study. The reported increase in carbohydrate reserves after harvest implies that one-year-old stems do not reflect the carbohydrate partitioning of the rest of the tree; it is possible that reserves were laid down in older wood and this requires further investigation. However, other evidence suggests that the high yields of New Zealand apple trees may not be due to increased carbohydrate storage after harvest, but due to the lower dark respiration rates in New Zealand trees brought about by cooler night temperatures resulting in less carbohydrates being used (Lakso, 1994).

Sorbitol made up the majority of total carbohydrates in the trees in this study. Interconversions between starch and sorbitol have been correlated to cold hardiness during the winter (Ichiki and Yamaya, 1982). However, no clear relationship between starch and sorbitol was seen in Waikato trees over the winter months, possibly due to reasonably mild winter temperatures (*c.f.* chapter 3, Hawke's Bay trees).

2.4.2. Starch storage

Apple bark may contain available reserves of 50-60% of its dry weight (Oliveira and Priestley, 1988). In this study, the bark always contained higher extracted concentrations of sorbitol and sugars than the wood, which has also been found by Keller and Loescher (1989) and Kandiah (1979b). That this occurred does not mean that the cellular concentration of sugars in the bark was higher than the wood, as the bark contains proportionally more living cells.

In this study, starch was stored throughout the summer, reaching a maximum in autumn. Starch was also found in higher concentrations in bark than in the wood. However, other studies have generally found that starch concentrations are higher in wood (Kandiah, 1979b; Keller and Loescher, 1989).

The results from this study suggest that starch was laid down close to where the sugars were unloaded from the phloem. Microscopic examination of iodine-stained apple wood sections showed starch grains present mainly in the xylem near the cambium at times of the year when starch concentrations were high. However when starch concentrations were low, starch was visualised closer to and in the pith region of the stem. This suggests that to begin with, starch nearest the phloem was broken down and used. The starch concentration increased over the summer months (see Fig. 2.1b), meaning that newly assimilated starch was being laid down over this period. This suggests that newly assimilated starch was initially laid down close to the phloem region. Sauter (1988) found that starch was fairly evenly distributed in poplar wood in autumn, a period when starch storage would be expected to be reaching a maximum.

It appears as if starch was initially both stored and used closest to the phloem region in the stem and this is consistent with the physical parameters operating in the stem. Starch production is influenced by the transient compartmentation of solutes in the vacuole (Kosegarten and Mengel, 1998). Vacuolar compartmentation of sucrose and sorbitol represents a storage pool that can regulate concentrations and so maintain a concentration gradient between phloem and storage cells (Kosegarten and Mengel, 1998). In this storage pool, starch synthesis has priority over sucrose and sorbitol storage in vacuoles (Kosegarten and Mengel, 1998). Highest concentrations of sorbitol and sucrose were also found near the phloem region of the stem (higher bark concentrations vs. wood concentrations) suggesting that there was a concentration of solutes from the phloem into the wood (Fig. 2.4). Because the concentration of these solutes was directly related to starch deposition, it is reasonable to believe that starch will be deposited where the concentration of these solutes is initially highest, i.e. close to the phloem. Starch was also likely to be exhausted from the region closest to the phloem initially, as the concentration gradient reversed. Other experiments (see chapter 6) show that all starch in the stem can be utilised by the stem if needed and

Kandiah (1979a) was able to find assimilated ^{14}C in older parts of the wood and in the pith suggesting that even older wood is still physiologically functional. Thus there is no reason to believe that any of the starch seen in the one-year-old stems was unavailable for use.

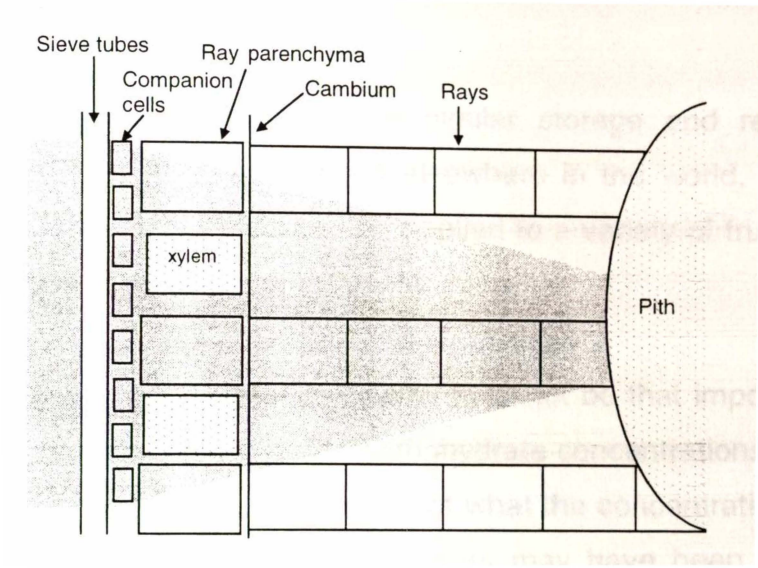

Figure 2.4. A diagrammatic representation of the starch concentration (dark grey area) in the apple stem during storage. Starch is initially stored closest to the phloem region. During remobilisation, starch is also initially depleted from the phloem region.

2.5. Conclusion

This study has shown that the seasonal trends in carbohydrate concentrations seen in New Zealand apple trees, growing in the Waikato region, are similar to those obtained by other authors throughout the world. This information has provided the foundation for further investigations into storage.

New Zealand apple trees have similar storage and remobilisation of carbohydrates as trees growing elsewhere in the world, suggesting that the findings in this thesis can be applied to a variety of fruit trees growing in a variety of locations.

The reserves in one-year-old stems may not be that important for growth of buds the next spring as the carbohydrate concentrations returned to the same level over winter, regardless of what the concentrations were earlier in the season. Some of the reserves may have been moved to older tissue or roots. Immediately before budbreak, the one-year-old stems in the entire tree contain approximately 43g glucose equivalents, which were enough reserves for the complete growth of less than two fruit.

In one-year-old stems, starch was initially both stored and used up closest to the phloem. This is a step towards understanding the underlying processes involved in the storage and utilisation of carbohydrates in apple stems.

Chapter 3: Carbohydrate Allocation[2]

3.1. Introduction

Carbohydrate storage within the perennial tissue of deciduous fruit trees is critical to new leaf and early fruit development (Hansen, 1971). Remobilisation of these reserves from roots and stems ensures an adequate supply of carbohydrate early in the season and this is critical for both fruit yield and quality.

Models of carbohydrate storage and remobilisation have been developed in annual herbaceous species, especially cereals where it has been shown that storage is the lowest priority sink (Wardlaw, 1990). In wheat and barley, carbohydrate storage in the stem is not competitive with grain filling (Schnyder, 1993). However, storage does occur when there are excess photosynthates caused by less demand from other sinks and carbohydrate stored in the stem has a buffering action, providing carbohydrate during deficits in photosynthesis production (Schnyder, 1993).

Models of carbohydrate storage and remobilisation in woody perennial species have not been developed. Studies into carbohydrate storage in trees have generally concentrated on measurements of the reserve pools over an annual cycle and there have been few studies on the mechanisms involved in carbohydrate storage and remobilisation.

The role of carbohydrate storage in determining the yield of fruit crops in the subsequent season is controversial. Some studies have questioned the importance of reserves for new growth (Avery *et al.*, 1979; Hennerty and Forshey, 1971; Priestley, 1981; Scholefield *et al.*, 1978). Apple yields in New Zealand of 100 t fruit ha^{-1} yr^{-1} (Bieleski, 2000) are almost twice as

[2] The starch data obtained in this chapter has been accepted for publication in Acta Horticulturae (see Appendix 1).

high as in New York (Lakso, 1994), but the physiological reasons behind this are not fully understood. This situation provides a model for testing various hypotheses regarding storage and yield. A current hypothesis for the higher yields is the long photosynthetic period after harvest in New Zealand trees, allowing accumulation of considerable reserves in autumn (Tustin *et al.*, 1997). Studies to test this hypothesis have involved premature defoliation of New Zealand trees in autumn (Tustin *et al.*, 1997). However, this method does not differentiate between low carbohydrate reserves and low nutrient reserves (see *Discussion*, chapter 2). Defoliation experiments, unless modified by adjusting nutrient levels (especially N) in a tree, are at best, equivocal in explaining the role of carbohydrate storage. The work described in this thesis attempts to describe carbohydrate storage more directly.

Another problem in apple tree fruit production that may have an origin in carbohydrate storage, is that of alternate bearing. All fruit trees, and apple in particular, suffer from alternate high and low yield years. This cyclical production is related to biannual carbohydrate fluctuation in which trees store large amounts of carbohydrate in a non-fruiting or 'off' year, which is made available the following heavy fruiting or 'on' year (Li *et al.*, 2003). In extreme cases, carbohydrate depletion causes collapse of trees at the end of an 'on' year (Smith, 1976). Alternate bearing is unquestionably related to carbohydrate storage, but whether as cause or effect has not been determined.

Alternate bearing reduces economic returns in both years from either the low yield and excessive fruit size in 'off' years or the reduced fruit size and increased thinning costs in 'on' years (McArtney, 1994). Alternate bearing is managed and the negative effects mitigated in the orchard by management practices such as reducing fruit set with chemical thinning agents. There is evidence that alternate bearing may be a hormonal response from seed produced gibberellins (Khurshid *et al.*, 1997; McArtney, 1994; McArtney and Li, 1998) in which a hormonal message from developing seeds inhibits flower bud formation in adjacent tissues.

However, the use of inhibitors to overcome alternate bearing in apples is not widely documented in the literature (McArtney and Li, 1998). There is no consensus or understanding of the true cause of alternate bearing. Is alternate bearing a carbohydrate initiated or a carbohydrate related problem? High fruit loads in one season deplete reserves to such an extent that there is little flower initiation and production in the subsequent season, resulting in a large build up of reserves continuing the cycle. This model merely describes the obvious; it does not explain the seasonal carbohydrate storage mechanism or the mechanisms behind alternate bearing. This study attempts to understand the mechanisms of carbohydrate storage in apple trees.

In apple trees, it is not clear what causes storage in the stem to occur. Storage of carbohydrates is likely to occur when there is less demand by other sinks as it does in cereal crops. However in apple trees, there is an interaction between sink strength and season making it hard to distinguish one from the other. In this study, the hypothesis that storage of carbohydrate is determined by sink demand in apple trees was tested.

To investigate the hypothesis, it was necessary to disrupt the seasonal aspect of sink demand. This was achieved by creating model systems (girdled stems) in which to test this hypothesis. At different stages throughout the season, fruit load was reduced to alter sink demand to determine if reserve accumulation was responsive to changes in sink demand.

Two possible outcomes were expected from this experiment. If storage is dependent on sink priority, then removal of fruit should cause an increase in storage, regardless of the time of year. But if this is not the case, storage of carbohydrates may be a seasonal phenomenon and at a certain time of the year, reserve carbohydrates should increase regardless of the number of sinks present.

3.2. Method

Carbohydrate concentrations in one-year old stems of 8-year old 'Braeburn' apple trees, grown on various rootstocks, were examined at the HortResearch Havelock North Research Centre, New Zealand (NZMS 260 V21 644 428), from December 2000 to July 2001. Trees were planted at a spacing of 5 x 3.5m in north-south oriented rows. Originally part of an interstem trial, the trees were planted in a randomised design of four replicates per interstem type. Three (MM.106, M.793, M.793 with 10cm M.9 as an interstem) of the original seven interstem treatments were used in this experiment. Twelve trees were selected based on there being four trees with the same interstem that looked similar in form. Within these groups of four, two trees were randomly selected to have stems girdled, while two trees were the ungirdled controls.

Dormant pruning, fertilisers, pesticides and herbicides were applied as required according to standard commercial practices, however no hand-thinning of fruit was carried out in order to keep fruit on the one-year-old stems (fruit on this wood is removed in normal orchard practice). 'Braeburn' was a convenient cultivar to use for this trial as it can flower and set fruit well on one-year-old stems. Full bloom occurred on the 26th of September 2000.

Disruption of the phloem tissue by stem girdles maintained an isolated system for the period of this study, so that no carbohydrates were removed via the phloem from the experimental system. In December 2000, 34 one-year-old stems carrying at least six fruit and of similar form, were girdled per tree. A 2cm wide strip of phloem, cambial tissue and connected bark was removed. An aluminium splint was taped to the girdled area for support and the girdle left exposed to the air. Twenty stems per tree were thinned to a high fruit load (6 fruit), eight stems to a low fruit load (2 fruit), and six stems to a zero load (no fruit). The fruit loads were reduced to low or zero in January and April (commercial

harvest time). Unless stated, fruit was not removed from the tree, but left to abscise naturally.

Sampling for carbohydrate extraction was carried out six-weekly from December 2000 until July 2001. Careful examination of the girdles was made during sampling to ensure phloem discontinuity was retained. Spurs, leaves and fruit were collected for biomass measurements at each sampling time. The entire stem was removed at the girdle and separated into this season's and one-year old growth. One-year-old stems were cut into small pieces and frozen in liquid nitrogen on site. The stem was not separated into components; bark and wood were analysed together. Frozen samples were then stored at -80^0C. After freeze-drying, samples were stored at -20^0C until carbohydrate extractions were carried out.

Stem samples were ground into a fine powder (mill grinder, General Electric Motors). Samples were then well mixed and re-freeze-dried before weighing. 100mg of powered sample was subsequently extracted for carbohydrates by heating in 80% ethanol for 60mins at 60^0C as described in chapter 2. After mixing well and centrifuging, sugars were assayed (in duplicate) from the supernatent as described by Jones et al. (1977) and starch was assayed from the pellet. 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.

Sucrose, sorbitol, glucose + fructose and starch were extracted from the one-year-old stems as described in chapter 2.

Photosynthesis was measured indirectly using a Delta T AP4 transit-time porometer (United Kingdom) to assess stomatal opening. Porometer measurements were made in December, January and February, starting at 10:00am and finishing by midday.

On the data from the ungirdled stems, analysis of variance (ANOVA) was performed and the assumption that parametric parameters were filled was tested before an analysis. The hypothesis that carbohydrate storage in apple stem tissue was a seasonal phenomena was tested by statistical analysis carried out by a linear mixed model fitted using GenStat (© 2002, Lawes Agricultriural Trust, Rothamsted Experimental Station). A linear mixed model was necessary because of missing data and to include ungirdled stems (which did not have the same treatments) in the same analysis as girdled stems. Stems that had broken and died, or lost significant amounts of fruit prematurely, were excluded from analysis. Missing data points were found to be random across girdled stems with different fruit load treatments and thus could be estimated by GenStat and included in the data set.

3.3. Results

Different effects between trees due to different interstems and rootstocks were allowed for by the statistical analysis, but there was no evidence for any such effects. Variation within stems of the same tree was greater than between tree variations.

3.3.1. Seasonal changes in carbohydrate concentration

Seasonal changes in starch, sorbitol, sucrose and glucose + fructose were determined in ungirdled one-year-old stems (fruit load not manipulated) from apple trees grown in the Hawkes Bay with an above average fruit load (Fig. 3.1). Total non-structural carbohydrate concentrations changed little before fruit harvest, but did increase slightly after harvest. Total non-structural carbohydrate concentrations were significantly higher ($P=0.003$) in May and July than in the previous months.

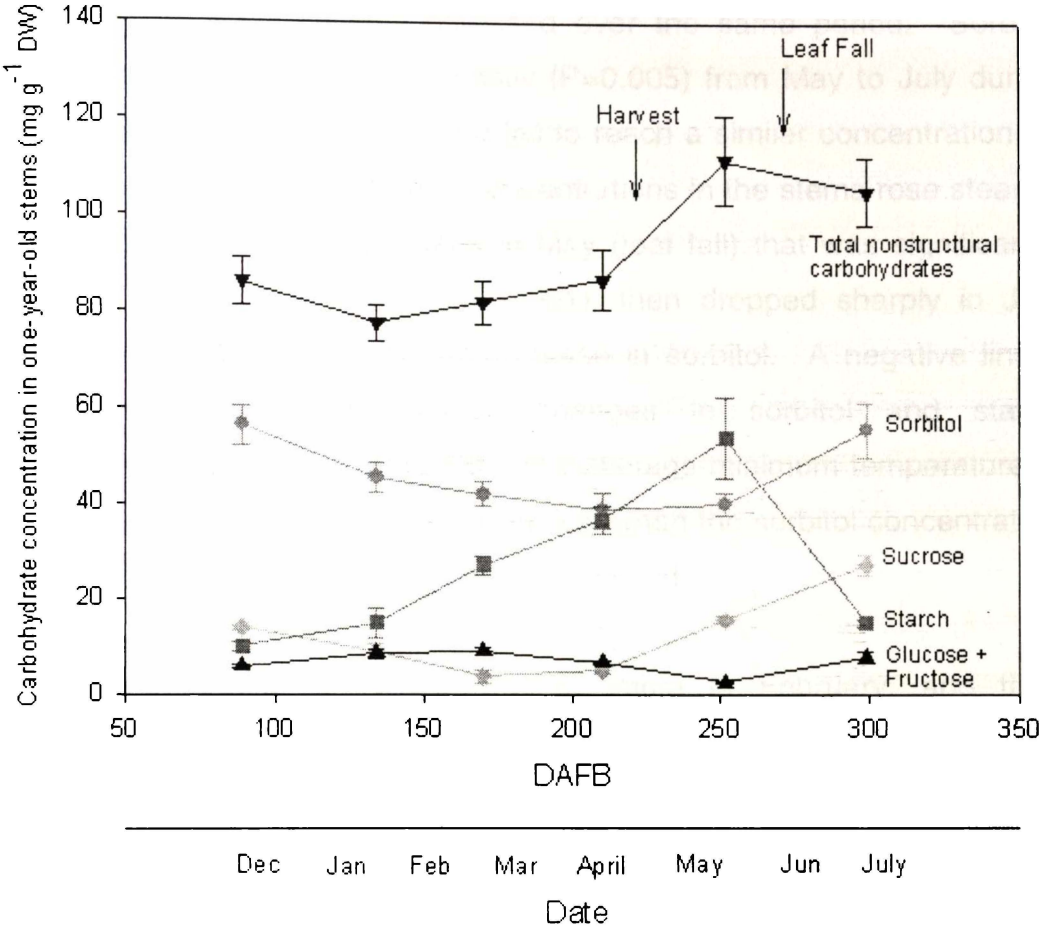

Figure 3.1. Seasonal changes in carbohydrate concentration (mg g^{-1} dry weight) of one-year-old stems from ungirdled branches from trees grown in the Hawkes Bay. DAFB = days after full bloom (full bloom was on 6th September 2000). Error bars are standard errors (n=6).

Sorbitol was the major carbohydrate in the stems, except in May (late autumn), when starch levels were slightly higher. The sorbitol concentration of the stems gradually declined from December to May, while starch concentrations increased over the same period. Sorbitol concentrations increased significantly ($P=0.005$) from May to July during the period of leaf senescence and fall to reach a similar concentration as was found in December. Starch concentrations in the stems rose steadily from December, reaching a peak in May (leaf fall) that was significantly higher than all other months ($P<0.001$), then dropped sharply in July (midwinter), corresponding to an increase in sorbitol. A negative linear correlation was found between changes in sorbitol and starch concentrations over time ($r^2=0.63$). The average minimum temperature in the orchard declined to around 1^0C in July when the sorbitol concentration increased and the starch concentration declined.

Sucrose concentrations reached a minimum in February, and then increased constantly to a significantly higher concentration in July ($P<0.001$) (Fig. 3.1). Glucose + fructose was always present in the stems in low, fairly constant concentrations and because the concentration was so low, was not analysed further.

3.3.2. Changes in carbohydrate concentration due to fruit load

Total non-structural carbohydrates in one-year-old stems that had been girdled and the fruit load adjusted were found to differ with fruit load (Fig. 3.2). All stems began with similar total carbohydrate concentrations in December, but after this time, stems with reduced terminal sink demand (low and zero fruit loads) had higher total carbohydrate concentrations than stems with high terminal sink demand.

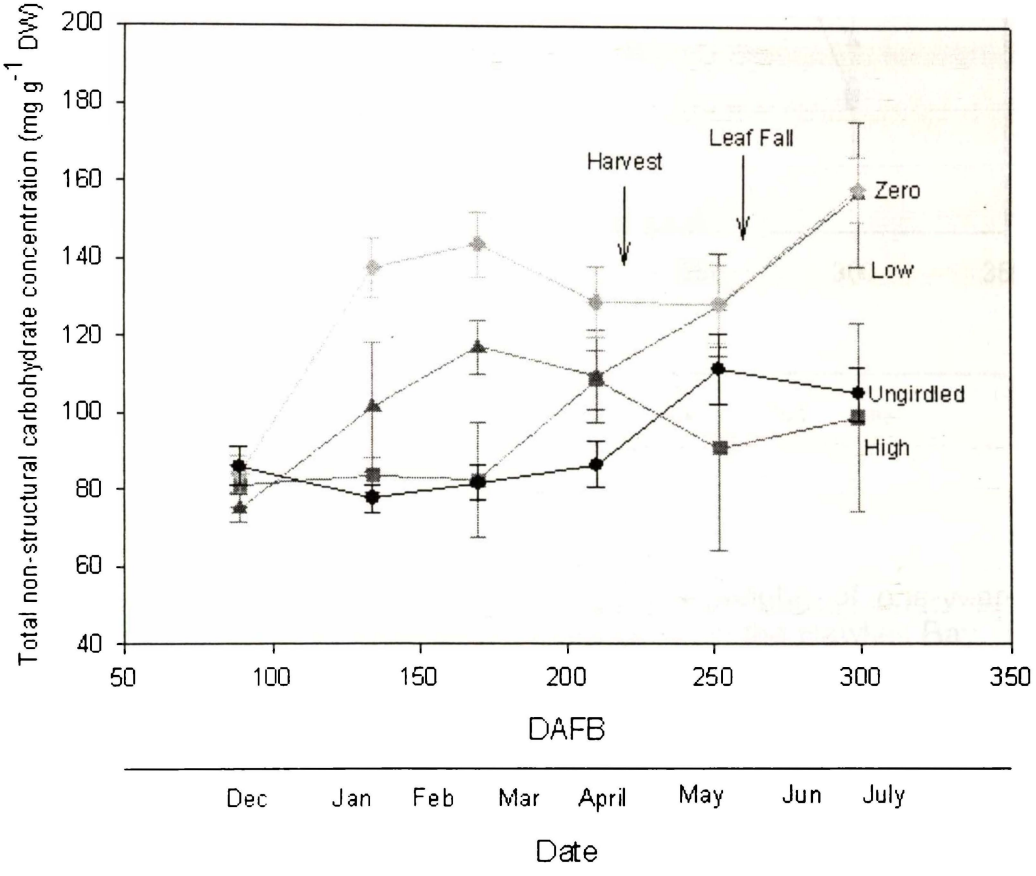

Figure 3.2. Total non-structural carbohydrate concentration (mg g^{-1} dry weight) of one-year-old stems under different fruit loads from trees grown in the Hawkes Bay. Full bloom was on 6th September 2000. Harvest and leaf fall refer to normal timings in commercial orchard trees. Harvest and leaf fall varied amongst the girdled stems. Error bars are standard errors (n=3 – 6). DAFB = days after full bloom. High = 6 fruit/branch Low = 2 fruit/branch Zero = no fruit/branch Ungirdled = phloem connections retained and fruit load not altered.

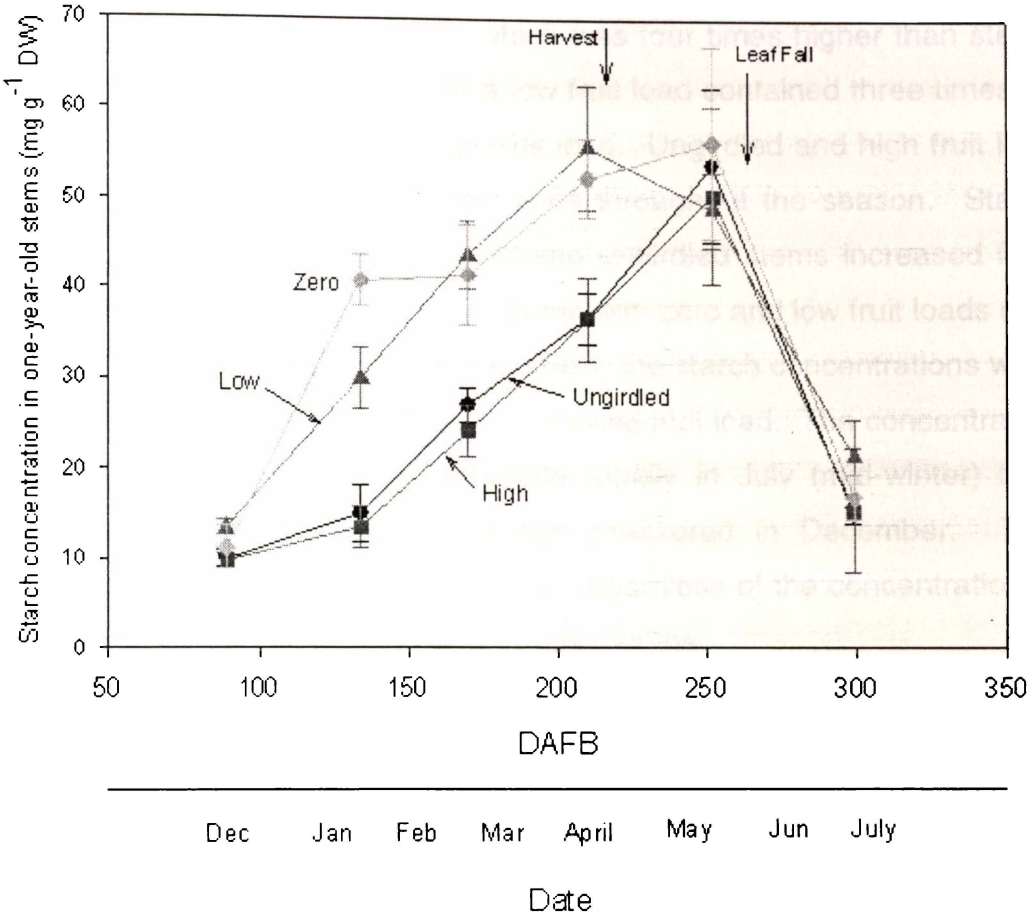

Figure 3.3. Starch concentration (mg g^{-1} dry weight) of one-year-old stems under different fruit loads from trees grown in the Hawkes Bay. Full bloom was on 6th September 2000. Harvest and leaf fall refer to normal timings in commercial orchard trees. Harvest and leaf fall varied amongst the girdled stems. Error bars are standard errors (n=3 – 6). DAFB = days after full bloom. High = 6 fruit/branch Low = 2 fruit/branch Zero = no fruit/branch Ungirdled = phloem connections retained and fruit load not altered.

The starch concentration of one-year-old stems was affected by fruit load. Starch concentrations were similar in all stems when girdling was carried out in December (Fig. 3.3). In January, stems with a zero fruit load had the highest starch concentration, which was four times higher than stems with a high fruit load. Stems with a low fruit load contained three times as much starch as stems with a high fruit load. Ungirdled and high fruit load stems had similar starch concentrations throughout the season. Starch concentrations in the high fruit load and ungirdled stems increased five-fold from January to May, whereas stems with zero and low fruit loads only increased by 1.5 fold, meaning that in May, the starch concentrations were the same in all stems, regardless of the initial fruit load. The concentration of starch in all stems then decreased rapidly in July (mid-winter) to a concentration only slightly above that measured in December. This decrease in July was seen in all stems, regardless of the concentration of starch that was measured in the previous months.

