Structure, function and quality development in apples

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Dedication

This thesis is dedicated to my family in recognition of the suffering they endured and the love they invested in me.

Abstract

Relationships between structure and function in apples were assessed in a series of experimental studies on different aspects of fruit quality development. A holistic model is presented that describes the major contributing elements. Special attention is directed to the mechanisms underlying the various physiological responses.

The study of the relationship between seed set, fruit shape and fruit minerals showed that fruit growth was modular and was strongly influenced by seed distribution and seed growth. This pattern influences the spatial distribution of minerals (e.g. calcium) within the fruit and thus is likely to impact on fruit storage quality. The nature of the developmental stimulus was investigated by *in situ* applications of the auxin-transport inhibitor, N-(1-Naphthyl)phthalamic acid (NPA). NPA application reduced vessel differentiation in the stalk, enhanced fruit abscission, and led to a reduced seed and flesh growth. Because of the specificity of the action of NPA, the identity of the developmental stimulus is most likely to be auxin.

The slowing and eventual stoppage of calcium import by apples is probably due to a decline in the functionality of its xylem. This decline in functionality was shown to be due to a physical disruption of the vascular bundles caused by flesh expansion. The extent of xylem dysfunction was governed by the structure and character of the tissue surrounding a particular bundle type. Flesh expansion also influences the textural properties of the fruit. The spatial distribution of intercellular air within the fruit and the shape and mutual disposition of the flesh cells were assessed using novel techniques. The radial pattern of intercellular air was not uniform indicating features that are likely to impact upon gas transfer within the fruit and thus on storage behaviour.

The mechanistic understandings gained in this thesis permit the elucidation of complex interrelations between the processes of fruit quality development. The work offers new insights into the origins of some physiological defects and indicates new lines for future research.

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Chapter 1. General introduction

1.1. STATEMENT OF THE PROBLEM

World production of apples is about 60 million tonnes of which *c.* 8% (5 million tonnes) is traded internationally. New Zealand exports contribute almost 6% (286,000 tonnes) to the international market although the total production of apples in New Zealand is less than 1% (486,000 tonnes) of world production (Anon., 2001). This emphasises the importance of the quality image of the New Zealand apple in international marketing.

Consumers demand consistent and uniform fruit quality whereas apple fruit are prone to a range of quality defects induced during the growth period or after the harvest. These defects are of serious concern to growers. Also, as market preferences can change quite quickly, the New Zealand apple grower must be able to respond. Because the international market is very competitive this imposes considerable pressure to develop strategies that accommodate these changes without impacting profit.

To develop the strategies required to efficiently manage a crop, it is necessary to elucidate the origins of quality defects. Knowledge of the factors that lead to the development of a particular quality defect might not only assist in adjusting management practices but might also help identify genotypes at risk. Market-selected quality attributes are, therefore, the primary target, and can only be optimised through an understanding of the key factors that affect fruit physiology.

However, the factors associated with development of some fruit quality attributes are either poorly understood or completely neglected. Management practices are often unable to remedy negative impacts or are applied in the final stages of fruit quality development with often small effects but at large expense. Hence, an understanding of the mechanisms underlying physiological responses that affect final quality attributes of an apple is essential to optimising both selection during breeding programmes and management practices.

Generally, quality assessment of a fruit is based on particular market preferences for particular quality attributes. Hence, fruit quality cannot be simply defined, and further is affected by genetic predisposition, management practices and environment. However, quality criteria generally include major organoleptic and visual preferences such as flavour, texture, appearance, size, shape and storage characteristics (Janick *et al.*, 1996).

Fruit development can be conceptualised as a series of physiological responses of constituent parts that establish the principal quality attributes of the apple fruit. The final characteristics of the fruit could, therefore, be aligned with development of a particular tissue such as proposed in Fig. 1.1.

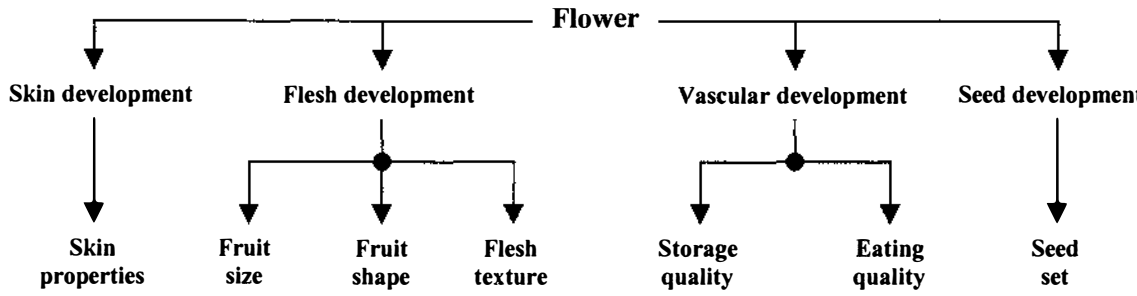


Fig. 1.1. General assessment of tissue growth and major quality attributes of apple fruit.

Fruit quality is dependent on many aspects that contribute to apple fruit development (Fig. 1.1). However, the emphasis in the following literature review is on those aspects that were investigated experimentally in this thesis. Of particular interest were the links between the elements of developmental quality paths towards fruit shape, flesh texture, storage quality and seed set. For completeness, other aspects of fruit quality development shown in Fig. 1.1 will also be considered.

1.2. STRUCTURE OF APPLE FRUIT

1.2.1. Structural origins of apple fruit

The basic anatomy of the apple fruit is well established and in the present review Esau (1977) was used as the general reference base. By definition, the fruit is a product of a matured ovary, although edible parts may also derive from extracarpellary tissues of the flower. An apple is a *pome*, which consists of two parts that originate from different floral structures but their interpretation initiates two hypothesis that favour different terminology (Pratt, 1988). The origin of the extracarpellary tissue outside the core line is subject to debate with one view favouring an origin from the flower receptacle (receptacular interpretation), the other an origin from the fused floral appendages (apendicular interpretation). For the sake of convenience, rather than supporting a particular concept, in this thesis the carpellary tissue (ovary) inside the core line will be referred to as the 'core' with a fleshy 'pith', whereas the extracarpellary tissue outside the core line will be referred to as the 'cortex'. The gross structure of apple fruit in transverse section is shown in Fig. 1.2.

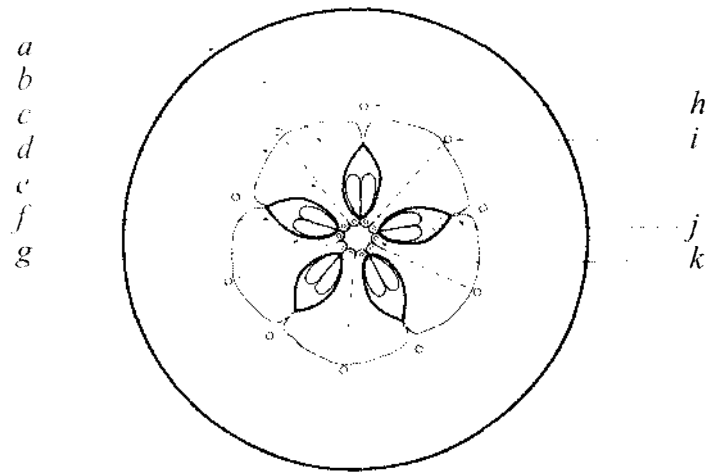


Fig. 1.2. Transverse section of mature apple fruit (*Malus domestica* Borkh.). The structure of the fruit is based on 'Braeburn' with carpel partitioning adapted from Esau (1977). To clarify the details of some structures (e.g. the ventral bundles) the relative proportions of these tissues have been slightly adjusted. The symbols are: *a*, skin; *b*, extracarpellary tissue ('cortex'); *c*, carpellary tissue ('core'); *d*, core line; *e*, carpel margins; *f*, cartilaginous endocarp ('locule'); *g*, seed; *h*, sepal (primary) bundle; *i*, petal (primary) bundle; *j*, dorsal (carpellary) bundle; and *k*, ventral (carpellary) bundle.

1.2.2. Structure of the skin

The apple skin is a multi-layered tissue, comprising epidermal hairs (absent in mature fruit), cuticle, epidermis and hypodermis (Bell, 1937). The cuticle is the non-cellular waxy coating of the epidermis (Miller, 1982) which provides the major transport barrier for the diffusion of permeants (Riederer and Schreiber, 1995). The epidermis and hypodermis are, however, cellular structures and their unmodified cellulose walls are permeable to water migration (Burton, 1982). All these features make the skin the ultimate protective structure of the fruit. Furthermore, skin components influenced by environment and commercial practices can induce peculiar changes to the external features of the skin (Pratt, 1988), that are used for cultivar identification (Bultitude, 1983).

1.2.3. Structure of the extracarpellary tissue

The extracarpellary tissue is made up of fleshy parenchyma permeated with vasculature and intercellular spaces. The major constituents, however, are the parenchyma cells that determine the overall character of the tissue (Harker *et al*, 1997). Mature parenchyma cells are large, vacuolated, multifaceted forms with thin, non-lignified walls under internal pressure (Pitt, 1982). Depending on their position within the tissue, parenchyma cells vary in their wall thickness, size, shape and packing arrangements (Harker *et al.*, 1997).

1.2.4. Structure of the carpellary tissue

The syncarpous ovary is differentiated into a cartilaginous endocarp defining five locules that are embedded in the fleshy part of the pericarp. Each locule has 2 – 4 ovules that become seeds after fertilisation (Pratt, 1988). The carpels of the ovary become fused along their margins in the later stages of growth (Esau, 1977), which indicates a degree of physiological autonomy in carpel development. The ovary extends from the centre of the fruit to the core line, which is a distinct sheet of parenchyma cells that demarcates the line of fusion with the adnate extracarpellary tissue.

1.2.5. Structure of the vascular tissue

The conducting framework of apple is made of vascular tissues, xylem and phloem. The xylem principally carries water and minerals while the phloem carries organic materials.

Both vascular tissues have highly specialised conduits, consisting of tube-like series of cells arranged at the ends to provide continuous flow. In the xylem, these are vessels, the files of finite length made of dead vessel elements with perforations at the end walls that enable free sap movement within the vessel. Intervascular pitting (bordered pits) on the walls enables sap movement between the vessels. In the phloem, however, sieve tubes are made of living sieve elements with porous plates that connect the modified protoplast of the cells. By analogy with the concept of the xylem path, the phloem source to sink path might be broken up into the number of sieve tubes of finite length (Lang, 1979).

The vasculature in apple is morphologically and physiologically organised into elongated strands known as vascular bundles, whose basic pattern of ramification has been assayed by many workers (McAlpine, 1912; Kraus and Ralston, 1916; MacArthur and Wetmore, 1939; MacDaniels, 1940). Vascular bundles are arranged within two distinct systems, 'cortical' and 'carpellary', in regard to their position and role within the fruit. The cortical vascular system has ten primary bundles that bend around the drupe-like carpels, giving off a skeleton of traces which ramifies across the flesh. Sepal bundles (opposite the locules) alternate with petal bundles in a ring, somewhat aligned with the core line. The carpellary vascular system is composed of ten ventral and five dorsal bundles that anastomose with each other. The paired ventral bundles emerge from the remaining stele after the separation of primary bundles to form a ring inside the locules, giving off lateral traces to the ovules. Dorsal bundles diverge from primary (sepal) bundles, swinging around the locules and terminating together with ventral bundles in the fused style of the pistil.

The vascular system of the fruit includes the stalk (Pratt, 1988). The stalk of an apple is a stem-like structure connecting the terminal fruit with a short spur. Vessels embedded in non-conducting structural components of the stalk provide bi-directional sap flow and mechanical support essential for fruit development. However, other than describing the essential structural features (Kraus and Ralston, 1916; MacArthur and Wetmore, 1939) or growth (Prive *et al.*, 1988; 1989), relatively little work has been done to quantify the dynamics of tissue development in the apple stalk (Barden and Thompson, 1963; Habdas *et al.*, 1982).

1.2.6. Structure of the seed

The seed is the primary organ of the apple as it provides the continuity of the species. The seed develops as the product of a fertilised ovule and differentiates into the embryo enclosed by stored nutrients (endosperm) and seed coat (testa). The embryo contained within the seed originates from a fertilised egg (zygote) and develops into the seedling at the expense of endosperm. The testa is derived from the integuments enclosing the nucellus of the ovule and protects the embryo (mechanically and biochemically) until germination.

1.3. THE ATTRIBUTES OF FRUIT QUALITY

1.3.1. Skin properties

The appearance of the skin strongly influences consumer preference while the actual area and structure of the skin are essential for gas exchange. The resistance of the skin to gas diffusion tends to exceed flesh resistance by around 10- to 20-fold, which makes the skin a primary barrier to the permeance of the gases in the apple fruit (Solomos, 1987). However, during the growth period, apple skin is meristematically active (Tetley, 1930; Harker and Ferguson, 1988a) and skin permeance to gases (O_2) increases as the fruit matures (Andrich *et al.*, 1989).

In addition, the permeance of the skin to water vapour regulates the weight loss of apples which is mainly due to water loss (Wilkinson, 1965; Maguire *et al.*, 2000). This appears to be an important aspect of quality development because inadequate weight loss during fruit ripening can lead to either physiological disorders or skin blemishes (Wilkinson, 1965; 1970; Martin *et al.*, 1967; Scott and Roberts, 1967, 1968; Wills and Scott, 1972; Johnson, 1976; Hatfield and Knee, 1988; Maguire *et al.*, 2000).

1.3.2. Fruit size

Fruit size is one of the physical growth attributes of a fruit and an important market preference (Opara, 2000). Growth towards final size is accompanied by significant changes in cellular structure that involve increase in cell number (cell division), cell size (cell expansion) and intercellular spaces (Pratt, 1988). The latter is indicated by a greater

increase in fruit volume than in fruit weight and a decrease in fruit density (Bain and Robertson, 1951; Skene, 1966; Westwood *et al.*, 1967). Larger fruit are usually softer (Harker *et al.*, 1997), having higher intercellular air volumes than smaller fruit (Reeve, 1953; Ruess and Stösser, 1993; Volz *et al.*, 2002).

Since the growth pattern is generally characterised by a short initial period of cell division followed by a long period of cell expansion (Bollard, 1970), there has been much discussion about the contribution of each stage to fruit size. A number of studies that often involved thinning and crop load effects on fruit size (including alternate bearing) showed high degrees of contradiction. In one view, cell number is the primary determinant of fruit size (Smith, 1950; Bain and Robertson, 1951; Pearson and Robertson, 1953; Sharples, 1968; Bergh, 1985; 1990; Goffinet *et al.*, 1995). Other reports favour cell enlargement as the major contributor to fruit size, (Martin and Lewis, 1952; Sharples, 1964). However, a combination of both processes prevails under certain conditions or treatments (Smith, 1940; Denne, 1960; Westwood *et al.*, 1967; Blanpied and Wilde, 1968).

The significance of air volume on fruit size is also a matter of controversy. Tetley (1930), MacArthur and Wetmore (1941) and Reeve (1953) claimed that fruit enlargement at the end of the season was mainly due to an increase in the size of intercellular spaces. In contrast, Skene (1966) and Goffinet *et al.* (1995) suggested that development of intercellular spaces alone was not sufficient to account for the majority of late fruit growth. Nevertheless, in both opinions, the air fraction of the flesh was a significant contributor to the volume of the fruit.

It appears that the growth responses of different cellular structures to different environmental and management stimuli are not well understood. Furthermore, the evidence cited above points to the physiological balance that can trigger a separate developmental process in regard to the nature, time period and strength of the stimuli. The assessment of the relationship between fruit size and character of growth is necessary to establish the timing and the nature of cultural practices that will balance size preferences with storage potential.

1.3.3. Fruit shape

Fruit shape is one of the key determinants of quality in apples. It is geometrically defined by a heart-shaped outline extended between two points of arrest (Thompson, 1966). Shape is a cultivar characteristic (Bultitude, 1983) and requires visual uniformity within a pack (ENZA, 1995). A significant effort was made to graphically visualise and identify principal shape traits from a broad population of genotypes, so as to determine the origins of shape defects (Currie *et al.*, 2000). Among five independent shape traits, 'asymmetric-sides' (lopsidedness) was determined to be mainly due to non-genetic effects as the heritability of the trait was close to zero.

Indeed, standard shape defects in lopsided apples includes dropped shoulder at the calyx end (ENZA, 1995), which was related to seed development (Brookfield *et al.*, 1996). The physiological basis of shape defects can, therefore, include the difference in the distribution of the growth within the developing apple. Skene (1966) showed that longitudinal growth was more pronounced around the calyx end which indicates why the most conspicuous change in tissue growth occurred at this part of lopsided fruit.

The asymmetry of lopsided apples indicates specific morphological and physiological features underlying this phenomenon. The ovary consists of five carpels that fuse with one another at a later stage of fruit development (Esau, 1977). This structural feature could be of great importance in elucidating the nature of shape defects because it implies that the growth of the ovary is modular in nature. This could change the holistic approach to assessing shape defects because the apple could than be viewed as an assemblage whose asymmetry is associated with uneven development of replicate parts. Spatial asymmetry confined to a particular part of the fruit (Heinicke, 1917; Simons, 1965; Way, 1978) conforms to the modular concept of the fruit.

In this thesis, the effect of the modular nature of growth of the ovary on extracarpellary growth and mineral composition is assessed in regard to seed set.

1.3.4. Flesh texture

Harker *et al.* (1997) concluded that the textural properties of apple are determined by the physical characteristics of the cells and the associated patterns of cell packing. Flesh is comprised of parenchyma cells with non-rigid walls so that tissue strength is the resultant of internal pressure (turgor) that develops in each cell, the properties of cell walls including the nature of bonds between adjacent cells and the network of intercellular spaces (Pitt, 1982).

1.3.4.1. *Cell wall.* The primary cell wall is a network of cellulose microfibrils impregnated by polysaccharides and protein (Knee and Bartley, 1981). The structural components of the cell wall can be grouped into the inner and the outer part, the latter, known as the middle lamella, is a gel-like structure where pectic substances provide the contact with adjacent cells (Demarty *et al.*, 1984). This composition enables cell expansion in rapidly growing fruit (through pectic and hemicellulosic components) and confers strength (through cellulose), which determines the shape of the cell (Harker *et al.*, 1997). The mechanical properties of cell walls (elasticity and rigidity) thus appear essential in determining the textural properties of the fruit. Furthermore, the cell walls are permeated with numerous pores radiating between pectic polymers that enable transport of solutes across the walls (Read and Bacic, 1996).

An important structural basis of texture includes the nature of cell-to-cell contact in apple parenchyma. Over half of the cell wall constituents are pectic substances (Jona and Foa, 1979), mainly galacturan chains with free carboxyl groups available for linkages (Ferguson, 1984). Calcium is thought to form the cross-links between the carboxyl groups of pectin molecules, both in the middle lamella (Knee and Bartley, 1981; Ferguson, 1984) and the inner part of the cell wall (Demarty *et al.*, 1984). Reversing the loss of firmness during ripening by infiltrating fruit with calcium confirms the essential role of calcium ions in the structural integrity of the cell wall (Stow, 1989; Glenn and Poovaiah, 1990; Siddiqui and Bangerth, 1996).

Fruit ripening is characterised by two major chemical changes in cell wall structure: the fractional increase of soluble pectin, presumably originating from the pectin depositions of the middle lamella (Knee and Bartley, 1981), and the loss of non-cellulosic neutral

sugar components of pectic polysaccharides, namely galactose (Gross and Sams, 1984). In addition, Ferguson (1984) suggested that calcium could affect postharvest changes in texture either through the structural stability (binding) to the cell wall or through functional interactions with components other than the cell wall. Electron microscopy of ripe apple fruit, however, showed that cell wall breakdown was principally encountered in the middle lamella (Fuller, 1976; Ben-Arie *et al.*, 1979; Glenn and Poovaiah, 1990; Siddiqui and Bangerth, 1996). These observations suggest that structural changes occurring in the region of cell-to-cell contact are likely to influence the cohesive strength of the parenchyma.

Indeed, the loss of cohesion in stored apples was associated with low adhesion between adjacent cells (Glenn and Poovaiah, 1990; Harker and Hallett, 1992) such that early season apples usually have lower cell adhesion and become mealy sooner than late season apples of the same age (Khan and Vincent, 1993). Furthermore, the lower stiffness of the apple parenchyma could be related to the reduced amount of cell wall material in early season cultivars (Khan and Vincent, 1993).

Considering that the walls of adjacent cells are held together by the calcium cross-links with pectic polymers (Knee and Bartley, 1981), it seems likely that calcium accumulation could promote the strength of the cell wall network. However, Siddiqui and Bangerth (1996) found no relationship between flesh firmness and the amounts of calcium associated with pectin fractions of purified cell walls. This raised the question of the mode of action of calcium in maintaining the structure and functioning of the cell walls. Saftner *et al.* (1998) found that the calcium binding capacity increased as the fruit softened which implied that only the ratio of wall-bound calcium to the total binding capacity of the cell wall could be taken to explain the maintenance of fruit firmness.

1.3.4.2. ***Cell shape.*** The shape of cells differs with respect to their position within the flesh and appears closely related to the increase in tissue organisation towards the centre of the fruit. Subepidermal cells are small and periclinally flattened. A marked increase in cell size with depth also brought change to the shape of cells that become spherical in the outer cortex. However, the increase in cell orientation towards the interior is followed by

the gradual change in aspect ratio and the cells become increasingly elongated (Bain and Robertson, 1951; Reeve, 1953; Khan and Vincent, 1990; Ruess and Stösser, 1993).

Parenchyma cells tend to be of a distinct 14-sided shape, known as tetrakaidecahedra (Lewis, 1923) that enables the most efficient packing (Korn, 1984). By analogy with plant cells, physical models approaching this form were established by compressing spherical lead shots in a cylinder (Marvin, 1939; Matzke, 1939). These assessments showed the importance of contact area and pressure of growth in multi-cellular tissues, although the intercellular spaces that normally would have developed in the tissue were abundant after the compression. However, parenchyma cells in the mature apple are loosely arranged and the area of cell-to-cell contact was estimated to be less than 40% of the overall cell wall area (Vincent, 1989), which signifies the importance of tissue growth dynamics in cell shape determination.

There is no progressive change in the aspect ratio of cortical cells in apple (Lang, unpublished) although the cells became more angular as the fruit mature (Blanpied and Wilde, 1968). The stability of the cell aspect ratio suggests that uniform physical force acts on the walls of rapidly growing parenchyma cells so that cell edges expand at a constant rate (Korn, 1980).

1.3.4.3. *Cell packing.* Subepidermal cells of apple consist of compact layers of cells, which change gradually towards the interior of the fruit. Progressing inwards, cortical cells initially appear randomly distributed and loosely packed that clearly indicates the presence of intercellular spaces. However, a transition from a random to a radial arrangement occurs deeper within the flesh. The cells become more elongated and organised into radial tiers with a network of markedly elongated intercellular spaces lying in between, which form air channels up to 3,000 μm long (Reeve, 1953; Khan and Vincent, 1990).

The mechanical properties of apple parenchyma closely reflect the patterns of cell packing. Apple parenchyma is highly anisotropic and heterogeneous (Vincent, 1989; Khan and Vincent, 1990) such that the specimen collapses in a single line when compressed radially but fails in shear when compressed tangentially (Khan and Vincent,

1993). Mechanical tests shows that parenchyma stiffness is a function of cell-to-cell contact. Hence, radially orientated cell tiers and intercellular spaces of the tissue are thought to influence the path of the crack (Vincent, 1989; Khan and Vincent, 1993).

It was proposed that the expanding cells tend to reduce mutual adhesion by turgor-driven cell rounding (Vincent, 1992). The same mode of action was thought to increase the volume of intercellular spaces in ripening apples (Hatfield and Knee, 1988; Harker and Hallett, 1992). However, the concept of cell rounding requires either progressive increases in cell turgor or decreases in contact area between the cells. The evidence suggests that cells maintain their osmotic properties and burst rapidly when incubated in water but leakage becomes more extensive as fruit mature (Simon, 1977). Cell shape appears constant during growth while the osmotic concentration of the cell wall free space increases (Lang, unpublished). The alternative view is that the cell-to-cell contact areas do not increase as fast as cell-to-air areas (Lang, unpublished). This may have the result that the intercellular space fraction increases relative to the cell fraction without changes in the pattern of cell packing.

