Vascular functioning and development of the kiwifruit berry (*Actinidia deliciosa*)

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Abstract

The aim of this study was to understand kiwifruit berry development and the role of cell turgor and the phloem unloading pathway in development. Important aspects of berry development include the size of the fruit and its composition. The fresh weight growth curve of the kiwifruit berry was shown to be double sigmoid in shape. Dry weight accumulated linearly for the initial 139 days after anthesis (DAA). At this time the soluble solids concentration began to increase. Berry firmness was measured using two methods, with the penetrometer and with a new non-destructive method, utilising skinfold callipers. Both methods exhibited similar results, indicating that the skin callipers may be useful in the future for non-destructive berry rheological measurements.

Cell turgor was measured indirectly from measurements of symplasmic and apoplasmic solute potentials, and the matric potential of the berry. Apoplasmic sap, required to measure the apoplasmic solute potential, was extracted using two different methods the pressure chamber and through centrifugation. Measurements of sap osmotic potential suggest that the sap extracted using the centrifuge was contaminated with symplasmic sap, resulting in a negative cell turgor estimate. However, the pressure chamber technique provided apoplasmic sap that produced a more accurate estimate of cell turgor. Direct estimates of cell turgor were only obtained from the midpoint of the growing season because of contamination with symplasmic sap, but the values obtained were comparable to literature values for developing grape and tomato berries.

The phloem unloading pathway in the fruit was investigated using a symplasmic tracer dye, carboxyfluorescein diacetate coupled with ^{14}C labelling and autoradiography. The phloem unloading pathway was symplasmic until 91 DAA when the dye was restricted to the phloem cells only, indicating a change to an apoplasmic pathway. However, due to the lack of functional unloading seen in radiolabelled samples, a change in the phloem unloading pathway could not be confirmed.

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The last two years have been an enduring yet enjoyable adventure.

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Contents

List of Figures

List of Abbreviations and Symbols

CF = carboxyfluorescein

CFDA = carboxyfluorescein diacetate

D = deformation during compression

D1 = narrowest diameter of berry equator

D2 = widest diameter of berry equator

DAA = days after anthesis

Df = berry diameter after compression

Di = berry diameter before compression

DM = dry matter

DW% = dry weight percentage of fresh weight

DWtotal = total dry weight

E = elasticity

F = external force applied

FW = fresh weight

L = length of berry

P = cell turgor

v = Poissons ratio (assumed = 0.5)

Wa = weight after drying

Wb = weight before drying

Chapter 1 – INTRODUCTION

1.1 Overview

Fruit development involves a complex system of physical and biochemical processes. Watada (1984) describes fruit development as the series of processes from the initiation of growth to death. These processes include; growth, maturation and ripening, with each stage representing changes in fruit characteristics. From a horticultural perspective, fruit development represents the pathway that determines the quality and composition of the end product. The horticultural industry has therefore identified the importance of fruit development, as producing high quality fruit returns the greatest profits. The patterns of development of economically important fruit such as apples (Bain & Richardson, 1951), tomatoes (Monselise et al., 1978) and grapes (Wada et al., 2008) have been extensively researched. For the same reason, it is vital that the development of the kiwifruit berry is further investigated.

Kiwifruit stands as one of New Zealand's most prolific horticultural export products. In 2010, kiwifruit accounted for nearly 70% of all fresh fruit exports, providing $995.7 million for the New Zealand economy (Aitken et al., 2010). With strong global competition from other fresh fruit producers it is vital that the New Zealand kiwifruit industry continues its research into this important crop.

The New Zealand kiwifruit industry is focused towards producing fruit that maximise satisfaction for the consumer as these fruit command a premium price on the overseas market. Traditionally, it has been thought that the physical characteristics of the fruit such as size and colour as well as fruit price have been the decisive factors that attract customer purchases. However, it is now accepted that other factors such as taste and internal quality are more important when it comes to repeat customer purchases (McMath et al., 1992; Richardson et al., 1997). Consumers prefer kiwifruit that have a high soluble solids concentration (Jaeger et al., 2003; Harker et al., 2009). Currently, growers use dry matter content (DM), defined as the fruit dry weight expressed as a percentage of the fresh weight, as an indicator of eating quality (Minchin et al., 2010). It has been shown that the DM content of fruit at harvest is a good predictor of the soluble solids concentration of the ripe fruit at the point of consumption (Jordan et al., 2000; Harker et al., 2008). Thus the challenge is to produce fruit with maximum dry matter.

In kiwifruit, dry matter accumulates primarily in the form of the storage carbohydrate, starch. This increases in the fruit until maturity when it is metabolised into soluble sugars. However, at present we are unable to identify the physiological processes that limit the accumulation of carbohydrates by the fruit (Morandi et al., 2010). To do this we must first understand the physiological components of fruit development. Other fruits, such as grapes have been examined in detail (Coombe & McCarthy, 2000; Keller et al., 2006, Matthews et al., 2009). The continually growing wine industry has driven the constant need for further research into grape vascular functioning and development, and there is similar interest in other economically important fruits such as tomato (Ruan &

Patrick, 1995) and apple (Zhang et al., 2004). Unfortunately, kiwifruit has received less attention in this field of study.

Understanding carbohydrate accumulation in the fruit requires prior knowledge of the delivery system, the phloem. The phloem functions to translocate photoassimilates from the autotrophic to the heterotrophic parts of the plant (Schulz, 1998). Photoassimilates are loaded into the phloem at the source (or collection) phloem and transported to the sink (or release) phloem (van Bel, 2003). According to the concept of Ernst Münch (Münch, 1930), the phloem system makes use of a turgor gradient along the sieve tubes as the driving force for mass flow. High turgor values resulting from photo-assimilate build up in the collection phloem at the source ends propel the sieve tube sap towards sites of low turgor values caused by the escape of photo-assimilates and the corresponding loss of water from release phloem at the sink ends. This source to sink pressure driven system provides an efficient way of transporting photosynthates long distances.

Phloem loading is the initial step required to transport photosynthates (generally sucrose) from source to sink. This involves transport of assimilates from their cellular sites of acquisition/storage to the lumens of se/cc complexes (Oparka & van Bel, 1992). This can occur via a symplasmic path through plasmodesmata or an apoplasmic pathway with the assistance of specific transmembrane-transporters (Lalonde et al., 2003). The final step, is the unloading of assimilates from the phloem into the recipient sink cells. This has been recognized as an important step in understanding carbohydrate accumulation by the fruit.

1.2 Berry Development

Changes in kiwifruit berry size and composition during development have been the interest of multiple studies (Richardson et al., 1997, Minchin et al., 2010). Fruit growth consists of an increase in size, changes in shape, and mobilization of reserves from other parts of the plant (Coombe, 1976). However, the extent of fruit growth from anthesis to maturity is extremely variable. In some species, fruit enlarge relatively little while in others they may increase in volume many times (Crane, 1964). For example, one of the largest is shown by the avocado which increases in volume 300,000 times from anthesis to maturity (Coombe, 1976). The interval from anthesis to ripeness also varies considerably between species, from 3 weeks (strawberry) to 60 weeks (Valencia orange) (Coombe, 1976) for example.

Fruit growth is associated with an increase in volume which is the result of cell division or enlargement, however most commonly both. Generally cell division pre-dominates in the early stages but overlaps the cell enlargement phase which continues until maturation (Crane, 1964). For example, in apple, rapid cell division occurs following pollination in all parts of the ovary and associated tissues for approximately three weeks, following which few or no further cell divisions occur. Subsequent increase in size results entirely from cell enlargement (Bain & Robertson, 1951).

The developmental pattern of fruit growth varies greatly between species. A single sigmoid growth curve has been observed in apple, pear, date, pineapple, banana, avocado, strawberry, orange and melon (Coombe, 1976). This pattern is characterized by rapid growth early in development followed by a lesser rate of growth later (Fig. 2). Other species exhibit a double sigmoid growth curve, e.g. stone fruits, fig, blackcurrant, raspberry, blueberry, grape and olive (Coombe, 1976) (Fig. 1). In the grape, the double sigmoid growth curve can be split into three stages (Wada et al., 2008). Stage I represents rapid growth early in development. Stage II is a period of slower growth, followed by Stage III that represents another period of faster growth before maturity. The transition from Stage II to Stage III is termed ‘veraison’ and is when the onset of ripening occurs.

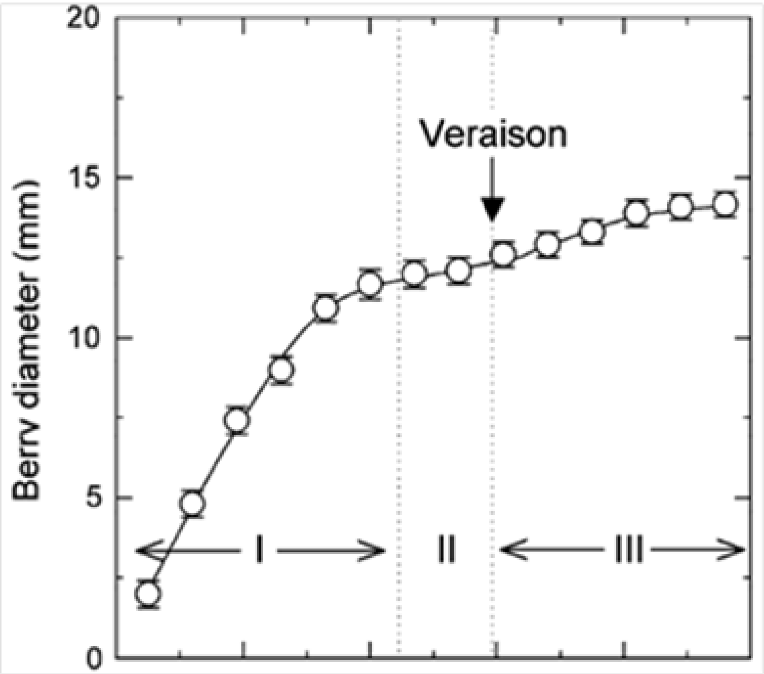


Figure 1: Double sigmoid growth curve of the grape berry. Growth is split into three stages, with veraison occurring between Stages II and III. (Wada et al., 2008).

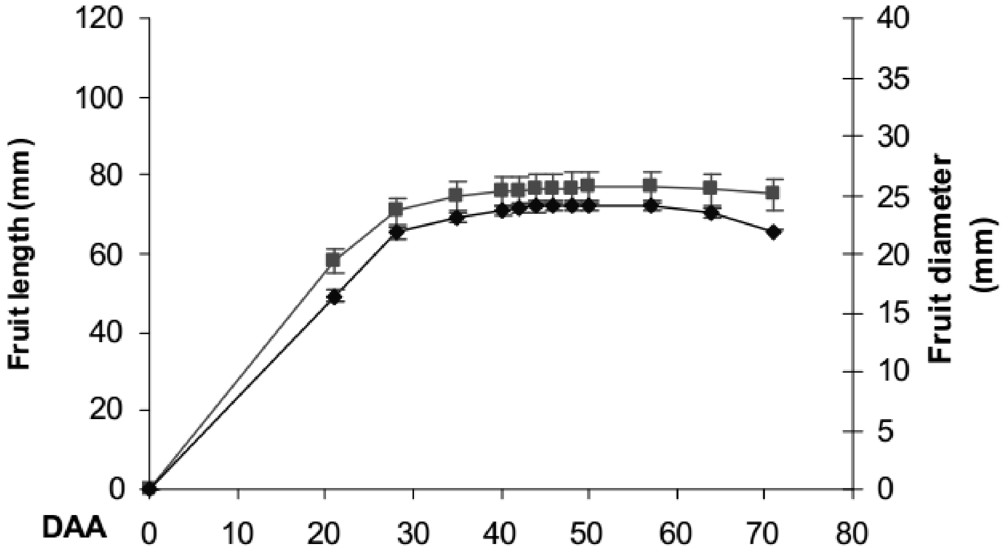


Figure 2: Single sigmoid growth curve of the capsicum. Increasing growth during the early stages of development before decelerating as the fruit reaches maturity. Grey squares represent fruit length, black diamonds represent fruit diameter (Thang, 2007).

1.3 Berry Growth

Kiwifruit growth curves have been described as double sigmoid (Hopping, 1976). However, this has been the subject of debate. Walton & De Jong (1990) argued that in fact kiwifruit has a single sigmoid growth curve and any variation from this is due to cultural conditions and/or sampling error. Pratt & Reid (1974) observed a triple sigmoid pattern. However this may be due to external factors such as temperature (Morgan et al., 1984), soil moisture (Judd et al., 1986), rainfall (Sale, 1981) hail, wind or frost (Hopping, 1986) affecting he standard curve.

Hopping (1976) divided the kiwifruit growth curve into 3 distinct phases (Fig 3):

- Stage I (0-58 days after flowering): A period of rapid increase in both weight and volume of the berry. This is due to cell division and then cell enlargement.

- Stage II (58-76 days after flowering): This period is characterized by a reduction in growth and weight gain due to a reduction in cell enlargement in the inner pericarp and central core.

- Stage III (76-160 days after flowering): A second period of growth and weight gain due to faster cell enlargement in the inner pericarp and core.

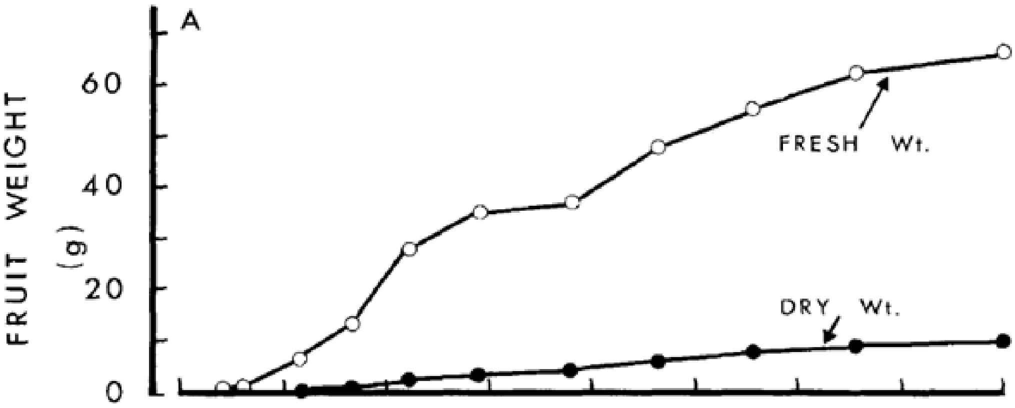


Figure 3: Fresh weight and dry weight curve of the developing kiwifruit berry. Fresh weight curve shows two stages of rapid growth separated by a period of slower growth (Hopping, 1976).

Hopping (1976) describes kiwifruit development as a series of acceleration and decelerations of growth; however Richardson (2004) divided the berry development according to processes occurring within the berry (Fig. 4). The first phase of development is a period of rapid cell division; this corresponds with the rapid growth phase shown in Fig. 3. The next phase includes the majority of fruit development and is characterized by starch accumulation. The final phase is a period of berry maturity. However, the three stages identified by Richardson (2004) on the basis of internal changes, do not correspond with the three stages identified by Hopping (1976) on the basis of growth in fresh weight.

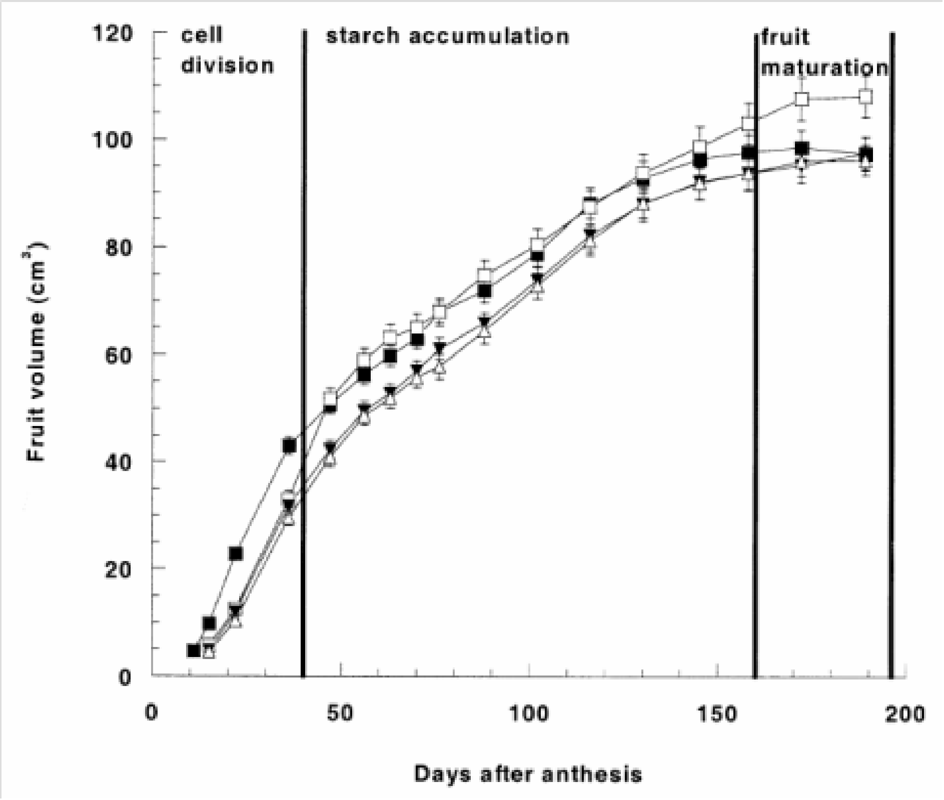


Figure 4: Berry growth curve showing the change in fruit volume over time. The growth curve is divided into three stages, each defined by changes occurring within the fruit. Lines represent different heat treatments applied to the fruit (Richardson 2004).