The response of sorbitol concentrations to manipulation of fruit loads was not as clear as starch or total carbohydrates (Fig. 3.4). The concentration of sorbitol in the stems was the highest of any single carbohydrate (sorbitol concentration was four-fold higher than the starch concentration in December). In the first three months of measurement, sorbitol concentrations followed a similar pattern to starch concentrations. Stems with a zero fruit load had the highest concentrations of sorbitol, low fruit load stems had intermediate concentrations, and ungirdled stems and those with a high fruit load had the lowest concentrations. Unlike starch concentrations, the ungirdled stems had less sorbitol than the high fruit load stems. The sorbitol concentration increased from 55 to 75 mg g^{-1} dry weight during the months of January and February in stems with a zero fruit load, but in ungirdled and low fruit load stems the concentration actually decreased over this time, while the concentration in high fruit load stems did not change. In April, the sorbitol concentration decreased significantly in stems with a zero fruit load and also declined in stems with other fruit loads. As a consequence, there were no significant differences between stems with different fruit loads in April and May. In May, starch

concentrations reached a peak five times higher than the December concentration, whereas the sorbitol concentrations were similar to the December concentrations and in ungirdled stems, the sorbitol concentration was actually lower. In July, concentrations of sorbitol increased significantly in all stems except those under a high fruit load. This increase in sorbitol concentration in July corresponded to a decrease in starch concentration.

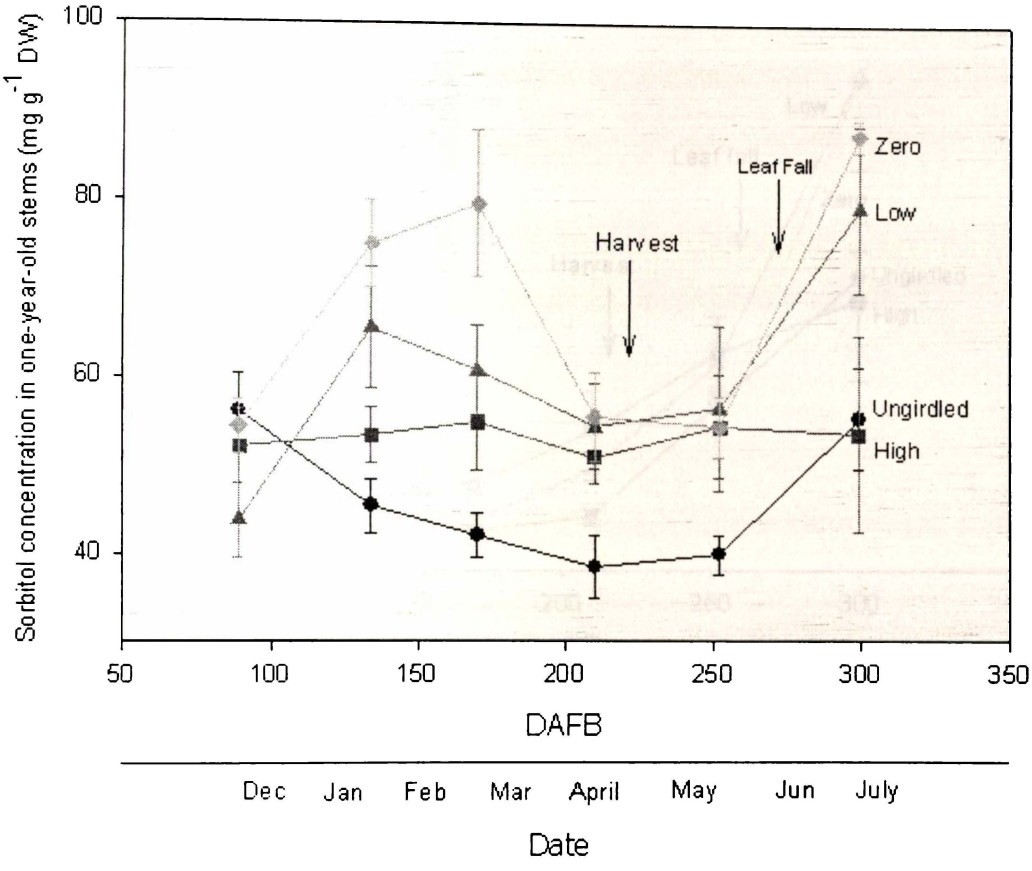

Figure 3.4. Sorbitol concentration (mg g^{-1} dry weight) of one-year-old stems under different fruit loads from trees grown in the Hawkes Bay. Full bloom was on 6th September 2000. Harvest and leaf fall refer to normal timings in commercial orchard trees. Harvest and leaf fall varied amongst the girdled stems. Error bars are standard errors (n=3 – 6). DAFB = days after full bloom. High = 6 fruit/branch Low = 2 fruit/branch Zero = no fruit/branch Ungirdled = phloem connections retained and fruit load not altered.

The sucrose concentration of one-year-old stems was not affected by fruit load (Fig. 3.5). In July, the sucrose concentration rose significantly ($P<0.001$) in all stems and this increase was greatest in stems that had zero or low fruit loads.

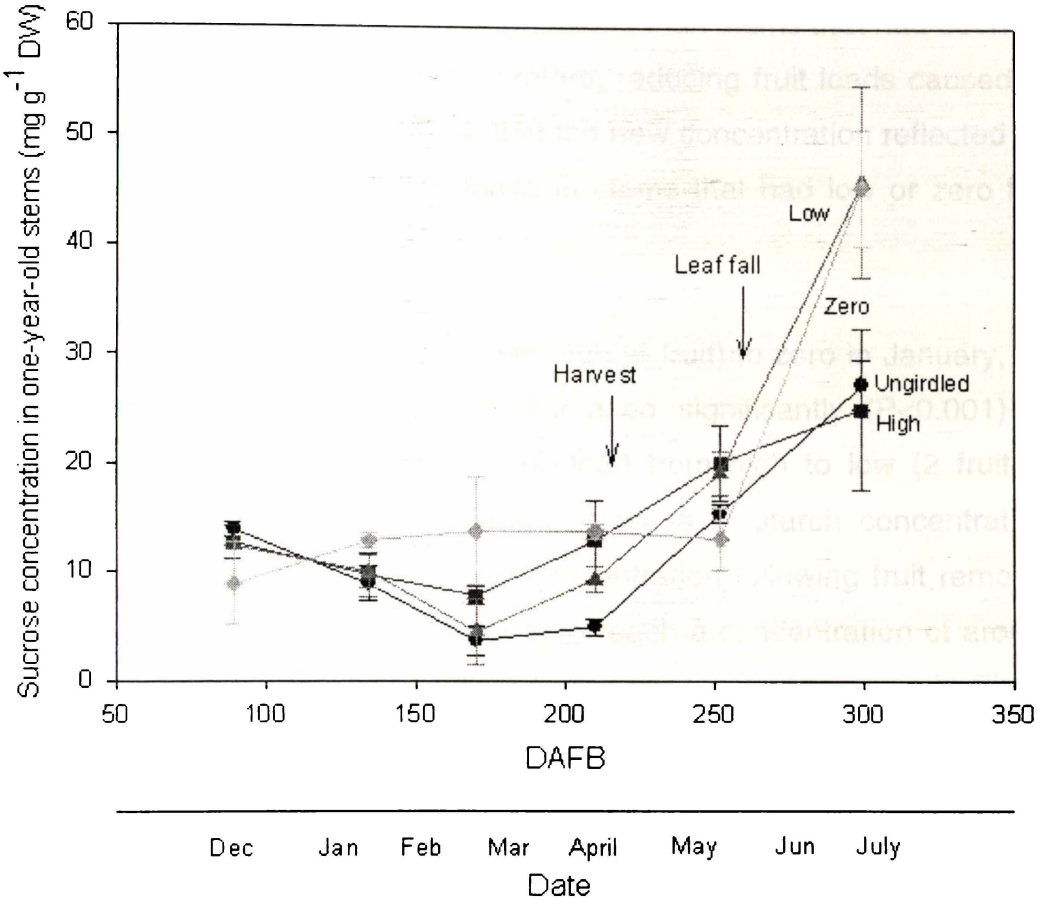

Figure 3.5. Sucrose concentration (mg g^{-1} dry weight) of one-year-old stems under different fruit loads from trees grown in the Hawkes Bay. Full bloom was on 6th September 2000. Harvest and leaf fall refer to normal timings in commercial orchard trees. Harvest and leaf fall varied amongst the girdled stems. Error bars are standard error (n=3 – 6). DAFB = days after full bloom. High = 6 fruit/branch Low = 2 fruit/branch Zero = no fruit/branch Ungirdled = phloem connections retained and fruit load not altered.

3.3.3. Out of season sink changes

When sink demand was reduced throughout the season, in all months, except for May, the total non-structural carbohydrate concentration depended on the number of fruit present during that month. This means that in stems in which the fruit load was altered, the total carbohydrates reflected the new altered load. However, in May, stems that had been reduced to a zero fruit load at some point prior had significantly higher ($P=0.027$[3]) total carbohydrate concentrations than stems that had been set at zero at the time of girdling. Therefore, reducing fruit loads caused an increase in total carbohydrates so that the new concentration reflected the concentration of carbohydrates found in stems that had low or zero fruit loads from the outset.

When the fruit load was reduced from high (6 fruit) to zero in January, the starch concentration in the stem increased significantly ($P<0.001$) by 400% (Fig. 3.6a). Reducing the fruit load from high to low (2 fruit) in January also resulted in a significant increase in starch concentration. However, after an initial increase in concentration following fruit removal, starch concentrations rose only slowly to reach a concentration of around 50mg g^{-1} in May, which was similar to the concentrations seen in Figure 3.3. Starch concentrations then declined in July to become the same as all other stems, regardless of fruit loads. Removing fruit from a high fruit load stem in April (normal harvest time) significantly increased the starch concentration the following month. However, removing fruit from a low fruit load stem in April did not result in a significant increase in starch. Stem starch concentrations consistently fell to similar levels in July, regardless of the size, or changes to fruit load earlier in the season.

[3] Values based on chi-square statistic. In data sets with fewer than 40 observations, the P-values may exaggerate significant effects.

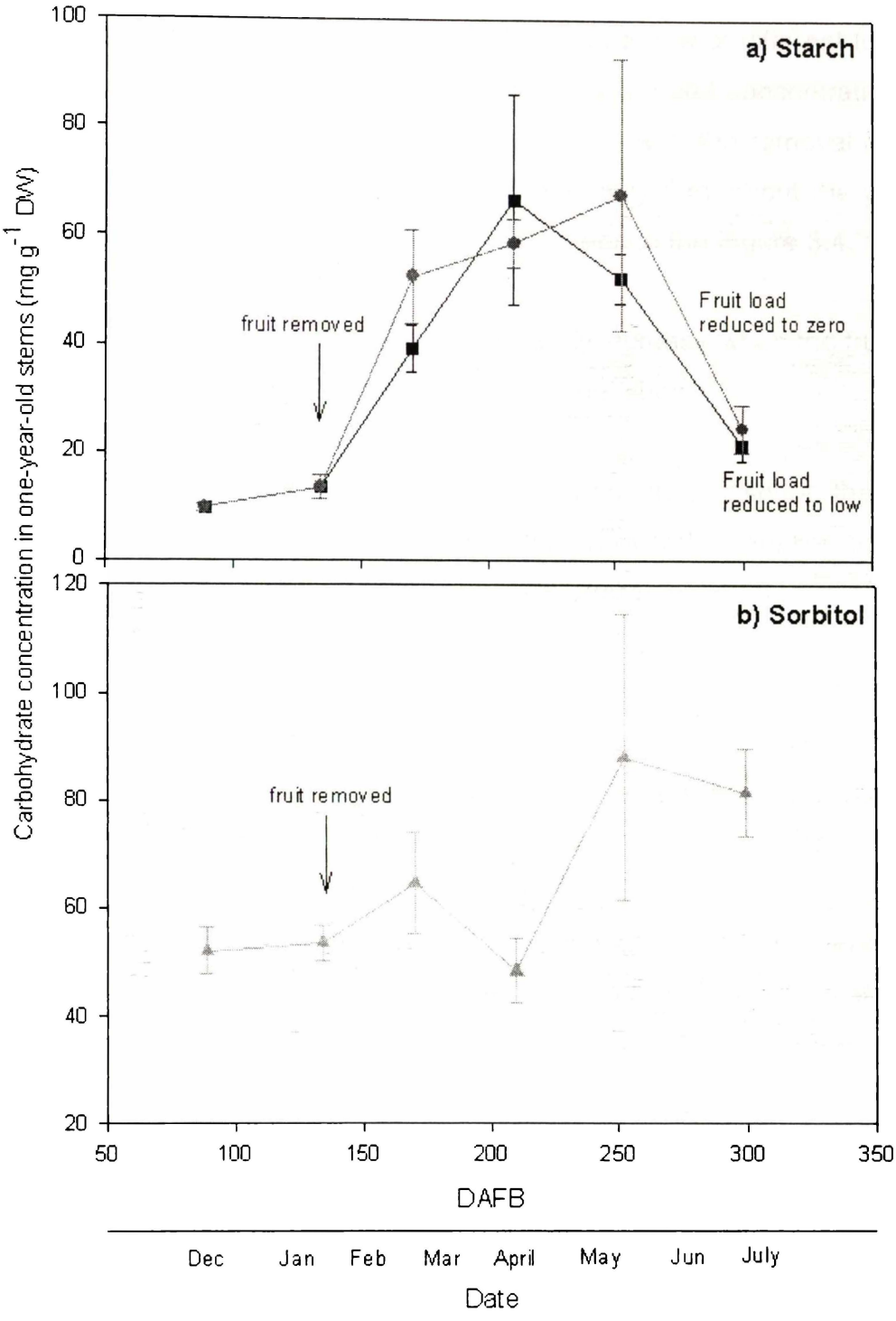

Figure 3.6. The starch (a) concentration (mg g^{-1} dry weight) of one-year-old stems when the fruit load has been reduced from high (6 fruit) in January to low (2 fruit) or zero (0 fruit). (b) the sorbitol concentration when the fruit load has been reduced from high to zero. Error bars are standard errors (n= 3-6). DAFB = days after full bloom.

In contrast to the increase in starch concentrations after the removal of fruit, the sorbitol concentration in the stem did not significantly increase when the fruit load was reduced from high (6 fruit) to zero in January (Fig. 3.6b). Reducing the fruit loads from either high or low at different times of the season also did not significantly affect the sorbitol concentration. In contrast to the abrupt rise in starch levels seen with the removal of fruit, sorbitol concentrations in the stem rose gradually throughout the year to reach similar concentrations in July as were seen in the Figure 3.4.

The sucrose concentration did not significantly increase when the fruit load was reduced at any time of the season (data not shown).

When results were expressed as absolute amounts per shoot, the same differences were seen between treatments, however the variation between limbs increased, resulting in larger standard errors.

Stomatal conductance measurements were made as an estimate of photosynthetic capacity and were found to be very low on shoots with a zero fruit load (data not shown). Leaves on stems with a zero fruit load were thick, xeromorphic, had red venation and chlorosis. These leaves senescenced months before leaves on ungirdled branches and also became very brittle (an indication that the starch levels were very high). Stems that were ungirdled or had a high fruit load retained a high stomatal conductance and healthy green leaves until normal senescence (late May). Stems with a low fruit load showed an intermediate set of characteristics to the high and zero fruit load stems. Where fruit were removed, stomatal conductance remained high for approximately another month before these stems began to show the characteristics seen in stems with zero fruit loads.

3.3.4. Summary of results

This experiment has highlighted a number of important observations of carbohydrate storage in one-year-old apple stems when fruit load (sink demand) was manipulated. The results are presented as a graphical summary (Fig. 3.7).

The main findings of this experiment are:

- Total carbohydrates were higher in stems with low and zero fruit loads than stems that were ungirdled or had high fruit loads
- Starch concentrations reached a peak of about 50mg g^{-1}. No stems ever had significantly higher starch concentrations.
- By May, all stems reached this starch peak, regardless of fruit load earlier in the season
- Starch concentrations declined in winter, generally corresponding to an increase in sorbitol
- Sorbitol concentrations were highest in low and zero fruit load stems at the beginning and end of the season, but these levels fluctuated during autumn
- The sorbitol concentration had a large influence on the total carbohydrate concentration
- Starch concentrations increased within six weeks on the removal of fruit
- Sorbitol concentrations did not increase with the removal of fruit
- Stems with zero fruit loads stopped photosynthesis and senescenced earlier than stems with high fruit loads

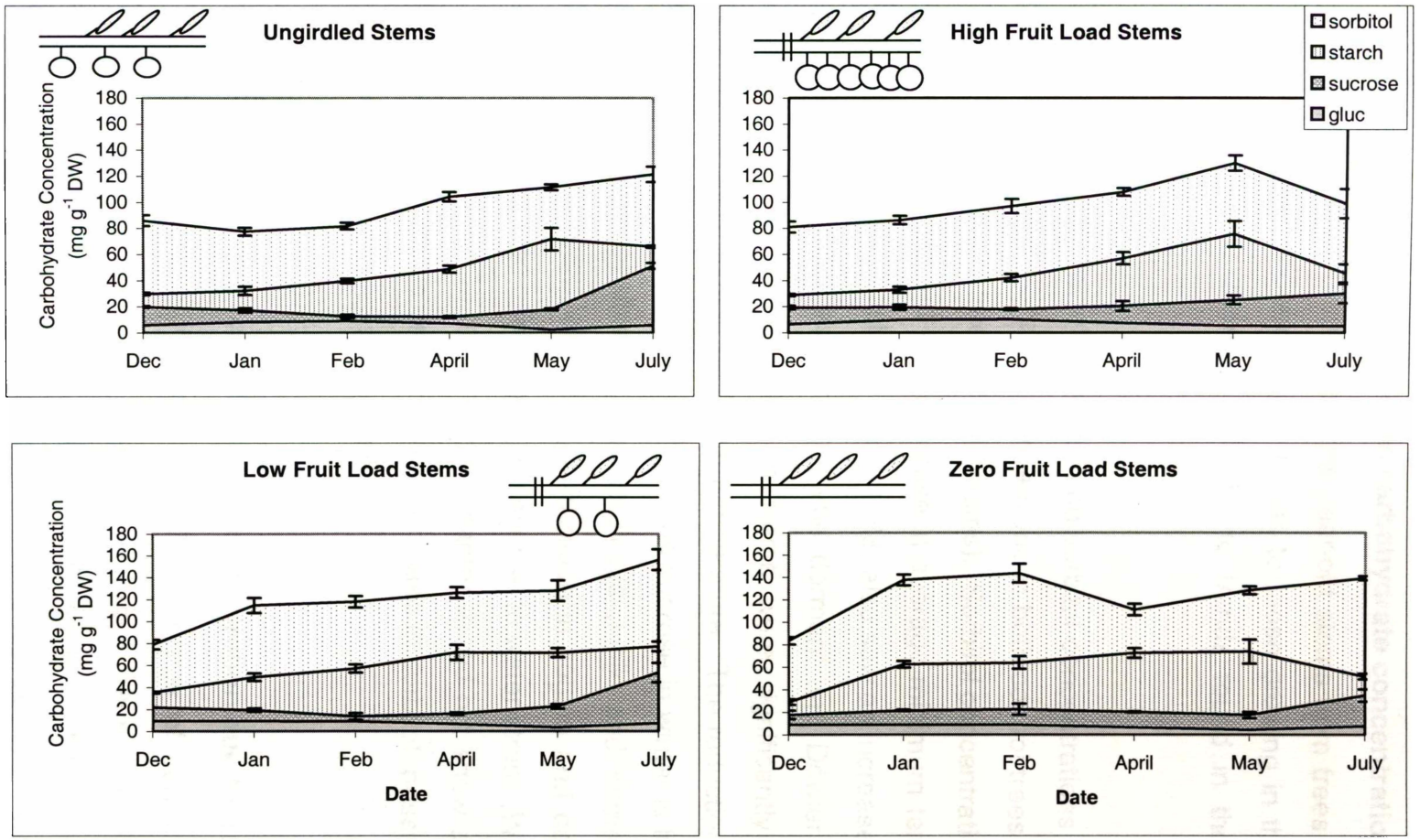

Figure 3.7. Summary of the components making up the total non-structural carbohydrate concentrations in one-year-old apple stems with various fruit loads.

3.4. Discussion

3.4.1. Seasonal changes in carbohydrate concentration

The carbohydrates in ungirdled one-year-old stems from trees growing in the Hawkes Bay showed similar trends to those growing in the Waikato (chapter 2), which were also similar to trends found in the Northern Hemisphere.

Starch concentrations fell in winter and sorbitol concentrations increased, suggesting starch-sorbitol interconversions in both control trees (ungirdled stems) and treatment trees (girdled stems). Sorbitol concentrations in July appeared to correspond to a decrease in average minimum temperature. Priestley (1960) and Yoshioka *et al.* (1988) also saw an increase in sugars and decline in starch as trees became dormant and Dowler and King (1966) found that starch levels in stems were significantly positively correlated to the average maximum temperature. The reduction of starch and corresponding increase in sorbitol and sucrose in winter is likely to be a mechanism responsible for cold hardiness (Ichiki and Yamaya, 1982; Yoshioka *et al.*, 1988), although an increase in sugars and decrease in starch levels has also been found in response to water stress (Wang *et al.*, 1995). Trees grown in the Waikato (chapter 2) did not show a dramatic decline of starch with a corresponding increase in sorbitol, possibly due to the milder, wetter winters in the Waikato.

3.4.2. Changes in carbohydrate concentration due to fruit load

This study investigated the hypothesis that storage of carbohydrate in stems is determined by sink demand in apple trees. In this study, developing fruit were the major carbohydrate sink and altering the number of fruit on a girdled stem was a way of altering the carbohydrate demand of the system. Reducing the fruit load resulted in an increase in stem starch concentrations. A reduction in fruit load in January resulted in a five-fold increase in starch concentration within six weeks (Fig. 3.6a).

Sorbitol concentrations did not respond in this manner (Fig. 3.6b) and possible reasons for this will be discussed below.

A number of other interesting observations were made in this study. Firstly, starch concentrations responded to fruit load early in the season, but in May (autumn), the starch concentration was the same in all stems regardless of their fruit load earlier. Secondly, starch concentrations declined significantly in July (mid-winter) to be the same as concentrations found in December. Finally, sorbitol concentrations only responded to fruit load during the first few months after girdling. These observations will be discussed in more detail below.

In annual herbaceous species, especially cereals it has been shown that storage is the lowest priority sink (Wardlaw, 1990) and is not competitive with grain filling (Schnyder, 1993). In this study, starch concentration increased after fruit removal demonstrating that storage of carbohydrate (at least of starch) in woody stems of apple is determined by sink demand, as it is in herbaceous annual plants. Thus the hypothesis posed in the introduction was correct: The mechanisms involved in laying down starch reserves could be utilised within six weeks, demonstrating that starch can be stored at any time of the season when there is a sudden increase in available photoassimilate. Environmental signals were not necessary to initiate storage of carbohydrates in apple stems, rather, storage was initiated by sink demand. This is consistent with Battey (2000), who states that Rosaceous fruit trees do not go dormant in winter in response to environmental cues. Carbohydrate partitioning into reserves is controlled by carbohydrate availability in apple trees.

The observation that storage in the stem is controlled by carbohydrate availability is consistent with the concept that storage is a low priority sink; stem starch concentrations increased when higher priority sinks (such as fruit) were removed or reduced. Growth of fruits, leaves and shoots is likely to have occurred before stem storage. This is consistent with Wardlaw's (1990) order of sink priority: growth of seeds > growth of fleshy

fruit parts = growth of shoot apices and leaves > growth of cambium > growth of roots > storage. This implies that starch is stored in the stem of apple trees only when competing sinks, such as fruit, are removed or their demand is saturated. In this study, storage has been shown to occur when these other sinks are removed or unavailable (through girdling), regardless of the time of year.

This study has demonstrated that the stem storage sink is functional at any time of the year, although reduced by its low priority, at times when there is little excess in carbohydrate supply. This shows that the mechanisms of carbohydrate storage in woody perennials are present, or at least able to be induced within the sampling of time of six weeks of this study, as they are in cereals; more storage occurs when there are excess photosynthates caused by less demand from other sinks (Schnyder, 1993). In this study, although storage in the stem is a low priority sink, storage occurred even in high fruit load and ungirdled stems at a consistent rate during times of strong sink demand from growing fruit. This suggests that in this system, carbohydrates were never limiting for fruit production and some carbohydrate storage in the stem was always able to occur.

Considering that carbohydrates were never limiting in this study, in spite of very high fruit production, conclusions regarding some aspects of carbohydrate storage are tenuous. For example, alternate bearing is related to carbohydrate storage, but whether carbohydrate storage is a cause or an effect of alternate bearing has not been determined, although there is strong evidence to suggest a role from hormones produced during seed development (Chan and Cain, 1967). Because branches were never limited in carbohydrate, conclusions about causes of alternate bearing cannot be made. In addition, possible reasons behind the increased crop yield of New Zealand trees are not understood, although one hypothesis is the long photosynthetic period after harvest in New Zealand trees, allowing replenishment of reserves in autumn (Tustin *et al.*, 1997).

Reserves were never depleted in this study and this makes it difficult to draw conclusions about the relevance of these reserves.

A maximum of about 50mg g^{-1} of starch was reached in May (Fig. 3.3), regardless of fruit loads. Stems with zero and low fruit loads did not end up with greater starch concentrations in their stems, even though they had much higher concentrations than other stems earlier in the season. No changes in sink demand caused substantial increases in starch concentrations above this value. Stems with a high fruit load were able to obtain maximum storage of starch in May, suggesting that there was ample time after fruit ripening for storage to be adequately fulfilled in one-year-old stems. Thus it was likely that a concentration of about 50mg g^{-1} was a maximum capacity, as instead of storing more starch, once this capacity was reached prematurely, the leaves on these stems stopped photosynthesising and underwent senescence much earlier than leaves on the rest of the tree. The stopping of photosynthesis when fruit were removed was consistent with findings by Schechter *et al.* (1994), who found that girdled stems with no fruit characteristically have decreased carbon exchange rate, red venation, leaf blade discoloration and visible starch grain formation. A reduction in photosynthesis in apple after fruit removal has also been observed in many studies (Wunsche *et al.* 2000 and references therein) including in this study and could be the result of feedback inhibition caused by accumulation of starch in the leaf chloroplast (Schechter *et al.*, 1994; Wunsche *et al.*, 2000). This suggests that photosynthetic capacity is controlled by sink demand for photosynthate.

Like starch concentrations, total non-structural carbohydrate concentrations reached similar levels in May, regardless of fruit load, but this was not a maximum level. This suggests that although starch storage pools were full, soluble carbohydrates were still dynamic and had a large influence on the total carbohydrates.