1.3.4.4. ***Tissue and air volume of the fruit.*** The ratio of tissue volume to air volume changes as a result of the collective dynamics of the physical properties and developmental patterns that take place during fruit growth. It is well established that the volume of intercellular spaces increases as fruit mature (Bain and Robertson, 1951; Skene, 1966; Westwood *et al*., 1967; Soudain and Phan Phuc, 1979; Goffinet and Maloney, 1987; Harker and Ferguson, 1988b; Yamaki and Ino, 1992; Ruess and Stösser, 1993) including during postharvest storage (Soudain and Phan Phuc, 1979; Hatfield and Knee, 1988; Harker and Hallett, 1992; Ruess and Stösser, 1993). This has the result of increased tissue aeration of developing fruit while the character of intercellular spaces influences the mechanical properties of the flesh (Vincent, 1989; Khan and Vincent, 1993).

The intercellular spaces of apples, like the cells, are distributed anisotropically. Progressing inwards, the intercellular spaces showed increased radial orientation that results in formations of elongated air channels, greater than the actual size of voids between the adjacent cells (Reeve, 1953; Khan and Vincent, 1990). The change in aspect

ratio from the randomly orientated and roughly isodiametric intercellular spaces of the outer flesh to the spatially arranged air channels of the interior lying in between the columns of cells may have general implications for the character of gas exchange. These air channels are enriched with CO_2 emanating from respiring cells and mainly serve to exchange gases with the surrounding atmosphere, either solely (Solomos, 1987) or in a combination with fluid/solid matrix of the flesh (Rajapakse *et al.*, 1990).

This signifies the critical role of the intercellular network in fruit development because the volume, distribution and degree of communication between intercellular spaces determine the physical limits of respiration across the apple flesh. Vincent (1989) claimed that the radial anisotropy of apple prompted specific patterns in communication between intercellular spaces that mainly occurred along the radius with negligible lateral contacts. Hence, a tissue with a small air volume is likely to develop steeper gradients in the concentration of gases along the radius of the fruit. This could affect both the respiratory metabolism of the fruit as well as oral perception of flesh texture.

In this thesis, the spatial character of air and cell alignments was assessed in stored apples. A gravimetric technique based on Archimedes' principle with calculated adjustment for air lost along the sample edges was used for accurate measurements of tissue air content.

1.3.5. Storage quality

A major contributing factor to the storage quality of fruit is mineral composition (Ferguson and Watkins, 1989). In this section, emphasis is given to the relationship between the structure and function of the xylem and how this influences the postharvest condition of the fruit.

1.3.5.1. ***Vascular development – induction and growth pattern.*** There is evidence that all of the classical plant hormones may be involved to some extent in complex patterns of vascular differentiation (Aloni, 1995). However, the emphasis will be given to the role of auxin in controlling xylem differentiation (xylogenesis).

The fact that vascular tissues are organised as distinctive longitudinal strands stretching from developing organs to roots points to a possible inductive signal that enables vascular differentiation along a file of cells. Jacobs (1952) demonstrated that auxin (IAA) produced by the young leaves or applied as a synthetic substitute after defoliation of the stem promoted basipetal xylem regeneration in *Coleus* plants. These results implied that xylogenesis was induced and limited by polar auxin movement. Sachs (1981) concluded that basipetal auxin diffusion through undifferentiated cells resulted in vascular strands that become the preferred path for further auxin flow. Furthermore, the increase in vessel size and decrease in vessel number along the plant axis was associated with a gradual decline in auxin concentration (Aloni and Zimmermann, 1983).

The induction of the two sorts of vascular tissues takes place at different auxin concentrations. Phloem differentiates at low auxin levels, often in the absence of xylem formation (Aloni, 1980; Aloni and Barnett, 1996), while xylogenesis occurs only at higher auxin levels (Aloni, 1980). Anatomical similarities in the structure of plants grown in similar environments fit with the proposed polar pattern of xylem differentiation. The short growth period characteristic of extreme habitats (desert, arctic and alpine) was thought to evoke vigorous auxin production that results in rapid differentiation of numerous but narrow vessels. However, longer duration of growth (humid tropics) favours lower auxin levels that, over the extended period, produce fewer and wider (therefore more efficient) vessels (Aloni, 1987). These effects are consistent with the patterns of vessel differentiation observed in transgenic plants with altered production of endogenous auxin (Klee *et al.*, 1987; Romano *et al.*, 1991).

There are two developmental stages of the vascular meristem: the procambium that initiates primary (proto- and meta-) formations and the cambium that initiates secondary formations of vascular tissue. The different forms of wall thickenings characterise specific ontogenetic series. In vessels, annular (ring) thickening occurs during initial development, followed by helical (spiral) thickenings, reticulate (netlike) thickenings and finally pitted elements. The unlignified walls of sieve tubes lack characteristic patterns of thickening. The proto-formations become dysfunctional early in development due to excessive stretching generated by the growth of surrounding tissues. The meta-

formations remain functional after primary growth but cease to function with the commencement of secondary development (Esau, 1977).

1.3.5.2. ***Xylem functionality – diurnal and seasonal changes.*** Xylem function exhibits specific diurnal and seasonal changes. The diurnal growth rhythm of apple fruit occurs as the result of daily cycles (Tukey, 1964; Jones and Higgs, 1982; 1985; Higgs and Jones, 1984; Tromp, 1984) where xylem inflow from the tree at night replaces xylem outflow to the tree during the previous day (Lang, 1990; Lang and Volz, 1998). Fruit volume not only varies with time of day but depends upon the severity of weather conditions (Jones and Higgs, 1982, 1985; Higgs and Jones, 1984; Tromp, 1984; Lang, 1990; Lang and Volz, 1998).

The seasonal pattern revealed an imbalance between the contributions of the xylem and phloem to apple growth. Lang (1990) demonstrated that sap flow into the fruit changed progressively in favour of the phloem and the change was more marked in the cultivar susceptible to the calcium-related physiological disorder, bitter pit. Subsequently, Lang and Ryan (1994) concluded that the progressive decline in xylem conductance was due to dysfunction of the vessels within the fruit, and that the decline in xylem conductance was again more severe in bitter pit susceptible cultivar.

These findings agree with changes of xylem function observed in fruit of other species (Findlay *et al.*, 1987; Düring *et al.*, 1987; Creasy *et al.*, 1993; Wolswinkel *et al.*, 1999). A plausible mechanistic explanation predicts the growth-induced rupture of fruit xylem (Findlay *et al.*, 1987), even the fragmentation of whole bundles (Düring *et al.*, 1987; Wolswinkel *et al.*, 1999). The physical disruption of the xylem coincided with the sudden expansion of the grape berry (Findlay *et al.*, 1987; Düring *et al.*, 1987) while the phloem remained functional (Findlay *et al.*, 1987). The cellular properties of the berry changed subsequently (Lang and Düring, 1991). These lines of evidence indicate that all inter-linked effects are serving to adjust for high sink demand in advanced maturity so that the change in fruit mineral composition is the consequence of the mechanism that controls assimilate partitioning.

In this thesis, temporal and spatial patterns of the xylem function in apple were assessed by infusing the fruit with dye. In addition, the dynamics of tissue growth and microscopic examination of dysfunctional bundles were carried out to determine the character of changes during the development of the fruit.

1.3.5.3. ***Xylem functionality - mineral accumulation and storage quality.*** The dynamics of growth and function of the conduits appear to determine the mineral status of the fruit, which strongly influences fruit storage quality. This relationship is of particular importance in long-distance transport of calcium and the occurrence of bitter pit.

Calcium nutrition is characterised by the limited ability of apple fruit to accumulate and distribute calcium. The xylem is considered to be the principal pathway for calcium transport. This transport is thought to occur with the convective flow of water in this tissue (Ferguson and Watkins, 1989) although ion exchange between the flowing sap (the mobile phase) and the cell walls (the stationary phase) through which it is flowing will slow the flow of calcium relative to that of water (Marschner, 1986). Hence, the accumulation of calcium in the fruit will be governed by the hydraulic conductance of the xylem vessels, the calcium concentration of the sap and the driving force for flow.

The potential conductance of a xylem vessel is proportional to the fourth power of the vessel radius, so that wide vessels have a disproportionately large effect on flow (Zimmermann, 1983). The progressive dysfunction of the xylem in apple (Lang, 1990; Lang and Ryan, 1994) will reduce the hydraulic capacity of this tissue to the cross-sectional area of just the functional vessels and thus reduce the amount of xylem sap entering the fruit. Indeed, most of the calcium is imported early on in fruit development (Wilkinson, 1968; Jones *et al.*, 1983), because water flow entering the fruit is diverted *via* the phloem during the later part of the season (Lang, 1990).

The calcium concentration of the sap is the measure of available calcium for fruit uptake. The common method of mineral assessment includes extraction of the sap from a stem segment (Bollard, 1953; Bradfield, 1976; Ferguson and Bollard, 1976; Tromp, 1979; Jones *et al.*, 1983; Lang and Volz, 1998). Xylem sap extracted from the shoot showed the highest calcium concentrations early in the season, before calcium reached maximum

concentrations in the fruit, but with a declining trend from then on (Tromp, 1979; Jones *et al.*, 1983). More importantly, around half of the calcium present in the shoot sap was in ionic form (Ca^{2+}), while the rest was uncharged or in negatively charged complexes with organic acids (Bradfield, 1976). These complexes may expedite the mobility of calcium in the xylem (Ferguson and Bollard, 1976) probably by reducing the degree of adsorption at negatively charged exchange sites in the wall (van de Geijn *et al.*, 1979).

Diurnal cycling becomes more pronounced when the weather favours a high transpirational loss of water (Jones and Higgs, 1982, 1985; Higgs and Jones, 1984; Tromp, 1984; Lang, 1990). This may affect calcium accumulation in the fruit because spur-wood sap carries markedly more calcium than fruit sap while the volume of xylem inflow exceeds that of xylem outflow (Lang and Volz, 1998). Hence, defoliation of subtending spur leaves reduces calcium accumulation in the fruit (Feree and Palmer, 1982; Jones and Samuelson, 1983; Proctor and Palmer, 1991; Volz *et al.*, 1994; 1996a), presumably as the consequence of diminished xylem sap cycling (Lang and Volz, 1998).

The significance of calcium movement into the fruit has been extensively studied in relation to the occurrence of storage disorders, such as bitter pit, that has serious consequences for the economics of apple production. Ferguson and Watkins (1989) provided arguably the most comprehensive review on the role of calcium in postharvest physiology of apple fruit. Poor mineral nutrition and, in particular, low fruit calcium was related to the incidence of bitter pit. The disorder is characterised by discrete necroses in the subepidermal zone of the cortex, and is more frequently located at the calyx-end of the fruit. The localised nature of the disorder complies with uneven calcium distribution in radial and longitudinal directions of the fruit. A number of different factors are thought to affect fruit calcium and development of bitter pit, such as transport routes, diurnal growth, canopy foliage, and fruit growth. Other factors involved in the incidence of bitter pit could be related to genetic predisposition (cultivar susceptibility), climate and soils (growing regions) or crop maintenance (management practices, postharvest conditions).

1.3.6. Eating quality

Increases in fruit dry matter content reflect the continued import of assimilates throughout growth. Although phloem-driven, high fruit dry matter content is profoundly influenced by changes in xylem conductivity during growth. The mechanism proposed for grapes states that the xylem breakdown in the berry around the onset of ripening prevents an outflow of sugary sap while the loss of membrane integrity in the berry (compartmentation breakdown) reduces the apoplastic osmotic potential (*viz* the osmotic potential becomes more negative). This attracts more assimilate into the fruit by reducing sieve tube turgor in the sink region (Lang and Thorpe, 1989; Lang and Düring, 1991).

The imbalance between xylem and phloem sap flow in apple may, in a similar way, serve to stimulate the flow of assimilates into the fruit. In young apple fruit, the osmotic concentration of the cell wall free space of the flesh (apoplast) is not insignificant and this concentration increases strongly during the season (Lang and Diack, unpublished). The very high values toward maturity must decrease cell turgor and thus will increase phloem sap flow into the fruit (sieve tube sap flow is proportional to the source:sink pressure difference).

Although a progressive decline of xylem conductance appears to be an integral part of fruit development (Lang and Düring, 1991), through physical disruption of the xylem strand (Findlay *et al.*, 1987; Düring *et al.*, 1987; Lang and Ryan, 1994; Wolswinkel *et al.*, 1999), there is no evidence that the phloem is able to withstand growth-induced stretching. However, during the latter part of the season, fruit growth is predominantly phloem driven (Lang, 1990), suggesting unchanged integrity of the phloem structures. The effect of stretching on a vascular bundle will depend on its stress:strain properties and its continued ability to grow.

1.3.7. Seed set

Seed set relies on many internal and external factors. In apple fruit, the number of ovules formed during flower development sets an upper limit for seed number (potential). However, irregularities in ovule and pollen development and unfavourable conditions for pollination can alter the number of seeds (actual).

1.3.7.1. *Ovule development.* The genetically determined limit for the number and biological characteristics of ovules is affected by various physiological and environmental factors that will impact on fruit setting. Ovule number, however, is more closely related to the dynamics of floral development that appears to be determined in the previous season (Williams, 1970).

The important characteristics of ovule development are the functional longevity and the fertility potential (Williams, 1970). Ovule longevity is essential for pollen transfer to stigmas and pollen tube growth in the style to be effective (Stott, 1972). Extended ovule longevity and the presence of more than one embryo sac in the nucellus of triploid ovules compensate for high ovule sterility and enables heavy cropping in triploid apple cultivars (Williams, 1970). In contrast, some diploid cultivars show a high proportion of ovule sterility resulting from irregularities in ovule development that are related to erratic fruit set (Howlett, 1928; 1938; Hartman and Howlett, 1954; Williams, 1970; Williams *et al.*, 1984; Costa Tura and MacKenzie, 1990).

The establishment of vascular tissues in the apple gynoecium is also related to ovule development. Inadequate vascularisation between ovule and placental tissue is associated with open calyx of the fruit, and favoured ovule abortion (Simons and Chu, 1968). The fact that early xylem lignification in the ventral carpellary bundles coincides with nucellus differentiation in the ovule (Costa Tura and MacKenzie, 1990; MacKenzie and Costa Tura, 1991) indicates a causative link between these events.

1.3.7.2. *Pollen development.* Commercial apple cultivars are generally self-incompatible and require cross-pollination to set fruit. Insect vectors, such as honey bees, usually carry pollen released from the anthers of the donor tree to the stigmas of the receptor tree (Janick *et al.*, 1996) so that close proximity of pollen source or hand pollination markedly improves seed and fruit set (Free, 1962; Free and Spencer-Booth, 1964; Williams and Smith, 1967; Maggs *et al.*, 1971; Way, 1978; DeGrandi-Hoffman *et al.*, 1990; Milutinović *et al.*, 1996; Schneider *et al.*, 2001). Similar patterns of distance-dependant pollen dispersal were also confirmed using marked pollen donors (Wertheim, 1991; Kron *et al.*, 2001a; 2001b). Hence, the main characteristics of pollen development that could affect cropping are availability and effectiveness of pollen (Williams, 1970).

Pollen availability is determined by the amount of pollen released from the anthers, and strongly depends upon climatic conditions, specifically temperature and humidity (Williams, 1970). In some cases, the effect of flower morphology on pollination is a matter of controversy as the presence of wide basal gaps between stamens permits honey bees to extract nectar from flowers without touching the stigmas (Roberts, 1945; Robinson, 1979; Robinson and Fell; 1981; Kuhn and Ambrose, 1982; DeGrandi-Hoffinan *et al.*, 1985). This outcome is of particular importance for adequate pollination that appears more dependant on insect than wind-borne dispersal of pollen (Williams, 1970).

The effectiveness of released pollen is characterised by viability, compatibility and growth rate of the pollen tube (Williams, 1970). Viability denotes the germinating capacity of pollen, which is usually high in diploid cultivars (Stott, 1972; Deckers and Porreye, 1984). On the basis of pollen tube compatibility, Williams (1970) divided apple cultivars into three groups: invariably self-sterile, invariably self-fertile and variably self-fertile. Kobel *et al.* (1939) had assigned compatibility to a single multi-allelic gametophytic locus S, which regulates cropping by preventing inbreeding if the pollen carries the same alleles as the pistil. This phenomenon has been extensively studied since, and many apple genotypes characterised for the presence of S alleles (Manganaris and Alston, 1987; Sassa *et al.*, 1994; Batlle *et al.*, 1995; Janssens *et al.*, 1995; Broothaerts *et al.*, 1995; 1996; Sakurai *et al.*, 1997; 2000; Matsumoto *et al.*, 1999; Bošković and Tobutt, 1999; Goldway *et al.*, 1999; 2001; Schneider *et al.*, 2001).

The growth rate of the pollen tube is, therefore, principally governed by the genetically-controlled compatibility between donor and receptor tissues (Modlibowska, 1945; Haasbrook *et al.*, 1967; Stott, 1972), although temperature may influence this process (Modlibowska, 1945; Child 1966; Williams, 1970; Stott, 1972; Visser and Marcucci, 1983). Furthermore, two successive pollinations where the first 'pioneer pollen' promotes the growth of the second pollen, can produce more seeds in apple (Visser and Verhaegh, 1980; Visser and Marcucci, 1983; Visser *et al.*, 1983). This implies that the contribution of later (second) pollen is dominant to seed set.

1.3.7.3. ***Seed development.*** The dynamics of seed development in apple are described in a comprehensive review by Pratt (1988). The development of the seed generally undergoes three stages and closely accompanies that of the fruit. During the initial stage, the embryo grows slowly while the other fruit and seed structures develop rapidly. At the same time, the first wave of abscission (drop) of fruitlets takes place as a consequence of irregularities during fertilisation. The second stage commences with the change from a free-nuclear to a cellular endosperm (cytokinensis), which enables the rapid growth of the embryo at the expense of the primary endosperm. Secondary endosperm, developed and constantly regenerated by a peripheral meristem, replaces digested primary endosperm and enables continuous growth of the embryo until it attains its full size. Concurrently, the maturing apple also attains its maximum size. The cessation of meristematic activity in the secondary endosperm marks the completion of the third stage of embryo development. The testa hardens and changes colour and the seed enters a period of dormancy in the mature fruit.

In many pome fruit, there is a range of seed classes at maturity that are associated with irregularities in seed development. Generally, seeds that have undergone normal development can be categorised as 'viable' and seeds in which abortion has occurred at some stage can be categorised as 'aberrant' (Luckwill, 1953; Rohitha and Klinac, 1990; Brault and de Oliveira, 1995; Brookfield *et al.*, 1996; Broom *et al.*, 1998). 'Aberrant' seeds vary in size and appearance having small and deformed locular traces (rudiments) and expanded but flattened seeds with empty interiors (Rohitha and Klinac, 1990). Morphological discrepancies among 'aberrant' seeds indicate that changes may have occurred at various stages of growth, such that smaller seeds represent early aberrations in seed development, as noted in grapes (Barritt, 1970; Ebadi *et al.*, 1996).

1.3.7.4. ***Seeds in fruit development.*** Nitsch (1970) emphasised that overall fruit development is primarily dependent on pollination and the resultant seed formation. Stimuli for fruit growth commence with the germination of pollen and continue throughout fertilisation and development of seed structures, which in turn govern the development of the fruit. Several lines of evidence support the principal role of seeds in fruit development.

The growth of apple fruit is dependent upon the continuous presence of seeds, such that physical damage to the ovules induces severe fruit shedding (Abbott, 1959; Marini and Byers, 1988; Ward *et al.*, 1999), while increases the incidence of fruit deformity and reduces fruit size (Proctor and Schechter, 1992). Hence, an over-seeded apple (more than ten seeds in the fruit) is usually heavy bearing (Simons, 1974). The final size and shape of apple fruit are, therefore, directly related to the number and distribution of the seeds it contains (Heinicke, 1917; Alderman, 1918; Murneek and Schowengerdt, 1935; Latimer, 1931; 1937; Einset, 1939; Roberts, 1946; Denne, 1963; Way, 1978; Tomala and Dilley, 1989; 1990; Brault and de Oliveira, 1995; Brookfield *et al.*, 1996; Broom *et al.*, 1998).

It appears that seed set must precede fruit growth. Because the stimulus for growth commences with the onset of pollination (Nitsch, 1970), any fertilised ovule will exert an influence upon fruit growth. Hence, aberrations in seed development at different stages during fruit development will impact differently on the fruit (e.g. grapes, Ebadi *et al.*, 1996). Indeed, the relationship between seed set and size and shape of mature apple fruit are better explained when 'aberrant' seeds are included (Einset, 1939; Denne, 1963; Brault and de Oliveira, 1995).

Furthermore, seed development and storage quality appear closely related. Poor seed set or artificially induced parthenocarpy are associated with lower fruit calcium and/or higher incidence of physiological disorders (Heinicke, 1920; Bangerth, 1976; Greene *et al.*, 1982; Tomala and Dilley, 1989; 1990; Bramlage *et al.*, 1990; Brookfield *et al.*, 1996; Volz *et al.*, 1996b; Broom *et al.*, 1998).

1.3.7.5. ***Seeds as sites of auxin production.*** Since the biosynthesis of auxin is thought to occur in meristematic tissues (Swarup *et al.*, 2000), an obvious site for auxin production would be developing seeds. Several lines of evidence indicate that this is the case: seeds generally contain higher auxin levels than other fruit tissues (Gustafson, 1939; Nitsch, 1950; Varga and Bruinsma, 1976), increase in seed number stimulates the amount of auxin exported from apple (Bangerth *et al.*, 1989; Callejas and Bangerth, 1997; Bangerth, 2000), and terminal ('king') fruit which set first export more diffusible auxin than subordinate (lateral) fruit (Gruber and Bangerth, 1990). The earlier appearance and faster growth of terminal-borne fruit is likely to parallel that of seed development, which could

be taken to explain why 'king' fruit on average produce twice as much auxin while having only a slightly higher seed number than lateral-borne fruit of the same cluster (Bangerth, 2000).

It also appears that fruit dominance within the cluster could be mediated by the amount of auxin emanating from the fruit. The concept of 'primigenic dominance' proposed by Bangerth (1989) refers to inter-fruit competition in which the earlier developed fruit is dominating by means of stronger polar auxin export that suppresses the weaker stimuli coming from later setting fruit. Furthermore, the removal of 'king' flowers at the time of full bloom led to a significant increase in diffusible auxin from the remaining lateral fruit and *vice versa* (Gruber and Bangerth, 1990). This evidence is supported by the fact that external auxin application can mimic the dominating effect of the 'king' fruit in a cluster (Bangerth, 1993; 1997). The altered dominance and, therefore, fruit set is a self-regulating process, which depends upon the strength of the polar auxin signal (Bangerth, 2000). This view is supported by the results from Quinlan and Preston (1971) that showed an increase in fruit set after the removal of shoot tips.

In addition, the importance of polar auxin movement for calcium inflow to the fruit is well established. Banuelos *et al.* (1987) demonstrated the dependence of ascending calcium transport on a steady auxin flow from the opposite direction. The decreased supply of calcium in fruit treated with auxin transport inhibitors was independent of the transpiration stream and the cation exchange capacity of the fruit. In a number of other studies, the relationship between application of auxin transport inhibitors and calcium deficiencies or the incidence of physiological disorders of the fruit has been observed (Bangerth and Firuzeh, 1971; Bangerth, 1976; Himelrick and Ingle, 1981; Stahly and Benson, 1982; Stahly, 1986; Bangerth *et al.*, 1989; Tomala and Dilley, 1989; 1990). In some cases, an inhibition of auxin transport markedly suppressed seed development and promoted a physiological disorder (Hamamoto *et al.*, 1998). Although the physiological mechanisms for the regulation of long-distance transport of nutrients are complex, it appears that auxin production associated with seed development influences calcium uptake by the fruit.

1.3.7.6. *Cellular mechanism of auxin transport and development of storage quality.*
Indole-3-acetic acid (IAA) is considered to be the principal form of auxin in higher plants that is synthesised in young apical tissues and transported in a polar fashion towards the basal target tissues (Swarup *et al.*, 2000). Auxin has been implicated as a key factor involved in a range of developmental processes including cell division and expansion, vascular differentiation, root initiation, tropistic responses, apical dominance, leaf senescence, leaf and fruit abscission, flower growth, fruit set, growth and ripening and assimilate partitioning (Davies, 1995). Understanding of the mechanisms regulating these processes is essential to govern and adjust plant responses influenced by interactions between the tissues and environmental (cultural) growing conditions.