1.4 Berry Quality

Fruit quality traits are strongly correlated with fruit composition. This is highlighted in recent studies examining the affects different berry attributes have on customer purchase intentions (Harker et al., 2009; Jaeger et al., 2011). These studies observed the relative importance consumers place on a variety of kiwifruit attributes including size, dry matter and price. The conclusion was that the dry matter content of the fruit was the most important factor influencing customer purchases. This result highlights the importance that fruit composition, in particular carbohydrate content has on fruit quality.

Dry weight can be defined as the weight of desiccated (dried) tissue. It is a measurement that avoids variation in water content when fresh weight measurements are made (Taiz & Zeiger, 2006). Dry matter embraces both the soluble (largely sugars) and insoluble (mainly the structural carbohydrates and starch) carbohydrate pools in fruit (Scott et al., 1986; Beever & Hopkirk, 1990; Hopkirk, 1991). Dry matter can therefore be taken as an indicator of the total fruit carbohydrate content, of which, in kiwifruit, at harvest, 40-70% may be starch. Starch builds up in kiwifruit rapidly from 50 to 120 days, generally reaching its maximum around the end of this period (Fig. 5). However during fruit ripening, starch is broken down into soluble sugars (Fig. 6) and it is these sugars that directly affect fruit flavour (Richardson et al., 1997).

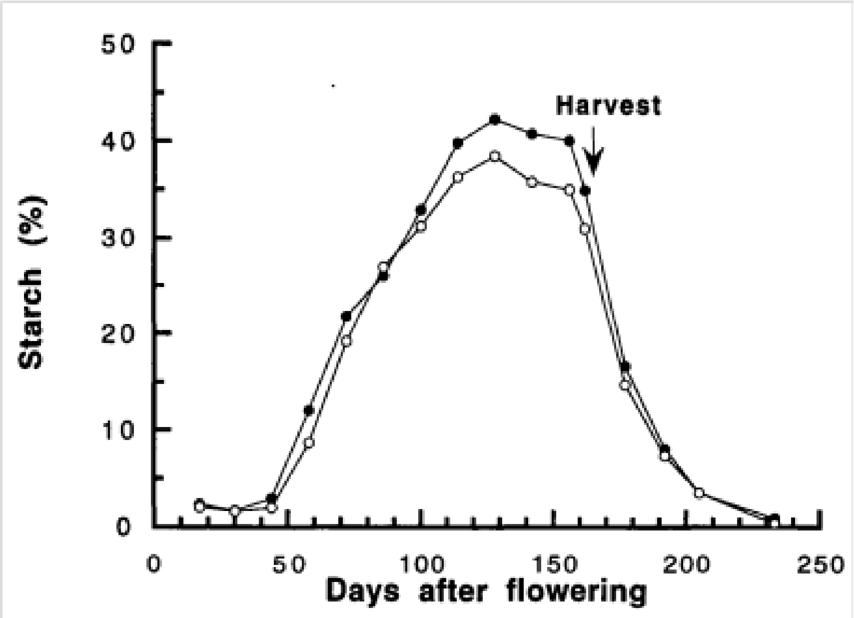


Figure 5: Changes in starch content of the kiwifruit berry during development. Starch reaches its maximum around 125 DAA before decreasing rapidly at 150 DAA. Black and white circles represent different crop loading treatments (Richardson 1997).

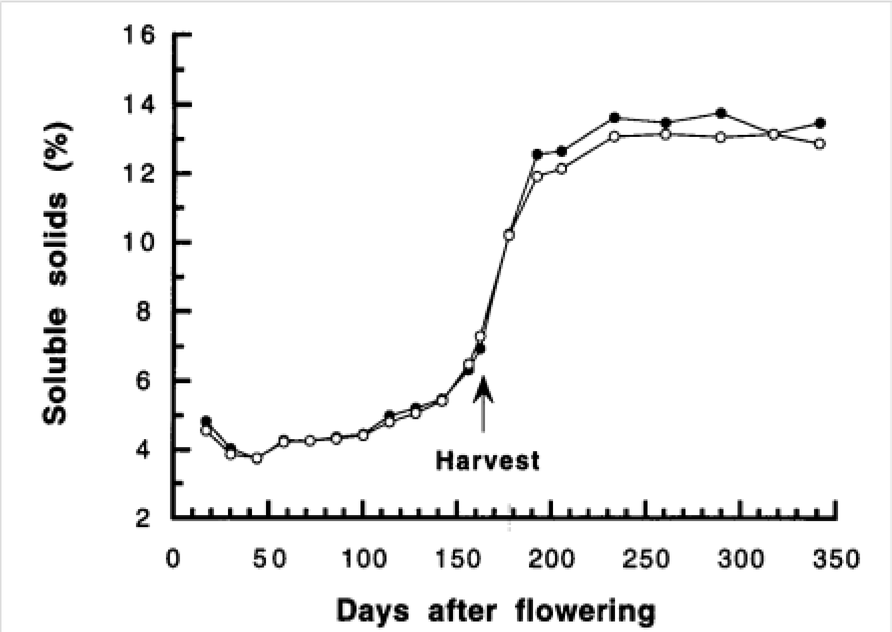


Figure 6: Changes in the soluble solids concentration during kiwifruit development. The concentration rises rapidly at 150 DAA, coinciding with the

decrease in starch (Fig. 5). Black and white circles represent different crop loading treatments (Richardson et al., 1997).

1.5 Phloem Unloading

Fruit gain their high flavour through the build up of sugars that travel in the phloem from source areas, such as the leaves, to the fruit, where they are unloaded into storage parenchyma cells and stored as starch. Phloem unloading is the movement of assimilate from sieve element-companion cell (se-cc) complexes to sites of utilization/storage in the recipient sink cells (Oparka, 1990; Patrick, 1997). This includes transfer across the se-cc complex boundary (SE unloading) and subsequent transport through a diverse range of sink parenchyma cells (post phloem transport) (Oparka, 1990; Patrick, 1997).

Over the last 25 years, the phloem unloading pathway of a range of different sink tissues have been studied, including vegetative apices (Oparka et al., 1994; Imlau et al., 1999), sink leaves (Roberts et al., 1997 ; Imlau et al., 1999 ; Haupt et al., 2001) and potato tubers that represent a typical terminal vegetative storage sink (Viola et al., 2001). There has also been an interest in phloem unloading in reproductive storage sinks such as developing fruit (Ruan & Patrick 1995; Pomper & Breen, 1995) and the maternal tissues of developing seeds (Wang et al., 1995; Wright & Oparka, 1997;). The unloading pathway operating in the kiwifruit berry is currently unknown.

Based on studies of sink organs in other species, there are two potential pathways for the unloading of sugars from the phloem into the surrounding storage cells of the fruit (Patrick, 1997) (Fig. 7). These are the symplasmic pathway and the apoplasmic pathway, however there may also be a combination of both pathways acting in series. The symplasmic pathway involves a connection from the sieve element/companion cell complex of the phloem to the surrounding parenchyma cells. The apoplasmic unloading pathway requires the direct transfer of assimilates across the sieve element/companion cell complex plasmalemma into the apoplasmic space between cells, followed by trans-membrane uptake into the storage parenchyma cells (Oparka, 1990).

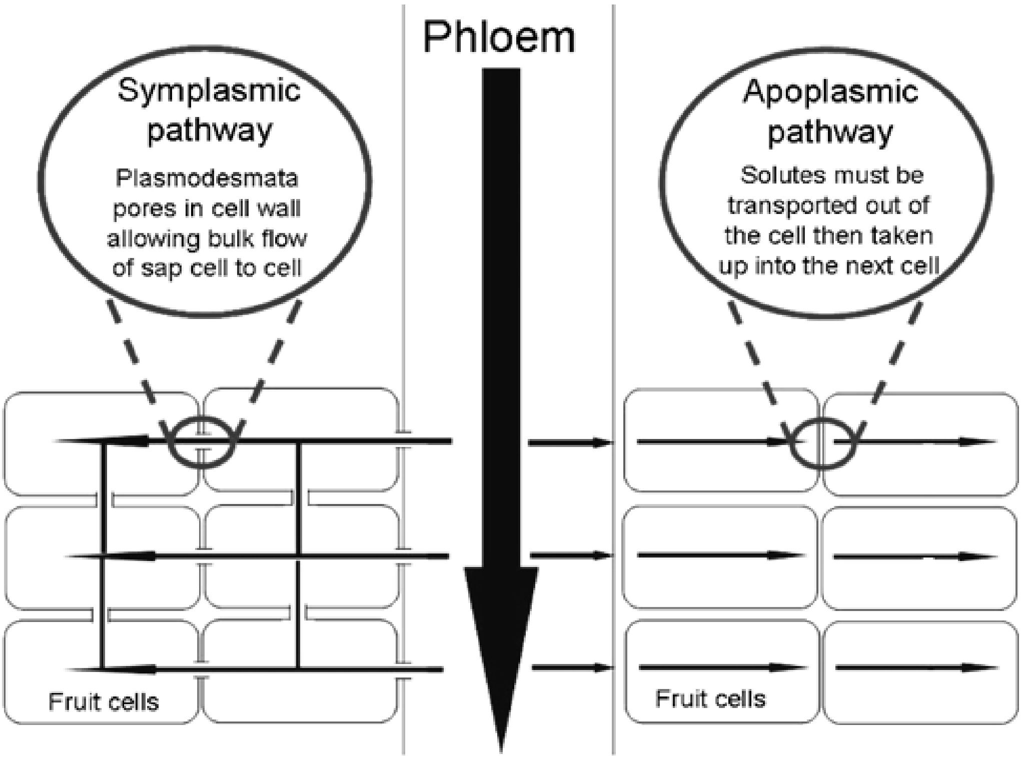


Figure 7: Potential phloem unloading pathways. The symplasmic pathway utilises the presence of plasmodesmata, but the apoplasmic pathway requires solutes to be transported out of the cell, into the apoplast before crossing the membrane into the next cell.

Recent studies have shown that a change in the phloem unloading pathway may occur during the development of some sinks (Ruan & Patrick, 1995; Roberts et al., 1997; Viola et al., 2001; Zhang et al., 2006). For example, Roberts et al., (1997) revealed that the symplasmic phloem unloading pathway may be blocked during sink-source transition in tobacco (*Nicotiana tabacum*) leaves. Viola et al., (2001) found that tuberization of potato (*Solanum tuberosum*) stolons is associated with a switch from apoplasmic to symplasmic phloem unloading pathway. The modification of the phloem unloading pathway may result from changes in the numbers or conductivity of plasmodesmata, a phenomenon that is of special importance to sink development and function (Patrick, 1997; Oparka & Turgeon, 1999; van Bel, 2003).

The pathway that predominates has important implications for the identity of the enzymes most likely to influence the rate of carbohydrate accumulation. In apoplasmic unloading, cell wall acid invertases usually cleave the sucrose within the apoplast and hexose transporters then transfer the hexose products into the storage cells. In symplasmic unloading, enzymes associated with the cytoplasmic sucrose synthase cleavage pathway are likely to be involved (Rotisch & Gonzalez, 2004). Of the sucrose-metabolising enzymes in the outer pericarp of the kiwifruit during development, sucrose synthase has the greatest activity in comparison to two invertase enzymes, acid invertase and neutral invertase (Moscatello et al 2011). Late in development, the activity of sucrose synthase is shown to be equal to that of the invertase enzymes (Moscatello et al., 2011).

The unloading pathway of sugars (symplasmic or apoplasmic) into fruit may also be linked to the form of carbohydrate which accumulates in the fruit cells. For example, grapes and tomatoes unload via a symplasmic pathway early in development when starch is accumulated in the fruit cells. However, later in fruit development, starch accumulation ceases, osmotically active soluble sugars accumulate directly, and fruit fresh weight increases rapidly. During this period the apoplasmic pathway predominates (Ruan & Patrick, 1995; Zhang et al., 2006). In contrast, kiwifruit accumulate high levels of starch throughout the period of rapid fruit growth (Boldingh et al., 2000; Nardozza et al., 2010) and hence sugar unloading may be expected to follow a symplasmic route until the time of cellular starch breakdown at fruit ripening.

Recent research has also revealed the disaccharide planteose as a major temporary sugar storage compound in kiwifruit leaves (Klages et al., 2004). Planteose has also been detected in significant quantities in kiwifruit phloem sap (H. Boldingh & S. Nardozza, Pers. Comm). Planteose comprises of galactose and sucrose and is similar to raffinose (the two differ in that galactose is attached to a different carbon of the sucrose). Planteose has previously been found in cyclamen (Rothe at al., 1999), ash (Jukes & Lewis, 1974) and sesame seed (Dey, 1980). This discovery in kiwifruit implies that enzymes, other than those that are sucrose-metabolising, may have a major role in the phloem unloading pathway.

1.6 Water Relations

The growth of fruit requires an adequate flow of water to the organ and sufficiently high turgor to drive cell enlargement (Pomper & Breen, 1997; Meyer & Boyer, 1981). Due to this high pressure, plant cells require walls that permit high turgor pressures to develop (Cosgrove & Cleland, 1983). These walls must expand with cell growth whilst retaining enough strength to withstand the mechanical stress arising from internal hydrostatic pressure (Peitruszka, 2011).

Plant cell growth mechanics govern the limits of fruit growth. The Lockhart (1965) model was developed to describe plant cell elongation as a combination of elastic, turgor driven extension and plastic deformation (yielding) of the cell wall. It assumes that cellular extension is driven by a constant turgor pressure due to osmotic uptake of water (Peitruszka, 2011). This model creates a correlation between the vascular flows into a fruit and the growth of its cells. Therefore, it is hypothesized that fruit growth is controlled not only by the limits of cell walls, but also through long distance transport of water and solutes through vascular tissues, and short distance transport and uptake at a cellular level (Matthews & Shackel, 2005).

The onset of ripening is an important step in the development of economically important fruit such as grapes, tomatoes and kiwifruit. It is a genetically programmed process that involves the upregulation of genes responsible for sugar transport and cell wall modification (Davies & Robinson, 2000; Goes de Silva et al., 2005) However, changes in cellular water relations have also been shown to be involved in the onset of ripening (Shackel et al., 1991; Wada et al., 2008).

More specifically, recent research has implicated changes in cell turgor as having a central role in fruit development (Wada et al., 2009).

Cell turgor changes significantly during the development of the grape berry (Thomas et al., 2008; Wada et al., 2008). Measuring cell turgor directly, Thomas et al., (2008) showed that a rapid decrease in turgor occurred ten days prior to veraison, the significant point of time in grape development that involves fruit softening (Coombe, 1992) and a large increase in phloem influx (Greenspan et al., 1994). It was speculated that the early decrease in cell turgor may act as a signal for gene expression and metabolic changes that occur at the onset of ripening. A similar pattern of cell turgor loss was seen by Wada et al., (2008). This study hypothesized that the loss of turgor before veraison was associated with an accumulation of apoplasmic solutes in the mesocarp tissue. This was thought to cause the reduction in cell turgor. Like Thomas et al., (2008), it was proposed that regulation of apoplasmic solutes is a regulatory event that leads to veraison. Veraison has also been identified as the time at which the phloem unloading pathway transitions from symplasmic to apoplasmic, thus connecting and important transition fruit growth and composition to changes in both cellular water relations and phloem unloading pathway.