Starch concentrations declined in July to concentrations similar to those found in December (Fig. 3.3). This suggests that there was a limited amount of starch available in one-year-old stems for remobilisation in the spring and this amount was the same, regardless of fruit load. In ungirdled systems, carbohydrates could have moved from the one-year-old stems into older parts of the tree. Sorbitol has been found in xylem sap during subfreezing temperatures in the dormant season (Noiraud *et al.*, 2001b), so it is possible that carbohydrates could have been transported through the girdle via the xylem. However, total non-structural carbohydrates did not decrease in ungirdled stems (Fig. 3.1) implying carbohydrates were not removed from the system. Girdled stems, where carbohydrates were unable to move out of the stems, showed similar trends to the ungirdled stems and total non-structural carbohydrate concentrations did not decrease in winter. In fact, the total amount of carbohydrate was substantially higher in winter than in early summer for low and zero fruit load branches and similar in high and ungirdled branches, reflecting the changes in sorbitol concentrations over this time. This suggests that starch was converted to sorbitol, possibly as protection from freezing.

Sorbitol contributed to a large proportion of the total non-structural carbohydrates and thus the concentration of sorbitol had a considerable influence on the total carbohydrate curve. The increase in sorbitol concentration in July in low and zero fruit load stems corresponded to a decrease in starch concentrations suggesting that the storage pools of starch and sorbitol are linked. However, it is interesting to note that starch concentrations in high and ungirdled stems also decreased in July, with no corresponding increase in sorbitol. Sorbitol may have moved out of the system via the xylem, or this result could simply be a consequence of a lack of replication in the stems with a high fruit load due to breakages throughout the season.

Reducing the fruit load in January (midsummer) did not cause dramatic increases in sorbitol concentrations. While extra starch was clearly stored

on the removal of fruit, the sorbitol concentration only increased slowly, maintaining a constant total carbohydrate concentration. This suggests that sorbitol is not regulated by sink demand in the same way starch is and possibly does not act as a long-term storage pool as starch does. This raises interesting questions as to the role sorbitol plays in the storage of carbohydrates in the stems of apple trees. Is sorbitol a temporary storage carbohydrate in apple stems? Sorbitol appears to fluctuate in the stem to maintain a reasonably constant level of total carbohydrates. Does it have a role in maintaining constant carbohydrate levels? Bieleski (1982) noted that sorbitol serves a storage role only in certain organs or under certain conditions, for example, sorbitol accumulates in leaves but is not stored in fruit tissue. The results obtained in this study imply that sorbitol is not a storage carbohydrate in the stem, but rather is present in a state of flux, moving between sink tissues and possibly being metabolised for stem maintenance and growth. Sorbitol could be sequestered in the vacuole where it has a buffering action on total sorbitol concentrations in the stem. Because there appears to be some pattern in sorbitol concentrations, it is possible that sorbitol is acting as a short-term (weeks) storage pool in apple stems.

3.4.3. A model of carbohydrate storage in apple stems

The results of this chapter allow a model of carbohydrate storage in apple stems to be proposed. The findings so far suggest that sorbitol may be in transit through the stem as sorbitol concentrations do not respond to sink demand and bark concentrations of sorbitol were very high (chapter 2) implying that sorbitol is concentrated around the phloem region. It is possible that sorbitol is not metabolised (broken down) in the stem and simply flows from source to sink. Sorbitol may be an inert compound in the stem, unavailable for chemical processes here (the hypothesis that sorbitol is not metabolised in the stem will be examined in chapter 6).

When terminal sinks are removed, sorbitol (as the major photosynthate) may continue to be produced and loaded into the transport phloem at the

source. Because there is no longer a terminal sink to receive this sorbitol, a sink of lower priority has a chance to receive the sorbitol, in this case storage of starch along the transport pathway. If there is no metabolism of sorbitol in the stem, this model implies that starch can be synthesised directly from sorbitol (it is not known if this is a feasible reaction, although it has been implied by other authors e.g. Tromp 1983). Once starch storage is saturated (in Hawkes Bay stems at a concentration of 50mg g^{-1}, but in Waikato trees, 70mg g^{-1} was reached), the concentration of sorbitol in the apoplast will rise. As the concentration in the apoplast rises, the concentration gradient between the sieve tube and apoplast will be reduced. If unloading of sorbitol is diffusional, the reduction in a concentration gradient will reduce the rate of unloading from the sieve tubes. A decrease in unloading will result in an increase in concentration in the sieve tubes, reducing the concentration gradient between source and sink. This will result in a reduction in mass flow of sugars from the source, resulting in accumulation of sorbitol in the leaves. This will result in accumulation of sorbitol in the leaves and thus stoppage of phloem flow, finally causing a reduction or shut down of photosynthesis. Once photosynthesis ceases, premature senescence occurs. In winter, to prevent freezing, some starch may be converted back to sorbitol.

This feasibility of this model of carbohydrate storage in apple stems, such as the concentration of sugars in the apoplast, sugar uptake from the apoplast and metabolism of sorbitol, will be explored in more detail in subsequent chapters.

3.5. Conclusions

This study has shown that the hypothesis 'storage of carbohydrate is determined by sink demand' is correct for starch. By altering sink demands throughout the growing season, starch can be made to accumulate in the one-year-old stems at any time. Storage of starch in woody perennial species has a low priority, as in herbaceous annuals, and

can occur at any time when higher priority sinks are removed or their demand saturated.

There appears to be a maximum concentration of starch that can be stored in one-year-old apple stems, which is reached in May in New Zealand. In New Zealand, there is ample time for this maximum to be reached following harvest and before leaf fall, regardless of the carbohydrate demand earlier in the season due to fruit load.

Storage of sorbitol in apple stems is not regulated by sink demand. Sorbitol appears to regulate total carbohydrate concentrations by inter-conversions with starch. Sorbitol may be a temporary (weeks) storage carbohydrate in apple stems.

A model of carbohydrate storage in apple stems can be proposed, in which removal of terminal sinks causes a back up of carbohydrates, eventually resulting in the stoppage of photosynthesis and early leaf senescence.

Chapter 4: Kinetics of Sugar[4] Uptake

4.1. Introduction

In the source-path-sink continuum of the translocation process, transport events contributing to the movement of sugars along the phloem are a key factor. Loading and unloading of sugars in collection and sink phloem are key processes in transport events and are often considered to be the rate limiting steps to crop production (Patrick, 1997; van Bel, 1993). Equally important must be the retrieval and release of sugars along the transport phloem as there is a balance between supplying terminal sinks with photosynthate and retention of photosynthate along the pathway (van Bel, 1996).

Loading of sugars into collection phloem in leaves can be either symplastic or apoplastic depending on the species (Komor *et al.*, 1996). Active uptake of sugars across membranes occurs when H^+ ions are pumped out of the cell to generate an electropotential gradient across the membrane (proton motive force) (Nelson, 1994). Phloem loading involves a set of sucrose/H^+ symporters and sugar transport across membranes is facilitated by these energy-dependent, cargo-specific transporters (Shakya and Sturm, 1998). An excess of 20 genes have been identified for these transporters in different herbaceous plant species (Patrick *et al.*, 2001). These transporters have characteristic uptake kinetics depending on the sugar they transport and their position in the plant.

The kinetics of sucrose uptake into collection phloem has been well characterised in herbaceous plants. In a number of sucrose transporting plants, sucrose has been found to exhibit biphasic uptake (Aked and Hall, 1993; Bowen, 1972; Daie, 1985; Diettrich and Keller, 1991; Eksittikul *et al.*,

[4] The term 'sugar' is used in this discussion for simplicity even though it is recognised that sorbitol is a sugar alcohol.

2001; Maynard and Lucas, 1982a), both in heterogeneous tissue and isolated cell types (e.g. vascular bundles of petiole celery phloem: Daie 1986). The explanation for this biphasic uptake is controversial (van Bel *et al.*, 1982), but has been explained as two separate transport systems operating in parallel; a saturable process exhibiting Michaelis-Menten kinetics and a non-saturable process exhibiting first order kinetics (Maynard and Lucas, 1982a). The transport system exhibiting Michaelis-Menten kinetics is thought to saturate when all transport binding sites become full. The non-saturable process is thought to represent facilitated diffusion where transporters act as shuttles for a net passive movement of molecules across the membrane towards regions of lower energy.

The uptake characteristics of sucrose into stem tissue have received less study than collection phloem, but have still been found to exhibit biphasic uptake, at least in herbaceous stems (Bowen, 1972; Daie, 1986). The uptake characteristics of sucrose into woody stem tissue have never been studied.

Retrieval of sugars in stem tissue of herbaceous species has been much less studied than phloem loading. Rigorous regulation of the release/retrieval balance is necessary to supply terminal sinks with photosynthate and still retain photosynthate to supply sinks such as storage along the pathway (van Bel, 2003). The strict control seems to require a degree of symplastic isolation of sieve element/companion cell complexes in transport phloem and symplastic isolation has been found in stems of *Phaseolus, Lythrum, Cucurbita, Vicia, Zinnia, Ricinus, Solanum,* and *Lupinus* (van Bel, 2003). This suggests that in some herbaceous plants, release and retrieval are apoplastic. Release has mostly been studied in sugar cane (Glasziou and Gayler, 1972) and bean (Hayes *et al.*, 1987; Minchin *et al.*, 1984) and has been found to be apoplastic, however in pea, there is evidence that release is symplastic (Schmalstig and Cosgrove, 1990). This contradictory data is probably due to differences in sink conditions and indicates that sieve element/companion cell

complexes are able to shift between symplastic and apoplastic routes in the stem (Patrick and Offler, 1996).

Apple trees have the additional complication of being sorbitol transporters. Only limited study into the mode of phloem loading has been carried out in polyol transporters. Moing et al. (1997) found that sorbitol loading into peach leaves was most likely apoplastic. However, the mode of phloem loading is likely to differ between species (Komor *et al.*, 1996). Sugar alcohols all probably have their own transporters and a mannitol transporter (AgMaT1) has recently been discovered in celery phloem (Noiraud *et al.*, 2001a). Two sorbitol transporters have recently been found in sour cherry fruit (*PcSOT1* and *PcSOT2*) (Gao *et al.*, 2003). These transporters are similar to uncharacterised sugar transporters found in *Arabidopsis*, sugar beet and to the celery phloem mannitol transporter (Gao *et al.*, 2003).

Sorbitol uptake into apple stem tissue has never been characterised. In growing apple fruit tissue, sorbitol uptake was found to be biphasic with increasing substrate concentration (Beruter and Kalberer, 1983). However, in older fruit tissue, sorbitol uptake was linear (Beruter and Studer - Feusi, 1995). Changing sink conditions in the fruit resulting in different sorbitol carriers being used may explain these differences. Mannitol uptake into petiole tissue of celery exhibited biphasic uptake (Daie, 1986; Daie, 1987; Keller, 1991).

The mode of retrieval and release of sugars in woody stems has not been studied. It can be hypothesised that the same mechanisms found in annual plants will apply to woody species; in other words, retrieval and release of sucrose and sorbitol is apoplastic in woody stems. Only retrieval will be investigated in this thesis, as release is very difficult to study (Patrick, 1997). The characteristics of sucrose and sorbitol uptake in woody stem tissue have also never been determined. One hypothesis is that sucrose exhibits similar uptake characteristics to those found in leaves of herbaceous plants, i.e. biphasic uptake, suggesting that similar

sucrose transporters are present. It can be hypothesised that sorbitol exhibits similar uptake characteristics as mannitol in celery, i.e. biphasic uptake, also suggesting that similar transporters are present. Other questions that can be asked about the characteristics of sucrose and sorbitol uptake include: do the uptake characteristics change with season? Do the uptake characteristics change with sink demand on the stems?

To test these hypotheses, a series of experiments was required. Whether retrieval of sucrose and sorbitol was symplastic or apoplastic was determined using stem segments. Although retrieval only occurs in several highly specialised cells, it is necessary to use intact segments of plants to maintain the metabolic network over the entire phloem pathway. Unfortunately the use of such plant segments is a major handicap towards providing unequivocal information on uptake as a number of different tissue types are present in such segments (van Bel, 1993); as such the results need to be treated with caution.

The metabolic inhibitors p-chloromercuribenzenesulphonic acid (PCMBS) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were used to help characterise the uptake of sugars into stem pieces. PCMBS is a non (or slowly) permeable thiol reagent that disrupts SH bonds (Bourquin *et al.*, 1990). All membrane proteins bearing free external SH groups are expected to be sensitive to PCMBS (Aked and Hall, 1993). CCCP blocks the formation of ATP, which is normally coupled with electron transport in cristae and thylakoid membranes (Luttge and Higinbotham, 1979). Sensitivity to PCMBS demonstrates the existence of an apoplastic step in retrieval because sucrose carriers are blocked. CCCP is used to show whether metabolic energy is required in the uptake process.

Other experiments included examining the uptake characteristics of sucrose and sorbitol when fruit load was manipulated, determining whether sucrose and sorbitol competed for the same uptake system and determining where in the stem uptake occurred. Seasonal variation in the uptake characteristics of the sugars was also examined.

The outcomes of these experiments should lead to understanding of the retrieval mechanisms in woody stems and help to show the feasibility of the model presented in chapter 3. Retrieval of sucrose and sorbitol may be apoplastic as it is in many herbaceous plants. However, there is no evidence that sorbitol will follow the same uptake mode as sucrose.

Sucrose and sorbitol may exhibit biphasic uptake as has been found in herbaceous tissue, or alternatively may exhibit a different uptake mechanism. If there were seasonal variations in the uptake characteristics of sugars, this would be seen in differences in uptake at different times of the year.

4.2. Method

4.2.1. Plants

One-year-old stems were taken from 'Braeburn' trees growing in the Waikato orchard (see chapter 2) for all experiments except where fruit loads were adjusted. Stems were freshly harvested on the day of measurement. Replication was made using different stems off different randomly selected trees. Stems were selected on the basis of uniformity.

In experiments involving different fruit loads, 'Braeburn' trees growing in Hawkes Bay were used (see chapter 3). Five stems were girdled on six trees in January 2002. A 2cm wide strip of phloem, cambial tissue and connected bark was removed and an aluminium splint taped to the girdled area for physical support. Fruit on these stems was reduced to either high (six fruit), low (two fruit) or zero (no fruit) at the time of girdling. In addition, fruit was completely removed four weeks after girdling on some high and low stems. Sugar uptake measurements took place eight weeks after girdling.

4.2.2. Sugar uptake measurements

To carry out sugar uptake measurements, leaves were removed from the excised stems in the laboratory. In chapter 1, some of the difficulties associated with working on woody tissue were outlined. The hardness of the wood made cutting of thin stem slices, necessary for the type of experiments carried out in this study, difficult. When stems were sliced, the bark and wood usually split and/or separated from one another. To overcome this problem, the woody internodal tissue was sliced longitudinally and cut into approximately 1-2mm thick half-round discs using a razor blade. In one experiment, the stems were sectioned into different tissue types (periderm, phloem + cambium, xylem + pith) before bathing in the incubation medium.

To measure the uptake of sucrose or sorbitol into apple stem pieces, 10 half-round discs were immediately placed into 10ml of continuously aerated medium containing 20mM 2(N-morpholino)ethanesulphonic acid (MES) buffer (pH 6.5), 1mM $CaCl_2$ and mannitol and sorbitol or sucrose at concentrations which varied with experiment. The osmolarity of the incubation medium was maintained by addition of appropriate amounts of mannitol. The discs were equilibrated at room temperature for thirty minutes, followed by a change of medium to remove any potential inhibition to uptake resulting from the contents of damaged cells at the tissue ends.

Where appropriate, inhibitors CCCP and PCMBS were added to the medium at concentrations of 5μM and 2mM respectively. PCMBS is a non (or slowly) permeable thiol reagent that disrupts SH bonds without effect on the proton pump of the plasmalemma at least for short-term treatments (Bourquin *et al.*, 1990). All membrane proteins bearing free external SH groups are expected to be sensitive to PCMBS (Aked and Hall, 1993). CCCP blocks the formation of ATP, which is normally coupled with electron transport in cristae and thylakoid membranes (Luttge and Higinbotham, 1979). Because CCCP was initially dissolved in ethanol, an

equivalent amount of ethanol was added to the control and PCMBS flasks. Flasks containing PCMBS were wrapped in dark cloth to prevent light degradation.

In competition experiments, two sugars (sucrose and sorbitol) were added together at different concentrations in the incubation medium with one radiolabelled sugar (either sucrose or sorbitol).

Measurements were started with the addition of ^{14}C-sucrose (7.4MBq ml^{-1}) or ^{14}C-sorbitol (7.4MBq ml^{-1}). Incubation was carried out at room temperature for two hours and was terminated by removal of the incubation solution. The stem discs were immediately washed to remove any radioactivity originating from the apparent free space of the tissue. Discs were washed three times for a period of approximately 10 minutes per wash, with 10ml of the initial equilibrium medium minus any ^{14}C and inhibitors. Discs were kept on ice during washing to prevent further metabolic uptake.

The soluble fraction was extracted from the stem pieces by addition of 6ml of 80% ethanol to each flask and heating to 60^{0}C for one hour. The radioactivity within the soluble fraction was determined by liquid scintillation (counted for one hour per sample or to a precision of 2%, whichever came first). The remaining discs were dried at 50^{0}C for 24 hours and weighed.

4.2.3. Autoradiography

Stem pieces exposed to ^{14}C-sucrose or ^{14}C-sorbitol (as described above) were either placed directly onto Kodak X-OMAT x-ray film before drying, or a tissue print was made onto nitrocellulose paper as described by Reid and Pont-Lezica (1992). The tissue print was then placed onto x-ray film for exposure. A variety of exposure lengths were tested, ranging from 1 – 28 weeks. The radiograms were processed in Kodak x-ray developer and fixer.

4.2.4. Sugar concentration in the apoplast

To determine the concentration of sugars in the stem apoplast, the efflux of sugars into a bathing solution was measured. Four, 2 and 1mm stem internodal pieces were placed in equilibrium medium (as described above) on ice and aliquots of the bathing solution were removed at various time intervals. The amount of sugar in the aliquots was determined using standard enzyme assays (see chapter 2).

The quantity of sugar leaked from the stem pieces came from both damaged cells at the cut surface and a contribution from the apoplast. If it is assumed that the contribution from the damaged cells was proportional to the area of the cut surface and there were N samples each of length L with cut ends of area A, and the fraction of the total tissue volume that is apoplastic is v, and apoplastic sugar concentration is s, then the total quantity of sugar which could be leached is:

$q = N2A + NALvs$ (Minchin and Thorpe, 1984).

Where q is the quantity of sugar leached and v is given a value of 0.04 (a typical value, based on apoplastic volumes found in bean (Minchin and Thorpe, 1984).
This equation can be rearranged to:

$q/N = 2A + AvsL$

so that a plot of q/N versus L will give a straight line: the slope (Avs) and intercept (2A) being estimated by means of a least squares fit.

4.2.5. Statistics

In experiments measuring uptake with fruit load and different tissue types, Analysis of Variance (ANOVA) was carried out. The assumption that parametric parameters were filled (i.e. the data has equal variance, is

independent and normally distributed) was tested before an analysis was performed.

4.3. Results

4.3.1. Uptake kinetics

A time-course of sugar uptake was carried out to ensure that uptake of sugar into the symplast of apple stems occurred over the entire time period used in the experiments and sugar availability was not limiting to uptake. Uptake was linear with time (measured over three hours) for sucrose at a concentration of 7.02×10^{-6} mM and 100mM and for sorbitol at a concentration of 9.67×10^{-6} mM and 100mM (data not shown). All subsequent kinetic studies were carried out for two hours, within this linear time period.

The optimum osmolarity (controlled by mannitol) for sucrose uptake into apple stems was found to be 125mOsM (data not shown). Thus all incubation solutions were adjusted to 125mOsM with the appropriate amount of mannitol.

The kinetic uptake curve for sucrose (Fig. 4.1) shows a saturable component at concentrations below 5mM and a linear component above 5mM. Estimates of K_m and V_{max} were made from Eadie-Hofstee plots of the kinetic data in the concentration range below 5mM. The values presented in Table 4.1, which are for stem pieces harvested at different times, show no significant differences in K_m between months. V_{max} was significantly higher in May than October. Time of year did not significantly alter the affinity of binding of sucrose (K_m), but did affect the number of sucrose binding sites present in one-year-old stem tissue (V_{max}).

In contrast to sucrose, sorbitol uptake did not show the two uptake phases; uptake was linear over the entire concentration range (Fig. 4.1).

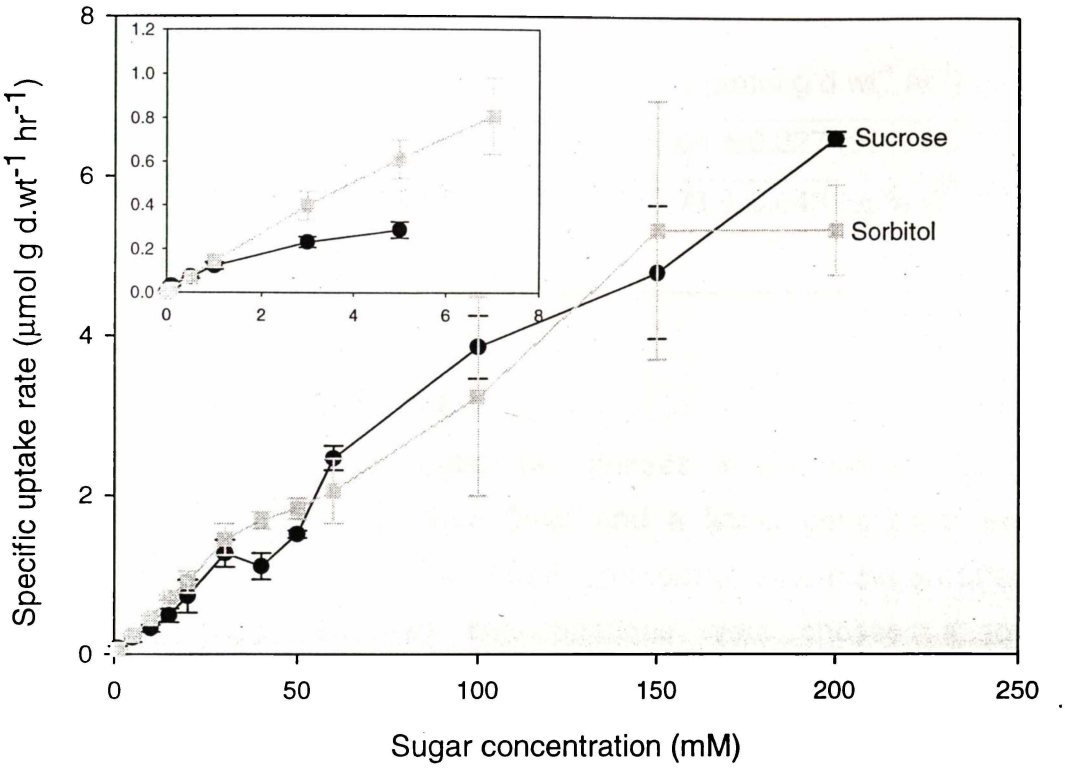

Figure 4.1. Uptake of ^{14}C-sucrose and sorbitol into stem pieces of apple trees over a wide range of sugar concentrations. A separate measurement was performed for each sugar with 2 duplicates per measurement. Two repeats were made for each sugar at each time. Results shown were obtained in March 2001. The inset graph shows the uptake of sucrose and sorbitol at concentrations less than 8mM. A linear uptake can be seen for sorbitol, while sucrose has a curvilinear uptake at low concentrations. These results were obtained in May 2002.

Table 4.1. Sucrose: Estimates of K_m (mM) and V_{max} (µmol g. dry weight^{-1} hr^{-1}) from Eadie-Hofstee plots of the kinetic data in the concentration range below 5mM. Errors at 95% confidence interval (CI).

Date	K_m (mM)	V_{max} (µmol g d.wt^{-1} hr^{-1})
October 2001	0.138 ± 0.141 (95% CI)	0.564 ± 0.227 (95% CI)
May 2002	0.363 ± 0.135 (95% CI)	0.171 ± 0.043 (95% CI)

4.3.2. Uptake and fruit load

The previous results demonstrated two phases of sucrose uptake; a saturable component at less than 5mM and a linear component that dominates at concentrations above 5mM. In order to determine an effect of fruit load on uptake, two concentrations were chosen: a low concentration (0.1mM) where the saturable component dominates and a high concentration (7mM) where the linear component dominates.

Uptake rates of sorbitol and sucrose into girdled one-year-old apple stems with different fruit loads are shown in Figure 4.2. A general pattern was seen in the uptakes with both sugars and at both concentrations: Stems that were under a high fruit load had the highest rates of uptake, with zero fruit load having the lowest uptake rates. Stems that had the fruit removed four weeks after girdling (HZ and LZ, Fig. 4.2.) did not have different uptakes compared to stems that were set at zero fruit load in the first place.

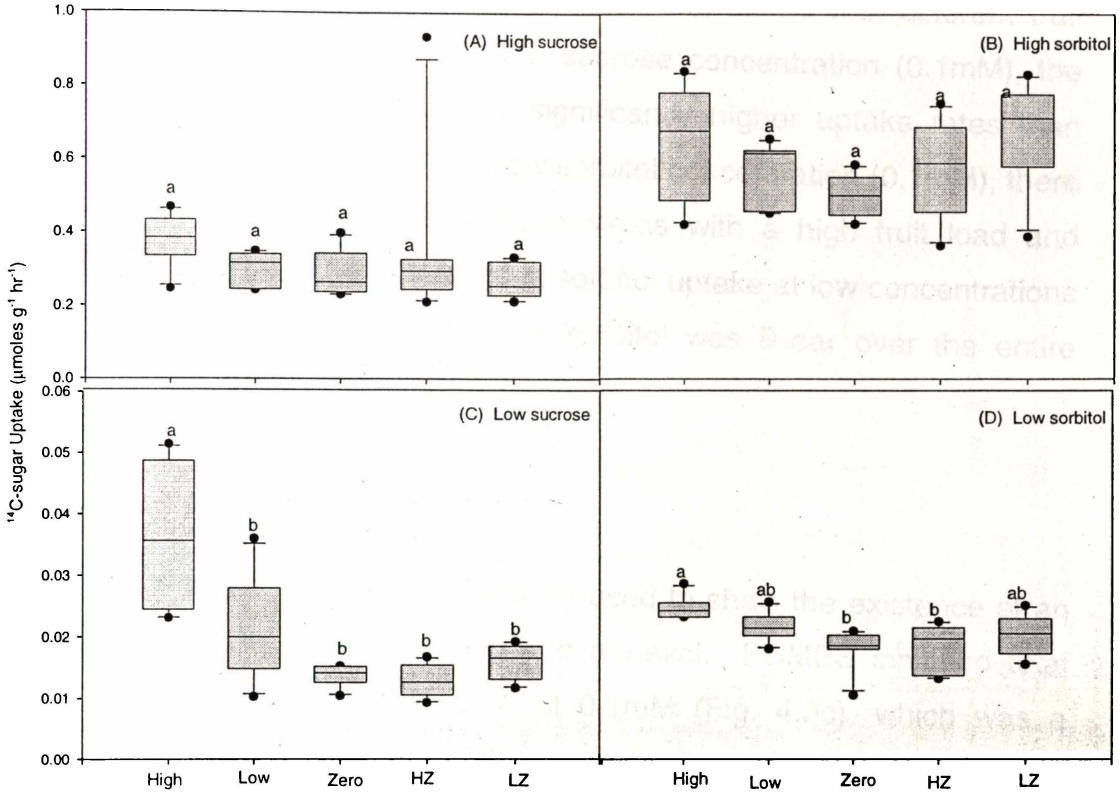

Figure 4.2. Uptake rates of sucrose or sorbitol into the one-year-old stems of apple trees with different fruit loads. (A) ^{14}C-sucrose uptake at 7mM sucrose; (B) ^{14}C-sorbitol uptake at 7mM sorbitol; (C) ^{14}C-sucrose uptake at 0.1mM sucrose; (D) ^{14}C-sorbitol uptake at 0.1mM sorbitol. n=6. High = 6 fruit/branch: Low = 2 fruit/branch: Zero = 0 fruit/branch: HZ = fruit load reduced from 6 to 0 four weeks after being set: LZ = fruit load reduced from 2 to 0 four weeks after being set. Letters denote significant differences. Note different y-axes.