The long-standing "chemiosmotic hypothesis" of polar auxin transport proposes a carrier-mediated and energy-dependent process that occurs in a cell-to-cell fashion. The difference in pH is believed to move undissociated lipophilic auxin molecules across the cell membrane (by diffusion) or, to some extent, *via* specific uptake symport carriers distributed around the membrane. Inside the cytoplasm, auxin (weak acid) dissociates as the result of higher internal pH. A specific efflux carrier drives auxin anions across the cytoplasm by excretion at the basal end of the cell, which provides the polar nature of auxin transport (Lomax *et al.*, 1995).

Because auxin transport in the cell is mediated by influx and efflux carriers, the existence of carrier-specific inhibitors provide a useful tool in understanding the nature of events that takes place during polar auxin movement. Manipulations with phytotropins, synthetic inhibitors of auxin transport, suggest a high affinity with the efflux carrier (Lomax *et al.*, 1995). However, the knowledge of influx carrier function is still scarce, and the recent discovery of a novel class of carrier-specific inhibitors will provide useful tools to investigate the function and properties of the influx carrier (Imhoff *et al.*, 2000).

Manipulations with auxin transport inhibitors have shown the relationship between basipetal transport of auxin and acropetal calcium uptake of the fruit appears insensitive to either transpiration or exchange adsorption mechanisms of transport (Banuelos *et al.*, 1987). This infers that auxin and calcium transport pathways are directly linked, possibly through the mutually dependent interaction that also occurs in the opposite direction

(sunflower, dela Fuente and Leopold, 1973; sunflower and corn, dela Fuente, 1984; de Guzman and dela Fuente, 1984; 1986; zucchini, Allan and Rubery, 1991).

Furthermore, the nature of interaction between auxin and calcium could be involved in the character of dominance between the organs. The influx of calcium may facilitate the reciprocal release of auxin that sustains the dominating position of the organ. This will further reduce the supply of calcium and the efflux of auxin from the dominated organs, thus affecting the overall calcium status of these organs (Tamas, 1995). Hence, the export of auxin from the major sites of biosynthesis within the fruit could be essential to the calcium status and thus the storage quality of the fruit.

In this thesis, the nature of developmental stimuli is assessed through the effect of auxin transport inhibition on vessel differentiation in the fruit stalk, abscission, and seed and fruit development during the early stages of fruit growth.

1.4. CONCEPTUAL MODEL OF FRUIT QUALITY DEVELOPMENT

The review of the literature provides a solid insight into the origins and the paths of fruit quality development. Based on this evidence, the initial assessment shown in Fig. 1.1 was developed into the conceptual model shown in Fig. 1.3. The path towards each quality attribute was structured to accommodate physiological processes (text) and factors (ovals) that influence various tissue properties (boxes) along the path. Every element of the model is arranged in time and function such that some tissue properties can became 'externality factors' across the paths of quality attributes.

The potential usefulness of this model is to provide a broad framework for conceptualising the major aspects of fruit growth and development. The model allows for multiple interactions at any level of path development, which describe the mutual dependence between the quality attributes. In that way, each element of the model can be assessed for the hierarchical position within the path, which is time dependent. The model could, therefore, be used to indicate the origins of physiological defects in apple fruit and provide mechanistic links between the elements involved.

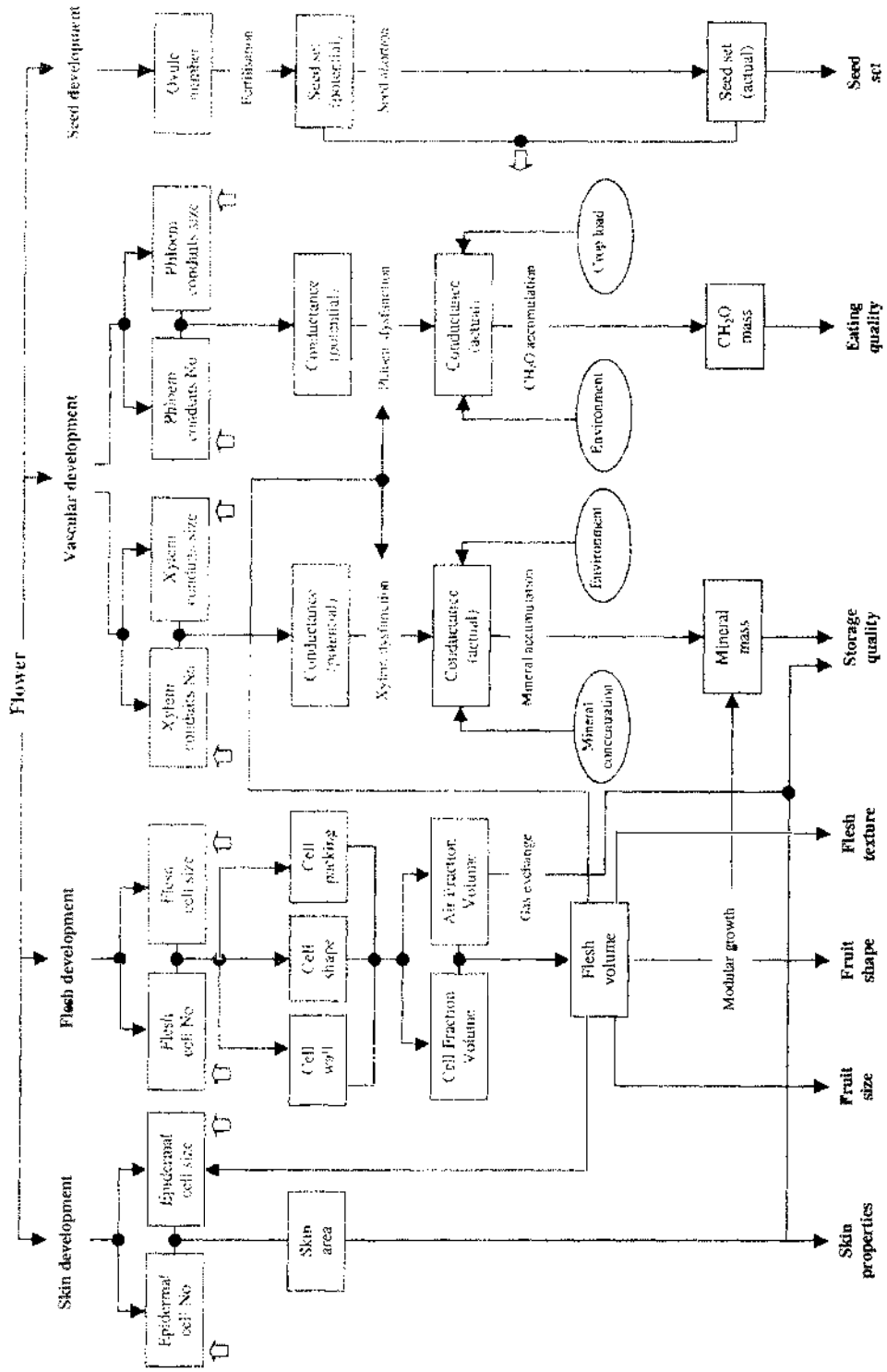


Fig. 1.3. Conceptual model of fruit quality development. The symbols are connecting the interactions between processes (text), factors (ovals) and properties (boxes) (-●-), and hormonal influences on tissue development (⏉).

1.5. RESEARCH OBJECTIVES OF THE THESIS

The aim of this thesis was to investigate the relationship between structure and function in different fruit tissues that impact on fruit quality development. The view was taken that some elements of developmental quality paths have not been fully examined in the light of current knowledge and their implications for both fruit physiology and apple industry have not been considered.

More specifically, the research objectives were:

- To determine the impact of seed set on fruit shape and fruit storage potential by recording its impact on modular flesh development and mineral distribution;
- To determine the nature and impact of developmental stimuli on fruit structure by manipulating auxin efflux from the fruit and recording vessel differentiation in the stalk;
- To determine the impact of different aspects of flesh growth and vascular morphology on xylem functionality during the season; and
- To develop an anatomical basis for the gas-exchange properties of apple tissue using a novel technique to determine the amount and disposition of intercellular air and, also, the shape and packing of the flesh-cells.

The primary research aims outlined above are the focus of the experimental studies presented in Chapters 2-5. As the experimental studies presented in this thesis differed from one another, a conceptual model of apple fruit growth was developed based on the literature (Fig. 1.3). The model identifies and links the various developmental and environmental elements that contribute to the overall development of fruit quality. In this way, each study falls within a path of the model which links to a particular quality attribute of the fruit.

Chapter 2. Seed set, fruit shape and fruit minerals

2.1. INTRODUCTION

The apple (*Malus domestica* Borkh.) is one of New Zealand's most important fruit crops with annual production of 486,000 tonnes of which *c.* 60% were exported and valued at NZ $339 million (fob), (Anon., 2001). International markets demand quality and consistency including uniform size, shape and blemish-free fruit. Significant quantities of fruit fail to meet critical shape criteria but the magnitude of these losses is unknown. Some calculations based upon losses in the packhouse suggest that *c.* 2.4% of the annual crop is rejected (Palmer, pers. comm.). This figure does *not* include lopsided fruit rejected by pickers in orchards.

The development of lopsided apple fruit has been attributed to poor and asymmetrical seed set (Heinicke, 1917; Latimer, 1931, 1937; Roberts, 1946; Way, 1978; Brault and de Oliveira, 1995; Brookfield *et al.*, 1996). Furthermore, a number of studies indicate a relationship between seed set and fruit calcium (Bramlage *et al.*, 1990; Tomala and Dilley, 1989; 1990; Brookfield *et al.*, 1996; Volz *et al.*, 1996b; Broom *et al.*, 1998), and the occurrence of physiological disorders (Heinicke, 1920; Bramlage *et al.*, 1990; Brookfield *et al.*, 1996). However, the rational relationship between seed set, fruit shape and fruit calcium (as distinct from a simple correlation) has not been properly examined either mechanistically or quantitatively.

Since the pomaceous ovary is made up of five fused carpels (Esau, 1977), the apple fruit can be viewed as an assemblage of replicate parts - fruit shape asymmetry suggests that each carpel tends to develop as a unit. If this is the case it is likely that the developing seed may have a localised effect on fruit development so that each carpel contributes somewhat autonomously to the overall fruit shape and calcium concentration. Therefore, the objectives of this study were to quantify seed variability, quantify seed asymmetry, relate seed set to fruit asymmetry, and relate fruit asymmetry to the spatial distribution of minerals. Preliminary data and analyses have been published (Drazeta *et al.*, 2000).

2.2. MATERIALS AND METHODS

2.2.1. Plant material

The study used mature trees of 'Granny Smith' apples grown at the Massey University Fruit Crops Unit, Palmerston North, New Zealand. Trees were planted on MM 106 rootstock and managed according to standard commercial practice including the usual calcium sprays. A total of 180 fruit were randomly collected at commercial harvest. Fruit were stored in a cool-store (0.5°C, *c.* 95% RH) for up to 8 months pending analysis. Day-to-day storage was in a cold room (2°C, no humidity control).

2.2.2. Seed variability, seed asymmetry and fruit asymmetry

To assess variability in seed weight, a total of 120 fruit without blemishes were processed. Fruit were weighed, then halved and the number and individual weights of the seeds were recorded. In addition, spatial variability of seed weights in the locules was obtained from a sample of 60 lopsided apples used for the assessment of fruit asymmetry.

In each locule, seeds can arise through fertilisation of either or both of two ovules, giving four possibilities - locules can contain (0,0), (1,0), (0,1), or (1,1) seeds. With five locules, there are, therefore, 4^5 or 1,024 possible seed arrangements. To quantify the asymmetry associated with uneven seed distribution an *index of seed asymmetry* (I) was calculated for each arrangement.

A value was calculated for I, for all possible seed arrangements amongst the five locules. To do this the fruit was treated as if it were a five-spoked wheel held in the horizontal plane with a number of uniform weights (corresponding to the seeds) variously distributed around its margin in line with the spokes. The asymmetry of any particular seed arrangement can be measured by the extent to which the centre of gravity of the wheel falls away from the axis. The index of asymmetry I is then quantified as the radial displacement of this imaginary centre of gravity from the geometrical centre. By analogy with the wheel:

$$x = \Sigma(m_i l_i \cos \alpha_i)/M \quad (1)$$

and

$$y = \Sigma(m_i l_i \sin \alpha_i)/M \quad (2)$$

from which, by Pythagorus,

$$I = \sqrt{(x^2 + y^2)} \quad (3)$$

where x and y are the orthogonal displacements of the centre of gravity from the geometric centre, being calculated by taking moments for seed masses m_i at radial distances l_i arranged at angles α_i. M is the total seed mass, or Σm_i. Assuming $l_1 = 1$ while m_1 is equal for all seeds, than I can range from 0 (perfect symmetry) to 1 (maximum asymmetry).

To quantify fruit asymmetry and spatial mineral distribution, 60 fruit (previously used to assess spatial variability of seed weights in the locules) were processed. The effects of uneven seed distribution were maximised by selecting only lopsided fruit. The most common form of fruit asymmetry was expressed as a severely dropped shoulder at the calyx end to make an angle greater than 15° (Fig. 2.1). Irregular enlargement of the base at the stem end with oblate fruit shape was also apparent.

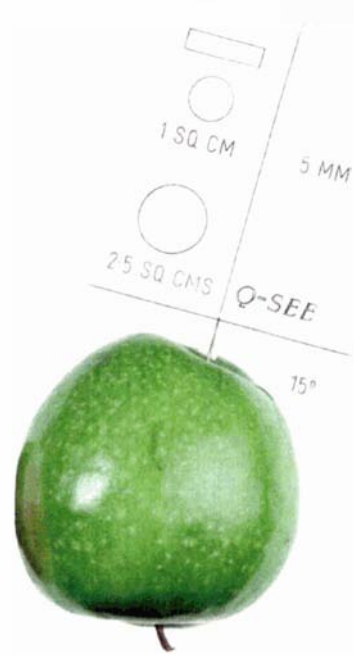


Fig. 2.1. Lopsided 'Granny Smith' apple. Asymmetry exceeds 15° divergence from the vertical axis at the calyx end. The fruit does not meet the 'Granny Smith' grade standard for class 1 apple (ENZA, 1995).

Apples were weighed and cut in half transversely near the equator to expose the locules without damaging the seed. The fruit halves were each carefully sliced longitudinally into five sectors along the carpel margins passing through the primary (petal) bundles and adnate (extracarpellary) cortex (Fig 2.2). The weight of each individual sector (recombining the two portions of each sector previously separated by the transverse cut and comprising of a locule with seeds, flesh with skin as shaded in Fig. 2.2) and of the seeds alone were recorded. Sub-samples for mineral analysis were taken from the middle of each sector using a cork borer, weighed, promptly sealed in polyethylene bags and frozen at -20°C pending analysis.

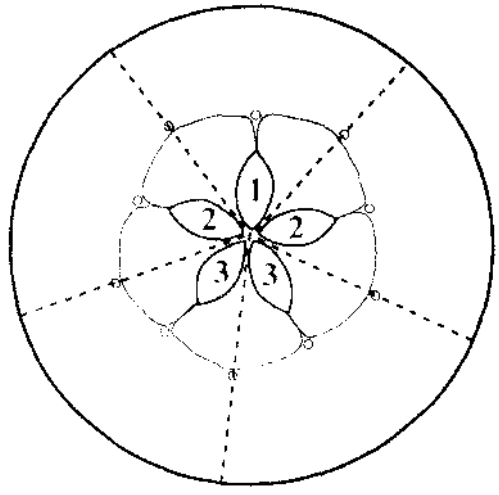


Fig. 2.2. Transverse section of fruit showing the direction of the longitudinal cuts to separate the five sectors. The weight of the sector (shaded) was related to the weight of all of the seeds as combined by a three-order model.

To relate actual seed weight to the sector weight, a *three-order model* was developed. In this model, the weight of a sector is related to the weight of the seed within it (as a first-order effect), to the weight of the seed in the two adjoining sectors (second-order) and to the weight of the seed in the two distant sectors (third-order), (Fig. 2.2). The model is expressed as:

$$S = a + b(w_i) + c(w_{i-1} + w_{i+1}) + d(w_{i-2} + w_{i+2}), \quad (4)$$

where S is sector weight, a is the intercept and b, c and d are the coefficients for the seed weights w_i in the various sectors. To test the data for the three-order model, analysis was

carried out using SAS Proc Mixed with a Toeplitz correlation structure (SAS, 1996), to allow for the expected correlation between data from different sectors in the same fruit. Separate intercepts were fitted for each fruit.

2.2.3. Mineral analyses

For each lopsided fruit, goodness of fit to the model (eq. 4) was estimated by calculating the ratio of the sum of the squared deviations about the model estimates to the sum of the squared deviations about the model sector weight. A subset of 20 fruit (100 samples) was selected for mineral analyses by choosing those fruit for which the sector weights best fit the model but omitting those fruit where the diversity of sector weights was too small.

Mineral analyses were carried out by atomic absorption spectrophotometry and spatial distribution of calcium, magnesium and potassium concentrations were evaluated using regression analysis. However, as the size of analysed fruit differed markedly (134-196 g), fruit effects, as determined by the analysis of variance, were removed before carrying out the regression.

2.3. RESULTS

2.3.1. Seed variability

Seeds were described as 'rudimentary' (≤ 10 mg), 'empty' (*c.* 10-30 mg) or 'viable' (*c.* 40-100 mg), (Fig. 2.3A). 'Rudimentary' and 'empty' seeds together were described as 'aberrant'. 'Rudimentary' seeds were characterised by completely arrested development, shrivelled testa and deformed shape, and consisted mainly of very small seeds (≤ 2 mg). 'Empty' seeds were bigger than 'rudimentary' seeds but the interior was resorbed giving the seeds a flattened appearance with testa curving inwards. Such seeds notably varied in their size. In contrast, 'viable' seeds were fully developed with a plump interior and weighed considerably more. The frequency of 'viable' seeds showed a normal distribution with a wider range of weights than for 'aberrant' seeds.

The frequency distribution of seed weights in the locules of lopsided 'Granny Smith' showed marked variability (Fig. 2.3B). A relatively small number of locules contained only 'aberrant' seeds (≤ 20 mg) and these were prevalent in the underdeveloped parts of lopsided fruit. The total seed weight in a locule within the range of *c.* 30-100 mg usually represented one 'viable' and one 'aberrant' seed, while locules with total seed weight of over 100 mg usually contained two 'viable' seeds and these were associated with larger sector development (Fig. 2.4).

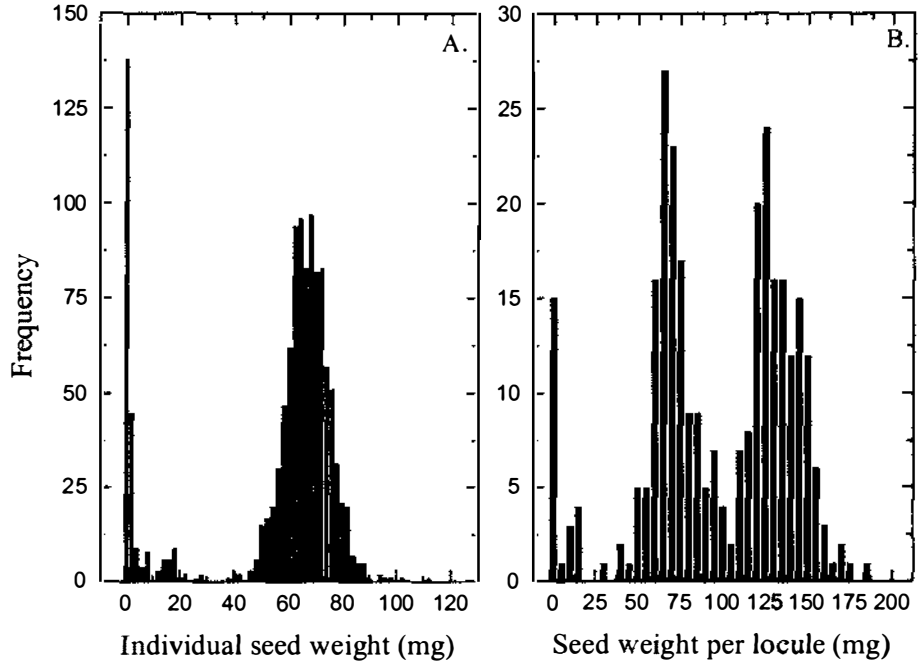


Fig. 2.3. Variability in seed weight. (A) Frequency distribution of individual seed weights from 120 fruit. Each bar represents 2 mg increments in seed weight. (B) Frequency distribution of combined seed weights in an individual locule from 60 lopsided fruit. Each bar represents 5 mg increments in seed weight.

Fig. 2.4. Excised fruit with seeds. The size of individual sectors relates to the seed size.

2.3.2. Seed asymmetry

The theoretical asymmetry of seed arrangements in the fruit is shown in Figure 2.5. The line was obtained by joining the means of I for each seed arrangement for a number of seeds in a fruit, weighting each data point by the number of seed arrangements that gave rise to it. The fewer seeds the higher the value of I. This reached a maximum ($I = 1$) in fruit with one seed, and in one arrangement in fruit with two seeds (both seeds in the same locule). In contrast, a perfectly symmetrical seed distribution ($I = 0$) occurred only for fruit with no seeds or with ten seeds and one arrangement in fruit with five seeds (having one seed in each locule). For all other seed arrangements asymmetry generally increased as seed number decreased.

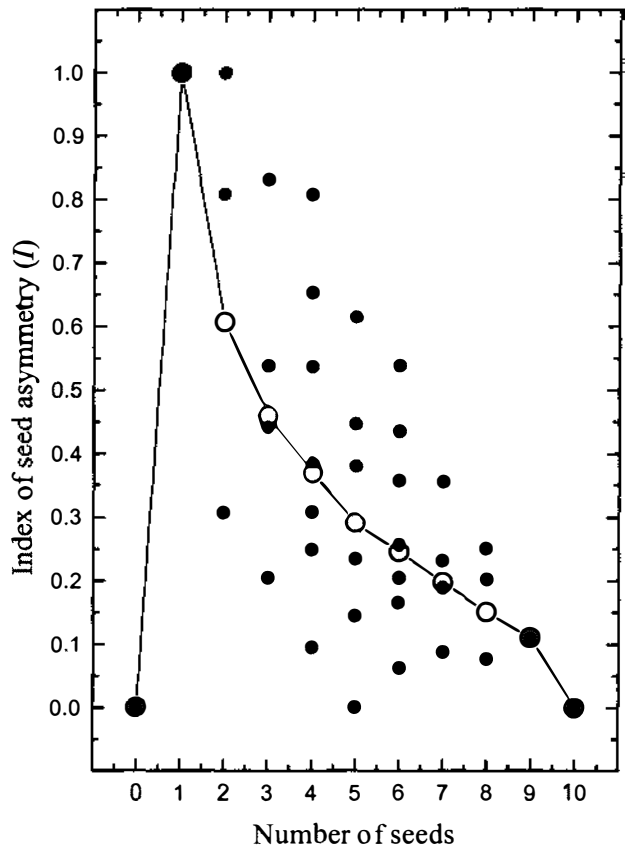


Fig. 2.5. Seed asymmetry *vs* the total number of seed per fruit. Results calculated for all possible combinations of seed number and seed arrangement. The line connects means (○) from the population of *I* (●).

2.3.3. Fruit asymmetry

Analysis of the data partially confirmed the three-order model. Sector weight was found to be principally influenced by its own seeds ($b = 75.36$; $p \leq 0.001$), less so, but still significantly, by the seeds in the two flanking locules ($c = 18.97$; $p \leq 0.05$) but not significantly by the seeds in the two distant locules ($d = 0.25$; $p = 0.97$). The contribution of fruit effects to the variance was found to be highly significant ($p \leq 0.001$), but the Toeplitz variance components were not significant.

A refined seed weight model with $d = 0$ was therefore fitted with separate intercepts for each fruit, but no Toeplitz structure. This seed weight model gave very similar values of b and c (75.56 and 18.86 respectively). Regression analysis shows a strong relationship between the combined seed weights (for the sectors own seeds plus those from the two flanking locules) and individual sector weight adjusted to the same average intercept (Fig. 2.6). The slope is significant ($p \leq 0.001$) while the fit ($r = 0.42$) suggests that the variation is partially explained by the model – remaining variability is probably the result of a range of factors such as vigour, crop load, position in the canopy.