1.7 Overall Aims

The objective of this research was to further our understanding of the development and vascular functioning of the kiwifruit berry. This research aimed to describe changes in the functioning of the vasculature of the berry during its development, including the phloem unloading pathway and changes in water relations, and determine how these changes may affect carbohydrate accumulation by the berry.

A developmental study of the berry was performed that analysed berry growth and composition. Attention was paid to the growth curve of the berry and the timing of any changes of growth rate. Also changes in carbohydrate accumulation was analysed, including dry matter content and changes in the soluble solids concentration.

The water relations of the berry was also examined. The aim was to study changes in cell turgor during development and monitor any changes that may correspond with changes development or phloem unloading pathway. Two methods of extracting apoplasmic sap for the indirect method of estimating cell turgor were compared.

Finally, the phloem unloading pathway was investigated. A symplasmic phloem tracer was used to identify the pathway at time points across development. Any changes in the pathway were noted and compared with developmental data to analyse why these changes were occurring.

1.8 Thesis Outline

Chapter 1:

This chapter provides a general introduction to the research. The topics of each of the following chapters are explained with an up to date review of the literature.

Chapter 2:

The development of the kiwifruit berry is analysed with monitoring of berry growth, dry matter accumulation and the soluble solids concentration. The growth curve of the kiwifruit berry is analysed. Two methods of measuring berry firmness, the penetrometers and skin callipers, are compared.

Chapter 3:

Kiwifruit water relations are investigated, in particular cell turgor. Two methods of apoplasmic sap extraction from the kiwifruit berry are compared and the resultant measures of apoplasmic solute potential are used to estimate cell turgor using a non-direct method.

Chapter 4:

The phloem unloading pathway of the kiwifruit berry is analysed using carboxyfluorescein diacetate (CFDA), a symplasmic tracer dye. A variety of loading methods are trialled to determine the most effective way of loading CFDA into the berry. $^{14}CO_2$ labelling and autoradiography are also used to visualise phloem transport.

Chapter 5:

This is a discussion chapter that provides an overall synthesis of kiwifruit development and vascular functioning. The relationships between the results presented in the experimental chapters are discussed.

Chapter 2 – KIWIFRUIT DEVELOPMENT

2.1 Introduction

A fruit is the product of determinate growth from an angiosperm flower or inflorescence (Coombe, 1976). Fruit growth is associated with an increase in volume which is the result of cell division or enlargement, however most commonly both. Different fruit have been shown to exhibit different growth curve patterns when the cumulative increase in weight, diameter, volume etc. is plotted as a function of time.

Kiwifruit growth curves have been described as double sigmoid (Hopping, 1976). However, this is a debated subject. Walton & De Jong (1990) argue that kiwifruit has a single sigmoid growth curve and any variation from this is due to cultural conditions and/or sampling error. Pratt & Reid (1974) observed a triple sigmoid pattern. However this may be due to external factors such as temperature (Morgan et al., 1985), soil moisture (Judd et al., 1986), rainfall (Sale, 1981) hail, wind or frost (Hopping, 1986) affecting the standard curve.

Changes in growth rate have been shown in grape to be correlated with quality attributes such as colour, texture and the accumulation of sugars (Coombe, 1976). The grape berry exhibits a double sigmoid pattern of growth (Wada et al., 2008). A time point called ‘veraison’ occurs at the beginning of a second period of rapid growth, generally around 45 DAA. This period is associated with the appearance of red colour in red and black grape varieties, fruit softening, and a decline in solute potential, with the decline reflecting the accumulation of substantial concentrations of sugars and a change in the phloem unloading pathway (Matthews & Shackel, 2005). In kiwifruit, it is important that any changes in growth rate are identified as these changes may be associated with similar changes in underlying developmental processes. Furthermore, an in depth analysis of kiwifruit growth will determine whether kiwifruit exhibit a single, double or triple sigmoid pattern of growth.

Changes in fruit composition during development may also affect fruit quality. The carbohydrate content of kiwifruit has been shown to have a major influence on the purchase intentions of potential customers (Harker et al., 2009; Jaeger et al., 2011). Dry matter is an indicator of total fruit carbohydrate, of which, in kiwifruit, at harvest 40-70% may be starch. Kiwifruit starch accumulation generally exhibits a pattern of linear rise from 50-120 DAA, when it reaches its maximum (Richardson et al., 1997). This time point is also significant as starch begins to break down into soluble sugars (Richardson et al., 1997), which directly influence fruit flavour (Harker et al., 2009).

Of particular interest are the time points of any significant changes during the pattern of starch accumulation and break down. The metabolism of starch influences the concentration of soluble sugars. This in turn may affect cell turgor and fruit firmness. High sugar concentrations may also affect the phloem unloading pathway into the fruit. In this study, dry matter content and the soluble solids concentration will be monitored during the development of the berry. Significant changes in these attributes will be examined and used as a developmental scale to better understand any changes in other physiological components such as cell turgor changes and phloem unloading pathway changes.

Berry firmness is a measure traditionally used to indicate berry quality. Fruit softening is correlated to fruit ripening which in turn is the period of time that involves an increase in the concentration of sugars. The penetrometer measures firmness with the use of a probe that is pushed into the fruit and measures the force required to push through the fruit cells (Harker et al., 2009). An alternate method is the use of skin callipers that can measure berry deformation without crushing cells (Lang & During, 1991). This method measures berry elasticity and may be more directly related to cell turgor. A comparison between the penetrometer and skin callipers will determine whether the use of the callipers and the calculated berry elasticity may be an accurate estimate of berry firmness.

Berry firmness can be used to predict changes in cell turgor (Thomas, 2008). In grapes, cell turgor and elasticity both begin decreasing approximately 10 days before the onset of veraison (Matthews et al., 2009; Thomas et al., 2008). In kiwifruit, changes in berry elasticity will be compared with changes in cell turgor to determine whether a similar correlation occurs.

This chapter aims to further our understanding of kiwifruit berry development. Berry growth will be analysed using both non-destructive and destructive methods to analyse whether a single, double or triple sigmoid growth pattern exists. The composition of the berry will also be examined. In particular, the dry matter content and soluble solids concentration. Any changes in these components during development will be used as time points to compare changes in cell turgor and potential changes in the phloem unloading pathway identified in Chapters 3 and 4. Berry firmness will be measured using two methods, the penetrometer and the skin callipers method which has not been trialled in kiwifruit. Elasticity measurements from the skin callipers will also be compared to cell turgor results to determine whether a correlation exists.

2.2 Methods

2.2.1 Plant Material

Trials were carried out during the 2010/11 growing seasons on kiwifruit vines (*Actnidia deliciosa* var.*deliciosa* C.F. Liang et A.R. Ferguson) (cultivar Hayward) growing on a T-bar trellis in an experimental orchard at Plant & Food Research, Hamilton, New Zealand.

A random selection of 300 flowers on determinate fruiting shoots, located on the horizontal part of the canopy were chosen. Once flowers were open they were labelled, indicating the day of anthesis. Flowers were pollinated by hand using freshly picked male flowers.

Sampling dates were decided from the observation of previously published kiwifruit growth curves and dry matter accumulation (Hopping, 1976). Particular attention was placed early in fruit development, when fruit growth is fast. After recording weekly for the first two weeks, from this point onwards, sampling dates were fortnightly. However in the fruit growth study, non-destructive measurements of fruit growth were performed weekly during the growing season.

2.2.2 Fruit Growth

A non-destructive method of measuring fruit growth was used. This enabled higher resolution estimates of growth to more easily identify growth stages. Thirty flowers were selected during anthesis and labelled. Weekly measurements were taken of the length of the fruit (L) and the narrowest (D1) and widest (D2) points around the equator (Fig. 8). These measurements were used to calculate the fruit LDD (Eq. 1). Berry LDD was converted to weight (grams) using the conversion developed by Snelgar (1992) (Eq. 2).

A destructive measure of growth was also used. Berry fresh weight was determined using 10 randomly selected berries. These were weighed on a three-decimal place scale and recorded.

$$\mathrm{LDD} = \mathrm{L} \times \mathrm{D1} \times \mathrm{D2} \qquad \text{(Eq. 1)}$$

$$\mathrm{FW} = 0.454 \times (\mathrm{L} \times \mathrm{D1} \times \mathrm{D2})^{1.05} \qquad \text{(Eq. 2)}$$

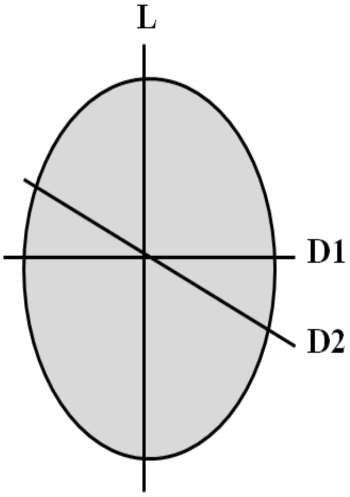


Figure 8: Kiwifruit berry showing the placement of LDD measurements. The length of the berry (L), and the widest and narrowest sides of the equator (D1 & D2).

2.2.3 Measuring dry matter and soluble solids

On each sampling date, 10 fruit were randomly selected and used for measurements of dry matter content and soluble solids concentration. Fruit dry weight was determined by taking a slice through the equator of the fruit, weighing (Wb), placing on a disposable petri dish and drying until no weight change was observed. The dry tissue was weighed again (Wa) to determine tissue dry weight. Fresh weight of the berry was used to estimate total dry weight $\mathrm{DW}_{\mathrm{total}}$.

$$\mathrm{DW\%} = \frac{\mathrm{Wa}}{\mathrm{Wb}} \times 100 \qquad \text{(Eq. 3)}$$

$$\mathrm{DW}_{\mathrm{total}} = \mathrm{FW} \times \mathrm{DW\%} \qquad \text{(Eq. 4)}$$

The soluble solid concentration of berry juice, extracted from a 2cm x 2cm segment of tissue was measured using a refractometer (ATAGO, Washington, USA).

2.2.4 Measuring fruit firmness

Berry firmness was measured using a penetrometer (Fruit texture analyser, GUSS, South Africa). A 1mm slice of skin was removed from both sides of the equator. The penetrometer probe (11mm) was inserted into the skinless window and the firmness reading was recorded. Ten berries were measured on each sampling date.

An alternative berry rheological measurement was taken using Harpenden skinfold callipers (Baty International, England) as described previously (Lang & During, 1991). Unlike the penetrometer which crushes cells, this technique measures berry deformation (D) without damaging fruit cells and has not previously been used on kiwifruit (Eq. 5). The callipers were placed around the berry equator and the initial diameter (Di) was recorded. The callipers were released to apply the force of the spring to the berry surface and a second diameter reading (Df) was recorded. This was repeated on both sides of the equator.

$$\mathrm{D(mm)} = (\mathrm{Di} - \mathrm{Df}) \qquad \text{(Eq. 5)}$$

The Hertz equation (Ravi et al. 2006) expresses the response of a fruit to an applied force in terms of elasticity (E). The elastic modulus is the description of an objects tendency to be deformed elastically when a force is applied. A higher elastic modulus equates to a firmer object. The Hertz equation corrects for the geometrical effects of both D and R on the area of contact between a flat plane and an elastic sphere. Measurements of D, taken from the skin callipers were used to calculate the elastic modulus (Eq. 6).

$$E(\mathrm{Mpa}) = \frac{3\mathrm{F}(1-\mathrm{v}^2)}{(2.\mathrm{Di.D})^{0.5}} \qquad \text{(Eq. 6)}$$

2.3 Results

The growth curve of the kiwifruit berry was double sigmoid (Fig. 9). LDD measurements, converted to fresh weight, show two periods of rapid growth separated by a period of slower growth. Growth rate, measured as change in fresh weight per week showed one major peak from 48 – 55 DAA. Another smaller peak was seen at 104 DAA (Fig. 10).

The fresh weight of the kiwifruit berry increased rapidly for the first 63 DAA. (Fig. 11A). After this date, fresh weight accumulation slowed down gradually until maturity. Percent dry weight initially decreased over the initial 26 DAA. From this lowest point it increased in a steady fashion until 139 DAA to reach the peak of 17.89% (Fig. 11B). Total dry weight, calculated using fresh weight and dry weight data showed a linear increase during the initial 139 DAA (Fig. 11C). At its maximum (20.39g) it stabilised and remained close to this value until the final sampling date. The soluble solids concentration remained relatively constant (4 - 4.5%) until 124 DAA (Fig. 12). After this point it began to rise at an increasing speed. The maximum soluble solids concentration value, (13%) was observed on the final sampling date.

Berry firmness, measured using the penetrometer initially increased until 38 DAA (Fig. 13A). Firmness remained constant for the majority of development, but decreased after 153 DAA as the berry matured. Berry elasticity gradually increased throughout development, but similar to the penetrometer readings, decreased from 153 DAA (Fig. 13B). There was no variation between measurements taken between sides D1 and D2.

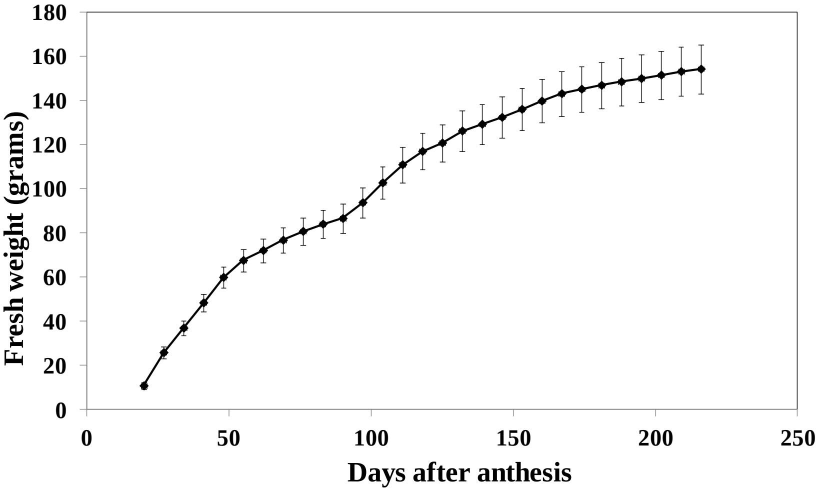


Figure 9: Showing the double sigmoid growth pattern of the kiwifruit berry. Fresh weight values have been estimated from LDD measurements using Eq. 2. Data are the means of 30 replicates (±SE).

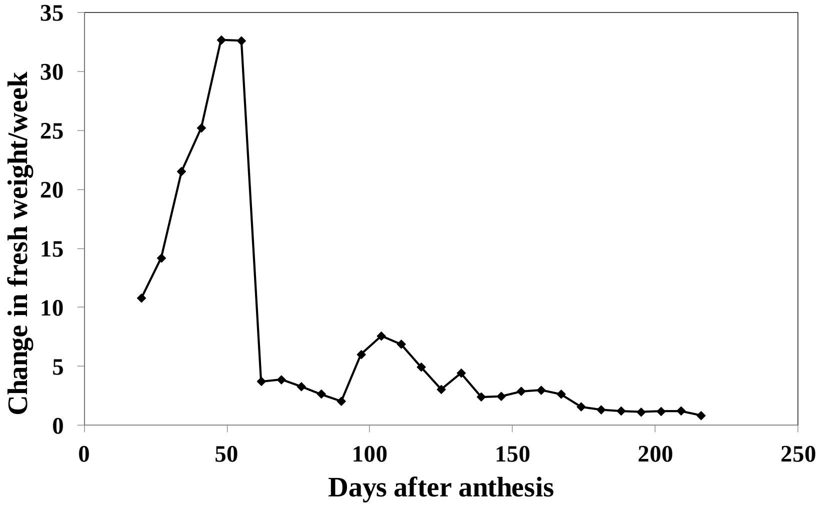


Figure 10: Change in fresh weight per week shows one major peak in growth early in development, 50 DAA, and a smaller peak at 120 DAA. Data are the means of 30 replicates.