At high concentrations (7mM) of both sucrose and sorbitol, no significant differences were observed in uptake rates into stems with different fruit loads (Fig. 4.2). However at a low sucrose concentration (0.1mM), the stems with a high fruit load had significantly higher uptake rates than stems with lower fruit loads. At a low sorbitol concentration (0.1mM), there was a significant difference between stems with a high fruit load and stems with no fruit. The difference in sorbitol uptake at low concentrations was unexpected because uptake of sorbitol was linear over the entire concentration range measured.

4.3.3. Effect of inhibitors

Inhibition of sugar uptake by PCMBS is used to show the existence of an apoplastic step in phloem loading and retrieval. PCMBS inhibited total ^{14}C-sucrose uptake by up to 80% at 0.1mM (Fig. 4.3c), which was a greater inhibition than at high concentrations (7mM). At high concentrations, PCMBS inhibited uptake by only 13% (Fig. 4.3a).

PCMBS inhibited ^{14}C-sorbitol uptake slightly less than it inhibited sucrose uptake at low concentrations. Inhibition by PCMBS was greatest at low sorbitol concentrations (0.1mM) and caused inhibition of 71% of uptake (Fig. 4.3d). At high sorbitol concentrations (7mM), uptake of sorbitol was inhibited by 55% (Fig. 4.3b).

Inhibition of sugar uptake by CCCP is used to show whether uptake of sugar is an active metabolic process. CCCP inhibited total ^{14}C-sucrose uptake by 44% at 0.1mM (Fig. 4.3c), which was a greater inhibition than at high concentrations (7mM). At high concentrations, CCCP inhibited uptake by 32% (Fig. 4.3a).

CCCP inhibited ^{14}C-sorbitol uptake to a greater extent at high concentrations, which was a contrast to sucrose inhibition. CCCP inhibited total ^{14}C-sorbitol uptake by 32% at 0.1mM (Fig. 4.3d). At high

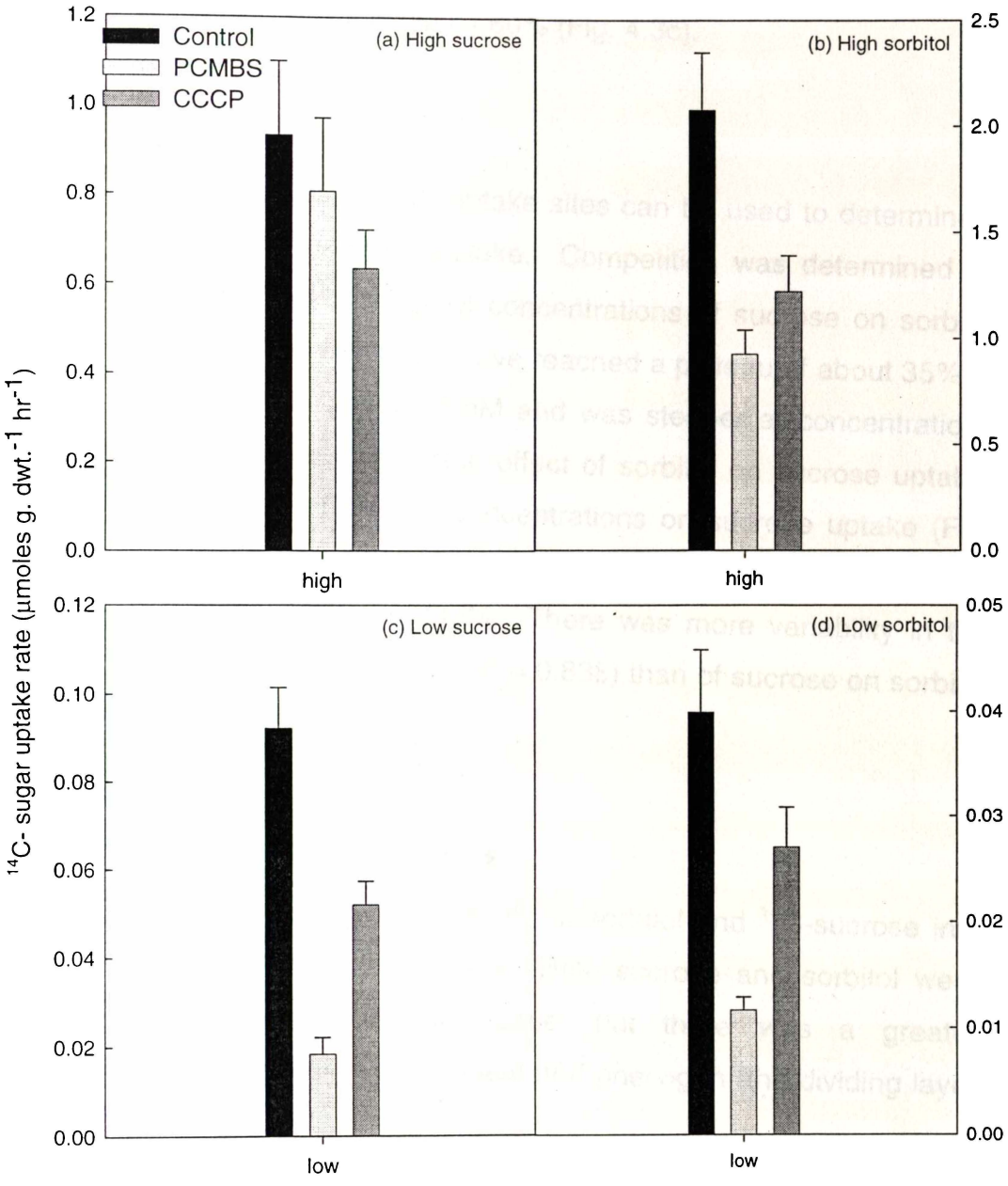

Figure 4.3. Effect of the inhibitors CCCP and PCMBS on sucrose and sorbitol uptake at high concentrations (a) & (b) and at low concentrations (c) & (d) (0.1mM and 7mM respectively). Note different y-axes.

sorbitol concentrations (7mM), there was greater inhibition of sorbitol uptake and uptake was inhibited by 60% (Fig. 4.3c).

4.3.4. Competition

Competition between sugars for uptake sites can be used to determine if separate carriers are used for uptake. Competition was determined by examining the effect of a range of concentrations of sucrose on sorbitol uptake (Fig. 4.4a). The inhibition curve reached a plateau of about 35% at sucrose concentrations above 10mM and was steeper at concentrations below 10mM than the curve for the effect of sorbitol on sucrose uptake. The effect of a range of sorbitol concentrations on sucrose uptake (Fig. 4.4b) showed that the inhibition curve reached a plateau of about 25% at sorbitol concentrations above 10mM. There was more variability in the effect of sorbitol on sucrose uptake (r^2 = 0.838) than of sucrose on sorbitol (r^2 = 0.941).

4.3.5. Autoradiography results

Autoradiographs showing the uptake of ^{14}C-sorbitol and ^{14}C-sucrose into stem pieces are shown in Figure 4.5. Both sucrose and sorbitol were taken up throughout the whole tissue, but there was a greater concentration of ^{14}C-sugar in the phloem and phellogen (the dividing layer of newly forming bark).

4.3.6. Uptake into different tissue types

When apple stem tissue was dissected into periderm, phloem + cambium and xylem + pith before bathing, no significant differences were found in uptake between tissue types expressed on a weight basis (Fig. 4.6).

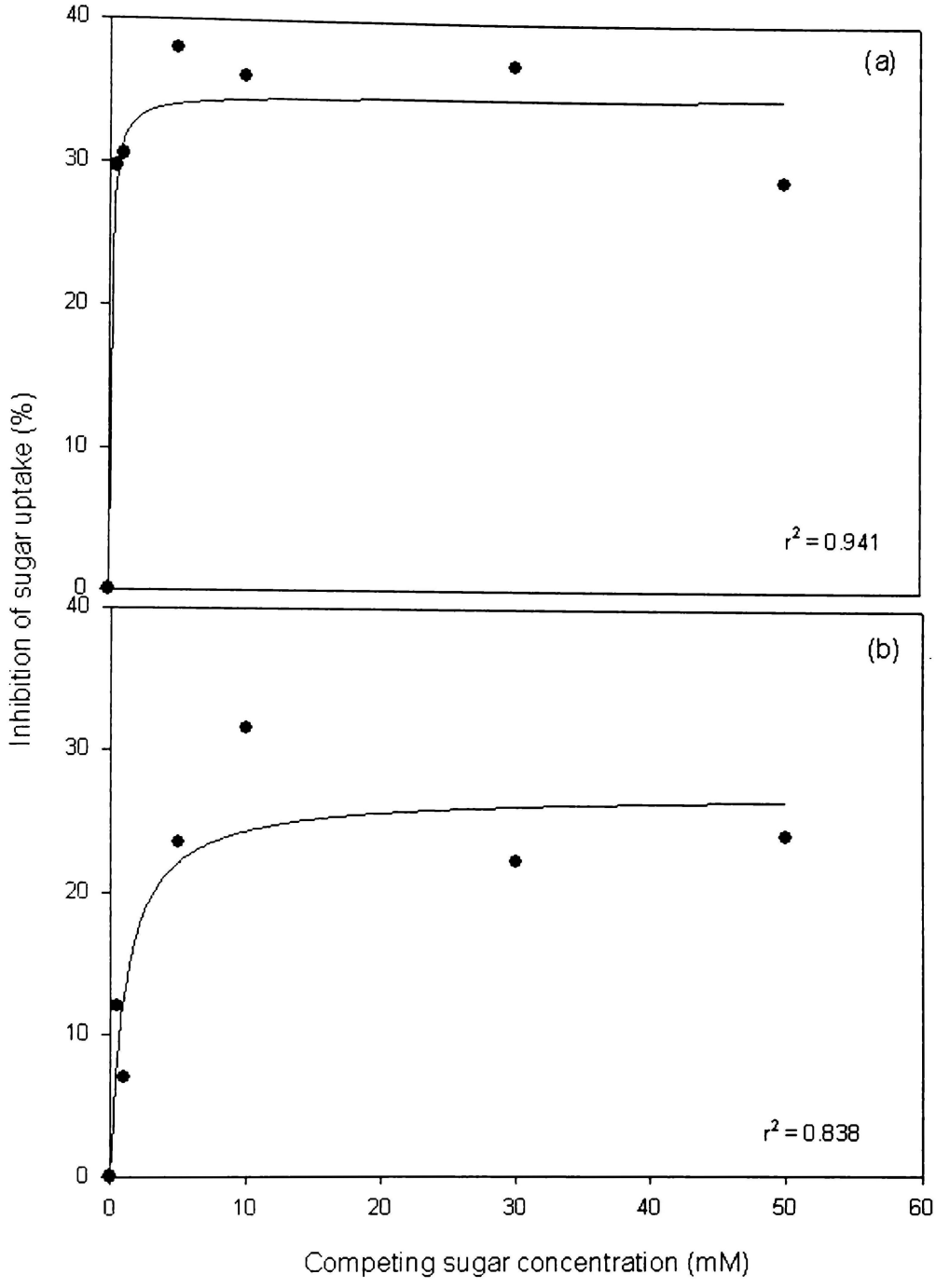

Figure 4.4. Competition between sucrose and sorbitol for uptake into the one-year-old stems of apple. (a) sorbitol uptake: (b) sucrose uptake. Each point represents the average uptake into ten half round discs. The competition effect is expressed as % inhibition of uptake from sorbitol (a) and sucrose (b) compared with uptake rates in media containing no competing sugar. n=3 (a) or 4 (b).

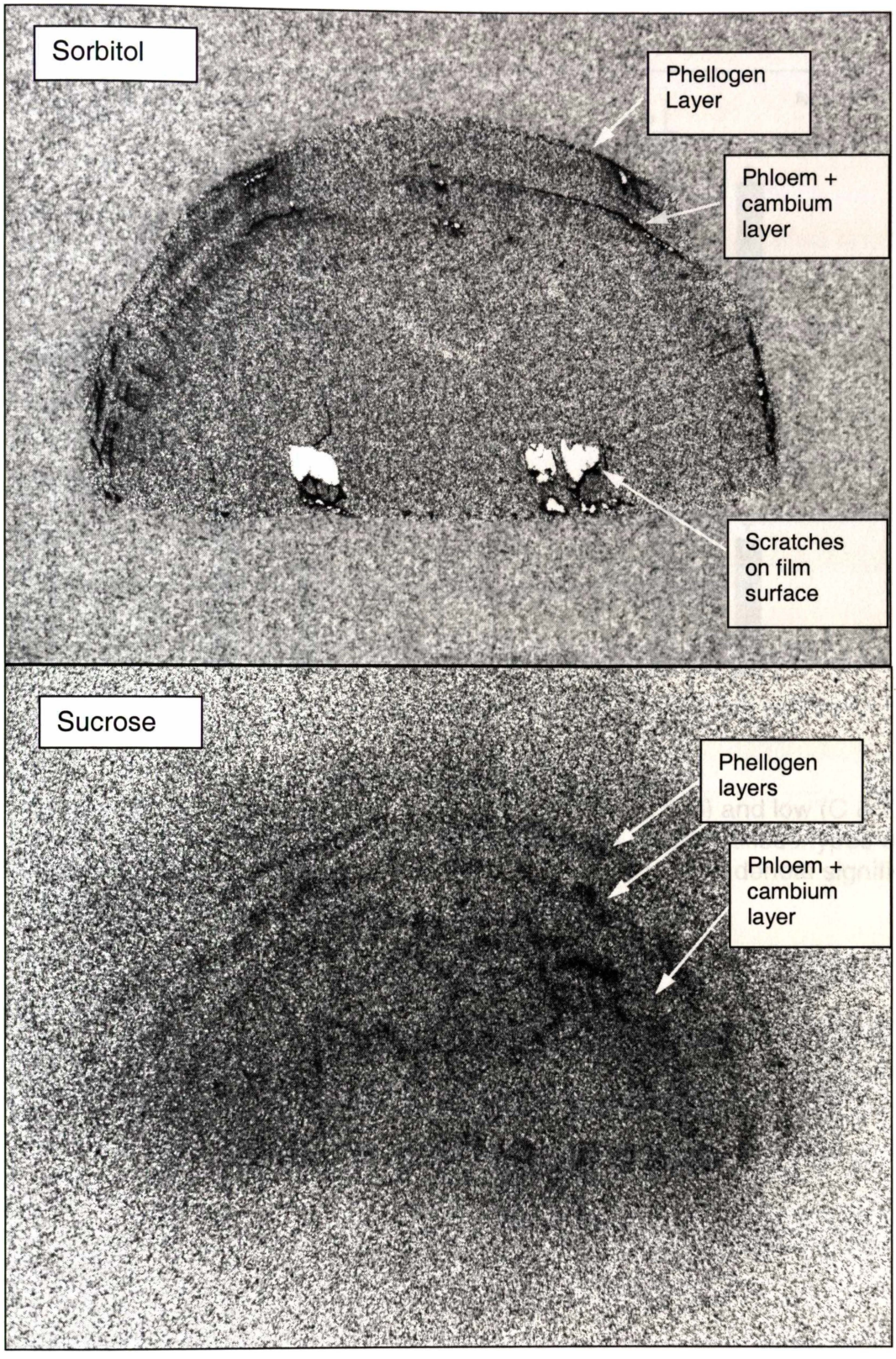

Figure 4.5. Autoradiographs of ^{14}C-sorbitol and sucrose uptake into apple stem tissue. The sorbitol image was obtained by placing the stem directly onto x-ray film (exposed for 4 weeks) while the sucrose image was obtained using a tissue print (exposed for 28 weeks) (see Methods).

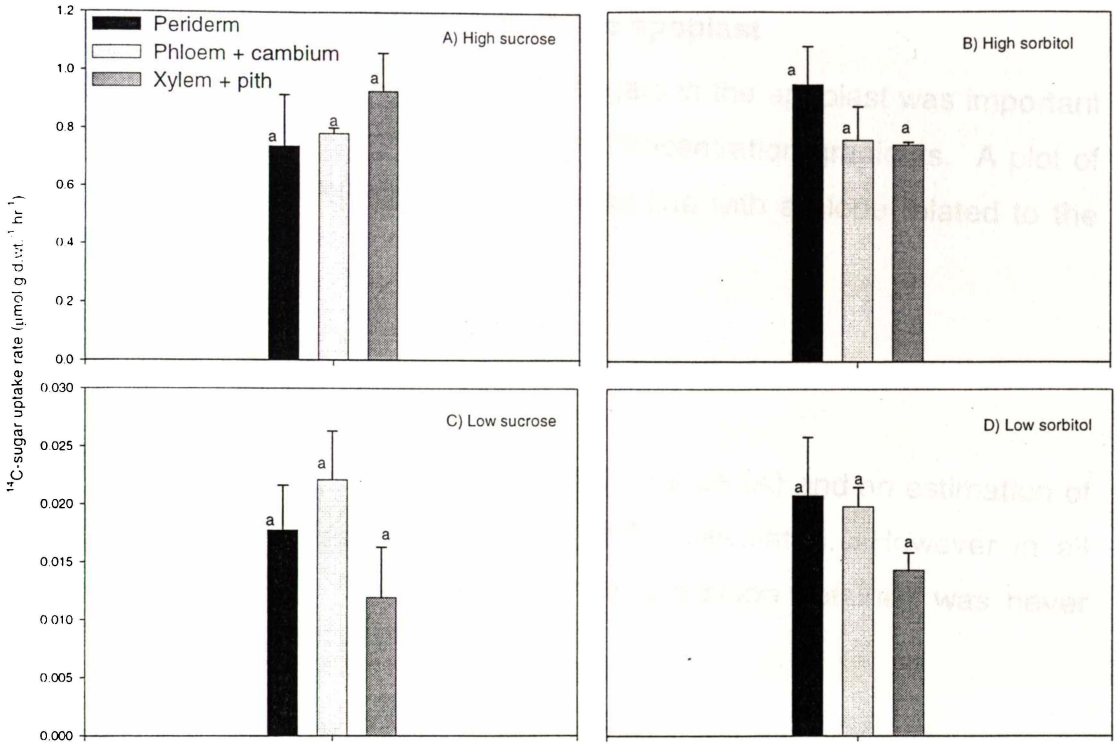

Figure 4.6. Uptake of sucrose and sorbitol at high (A & B) and low (C & D) concentrations (0.1mM and 7mM respectively) into different tissue types of the apple stem. Results are averages from 3 trees. Letters denote significant differences.

4.3.7. Sugar concentration in the apoplast

Determining the concentration of the sugars in the apoplast was important as uptake into the phloem depends on concentration gradients. A plot of q/n versus L is expected to be a straight line with a slope related to the tissue parameters of:

Slope = Avs

By knowing the cross sectional area of the tissue (A) and an estimation of apoplastic volume (4% in bean), s can be calculated. However in all experiments carried out (12 replications), the slope obtained was never significantly different to zero.

Although the apoplastic volume was based on an assumed volume based on volumes found in bean, this had no effect on the slope values. Because area was known and there unquestionably was some apoplastic volume, the concentration of sugar in the apoplast must be zero. The actual apoplastic volume in apple stem was not determined, as with no appreciable sugar concentration, this parameter was not needed.

Experiments using ^{11}C (chapter 5) suggested that time of day may have a bearing on sugar concentration in the apoplast. However, similar measurements carried out late in the day also failed to show a significant apoplastic sugar concentration.

4.4. Discussion

4.4.1. Uptake kinetics

This study has, for the first time, characterised the uptake of sucrose and sorbitol in woody perennial stem tissue. The hypothesis that sucrose exhibits similar uptake characteristics to those found in leaves of herbaceous plants, i.e. biphasic uptake was correct. Biphasic uptake was seen for sucrose uptake into apple stems and this suggests that sucrose transporters, similar to those found in herbaceous plants, were involved. The hypothesis that sorbitol exhibits similar uptake characteristics as mannitol in celery, i.e. biphasic uptake, was found to be incorrect. Sorbitol uptake was linear with time, suggesting that different transporters are involved for sorbitol and mannitol, which has now been shown by Gao *et al.* (2003).

A biphasic uptake system for sucrose uptake into plant tissue has been well studied (e.g. Sovonick et al. 1974, Maynard and Lucas 1982a, Setter and Meller 1984, Daie 1985, Diettrich and Keller 1991, Aked and Hall 1993, Hole and Dearman 1994). The two phases of sucrose uptake present in apple stem pieces can be interpreted as saturable and non-saturable components of transport (Maynard and Lucas, 1982a). The saturable component conforms to Michaelis-Menten kinetics and the non-saturable component operates as a first order kinetic reaction (Maynard and Lucas, 1982a). Whether Michaelis-Menten kinetics is the correct explanation for the uptake curves found is a matter of debate (Reinhold and Kaplan, 1984). Even so, the saturable component of sucrose uptake is widely accepted to be a carrier-mediated H^+ co-transporter (Daie, 1987).

V_{max} was found to be higher in October than in May for sucrose uptake. This indicates that the number of binding sites for sucrose, i.e. the concentration of the sucrose transporter(s) was higher in spring (a period of rapid growth) than in autumn. This is consistent with greater sucrose

retrieval in spring to provide sucrose to actively growing terminal sinks and down-regulation of these transporters in autumn when retrieval is reduced, allowing greater storage of carbohydrate to take place in the stem. There is evidence in citrus roots that the sucrose transporter, SUT1, was up-regulated by carbohydrate depletion (Li *et al.*, 2003), suggesting that sucrose transporter activity can be altered by sink demands. High sucrose levels in the phloem resulting from decreased sink demand have been shown to down regulate transporter activity (Bush, 1999), which is consistent with the results reported here.

Sucrose transporters are responsible for phloem loading and retrieval (Lalonde *et al.*, 2003) and have been named SUT or SUC. A large number of these transporters have been found in plants and it is difficult to assign precise and unequivocal functions even for the best studied (Delrot *et al.*, 2001). SUT1, a high affinity transporter, (isolated from potato sieve elements, equivalent to SUC2 from Arabidopsis) is essential for phloem loading and long-distance transport and may also play a role in retrieval of sucrose leaked from sieve elements along the translocation pathway and in phloem unloading (Kuhn *et al.*, 2003; Williams *et al.*, 2000). Similar transporters may play a role in retrieval in apple stems.

The K_m values obtained for sucrose (0.138mM ± 0.141 in October, 0.363mM ± 0.135 in May) were similar to those found *in situ* in Chlorella cells (0.3mM) by Komor and Tanner (1975), suggesting a common transport system operates in all photosynthetic organisms. All members of the SUT family characterised to date have K_m values between 0.3 and 2mM for sucrose (Weise *et al.*, 2000). Estimation of K_m is dependent on the concentration range examined (Reinhold and Kaplan, 1984) and this may account for the range of K_m's reported in the literature.

The nature of the linear (non-saturable) component of sucrose uptake is not well understood although it is commonly believed to be facilitated diffusion (Aked and Hall, 1993; Hole and Dearman, 1994). The role and

importance of the diffusion-like uptake component is controversial (Stanzel *et al.*, 1988). A linear phase in uptake rate versus concentration can be obtained as an artefact when the radioactive uptake compound is contaminated by just a few percent with a substance that is readily taken up (Stanzel *et al.*, 1988), although this implies poor experimental technique.

The stem apoplastic sugar concentration was found to be zero in this study. The lack of apoplastic sugar may be due to efficient sugar retrieval mechanisms in the apple stem. Very young stems were used in this experiment for ease of cutting into exact lengths, meaning that the cells were likely to be rapidly growing and scavenging all available sugar from the apoplast as soon as it was unloaded. Stem apoplast pools are quite small and rapidly depleted if there is large sink demand (Patrick, 1991). Additionally, in leaves, water-impermeable layers in the wall interface between the mesophyll and mesophyll sheath could result in poor access to the apoplast pool (van Bel, 1993); water impermeable layers may also be present in stem tissue. This requires further investigation, but due to the progressive hardening of the apple stem throughout the season, no further experiments could be carried out. Once the wood hardened, precise cutting of the stem, necessary to account for the damage of the cut ends, became impossible.

Sucrose concentrations in the apoplast of various plant tissues have been found to range from 25-30mM in bean (Minchin and Thorpe, 1984), 1.6mM in barley (Tetlow and Farrar, 1993), 13mM in *Cyclamen* (Rothe *et al.*, 1999), 1-8mM in *Gossypium* hypocotyls and 10mM in *Vica* stems (Rothe *et al.*, 1999). Sucrose uptake into the apple stem tissue was linear at a concentration range which is typical to that found in the apoplast of other species. If the zero apoplast concentration is true in apple tissue (and this should be investigated further), the saturable process of uptake may have the most importance in this tissue. Higher apoplastic concentrations would indicate that linear uptake is important in this tissue.

Some authors argue that the biphasic nature of sucrose uptake may not be the result of two uptake processes, but rather a phenomenon resulting from a mixture of tissue types (van Bel, 1993); a high affinity uptake in one cell type, e.g. parenchyma and a low affinity uptake in a different cell type. However, some isolated tissue, such as pea epidermis (Aked and Hall, 1993), does have a biphasic uptake, while some tissue consisting of multiple cell types has been found to have a single saturating component (isolated phloem strands of celery (Daie, 1987)).

In apple stems, there were no significant differences in uptake rates when the stem was separated into different tissue types, but because cell types could not be separated, the possibility that biphasic sucrose uptake was a result of multiple cell types cannot be discounted. Autoradiographs showed that ^{14}C-sugar was taken up into all cell types, although there appeared to be greater uptake into the phloem and phellogen regions. It is possible that influx into the phloem could be overestimated by autoradiography, as without using a freeze-substitution method to fix sugars in place, sugar could move from the parenchyma to the phloem before drying occurred.