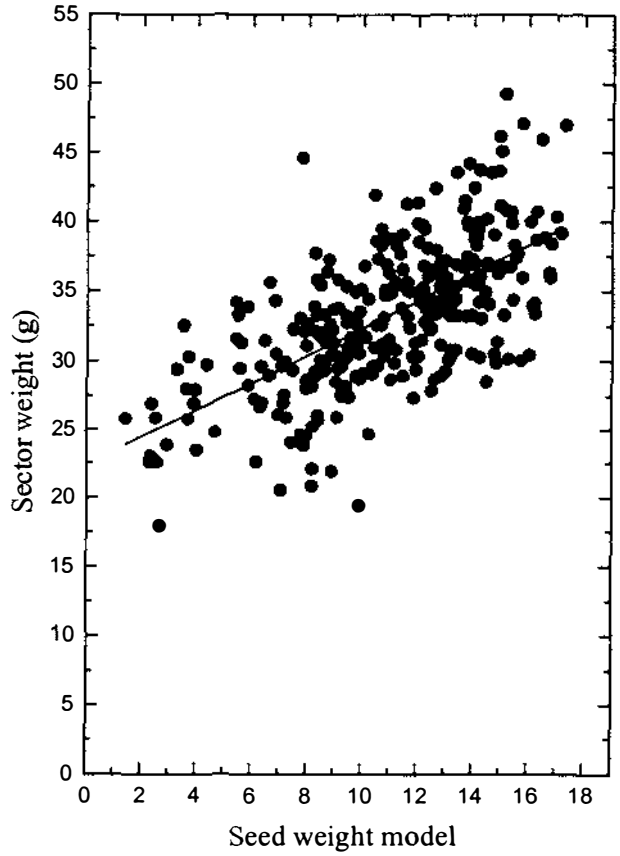


Fig. 2.6. Fruit asymmetry in apple. The influence of seed weight on sector weight as combined in the seed weight model (from the sectors own and the two flanking locules). Each data point represents an individual sector weight.

2.3.4. Fruit minerals

The mineral composition of lopsided 'Granny Smith' showed different distribution patterns for different minerals. The concentration of calcium declined with increasing sector weight ($p \leq 0.05$), (Fig. 2.7), while the concentrations of magnesium and potassium were not significantly affected (Fig. 2.8).

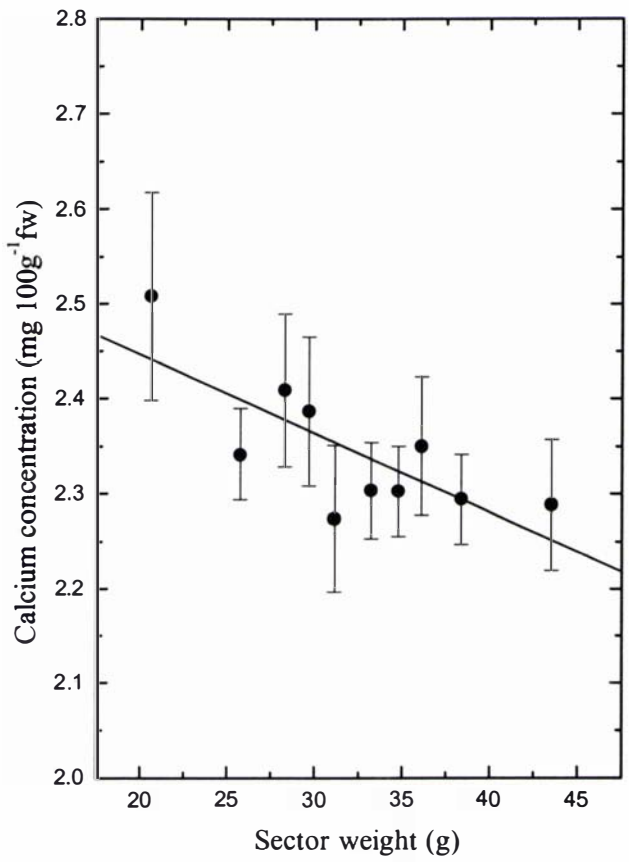


Fig. 2.7. Spatial distribution of calcium in 20 lopsided fruit. For clarity, each data point represents the mean of a successive grouping of ten values. Vertical bars represent SE.

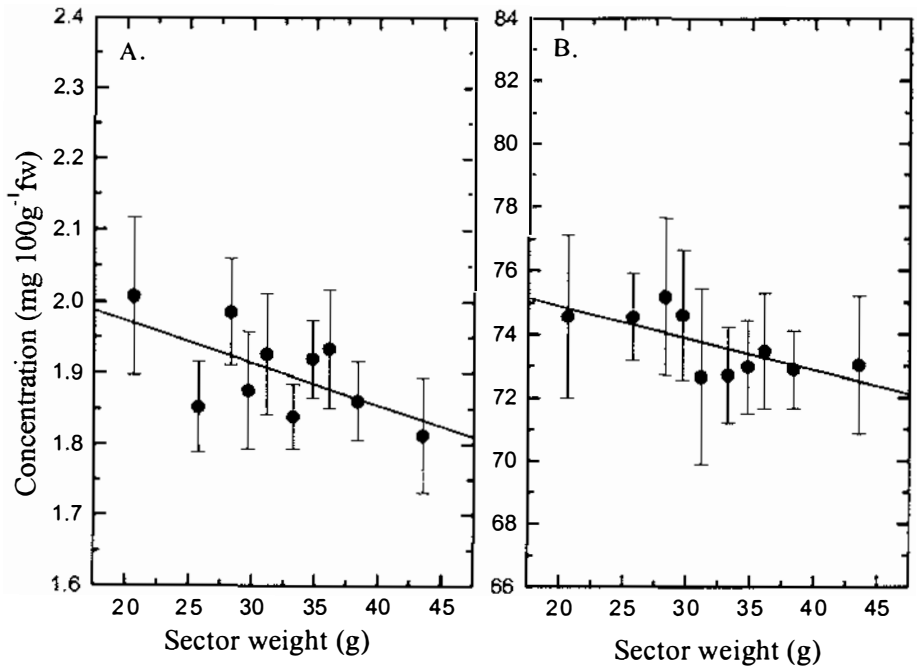


Fig. 2.8. Spatial distribution of magnesium (A) and potassium (B) in 20 lopsided fruit. For clarity, each data point represents the mean of a successive grouping of ten values. Vertical bars represent SE. These regressions showed no significant slope at $p \leq 0.05$.

2.4. DISCUSSION

2.4.1. Seed variability

The three types of seeds described for 'Granny Smith' are similar to those described for other apple cultivars (Brault and de Oliveira, 1995; Brookfield *et al.*, 1996; Broom *et al.*, 1998). The variability in the size of 'aberrant' seeds probably indicates that seed abortion occurred at different developmental stages, as suggested for grapes (Barritt, 1970; Ebadi *et al.*, 1996). For the collapsed 'rudimentary' seeds abortion presumably occurred at the earliest stage of development. However, in this study, no histological analysis was carried out to determine whether the trace formations in lopsided 'Granny Smith' represent aborted (unfertilised) ovules or aborted (fertilised) seed. Locular traces were regarded as seed rudiments based on their microscopic appearance with the desiccated

testa-like surface indicating that fertilisation of the ovule may have take place even though the testa was incomplete (Ebadi *et al.*, 1996). From a botanical standpoint, a true seed is a fertilised ovule with a developed embryo, stored nutrients and testa (Abercrombie *et al.*, 1981). Hence, ovular structures that abort after fertilisation can be still regarded as seeds.

Abortion at a later stage of development presumably leads to the formation of 'empty' seeds since the main body of the seed (as measured by growth in length) is attained within 4-5 weeks after petal fall (Luckwill, 1953; Sato and Kanbe, 1984). On the other hand 'empty' and 'viable' seeds could attain similar size but the variability in weight is considerable. The difference is the result of a degenerated interior in an 'empty' seed - mainly the lack of an embryo and endosperm (Olmo, 1934; Stout, 1936; Luckwill, 1953; Ebadi *et al.*, 1996) that occupy the largest volume of the seed (Esau, 1977).

Because most of the locules in lopsided 'Granny Smith' apples were fully seeded (containing two seeds), any aberrations in seed development create a considerable discrepancy in seed weights. A locule having a single large seed can have a similar weight of seed as one with two small seeds. As a result, only a seed weight distribution of over 100 mg is indicative of normal seed development in a locule (Fig. 2.3B). The remaining locules with lesser seed weights were likely associated (to a different degree) with irregularities in seed and flesh development (Fig. 2.4).

Seed number indicates the degree of fertilisation in the fruit but seed weight reflects the dynamics of post-fertilisation development. Indeed, seed weight rather than seed number was better correlated with fruit shape in nashi pear (Rohitha and Klinac, 1990) while the array of seed classes (including aberrant seeds) influenced apple fruit shape (Brault and de Oliveira, 1995). Hence, the effect of seed on fruit growth should be assessed within the same seed class based on the morphological appearance of the seed. Considering the role of seeds in sector development of 'Granny Smith' (Fig. 2.6) it seems likely that larger seeds generate a stronger developmental signal.

2.4.2. Seed asymmetry

The index of seed asymmetry (I) quantifies the deviation of a particular seed arrangement from a perfectly symmetrical distribution. Fruit with fewer seeds will generally have a more asymmetrical seed arrangement, which is reflected as a shape defect because flesh development is less near locules with 'aberrant' seed (Fig. 2.4). In practice, Latimer (1937) showed that apple fruit with one or two seeds were lopsided whereas fruit with no seeds developed a symmetrical fruit shape. This conforms to the predicted trend of seed asymmetry where I increase steadily with decreasing seed number, but suddenly drops when fruit contained no seed (Fig. 2.5). Hence, the mathematically generated I indicates the actual effect of seed set on fruit shape.

The theoretical model assumes identical seed masses (m_i) distributed around the five spokes (locules) of the light wheel (fruit) but a wide range of seed number. However, in practise, seed weights varied considerably (Fig. 2.3B) but the average number of seeds in lopsided 'Granny Smith' was fairly high (8.63 ± 0.2). However, 'aberrant' seeds are little more than aborted ovules and if omitted from seed asymmetry assessment on the basis of having a small influence on fruit development, the omission could create the range of seed distribution seen in Fig. 2.5. In applying the theoretical model, an 'aberrant' seed should be scored as 'no seed' and all 'viable' seeds should be assumed to be of equal weight. Since variability in seed weight has not been included in the 'light-wheel' model, the distribution of I represents solely the property of uneven seed arrangement.

2.4.3. Fruit asymmetry

Sector weight was influenced by the weight of the associated seeds as combined in the seed weight model (Fig. 2.6). A significant physiological effect on the growth of a particular sector occurred not only from the seeds in the sector's own locule (first order) but also from those in the two flanking locules (second order). Clearly, fruit asymmetry is the result of unbalanced sector growth associated with an asymmetrical seed weight distribution. This indicates that the course of seed development, as shown by the variability in seed weight, and seed distribution are the principal determinants of fruit asymmetry. Failure to form a symmetrical shape early in the season has previously been attributed to 'ovule' abortion (Simons, 1965).

For many years the asymmetry of pome fruit was simply correlated with uneven seed set (Heinicke, 1917; Latimer, 1931, 1937; Roberts, 1946; Way, 1978; Brookfield *et al.*, 1996). Other attempts were made to quantify fruit asymmetry by assessing fruit sector dimensions through the carpel (Rohitha and Klinac, 1990) or by calculating an asymmetry index from radial measurements taken along the carpellary margins (Brault and de Oliveira, 1995). In both studies the relationship between fruit asymmetry and seed set was investigated at the carpel level although they did not view the fruit from a modular standpoint. Furthermore, their results were based on one- or two-dimensional measurements, whereas the tissue weight assessed in this study is a more integrated parameter that effectively gives a three-dimensional perspective (i.e. tissue volume) of tissue development. An additional benefit of using weight as the measured variable (rather than radius or height) is that weight can be recorded with very high accuracy.

Based on the seed weight model (Fig. 2.6), seed set influences fruit shape in apple *via* an influence on fruit sector development. This relationship supports a modular view of an apple as an assemblage of five replicate sectors (extended carpels), where each develops as a unit although influenced to some extent by the immediately adjacent units. The morphology of the apple fruit itself supports such a modular view. The ovary (core) is composed of five carpels fused along the margins to include a locule with seeds and a fleshy pericarp while the extracerpellary tissue (cortex) encloses the ovary and forms the majority of the fruit flesh (Esau, 1977).

Clearly, the core structure implies autonomous growth at the level of the single carpel. However, the fruit sector margins were extrapolated through the outer cortex on the basis of the positions of the carpellary partitions (Fig. 2.2). They do *not* represent a strict morphological division of the extracarpellary tissue. Seeds clearly influenced the development of the two flanking sectors indicating that their principle affect is in the centrifugal direction (towards the skin) – a first-order effect. Their radial influence is somewhat weaker – a second order effect.

2.4.4. Fruit minerals

The distribution of minerals within the fruit was affected by the distribution of fruit growth. The stronger growth of the larger sectors was associated with lower concentrations of minerals (Figs. 2.7 and 2.8), but only that of calcium was significantly affected. The consequence of such a pattern of mineral composition is a changing ratio of calcium to magnesium and potassium that could promote the occurrence of calcium-related physiological disorders, such as bitter pit (Ferguson and Watkins, 1989). The data provide a plausible physiological link to increased frequency of bitter pit lesions in larger and well seeded parts of lopsided apple fruit as first suggested by Heinicke (1920).

Spatial trends in fruit mineral status and bitter pit development somewhat contradict the mechanism underlying the relationship between seed set and fruit calcium. If seed set directly influences calcium uptake into fruit (Tomala and Diley, 1989; 1990; Bramlage *et al.*, 1990; Broom *et al.*, 1998), it follows that fruit mineral concentration could be maximised simply by improving seed set. However, the overly simplified explanation (more seed → more calcium) does not fully account for the significance of other factors involved in fruit mineral nutrition. For example, the larger sectors usually have a full complement of seed, yet calcium concentration declines with increased sector weight (Fig. 2.7).

Bangerth (1973) considered fruit size was one of the factors secondary to fruit calcium in the development of bitter pit. In apples, the xylem sap, as the principal carrier of calcium (Marschner, 1986), undergoes a progressive decline in flow rate relative to the phloem (Lang, 1990). The changing balance in transport pathways favours the accumulation of phloem-borne minerals, while reducing the amount of available calcium in the tissue. In such a way, the ratio of calcium to magnesium and potassium becomes less favourable in rapidly growing or larger fruit (Ferguson and Watkins, 1989). By analogy, the uneven spatial development in lopsided fruit could lead to relatively lower calcium concentrations in the most vigorously expanding tissues, which in turn may lead to a localised occurrence of bitter pit, presumably more often confined to the bigger sectors.

2.5. CONCLUSIONS

Seed weight was variable and the distribution of seed weights tended to follow their morphological appearance, presumably as the result of aborted seed development. Because seed formation precedes fruit set, seed variability is reflected in two important attributes of quality, namely fruit shape and fruit mineral composition.

The nature of fruit asymmetry itself is closely related to the morphology of the apple fruit so that each carpel contributes somewhat autonomously to overall fruit growth. Sector growth is principally influenced by the complement of seed in its own locule (first order) and to a lesser extent by the seed in the two flanking locules (second order). The distribution of seed weight appears to be a key in defining fruit symmetry with 'aberrant' seeds generally confined to the poorly developed sectors, while fully formed seeds prevail in the bigger sectors. Consequently, as seed weight affects sector weight, seed arrangement affects fruit shape. Asymmetry in seed distribution increases when seeds are few.

Variations in spatial fruit development result in variable mineral composition. A change in the ratio of essential fruit minerals was associated with uneven growth. Calcium concentrations in the larger fruit sectors were lower than the calcium concentrations in the smaller fruit sectors while magnesium and potassium concentrations were largely unaffected. This pattern favours calcium deficiency and the occurrence of physiological disorders in the fruit. Seed set, either directly or indirectly, is thus one of the key factors affecting final mineral status of the fruit.

Although fruit quality depends on various growth factors, improved seed set (attained through good pollination) provides a relatively inexpensive and efficient practice to improve fruit quality. However, seediness could be less responsive to pollination in apples prone to irregularities in pollen and ovule developments. This suggests that a more comprehensive study would be worthwhile to quantify possible genetic differences between apple cultivars.

Chapter 3. Xylem development in the stalk of apple and the effects of auxin transport inhibition

3.1. INTRODUCTION

The fact that xylem tissue differentiates as a strand of elongated cells, with a gradual change in diameter and density of vessels along the plant axis, suggests that an inducting stimulus moves in a polar fashion between source and sink. Since Jacobs (1952) demonstrated that the auxin from developing leaves in *Coleus* was the limiting factor for xylem regeneration in the stem internode, it has been generally accepted that auxin plays a major role in controlling xylogenesis. The young developing organs are regarded as the primary sites of auxin synthesis and it is the steady polar flow of auxin towards the roots which is thought to promote development of conductive tissues (Aloni *et al.*, 2000).

The major operating conduits of the xylem are vessels (Esau, 1977) which, due to the existence of open perforation plates at the end walls of the vessel elements, provide a low-resistance pathway for xylem sap (Nobel, 1999). The measured hydraulic conductances in several dicotyledons with different perforation plates were close to estimated values based on Hagen-Poiseuille's law (Schulte *et al.*, 1989). For this reason, the relative flow rate (efficiency of conductance) depends upon the cross-sectional area of the xylem capillaries (Zimmermann, 1983). The pattern of vascular differentiation, which determines relative frequency:size distribution of vessels, is thus a critical factor in the hydraulic architecture of a particular organ.

Cross-sectional area and the length of time when the vessels are functioning could be of particular importance to mineral nutrition, and thus keeping quality, of fruit such as apples (Lang and Ryan, 1994). This study was undertaken to determine the structural changes of vessels in the developing stalk, and the effects of auxin transport inhibition imposed after bloom. For this, N-(1-Naphthyl)phthalamic acid (NPA) was used, based on its specific ability to inhibit cellular carrier-mediated auxin transfer (Lomax *et al.*, 1995), without itself being transported in a polar manner (Thomson *et al.*, 1973). The hypothesis

being tested was that if auxin export from the fruit was the determining factor in xylogenesis, then NPA applied to the fruit stalk just after bloom would suppress proximal post-bloom vessel differentiation in the stalk. Formations of primary xylem laid down during the initial growth of the apple flower stalk were not expected to be affected, but the secondary differentiation that occurs largely after anthesis (Barden and Thompson, 1963) was expected to be affected.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Measurements to assess stalk development were carried out by Dr Alexander Lang on mature 'Royal Gala' apple trees grown at the Massey University Fruit Crops Unit, Palmerston North, New Zealand. The trees were planted on MM 106 rootstock and subjected to standard commercial management.

The NPA experiments were carried out on 'Granny Smith' apple trees. Six mature trees planted on MM 106 rootstock (three trees for each of the two NPA experiments) were chosen from a private organic orchard on the outskirts of Palmerston North, New Zealand. Clusters were randomly selected around the canopy at 1-2 m height, mainly on 2-year old spurs. To minimise possible effects of uneven seed set on vessel differentiation in the stalk, clusters were hand-thinned to one lateral flower and hand-pollinated with 'Red Delicious' pollen collected the same season.

3.2.2. Preparation and application of auxin transport inhibitor (NPA)

N-(1-Naphthyl)phthalamic acid (NPA) was purchased from Tokyo Kasei Kogyo Co Ltd, Tokyo, Japan. In both experiments anhydrous lanolin was used as a carrier for exogenous application. Since NPA powder may not dissolve completely in lanolin resulting in uneven application (Haga and Iino, 1998), NPA was dissolved in a mixture of ethanol (99%) and 1M NaOH before being added to the lanolin. The NPA/lanolin pastes were prepared in four logarithmically scaled concentrations: 0.01 mg g^{-1}, 0.1 mg g^{-1}, 1 mg g^{-1} and 10 mg g^{-1}. To obtain 10 mg NPA g^{-1} lanolin, 200 µl 1M NaOH and 400 µl ethanol were mixed with 50 mg NPA. Solution was slowly added to 4950 mg of melted lanolin

resting on a hot plate to avoid an explosive reaction of ethanol and stirred with a glass rod until homogenised. Similar amounts of solvent added to lanolin served as the control. Prepared pastes were kept in a refrigerator for a short period (several days) until their application in the orchard.

Two different applications of NPA were carried out in the earliest stage of fruit development, 2 - 4 d after petal fall. In the first experiment, 10 mg NPA g^{-1} lanolin was applied in a horizontal ring (about 2 mm wide) to the surface of the stalk using the tip of a wooden toothpick. Two distinct positions along the stalk were chosen – proximal, at the base of the stalk adjacent to the spur and distal, at the base of the fruit junction with the stalk. This created four different treatments: (a) NPA/lanolin paste to the proximal end, (b) NPA/lanolin paste to the distal end, (c) NPA/lanolin paste to both proximal and distal ends, and (d) lanolin paste (with solvent only) to both proximal and distal ends. There were 60 samples for each treatment, giving a total of 240 samples. In the second experiment, four different NPA/lanolin pastes (0.01 mg g^{-1} to 10 mg g^{-1}) and control (with solvent only) were placed with a wooden toothpick as a horizontal half-ring (again some 2 mm wide) at the midpoint of the stalk. For each treatment there were 40 samples, giving a total of 200 samples.

3.2.3. Vessel measurements

To monitor extension growth and vessel differentiation in 'Royal Gala' stalks, 22 periodic measurements were taken between bud break and maturity. At each sampling time, five average sized fruit were randomly selected from the canopy, which provided a total of 110 fruit to be analysed.

To assess the effects of NPA on vessel differentiation, all treated 'Granny Smith' fruit were collected 26 and 30 d after petal fall. Fruit were picked from the orchard in the morning and held in promptly sealed polyethylene bags pending measurements on the same or following day. In the laboratory, fruit and seeds were weighed. Seeds were classified into two broad categories: 'viable' and 'aberrant' and their number recorded. The parameter for separating seeds was the overall appearance, with the 'viable' seeds being fully formed and fleshy while the 'aberrant' seeds were considerably smaller and often flattened with empty interiors.

Stalks were removed with a small amount of fruit flesh still attached at the distal end and immediately placed in Formol-Acetic-Alcohol (FAA) fixative (5ml 38% w/w aqueous formaldehyde and 5 ml glacial acetic acid in 90 ml 70% v/v aqueous ethyl alcohol) pending microscopic examination. PROC GLM (SAS, 2000) was carried out to evaluate the effects of NPA treatments from both experiments through a) fruit abscission and b) seed set (number and weight) and fruit weight. Tree and branch effects were tested for significance (as class variables).

In both studies, sectioning was done by hand using a sharp single-edged blade (Leica Model 818) to produce transverse sections suitable for examination after staining. For microscopy, excised transverse sections were soaked on a glass slide with phloroglucinol solution (2.5 g in 50 ml of 70% v/v aqueous ethyl alcohol) for about 60 s and then flooded with concentrated HCl. Once the lignified walls had stained red the sections were mounted in 80% (w/w) glycerol.

The size and frequency of vessels within any size class were assessed immediately after slide preparation because the stain used to highlight the lignified cell walls fades after a few hours. The image from a compound light microscope (Kyowa Microlux-11 coupled to a Panasonic GP-KR222 video camera) was presented on a 17" flat VDU screen (Sony Trinitron Multiscan G400) at ×475 magnification using an image-grabber card and *Video Master*™ software (Fig. 3.1). Based on the method described by Lang and Ryan (1994), the internal diameters of vessels were evaluated by eye using custom mouse-controlled software that presented a moveable bitmap image on the screen. This image comprised a set of five graded rings (sizes 1, 3, 5, 7 and 9) allowing estimates to be made of four intermediate sizes by interpolation (sizes 2, 4, 6 and 8). That arrangement allowed nine vessel-size classes to be recorded using custom software that converted the PC's numeric keypad into a nine-channel tally counter. Each ring size was calibrated to a known diameter on the slide (3, 5, 7, 9, 12, 14, 16, 18 and 21 μm).