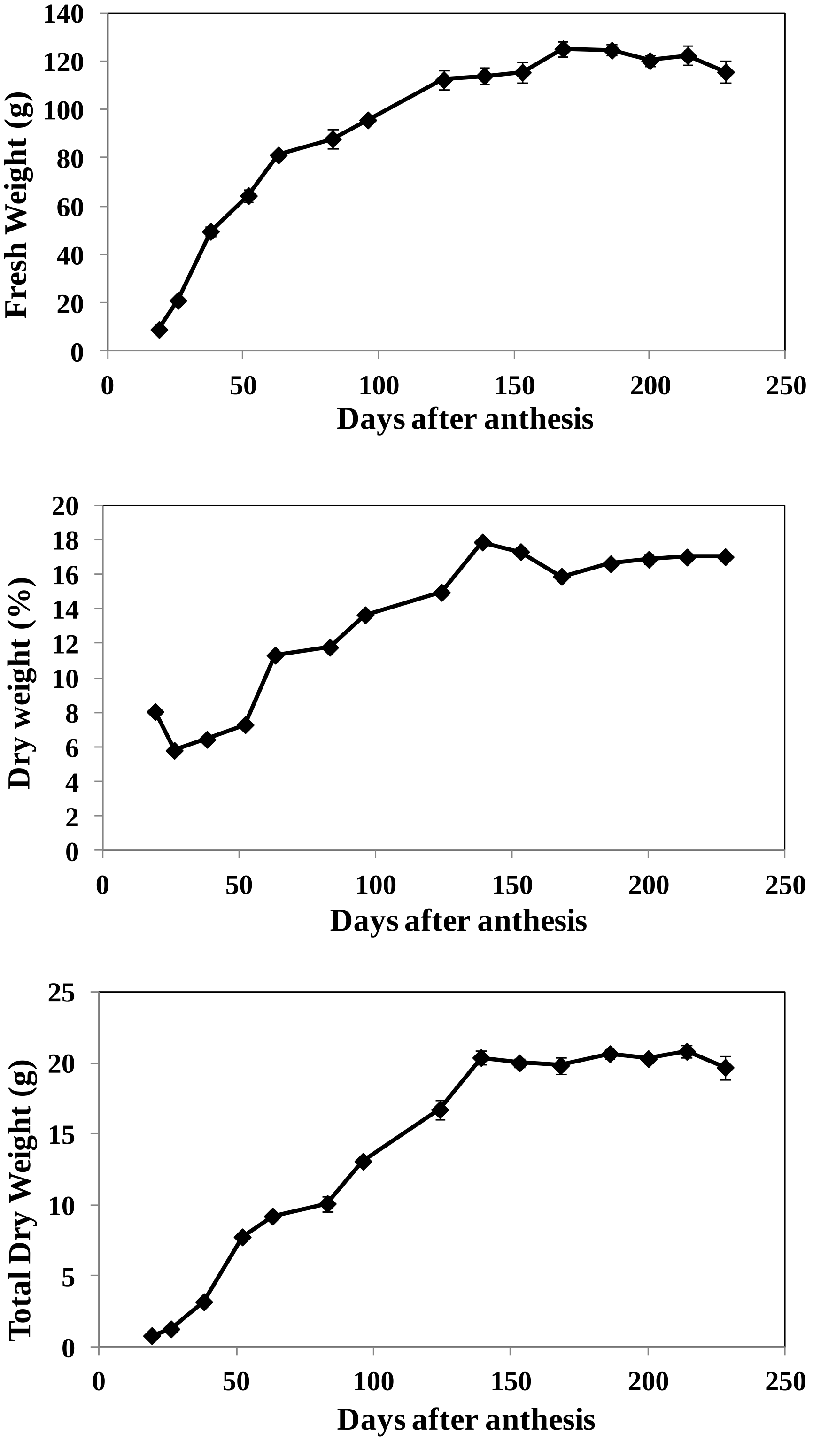


Figure 11: A, Berry fresh weight measurements sampled using destructive random sampling. Berry growth is rapid early in development, but slows down as the berry matures. B, Berry dry matter declines very early in development, before rising to its peak 139 DAA. After this time dry weight stabilises. C, Total dry weight, calculated using Eq. 4, shows a linear rise until 139 DAA when it reaches its maximum. Data are the means of 10 replicates (±SE).

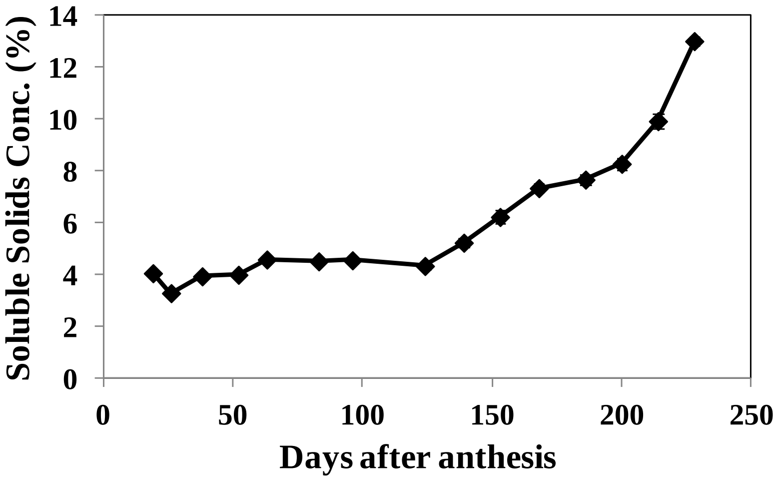
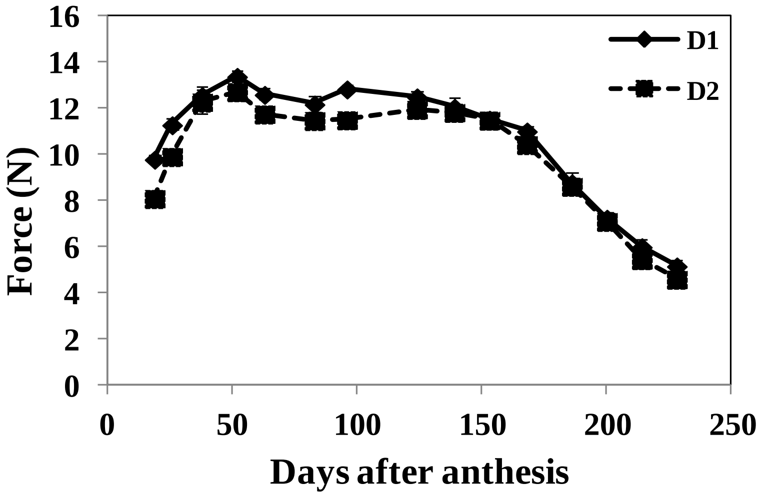


Figure 12: Change in the soluble solids concentration during development. It begins to rise at 124 DAA with rapid accumulation late in development. Data are the means of 10 replicates (±SE).



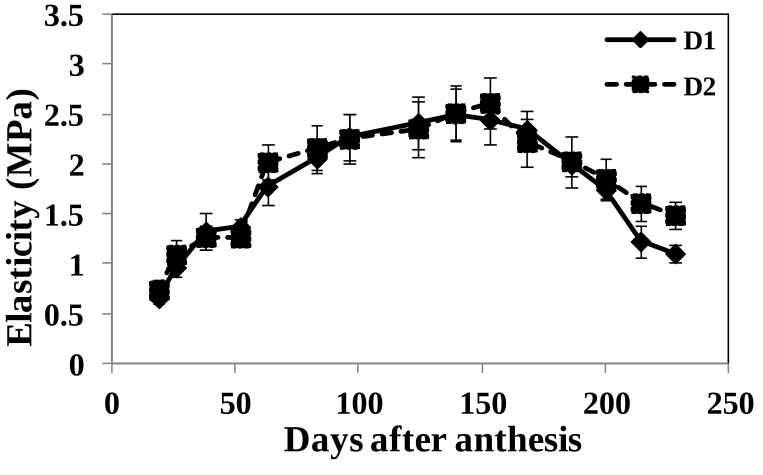


Figure 13: A, Berry firmness measured using the penetrometer, shows a decline at 153 DAA. B, Elastic modulus is low early in development but reaches its

maximum at 153 DAA. It then decreases as the fruit matures. D1 and D2 refer to the narrowest and widest sides of the equator respectively. Data are the means of 10 replicates (±SE).

2.4 Discussion

The kiwifruit berry has a double sigmoid growth curve. The non-destructive method of growth measurement, using LDD, shows two periods of rapid growth separated by a period of slower growth. The growth rate is fast until 90 DAA and is due to cell division followed by cell enlargement (Hopping, 1976) This pattern of cell division followed by enlargement is typical of the early development of drupes such as sour cherry (Tukey, 1934). From 90 DAA until 110 DAA the growth rate slows down. Cell division has stopped by this time and the rate of cell enlargement also diminishes (Hopping, 1976). The final period of rapid growth, after 110 DAA, is due to cell enlargement again. In particular, the elongation of the septum cells of the inner pericarp (Hopping, 1976). The grape berry exhibits a similar pattern, however the lag period is more pronounced.

Measuring berry fresh weight using a destructive harvest fortnightly revealed a different pattern of growth to that of the weekly LDD measurements. Fresh weight measurements showed a single sigmoid growth curve. The difference between the two methods of growth measurement can be explained by the error associated with sampling different fruit on each date. The LDD measurements were on the same fruit throughout the entire season, but the fresh weight measurements were carried out on different fruit on each sampling date. The lag in growth, associated with a double sigmoid growth curve may not have been seen by the fresh weight measurements due to randomly selecting larger fruit during that period of time. Also, LDD measurements were taken weekly during the growing season, whereas fresh weight measurements were taken fortnightly. The higher frequency amde it possible to see changes in growth rate that occured between the sampling dates of the destructive, fresh weight measurements. Due to these reasons, the non-destructive LDD measurements were used to classify the growth curve pattern of the kiwifruit berry.

Berry dry weight rose in a linear fashion and reached its maximum at 139 DAA. It remained close to this maximum dry weight until the final sampling date (228 DAA).The dry matter content is important as later in development, the accumulated carbohydrates are metabolised into soluble solids, which influence the taste of the fruit (Harker et al., 2009). This work showed that the soluble solids concentration began to rise at 124 DAA. The soluble solids concentration rose rapidly until the final sampling date. This is significant as high concentrations of sugars in the fruit cells can have an effect on cell turgor, which in turn can play a role in phloem unloading. These implications will be discussed in later chapters.

The penetrometer and skin callipers show a similar pattern of the changes in berry firmness during development. The force required to push the penetrometer probe into the berry reached its maximum early on development, however this remained relatively stable. After 153 DAA this force required decreased showing that the berry was softening. Berry elasticity, measured using the skin callipers, is a different type of firmness, but exhibited a similar result. A gradually increasing elastic modulus was seen for the initial 153 DAA. However after this time point, similarly to the penetrometer values, the elastic modulus decreased. This is significant as it shows that the results of both the penetrometer and skin callipers are consistent. Using skin callipers as a berry rheological measurement tool has not previously been trialled in kiwifruit. This study shows, for the first time, that skin callipers are a useful method for kiwifruit and are able to measure berry firmness non-destrcutively with similar results to the traditional penetrometer method.

2.5 Conclusion

This work has provided an in-depth look at kiwifruit berry development. The previously debated kiwifruit growth curve has been confirmed as double sigmoid in nature. Furthermore, the major fruit quality attributes of, berry firmness, berry dry matter, and the soluble solids concentration were recorded throughout development. Most importantly, the results of this study give significant time points in the development of the berry, for example when the onset of ripening occurs. These time points are transitions that may be related to transitions in other physiological functions such as the regulation of cell turgor and changes in the phloem unloading pathway, processes that will be addressed in the same season and population of fruit in later chapters.

Chapter 3 – FRUIT WATER RELATIONS AND TURGOR

3.1 Introduction

Fruit ripening refers to the processes that transform the mature fruit as it reaches the end of its growth period (Leopold, 1964). Changes generally include tissue softening, with associated changes in colouration, and flavour (Opara, 2000). More specifically, ripening is a regulated developmental program that involves the upregulation of genes responsible for sugar transport and cell wall modification (Brady, 1987; Davies & Robinson, 2000; Goes de Silva et al., 2005). Changes in cellular water relations are a result of changes in gene expression and help drive both growth and ripening.

Turgor pressure, a central part of plant water relations is actively controlled through osmotic adjustment (Pomper & Breen, 1997). The accumulation of solutes within cells is a known strategy used by plants to tolerate water or salt stress (Morgan, 1984). Regulation of cell turgor may also be involved during fruit ripening. It is hypothesized that during ripening, solutes accumulate in the apoplast of sink tissue (Shackel et al., 1991). Evidence for this has been found in growing stems of peas, soybean and cucumber (Cosgrove & Cleland, 1983) and in stems of sugarcane (Welbaum & Meinzer, 1990), which like normal fruit tissue accumulate large concentrations of sugars as a normal part of development. This theory of apoplasmic solute accumulation is consistent with the Münch pressure-flow hypothesis in that phloem turgor will be reduced, and hence phloem transport increased (Matthews & Shackel, 2005).

Cell turgor has been examined in the mesocarp cells of grape berries during development (Thomas et al., 2008). Grape berries are representative of fruit that exhibit a dynamic, double sigmoid growth curve. Growth is able to be divided into two growth phases, Stage I and III, separated by a period of little or no fruit expansion, Stage II (Coombe, 1976). The initiation of Stage III is known to be the onset of ripening and is termed 'veraison'. This time point involves a range of physiological changes including fruit softening (Coombe, 1992), upregulation of hexose and sucrose transporters (Davies et al., 1999), and a large increase in phloem influx (Greenspan et al., 1994). The results from this study illustrated that in grapes, cell turgor rapidly decreased (from 0.35MPa to less than 0.1 MPa) ten days before veraison. Thomas et al., (2008) speculate that the early decrease in cell turgor may act as a signal for gene expression and metabolic changes that occur at the onset of ripening.

How cell turgor changes during the development of the kiwifruit berry is of interest. The aim of this study was to estimate cell turgor during development and examine whether any changes occur. Potential transitions in cell turgor may have implications for the phloem unloading pathway and fruit firmness.

Cell turgor can be measured directly or indirectly. The direct approach requires the use of a cell-pressure probe, as used to measure cell turgor in the grape berry (Thomas et al., 2008). This method may be inappropriate for use on kiwifruit as the thick skin will need to be removed to allow the glass needle of the pressure probe to enter the cells. It is possible that damaging the skin and associated cells will alter the water relations in that region. The indirect method requires estimates of the apoplasmic solute potential, fruit matric potential and tissue solute potential. These three components may be used to estimate cell turgor. The difficulty with this method involves the extraction of samples of apoplasmic sap, as contamination or dilution are common problems. In the work presented here, apoplasmic sap samples extracted early on in development were contaminated with symplast contents; therefore this study was unable to examine cell turgor throughout the entire kiwifruit growing season. The aim of this study therefore was to identify the best method of extracting pure apoplasmic sap samples from kiwifruit, in order to reliably estimate cell turgor. A comparison of two common methods, the pressure chamber technique (Jachetta et al, 1986; Wada et al, 2005) and the centrifuge (Cosgrove & Cleland, 1983; Meinzer & Moore, 1988; Pomper & Breen, 1995; Zhang et al., 1996) was carried out.

3.2 Methods

3.2.1 Plant Material

Trials were carried out during the 2010/11 growing seasons on kiwifruit vines (*Actnidia deliciosa* var.*deliciosa* C.F. Liang et A.R. Ferguson) (cultivar Hayward) growing on a T-bar trellis in an experimental orchard at Plant & Food Research, Hamilton, New Zealand.

Fruit age was determined by tagging each flower at anthesis. A random selection of 300 flowers on determinate fruiting shoots, located on the horizontal part of the canopy were chosen. Flowers were pollinated by hand using freshly picked male flowers.

3.2.2 Calculating cell turgor

Total water potential of a cell is usually described as:

$$\Psi_{\mathrm{W}} = \Psi_{\mathrm{S}} + \mathrm{P} \qquad \text{(Eq. 1)}$$

where Ψ_{S} can be equivalent to Ψ_{s}^{T}, the tissue solute potential. P is cell turgor.

Cells form two compartments separated by a plasma membrane. The water potential of the protoplast (Ψ_{w}^{P}) and apoplast (Ψ_{w}^{A}) in tissue can be described separately as,

$$\Psi_{\mathrm{W}}^{\mathrm{P}} = \Psi_{\mathrm{S}}^{\mathrm{P}} + \mathrm{P} \qquad \text{(Eq. 2)}$$

$$\Psi_{\mathrm{W}}^{\mathrm{A}} = \Psi_{\mathrm{S}}^{\mathrm{A}} + \Psi_{\mathrm{M}} \qquad \text{(Eq. 3)}$$

where Ψ_{M} is regarded as the tissue-averaged matric potential. Ψ_{s}^{P} is the protoplast (symplast) solute potential. Ψ_{s}^{A} is the apoplast solute potential.