This study examined the uptake characteristics of sorbitol into stem tissue. Sorbitol uptake was linear with concentration and did not display the biphasic uptake seen with sucrose. A possible explanation for this is that the concentration range used in this experiment was not sufficient to show two phases of uptake (i.e. not low or high enough). Alternatively, it could suggest that only one transport system or conformation of protein is present for the uptake of sorbitol in the stem. In other tissue, such as fruit, more than one transporter may operate as suggested by Gao *et al.* (2003) finding of two transporters in sour cherry fruit. If the linear phase seen is similar to the linear component of sucrose uptake, it may represent facilitated diffusion. Linear uptake of sorbitol was also seen in intact and exercised half-grown apple fruit (Beruter and Studer - Feusi, 1995). However, in younger apple fruit, saturation characteristics were seen (Beruter and Kalberer, 1983), suggesting a carrier-mediated system for

sorbitol transport. These contradictory findings could suggest that uptake of sorbitol changes with sink demand. However in this study, there were no differences in sorbitol uptake kinetics in the stem with different situations of sink demand during the season. Beruter and Kalberer (1983) found that saturable sorbitol uptake correlated with conversion of sorbitol to fructose, suggesting that uptake was controlled by sorbitol metabolism. In the stem, a lack of sorbitol metabolism (see chapter 6) could be why only a non-saturable system was seen. This suggests that there is potential for two sorbitol uptake systems and only the non-saturable one operates in the stem. Mannitol, which is the major carbohydrate of celery plants, was found to exhibit biphasic uptake in a number of different cell types (Daie, 1986; Daie, 1987; Keller, 1991). It would be interesting to carry out further experiments to determine whether a single linear uptake system operates in celery for mannitol, given the right sink conditions, or whether a single linear uptake is only a feature of sorbitol.

4.4.2. Uptake and fruit load

In this study, the uptake characteristics of sucrose and sorbitol into apple stem tissue with changes in sink demand were investigated. At low external sucrose concentrations, uptake was greatest into stems with high fruit loads. This suggests that when there is greater demand for carbohydrate (such as when there is growing fruit), the sucrose transporter system represented by the saturable phase (SUT1) is up regulated. Where there is little demand for carbohydrate, such as when there is no fruit, the transporter system is down regulated. A similar observation was made when the uptake characteristics were examined at different times during the season.

At a high external sucrose concentration, there was no difference in uptake of sucrose at different fruit loads. This suggests that the sucrose transporters that act in the linear component, possibly via facilitated diffusion, are not regulated by carbohydrate demand.

Sorbitol uptake did not change with fruit load at a high external sorbitol concentration. However at a low external sorbitol concentration, there was greater uptake into stems with a high fruit load in spite of the linear sorbitol uptake. This suggests that even though uptake is entirely linear and thus could be via facilitated diffusion, at low concentrations of sorbitol there is some degree of regulation. Why this regulation was seen when uptake may be via facilitated diffusion throughout the concentration range tested is not clear, although it could mean that a saturable sorbitol carrier (described earlier) was induced in stem tissue under these conditions. This requires further investigation.

4.4.3. Retrieval pathways

Retrieval of sugars into the sieve elements and phloem parenchyma could follow an apoplastic or symplastic pathway or both. In annual species, retrieval of sucrose is apoplastic. The hypothesis that the same pathways are used in woody species was tested in this chapter. The potential for apoplastic uptake into stem tissue was seen as sucrose was taken up into the plant tissue from an apoplastic solution. Uptake could be apoplastic into one cell type and then symplastic after that. Further evidence and characterisation of the uptake pathways was obtained using the inhibitors PCMBS and CCCP.

PCMBS is a non (or slowly) permeable thiol reagent, which is used to inhibit active loading from the apoplast by disrupting SH bonds in membrane bound carriers. PCMBS was found to inhibit sucrose uptake at low external sucrose concentrations. At higher external sucrose concentrations, PCMBS only slightly inhibited uptake (13%) suggesting that this facilitated diffusion step does not utilise SH groups. These observations are consistent with the specific inhibition of a sucrose-H^+ co-transport system in the saturable component, reported by other investigators for different tissue types (Daie, 1985; Delrot *et al.*, 1980; Hole and Dearman, 1994; Maynard and Lucas, 1982b). These findings provide

further evidence that sucrose transporters in apple stem tissue are similar to those found in other plants.

PCMBS was found to inhibit sorbitol uptake at both concentrations, indicating that a membrane bound carrier with SH groups may have been involved in sorbitol uptake at all concentrations. In intact systems, PCMBS can be used to infer a pathway of phloem loading through its inhibition of apoplastic sugar loading.

Apoplastic phloem loading of sorbitol in peach leaves has been suggested by PCMBS inhibition (Moing *et al.*, 1997). By contrast, Marquat et al. (1996) found that sorbitol uptake into peach buds was only weakly inhibited by PCMBS. Gao *et al.* (2003) also found that the sorbitol transporters they isolated were sensitive to PCMBS. These contradictory results may be due to the different tissue types used, or the time of year of the measurements (Marquat et al. (1996) used dormant tissue), but could also be explained by the nature of the inhibitor. Recent data shows that water uptake through aquaporins is highly sensitive to PCMBS (Noiraud *et al.*, 2001b). Uptake of sorbitol in animals has been found to occur through channels related to aquaporins (Noiraud *et al.*, 2001b); seeing as a number of water channels are present in plant cells it is possible that uptake of sorbitol is through one of these. A decrease in water transport or a leaky membrane as a result of PCMBS interference may account for the inhibition of sorbitol seen and symplastic retrieval of sorbitol into sieve tubes cannot be ruled out.

Beruter and Studer - Feusi (1995) concluded that sorbitol uptake into intact apple fruit probably follows a symplastic route. Evidence for symplastic phloem loading of sorbitol in peach leaves was provided from estimation of concentration gradients and apoplastic volumes (Moing *et al.*, 1997) but sensitivity to PCMBS led the authors to conclude that loading was apoplastic. In light of recent knowledge of PCMBS interactions, there is a strong possibility that loading was symplastic. Plant families that translocate 20-80% of their sugars in the form of raffinose-

related compounds exhibit symplastic phloem loading (van Bel, 1994). It could be that polyol transporting groups also exhibit symplastic loading of the polyol. According to Noiraud et al. (2001b), mannitol and a number of other polyol transporters are not sensitive to PCMBS, suggesting a symplastic pathway. The results obtained in this experiment show that sorbitol uptake in apple stem tissue can be sensitive to PCMBS, but it is uncertain exactly what effect the inhibitor may be having (e.g. on aquaporins, making the cell leaky) and at what point in the tissue it is binding to. If the sorbitol carrier is part of a general retrieval system on all plant cells, other cells could have as much capacity as the phloem for sorbitol uptake even if retrieval into the phloem is symplastic (Turgeon and Beebe, 1991). The low sugar concentrations found in the apoplast support a symplastic pathway.

CCCP is a metabolic uncoupler and is used to show whether metabolic energy is required in the uptake process. CCCP was found to inhibit sucrose uptake, indicating that energy is required for uptake, especially at low sucrose concentrations. The greater inhibitory effect of CCCP on sucrose uptake at low concentrations has also been reported by Delrot and Bonnemain (1981). The sensitivity of the saturable component to CCCP supports the hypothesis that the carrier for sucrose requires energy, presumably via its coupling to the plasmalemma H^+-ATPase (Wright and Oparka, 1989). The CCCP insensitive component of uptake can be considered as a diffusive one.

CCCP was found to inhibit sorbitol uptake at both high and low concentrations. Sensitivity of sorbitol uptake to CCCP suggests that there is an energy dependent component (proton electrochemical potential) to sorbitol uptake that is independent of solute concentration. This result is in agreement with Gao *et al.* (2003) who found that isolated sorbitol transporters were inhibited by CCCP. In these experiments, CCCP inhibited uptake at low and high concentrations of sorbitol, providing further support that only one sorbitol transporter is present in apple stems.

However, Gao *et al.* (2003) isolated two sorbitol transporters and both were sensitive to CCCP.

4.4.4. Competition

Competition between sucrose and sorbitol for carriers across membranes in stem tissue was examined in this experiment. The results of the competition experiments between sucrose and sorbitol are very similar to the results obtained by Aked and Hall (1993) who examined the competitive nature of glucose and fructose against sucrose uptake into the epidermis of Argenteum leaves.

Aked and Hall (1993) found inhibition of sucrose uptake reached a plateau at around 60%, but interpreted these results by suggesting that there are separate carriers for sucrose and glucose and fructose. Molecular biology has now shown the existence of two distinct transporter families for sucrose and hexoses (Williams *et al.*, 2000).

The results of competition between sucrose and sorbitol uptake into apple stem pieces show that inhibition reached a plateau at 25% and 35% respectively. These results suggest that there is a competitive interaction between sucrose and sorbitol at high concentrations. A true competitor would be expected to show a positive correlation between inhibition and concentration. At low concentrations there was no inhibition of uptake, suggesting that sorbitol carriers in apple are different to the sucrose SUT carriers isolated to date (Lalonde *et al.*, 1999; Lalonde *et al.*, 2003; Lemoine, 2000). At higher concentrations, different carrier systems may be operating which may not be as sugar specific.

Marquat et al. (1996) found no competitive effect on sorbitol from sucrose in peach buds, but Beruter and Studer - Feusi (1995) did find that sucrose competitively reduced uptake of sorbitol in intact apple fruit. Bieleski (1977) found that in pear leaf slices, sucrose inhibited sorbitol uptake by less than 15% and suggested that sorbitol uptake occurs by a specific

sorbitol accumulation mechanism. Separate carriers for mannitol and sorbitol have recently been identified (Gao *et al.*, 2003; Noiraud *et al.*, 2001a).

4.4.5. Application to the model

This chapter has investigated some of the details pertaining to the model proposed in chapter 3 for storage of carbohydrate in apple stem tissue. In this model it was proposed that, after a reduction in sink demand, sorbitol saturates the apoplastic space once starch storage is full. This in turn reduces concentration gradients creating a back up of sorbitol in the leaves, resulting in a reduction or complete stoppage of photosynthesis.

In this chapter, the sugar concentration of the apoplast was found to be zero. This does not seem to fit with a model in which the apoplastic space was important for storage controls. However, very young stem tissue was used to determine the apoplastic sugar concentration and in young tissue it is unlikely that the model of storage would apply. Any sugar released into the apoplast would probably be retrieved to supply the high sink demand of growing buds and shoots.

Evidence in support of the model was gained by the finding that sucrose and sorbitol carriers exist in the stem tissue for retrieval of sugars from the apoplast. The sucrose carrier is possibly down-regulated when sink demand is reduced. The sorbitol carrier may also be regulated by sink demand and the characteristics of the sorbitol carrier may change with sink demand. These findings support the proposal that sorbitol (and potentially sucrose as well) exist in the stem while they are in transit from the source to terminal sink. In periods of high sink demand, it is likely that the up-regulated carriers retrieve any sorbitol or sucrose lost from the phloem. Conversely, during periods of low sink demand, it is likely that sorbitol and sucrose lost from the phloem is less likely to be retrieved, beginning the cycle of storage feed back.

Evidence that the apoplast was involved in retrieval of sucrose and sorbitol was gained from the inhibition of uptake by PCMBS. However, evidence was also presented that suggested sorbitol uptake may be symplastic, which does not tie in with the present model. More experiments are needed to conclusively determine the uptake mode of sorbitol. Further evidence for the role of the apoplast will be presented in chapter 5.

4.5. Conclusion

Loading and unloading of sugars in collection and sink phloem are key processes in transport events and are often considered to be the rate limiting steps to crop production (Patrick, 1997; van Bel, 1993). Retrieval and release of sugars along the transport phloem must also be important as there is a balance between supplying terminal sinks with photosynthate and retention of photosynthate along the pathway (van Bel, 1996). Studies into the retrieval of sugars in stem tissue are limited and have only been carried out in herbaceous species. This study has characterised the uptake of sorbitol and sucrose into a woody perennial stem and begun to provide us with an understanding of these processes. This study has also shown that it is possible to manipulate most of the experimental techniques used to study sugar uptake to suit woody stem tissue.

It was hypothesised that retrieval of sucrose and sorbitol is apoplastic in woody stems, as it has been found to be in annuals. Sucrose uptake was found to be biphasic and sensitive to PCMBS, as it is in annuals. This shows that there is potential for apoplastic uptake of sucrose in apple stems. The saturable component of sucrose uptake was likely to be a carrier-mediated H^+ co-transporter, similar to members of the SUT family, while the non-saturable was likely to represent facilitated diffusion. Seasonal differences were seen in the amount of sucrose transporters, with more transporters in the spring than in autumn. The transport system was also up-regulated when there was high sink demand.

Sorbitol uptake was found to be linear over the entire concentration range measured. This suggests uptake was by carrier-mediated diffusion. However there is evidence that two sorbitol uptake systems may be operating, depending on sink demands. Uptake of sorbitol was sensitive to PCMBS, suggesting a membrane bound carrier with SH groups was involved in sorbitol uptake. However, PCMBS may have different effects on sorbitol uptake if uptake is through aquaporins, as has been found in animal cells. A symplastic pathway of sorbitol retrieval may be followed in intact tissue and this was supported by the very low apoplastic concentrations found.

Sucrose and sorbitol compete for carrier sites at low concentrations, but at higher concentrations this competitive interaction saturates. From these results it is likely that sucrose and sorbitol have related but separate transporter mechanisms.

The results of this chapter show that sugar uptake (retrieval) from the apoplastic space is feasible and this ties in with the model of carbohydrate storage in apple stems proposed in chapter 3. Sugar carriers were found to be regulated by sink demand, which also fits in with the proposed model. However, no sugars were found in the apoplast, which is inconsistent with the proposed model, but this may be due to the age of wood used in the study.

5.1. Introduction

Short-term storage of sugars in the stem plays a major role in the carbohydrate status of a plant. Sugars can accumulate in phloem parenchyma cells for temporary or long-term storage (as starch) and then be distributed back to the sieve element/companion cell complex when there are fluctuations in photoassimilate supply. In addition, short-term changes in phloem sugar concentration can be buffered against by movement of sugars from the apoplast (Minchin and Thorpe, 1987).

The sieve tubes in the stem are not hermetically sealed pipes; they actually lose appreciable amounts of photosynthate, part of which is retrieved (Minchin and Thorpe, 1987). In bean, sieve tubes lose 6% of photosynthate per centimetre of stem, of which, two thirds is retrieved (Minchin and Thorpe, 1987). This passive leakage of photosynthate occurs along the entire length of the stem and is continuously retrieved (Minchin and Thorpe, 1984).

In bean stems, it has been found that the storage of carbohydrate in the stem apoplast along the length of the pathway has a buffering action on the fluctuations in short-term changes to the supply of photosynthate available for growth of e.g. fruit (Minchin *et al.*, 1984).

Thus far, the stem buffering response has only been studied in herbaceous annual species. It is not known whether there is continuous leakage and retrieval of sugars in perennial woody species. If woody perennials have the same transport phloem mechanism(s) as found in annuals, then a similar buffering response to short-term changes in photosynthate supply or demand to that in bean should be observed. In this study, the hypothesis that in apple stems, the transport phloem

involves leakage and retrieval into the apoplastic space, which acts as a buffer against short-term changes in photosynthate supply, was tested. Given that the apoplastic concentration of sugars was found to be zero in chapter 4, it is uncertain whether buffering does involve the apoplastic space in apple stem tissue.

To determine whether buffering occurred in apple stems, experiments were carried out using the short-lived (20.4 minutes) radioisotope, carbon-11 (^{11}C). ^{11}C allows measurements to be made *in vivo* because gamma rays are released that have a high energy, which can be detected through several centimetres of plant tissue (Minchin, 1985). This type of monitoring is not possible using ^{14}C, which has a much weaker radiation decay that can only travel a distance of about 0.3mm in plant tissue.

Plants took up ^{11}C as $^{11}CO_2$ and the resulting photosynthate was then monitored moving down the length of the stem. Chilling a short length of stem region was used to interrupt the flow of photosynthate along the stem length. Chilling causes a local phloem tube blockage, resulting in no further tracer moving through the chilled region, and in the presence of buffering, tracer will continue to accumulate at the stem apex. This can be interpreted as the falling pressure and sugar concentration, just below the chilled region, causing sugar movement into the sieve tubes from the surrounding storage pools (Minchin and Thorpe, 1984), thus maintaining a hydrostatic pressure gradient and hence flow. The lateral flow of carbohydrate buffers the falling sucrose concentration, maintaining flow at the stem apex until these pools are depleted and ensures a supply of photosynthate continues to reach the sink (Minchin and Thorpe, 1984).

If buffering occurs, PCMBS can be used to inhibit apoplastic loading, illustrating the location of the lateral carbohydrate storage pool involved in buffering. A buffering response would provide support for the existence of lateral flows of photosynthate along the length of the transport phloem within apple stems. If no buffering occurs, then woody plant species either have different mechanisms to herbaceous annuals to cope with short-term changes in photosynthate supply, or simply do not buffer against short-term changes in photosynthate supply.

5.2. Method

5.2.1. Plant material

Apple seeds (Baujade x A151R4T128) were sown in potting mix and chilled at 0^0C for eight weeks. Different cultivars were used from previous experiments as small seedlings were required for the experimental technique used. Germination occurred after trays were put into a glasshouse. Seedlings were transplanted to 10cm pots once 2-3cm high. Plants were watered daily and spraying every 10 days was carried out with copper oxychloride (Copperox®), triforine (Saprol Disease Spray®), maldison (Malathion 50 EC®) and chlorthalonil (Bravo fungicide®) to prevent fungal and insect problems.

5.2.2. Solution uptake

Minchin and Thorpe (1987) have peeled the epidermis of bean stems as a means of getting solutions into the apoplast. Experiments have also been carried out with split stems as a means of getting solutions into the apoplast e.g. moonflower (Pickard *et al.*, 1978). However, when dealing with apple seedlings the epidermis cannot simply be peeled off and splitting the stem stops all phloem transport (P.E.H. Minchin, HortResearch, New Zealand, *pers comm.*). Thus various methods were tested as a way of getting solutions into the apoplast of apple stems.

A piece of fine cotton wicking material (Fiesta Flarewick) was threaded through the stem of the plant using a darning needle. To increase the amount of stem tissue in contact with the wick, it was threaded through the stem twice at right angles to one another, with the ends of the wick left long to enable them to be bathed in the uptake solution of interest. The ability of stem tissue to take up solutions in this way was tested by applying a solution of blue dye (China Blue, National Aniline division, Allied Chemical and Dye Corporation, New York) to the wick. The stem and leaf veins above the wick turned blue proving that solutions were taken up into the stem (Fig. 5.1). In addition, aphids feeding on these plants also turned blue, indicating that dye had moved into the phloem of these plants. Plants were able to survive with a wick in place indefinitely. Thus using a wick system appeared to be a way of getting solutions into the apoplast.

Figure 5.1. The effectiveness of the wicking system used to get solutions into the apple stem. Blue dye was taken up by the wick and has turned the stem and the main veins of the leaves blue.

5.2.3. Experimental set up

A wick was threaded through the stem of 14-week-old apple seedlings 24 hours before tracer experimentation. The stem was girdled (bark, phloem and cambial tissue removed) near the base of the stem (below the wick) prior to experimentation and all but one mature leaf was removed above the girdle. Leaves below the girdle supplied carbohydrate to the roots. This produced a simple plant system comprising a single mature leaf as the source of carbohydrate supplying a sink at the shoot apex (Fig. 5.2).

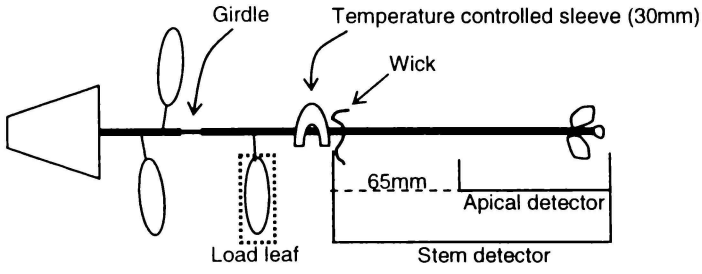

Figure 5.2. The experimental set up. Two apple seedlings were set up as depicted above in environmental cabinets.

Temperature treatments were applied by passing water (at the appropriate temperature) through a piece of copper pipe bent so as to be in contact with the stem for a length of 3cm. Lanolin provided good thermal contact between the plant stem and the copper pipe. Initially water at 25^0C was circulated and at the appropriate time this was changed to water from a second temperature controlled bath at 6^0C. The stem temperature was measured using a thermocouple attached to the stem.

The ends of the wick threaded through the plant were placed in apoplastic bathing solution (ABS) consisting of 20mM 2(N-morpholino) ethanesulphonic acid (MES) buffer (pH 6.5) and 1mM $CaCl_2$. During some

experiments, the bathing solution was changed to 2mM p-chloromercuribenzenesulphonic acid (PCMBS) in ABS (a non (or slowly) permanent thiol reagent that is used to demonstrate the existence of an apoplastic step in retrieval; see Chapter 4) or 20mM dithioerythritol (DTE) in ABS (a sulfhydryl-containing reagent that can be used to reverse the effects of PCMBS: M'Batchi and Delrot 1984).

The leaf chamber formed part of a closed loop system through which air was circulated at 1000cm^3 min^{-1}. Dew point was controlled to 10^0C and carbon dioxide concentration was held at 320ppm (±5%). Approximately 1 GBq of $^{11}CO_2$ was produced every 2-3 hours and used to replenish a reservoir connected to the closed loop system. $^{11}CO_2$ was bled from this reservoir into the circulating gas stream as required (Pickard *et al.*, 1993). The ^{11}C as $^{11}CO_2$ was prepared as described by More (1985).

The sensitivities of all the radiation detectors, necessary for comparison between detectors, was determined using a piece of the labelled load leaf contained within an eppendorf tube to act as a β^+ shield (this ensured all emitted positrons were converted into x-rays). The piece of leaf was positioned where the stem had been and the count rate of this known amount of ^{11}C recorded. Allowing for decay, this gave the count rates at all the detectors for the same amount of tracer within their respective fields of view (Pickard *et al.*, 1993).

During experiments, an on-line computer continuously read the total number of counts in 60 seconds from each detector. All data presented have been corrected for background and radioactive decay.

5.2.4. Measurement of buffering

Pulse labelling with ^{11}C and a half-life correction of the data enabled the arrival of tracer to be monitored in the sink. Upon chilling a short length of stem using the cold block, tracer stopped moving into the sink region as monitored by the stem detector (Fig. 5.2). If buffering occurred, a small amount of tracer moving into the plant apex would still be seen by the apical detector. Plotting tracer movement into the plant apex before and after the application of the cold block allowed the extent of buffering to be determined. A ratio of the pre and post cold block slopes gave an indication of the buffering response. A ratio of 1 would indicate complete buffering while a ratio of zero would indicate no buffering occurred. Errors in these values are associated with errors in the estimation of slopes pre and post cold block.

5.3. Results

5.3.1. Response to chilling

In order to exam a buffering response in stem tissue it is imperative that chilling a short segment of stem causes complete stoppage of tracer flow, as seen by the stem detector. Flow of tracer into an apple stem did stop on cooling and a typical effect of cooling an apple stem to 6^0C is shown in Figure 5.3. Within 1 minute of cooling the stem to 6^0C, the accumulation of ^{11}C in the sink stopped (Fig. 5.3) as seen by the stem detector (the entire stem region above the cold block: Fig. 5.2). On rewarming to 25^0C sink accumulation resumed.

Accumulation of ^{11}C tracer as seen by the apical detector (plant apex) did not completely stop on chilling, but on rewarming to 25^0C, tracer was immediately seen moving into here at the pre-chilling rate (Fig. 5.3). This demonstrates a buffering response and was found in all apple stems examined. Tracer flow into the apical detector was not completely stopped by the cold block as would be expected if the transport phloem

behaves as a perfect leak-free pipe. This continuation of flow (even at a reduced rate) is what has been termed buffering.

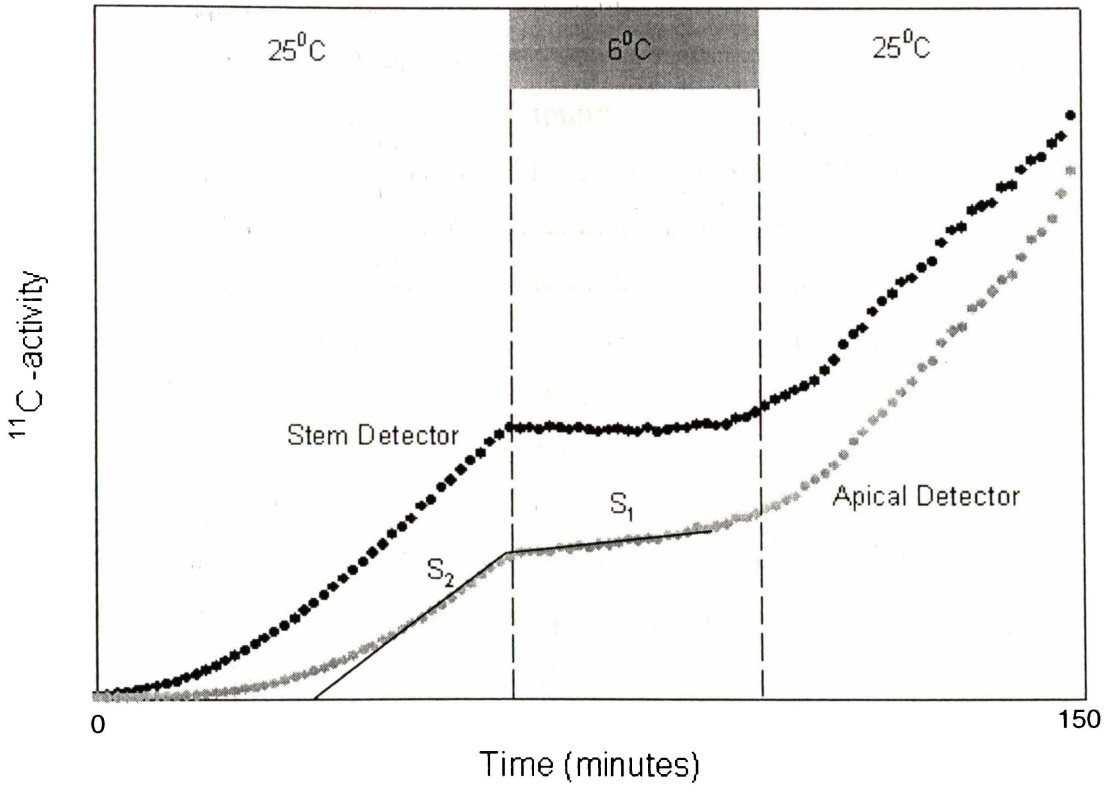

Figure 5.3. Accumulation of ^{11}C-photosynthate in the shoot apex (the sink region). ^{11}C-activtity half-life corrected to time zero.

To quantify the buffering response of apple stems, the ratio of the slopes S_1 and S_2 (Fig. 5.3) was used. These slopes were estimated by a least squares regression. A S_1 slope greater than zero indicated that buffering had occurred. A slope ratio of 1 would be expected from complete buffering and a value of 0 would indicate that no buffering had occurred.

In order to characterise this buffering response, the stem needed to be subjected to multiple chilling and rewarming cycles, so it was necessary to check that stoppage in tracer flow still occurred after multiple chill/rewarm

cycles. A second cycle of chilling and rewarming caused a similar complete stoppage and start to flow to be seen by detector 1 and this result was achieved with up to 4 chilling/rewarming cycles (Fig. 5.4).

5.3.2. Buffering

When the buffering response of apple stems was measured over multiple chilling/rewarming cycles, it was found to increase throughout the photoperiod (Fig. 5.5). Replication in ^{11}C experiments is usually very low due to the expense and time involved in experimentation and it can be argued that each plant is its own control due to the uninvasive nature of this technique. Despite this, in these experiments, we attempted to obtain more quantitative comparisons between plants and the increase in buffering throughout the photoperiod was seen in five replicates.