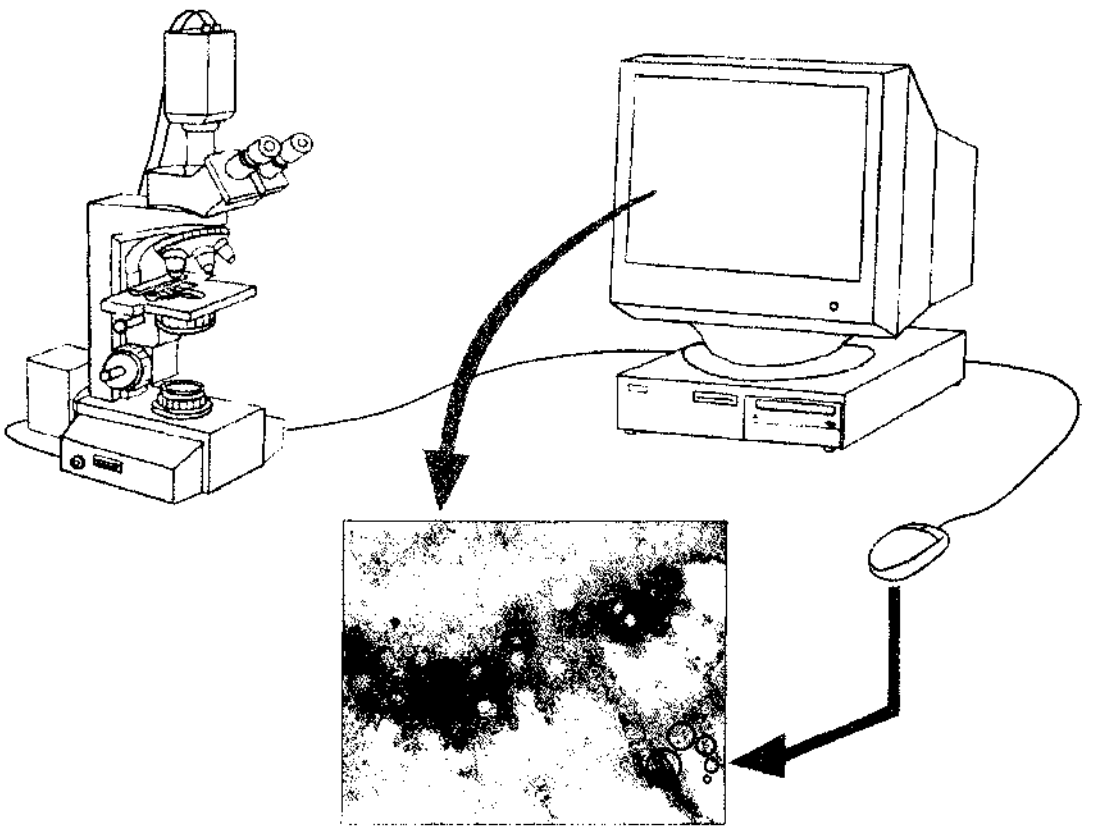


Fig. 3.1. Diagram showing the set-up for quantitative video-microscopic analysis. Sizing was by comparison with the set of five mouse-controlled reference images (visible at bottom right of the screen image) and their four (estimated) half sizes. The data were directly stored through the proprietary software to disc.

The hydraulic conductance (C) was estimated using the Hagen-Poiseuille law (Nobel, 1999) as the product of the sum of vessels (n) and the fourth power of radius (r) for a particular vessel size class ($C \propto n\, r^4$).

Because of the nature of the NPA experiments, it was essential to determine the precise positions from which transverse sections were to be taken. Three relatively long stalks (~ 30 mm in length) of control fruit taken from the first NPA experiment were hand-sectioned every 2 mm along the stalk, starting from the spur end. This provided 14 positional cuts per stalk. Immediately after the completion of serial vessel area

measurements, the data were analysed for the spatial distribution of vessels. The result determined two regions with similar hydraulic conductance (*c.* 20% of the stalk length in from either end) from which proximal and distal sections were obtained in the second NPA experiment.

The analysis showed that stalks treated with 1 mg NPA g^{-1} lanolin revealed no significant effect on vessel differentiation. Therefore, sectioning of the 0.01 and of 0.1 NPA g^{-1} lanolin samples was not done and that of the 1 mg NPA g^{-1} lanolin samples was discontinued after just five fruit stalks had been assessed. All of the thirteen available samples treated with 10 mg NPA g^{-1} lanolin were analysed whilst for the control the analysis of ten samples (including three samples used for serial sectioning) was considered sufficient.

PROC GLM (SAS, 2000) was carried out to evaluate the significance of differences between the two positions on the stalk. The data were normalised by taking the distal:proximal ratio to remove fruit-to-fruit variability and tested to determine if the ratio differed:

- between NPA-treated fruit and control, and
- from 1 for each treatment.

Tree and branch effects were tested for significance (as class variables).

3.3 RESULTS

3.3.1. Spatial distribution of the vessels

Serial sectioning indicated an even pattern of vessel frequency along the fruit stalk (Fig. 3.2A). However, estimated hydraulic conductance exhibited a conspicuous bell-shaped distribution (Fig. 3.2B), producing regions with higher hydraulic resistance closer to the two ends of the stalk. The extent of xylem development at the distal end was reduced compared to that in the middle of the stalk, but there was a more significant reduction in the conductance at the proximal (spur) end (*c.* 3-fold decrease).

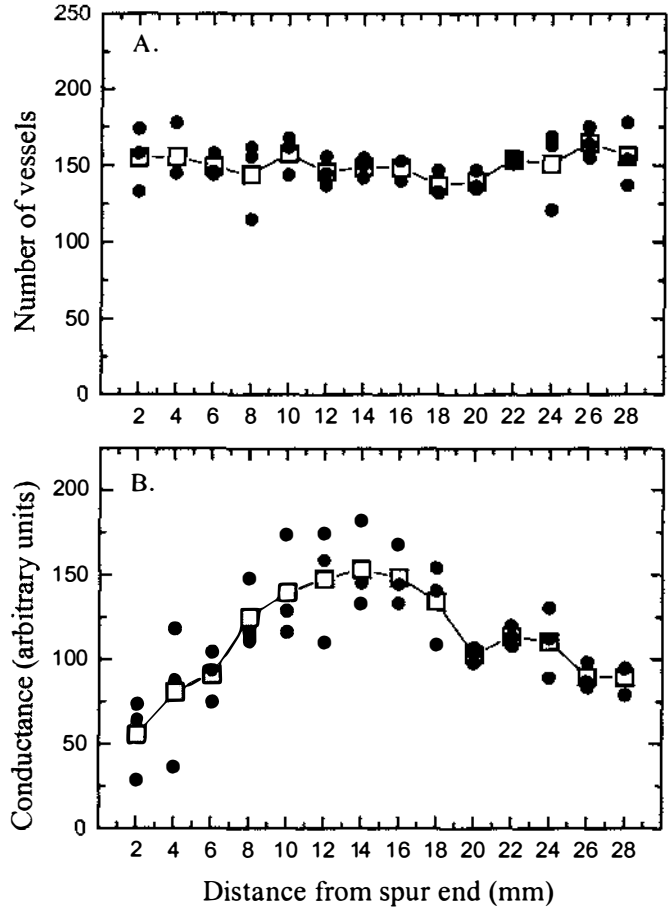


Fig. 3.2. Spatial distribution of the number of vessels (A) and estimated conductance (B) in the stalk of 'Granny Smith'. For each position along the stalk, the mean value (□) was derived from three measurements (●).

3.3.2. Frequency:size distribution of the vessels

The frequency of a particular size class of vessel showed a distinct pattern in the stalk of 'Granny Smith' (Fig. 3.3A). The 'narrow' vessels (≤ 9 μm in diameter) dominated in the cross-sectional area of the xylem tissue, comprising over two-thirds of the existing conduits with the most frequent diameters being 5 and 7 μm. The drop in frequency of the 9 μm size class, relative to the 7 and 12 μm classes, was taken arbitrarily as the boundary between vessels of different derivation. The 'wide' vessels (≥ 9 μm in diameter) contributed relatively little to the overall vessel frequency. The majority of

these vessels fell into the 12 μm and 14 μm classes with a progressive decrease in the frequency of vessels with larger diameters.

The mean number of vessels contributed by each size class in the stalk was used to calculate their fractional mean conductance (Fig. 3.3B). Contrary to the previous trend, most of the conductance in the stalk was attributed to a relatively small number of 'wide' vessels. The largest proportion of the conductance (37%) occurred through the vessels in the 14 μm size class. However, the larger vessels (16, 18 and 21 μm diameter) were relatively few, and together contributed less (33%) than the single 14 μm size class, while 'narrow' vessels were too limited in diameter to contribute significantly.

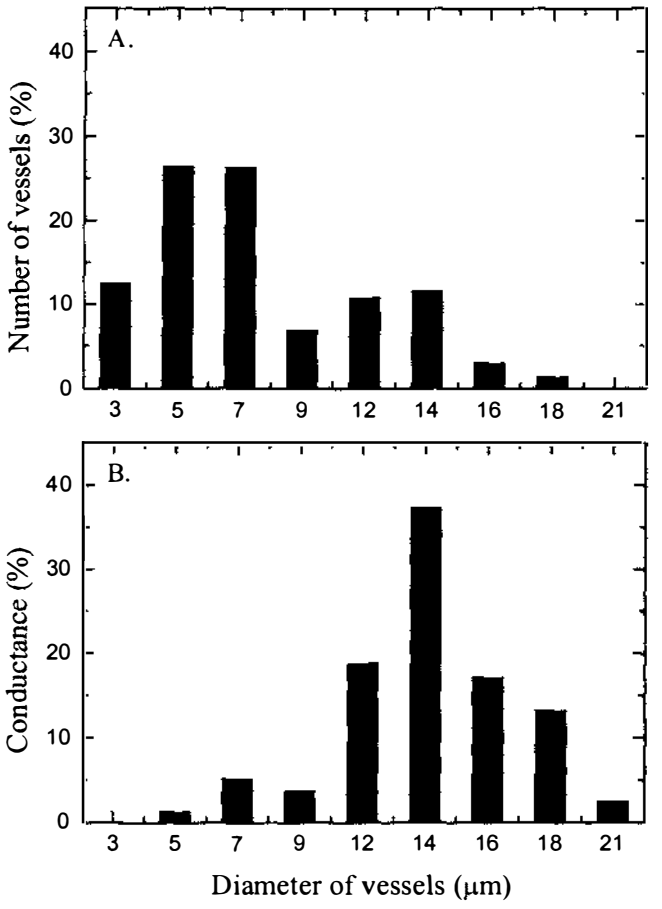


Fig. 3.3. Fractional contribution by vessel size classes averaged from cross-sections of three excised stalks of 'Granny Smith': (A) for the mean number of vessels; (B) transformed to reflect the mean conductance.

3.3.3. Development of the stalk

The apple stalk extended rapidly over a brief period at the beginning of the season. Extension started pre-bloom and had largely ceased by 12 days after full bloom (DAFB), (Fig. 3.4). Consequently, extension growth of the stalk of 'Royal Gala' was completed within 4-5 weeks after extension growth started at bud break.

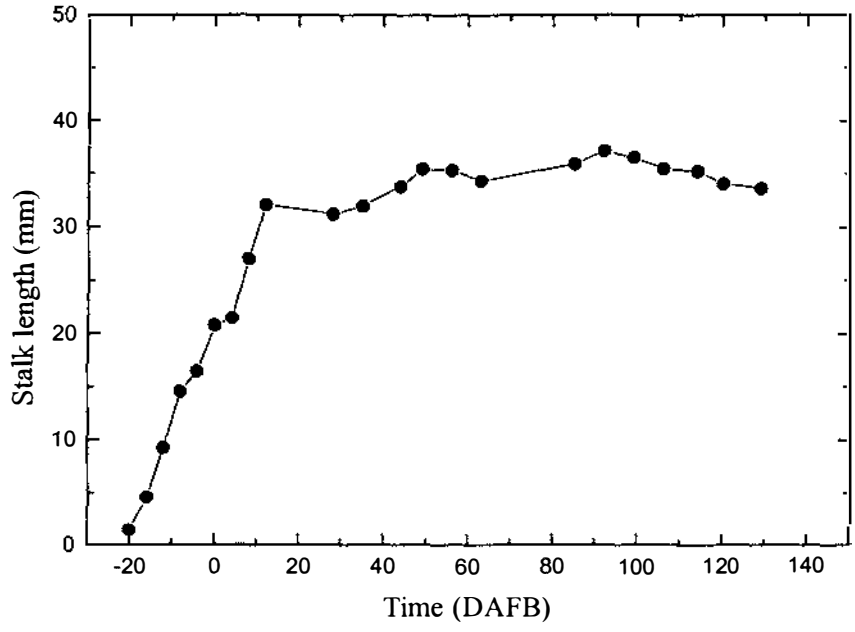


Fig. 3.4. Stalk extension growth in 'Royal Gala'. For each time period, the point (●) represents the mean of five individual measurements (unpublished data, A. Lang).

Vessels differentiated over a relatively short period of time during the extension of the stalk (Fig. 3.5A). The differentiation of 'narrow' vessels (≤ 10 μm in diameter) ceased at –8 DAFB by which time 'wide' vessels had emerged in the xylem. During the period of bloom, there were no new 'wide' vessels (a lag phase) but soon after vessel differentiation proceeded in an exponential fashion. Their differentiation started from 4 DAFB and continued rapidly for several weeks during which time 'wide' vessels fully differentiated in size. By 28 DAFB vessel differentiation in the stalk was effectively completed.

When assessed for conductance (Fig. 3.5B), 'narrow' vessels exhibited an initial increase but contributed very little to the flow due to their limited cross-sectional area. They reached a peak contribution around –4 DAFB. At that time, a small number of larger vessels had differentiated but no further differentiation occurred during bloom. From 4 DAFB vessel differentiation increased rapidly and by 28 DAFB the flow capacity in the stalk was essentially established. Conductance continued to increase after that but at a much-reduced rate due to the very limited additional vessel differentiation.

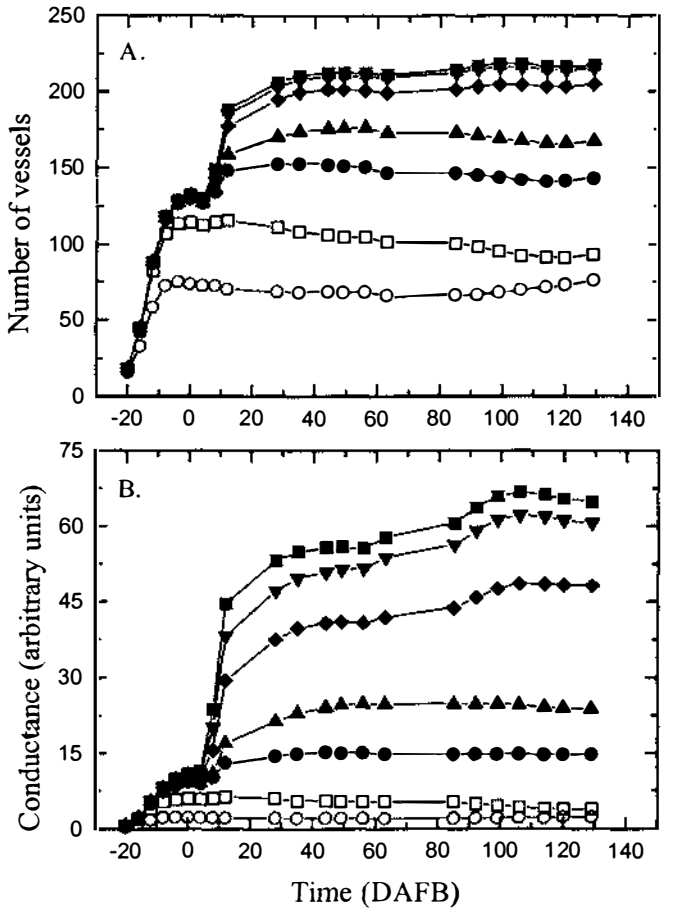


Fig. 3.5. Vessel differentiation in the stalk of 'Royal Gala'. Cumulative distributions of vessel frequency (A), and estimated conductance (B), contributed by different size classes: ≤ 7.5 μm (○), ≤ 10 μm (□), ≤ 12 μm (●), ≤ 14 μm (▲), ≤ 16 μm (◆), ≤ 18.5 μm (▼) and ≤ 21 μm (■). For each time period the points represent the means of five individual measurements (unpublished data, A. Lang).

3.3.4. Effect of NPA on fruit abscission

Post-bloom observations in the orchard revealed a severe drop of NPA-treated fruitlets from the first experiment only two weeks after application of NPA. The remaining fruitlets were harvested after the estimated completion of vessel formations in the stalk, between 26 and 30 days after petal fall. A small number of fruitlets from both experiments whose size was markedly reduced were held weakly in the canopy with obvious signs of partial disconnection at the abscission zone. Byers *et al.* (1991) and Ward and Marini (1999) observed cessation of fruit growth prior to abscission, while Avanzi *et al.* (1988) noted that the ovule structures of abscised and abscising apples showed no histological differences. On this basis, abscising fruit were excluded from further analyses.

The number of abscised fruitlets from the control treatment was very low and differed significantly from all NPA treatments ($p \leq 0.001$), (Table 3.1). However, the comparison between NPA treatments showed that distal applications caused significantly less abscission ($p \leq 0.01$), while the proximal application bore no significant difference from 10 mg NPA g^{-1} lanolin applied to both distal and proximal ends of the stalk.

Table 3.1. The effect of 10 mg NPA g^{-1} lanolin on fruit abscission when applied to the distal (D), proximal (P), and distal and proximal ends of the stalk (D + P). There were 60 fruit per treatment. Numbers followed by the same letter are not significantly different from one another.

Position	Fraction abscised (%)
D	83.4 *b*
P	91.6 *c*
D + P	100.0 *c*
Control	18.4 *a*

Drop was not as severe in the second experiment, but again the highest concentration of NPA resulted in a significant increase in fruitlet abscission ($p \leq 0.01$), while the other treatments with lower NPA concentrations were no different from the control (Table 3.2).

Table 3.2. The effect of four NPA concentrations on fruit abscission when applied to the mid-point of the stalk. Numbers followed by the same letter are not significantly different from the control.

NPA (mg g^{-1} lanolin)	Fraction abscised (%)
0.01	32.5 *a*
0.1	35.0 *a*
1	25.0 *a*
10	67.5 *b*
Control	35.0 *a*

3.3.5. Effect of NPA on seed and fruit development

Data showed no difference in either 'viable' seed number or total seed number between the NPA-treatments and controls from both experiments. However, the proximal application of 10 mg NPA g^{-1} lanolin in the first NPA-experiment caused a significant reduction in seed weight ($p \leq 0.01$) and fruit weight ($p \leq 0.001$), (Table 3.3).

Table 3.3. The effect of 10 mg NPA g^{-1} lanolin on fruit and seed development when applied to the distal (D), and proximal (P) ends of the stalk. Numbers of fruit per treatment are in brackets and are those from the first experiment shown in Table 3.1. Numbers followed by the same letter are not significantly different from the control.

Position	Fruit weight (g)	Seed weight (mg)	Seed number ('viable')	Seed number (total)
D (10)	3.1 *a*	81 *a*	8.6 *a*	10.2 *a*
P (5)	1.8 *b*	59 *b*	9.2 *a*	10.2 *a*
Control (49)	3.8 *a*	97 *a*	8.7 *a*	10.1 *a*

The analysis of data from the second experiment showed that 10 mg NPA g^{-1} lanolin effectively suppressed seed weight and fruit weight ($p \leq 0.001$), (Table 3.4). It should be noted, however, that 0.01 and 1 mg NPA g^{-1} lanolin treatments stimulated fruit growth ($p \leq 0.05$) but had no effect on seed growth (except for the slightly higher total seed number from 1 mg NPA g^{-1} lanolin treatment, $p \leq 0.05$).

The overall picture was essentially the same when ready-to-abscise fruit were added to the data, only the increased fruit-to-fruit variability amongst the treatments changed the *p*-values (data not shown). In addition, comparison of controls from the two NPA-experiments showed uniform seed number but a highly significant reduction in the weight of seed and fruit picked only 4 days apart ($p \leq 0.001$). Whenever fruit or seed weight effects were significant, so too were tree or branch effects ($p \leq 0.05$). Hence, these class variables were retained in all analyses.

Table 3.4. The effect of four NPA concentrations on fruit and seed development when applied to the mid-point of the stalk. Numbers of fruit per treatment are in brackets and are those from the second experiment shown in Table 3.2. Numbers followed by the same letter are not significantly different from the control.

NPA (mg g^{-1} lanolin)		Fruit weight (g)	Seed weight (mg)	Seed number ('viable')	Seed number (total)
0.01	(27)	6.6 *b*	145 *a*	8.2 *a*	10.1 *a*
0.1	(26)	5.8 *a*	127 *a*	7.9 *a*	10.1 *a*
1	(30)	6.3 *b*	143 *a*	8.4 *a*	10.3 *b*
10	(13)	3.4 *b*	100 *b*	8.5 *a*	10.0 *a*
Control	(26)	5.5 *a*	132 *a*	8.7 *a*	10.0 *a*

3.3.6. Effect of NPA on vessel differentiation

When the frequency of different vessel sizes was computed proximally and distally to the point of NPA application in the second experiment, two peaks were seen, representing populations of 'narrow' and 'wide' vessels in the stalk. This is apparent both for the number of vessels (Fig. 3.6A) and for the conductance (Fig. 3.6B) contributed by each

size class of vessel. The effect of auxin transport inhibition was statistically assessed in 'wide' vessels only, since it was assumed that the 'narrow' vessels had differentiated pre-bloom, as in the stalk of 'Royal Gala' (see Fig. 3.5A). In addition, branch and tree effects were not significant and so were omitted from the analyses.

Assessment of the data showed that 1 mg NPA g^{-1} lanolin caused a slight increase in the number of vessels and in conductance (Fig. 3.6) with changed distal:proximal (d/p) ratio (0.84 and 0.85 respectively) indicating promotion of growth in the proximal part of the stalk. While analysis showed these trends to be statistically non-significant, there was an effect on subsequent seed and fruit growth (Table 3.4).

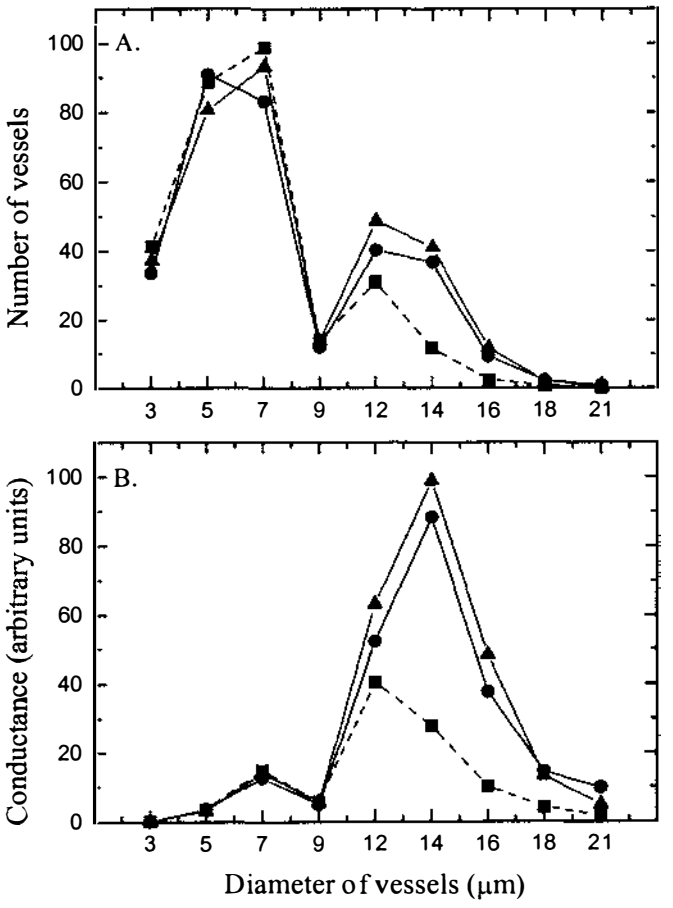


Fig. 3.6. The effect of 1 mg NPA g^{-1} lanolin (▲), 10 mg NPA g^{-1} lanolin (■) and control (●) on the number of vessels (A), and estimated conductance (B) in the stalks of 'Granny Smith'. Each point represents the average of proximal and distal sections.

The application of 10 mg NPA g^{-1} lanolin showed a significant increase in d/p ratio for vessel number relative to the control ($p \leq 0.01$), (Figs. 3.7A and 3.7B). The d/p ratio in NPA-treated stalks (2.31) differed from 1 ($p \leq 0.001$) suggesting greater suppression of vessel differentiation proximal to the point of NPA application. In comparison, the d/p ratio in the control stalks (1.05) was not significantly different from 1.