As described elsewhere (Nonami & Boyer, 1987; Wada et al., 2005) the Ψ_{w} of the two compartments are locally equilibrated in the mesocarp.

$$\Psi_{\mathrm{W}}^{\mathrm{P}} \approx \Psi_{\mathrm{W}}^{\mathrm{A}} \qquad \text{(Eq. 4)}$$

By equating equations 2 and 3 and measuring Ψ_{s}^{T}, Ψ_{s}^{A} and Ψ_{M}, P is able to be calculated:

$$\mathrm{P} = \left(\Psi_{\mathrm{S}}^{\mathrm{A}} + \Psi_{\mathrm{M}}\right) - \Psi_{\mathrm{S}}^{\mathrm{P}} \qquad \text{(Eq. 5)}$$

3.2.3 Matric Potential

Kiwifruit shoots with berry and leaves attached were removed from the vine at dawn and sealed within a black plastic bag to prevent transpiration. Assuming the fruit are not transpiring and growth has also stopped, a pressure gradient will not exist within the apoplast. The leaf matric potential will be in equilibrium with the fruit matric potential. Once in the lab, a leaf from the shoot was excised and the pressure required to balance the negative pressure in the shoot was determined using a pressure chamber.

3.2.4 Symplasmic solute potential

The symplasmic solute potential was estimated using a tissue sample taken from the berries used to extract apoplasmic sap. Berry tissue was initially frozen, then thawed, crushed and juice separated using a transfer pipette. The juice was spun for 30 seconds in a centrifuge and the clear supernatant was collected. The solute potential was determined by vapour pressure osmometry (Vapro 5520, Wescor, Logan, Utah).

3.2.5 Apoplasmic solute potential

Apoplasmic sap was extracted using two methods, the pressure chamber technique and the centrifuge. Once extracted, the sap was rapidly frozen in liquid nitrogen and stored at -80°C. On the day of measuring the solute potential, sap was thawed, and measured by vapour pressure osmometry as described above. The comparison between the pressure chamber and centrifuge apoplasmic extraction methods was carried out during a 40 day period from 119 DAA to 159 DAA.

3.2.6 Pressure chamber extraction

A berry was placed in the pressure chamber with its pedicel attached and exposed through the pressure chamber lid. The pedicel had been cut with a razor blade and washed with distilled water and blotted dry. A small tube was attached to the end of the pedicel, with the other end placed above an open eppendorf tube. A 0.6MPa pressure was applied to the berry for ~1 hour, enough time to extract 200-250µL of sap. To prevent any contamination of the apoplasmic sap collected from the cut end of the pedicel, early sap samples were not mixed with later samples.

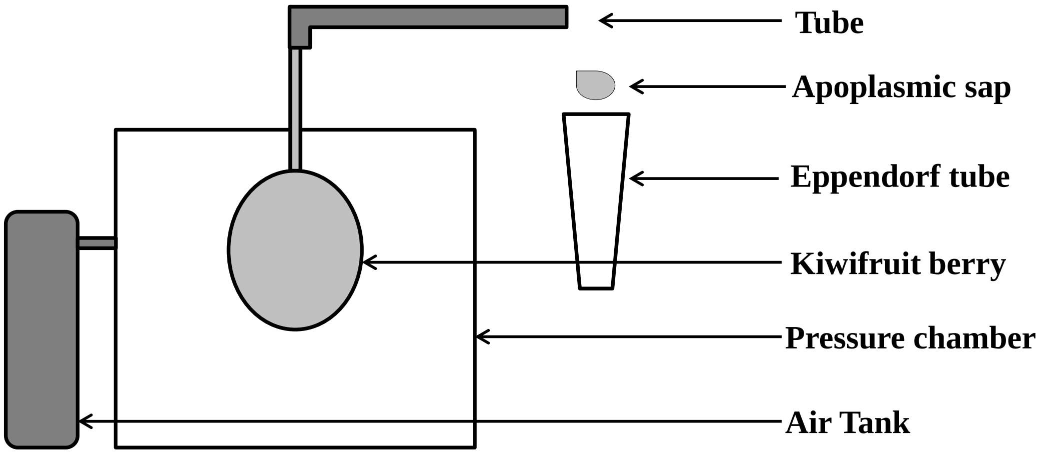


Figure 14: The pressure chamber apparatus for extracting apoplasmic sap. Pressure, applied from the air tank forced apoplasmic sap through the tube and into the eppendorf tube.

3.2.7 Centrifuge extraction

The centrifuge method for apoplasmic sap extraction, used for grapes by Wada et al., (2009), was modified for kiwifruit as described below. The berry was prepared for spinning by cutting through the equator, washing the cut surface and blotting dry. One half of the berry was placed, cut surface down into a cylinder with a mesh bottom. This cylinder was placed within a large centrifuge tube leaving ample space at the bottom to collect sap. The berry was spun for 10 minutes at a range of spin speeds, (25g - 2000g). An appropriate spin speed was required to extract sufficient volumes of sap, but not crush the fruit tissue and introduce symplasmic contamination. Trials were performed with and without washing and blotting the cut surface of the berry. After spinning, the sap was removed from the centrifuge tube, transferred to an eppendorf tube and snap frozen in liquid nitrogen.

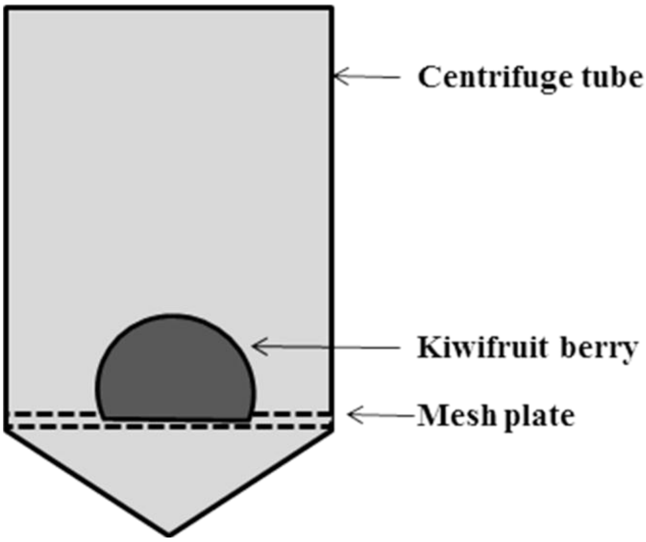


Figure 15: The centrifugation technique used a tube with a mesh bottom inserted within a large centrifugation tube. The half berry was placed cut-side down against the mesh.

3.2.8 Apoplasmic sap contamination

In addition to comparing the solute potentials of symplasmic and apoplasmic sap samples, glucose-6-phosphate dehydrogenase activity in apoplasmic sap samples was assayed as a marker of contamination by symplasmic sap Weimar & Rothe (1986). The enzyme activity was measured spectrophotmetrically at 334 nm and 25°C by continuously monitoring the change in absorbance of NAD(P)H. Glycine-NaOH buffer (500µl, 200m*M*, pH 9.5), $NADP^+$ (200µl, 4m*M* in distilled water) and 50µl desalted enzyme extract were combined. The reaction was started by adding glucose-6-phosphate (200µl, 30 mM in glycine-NaOH).

3.3 Results

Washing and blotting the cut surface of the kiwifruit berry before centrifugation extracted apoplasmic sap that had a lower solute potential, compared to not washing and blotting (Table 1). On average the difference between the two methods was -0.07MPa.

The g-force applied to the berry also affected the resultant apoplasmic sap solute potential of the sap extracted (Fig. 4). Spinning at very low g-force extracted sap with a similar osmolarity to symplasmic sap. As greater g-forces were applied, the solute potential rose (became more dilute); suggesting a higher proportion of the extracted sap was from the apoplast. However, there was a limit to the maximum g-force that could be exerted on the berry. At very high g-force (1412g), the berry tissue was crushed, contaminating the extracted sap with symplasmic content. The resultant solute potential of the extracted sap was close to the symplasmic solute potential.

The results from the enzyme assay to measure contamination of apoplasmic sap samples showed no contamination, even on samples believed to be highly contaminated. This may be due to the period of time spent in the freezer after extracting since trials on fresh, non-frozen apoplasmic sap showed some evidence of enzyme activity. Kiwifruit tissue is known to contain high levels of inhibitors and proteinases which sometimes contribute to the degradation of samples in storage and interfere with enzyme assays (Nardozza S, Pers. Comm). As all apoplasmic sap samples had been frozen during the course of kiwifruit berry development, the enzyme assay could not be used to quantify the level of contamination of apoplasmic sap by sap of symplasmic origin.

The pressure chamber method of apoplasmic sap extraction produced sap with varying levels of osmolarity (Fig. 5). The initial exudate samples (0-150µL) had low solute potentials, and this could have been due to contamination from the damaged cells at the cut end of the pedicel. However, after this initial volume was removed, a high, constant solute potential was produced. The solute potentials of the final two collections of apoplasmic sap from each fruit were used to estimate cell turgor.

Using Eq. 5 and the data from Fig. 6, cell turgor was estimated over four dates and is illustrated in Fig. 7. This pressure chamber extracted apoplasmic sap that, along with the symplasmic sap and matric potential values, estimated cell turgor to be in the range of 0.23 to 0.36MPa. Alternatively, apoplasmic solute potential values from the centrifuged apoplasmic sap estimated cell turgor to be negative.

	Apoplasmic sap solute potential (MPa)
Washing and blotting	-0.653 ± 0.006
No wash or blot	-0.582 ± 0.039

Table 1: Difference in solute potential of apoplasmic sap when washing and blotting the cut surface of the berry prior to spinning, in comparison to not washing or blotting.

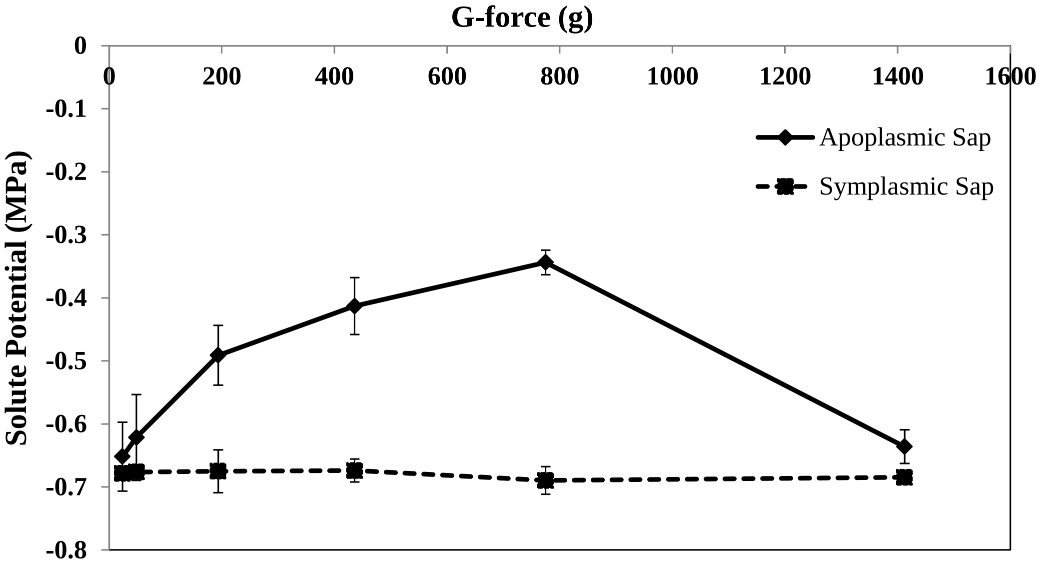


Figure 16: Solute potentials of apoplasmic and symplasmic sap over a range of g-forces. The apoplasmic solute potential increased with greater force, reaching its maximum at 775g. Spinning at greater than this crushed the berry, contaminating the apoplasmic sap.

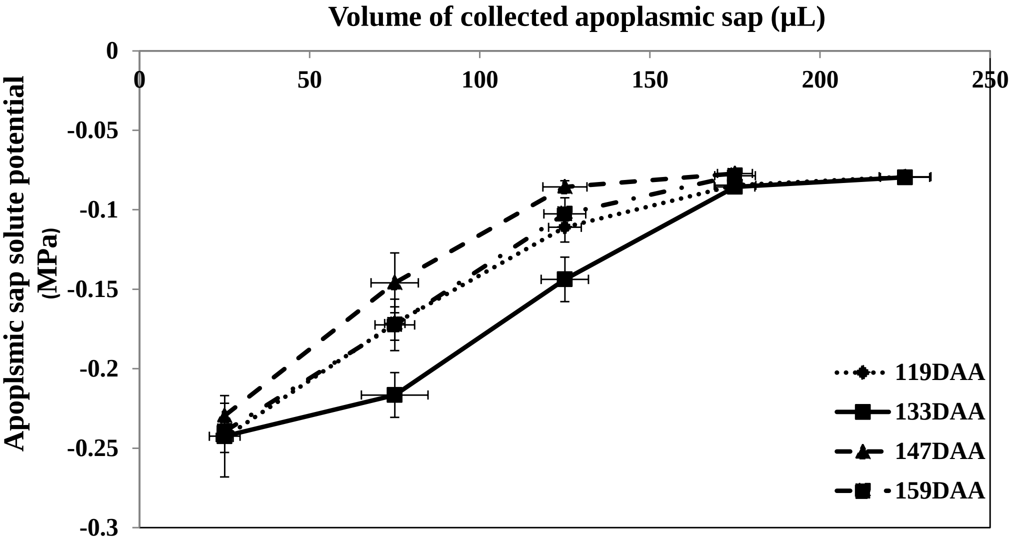


Figure 17: The solute potential of apoplasmic sap extracted using the pressure chamber method changes as the volume of sap increases. The initial volumes of sap extracted have a low solute potential. As more sap is produced, the solute potential rises and generally levels out after 150µL. Four trials were performed from 119DAA to 159DAA.

DAA	Sym. Solute Potential (MPa)	Apo. Solute Potential (Mpa)	Matric Potential (MPa)
119	-0.786	-0.082	-0.349
133	-0.778	-0.083	-0.452
147	-0.767	-0.077	-0.427
159	-0.779	-0.079	-0.467

Table 2: Shows the average apoplasmic and symplasmic solute potential and the matric potential values for the centrifuged expermient over four dates. These values were used, in Eq. 5 to estimate cell turgor.

DAA	Sym. Solute Potential (MPa)	Apo. Solute Potential(Mpa)	Matric Potential (MPa)
119	-0.823	-0.534	-0.482
133	-0.757	-0.637	-0.461
147	-0.764	-0.553	-0.530
159	-0.759	-0.586	-0.496

Table 3: Shows the average apoplasmic and symplasmic solute potential and the matric potential values for the pressure chamber experiment over four dates. These values were used, in Eq. 5 to estimate cell turgor.

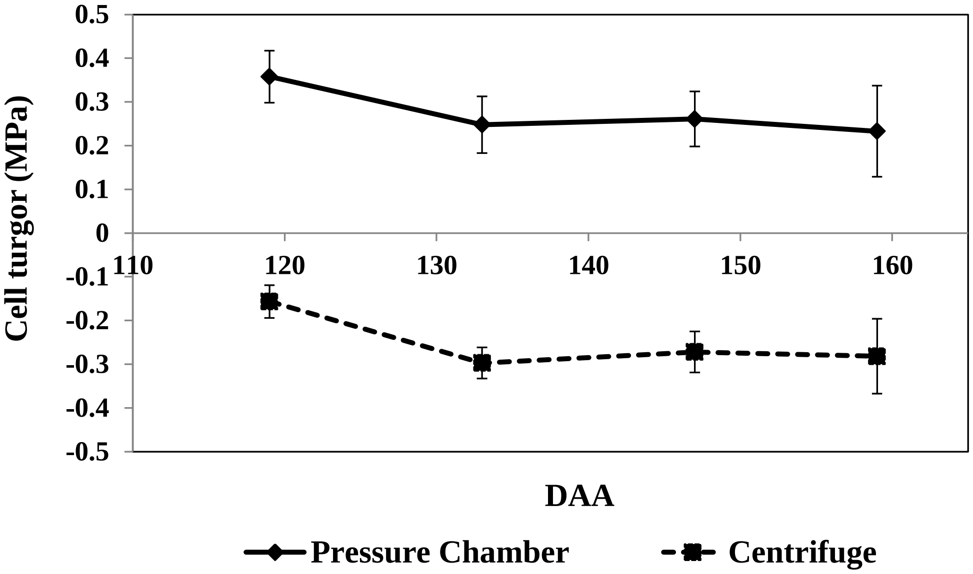


Figure 18: Estimated cell turgor using apoplasmic sap extracted using two methods, the pressure chamber and the centrifuge. Cell turgor, estimated using data from the pressure chamber experiment was positive. However the centrifuge experiment estimated cell turgor to be negative.