Determining the characteristics of the buffering capacity in apple stems required further experiments involving manipulation of buffering. The bigger the buffering capacity at the beginning of manipulation experiments, the more obvious any changes to buffering would be and there would be a greater chance of seeing small changes to buffering. The finding that the buffering response increased throughout the photoperiod resulted in adjustment of lighting so that plants were exposed to light for 10 hours prior to experimentation. This meant that a higher ratio between S_1 and S_2 was seen at the beginning of ^{11}C labelling (around 8am), allowing a full day for the characteristics of buffering to be examined.

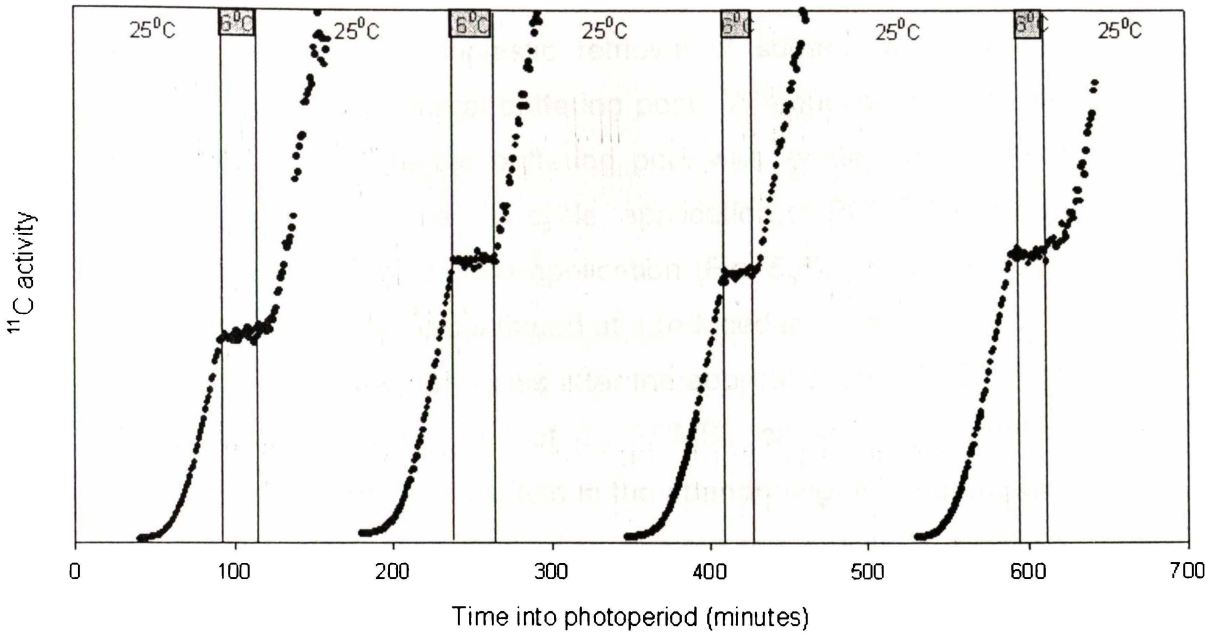

Figure 5.4. Accumulation of ^{11}C-photosynthate as seen by the stem detector in the shoot apex showing multiple chilling/rewarming cycles. ^{11}C-activity half life corrected to time zero.

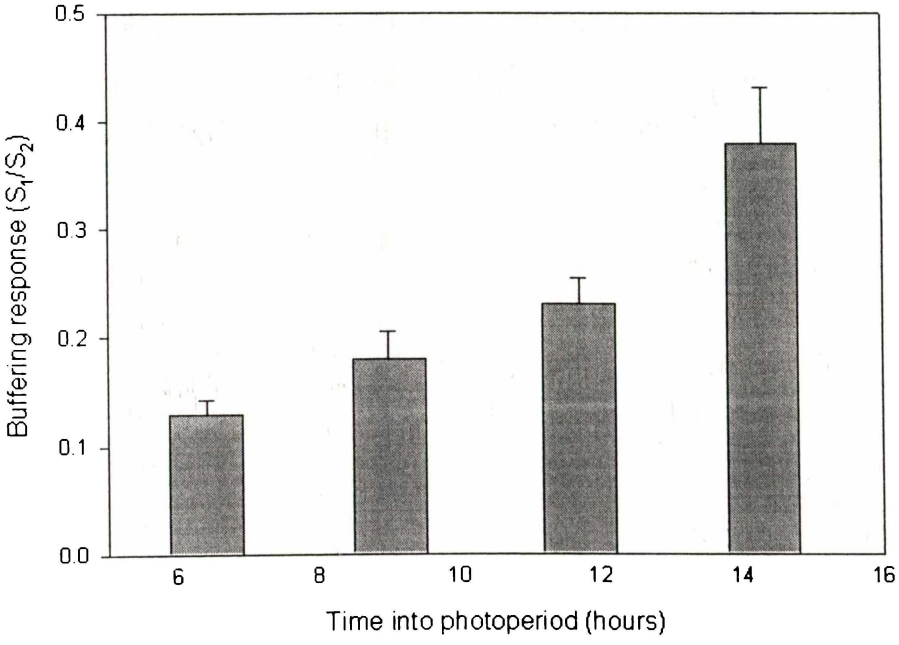

Figure 5.5. The buffering response of an apple seedling throughout the day. Error bars are the error associated with an estimation of the buffering response. This result was seen in five replicates.

To examine some of the characteristics of buffering, PCMBS was applied to the ends of the wick that was threaded through the plant stem. PCMBS was used to prevent any apoplastic retrieval of sugars, which would indicate the location of the lateral buffering pool. A reduction in buffering with PCMBS would indicate the buffering pool was located in the stem apoplast. After a delay of one ^{11}C cycle, application of PCMBS reduced the buffering response seen before application (Fig. 5.6). In some cases (in 4 out of 6 plants), buffering continued at a reduced level, as shown by a reduced S_1/S_2 ratio, for several cycles after the application of PCMBS (Fig. 5.6), while in some plants (2 out of 6), PCMBS caused the complete stoppage of ^{11}C translocation, resulting in the abandoning of that particular experiment. When DTE was applied to reverse the effects of PCMBS, there was an increase in the buffering response within the next ^{11}C cycle (Fig. 5.6).

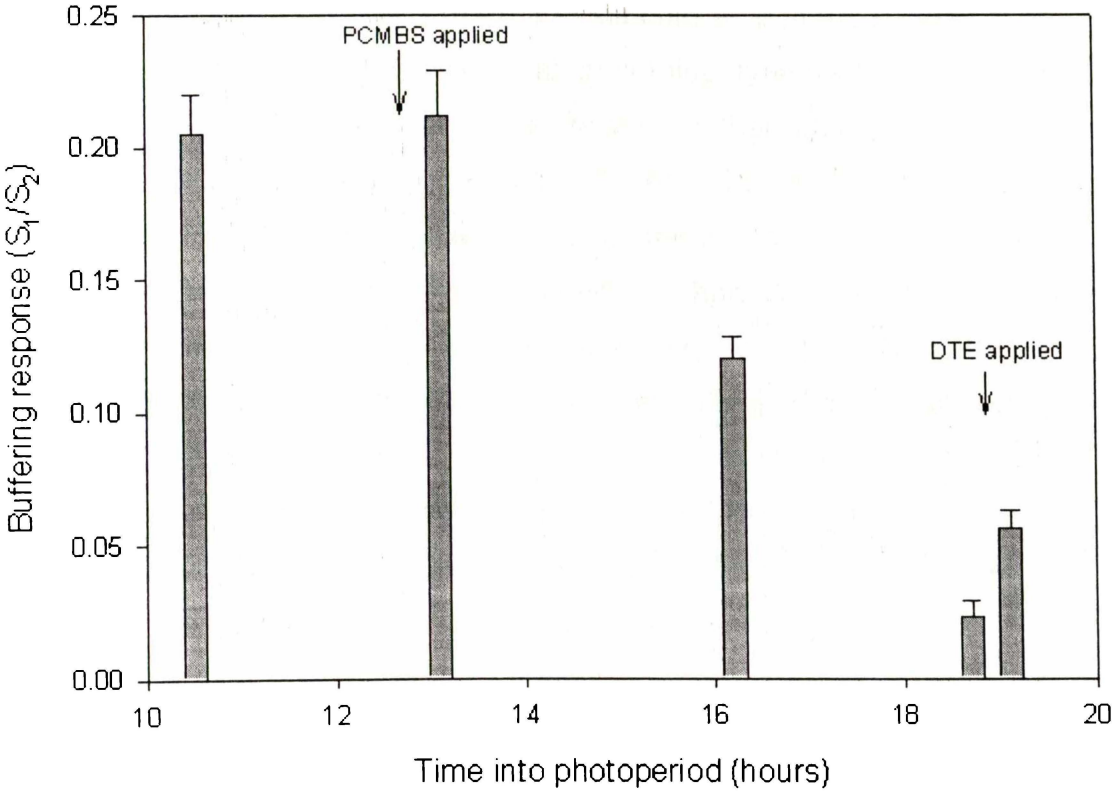

Figure 5.6. Effect of PCMBS and DTE on the buffering response of an apple stem. Data shown is for one plant only.

5.4. Discussion

To date, the stem buffering response has only been studied in herbaceous annual species and it was not known whether there was continuous leakage and retrieval of sugars in perennial woody species. It was hypothesised that that in apple stems, the transport phloem involves leakage and retrieval into the apoplastic space, which acts as a buffer against short-term changes in photosynthate supply. This hypothesis was found to be true in apple trees.

5.4.1. Response to chilling

In these experiments, the speed of the temperature change is used to disrupt phloem transport for a short length of time. Phloem blockage on rapid chilling has been shown in numerous plant species by Geiger (1969); Geiger and Sovonick, (1970) and Minchin *et al.*, (1983). However, different plant species can exhibit an 'all or nothing' type response to short lengths of stem chilling, where translocation either stops abruptly or continues unaffected (Lang and Minchin, 1986). Thus it was important to establish that rapid stem chilling did stop tracer flow in apple stems. Multiple chill/rewarm cycles were needed to characterise the buffering response seen in apple and it was necessary to establish that after multiple cycles, flow of tracer still stopped on chilling. Some plants (e.g. sorghum) have been found to only be sensitive to the first chill/rewarm cycle (Lang and Minchin, 1986). Apples were found to be sensitive to multiple chill/rewarm cycles, which allowed the buffering response seen in apple to be characterised.

5.4.2. Buffering in apple

The buffering response seen in apple stems was measured by determining the slopes of S_1 and S_2. This allowed a quantitative measure of buffering to be made. It was found that the magnitude of buffering

depended on the demand for carbon in the plant and the amount and availability of carbon in storage pools supplying the buffer.

The majority of previous work into the short-term storage of the stem has been carried out in bean, a herbaceous annual. Apple is a difficult plant to use in this work due to the difficulty of getting solutions into the apoplast while still maintaining phloem transport. The wicking system developed for apple stems was a successful way of getting solutions into the apoplast.

In bean, chilling causes a local blockage, resulting in no further photosynthate moving through the chilled region. Outside of this region, flow continues until the hydrostatic pressure gradient is dissipated (Minchin and Thorpe, 1984). Below the chilled region, flow continues to move photosynthate towards the sink. This can be interpreted as the falling pressure and sugar concentration causing sugar movement into the sieve tubes from the storage pools surrounding the sieve tubes (Minchin and Thorpe, 1984). This buffers the falling sucrose concentration, maintaining flow until these pools are depleted. This buffering ensures a supply of photosynthate still reaches the sink. Chilling also caused a local blockage of tracer in apple stems and flow continued outside of this region. This suggests that the mechanisms proposed to explain buffering in bean apply to apple.

Buffering in bean has been found to follow an apoplastic path of retrieval and release (Minchin and Thorpe, 1984). In apple stems, an apoplastic path of buffering was also found, as PCMBS (which blocks sugar uptake from the apoplast) reduced buffering. Retrieval of sugars, necessary for the buffering response was via the apoplast. These results may provide further evidence (see chapter 4) that there is an apoplastic step involved in the movement of photosynthate in apple stems. However, PCMBS may also affect aquaporins; water leaking out of the sieve tubes could affect the buffering response by reducing pressure gradients necessary to move tracer to the sink. A loss of water along the pathway after the application of PCMBS would cause the turgor pressure to drop and also stop tracer

flow. The loss of turgor pressure would prevent retrieval of sugars, as uptake and unloading of sugar is probably turgor sensitive (Aloni *et al.*, 1986; Grimm *et al.*, 1990). This situation might be seen in outputs of the tracer flow through a fall in tracer as sink sugars flowed backwards towards the source. This was not seen in any of these experiments. A similar situation could be expected to occur if sorbitol (which may be transported through aquaporin like channels) also leaked as a consequence of PCMBS use.

In apple, the reduction in buffering after application of PCMBS took one cycle of ^{11}C before occurring. This was probably a result of the time taken for solution to be taken up by the wicking system. Complete stoppage of buffering by PCMBS did not occur, as it was likely that not all sieve tubes were exposed to the inhibitor. While the wicking system was shown to be a way of getting solution into apple stems, no data was obtained on the distribution of solution within the stem.

DTE was found to reverse the effect of PCMBS on buffering in apple to some degree. This indicates that the SH groups affected by PCMBS were able to be re-established to some extent. DTE may also reverse other affects of PCMBS such as aquaporin function. Further evidence is necessary (such as disruption of symplastic connections by plasmolysis) to provide unequivocal data on the buffering pathway.

5.4.3. Diurnal variation

An interesting observation seen in the apple stem was that the buffering response increased into the photoperiod. This is consistent with a pool of sugar providing this buffering response, as has been proposed in bean. It was likely that this pool depleted overnight and slowly replenished throughout the day. A depletion of a storage pool during the night and replenishment during the day is consistent with studies in other plants. For example, Williams et al. (1979) found that a short-term pool in sunflower, used to provide a consistent flow of photosynthate into the

transport path, was more or less empty at the beginning of the day and much fuller at the end of the day. Buffering pools have also been depleted experimentally by increasing buffering demand through warming a sink (Thorpe *et al.*, In press).

The results obtained in this experiment are consistent with the apple stem acting as a short-term storage organ to buffer against changes in phloem concentration when there are fluctuations in photoassimilate supply. The mechanisms proposed by Minchin et al. (1984) for bean apply to deciduous fruit trees. This suggests that apple stem tissue has the ability to buffer short-term changes in photosynthate supply by drawing on a pool of carbohydrate to maintain phloem concentrations. Retrieval of sugars from this pool is probably apoplastic. The size of this pool is evidently reduced throughout the night so that early in the photoperiod, little buffering occurs. This pool is slowly recharged throughout the day, resulting in a bigger buffering capacity.

5.4.4. Application to the model

The buffering pool associated with the apoplast lends support to the model proposed in chapter 3. In chapter 4, the size and importance of the apoplast was questioned because no sugars were found in it. This implied that the apoplastic space was either very small, or that, because the tissue was very young, it was actively growing and scavenging all available sugar. The results of this chapter show that there is leakage from the sieve elements into the apoplast and retrieval of sugars from here. This suggests that the apoplast may be larger in older tissue and does play an important role in the regulation of sugar flow to the sink.

The apoplastic pool in this case was depleted overnight and recharged throughout the day. Without strong sink demand from growing buds or fruit, this apoplastic pool may not be depleted overnight, leading to the feedback mechanisms proposed in chapter 3. Sugars are released from the sieve elements into the apoplastic space because the sieve elements

behave as leaky pipes. This sugar is reloaded into the sieve elements to ensure a continued supply of photosynthate to the sink when there are short-term reductions in this supply. In the longer term, it can be proposed that if sinks are removed, sugar is still leaked from the sieve elements, but is not retrieved as efficiently. Once other storage pools are full, a back up of sugar results in a reduction of photosynthesis.

The reduction in buffering seen after the application of PCMBS suggests that sugar (potentially sorbitol, although this study did not differentiate between sugar types) is unloaded and reloaded into the apoplast. This shows that there is potential for a pool of sorbitol to exist in the apoplast and this is consistent with the proposed model.

5.5. Conclusion

The hypothesis that woody perennials exhibit a buffering response to overcome short-term changes in photosynthate supply was found to be correct. Buffering does occur in apples and probably involves retrieval from the apoplast. The buffering mechanisms proposed in bean can be applied to apple; the stem acts as a short-term storage organ to buffer against changes in photoassimilate supply thus maintaining a constant flow to the sink.

Buffering in apple exhibited a diurnal response, which is consistent with the concept of the buffering pool being depleted at night and replenished during the day.

The apoplast is involved in sugar buffering and this is consistent with the model proposed in chapter 3.

6.1. Introduction

Sugar alcohols are sugars with an extra alcohol grouping, also called polyols, polyalcohols or polyhydric alcohols (Bieleski, 1982). Sugar alcohols were shown to be primary photosynthetic products in higher plants only forty-four years ago and it has been estimated that about 30% of the primary carbon production on earth goes through sugar alcohol synthesis (mainly in algae) (Bieleski, 1982). Sugar alcohols are thought to play a variety of roles in protecting metabolism during stress (see chapter 1) and thus there could be economic benefit in the production of sugar alcohols in plants that only produce sucrose (e.g. tobacco 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 (transgenic tobacco plants accumulating high concentrations of sorbitol were infertile and unable to form roots: Sheveleva et al. 1998).

Sorbitol is a sugar alcohol, present in a number of higher plants, but found most often in the Rosaceae (Loescher, 1987) (see chapter 1). Apple leaves contain sorbitol as a major carbohydrate (60-70% of total sugar or 50-150mM) and a lesser proportion of sucrose (16-24%) (Bieleski, 1982). As shown in chapter 2, sorbitol is the major carbohydrate present in the stems of apple (approximately 50% of the total). Sorbitol is also a major translocation product (Bieleski, 1969) and in the phloem is found to comprise 65-70% of the sugars (Klages *et al.*, 2001). The ratio of sugars in the phloem resembles their ratio in the leaf (Bieleski, 1969) and there is very little diurnal change in these phloem ratios (Klages *et al.*, 2001).

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 and understood. Sorbitol is formed in the leaves via aldose 6-phosphate reductase by the reaction:

Glucose 6-P + NADPH + H^+ $\longleftrightarrow$ sorbitol 6-P + $NADP^+$
Sorbitol 6-P + $NADP^+$ → Sorbitol + Pi
(Loescher and Everard, 1996)

In the sink, sorbitol is metabolised by the enzyme sorbitol dehydrogenase (SDH) in the reaction:

Sorbitol + NAD^+ → Fructose + NADH + H^+
(Loescher and Everard, 1996)

Sorbitol can also be metabolised by sorbitol oxidase, which converts sorbitol to glucose in the absence of NAD and NADP (Loescher and Everard, 1996). Sorbitol oxidase activity has been found in apple leaves and developing peach fruit but only in concentrations too low to explain *in vivo* sorbitol interconversions (Loescher and Everard, 1996).

The majority of degradation of sorbitol in Rosaceae sink tissue depends on the presence of the enzyme SDH. In terminal 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 degrade sorbitol is lost at maturity (Zhou *et al.*, 2001). Surprisingly, very young leaves cannot degrade sorbitol either and in fact only expanding leaves have the ability to both use and make it (Bieleski and Redgwell, 1985). Sorbitol is also not extensively metabolised by nectaries in a variety of Rosaceae (Bieleski and Redgwell, 1980), although here, the major conversion of sorbitol may occur in the phloem (Bieleski and Redgwell, 1980).

Whether sorbitol is degraded in stem tissue, which is a weak sink, remains to be determined. Sorbitol appears to serve a temporary storage role in the stem; its concentration rises when there is excess carbohydrate and

declines when there is strong demand for carbohydrate, at least some of the time (chapter 3). A major question addressed in this chapter is whether sorbitol can be degraded for use in metabolism in the stem, or is it there as a carbon store purely for use by higher priority sinks such as fruit that have the ability to metabolise it.

Given that sorbitol is not degraded in all Rosaceae sink tissues examined to date, it is not clear whether there is metabolism of sorbitol in stem tissue. In this study, the metabolism of sorbitol in apple stem tissue was investigated. The high concentrations of sorbitol found in the stem and the inconsistent behaviour after manipulation of sinks led to the hypothesis that apple stem tissue has no ability to metabolism sorbitol.

To attempt to determine whether stem tissue can metabolise sorbitol, an experiment was carried out to determine whether the stem could be forced to use sorbitol by attempting to deplete all the carbohydrates. Secondly, metabolism of exogenously applied ^{14}C-sorbitol was investigated. Finally, SDH, the major enzyme necessary for sorbitol metabolism was looked for in the apple stem.

The results of this chapter will provide another step towards creating an integrated picture of sorbitol movement along the pathway from source to sink. Sorbitol may be metabolised in the stem and there may be continuous breakdown and reformation. Alternatively, there may be no sorbitol metabolism in the stem because the enzymes responsible for degradation are not present. Or, there may be no metabolism because sorbitol is metabolically segregated; in other words, able to be used in some tissue and not others. In leaves, sorbitol might be rapidly removed from or prevented from entering the metabolic pool and put into a storage

pool instead (Bieleski, 1977). A similar phenomenon may occur in the pathway; the mechanisms to metabolise sorbitol may exist, but sorbitol may be prevented from being metabolised by being placed in a storage pool.

6.2. Method

6.2.1. Depletion of carbohydrates

To determine whether all the carbohydrates (including sorbitol) in a one-year-old shoot could be utilised, 7 stems were girdled on one 'Braeburn' tree in the Waikato orchard (see chapter 2). Girdling involved the removal of a 2cm wide strip of phloem, cambial tissue and connected bark on 6th November 2000 (an aluminium splint was taped to the girdled area for physical support). Leaves were removed at $^{1}/_{3}^{rd}$ their final size before they could become carbon exporters (Hansen, 1971) and thereafter checked and removed if necessary every 3 days. The experiment was terminated on 11th December 2000 when no new leaves grew from the girdled stems. Stems were then removed at the girdle and analysed for starch, sucrose, sorbitol and glucose + fructose as described in chapter 2. The concentrations found in ungirdled non-defoliated stems were used for comparison.

To further determine if all carbohydrates in the stem could be utilised, one-year-old stems of 'Braeburn' apple trees were removed from the tree and kept in the dark as a means of depleting the carbohydrates. Six replicate one-year-old stems were harvested from trees growing in the Waikato orchard (foliage was retained on these stems) and placed in vases containing 50ppm hypochlorite (replaced every second day) in the greenhouse under full natural light or in darkness. Pieces of stem were removed at approximately weekly intervals for ten weeks for carbohydrate extraction. In addition three replicate whole seedling plants (same batch of seedlings as described in chapter 5) were placed in full natural light or

in darkness and harvested ten weeks later. Extraction of starch, sorbitol, sucrose and glucose + fructose was carried out as described in chapter 2.

6.2.2. Metabolism of sorbitol

To determine whether ^{14}C-labelled sorbitol or sucrose were metabolised when taken into the stem via a bathing solution, samples were bathed for two hours as described in chapter 4. In addition, a longer-term bathing was carried out for five hours. Blank samples and samples containing 3% hypochlorite solution were included to ensure that bacterial activity did not affect results. 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 6ml ethanol, samples were dried down and redissolved in 200µL H_2O. Sugar fractions were passed through a membrane filter before HPLC analysis using an ion-exchange lead column (Shodex SP 0810) with water as eluent (0.6ml min^{-1}) 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 one-minute intervals. The radioactivity of these samples was counted by liquid scintillation for 60 minutes or to a precision of 2% per sample, whichever came first.

6.2.3. Sorbitol dehydrogenase extraction

Sorbitol dehydrogenase (SDH) was extracted from stem tissue in April and December 2002, using the method of Lo Bianco and Rieger (1998). In summary, tissue was collected from the orchard and transferred on ice to the laboratory where extraction was carried out immediately. Tissue was homogenised (at 2 - 4^0C) in tris-HCl buffer (pH 9), containing 8% (v/v) glycerol, 0.1% (v/v) Tween 20 and 1% (w/v) PVPP. The homogenate was filtered through a layer of miracloth and centrifuged at 3000g for 15 minutes. The supernatent was desalted using a Sephadex G – 25 column

at 4^0C. The assay for SDH activity was carried out immediately. The assay volume consisted of desalted extract, 0.1M tris-HCl buffer (pH 9.5), 1mM NAD^+ and 300mM sorbitol. The assay mixture was incubated at 25^0C for 5 minutes minus the sorbitol and the reaction was started with the addition of sorbitol. Reading the absorbance on a spectrophotometer as Lo Bianco and Rieger (1998) did was found not to give high enough sensitivity. Thus the sensitivity of the method was increased by reading the production of NADH per minute at 25^0C over a 5-minute period on a fluorometer. In addition to stem tissue, tissue known to contain SDH such as roots and growing shoot tips (including expanding leaves) was also extracted to ensure the method was successful. Samples were also spiked with known quantities of SDH at the assay stage to ensure there was no SDH inhibition induced by the plant extract. Enzyme activity (nmol $min^{-1}g^{-1}$ fresh weight) was calculated from the net change in fluorescence per minute.

6.2.4. Statistics

The data was analysed statistically using Analysis of Variance (ANOVA). The assumption that parametric parameters were filled (i.e. the data has equal variance, is independent and normally distributed) was tested before an analysis was performed.

6.3. Results

6.3.1. Depletion of carbohydrates

Little starch remained in the girdled stems after a five-week period of leaf removal (Fig. 6.1). The starch concentration remaining in the defoliated girdled stems was significantly lower ($P < 0.05$) than the control (ungirdled, non-defoliated) stems. Concentrations of sucrose and glucose + fructose in the defoliated girdled stems corresponded to the concentrations normally found in one-year-old stems at that time of year. Sorbitol was present in high concentrations in defoliated girdled stems and was significantly increased by the defoliated girdling treatment. Total carbohydrates in the defoliated girdled stems were surprisingly not significantly different from control stems. The loss of starch in the defoliated girdled stems was paralleled by a gain in sorbitol concentration.

In the alternative experiment where foliated one-year-old stems were kept in the dark for ten weeks, there was a reduction in glucose + fructose (Fig. 6.2a) and sucrose (Fig. 6.2b) compared to stems kept in the light. Concentrations of these sugars fell to zero in the dark treatment, but then increased after ten weeks in the dark. Starch was present in higher amounts in the stems kept in the dark compared to stems in the light, while sorbitol was found in lower amounts in dark stems (Fig. 6.2c & d). All leaves were lost from both the light and dark stems only a week after removal of the branch from the tree. Blossom then formed three weeks after the start of the experiment in both light and dark stems, but died on the stems kept in the dark within a few days, whereas the stems kept in the light retained the blossom and started to grow new leaves.