When expressed as conductance, 10 mg NPA g^{-1} lanolin also showed a significant increase in d/p ratio relative to the control ($p \leq 0.05$), (Figs. 3.7C and 3.7D). The d/p ratio in NPA-treated stalks (2.83) differed from 1.0 ($p \leq 0.001$) but not in control stalks (1.25). The most pronounced differences occurred in 12, 14 and 16 μm vessel size classes that contribute over two thirds of the flow capacity in the stalk (see Fig. 3.3B).

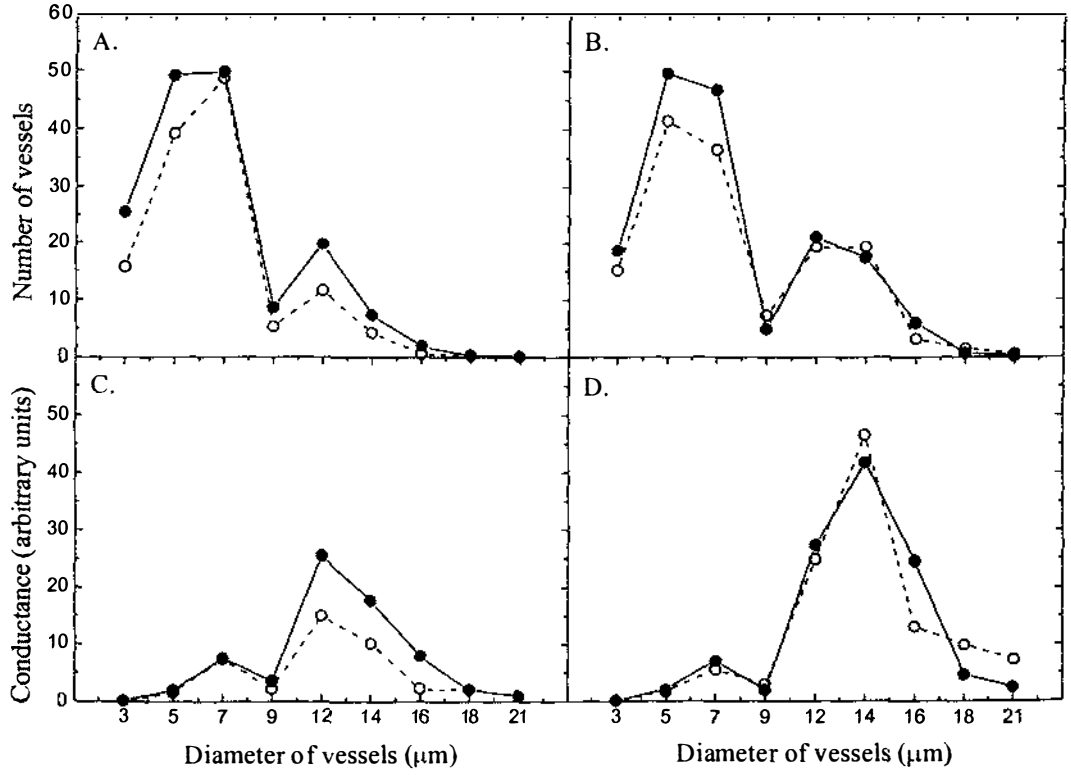


Fig. 3.7. Number of vessels and estimated conductance contributed by different size classes in the stalks of 'Granny Smith' treated with 10 mg NPA g^{-1} lanolin (A, C) and control (B, D). The points represent the mean values from distal (●) and proximal (○) sections.

3.4. DISCUSSION

3.4.1. Vessel measurements and estimated conductance

The method used to measure the numbers and internal diameters of vessels proved simple in concept and accurate. The cut vessels were clearly seen on the PC screen, as bright perforations with distinguishably stained walls. Oblique or over-thick sections were easily rejected because light only illuminated part of the vessel areas, and vessel margins were indistinct. Measurements were quick and straightforward using a mouse-controlled selective template with direct data acquisition to disc. Hence, every vessel in transverse section was processed only once without risk of repeating the same measurement and with minimal operator error because the eye quickly adopts an accurate and consistent pattern during evaluation.

Vessels were rarely circular in their cross-section, and the inner and the outer limits were matched by superimposed rings making allowance for irregularities in a perfect circle. This non-ideal geometry of conduit lumen may contribute to the differences between theoretical (estimated) and actual conductivity as noted by some authors (Tyree and Zimmermann, 1971; Calkin *et al.*, 1986; Tyree and Ewers, 1991). The agreement between the theoretical and actual conductance could be improved by using a correction factor for reduced conductance through non-cylindrical cross-sections of the xylem conduits (Calkin *et al.*, 1986). However, when such a correction to the Hagen-Poiseuille equation for laminar flow through ideal (perfectly cylindrical) capillaries was applied to the elliptical shape of the xylem conduits in the internodes and petioles of the grape it resulted in a very small reduction of the predicted conductance ranging from only 1.5 to 5.4% (Schultz and Matthews, 1993). Since the difference appears small, the conductance in the stalk of the apples used in this study was estimated by way of the original Hagen-Poiseuille equation (Nobel, 1999).

3.4.2. Spatial distribution of the vessels

The stalk seems to be an obvious site of increased resistance to water flow due to the limited cross-sectional area of conduits branching from the more developed fruit-bearing spur beneath and transporting sap into the much larger fruit above. While the number of vessels along the length of the stalk was consistent (Fig. 3.2A) their cross-sectional areas

were less developed towards the ends producing structural 'bottleneck' regions with markedly reduced conductance. Moreover, vessel development was more arrested at the proximal end of the stalk adjacent to the abscission zone (Fig. 3.2B). This is in agreement with the existence of distinct xylem constrictions at or near the abscission zone of the fruit (MacDaniels, 1937; Habdas *et al.*, 1982; MacKenzie, 1988; Lee, 1989) with increased hydraulic resistance of the bottleneck regions just distal to the branch points (Düring and Lang, 1993).

Whether vessel formations terminate at the junction and develop as adjacent vessels from the other side (arranged end-to-end) or simply reduce in frequency across the zone, it certainly indicates a natural cleavage site for organ separation. Furthermore, restrictions of xylem water flow could play an important role in the hydraulic architecture of the plant. It has been argued that hydraulic bottlenecks at branch points may provide a safety feature against xylem cavitation of the parent body after the abscission of terminal organs (Zimmermann and Milburn, 1982); or thus may lead to the development of a water potential gradient between the stem and the fruit to maintain the ascent of sap (Lee, 1989); or thus may cause the diversion of xylem water flow into the phloem (Lee, 1989; Düring and Lang, 1993).

The change of water flow carrier across the bottleneck regions could be of particular importance for fruit. Apple fruit growth is mainly phloem dominated (Lang, 1990) and this pathway is known to be calcium immobile (Marschner, 1986). Assuming that hydraulic constrictions along the xylem strand do promote a shift in the balance in favour of the phloem-borne transport, this may contribute to the relatively limited capability of plants to regulate calcium uptake by developing fruit. However, the convoluted pattern of xylem and phloem flow in regard to fruit development requires further research to confirm this assumption.

3.4.3. Frequency:size distribution of the vessels

The distribution of vessel size classes in the stalk of 'Granny Smith' (Fig. 3.3A) suggests two stages in vessel differentiation. The frequency of size classes correlated with the arbitrary division between 'narrow' and 'wide' vessels, the former representing the greater number of vessels in the stalk. In contrast, Lang and Ryan (1994) reported a bell-

shaped vessel distribution in two different apple cultivars but their distinctive profiles indicate that the course of vessel differentiation is a particular feature of a given cultivar.

When the frequency data of Fig. 3.3A were transformed to represent the conductance contributed by each vessel size class, it could be seen that the large number of 'narrow' vessels could carry only a small proportion of the sap (*c.* 10%); most of the conductance was contributed by the relatively small number of 'wide' vessels (Fig. 3.3B). However, the calculations assume that all the vessels in the stalk are functional. This is probably not the case. Actual flow is certainly smaller than estimated conductance because the earliest xylem formed is stretched and broken during the rapid early growth (Barden and Thompson, 1963; Esau, 1977; Mapfumo *et al.*, 1993), and because some large vessels could retain degenerated protoplasm which would restrict the effective vessel diameter (Mapfumo *et al.*, 1993). Xylem sap flow through the stalk will thus depend upon size and frequency of large and functional vessels, as suggested by other authors (Zimmermann, 1983; Tyree and Ewers, 1991; Lang and Ryan, 1994).

3.4.4. Development of the stalk

Stalk extension in 'Royal Gala' is effectively linear (Fig. 3.4) and matches that observed in other apple cultivars (Prive *et al.*, 1988). Final stalk length is apparently a cultivar characteristic and although it can be manipulated by the application of exogenous hormones (paclobutrazol and gibberellins), the dynamics of growth do not change (Prive *et al.*, 1989).

Vessel formation, and thus the hydraulic conductance of the stalk, start before flowering (Fig. 3.5A) as also shown in previous studies of apple (Barden and Thompson, 1963), pear (Nii, 1980) and grape (Theiler and Coombe, 1985). The physiologically significant increase in the cross-sectional area of the vessels was due to a relatively short post-bloom development of 'wide' vessels, which determines the hydraulic capacity of the stalk by around four weeks after bloom (Fig. 3.5B). It should be noted that the rapid post-bloom vessel differentiation starts to plateau when the stalk has achieved final length (12 DAFB) suggesting a correlative link between extension and structural development of the stalk. Furthermore, the timing and dynamics of vessel differentiation is in line with the rate of auxin transport from developing apple fruit (Gruber and Bangerth, 1990).

The growth dynamics of the vessels and corresponding conductance in the stalk indicate distinct stages in vessel differentiation. Vessels laid down after the initiation of flower development (≤ 10 μm in diameter) clearly mature before bloom (Fig. 3.5A) and could develop only as early formations of the primary xylem (protoxylem). The remaining vessels that start differentiating before bloom but largely developed after bloom, therefore, represent later stages of the primary xylem (metaxylem) as well as the secondary xylem formations in the stalk (Barden and Thompson, 1963). This bears close resemblance with the frequency distribution of vessels size classes in 'Granny Smith' apple (Fig. 3.3A).

3.4.5. Effect of NPA on fruit abscission

Young developing apple fruit are a source of diffusible auxin (Gruber and Bangerth, 1990), such that seed number greatly influences polar auxin efflux (Bangerth *et al.*, 1989; Callejas and Bangerth, 1997; Bangerth, 2000). The synthetic phytotropin NPA is thought to inhibit transport of auxin by blocking the sites of auxin efflux at the basal end of the cells (Lomax *et al.*, 1995) or even disrupting the influx carriers at higher concentrations (Delbarre *et al.*, 1996). Hence, sufficient NPA concentration that inhibits carrier-mediated auxin transport at the site of application will deplete the level of auxin at the abscission zone of the stalk.

Indeed, the highest NPA concentration (10 mg g^{-1}) induced significant abscission, which was more pronounced when a greater amount of NPA-paste was applied to the stalk as a complete ring (Tables 3.1 and 3.2). Although the abscission was complete when NPA was applied to both ends of the stalk (Table 3.1) all 10 mg NPA g^{-1} lanolin treatments caused significantly greater drop than the controls, suggesting that disruption of auxin transport distal to the abscission zone promoted shedding of the young fruit.

The results support the view that changes in the normal auxin status of a tissue initiate fruit abscission (Sexton and Roberts, 1982; Brown, 1997; Bangerth, 2000) at a pre-determined position (Osborne, 1989). Auxin has been shown to antagonise abscission (del Campillo and Bennett, 1996) by inhibiting the accumulation of the hydrolytic enzyme cellulase (Tucker *et al.*, 1988) in the abscission zone of the apple fruit stalk (Pandita and Jindal, 1991; Ward *et al.*, 1999; Ward and Marini, 1999). Hence, the lower

NPA concentrations (0.01, 0.1 and 1 mg g^{-1}) were presumably insufficient to counter the endogenous auxin transport at the site of efflux (Table 3.2).

Seeds appear critical in determining fruit retention on the tree. Abscised fruit may show degenerated ovule structures (Forino *et. al.*, 1987; Avanzi *et al.*, 1988), reduced seed set (Heinicke, 1917) or marked inhibition of seed growth (Wertheim, 1971; Abruzzese *et al.*, 1995; Knoche, *et al.*, 2000). Furthermore, damage to ovules leads to severe fruit drop in apple (Abbott, 1959; Marini and Byers, 1988; Ward *et al.*, 1999) that increases if injury is imposed earlier (Proctor and Schechter, 1992). This emphasises the significance of dynamics of 'hormone' production by developing seeds that coincides with the rate of fruit abscission (Luckwill, 1948; 1953).

3.4.6. Effect of NPA on vessel differentiation

Post-bloom application of 10 mg NPA g^{-1} lanolin induced significant changes in the xylem but only the ongoing differentiation of 'wide' vessels was affected (Fig. 3.6). Because 'wide' vessels principally govern the conductance in the stalk (Fig. 3.3B), any change in their frequency will amplify the difference between the treatments (Fig. 3.7). With respect to the point of NPA application on the stalk, the xylem at the proximal position developed fewer vessels indicating that the critical signal was travelling from the developing fruit along the stalk and not coming from the other direction.

This result points to the auxin-like signal, which can regulate the induction and formation of the vascular tissues (Aloni and Zimmermann, 1983). A good body of evidence has shown the close relationship between growth dynamics of the fruit and vascular development in the stalk (Nii, 1980; Habdas *et al.*, 1982; Bustan *et al.*; 1995). Hence, the amount of vascular formation in the stalk relies on the ability of the fruit to supply a growth-promoting stimulus.

The pattern of vessel differentiation signifies the mechanism underlying vascular changes in NPA-affected stalks. Because auxin movement is preferentially basipetal from the apple fruit (Bangerth, 2000), NPA was expected to alter vessel formations proximal to the point of the blockage. However, post-bloom vessel differentiation appears affected (to a different degree) along the entire stalk when compared to the control (Fig. 3.7). In

other words, 10 mg NPA g^{-1} lanolin suppressed developing formations of 'wide' vessels at both proximal and distal ends of the stalk although the nature of NPA was likely to inhibit polar auxin movement only at the site of action (Thomson *et al.*, 1973).

This could arise because the application of NPA was exogenous, in the orchard, and the lanolin paste may have migrated along the exterior surface of the stalk (both lanolin and the stalk surface are lipophilic). However, a more likely scenario involves inhibition of auxin biosynthesis due to severe disruption of auxin efflux at a point along the stalk. Since vessels resume rapid differentiation in the short space of time after anthesis (Fig. 3.5A), NPA principally affected ongoing development of 'wide' vessels at the proximal end of the stalk deprived of the continuous auxin signal (Fig. 3.7). This, in turn, could have stimulated auxin accumulation upstream (*c.f.* membrane vesicles isolated from *Cucurbita*, Michalke *et al.*, 1992). Once the blockage of polar auxin transport was completed and/or auxin levels in the tissue exceeded an upper concentration threshold it seems likely that auxin biosynthesis in the fruit would have ceased, which then affected vessel formations at the distal end of the stalk. The time lag between NPA-induced auxin inhibition at the mid-point of the stalk and negative feedback of auxin biosynthesis could explain positional differences in vessel differentiation on the same stalk. In addition, the stimulating effect of the lower NPA concentration on vessel differentiation (Fig. 3.6) and fruit growth (Table 3.4) conform with the idea that subtle rebalancing of hormones determines the nature of the physiological response.

3.4.7. Effect of NPA on seed and fruit development

Hand-pollination caused a high seed complement in all fruit (Tables 3.3 and 3.4). Analysis of variance showed fairly uniform seed number between NPA-treatments and controls suggesting that changes in the dynamics of fruit abscission and post-bloom vessel differentiation in the stalk were not influenced by inadequate or uneven seed formation. This is in line with evidence that pollination, as a precursor of seed formation in the fruit, controls the extent of the 'first drop' shortly after flowering (Wertheim, 1971)

The most significant effect on seed and fruit growth occurred when 10 mg NPA g^{-1} lanolin was applied either at the proximal end of the stalk (Table 3.3) or mid-stalk

(Table 3.4). Basipetal auxin export is obviously an initial step in the physiological mechanism that controls growth. This suggests that a continuous growth-promoting signal from the actively growing fruit structures is critical not only for vessel differentiation but also for the normal development of the fruit itself. It has been shown that an inhibition of auxin transport from fruit caused substantial reduction in seed weight and partially seed number (Hamamoto *et al.*, 1998). However, the assessment of 10 mg NPA g^{-1} lanolin treatments between two experiments was not possible because of the substantial differences in seed and fruit weight of the control treatments, which had slightly different durations of growth.

These observations point to the interaction between hormones and assimilates in developing fruit. During initial growth of apple, the seed undergoes a prominent increase in size (Harley, 1942; Luckwill, 1953; Sato and Kanbe, 1984), but changes in this period are not restricted to seed set only. Arrested seed development leads to inadequate hormonal production (Bangerth *et al.*, 1989; Callejas and Bangerth, 1997; Bangerth, 2000) and diminished sink capacity (Weinbaum *et al.*, 2001), which could affect fruit growth. The NPA-induced reduction in the size of seed and fruit suggests that movement of nutrients into the developing fruit is under hormonal control. The physiological significance of these effects is probably to promote the redistribution of nutrients into the dominant fruit. However, the exact mode of action or possible nature of the self-inhibiting signal remains uncertain.

Whether early seasonal vessel differentiation is simply a result of a continuous hormonal stream that supports fruit growth by expanding the conductive structures or as an auxiliary outcome of actively developing fruit structures is not yet clear. On the other hand, an increase in fruit size requires a suitable vascular capacity to meet the increasing nutritional demands of the sink. Limited development of conducting tissues due to reduced supplies of growth promoting substances could in return limit the rate of ascent of sap into the growing fruit and alter its sink capacity. As a consequence, weaker fruit may lose the ability to compete for nutrients supplied from the tree, which might lead to shedding. It seems obvious that further studies are needed to elucidate the mechanisms of hormonal action in the control of the physiological response of the vascular tissues and its possible influence on the nutritional status of the fruit.

3.5. CONCLUSIONS

Growth dynamics of vessel formations were determined periodically throughout the season in the stalks of 'Royal Gala'. Quantitative microscopic analysis showed that a majority of vessels are differentiated pre-bloom with the commencement of stalk extension but that the full conducting capacity of the xylem is attained shortly after bloom. These results suggest two distinct stages in vessel differentiation in the stalk.

The influence of auxin transport inhibition in post-bloom vessel differentiation of 'Granny Smith' apple stalk was investigated. Vessel-size frequency (that yields a potential conductance) was assessed using a quantitative microscopic analysis of the stalk of young fruit harvested around four weeks after petal fall. The evidence is presented that an auxin-like signal emanating from the young fruit not only stimulates vessel differentiation in the stalk but also controls the abscission and development of seed and fruit. The effects were discussed through the hormonal and nutritional aspects of fruit development and a proposed physiological link was related to the dynamics of vessel differentiation by auxin.

In addition, vessel constrictions were identified near the junctions of the stalk suggesting structural modifications of the xylem strand in the plant. Physiological consequences were discussed based on reports from the literature. Although the physiological mechanism for the regulation of long-distance transport is more complex, hydraulic adaptations along the xylem strand will contribute to the efficiency of the mineral uptake of the fruit.

Chapter 4. Causes and effects of changes in xylem functionality in apple fruit

4.1. INTRODUCTION

A number of fruit species undergoes a progressive xylem dysfunction (Findlay *et al.*, 1987; Düring *et al.*, 1987; Lang, 1990; Creasy *et al.*, 1993; Lang and Ryan, 1994; Wolswinkel *et al.*, 1999; Drazeta *et al.*, 2001). For example, in wine grapes, vascular dysfunction coincided with the sudden resumption of berry growth after the onset of ripening (Findlay *et al.*, 1987; Düring *et al.*, 1987). Viewed in these terms, the progress of xylem dysfunction is determined by a growth-induced damage of the bundles as the fruit expands.

Progressive breakdown of conducting tissue in apple has been attributed to the failure of a number of vessels in the xylem (Lang and Ryan, 1994). This is thought to result in the reduction in rates of xylem inflow to the fruit and a shift in the relative contributions of xylem and phloem saps to fruit growth (Lang, 1990). This may have consequential effects on the balance and amounts of key fruit minerals and thus on fruit quality. Calcium, a xylem-mobile element (Marschner, 1986), shows a progressive decline in its rate of accumulation during the season (Wilkinson, 1968; Jones *et al.*, 1983). A cultivar that is more susceptible to bitter pit (a low calcium induced disorder) showed an earlier slowing of xylem flow rate than a less susceptible one (Lang, 1990; Lang and Ryan, 1994). A more recent analysis of 10 apple cultivars showed a significant relationship between the susceptibility of a cultivar to bitter pit and the extent of xylem dysfunction taken at a single time point (Drazeta *et al.*, 2001).

The aim of this study was to elucidate the dynamics and the nature of xylem failure in developing apple fruit using a water-soluble dye as an indicator of functionality coupled with a conventional light microscopic examination of dysfunctional bundles. Two apple cultivars were tested, these having different susceptibilities to bitter pit. It was hypothesised that the accumulation of vessel damage in the fruit might explain a

progressive reduction in xylem conductance. In addition, by recording the growth of the internal structures of the fruit, it may be possible to relate aspects of their growth to particular functional outcomes. These hypotheses may together be able to explain cultivar differences in bitter pit susceptibility in terms of differences in xylem functionality, and these in terms of differences in the growth of the internal structures of the fruit. The study should lead to an improved understanding of the mechanisms of bitter pit susceptibility in apple.

4.2. MATERIALS AND METHODS

4.2.1. Plant material

The study used mature trees of 'Braeburn' and 'Granny Smith' apple cultivars from a private organic orchard on the outskirts of Palmerston North, New Zealand. The trees were planted on MM 106 rootstock in three-row blocks alternating with pollinator blocks. The trees had not been managed according to standard commercial practices and the fruit was not selectively thinned. The flowering period was briefer than usual, with full bloom starting about three days earlier in 'Granny Smith' than in 'Braeburn'.

4.2.2. Temporal changes in xylem functionality

Functionality of fruit xylem was recorded at weekly intervals until mid-season (113 and 116 days after full bloom, DAFB) and then fortnightly until harvest (183 and 200 DAFB). Average-sized fruit without visible blemishes were selected. Whole fruit-bearing spurs were collected from the orchard in the early morning (transpiration negligible, therefore, tree water potential close to soil water potential, ~ zero) and held in closed polyethylene bags pending investigation later the same day. On each occasion, fifteen fruit of each cultivar were assessed for xylem functionality. A total of 555 fruit were assessed throughout the season.

Xylem function in the fruit was examined using a simple dye-infusion technique to stain the vasculature along the path of dye movement (Findlay *et al.*, 1987; Düring *et al.*, 1987; Creasy *et al.*, 1993; Wolswinkel *et al.*, 1999; Drazeta *et al.*, 2001). To avoid air embolism of the xylem, each fruit was cut from the spur at the base of the stalk. This was

carried out under distilled water prior to immersion in a small vial (a 2 cm^3 Ependorf tube) containing a dye solution. The apoplastic dye (1% aqueous acid fuchsin) was drawn up into the fruit through the stalk for 2 h under standard transpiration conditions (*c.* 22 °C and 65% RH) with a brisk airflow from two fans to remove boundary layer effects (Fig. 4.1).

Fruit were then sectioned equatorially using a microtome blade to minimise tissue damage and to produce a smooth cut surface. The vascular bundles were clearly visible in these transverse sections and were scored for dye intensity (*D*) as 0 = nil, 1 = trace, 2 = medium and 3 = high. Because developmental changes in xylem functionality may differ between the classes of vascular bundles, the presence of dye was assayed separately for the ten primary, five dorsal and ten ventral bundles. An averaged *D* value was computed for each fruit from the number of scores obtained for a particular class of bundle at any time.