3.4 Discussion

Extracting apoplasmic sap using the pressure chamber produces a sap sample that is less contaminated by symplasmic sap, compared to the centrifuge, resulting in more realistic estimates of cell turgor. Cell turgor estimates using centrifuged apoplasmic sap were negative. Negative pressures do not normally occur in unlignified plant cells (Tyree, 1976). The consequences of negative turgor pressure would be risk of cavitation and/or collapse of the cell walls (Oertli, 1993). Although some have argued the theoretic possibility of negative turgor pressures (Rhizopoulou, 1997) it is highly unlikely that they would be occurring in kiwifruit as the fruit is growing and are extremely firm. It can therefore be concluded that contamination from the cut surface of the berry has affected the apoplasmic solute potential, thereby affecting the calculated cell turgor. The centrifuge method of extracting apoplasmic sap is not suitable for use on the kiwifruit berry, even though it has been used successfully with grape berries (Wada et al., 2009).

Cell turgor results from the apoplasmic sap extracted from the pressure chamber were positive in value (0.23 to 0.36MPa). In grapes, cell turgor values have been found to range from 0.05 to 0.3MPa during development (Thomas et al., 2008; Wada et al., 2009). Using the cell-pressure probe, a direct measure, the average cell turgor of a mature green tomato is 0.14MPa (Shackel et al., 1991). In comparison to these, the cell turgor values estimated from apoplasmic sap extracted via pressure chamber appear to be within a suitable range, although potentially a little high. It is hypothesized that a high estimate of cell turgor could be the result of dilution of the pressure chamber extracted sap by plasmalamella-filtered symplasmic sap (Jachetta et al., 1986). However, due to the consistency of the results and comparative values of other fruit species, the pressure chamber is recommended as the best method to extract a pure sample of apoplasmic sap to indirectly estimate cell turgor.

Cell turgor was unable to be estimated across all kiwifruit developmental stages. Early on in development, only the centrifuge method was used to extract apoplasmic sap. This method produced sap that was contaminated with symplasmic contents across all spin speeds, therefore cell turgor could not be estimated. The centrifuge method has been used on grapes with a general trend of increasing g-force equating to a more contaminated apoplasmic sap sample (Wada et al., 2008). In kiwifruit the opposite pattern was observed. Low g-forces produced very low solute potentials, indicating contamination, whereas high g-forces resulted in higher solute potentials. It is hypothesized, that in kiwifruit, washing and blotting the cut surface of the berry does not remove all the symplasmic contamination from the cut cells. When applying a low g-force, a small amount of apoplasmic sap was extracted with the remaining contaminants. When a larger force was applied, a greater volume of apoplasmic sap was extracted, diluting the effect of the remaining damaged cell contents. However, there is a limit to the extent of force that can be exerted on the berry. Very large forces resulted in cells being crushed against the mesh plate, producing more symplasmic contamination. The reasons for the contrasting results between grapes and kiwifruit are unknown, but the two fruits are very different in size, internal anatomy and composition.

The pressure chamber extracted apoplasmic sap of gradually increasing solute potential as greater volume was produced. All samples exhibited a pattern of a gradual increase in osmolarity for the initial 150μL. However after this volume was removed the solute potential remained constant. This change in osmolarity may be due to the effect of the pedicel. The first exudate samples collected from the fruit likely contained solutes released from damaged cells at the cut surface of the fruit pedicel. After 150μL the exudate solute potential appeared unaffected by contamination.

3.5 Conclusion

This work has compared the effectiveness of two methods of apoplasmic sap extraction to indirectly estimate kiwifruit berry cell turgor. The results have been successful in showing that the pressure chamber method extracts apoplasmic sap that is more pure with less symplasmic contamination. These sap samples can therefore be used to reliably estimate cell turgor. However, the centrifuge method produces apoplasmic sap that is contaminated and therefore this method should not be used in the future. Unfortunately, due to the centrifuge method being used for the majority of this developmental study, cell turgor was unable to be estimated throughout berry development. But with the knowledge gained from this work, a study of kiwifruit berry cell turgor during development can be performed in the future.

Chapter 4 - PHLOEM UNLOADING PATHWAY

4.1 Introduction

Photoassimilate partitioning between the sink tissues of the plant is governed by a number of individual physiological steps such as phloem loading, long-distance translocation, photosynthetic rate, unloading in sink cells, post phloem transport and metabolism of imported sugars (Oparka, 1990; Patrick, 1997). Phloem unloading is one of the important steps influencing partitioning of photoassimilate between competing sinks (Fisher & Oparka, 1996; Patrick, 1997; Viola et al., 2001). This claim is supported by the Münch pressure flow hypothesis for phloem translocation (Patrick, 1997). Translocation through the phloem is driven by positive hydrostatic pressures. The rate of flow is the product of the volume flux, path cross sectional area, and the concentration of the transported solute (Patrick, 1997). Phloem unloading in terminal sinks influences the volume flux by altering the hydrostatic pressure between the sieve element and sink tissue. Therefore phloem transport is able to be controlled (Patrick, 1997). Understanding the processes of phloem unloading and the particular pathways involved can lead to the development of new fruit varieties and improved orchard management processes that aim to increase the partitioning of carbohydrate into the fruit.

Fleshy fruit, such as kiwifruit, which are a class of terminal reproductive storage sinks, have received little attention regarding understanding their phloem unloading mechanism. Phloem unloading has been studied in tomato fruit, in which a symplasmic pathway operates at early stages, but an apoplasmic pathway occurs later in fruit development (Ruan & Patrick, 1995; Patrick & Offler, 1996).

The pathway transition occurs during the phase of rapid sugar accumulation and is believed to maintain a favourable turgor pressure gradient between source and sink. The grape berry has also been shown to be a sink that involves a shift in the unloading pathway (Zhang et al., 2006). Results from a symplasmic tracer dye (CF) experiment gave evidence that a symplasmic pathway is in operation early in development. At berry veraison, a high cell wall invertase activity, a high soluble sugar concentration, a decrease in plasmodesmata conductivity and the hydraulic isolation of the berry coincide with the commencement of an apoplasmic phloem unloading pathway (Zhang et al., 2006). During potato development there is a shift from an apoplasmic to symplasmic unloading pathway at the transition from stolon to tuber. Viola et al., (2001) showed that this transition corresponded with a decrease in cell wall invertase activity and a rise in soluble invertase activity. The kiwifruit berry differs from both the grape and tomato berries in that it is a starch storing sink that accumulates low levels of soluble sugars during fruit development (Boldingh et al., 2000; Walton & de Jong, 1990). The aim of this research was to determine the phloem unloading pathway that is occurring in kiwifruit during development as it is currently unknown.

The phloem unloading pathway that predominates in the kiwifruit berry has important implications for the identity of the enzymes most likely to influence the rate of carbohydrate accumulation. In most plant species, assimilated carbon is transported as sucrose, a disaccharide in which glucose and fructose are linked via an *O*-glycosidic bond. Cleavage of this bond initiates sucrose utilization and in plants this reaction is catalyzed by two types of enzymes with entirely different properties: invertases (EC 3.2.1.26) and sucrose synthases (EC 2.4.1.13) (Koch, 2004). Invertases are hydrolases which, cleave sucrose into the two monosaccharides, and when present in the cell wall are associated with an apoplasmic unloading pathway. By contrast, sucrose synthase is a glycosyl transferase, which, in the presence of UDP, converts sucrose into UDP-glucose and fructose and is associated with symplasmic unloading. Importantly, sucrose synthase and invertases differ in their cellular localization. Sucrose synthase is found solely in the cell cytoplasm, whereas the invertases are found in a variety of locations. (Koch, 2004). Soluble neutral invertases are located in the cytosol, soluble acid invertases are located in the vacuole, and insoluble extracellular invertases are bound to the cell wall (Moscatello et al., 2011). Identification of which unloading pathway is operating in kiwifruit will also give an indication of the type of enzyme responsible for sucrose metabolism.

The aim of this study was to determine the phloem unloading pathway functioning in the developing kiwifruit berry, from anthesis to maturity. Our hypothesis was that the unloading pathway is symplasmic during the initial periods of berry development, however, once ripening begins an apoplasmic unloading pathway dominates. The phloem mobile tracer dye, 6(5)carboxyfluorescein (CF), was used to determine which unloading pathway was in operation. Unloading of CF in sink tissues is indicative of a symplasmic unloading pathway because it moves only through the symplasm, from cell to cell via plasmodesmata. A number of CF labelling techniques were tested because introduction of the dye into the phloem of kiwifruit proved difficult. Results suggest that a transition in the unloading pathway may occur during kiwifruit development.

4.2 Methods

4.2.1 Plant Material

Trials were carried out during the 2010 & 2011 growing seasons on kiwifruit vines (*Actnidia deliciosa* var.*deliciosa* C.F. Liang et A.R. Ferguson) (cultivar Hayward) growing on a T-bar trellis in an experimental orchard at Plant & Food Research, Hamilton, New Zealand.

Fruit age was determined by tagging each flower at anthesis. A random selection of 300 flowers on determinate fruiting shoots, located on the horizontal part of the canopy were chosen. Once flowers were open they were labelled, indicating the day of anthesis. Flowers were pollinated by bees and by hand using freshly picked male flowers.

4.2.2 CFDA Preparation

The membrane-permeable 5(6)carboxyfluorescein diacetate (CFDA) was prepared as a 2.0% (w/v) stock solution in acetone and stored at -20°. Before use, it was diluted to 0.05% (w/v) (Ruan & Patrick, 1995).

4.2.3 Loading via wick through pedicel

CFDA was introduced into the berry via the pedicel. The fruit pedicel was pierced with a needle and cotton was thread through the outer phloem region. The wick was left in place, through the pedicel while the other end was immersed into a microcentrifuge tube containing 1ml of 0.05% w/v CFDA aqueous solution. Capillary action drew the CFDA into the pedicel. After allowing the plant 24-48 hours to transport the dye, the fruit was removed and immediately taken to the lab for sectioning and microscopy. On each experimental date, five fruit were loaded with CFDA and examined.

4.2.4 Loading via leaf abrasion

The method used by Roberts et al., (1997) to load sink leaves of *Nicotiana benthamiana* was trialled on kiwifruit. CFDA dye was introduced into the berry via surface abrasions on a source leaf. Shoots were selected seven days prior to CFDA labelling and prepared by removing the growing tip and phloem girdling the stem with a razor blade below the first leaf. Excess berries and leaves were removed to leave a shoot with one berry, its subtending leaf and 1-2 other leaves. The adaxial side of each leaf was lightly abraded with fine sand paper to remove the outer cuticle and dermal tissue. CFDA was painted onto the surface of the leaf before they were wrapped in plastic film to reduce evaporation. The shoots were allowed 24-60 hours to translocate the dye. On each date, five fruit were loaded with CFDA and examined.

4.2.5 Loading via leaf flap

Shoots were selected seven days prior to CFDA labelling and prepared by removing the shoot tip and girdling below the first leaf. Excess berries and leaves were removed to leave a shoot with one berry, its subtending leaf and 1-2 other leaves. CFDA was introduced into the berry via the leaves. In each leaf, a flap was cut on the primary leaf vein and on 1-2 secondary veins using a razor blade (Fig 21A). Excess mucilage was washed from the cut surface with distilled water before the flap was immersed into a microcentrifuge tube containing 1ml of CFDA solution. CF was allowed to load for 24 hours the flaps before the same flaps were re-cut and immersed again in fresh solution of CFDA. Shoots were left for a further 48 hours before being removed and immediately brought to the lab for sectioning and microscopy. The experiment was carried out weekly from 13 days to 165 days after anthesis. Each week a total of five berries were analysed, as well as control berries that had not been labelled with CF dye.

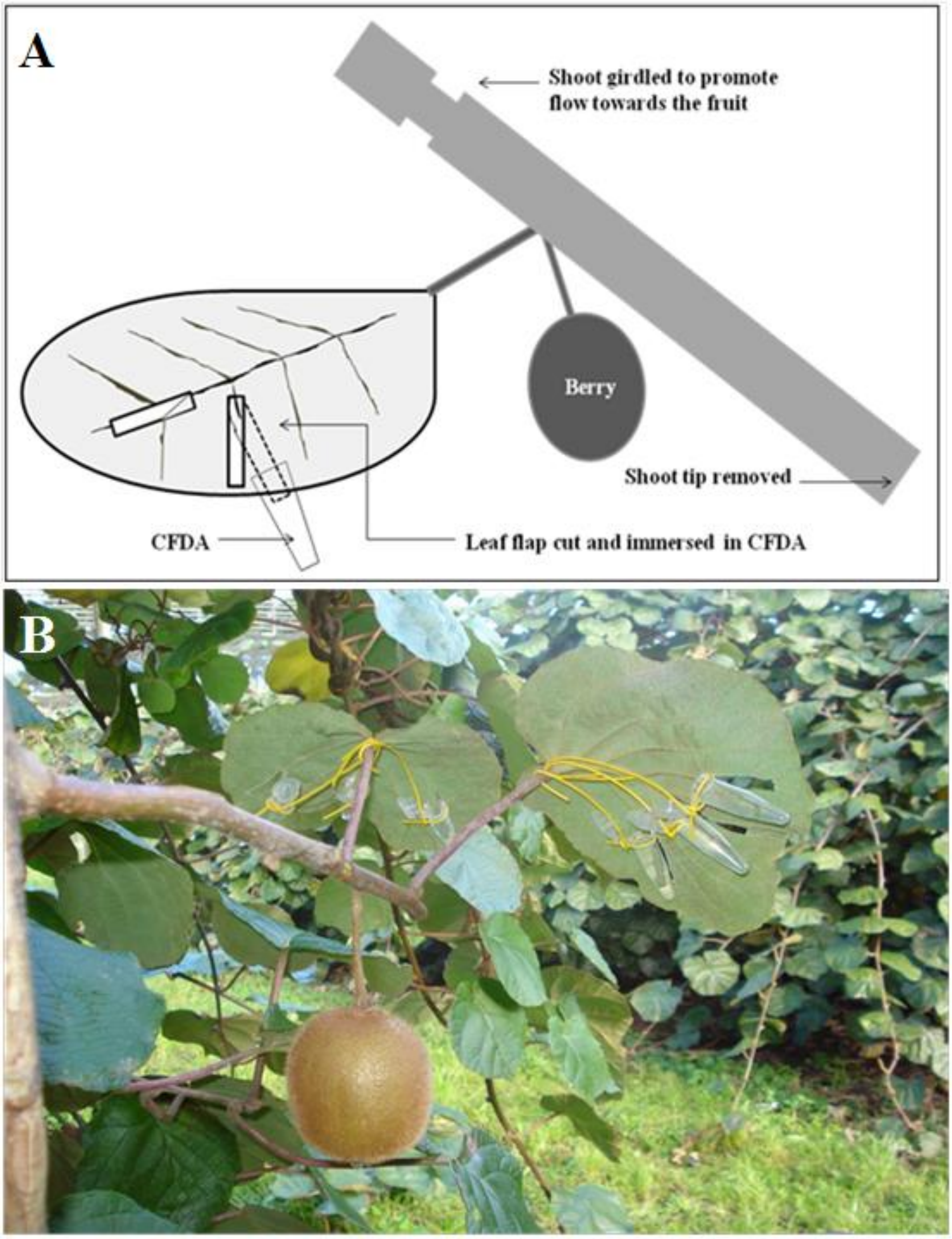


Figure 19: **A**, Diagram of the leaf flap loading method. Dye was loaded by immersing three sided leaf flaps, cut to include a major vein, into microcentrifuge tubes filled with CFDA solution. **B**, Image of leaf flap loading in situ, showing the microcentrifuge tubes secured with wire. Two leaves each with three leaf flaps are being used to label phloem transport into one kiwifruit.

4.2.6 Microscopy

Transverse hand sections of the leaf petiole, fruit pedicel and berries from CFDA loaded shoots were taken and mounted in water. A compound epifluorescence microscope (Leica DMRE) with a Plan Fluotar lense and a 50W Hg excitation light source used to image CF transport (optimum excitation at 490 nm and emission at 515 nm) as quickly as possible.