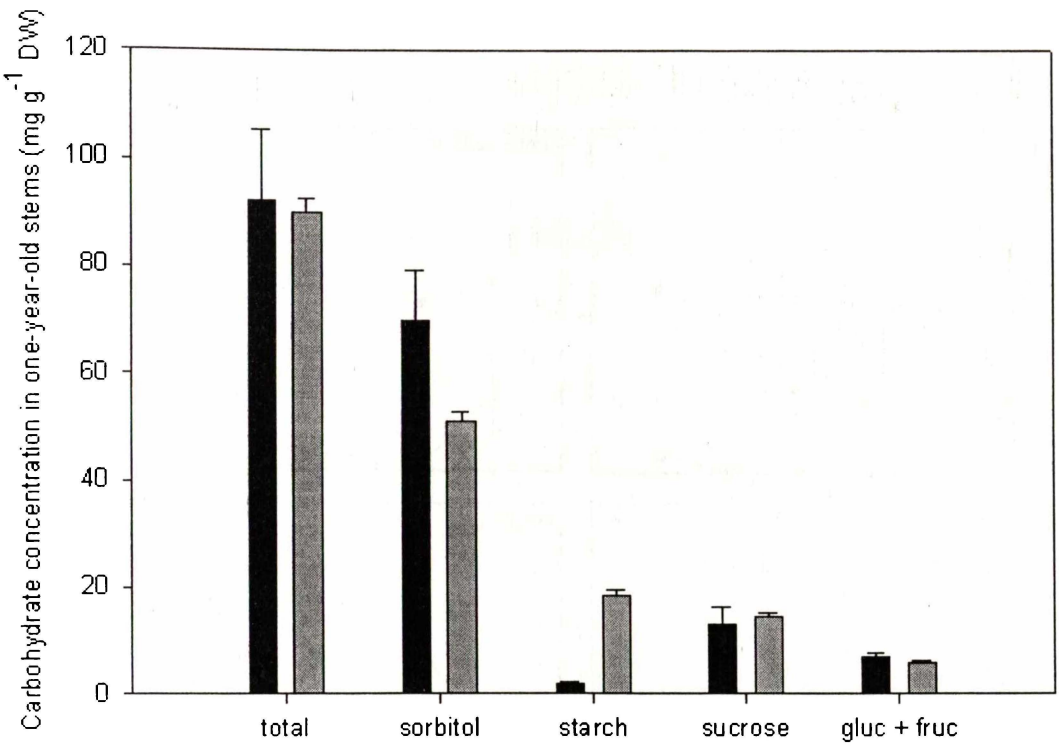

Figure 6.1. Carbohydrate concentration of girdled one-year-old stems when new leaves were removed before becoming net exporters of photosynthate (dark bars). Carbohydrate concentrations of control stems (not girdled) are also shown (light bars). Error bars are standard errors (n=7).

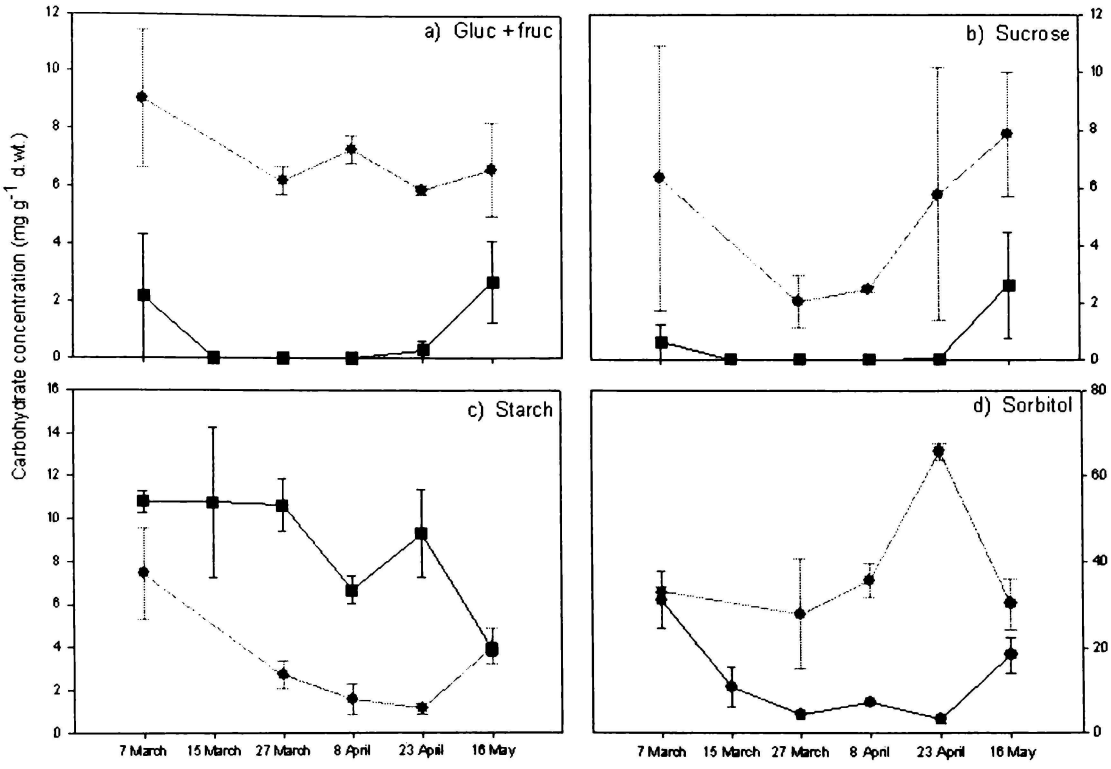

Figure 6.2. Carbohydrate concentrations of excised apple stems kept in either light (light coloured symbols) or dark (black symbols). Apple stems were removed from the tree on 28 February 2002 and put into the dark on the same day. Error bars are standard errors (n=6).

Starch concentrations were significantly lower ($P < 0.05$) in whole seedling plants kept in the dark for ten weeks than whole seedlings kept in the light (Table 6.1). The concentration of sorbitol was significantly higher in dark plants than in light. Glucose + fructose was also significantly higher in dark plants than in light, while the sucrose concentration was not significantly different. Plants kept in the dark lost their leaves within two weeks.

Table 6.1. The carbohydrate concentration of whole plants kept in either the dark or light for ten weeks. The letters denote significant differences ($P<0.05$).

	Carbohydrate concentration (mg g^{-1})			
	Starch	Sorbitol	Sucrose	Gluc+fruc
Light	67.2a	25.1a	8.9a	4.8a
Dark	41.8b	38.3b	10.5a	8.6b

6.3.2. Metabolism of sorbitol

To determine whether sorbitol and sucrose could be metabolised by the stem, stem pieces were bathed in labelled sugars and the radioactivity of the products measured. In stems that had been bathed in ^{14}C-sorbitol, no metabolism took place as only radioactive sorbitol was found. In stems bathed in ^{14}C-sucrose, metabolism of sucrose was confirmed by the presence of labelled glucose and fructose. Samples containing no plant material (blank samples) were free of any metabolism indicating that the metabolism seen was not due to microbial activity.

The above experiment was conducted with an in-line solid cell radioactivity detector with limited sensitivity. Therefore, the experiment was repeated using scintillation counting (which enabled longer counting of the

radioactivity of samples and therefore increased sensitivity) before samples passed through the radioactivity detector. This approach confirmed the non-metabolism of sorbitol and the metabolism of sucrose into labelled glucose, galactose and fructose. Labelled inositol and sorbitol were also products of sucrose metabolism.

6.3.3. Sorbitol dehydrogenase activity

The validity of the method for extracting SDH was tested using freshly harvested shoot tips and expanding leaves from trees of various ages and varieties growing at the Ruakura Research orchard. SDH activity was found in these shoot tips and leaves (Table 6.2). SDH was also detected in the roots of potted plants, but never found in stems at any time of the year (Table 6.2). SDH RNA has been found in fruits and leaves (HortResearch Plant EST_95 database), but there is no record of it being found in stem tissue.

Table 6.2. SDH activity (fresh mass basis) in various organs of apple at two different times of the year.

Time	Organ	SDH activity (nmol $min^{-1}g^{-1}$ f.wt)	Average
April	Root	50	
April	Root	47	
			48.5±1.5
April	Stem	0	
April	Stem	0	
April	Stem	0	
April	Stem	0	
			0.0±0.0
December	Shoot tips	40	
December	Shoot tips	25	
December	Shoot tips	29	
December	Shoot tips	27	
			30.3±3.4
December	Stem	0	
December	Stem	0	
December	Stem	0	
December	Stem	0	
December	Stem	0	
			0.0±0.0

6.4. Discussion

6.4.1. Depletion of carbohydrates

Depletion of carbohydrates by defoliation did not occur to the extent expected. Total carbohydrates were similar in both defoliated girdled and control branches, confirming the findings of chapter 2 that there is a degree of internal control of carbohydrate use within apple trees. This also suggests that there were limited sinks available to cause carbohydrate depletion. Removing leaves on ungirdled branches showed that in spite of 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, excised stems and whole plants were kept in the dark for ten weeks. Stems and plants kept in the dark did show a reduction in total carbohydrates compared to their light equivalents. However, even in the dark, starch levels were not depleted completely in either whole plants or stems. The sorbitol concentration was unaffected in whole plants but was reduced in stems kept in the dark. These contradictory data suggest that the apple stem, depending on the stress circumstances, can sometimes draw on the sorbitol pool of carbohydrate.

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 (Bieleski *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 (1962b) 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 ten weeks in darkness, the whole plants used 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 the author is unable to find any more information about this particular experiment, and sorbitol levels were not discussed.

6.4.2. Metabolism of sorbitol

No metabolism of sorbitol occurred when apple stem pieces were bathed in ^{14}C-sorbitol. This is consistent with the findings of Bieleski (1969) who found that ^{14}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 and Quebedeaux (1997) concluded that sorbitol can be converted into other carbohydrates in apple stems. They found that when ^{14}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}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}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 approximately 70% of the ^{14}C-sucrose taken into apple phloem tissues stayed in the form of sucrose and less than 0.5% was recovered as sorbitol. Apple root tips, bark and wood of stems and root tissues have been found to only synthesize a little sorbitol from fructose (Loescher and Everard, 1996). Using the cut stems in solution described above, Wang and Quebedeaux (1997) found that 7% of ^{14}C-glucose was converted to sorbitol in the stem (although this also could have been converted in the leaves and retransported).

6.4.3. SDH activity

SDH was recovered from shoot tips and roots of apple trees and ranged from 25 - 50 nmol. $min^{-1}g^{-1}$. This is consistent with the range that Lo Bianco and Rieger (1998) found for shoot tips of different Prunus species (10 - 311 nmol. $min^{-1}g^{-1}$), but is lower than reported values for root tips (164 - 244 nmol. $min^{-1}g^{-1}$). Lo Bianco et al. (1999) found SDH only in the first 13mm of root tip of 15-day-old peach seedlings and activity decreased from 300 – 25 nmol. $min^{-1}g^{-1}$ in a distance of only 5mm. Because root tissue was taken from more than 13mm of root tip in this study, the results were possibly 'diluted' to some extent by tissue that did not contain SDH.

No SDH was recovered from apple stems at either early or late times of the growing season. This is consistent with the stem not being able to metabolise sorbitol into other products, as the enzyme necessary for cleavage of sorbitol to fructose is not present. This appears to be the first time that attempts have been made to extract SDH from stem 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 and Everard, 1996), is not present in higher amounts in apple stem 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. No SDH was found in stem tissue providing further support that the stem is a low priority sink, as suggested in chapter 3.

From these experiments, it appears that sorbitol has limited metabolism in apple stems. Unfortunately, attempts to extract sorbitol oxidase from stem tissue were not made in this study, leaving the possibility open for sorbitol metabolism via this pathway. However, based on the other evidence presented in this chapter, it is a reasonable assumption that sorbitol metabolism in the stem is extremely limited, if not non-existent. Thus sorbitol may act entirely as a translocation product in the stem, where it is blocked from utilisation by the absence of the appropriate metabolic pathway. Bieleski and Redgwell's (1985) finding that sorbitol acts only as 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).

It has been noted that sorbitol serves a storage role only in certain organs or under certain conditions (Bieleski 1982). The lack of a precise storage role in apple stems helps to explain the results of chapter 3 where sorbitol concentrations were found to be independent of sink demand at certain times of the year. This suggests that sorbitol could play a buffering role in apple stems in maintaining a constant flow of sorbitol to sink tissue, by having a temporary (days to week) storage role in apple stems as has been shown in mature apple leaves (Zhou *et al.*, 2001).

6.5. Conclusion

The evidence presented in this chapter provides support to the hypothesis that sorbitol cannot be metabolised in stem tissue of apple. Sorbitol did not appear to be utilised when carbohydrates were depleted, there was no evidence for metabolism in stem pieces taking sorbitol up from the apoplast and SDH the major enzyme for degradation of sorbitol was not

found. However, more experiments are required to prove unequivocally that sorbitol is not metabolised in apple stems.

In the model proposed in chapter 3, it was suggested that sorbitol is not metabolised in the stem. Given that the greater body of evidence in these experiments suggests that sorbitol is not metabolised, the results can be applied to this model. It is possible that sorbitol, once produced in the leaf, travels through the stem to the terminal sink, without being reduced by metabolism along the way.

This study has provided another link towards an integrated picture of what is happening to sorbitol in the plant as a whole (Fig. 6.3). Therefore, sorbitol is probably not utilised by the stem, but is most likely to be a translocatory substance that can play a temporary storage role.

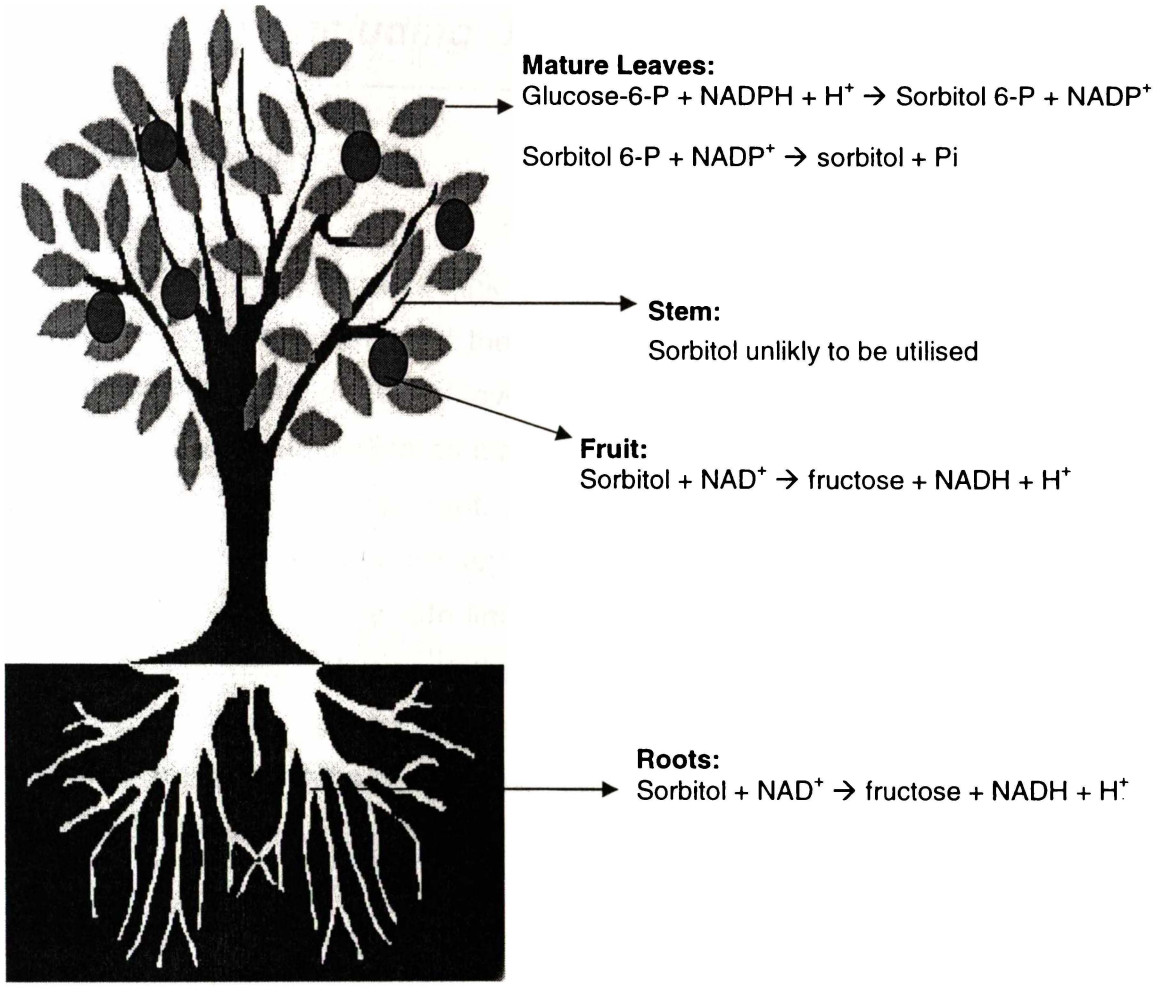

Figure 6.3. Pathways for synthesis and degradation of sorbitol in an apple tree (modified from Loescher and Everard 1996).

Chapter 7: Concluding Discussion

7.1. Introduction

In order to manipulate source-sink relationships in plants to increase crop yields, we need to understand the physiological processes involving the movement of carbohydrates. Movement of carbohydrates in stem tissue has not been as well studied as it has been in source or sink tissue, even though it is extremely important. Loading and unloading of sugars in collection and sink phloem are key processes in transport events and are often considered to be the rate limiting steps to crop production (Patrick, 1997; van Bel, 1993). However the retrieval and release of sugars along the transport phloem is just as essential, as there is a balance between supplying terminal sinks with photosynthate and retention of photosynthate along the pathway (van Bel, 1996).

There is extensive literature on the utilisation of reserve carbohydrates in cereals and grasses (Wardlaw, 1990) where carbohydrates in storage along the stem account for a significant amount of grain growth. There is some understanding of carbohydrate pathways (Hayes *et al.*, 1987; Patrick and Offler, 1996) and of enzyme activities associated with storage and remobilisation in stem tissues of these annual species (Wardlaw and Willenbrink, 1994).

This thesis has tested the theories on carbohydrate accumulation and remobilisation in stem tissue, developed in herbaceous annuals, in woody perennial plants. There is little understanding of what controlls the timing of storage and mobilisation of reserves in perennial species, the movement of carbohydrates in the stem and the buffering of short-term changes in photosynthate supply. This thesis has addressed some of these gaps in our knowledge, using apple as the experimental plant. This

thesis has also shown that it is possible to experimentally manipulate woody plant tissue, despite the difficulties imposed by the woody structure.

Physiological studies on apple trees are sparse, with much of the published literature having an empirical focus. It was noted in chapter 1 that it was important to go to the next stage of carbohydrate studies in apple trees, beyond orchard management and understand the physiological, fundamental processes involved in carbohydrate movement and remobilisation. Apple is a high yielding crop and knowledge of the carbohydrate processes in this plant could help to increase yields in other commercially important tree species.

The study of carbohydrates in apple tree stems had the additional complication of sorbitol as a major transport carbohydrate, about which relatively little is known. In spite of the advantages associated with sorbitol transport, understanding of the transport processes involving sorbitol remains limited. Sorbitol production has the potential to increase crop yields and provide resistance to stresses such as increasing boron mobility (Noiraud *et al.*, 2001b). Boron deficiency symptoms were reduced in transgenic tobacco and rice plants genetically engineered to produce sorbitol (Bellaloui *et al.*, 2003; Noiraud *et al.*, 2001b). Sorbitol production also provides resistance to cold and drought stress through its ability to regulate osmotic potentials (Bieleski, 1982). Understanding the sorbitol transport processes is important so that sorbitol synthesis can be successfully incorporated into other crop species. Knowledge of the transport processes of sorbitol in plants in which it occurs naturally will help to ensure its successful incorporation into non-synthesising plants to improve resistance to stress. For example, tobacco plants expressing high concentrations of sorbitol showed adverse effects (at the most extreme, plants were unable to form roots) (Sheveleva *et al.*, 1998). An increase in plant productivity could well be the outcome of increased understanding of sorbitol metabolism and transport (Loescher and Everard, 1996). To date, few studies into polyol transport have been carried out by a limited number of groups (Noiraud *et al.*, 2001b). Celery

is now a model plant for mannitol transport. Eventually, apple may become a model plant for sorbitol transport in trees.

7.2. Main Findings

The aim of this thesis was to understand the mechanisms of storage and remobilisation of carbohydrates in woody tissue of apple. A number of hypotheses were addressed in this thesis (outlined in chapter 1).

Storage of carbohydrates in herbaceous plants occurs when there is less demand by other sinks. In trees, there is little fundamental understanding of the storage process and there is an interaction between sink strength and season making it hard to distinguish one from the other. The hypothesis that storage of carbohydrate in the stem is determined by alternate sink demand in apple trees was tested in chapter 3 and found to be true. When seasonal cues are removed from the system by altering sink demand at different times of the year, additional starch can be stored in the stem. However, sorbitol, the major carbohydrate, did not respond to changes in alternate sink demand and may act as a temporary storage pool of carbohydrate in the stem.

Sugar transporters have been characterised in herbaceous annuals and exhibit specific uptake behaviours and responses to inhibitors. These transporters may be ubiquitous and this hypothesis was tested in chapter 4. Similarities to known transporters were tested by examining uptake kinetics and responses to inhibitors. In addition, some aspects of the sorbitol transporter(s) operating in the stem tissue were also characterised. Sucrose transporters were found to be similar to those in herbaceous species; sucrose uptake was biphasic, sensitive to PCMBS and up-regulated by carbohydrate demand at terminal sinks. Literature on sorbitol uptake for comparison was sparse. Sorbitol transporters were found to have different uptake kinetics to mannitol transporters (the only comparison available for stem tissue). Sorbitol uptake was linear, but two

uptake systems may be operating depending on sink demand. Sorbitol uptake was sensitive to PCMBS and CCCP.

In herbaceous plants, there is leakage and retrieval of sugars along the length of the transport phloem, which can buffer short-term changes in photosynthate supply. The hypothesis that the transport phloem in apple stems involves leakage and retrieval into apoplastic space was tested in chapter 5 and found to be true. Leakage and retrieval of sugars into the apoplastic space buffers against short-term changes in photosynthate supply in apple stems.

The role of sorbitol as a storage carbohydrate is ambiguous. It is possible that sorbitol is present in the stem purely for use by higher priority sinks such as fruit that have the ability to metabolise it. In chapter 6, it was found that sorbitol is unlikely to be metabolised in apple stem tissue.

7.3. Models of Carbohydrate Storage and Remobilisation in Apple Stems

The findings in this thesis have begun to form a picture of what the carbohydrates in an apple stem are doing at any one time. Models can now be developed, expanding on that proposed in chapter 3 and explaining to some extent, the storage and remobilisation of carbohydrates in one-year-old apple stems. The remobilisation of carbohydrate in spring (Fig. 7.1) and the storage of carbohydrate in autumn (Fig. 7.2) are summarised in schematic diagrams.

In the phloem, assimilates are composed of 65-70% sorbitol and 30-35% sucrose (Klages *et al.*, 2001). When sink demand is high (Fig. 7.1) the majority of assimilates in the translocation stream end are likely to end up at the terminal sink (growing fruit etc.) where they are used in metabolism and growth.

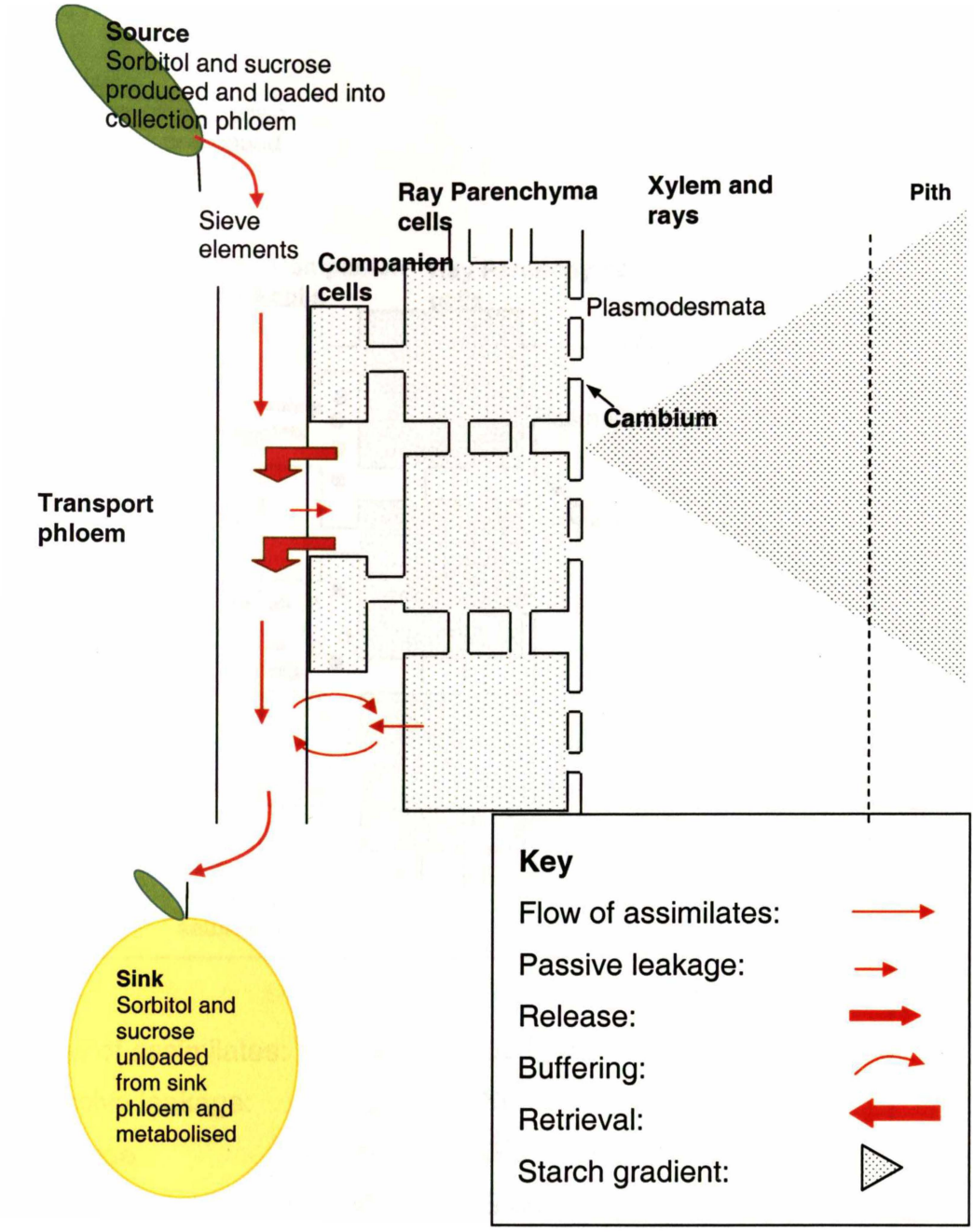

Figure 7.1. Remobilisation. A schematic diagram showing the fate of assimilates travelling through the stem tissue from source to sink in spring. Flow of assimilates is indicated by red arrows. A small amount of assimilates may be lost from the sieve elements through passive leakage, or through active unloading (release), probably into the apoplast. Retrieval of sugars is probably an active process and is likely to occur along the stem length, probably from the apoplast. In the spring, the carriers involved in retrieval are possibly up-regulated to ensure maximum amounts of sugars reach the terminal sinks. Along the length of the stem, buffering occurs to provide a continuous flow of sugar to the sink during short-term changes in photoassimilate supply. Within the stem, sorbitol may not be metabolised. Starch is likely to be remobilised from storage cells (phloem parenchyma cells, ray cells and pith) to provide adequate supplies of carbohydrate to sink tissue before photosynthesis of the new leaves reaches maximum potential.