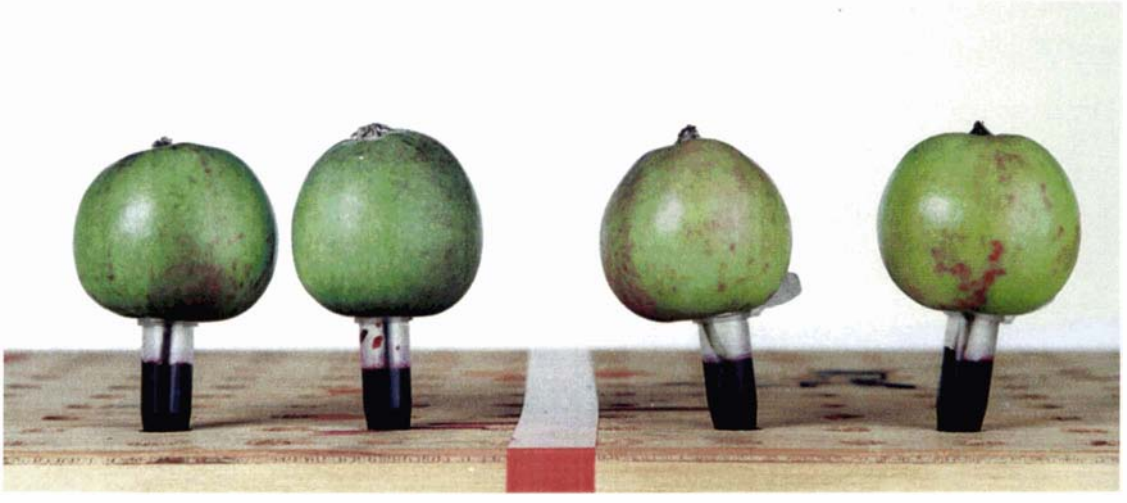


Fig. 4.1. Dye infusion of 'Granny Smith' (left) and 'Braeburn' (right) apples, *c.* 70 DAFB. Stalks were immersed in vials containing 1% aqueous acid fuchsin and exposed to the draught of two small fans. Some patchy dye accumulation can be seen at the fruit surfaces.

Dye was assessed in four categories with each being assigned an integer value (i.e. 0, 1, 2, and 3). For such data, that is constrained between two extreme values (0 and 3), a logistic transformation can be applied to both linearise the data and to make the residuals more uniform

$$\ln\left(\frac{y}{y_0 - y}\right) = a + bx \qquad (1)$$

where y_0 is the upper asymptote for D and was assessed as 3.1 (arbitrary units) for all data.

Following this procedure, however, the transformed data failed to summarise D from the sigmoid-like curves. Therefore, for each bundle type and cultivar, a model was chosen which described the approximate shape of the curve of changing D with time. Each model was fitted by non-linear, least-squares, using PROC NLIN (SAS, 2000). For each model, F-tests were applied to determine whether selected features of the shape of the fitted curve were significant, and whether differences between the fitted curves for each cultivar could be adequately described by a simple difference in time scales.

4.2.3. Exposure time

Preliminary staining trials conducted early in the season showed satisfactory dye distribution in the bundles of the fruit after *c.* 2 h. However, it was feared that changes in xylem functionality might alter the amount of dye appearing later in the season and so change the optimum period of exposure. Hence, it was worthwhile to assess dye accumulation as a function of time. For this, some 50 fruit of each cultivar were chosen in the age range of 93-104 DAFB and infiltrated with dye for 30, 60, 120, 240 and 480 min. The three classes of vascular bundles were assessed for D and averaged separately for each fruit as described previously.

4.2.4. Spatial changes in xylem functionality

Longitudinal dye accumulation in the fruit was investigated in ten fruit of each cultivar at a more advanced stage (160 – 163 DAFB). Dye-infiltrated fruit were sliced transversally using a domestic food slicer to form a contiguous series of *c.* 2.5 mm thick tissue disks, starting from the calyx end. Due to a slight variability in fruit size, different numbers of

disks were obtained from each fruit. *D* was assessed in each disk (not all classes of bundles were visible in the disks obtained near the ends of the fruit).

PROC GLM (SAS, 2000) was used to evaluate the significance of any longitudinal gradient in *D* for each bundle class and cultivar then, secondly, to test whether the gradients differed:

- between bundle classes for each cultivar, and
- between cultivars for each bundle class

The high natural variability between fruit was taken into account by including a fruit effect within each cultivar.

4.2.5. Anatomy of xylem breakage

Several fruit were collected at 85 and 141 DAFB for microscopic study. Fruit from both collection dates were infiltrated with dye and cut transversally into three, approximately equal, disks (using a sharp knife): disc 'A' was from near the stalk-end of the fruit, 'B' was from the middle and 'C' from near the calyx-end. In each disc, randomly chosen dysfunctional (unstained) bundle was marked on the skin along assumed path and excised with a small amount of attached flesh so as to produce an elongated sample.

Fruit picked at 85 DAFB were used to identify structural changes in the xylem. Each sample was soaked for several minutes in an excess of phloroglucin solution (2.5 g in 50ml of 70% v/v aqueous ethyl alcohol) and flooded afterwards with concentrated HCl to visualise lignified vascular elements. This procedure yields distinctively red-stained xylem strands embedded within a reasonably transparent parenchyma. To avoid rupture of xylem during the final tissue preparation, the bulk of the flesh was carefully dissected away with a microtome blade and fine forceps working under a dissecting microscope (Wild M3Z). The excised bundle was mounted in 80% w/w glycerol on a glass slide and examined with a compound light microscope (Microlux-11) attached to a video-camera system.

Fruit picked at 141 DAFB were used to investigate the nature of xylem disruption by a more sophisticated light microscopic procedure. To prepare dysfunctional bundle for fixation, intercellular air was first removed by evacuation. To do this the sample was

placed in a 50 cm^3 vial containing water and wetting agent (0.1% w/w Silwet L-77), and exposed to reduced atmospheric pressure (*c.* 18 mmHg at 20°C) for 2 min. After the vacuum had been released, water flowed in to replace the extracted air over a 2 min period. This treatment rendered the parenchyma reasonably transparent and suitable for dissection. Samples were trimmed with a sharp blade under the dissecting microscope (Wild M3Z) until the bundle was exposed with a very thin layer of surrounding flesh. It was then cut transversally every 4 – 5 mm. A total of five, four and three specimens were obtained from disks A, B and C respectively.

Once ordered, (A_1 to A_5) the specimens were preserved separately in sealed glass vials containing half-strength Karnovsky's fixative (3% v/v glutaraldehyde and 2% w/v formaldehyde in 0.1 M phosphate buffer at pH 7.2). Secondary fixation was in 1% osmium tetroxide using the same buffer. Specimens were dehydrated in a graded acetone series, and infiltrated and embedded in Procure 812 epoxy resin. Cured resin blocks were trimmed and longitudinal sections (1μm thick) were cut from the middle of the bundle, heat mounted on glass microscope slides and stained with 0.1% toludine blue. Tissues were prepared for microscopy and sections by Mr Douglas Hopcroft, EM Unit, HortResearch, Palmerston North. Slides were examined and photographed using a Zeiss Axioplan compound light microscope with attached MC 100 camera system.

A view of xylem breakage in primary bundles was obtained from the same fruit used for photomicrograph examination (141 DAFB). Fruit were cut transversally (with a sharp knife) into three uniform slices and each was re-cut longitudinally (with a microtome blade) along the path of unstained bundles so as to expose regions where dye abruptly stopped. Specimens were photographed using a Wild M3Z dissecting microscope with attached 35 mm Leica-Wild MPS52 camera system.

4.2.6. Growth of the different fruit tissues

To evaluate the growth of the different fruit tissues, dimensional measurements relative to the axis were made in the fruit of both cultivars (Fig. 4.2). These were measured in the same fruit used for dye infusion. One extra fruit of each cultivar at each time was cut equatorially using a microtome blade. Measurements were made using digital callipers (precision 0.01 mm).

Radial distances were taken from the fruit axis to the skin through the primary (sepal and petal) bundles. These gave a measure of the absolute tissue growth (ATG) of the core and cortex (assuming that the vascular ring delineates the boundary between two fleshy portions of the fruit) and of the fruit itself. The length of the cartilaginous walls defining locules was also recorded. The dimension of the fleshy region between the tip of the locule and the adjacent sepal bundle was obtained by difference. Relative tissue growth (RTG) was obtained by normalising the dimensional tissue measurements with the corresponding fruit radii to determine how the various structural components contributed to the growth of the whole.

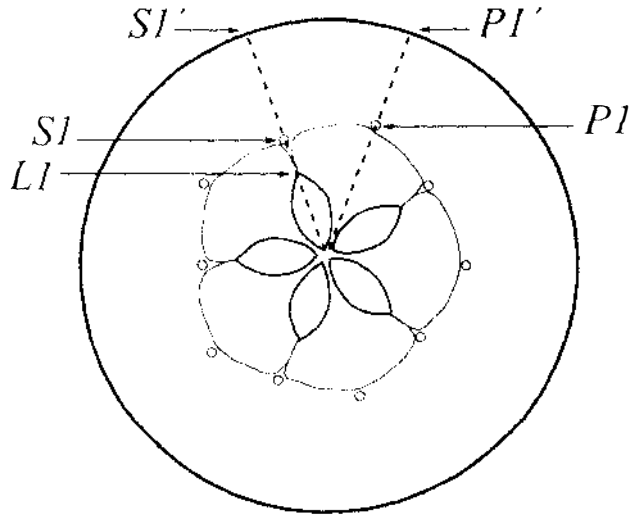


Fig. 4.2. Transverse section of fruit showing radial dimensions measured from the fruit axis to the skin. Here S1 refers to sepal bundle whereas P1 refers to petal bundle. Hence, distance from the fruit axis to L1 gives a measure of locule, to S1 and P1 gives a measure of core (ovary) and to S1′ and P1′ gives a measure of fruit dimensions respectively. The difference between fruit and core dimensions quantifies the size of the cortex. Following this approach, five locule dimensions and ten core, cortex and fruit dimensions were obtained for each fruit. In addition, the distance between the locule tip (L1) and the sepal bundle was assessed.

Observations of vascular functionality made about midway through the season revealed that the sepal bundles of the cortical vascular system of 'Granny Smith' retained functionality for longer than the petal bundles. This distinction was not apparent in 'Braeburn'. The spatial arrangements of the petal and sepal bundles were assessed in both cultivars. Each point was defined by the ratio of the distances of the petal and sepal bundles to the fruit axis (ten individual measurements) and so it was necessary to carry out analysis by assessing the mean ratio for a single fruit. For each cultivar PROC GLM (SAS, 2000) was used to evaluate the significance of the slope of the mean ratio of the radial distances to petal and sepal bundles in the fruit (P/S) *vs* DAFB.

It seemed reasonable to assume that the degree of sepal bundle drift outward was related to the magnitude of ovary growth in the same direction. This emphasised the importance of the proximity of the sepal bundle to the neighbouring locule (S-L) normalised with the corresponding fruit radii. The significance of cultivar differences in the time course for the mean ratio was compared using PROC GLM (SAS, 2000).

4.3. RESULTS

4.3.1. Exposure time

Dye infusion induced a rapid spread of dye throughout the functional portions of the vascular network enabling an assessment of functionality for the range of exposure times used. In all bundle classes *D* initially rose but reached a plateau after 120 minutes (Fig. 4.3). However, different bundle classes accumulated different amounts of dye. In both cultivars, the dorsal bundles appeared more functional than the primary or ventral bundles. Furthermore, the intensity of dye in the primary bundles of 'Braeburn' was less than in 'Granny Smith'

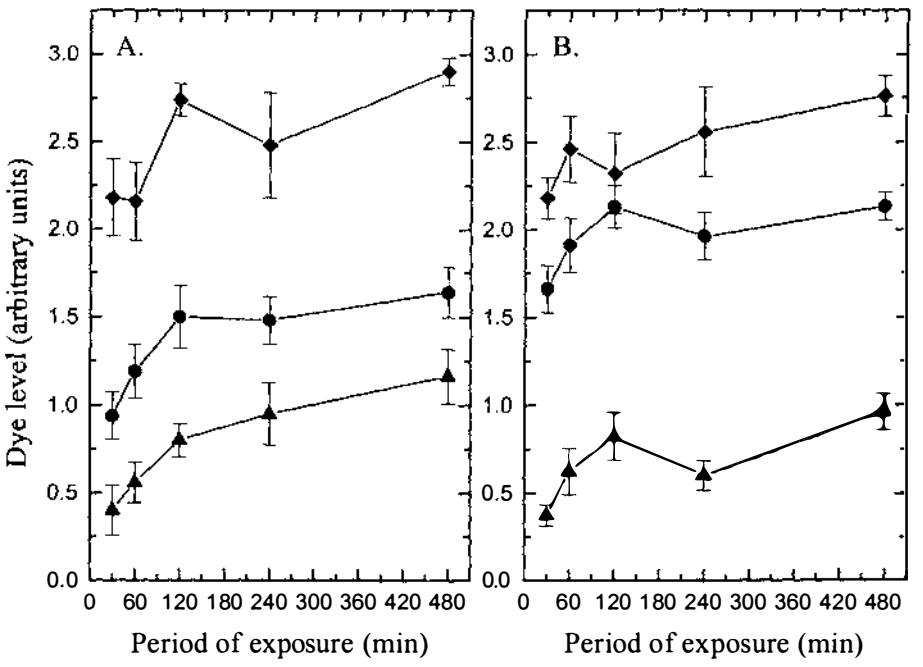


Fig. 4.3. Dye accumulation in the vascular systems of 'Braeburn' (A) and 'Granny Smith' (B) as a function of the period of exposure. The points are mean values (n = 10) for the primary (●), dorsal (◆) and ventral (▲) bundles of the fruit with the corresponding SE indicated by the vertical bars. Fruit were assessed at 93 DAFB ('Braeburn') and 104 DAFB ('Granny Smith').

4.3.2. Temporal changes in xylem functionality

Changes in dye accumulation were observed initially in the anastomosing vasculature, which ramifies into numerous branches to form a dense network of fine bundles that scatters across the flesh and terminates just beneath the skin. Early in the season, dye spread freely through the numerous fine traces leading to a somewhat diffuse appearance. Later, the fine vascular network was less strongly coloured. The reduction in colour took place before any noticeable change in the functionality of the primary bundles and followed a similar pattern in both cultivars, although it was more pronounced in 'Braeburn' (Fig. 4.4). Periodic observations of the stalk apex revealed no signs of dysfunction with the bundles appearing completely stained all through the growth period. This strongly suggests that xylem dysfunction occurs exclusively in the fruit.

Fig. 4.4. Transverse sections of dye-infused 'Braeburn' (left) and 'Granny Smith' (right). Fruit were assessed at 64 and 67 DAFB respectively. The degree of coloration in fine vascular anastomoses indicates a difference in vascular function between the two fruit.

The dye level in the primary bundles was at the maximum measurable value of 3.0 early in the season. After this colouration declined exponentially with time (Fig. 4.5), so that a model was chosen of the form

$$D_{\text{prim}} = \begin{cases} 3, & t \le t_0 \\ 3\exp(-k(t-t_0)) & t > t_0 \end{cases} \qquad (2)$$

where t_0 (the time, DAFB, when D first drops below 3.0) and k are parameters to be fitted. The exponential decay parameter k can be related to a 'half-life' ($t_{½}$), the time interval for D to be halved from any point on an exponential curve by

$$t_{½} = \frac{\log(2)}{k} \qquad (2a)$$

For both cultivars, the decrease in D with time was highly significant ($p \le 0.001$), i.e. $k \neq 0$, and by harvest fruit have lost most of their xylem function. Differences in the extent of xylem dysfunction between the two cultivars were well described by a single time-scaling factor, with the difference being highly significant ($p \le 0.001$), and with 'Braeburn' taking just 0.67 times as long as 'Granny Smith' to decay at any given D.

Using this scaling factor, fitted parameter values were estimated at t_0 = 67 DAFB and $t_{½}$ = 61 days for 'Granny Smith', and t_0 = 45 DAFB and $t_{½}$ = 41 days for 'Braeburn'. The sudden collapse in xylem function occurs earliest in 'Braeburn', whereas in 'Granny Smith' function continues for longer and the fruit still retains some functionality at harvest.

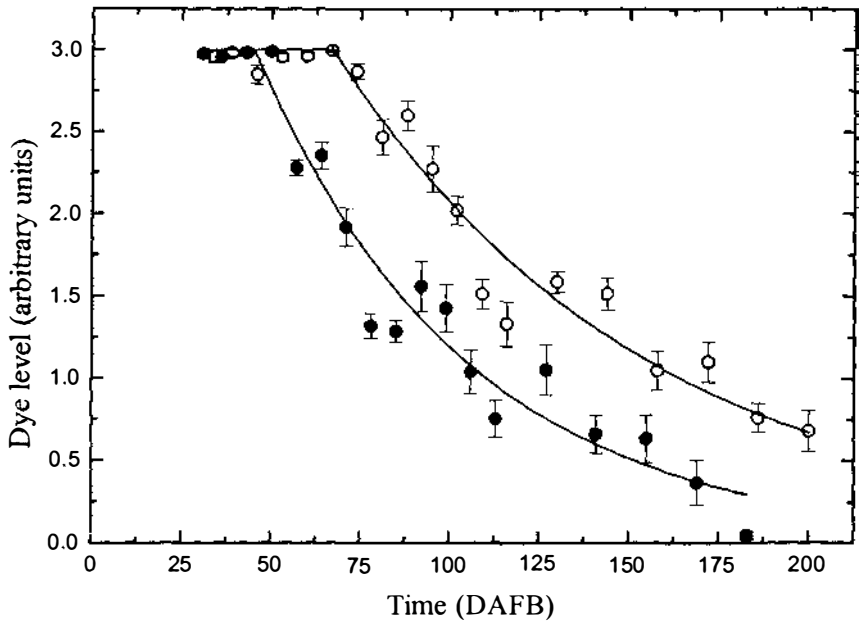


Fig. 4.5. Dye level in the primary (cortical) bundles of 'Braeburn' (●) and 'Granny Smith' (○) as a function of fruit age (DAFB). The mean dye level for each time period was obtained by assessing 15 fruit per cultivar. Vertical bars represent SE.

The dye level in the dorsal bundles (Fig. 4.6A) rose initially to a maximum value (*A*), but then followed a somewhat complex path. The conductance in both cultivars oscillated markedly but the xylem remained fairly functional throughout the season. As there was no obvious mechanism by which bundle conductance, and therefore *D*, could increase around day 125, this fluctuation was ignored and the path was described by a declining logistic curve

$$D_{\text{dors}} = \begin{cases} A + m(t - t_0), & t \le t_0 \\ \dfrac{A'}{1 + \exp(k(t - t_h))} & t > t_0 \end{cases} \quad (3)$$

where A, t_0 (the time, DAFB, at which D starts to decay), m (the slope up to the maximum D), t_h (the time, DAFB, when D drops to half of A'), and k are parameters to be fitted. To ensure continuity, the upper asymptote of the logistic curve $A' = 1 + \exp(k(t_0 - t_h))$. For a logistic, parameter k can be related to the times (DAFB) at which D drops to two thirds or one third of A' ($t_{⅔}$ or $t_{⅓}$), by

$$t_{⅔}, t_{⅓} = t_h \pm \frac{\log(2)}{k} \quad (3a)$$

As was the case for the primary bundles, the drop in D was significant ($p \le 0.001$), and using separate values for every parameter in each cultivar provided no significant improvement over using a simple scaling factor. The difference in time scales was significant ($p \le 0.05$), with the fitted scale factor of 0.85. With this scaling factor, fitted parameter values for 'Granny Smith' were m = 0.19, t_0 = 42 DAFB, and t_h = 172 DAFB (with $t_{⅔}$ and $t_{⅓}$ 25 days before and after this), and for Braeburn were m = 0.23, t_0 = 36 DAFB, and t_h = 146 DAFB ($t_{⅔}$, $t_{⅓}$ ± 21 days). For both cultivars, A = 2.2.

The dye level for ventral bundles (Fig. 4.6B) also rose initially to a maximum value (A), but dropped roughly exponentially to a non-zero final value (B). Hence, a model to describe these characteristics was

$$D_{\text{vent}} = \begin{cases} A + m(t - t_0), & t \le t_0 \\ B + (A - B)\exp(-k(t - t_0)) & t > t_0 \end{cases} \quad (4)$$

Parameters to be fitted are A, B, m, t_0, and the exponential decay parameter k, which is related to a 'half-life' ($t_{½}$), the time interval for D to halve along exponential curve relative to the final level B by (2a).

For both cultivars, the drop in D with time following t_0 was highly significant ($p \le 0.001$), and the final level reached (B) is significantly greater than zero ($p \le 0.001$). Nevertheless, there were no significant differences between parameters for the two cultivars, with the fitted scaling factor (0.97) not differing significantly from one. For

both cultivars, fitted parameter values were approximately $A = 2.3$, $B = 0.6$, $m = 0.1$, $t_0 = 43$ DAFB, and $t_{½} = 12$ days.

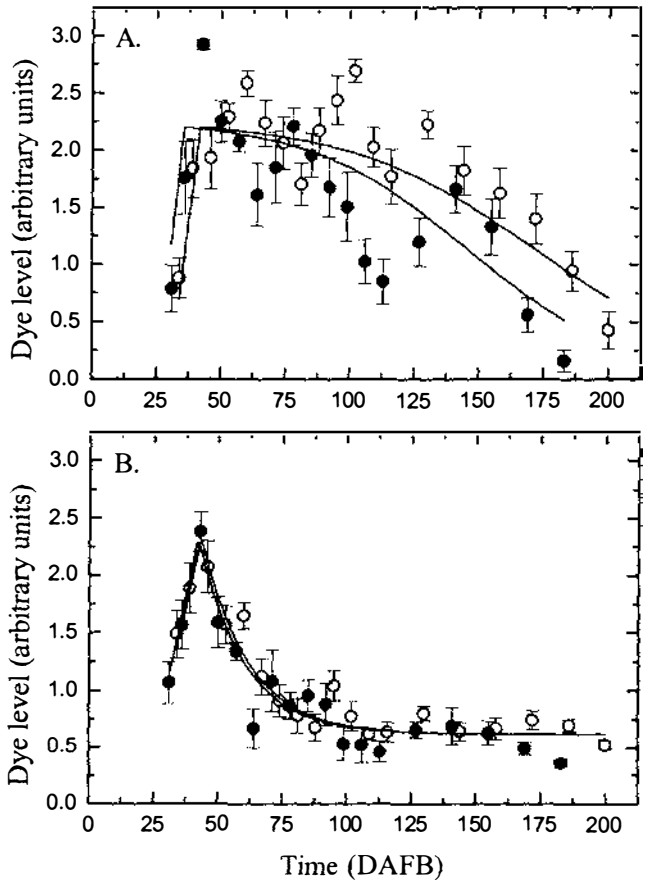


Fig. 4.6. Dye level in the dorsal (A) and ventral (B) carpellary bundles of 'Braeburn' (●) and 'Granny Smith' (○) as a function of fruit age (DAFB). The mean dye level for each time period was obtained by assessing 15 fruit per cultivar. Vertical bars represent SE.

4.3.3. Spatial changes in xylem functionality

Serial (lengthwise) sectioning of dye-infused fruit showed a strong spatial gradient in D with increasing distance from the stalk end of the fruit ($p \leq 0.01$), except for ventral bundles in 'Braeburn' (Fig. 4.7). Mean dye level is plotted against longitudinal position in the fruit, where '0' refers to the point near the stalk end where the vascular bundles were first apparent, and '10' refers to the last such point close to the calyx end. However,

the ventral bundles in 'Braeburn' were not clearly distinguishable at the '0' position in the fruit (missing value, Fig. 4.7A) while some data points in other vascular bundles of the same cultivar were determined only by a single value and lack error bars.