4.2.7 $^{14}CO_2$ Labelling

The $^{14}CO_2$ labelling and autoradiography were performed as described previously (Zhang et al., 2004). Shoots were removed from the vine and excess berries and leaves were removed to leave a shoot containing one berry and its subtending leaf. The leaf was enclosed in a plastic bag with a vial of the radiolabel (Fig. 22). Each leaf received 1.85 MBq of $^{14}CO_2$ released from [^{14}C]sodium-bicarbonate by the addition of a saturated citric acid solution. The leaf was exposed to the $^{14}CO_2$ for 20 minutes before the bag was removed and the shoot was left for 4-6 hours to translocate the ^{14}C to the berry. $^{14}CO_2$ labelling was carried out five times over the development of the berry from 18 to 207 days after flowering. On each date, between four and ten berries were labelled with $^{14}CO_2$.

Figure 20: Set up of ^{14}C labelling. Shoots with berry and leaves attached were held in flask of water. The leaf was sealed in a plastic bag with $^{14}CO_2$ being released from [^{14}C]bicarbonate by the addition of a saturated citric acid solution

4.2.8 Autoradiography

After $^{14}CO_2$ labelling, transverse fruit slices were rapidly frozen in liquid nitrogen between sheets of paper. The frozen sample was then gently compressed between aluminium plates and freeze dried. After drying, the tissue was pressed flat and autoradiographed using Kodak BioMax MR-1 film (Sigma, Poole, UK) at -80°C for five days.

4.3 Results

4.3.1 Wick through pedicel method

This method resulted in CF dye located in both the phloem and xylem (Fig 24A). As a consequence, the fruit was flooded with CF and the phloem unloading pathway was could not be observed.

4.3.2 Leaf abrasion technique

The leaf abrasion method resulted in no loading of dye into the phloem. Dye was instead restricted to the outer epidermal cells only (Fig. 24C). Hence no dye reached the fruit pedicel (Fig. 24B). This method could not be used to determine the phloem unloading pathway in the kiwifruit berry.

4.3.4 Leaf flap method

The leaf flap was the most successful method for loading CF dye into the phloem of the source leaves. After leaf flap loading the dye often translocated to the fruit in large volumes (Fig. 23A). Examination of both the leaf petiole and fruit pedicel indicated that the CF dye was travelling in the phloem only. No dye was seen in the xylem of the leaf petiole (Fig. 23A) or fruit pedicel (Fig. 23B). Based on this evidence, this method was chosen for future experiments examining the phloem unloading pathway of the kiwifruit berry.

4.3.5 Evidence for a shift in the unloading pathway

Fluorescence microscopy images of CF movement in kiwifruit show that there was a change in the phloem unloading pathway during development. Early in development (22 DAA), the symplasmically isolated CF spread throughout the berry tissue from both ventromedian and median dorsal carpellary bundles (Fig. 25A-B). At 54 DAA the dye moved through symplasmic connections, however this was visibly less than 22 DAA (Fig. 25C-D). By 71 DAA the dye was restricted to the sieve elements (Fig. 25E-F). No dye was visible in the fruit after 126 DAA (Fig 25G-H).

4.3.6 Decline in phloem function

The behaviour of CFDA dye is similar to the pattern of assimilate unloading determined by autoradiography (Viola et al., 2001). Early in development, large quantities of ^{14}C-labelled assimilates were unloaded from both ventromedian and median dorsal carpellary bundles (Fig. 26A). However, during berry development the quantity of ^{14}C-labelled assimilates decreased, and late in development, no assimilates are unloaded (Fig. 26B). The proportion of shoots from 14C labelled shoots that accumulated detectable ^{14}C in the pericarp decreased steadily during development (Fig. 27). Furthermore, the spread of label away from the major vascular bundles decreased during development, suggesting a reduction in the rate of unloading over time (Fig. 28).

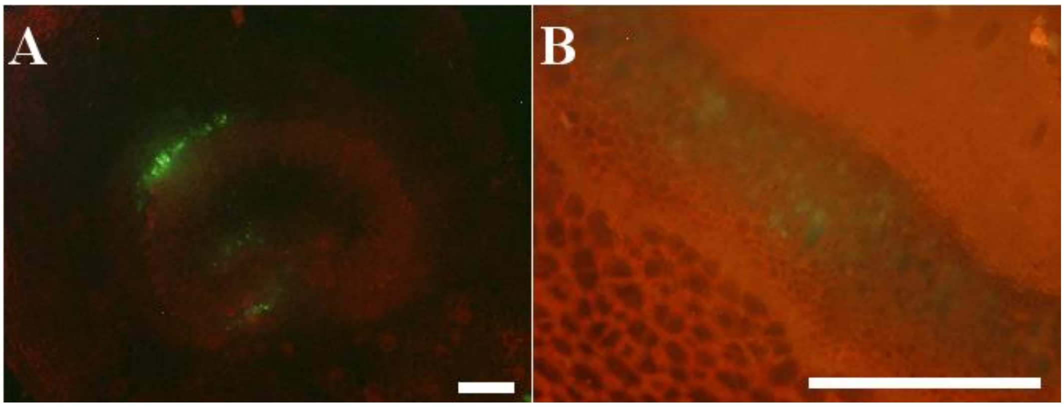


Figure 21: Epifluorescence microscopy images of CF localization in leaf petiole and fruit pedicel using the leaf flap loading method. A, Tranverse section through leaf petiole. CF is evident in phloem regions only, not in the xylem. B, Tranverse section through fruit pedicel. Fluorescent green CF is localized in the sieve elements and absent from the xylem. Scale bar represents 0.5mm.

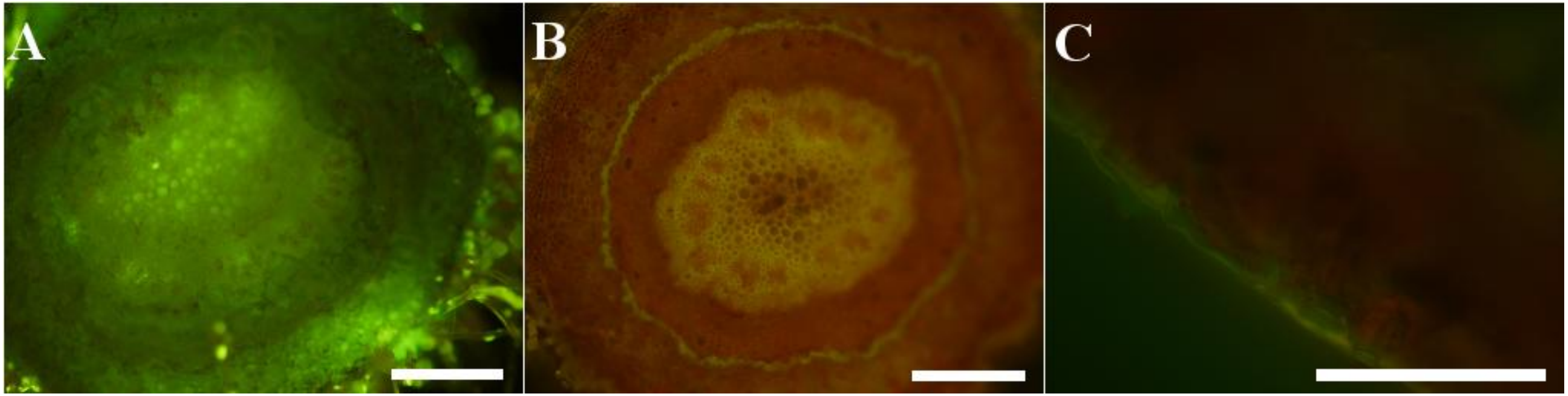


Figure 22: Epifluorescence microscopy images of CF localization in fruit pedicel and leaf. A, Tranverse section of fruit pedicel showing CF dye throughout all tissues, including xylem. CF was loaded via a wick through the pedicel. B, Tranverse section through fruit pedicel loaded using the leaf abrasion method. CF dye is absent from all tissues, including the phloem. Yellow colour seen in pedicel is due to auto-fluorescence. C, tranverse section through leaf, loaded using leaf abrasion. CF dye is seen on the outer layer epidermal cells only, therefore no penetration to the phloem. Scale bars represent 0.25mm.

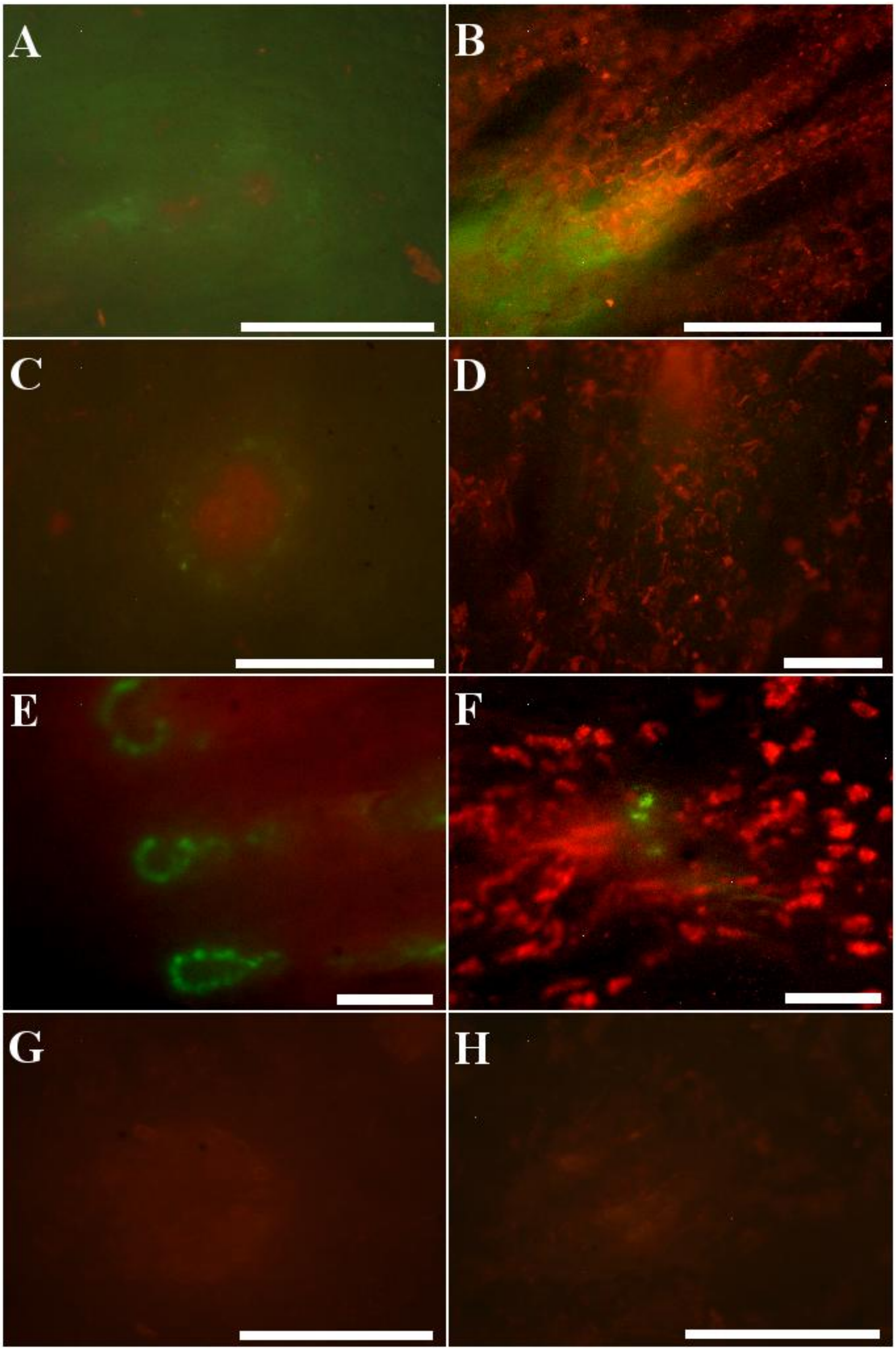


Figure 23: Epifluorescence microscopy images of transverse sections taken through kiwifruit of varying age, loaded with CF dye using the leaf flap method. A, C E & G show ventromedian vascular bundles. B, D, F & G show median dorsal vascular bundles. A, 22 DAA showing significant symplasmic unloading from sieve elements to surrounding sink cells. B, 22 DAA, with spread of CF from sieve elements towards the epidermis of the fruit. C, 54 DAA with spread of

CF dye. D, 54 DAA with spread from sieve elements, however significantly reduced. E, 71 DAA with CF dye restricted to the sieve elements. F, 71 DAA with CF dye restricted to the sieve elements. G, 126 DAA with no dye seen in any fruit tissue. H, 126 DAA with no CF dye. Scale bars represent 0.25mm.

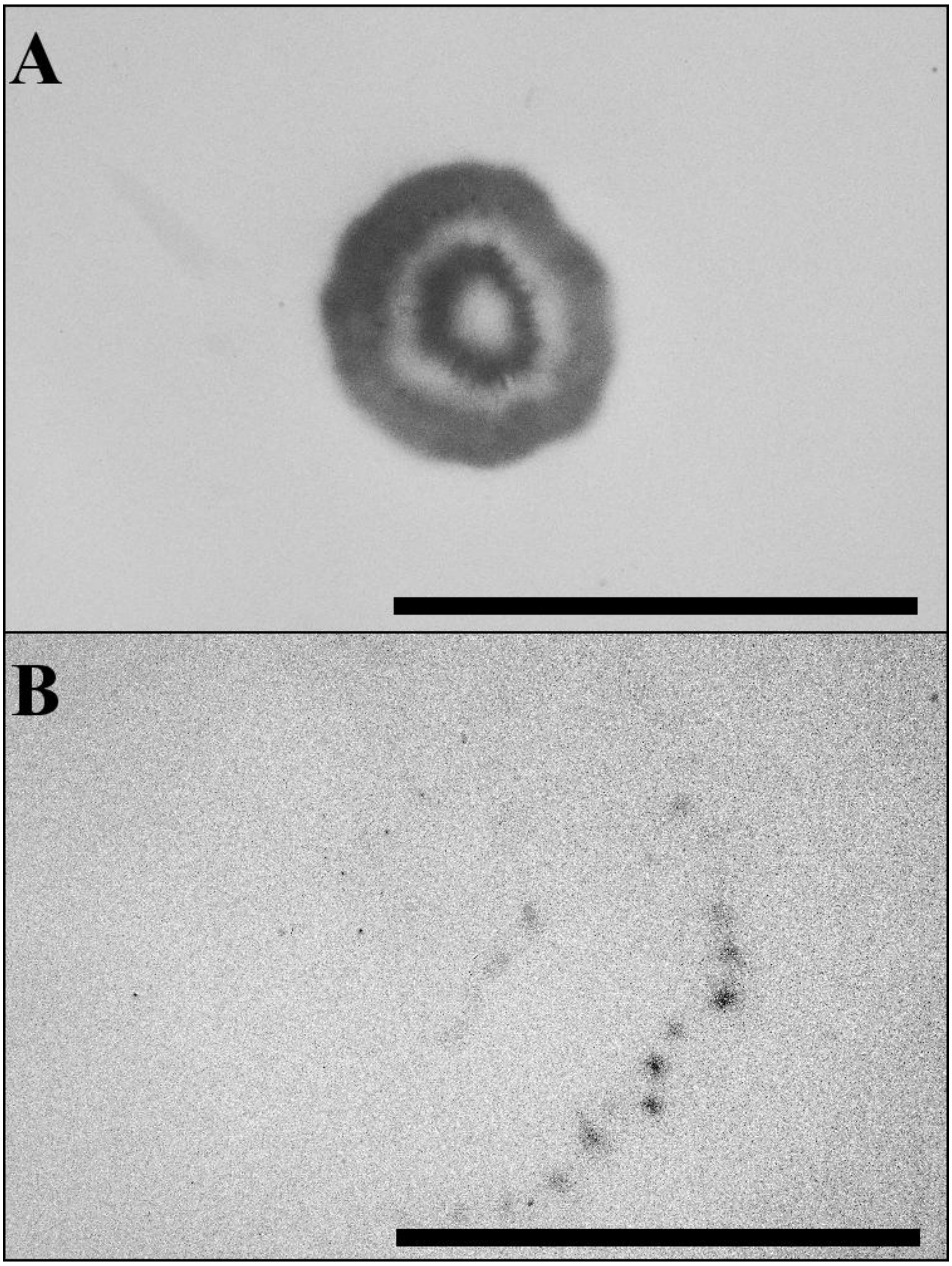


Figure 24: Autoradiography images showing vascular bundles in a kiwifruit berry and spread of ^{14}C label as it is unloaded. A, Autoradiography at 18DAA. Evident unloading of ^{14}C-labelled assimilates from both median dorsal and ventromedian carpellary bundles. B, Autoradiography at 135 DAA. Shows some vascular bundles with ^{14}C-labelled assimilates but no unloading. Scale bars represent 50mm.