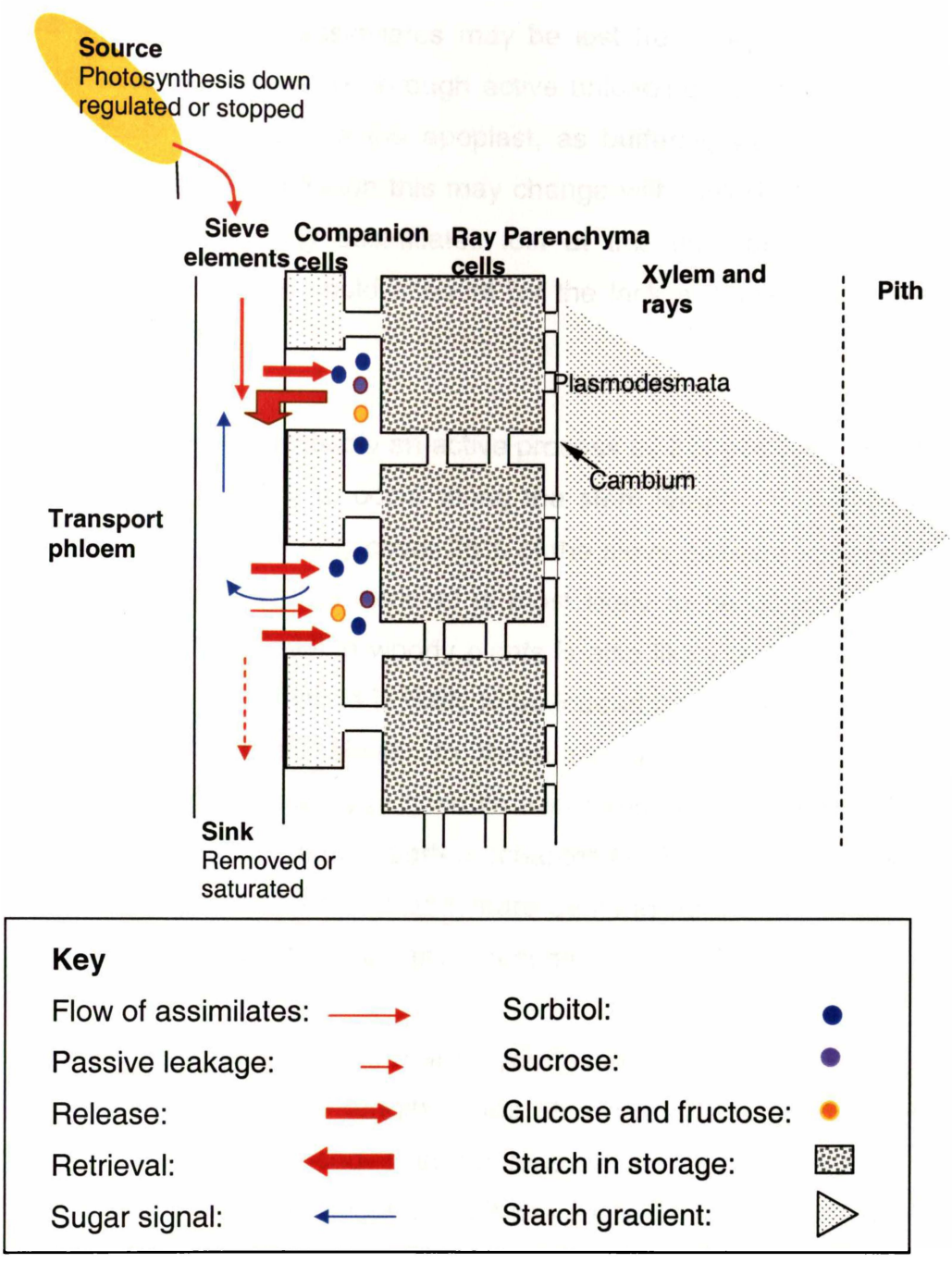

Figure 7.2. Storage. A schematic diagram showing the same piece of stem as in Figure 7.1. but in autumn (or when sinks are prematurely removed). Sink tissue is either removed or saturated by this time. Assimilates may be actively unloaded (released) into the stem apoplast or lost by passive leakage. Little retrieval of these leaked sugars is likely to occur. This could result in a build up of sugars in the stem apoplast causing storage of starch. Starch is likely to be stored in cells closest to the phloem region first, but eventually starch storage may become saturated. The apoplastic concentration of sugars probably rises until a signal is sent back to the leaves to stop photosynthesis. This signal is probably the reduction in concentration gradients. The reduction in concentration gradients probably causes a build up of sugars in the leaves, stopping assimilate flow. This probably results in a reduction or stoppage of photosynthesis, followed by leaf senescence.

However some of the assimilates may be lost from the sieve elements through passive leakage, or through active unloading (release). Release is most likely to occur into the apoplast, as buffering was disrupted by PCMBS (chapter 5), although this may change with sink demand (Patrick and Offler, 1996). Any assimilates lost at this time are likely to be retrieved rapidly, which could account for the lack of sugar found in the apoplast (chapter 4).

Retrieval of sugars is probably an active process as it is sensitive to CCCP (chapter 4) and is likely to occur along the stem length. In herbaceous plants, companion cells only cover 25% of the sieve element surface (van Bel, 2003) suggesting that retrieval is most likely to be directly into the sieve elements. Retrieval in woody plants is also likely to be directly into the sieve elements. Sucrose is likely to be loaded into the sieve elements by sucrose transporters similar to those found in herbaceous plants (chapter 4). Sorbitol is likely to have its own transporters, which could be closely related to sucrose transporters (chapter 4). These transporters are probably up-regulated when carbohydrate demand from terminal sinks is high (chapter 4) suggesting that any lost sugar is rapidly retrieved.

When there is high demand for carbohydrates at terminal sinks, buffering probably occurs along the length of the stem preventing interruption to flow from short-term changes in photoassimilate supply (chapter 5). Buffering may involve the apoplast as it was PCMBS sensitive. From this finding, it can be inferred that retrieval of sugar may be from the apoplast. The buffering capacity in apple stems appears to increase as more sugars become available during the photoperiod (chapter 5).

Within the stem, sorbitol is may not be metabolised due to a lack of SDH (chapter 6). This could allow sorbitol to exist in high concentrations in the stem where it may act as a temporary (days to weeks) storage pool and could play a buffering role, maintaining a constant flow of sorbitol to sink tissue (chapters 3 & 6). Sucrose can be hydrolysed by invertases into glucose and fructose, which can be metabolised by the stem. Because

only a small number of enzymes are able to hydrolyse sucrose, it is used by the majority of plants for phloem transport. Sorbitol appears to be a better translocation substance, as it may not be metabolised at all by tissue along the pathway, leaving only the smaller quantity of sucrose available for metabolism by the stem. Sucrose, glucose and fructose are also present in the stem, but have the ability to be metabolised. It is likely that they are present in the apoplast for a very short time before being scavenged by surrounding cells during stem growth, or returned to the translocation stream. This could be the reason why no apoplastic sugar was found in the stem tissue (chapter 4).

When sink demand is reduced because terminal sinks are saturated or fruit is harvested (autumn) (Fig. 7.2), retrieval of assimilate lost from the sieve elements is likely to be down-regulated. The potential build up of sugars in the apoplast caused by less retrieval, results in storage of starch, probably beginning in the cells closest to the phloem region (chapter 2). This implies that sorbitol can be converted to starch directly or is using another enzyme system in the stem, such as sorbitol oxidase. Eventually, starch storage may saturate when all cells are carrying their maximum capacity of starch (a concentration of around 50mg g^{-1} in Hawkes Bay trees). Once starch storage is saturated, the apoplastic concentration also probably rises until it is saturated. At this point a feedback message may be sent to the leaves to cease photosynthesis. This signal may not be a signal *per se* but simply the removal of pressure gradients once the apoplast is full. This is likely to stop diffusion of sorbitol out of the phloem, resulting in accumulation of sorbitol in the leaves and a reduction in phloem flow. This finally results in a reduction or shut down of photosynthesis. Once photosynthesis ceases, senescence occurs.

Storage in the stem has the lowest priority of all available sinks in terms of accumulating available carbohydrate. Storage of starch appears to occur when there is an excess of photoassimilate, which can occur when there is removal or saturation of higher priority sinks such as fruit (chapter 3).

Carbohydrate reserves in apple stems were never fully reduced in this study and in New Zealand trees there aapears to be some capacity for storage even during rapid fruit growth (chapters 2 & 3). Stem reserves are replenished during autumn, regardless of sink demand earlier in the season (chapter 3).

During winter, starch concentrations declined in this study and starch may either be converted to sorbitol (possibly as a cryoprotectant), or converted to sugars and translocated to other regions of the tree (chapters 2 & 3).

The findings in this thesis have helped to increase our knowledge of basic plant processes in perennial species. Understanding of these basic plant processes, involving the use of carbohydrate reserves, is important in order to begin to manipulate these processes, which could ultimately lead to increased crop yields. This thesis has also increased our knowledge of sorbitol transport processes. This understanding will be of value when sorbitol is expressed in transgenic plants.

7.4. Future Research

While this research has contributed substantially to our knowledge of carbohydrate movement in the apple stem, there are still many areas requiring further study.

Firstly, a paradox can be seen between the findings presented in this thesis showing buffering and retrieval of sucrose and sorbitol along the length of the phloem pathway, and the finding of Klages et al. (2001) that the sucrose to sorbitol ratio in the phloem does not change diurnally. This suggests some sort of control process maintaining the carbohydrate composition of the phloem. This raises interesting questions about how this is achieved, given that sorbitol and sucrose have different uptake carriers. Further study is needed firstly into the phloem composition (there was an extremely short pathway from source to sink in the study by Klages

et al. (2001); does the composition change with length of the pathway?) and secondly into possible control processes.

This research was carried out only on the one-year-old stems and the next logical step would be to see what happens to carbohydrate movement in older parts of the tree and in the roots. It can be hypothesised that the processes found in one-year-old apple stems apply to older tissue and to other sorbitol translocating species. Carbohydrate extractions could be carried out relatively easily on older woody tissue, but would involve sacrificing the tree. How important are the reserves in the one-year-old stems on a whole tree basis? Woody plant root reserves have received very little study (Loescher *et al.*, 1990), how important are these reserves?

Further evidence is required to ascertain the mode of retrieval for sorbitol and sucrose into the apple stem. All techniques used to determine the mode of retrieval have pitfalls that may invalidate results. PCMBS may interfere with water uptake (chapter 4). One way to conclusively demonstrate a mode of retrieval is to get the same results using a number of different techniques. No single experimental protocol yet devised can be used to distinguish unequivocally between phloem loading routes (Turgeon and Beebe, 1991). A first step could be to look for plasmodesmatal connections between cells to see if a symplastic pathway of sorbitol retrieval is feasible. Another logical step would be to carry out similar experiments as described in this thesis on other Rosaceae fruit trees. When sufficient data from several species is available we may be able to solve the controversies over loading pathways (Noiraud *et al.*, 2001b).

Other questions that have arisen during the course of this study include the mode of unloading of sugars; how are sugars unloaded at the sink? If the goal for understanding carbohydrate partitioning in apple trees is to improve crop yield or to transfer the high yields achievable in apples to other species, then the most important aspect that needs to be determined is the unloading pathway. Sink strength probably plays an extremely

important role in carbohydrate partitioning and phloem unloading at the sink is a key factor of this (Patrick, 1997).

More and more evidence is accumulating that sucrose is a signal as well as a substrate (Farrar *et al.*, 2000). Does sorbitol have a signalling role? Can we identify the sorbitol transporter in the stem? Answers to these questions will be valuable in understanding sorbitol expression in transgenic plants.

More research should be carried out into causes of alternate bearing in fruit trees and the reason New Zealand apple trees have such high yields. This research has touched on the potential role of carbohydrates in these processes, but to fully understand the interactions between carbohydrates and hormones, the carbohydrate flux for a whole tree is needed.

Some parasitic plants synthesize mannitol from host carbohydrates, presumably as a method to prevent the host recovering the carbohydrates (Williamson *et al.*, 2002). Because the host plants do not have the enzyme system required for the synthesis of mannitol, this presents a potential target for control. Do some parasitic plants utilise sorbitol in this way? Is there potential application in control of unwanted pest species? Application requires understanding of sorbitol transport.

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Appendix 1

CARBOHYDRATE ALLOCATION IN APPLE STEMS CAN BE ALTERED BY FRUIT LOAD

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Keywords: *Malus domestica*, starch, storage, reserves, girdling, one-year-old limbs

ABSTRACT

Carbohydrate allocation is a key process in deciduous fruit trees. In this study, our aim was to determine whether reserve accumulation in one-year-old apple stems is controlled by carbohydrate availability. We altered the carbohydrate balance of one-year-old stems during fruit development by altering the fruit load on girdled branches. One-year-old branches of 'Braeburn' with fruit were girdled early in the season and the fruit load adjusted to high (6 fruit), low (2 fruit) and zero (no fruit). This fruit load was subsequently reduced at different times throughout the season. Starch concentrations in one-year-old wood were found to be inversely correlated to fruit load; limbs with zero fruit load had the highest concentrations of starch. By winter, however, all girdled branches had the same concentrations of starch, regardless of fruit loads throughout the season. This implies that there is a maximum capacity for starch storage in stems and that all branches can reach this maximum, regardless of their carbohydrate status earlier in the season. Reducing fruit loads throughout the season caused starch concentrations in one-year-old wood to increase rapidly. The results show that starch can be forced into storage out of season, implying that its accumulation is dependent on carbohydrate availability. Storage is a low priority sink, and starch is only stored when other higher priority sinks are removed or become saturated.

INTRODUCTION

Carbohydrate storage within the perennial tissue of deciduous fruit trees is important in apple trees where new leaf and early fruit development depend on carbohydrates remobilized from roots and stems (Hansen, 1971). An adequate supply of carbohydrate early in the season from reserves is critical for both fruit yield and quality. Thus an understanding of carbohydrate allocation processes is important to ensure appropriate orchard management practices are applied.

In this study, we investigated whether reserve accumulation in one-year-old apple stems (i.e. last season's extension shoots) is controlled by carbohydrate availability. To alter carbohydrate accumulation into one-year-old limbs, limbs were girdled to maintain an isolated system and fruit load adjusted. During the

season, fruit load was reduced to see if reserve accumulation could be altered by sudden changes in fruit load.

Ungirdled limbs were used to follow non-structural carbohydrates (starch, sorbitol, sucrose and glucose + fructose) in one-year-old limbs. While similar data has been collected previously (Hansen and Grauslund, 1973; Kandiah, 1979; Murneek, 1933; Priestley, 1962, 1964), no studies of this kind have examined in detail the full range of non-structural carbohydrates present in apple tissue. Due to space limitations, in this paper only starch results will be reported.

MATERIALS AND METHODS

Carbohydrate concentrations in one-year-old limbs of 8-year-old 'Braeburn' apple trees (*Malus domestica*), grown on various rootstocks, were examined at the HortResearch Havelock North Research Centre, New Zealand, from December 2000 to July 2001 with sampling being carried out six weekly. Full bloom occurred on the 6th of September 2000. Trees were planted at a spacing of 5 x 3.5m in north-south oriented rows. Originally part of an interstem trial, the trees were planted in a randomised design of four replicates per interstem type. Three (MM.106, M.793, M.793 with 10cm M.9 as an interstem) of the original seven interstem treatments were used in this experiment. Twelve trees were selected based on there being four trees with the same interstem with similar form. Of these, two trees were randomly selected for girdling, while two trees were the ungirdled controls. 'Braeburn' was a convenient cultivar to use for this trial as it can flower and set well on one-year-old wood.

Dormant pruning, fertilisers, pesticides and herbicides were applied as required according to standard commercial practices. No hand-thinning of fruit was carried out resulting in fruit loads above commercial levels.

In December 2000, 34 one-year-old limbs carrying at least six fruit and of similar form, were girdled per tree. A 2cm wide strip of phloem, cambial tissue and connected bark was removed. An aluminium splint was taped to the girdled area for support and the girdle left uncovered. Twenty limbs per tree were thinned to a high fruit load (6 fruit), eight limbs to a low fruit load (2 fruit), and six limbs to a zero load (no fruit). These initial fruit loads were subsequently reduced to low or zero in January and/or April (commercial harvest time). Unless stated, fruit were not removed from the tree, but left to abscise naturally.

Girdles were examined at each sampling time to ensure phloem discontinuity was retained. The entire stem was removed at the girdle and the one-year-old wood was cut into small pieces and frozen in liquid nitrogen immediately. After freeze-drying, samples were ground into a fine powder and 100mg dry weight of sample was then analysed for glucose + fructose, sucrose and sorbitol as described by Jones et al. (1977). Starch was measured as described by Jones (1979), extraction optimised by heating with amyloglucosidase for 60 min at 55^{0}C.

Photosynthesis was measured indirectly using a Delta T AP4 transit-time porometer (United Kingdom) to assess stomatal opening. Porometer measurements were made in December, January and February, starting at 10:00am and finishing by midday.

Differences in limbs with different fruit loads were tested by statistical analysis carried out by a linear mixed model fitted using GenStat (© 2002, Lawes Agricultural Trust, Rothamsted Experimental Station). A linear mixed model was necessary to because of missing data and to include ungirdled limbs (which did

not have the same treatments) in the same analysis as girdled limbs. Limbs that had broken and died, or lost significant amounts of fruit prematurely, were excluded from analysis.

RESULTS

Different effects between trees due to different interstems and rootstocks were allowed for by the statistical analysis, but there was no evidence for any such effects. Variation within limbs of the same tree was greater than between tree variations.

Seasonal changes in starch concentration were determined in one-year-old wood of ungirdled limbs (fruit load not manipulated) (Fig. 1). Starch concentration in the ungirdled stems rose steadily from December, reaching a peak in May (leaf fall), then dropped sharply in July (midwinter).

Starch concentration of one-year-old wood responded to fruit load. Starch concentrations were similar in all limbs when girdling was carried out in December (Fig. 1). From January to April, limbs with zero fruit load had the highest starch concentrations while limbs with high fruit load had the lowest. Limbs with low fruit load had intermediate concentrations of starch. During this period, starch concentrations increased four-fold. An increase in vegetative growth was seen in the low and zero limbs *c.f.* limbs with high fruit load (data not shown). Over the next six week period, limbs with low and zero fruit loads did not increase in starch concentration, while limbs with high fruit loads showed a considerable increase so that by May, starch concentrations in the stem had reached a peak and all limbs had similar starch concentrations, regardless of the initial fruit load. The concentration of starch in all limbs then decreased rapidly in July (mid-winter) to a level only slightly above that measured in December.

Reducing the fruit load from high to zero in January caused an immediate increase of 400% in the concentration of starch measured in the stem (Fig. 2) with a further 130% increase by May. Starch concentrations then declined in July to become the same as all other branches. Reducing the fruit load from high to low in January caused a similar response (Fig. 2). Removing the fruit in April (normal harvest time) always caused a slight increase in the concentration of starch found in the stem (data not shown), however with all fruit loads, stem starch concentrations consistently fell to similar levels by July.

The leaves on limbs with a zero fruit load looked very different to those under a high fruit load or those that were not girdled. Leaves were thick, xeromorphic, chlorotic, had red veination and senescenced months before the rest of the tree. Starch levels in these leaves, determined by the brittleness of the leaves, was also very high. Stomatal conductance measurements were made as an estimate of photosynthetic capacity and were found to be very low on limbs with a zero fruit load, intermediate in limbs with a low fruit load and high in limbs with high fruit loads. High fruit load and ungirdled limbs retained healthy green leaves until normal senescence (late May). Limbs with a low fruit load showed an intermediate set of characteristics to the high and zero fruit load limbs.

DISCUSSION

This work demonstrates that by altering fruit loads at any time during the growing season non-structural carbohydrates accumulate as starch in the one-year-old wood, within six weeks. This implies that accumulation is dependent on carbohydrate availability. It also suggests that even for high fruit load and

ungirdled branches there is surplus carbohydrate available during the major phase of fruit growth in February and March to divert into storage. Where there was an excess of carbohydrates, such as in the limbs with zero fruit load, starch levels in the stem quickly reached a high level. This was likely to be a maximum capacity, as instead of storing more starch, the leaves on these limbs stopped photosynthesising and senescenced much earlier than leaves on the rest of the tree. Differences in vegetative growth of girdled branches induced by differences in fruit load were indicative of a compensatory response of these limbs, with lower fruit numbers resulting in increased shoot mass and spur growth (data not presented). When these data are presented as absolute amounts per shoot, the same differences are seen between treatments, however the errors associated with variations between limbs increases.

Schechter et al. (1994) also found an increase in wood dry weight accumulation in non-fruiting limbs – girdling caused a larger weight accumulation than non-girdled, non-fruiting branches. This indicates that limbs with a low or zero crop load could have apportioned larger amounts of photosynthate into these alternative vegetative sinks before beginning to store the extra carbohydrate.

A maximum of starch was reached in May (Fig. 1), regardless of fruit loads. Limbs with zero and low fruit loads did not end up with greater starch concentrations in their limbs, but reached a high concentration earlier than stems with high fruit loads. The maximum starch concentration reached in all fruit loads implies that in one-year-old limbs there is a maximum capacity for starch storage that cannot be exceeded by making more carbohydrate available (*c.f.* Fig. 2 low fruit load data). Limbs with a high fruit load reached a starch maximum capacity in May, demonstrating that in New Zealand, there is ample time after fruit ripening for storage to be fulfilled in one-year-old wood. It would be interesting, however, to carry out further investigations in trees in a different environment, which do not have such a long period of photosynthesis after fruit harvest.

Starch concentrations declined in July to concentrations similar to those found in December (Fig. 1) when the girdles were first applied. This means that there is a limited amount of starch available in the one-year-old wood for remobilization in the spring and this amount is the same, regardless of previous fruit load. This implies that starch storage in one-year-old wood is not that important for new leaf and bud development in spring.

Other studies have also questioned the importance of reserves for spring growth in apples; Tromp (1983) concluded that although apple roots supply above-ground parts with carbohydrates in early spring, it was doubtful whether such contributions were significant. Hansen and Grauslund (1973), Hansen (1967) and Hansen (1971) found that reserves act as a building material for new growth only to a slight extent. In ungirdled systems, carbohydrates could have moved from the one-year-old wood into older parts of the tree. This could make sense in terms of adaptation to the environment throughout evolution, as one-year-old wood could be the most vulnerable to loss during winter. Data on the soluble carbohydrates (not presented here) should help to complete this picture. Girdled limbs, where carbohydrates were unable to move out of the one-year-old wood showed similar trends to the ungirdled limbs, thus it is likely that starch was converted to sorbitol, possibly as a way of preventing freezing (Ichiki and Yamaya, 1982).

In annual species, storage is the lowest priority sink (Wardlaw, 1990). In wheat and barley, carbohydrate storage in the stem is not competitive with grain

filling, but storage does occur when there are excess photosynthates caused by less demand from other sinks (Schnyder, 1993). In this study, it was shown that a reduction in fruit load, which was a reduction in sink demand, resulted in a five-fold increase in starch concentration in the one-year-old wood within six weeks (Fig. 2). This demonstrates that in apple, a woody perennial, similar storage mechanisms as herbaceous species apply. Storage in apple stems is determined by carbohydrate supply and only occurs when there are excess carbohydrates available at any time of the season. Environmental signals are not necessary to initiate storage of carbohydrates in apple stems; rather, storage is initiated by sink demand. This is consistent with Battey (2000), who states that Rosaceous fruit trees that go dormant in the winter do not do so in response to environmental cues. Carbohydrate partitioning into reserves is controlled by carbohydrate availability in apple trees.

The observation that storage in the stem is controlled by carbohydrate availability is consistent with the concept that storage is a low priority sink, as stem starch concentrations increased when high priority sinks were removed. There was also a lack of starch in stems with high fruit loads, intermediate concentrations in stems with low fruit loads and high concentrations of starch in stems with zero fruit loads. Growth of fruits, leaves and shoots is likely to have occurred before stem storage. This is consistent with Wardlaw's (1990) order of sink priority: growth of seeds > growth of fleshy fruit parts = growth of shoot apices and leaves > growth of cambium > growth of roots > storage. This implies that starch is stored in the stem of apple trees only when competing sinks, such as fruit, are removed or their demand is saturated. In this study, storage has been shown to occur when these other sinks are removed or unavailable (through girdling), regardless of the time of year.

CONCLUSIONS

There is a maximum concentration of starch that can be stored in one-year-old apple wood, which is reached in May in New Zealand. There is ample time for this maximum to be reached following harvest and before leaf fall, regardless of the carbohydrate demand earlier in the season due to fruit load.

The findings suggest that starch storage in one-year-old wood was not very important for the growth and development of new buds and leaves the next season, due to similar concentrations of starch being found in winter as were seen the previous summer.

Carbohydrate partitioning in apple trees is controlled by carbohydrate availability and not environmental cues, as was seen by the accumulation of starch within six weeks of fruit removal, regardless of the time of the year.

Storage in one-year-old wood is a low priority sink as starch was only laid down once other competing sinks such as fruit were removed or saturated.

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Figures

Figure 1. Starch concentration of one-year-old wood under different fruit loads from trees grown in the Hawkes Bay, New Zealand ± SE (n = 3-6). DAFB = days after full bloom. Harvest and leaf fall refer to normal timings in commercial orchard trees. Harvest and leaf fall varied amongst the girdled limbs.

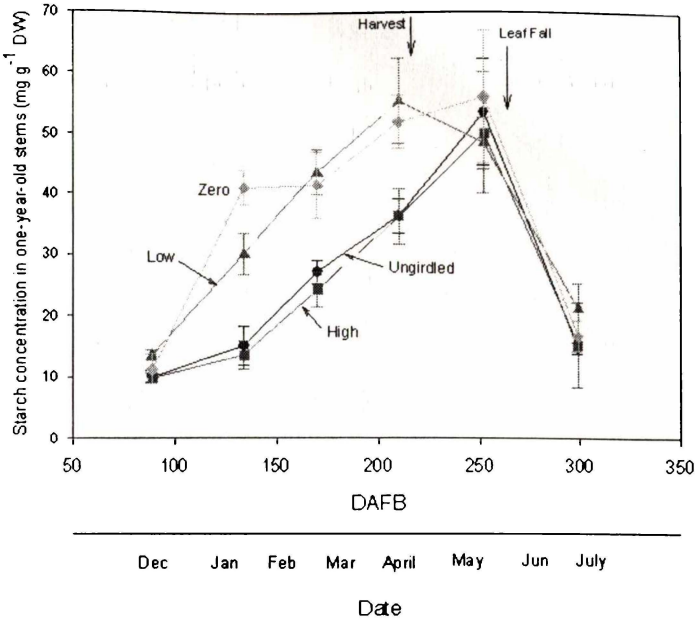

Figure 2. Starch concentrations of one-year-old wood when fruit load has been reduced from high (6 fruit) in January to zero or low (2 fruit) ± SE (n = 3-6).