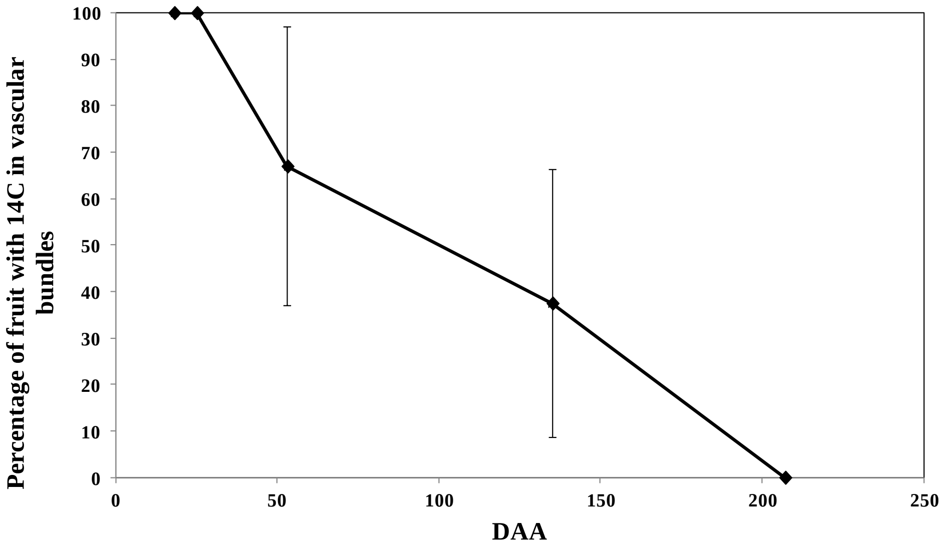


Figure 25: Change in the percentage of berries with ^{14}C in the vascular bundles. Early in development all berries have ^{14}C transported to the fruit, however over development the amount of berries with ^{14}C decreases. Error bars indicate the 95% confidence interval using the binomial distribution, n = 8.

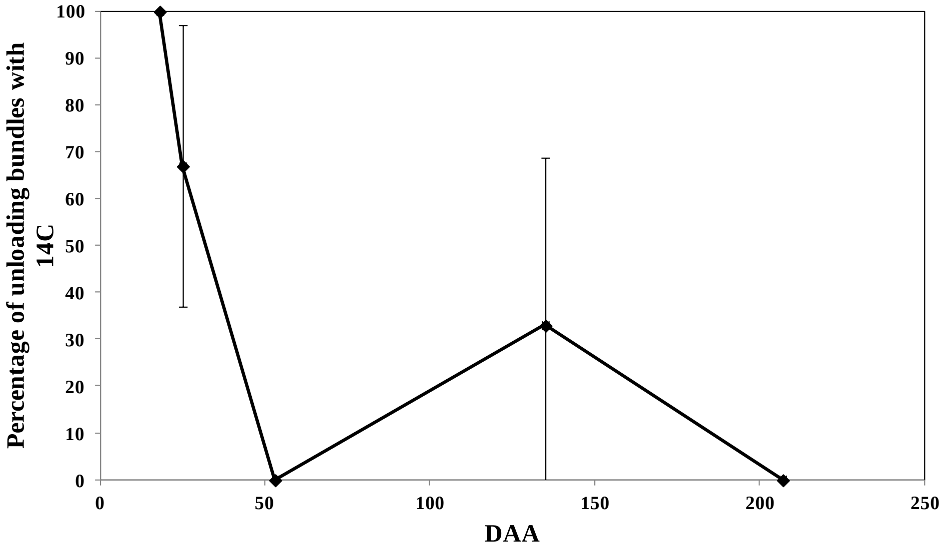


Figure 26: Graph showing the percentage of berries that had ^{14}C located in the vascular bundles that were actively unloading into the surrounding tissues. Error bars indicate the 95% confidence interval using the binomial distribution, n = 8.

4.4 Discussion

4.4.1 Leaf flap method provides effective translocation of CF

Carboxyfluorescein (CF) dye is a commonly used symplasmic fluorescent marker. When loaded into cells, the membrane-permeable and nonfluorescence carboxyfluorescein diacetate (CFDA) is degraded to CF, a membrane-impermeable fluorescent dye. However, attempts to load CF into the kiwifruit berry using most previously reported methods proved unsuccessful. Loading CF via a wick through the pedicel of a fruit is a popular method. It has been used to study phloem unloading in grape (Zhang et al., 2006), apple (Zhang et al., 2004), walnut (Wu et al., 2004), and cucumber (Hu et al., 2011). Due to the kiwifruit pedicel's small size, this method proved to be very difficult. In all experimental trials, CF was found in the xylem vessels of the pedicel. This resulted in xylem uptake into the fruit and the phloem unloading pathway could not be observed. Leaf abrasion has been used to load CF and determine the phloem unloading pathway of sink leaves of *Nicotiana bentamiana* (Roberts, 1997). Due to poor dye uptake this method could not be used to determine the phloem unloading pathway in kiwifruit. A novel CF loading system was therefore developed to be used in kiwifruit. The leaf flap method was very successful at getting sufficient quantities of dye into the fruit through the phloem.

4.4.2 The phloem unloading pathway

The present study reveals that the se-cc complexes in the phloem of vascular bundles in the outer pericarp and inner pericarp unload symplasmically early in development. This was shown by the movements of CF dye which can only be transported through plasmodesmata and not transmembraneously (Nie et al., 2010). The dye was able to symplasmically spread early in development Autoradiography indicated that the phloem strands were functional for assimilate unloading. This data provides evidence for a symplasmic phloem unloading pathway into the kiwifruit berry early in development. Symplasmic unloading also occurs during the early development of the berries of tomato (Ruan & Patrick, 1995) and grape (Zhang et al., 2006).

However, CF dye results also indicated that a change was occurring within the berry vasculature during development. At 91 DAA the CF dye was confined strictly to the phloem strands in both the ventromedian and median dorsal carpellary bundles. This suggests the assimilates were symplasmically restricted and an apoplasmic unloading pathway may be in use. However, due to the lack of assimilate unloading witnessed in the autoradiographs taken late in development, and the lack of any CF dye seen in the fruit after 126 DAA, it is also possible that this restriction in CF unloading was not the result of a transition to an apoplasmic unloading pathway, but rather part of a general trend in declining phloem function.

4.4.3 Significance of the unloading pathway

A change in the phloem unloading pathway from symplasmic to apoplasmic has been shown to occur during the development of the grape and tomato berries (Zhang et al., 2006). It has been hypothesised that the pathway alters so that fleshy fruit, such as grape, can accumulate high levels of soluble sugars (Patrick, 1997). Zhang et al., (2006) showed that the transition from a symplasmic to apoplasmic pathway occurred during the developmental transition from growing phase to ripening phase. Importantly, this transition concurred with the onset of ‘veraison’, a period marked by the rapid accumulation of soluble sugars. This high level of soluble sugars could theoretically result in a considerable rise in turgor pressure in parenchyma cells. If the se-cc complex of the phloem were symplasmically connected to parenchyma cells via plasmodesmata channels, an increased turgor pressure would impact on bulk flow (Patrick, 1997). Enhanced turgor pressure in the terminal release sieve tubes would inhibit flow through the phloem of the necessary soluble sugars (Patrick, 1997). An apoplasmic unloading pathway breaks the symplasmic linkage between sieve element and parenchyma cell, therefore separating the turgor pressure of sieve tubes from the surrounding ground tissues. This allows a pressure difference to continue to occur between the import phloem in source leaves and the terminal release phloem in sink berries, and ensures efficient long-distance phloem transport from source leaves to ripening berries (Zhang et al., 2006). The accumulation of sugars in the apoplast may also function to reduce turgor pressure and fruit firmness during ripening (Matthews & Shackel, 2005). The conductivity of grape berry xylem vessels has been found to be reduced at the time of onset of ripening (During et al., 1987; Choat et al., 2009). This results in some degree of hydraulic isolation of the berry xylem and may function to prevent apoplasmic sugar flow from the berry back to the vine.

The CF dye results of this study suggest a transition from symplasmic to apoplasmic unloading at 91 DAA. It was hypothesised that, like grapes, this transition in unloading pathway would occur at the onset of ripening in kiwifruit.

However, the beginning of the increase in soluble solids concentration, normally interpreted as indicative of the beginning of fruit maturation or ripening, did not occur until 125 DAA. Because of this variance, this hypothesis was not supported. Although the CF dye results suggest a reduction in symplasmic unloading, it is possible that this was not a transition to apoplasmic unloading but perhaps a decline in overall phloem function. This is supported by the insufficient dye transport witnessed after 126 DAA and the reduction in transport seen in the ^{14}C radiographs. However, it is very unlikely that phloem functioning ceased entirely because dry matter continued to rise after 91 DAA. ^{14}C loading may have been affected by the use of excised shoots. This may have altered the normal phloem function of the shoot, and the shoot response to excision and loading may have changed as the season progressed. If this experiment was performed *in vivo* and for a longer period of time than six hours, the ^{14}C may have been unloaded normally into the fruit later in fruit development.

4.5 Conclusion

This study has provided an insight into the phloem unloading pathway of the developing kiwifruit berry. A unique method of loading the symplasmic tracer dye, CF into the phloem of the fruit was developed and was successfully used to observe phloem unloading. Early in berry development the dye spread through symplasmic linkages in the fruit, suggesting that unloading was predominantly symplasmic. Later in development the dye was restricted to the vascular bundles, suggesting a possible transition to predominantly apoplasmic unloading. Unfortunately, because CF loading and ^{14}C unloading both became less effective later in development, the transition in unloading pathway could not be confirmed.

Chapter 5 - DISCUSSION

The objective of this research was to further our understanding of the development and vascular functioning of the kiwifruit berry. Analysis of developmental processes such as growth, firmness and carbohydrate accumulation has provided an overall picture of how the kiwifruit berry changes from anthesis to maturity. The function of the phloem, in particular the unloading pathway was examined as well as changes in cell turgor during development.

Chapter Two investigated the changes in berry size and composition during development. One of the main purposes of understanding these changes was to identify when these changes occurred, and how they may be related to berry water relations, and phloem transport. Clear patterns were observed amongst all the developmental components of the berry including berry growth. This work presented evidence that the kiwifruit exhibits a double sigmoid growth pattern. This is significant as the growth curve pattern of the kiwifruit berry has been debated as to whether it follows a single, double or triple sigmoid curve. It can be confirmed that using a non-destructive method of growth measurement, a double sigmoid pattern is clearly shown when berry LDD is plotted against time.

Dry matter accumulation and the soluble solids concentration, both understood to be vital to the flavour of the kiwifruit showed patterns that had clear transitional times during development. The aim was to monitor these berry traits and determine how they change during development. This experiment was performed and successfully provided a number of important transitional times during carbohydrate accumulation and metabolism.

Berry firmness was measured using two methods, one of which, the skin callipers had previously never been used on kiwifruit. This study has shown that the callipers measure of elasticity had a similar seasonal time course to the traditional penetromter measure of firmness. In may therefore be useful in the future as a non-destructive measure of fruit firmness. Future research is required to show how callipers firmness measurements relate to internal fruit properties turgor and cell wall degradation.

Chapter Three investigated changes in cellular water relations during development, in particular cell turgor. Plants adjust cell turgor osmotically during fruit ripening as a strategy to allow large phloem influxes (Matthews & Shackel, 2005). Due to contamination of extracted apoplasmic sap using the centrifuge method, cell turgor was unable to be estimated during the early stages of kiwifruit development in this study. However, similar work on grapes has shown a linear relationship between berry elasticity and cell turgor (Thomas et al., 2008; Wada et al, 2009). Both cell turgor and berry elasticity decreased significantly between 50–59 DAA (Thomas et al., 2008). This is known as Stage II in the development of the grape, the time before the beginning of veraison. The correlation between the two factors was inferred to be due to cell turgor being the primary component that determines berry firmness. This point of time is also important as the reduction in cell turgor was shown to be related to rapid phloem influx (Greenspan et al., 1994) and sugar accumulation (Wada et al., 2009).

Using this relationship between cell turgor and firmness, we are able to estimate the potential changes of cell turgor in kiwifruit, using berry firmness information. In kiwifruit, berry firmness, was measured using two separate techniques, the penetrometer, and skin callipers, however both methods returned similar results. Berry firmness, was relatively stable for the majority of development, but decreased after 153 DAA and continued to fall until the final sampling date, 228 DAA. Therefore, we can estimate that cell turgor may also have decreased at this same point in time, 153 DAA. This time point relates to the rapid accumulation of soluble solids. At 153 DAA, the soluble solids concentration of the fruit was beginning to rise rapidly.

In grapes, during the period of rapid sugar accumulation, it has been shown that solutes accumulate in the apoplast (Wada et al., 2009). In theory, this will reduce cell turgor permeating into the phloem, therefore allowing bulk flow to continue delivering high concentrations of solutes (Matthews & Shackel, 2005). Further evidence of this is the transition from a symplasmic to an apoplasmic pathway of phloem unloading at the beginning of veraison (Zhang et al., 2006). Unloading apoplasmically, takes solutes out of the phloem into the apoplast thereby dampening the pressure difference between the import phloem and terminal release phloem. This ensures phloem transport will continue from source leaf to the ripening fruit. In kiwifruit, the symplasmic tracer dye, CF was restricted after 91 DAA, evidence of a change from a symplasmic to apoplasmic pathway of unloading. However, due to $^{14}CO_2$ labelling results, this could not be confirmed. Although CF gave evidence of a transition, it is unlikely that a change to an apoplasmic pathway occurred at this point in time. At 91 DAA, fruit were very firm, dry matter was still accumulating and the soluble solids concentration hadn’t began to rise. In grapes, a change to an apoplasmic pathway at veraison correlated with a reduction in cell turgor, however this was not the case in kiwifruit.

Future Research

It would be valuable to estimate cell turgor in kiwifruit for the duration of development. This study compared two methods of apoplasmic sap extraction, used for the indirect estimation of cell turgor. Results showed that the pressure chamber method was the best method to extract sap in comparison to the centrifuge, which produced highly contaminated samples. Using the pressure chamber extraction method throughout development will give better estimates of cell turgor, and it can be determined whether a relationship between cell turgor and fruit firmness exists, as in grapes.

Further research is required in order to confirm the unloading pathway. Previous phloem unloading studies used electron microscopy to visualise the presence or absence of plasmodesmata between the sieve element-companion cell complex and surrounding sink cells (Zhang et al., 2004; Zhang et al., 2006; Hu et al., 2011). Furthermore, electron microscopy can also be used to determine whether plasmodesmata frequencies have changed, or whether they have branched, as it is hypothesized that branching may limit symplasmic connectivity (van Bel, 2003). Gene expression of apoplasmic unloading related enzymes could also be investigated further to determine whether this pathway exists (Viola et al., 2001; Nardozza, 2007).

CONCLUSION

The kiwifruit berry is recognized as one of New Zealand’s most important horticultural products. Therefore, it is important that research is carried out that will continue to expand our knowledge of this valuable fruit. This study has done this. The components that affect fruit quality such as size, firmness and dry matter accumulation have been analysed throughout berry development. Berry growth has been shown in this study to be double sigmoid. Berry firmness was successfully measured using a novel method, the skinfold. Dry weight accumulated with the expected temporal pattern, and was compared to the results of later experiments involving phloem unloading and cell turgor.

One aim of this study was to determine the best method of apoplasmic sap extraction, which could be used to estimate cell turgor using the symplasmic, apoplasmic and matric potentials. This work was successful in doing this. The centrifuge method contaminated the apoplasmic sap therefore giving unreliable estimates of turgor. However the pressure chamber method extracted apoplasmic sap that was less contaminated. Cell turgor calculations using this sap were more realistic. For future estimates of cell turgor in kiwifruit using the indirect method, the pressure chamber should be used to extract apoplasmic sap.

Carbohydrate accumulation in the fruit relies upon the phloem network to transport sugars from source to sink. The analysis of the phloem unloading pathway, believed to be a limiting factor to carbohydrate accumulation showed that a transition from a symplasmic to apoplasmic pathway probably does occur during development.

This study has answered a range of questions regarding the development and vascular functioning of the kiwifruit berry. New methods have been developed to assist apoplasmic sap extraction, CF dye loading and the measurement of berry firmness. Although future research is required to fully understand all of the limiting factors of carbohydrate accumulation by the kiwifruit berry, this study has provided some of the building blocks for which further research can utilise.

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