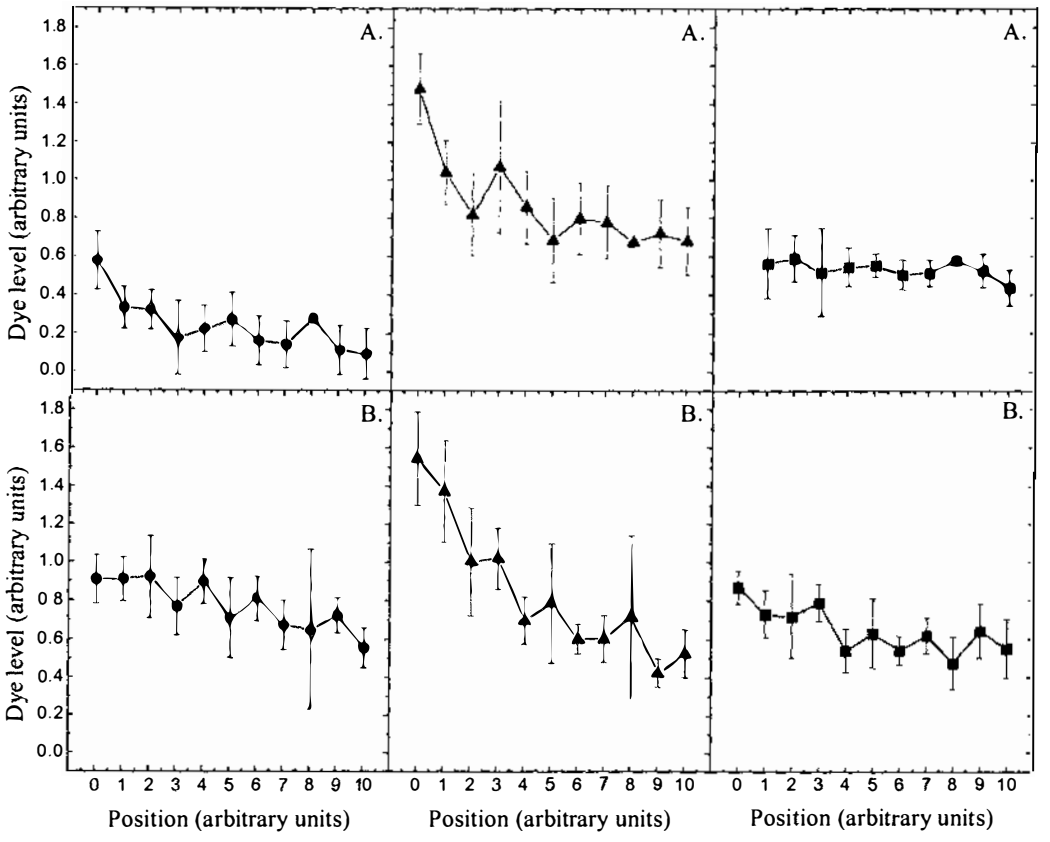


Fig. 4.7. Longitudinal changes in the functionality of xylem in 'Braeburn' (A) and 'Granny Smith' (B), assessed at 160 and 163 DAFB respectively. Each point represents the means dye level for the primary (●), dorsal (▲), and ventral bundles (■) falling within the same fractional distance down the fruit. Vertical bars represent SE.

For both cultivars, there were significant differences among the gradients for the bundle classes ($p \leq 0.01$), with dorsal bundles having the steepest gradient for both. The gradient for dorsal bundles in 'Granny Smith' was significantly greater than the gradient for 'Braeburn' ($p \leq 0.01$), but there were no significant differences in the gradients between cultivars for primary or ventral bundles.

4.3.4. Anatomy of xylem breakage

Longitudinal image of the dysfunctional primary bundle of 'Braeburn' shows interruption of dye movement close to the stem-end of the fruit (Fig. 4.8). The collapse of xylem function was evident through the sudden cessation of dye import beyond the point of disruption leaving the bundle completely unstained (dysfunctional) when assessed in transverse section. Diffuse dye in the flesh around the distinctively stained part of the bundle confirms that a substantial amount of apoplastic dye enters the fruit but that dye passage in the bundle can be blocked.

Fig. 4.8. A dysfunctional primary bundle in 'Braeburn' (141 DAFB). Discontinuity of dye distribution through the bundle indicates the point of dysfunction.

Microscopic examination of dysfunctional primary bundles confirmed a localised area of damage in the xylem tissue (Fig. 4.9). The conducting elements of the xylem consist mainly of vessels organised into parallel columns with spiral thickening of the walls, although occasional annular thickening is also apparent. The most conspicuous damage appears as an irregular spacing and deformation of the vessel files. Annular structures sometimes collapse entirely leaving occasional rounded elements completely isolated. At other times, spirals appear to be overly stretched and partially fragmented (Fig. 4.9A-B). That this characteristic was an artefact of sample preparation is judged unlikely due to the existence of vessels with intact walls adjacent to the deformed and fragmented structures. Furthermore, many vessels were seriously damaged with helices very stretched (Fig. 4.9C-D).

Additional structural changes were apparent when longitudinal sections of dysfunctional primary bundle were assessed for xylem damage in the advanced stage of fruit growth. The evidence showed that complete breakage of the xylem strand occurred randomly along the bundle. Cells of parenchyma filled the spaces between files of vessels indicating total discontinuity and collapse in the function (Fig. 4.9E). Within a xylem strand, stretched vessels with spiral thickening were frequent, often sustained in a broken line, which reflects the high degree of strain (Fig 4.9F).

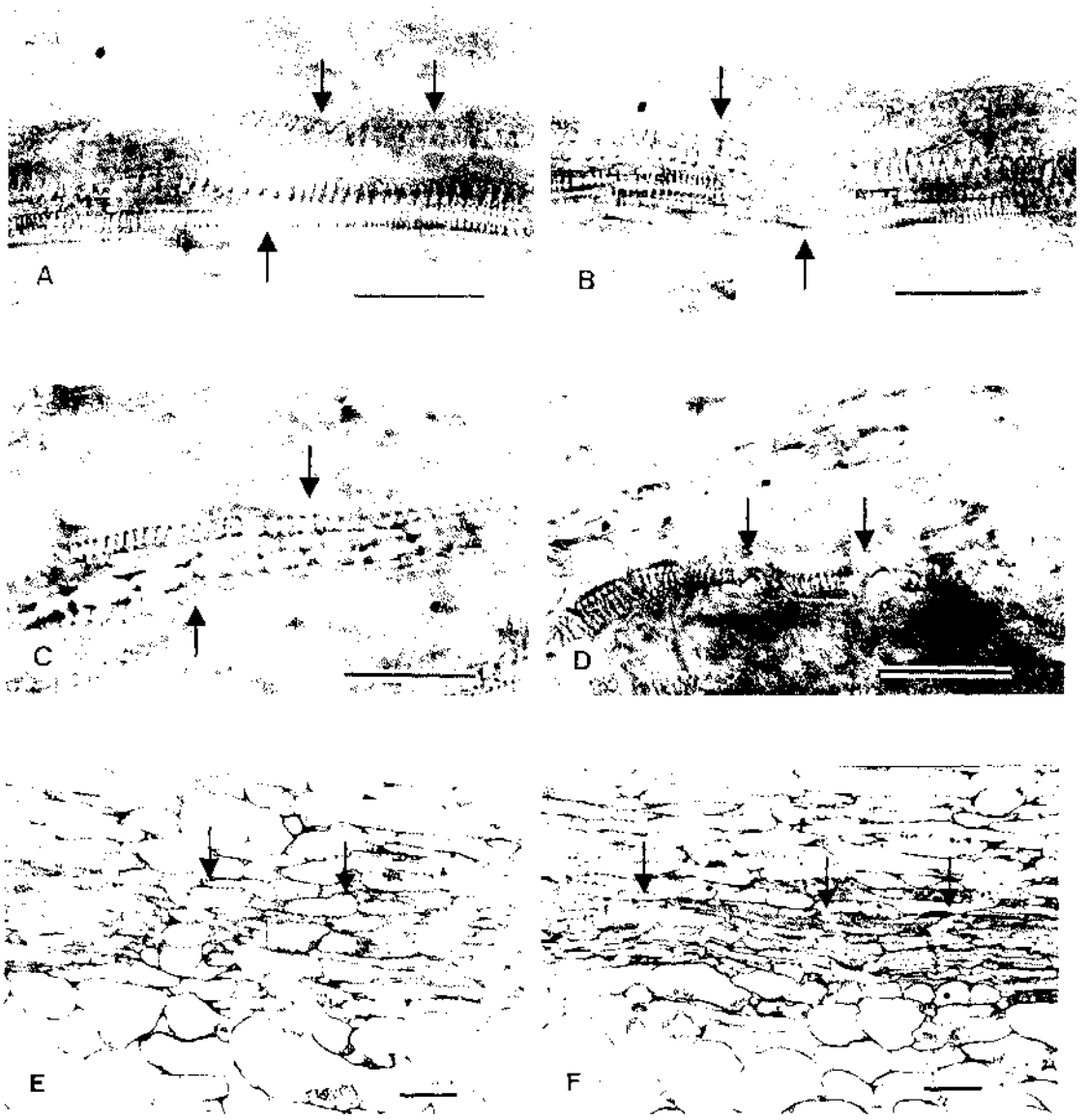


Fig. 4.9. Longitudinal sections of a primary (sepal) bundle in the fruit of 'Braeburn', 85 DAFB (A-D) and 141 DAFB (E-F) respectively. (A-B) Irregular spacing of the annular and spiral wall thickenings exists alongside undamaged cell structures. (C-D) Deformed and fragmented vessels. Arrangement of helices in the broken segments indicates stretching. (E) Breakage of the xylem strand. Shattered parts are displaced so that parenchyma cells occupy the resulting void. (F) Ruptured string of vessels. Multiple breaks signify excessive change in length of the stressed walls. The bar in A-F represents 50 μm. Arrows indicate the structural changes being described.

4.3.5. Growth of the different fruit tissues

4.3.5.1. ***Absolute Tissue Growth (ATG).*** Fruit structures in both cultivars exhibited a sigmoidal growth pattern (Fig. 4.10). A linear increase in the earlier stages was followed by a gradual decline in growth rate as the various dimensions of the tissues approach their maximum values. The curves of the various fruit tissues illustrate the difference in their growth dynamics. Increments in the locule dimensions flatten earlier in comparison with other tissues. Locule growth, therefore, makes the least contribution to overall fruit growth. The growth of the core continues for some time after the slope of the locule growth flattens as a result of prolonged flesh (pith) enlargement within the core. Overall fruit growth continues almost until harvest. The two cultivars exhibit similar growth patterns, although the slopes of 'Braeburn' growth are somewhat steeper.

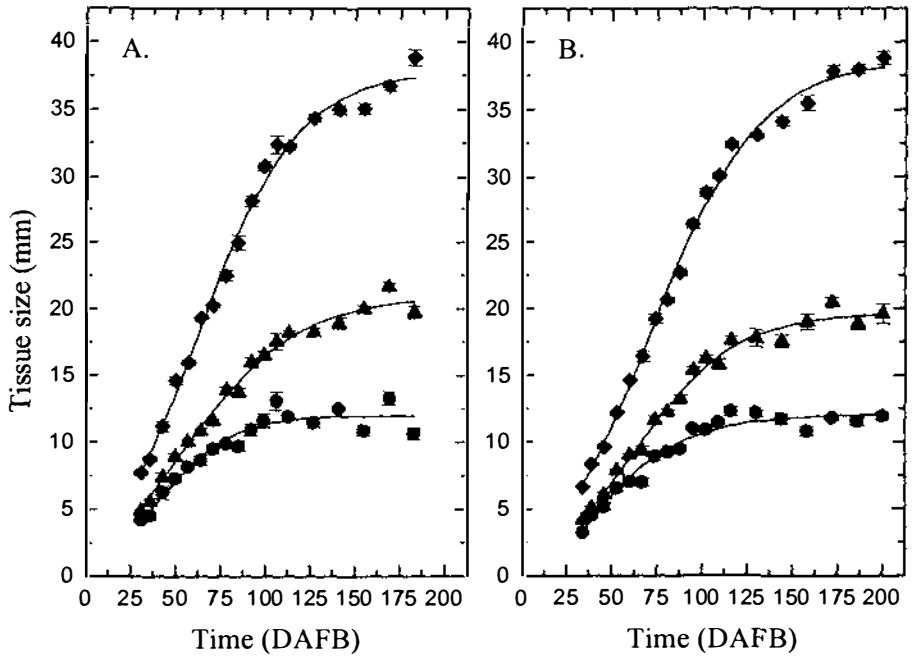


Fig. 4.10. Absolute growth of various tissues in the fruit of 'Braeburn' (A) and 'Granny Smith' (B). The points are mean values for a single fruit at each time period derived from five locule (●), or ten core (▲) and fruit dimensions (◆). The difference between core and fruit curves represents the cortical tissue. Vertical bars represent within-fruit SE.

4.3.5.2. *Relative Tissue Growth (RTG).* The data on ATG were transformed to represent the contributions of a particular tissue to the growth of the whole. Although absolute fruit growth involves rapid increases in the core structures, it is the cortical tissue that accounts for most of the enlargement of the fruit in both cultivars (Fig. 4.11). The difference between relative locule and core sizes shows the magnitude of changes in the size of the fleshy pith. Nevertheless, the declining trend in the relative size of the core, compared with the steady increase in the relative size of the cortex, provides key evidence of the relative contribution of these tissues to overall fruit growth.

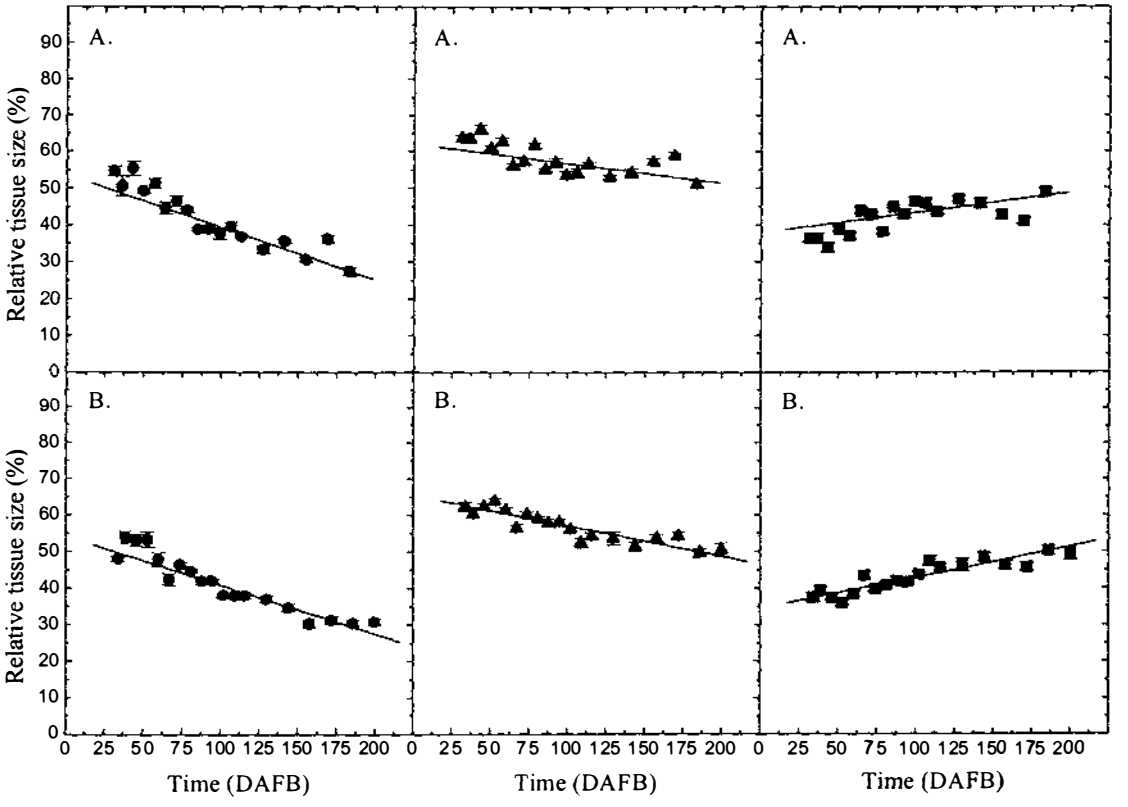


Fig. 4.11. Relative contribution of various tissues to the growth of the fruit in 'Braeburn' (A) and 'Granny Smith' (B). For each time period, the points represent the means of locule (●), core (▲) and cortex (■) size for a single fruit that have been normalised with the corresponding fruit radii. Vertical bars represent within-fruit SE.

4.3.5.3. ***Spatial arrangement of petal and sepal bundles in the flesh (P/S).*** The ratio of the radial distances to petal and sepal bundles provides a suitable measure of variation with time regardless of the differences in the growth potential of the cultivars. The result confirms that petal and sepal bundles move differently during fruit development (Fig. 4.12). In 'Braeburn', petal bundles move somewhat further from the axis of the fruit than the sepal bundles although this discrepancy is not significant. In contrast, the petal bundles in 'Granny Smith' drifted significantly closer to the skin than the sepal bundles ($p \leq 0.05$). These characteristics give rise to a distinctive cultivar layout in transverse sections of mature fruit.

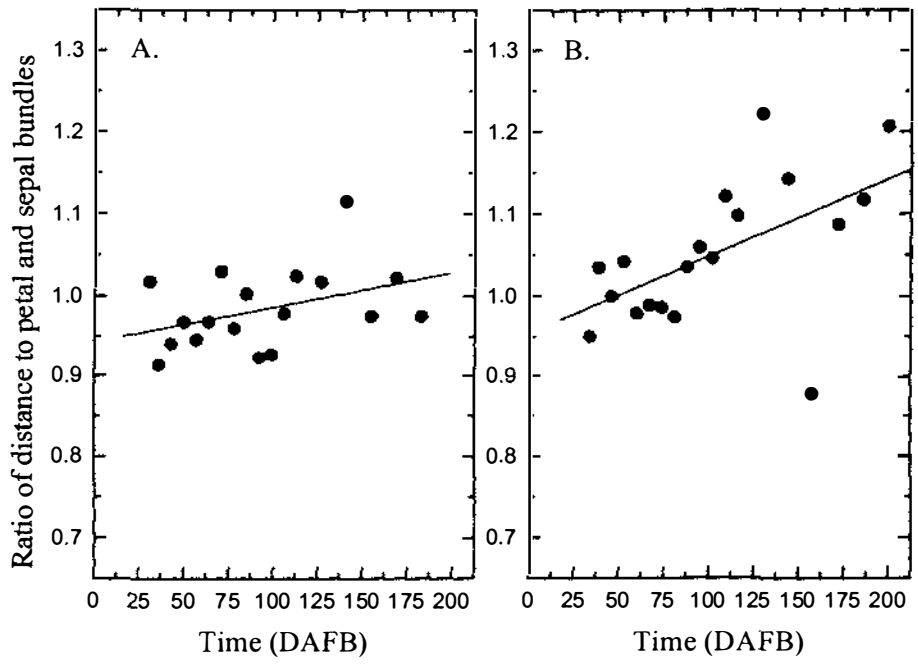


Fig. 4.12. Comparison of the relationship between the relative movements of petal and sepal bundles in 'Braeburn' (A) and 'Granny Smith' (B) during fruit growth. Each point (●) represents the mean ratio of the radial distance to petal and sepal bundle for a single fruit at each time period.

4.3.5.4. ***The layout of sepal bundle relative to the locule (S-L).*** Analysis of the normalised data on S-L does not show a significant overall difference in average rates of sepal bundle drift between the two cultivars. However, it does show that by the latter half of the season (*c.* 100 DAFB), sepal bundles have drifted significantly further from the locules in 'Braeburn' than in 'Granny Smith' fruit ($p \leq 0.05$), (Fig.4.13). Given that sepal bundles in 'Granny Smith' retain functionality for longer than in the fruit of 'Braeburn' (Fig. 4.14), this suggests an influence due to the proximity of the rigid (cartilaginous) walls of the locules.

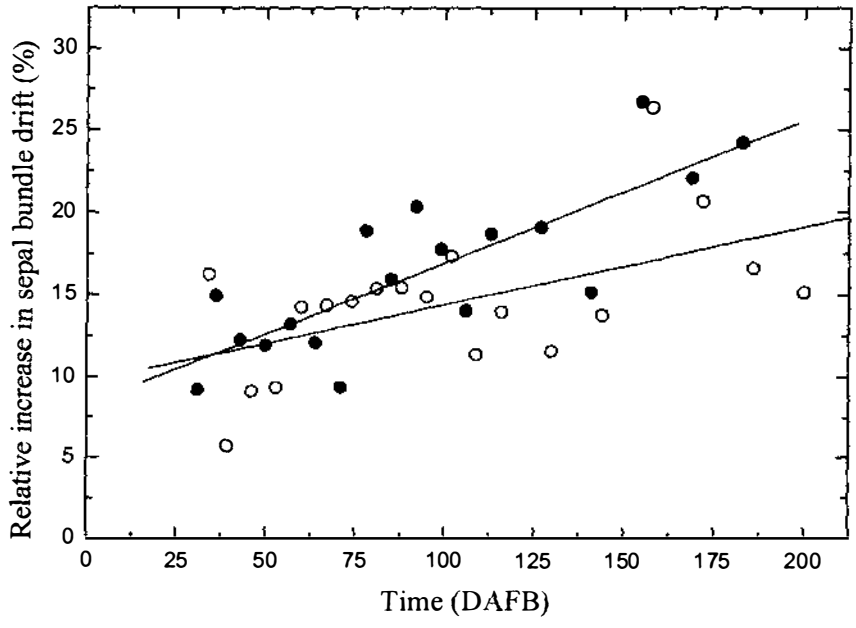


Fig. 4.13. Relative increase in the distance between locule tip and the sepal bundle normalised with the corresponding fruit radii in 'Braeburn' (●) and 'Granny Smith' (○). The points are mean values for a single fruit at each time period.

Fig. 4.14. Dye distribution in 'Braeburn' (left) and 'Granny Smith' (right) apples infiltrated with dye at 169 and 172 DAFB respectively. Note that more sepal bundles in 'Granny Smith' contain more dye and hence presumably remain functional in this fairly advanced stage of maturity.

4.4. DISCUSSION

4.4.1. Exposure time

Because the dye level in the vascular bundles appeared stable after 120 minutes (Fig. 4.3) any differences in D can be taken to reflect a physical property of the bundle itself rather than of the measuring technique.

4.4.2. Temporal changes in xylem functionality

For the primary and ventral bundles in the fruit, the model developed provided a good fit to the mean curves for both cultivars (Figs. 4.5 and 4.6B). For dorsal bundles, the model could not capture the later rise in D, but otherwise fitted reasonably well (Fig. 4.6A). The decline in D was significant for all three bundle-classes. Nevertheless, dysfunction was irregular, with some fruit losing the greater part of their xylem conductance relatively early on. The majority of bundles exhibited reduced dye movement, which indicated random damage to the xylem at any one time. Hence, the trend in D at any stage of the

growth period reflected averaged xylem conductance based on the prevailing condition of individual bundles. Consequentially, the few operational bundles close to harvest often masked the actual extent of xylem collapse in nearly all bundles of the fruit.

The most marked change in xylem conductance occurs in the primary bundles (Fig. 4.5) that are the principal nutrient suppliers of the flesh (MacDaniels, 1940). The highest xylem flow of these bundles was obtained at the beginning of the season. The early-season plateau in dye accumulation could be seen as an artefact of the arbitrary dye assessment that limits the maximum to which D can rise to an integer value of 3. Nevertheless, D in both cultivars exhibited identical trends indicating that the primary bundles were fully functional at the beginning of fruit development. A similar pattern in functionality of the fruit xylem was obtained in different apple cultivars examined using a pressure-bomb technique (Lang and Ryan, 1994; Drazeta *et al.*, 2001).

As the season progressed, an increasing proportion of primary bundles failed to transport apoplastic dye. For 'Braeburn', changes in D were more extreme, and xylem conductance became negligible over the final weeks of fruit development. A fitted time-scaling factor of 0.67 suggests that xylem conductance in 'Braeburn' breaks down in roughly 2/3 of the time it takes for conductance breakdown in 'Granny Smith'. The sharp decline in D from around 45 DAFB ('Braeburn') and 67 DAFB ('Granny Smith') closely matches the completion of vessel differentiation (*c* 50 DAFB) in the primary bundles of 'York Imperial' apple (Barden and Thompson, 1963). This indicates that cessation of cambial activity in the bundles of the fruit coincides with the onset of xylem dysfunction.

A mechanistic explanation for the reduction in xylem conductance proposes growth-induced damage to the xylem strand in the bundle (Findlay *et al.*, 1987; Düring *et al.*, 1987; Lang and Ryan, 1994; Drazeta *et al.*, 2001). However, the function of the phloem remains unchanged relative to the xylem flow (Lang, 1990). The hypothesis that fruit expansion stretches and ruptures the xylem provides no elucidation of the mechanism that maintains phloem function in the bundle. Because phloem tissue is composed of living conducting elements (Esau, 1977), the persistence of functional phloem could be attributed to:

- persistent sieve tube differentiation that bypasses the broken elements, since the phloem differentiates in the absence of the xylem and at lower auxin levels (Aloni, 1980; Aloni and Barnett, 1996),
- the more extensible cell walls of the sieve tubes (Lee, 1981) with a more prolonged synthesis of cell wall depositions enabling the sieve tubes to accommodate more strain, and
- continuous symplastic pathway through parenchyma cells that occupy the voids between the broken structures.

If fruit growth principally creates stress on conductive tissues it is likely that any one, or some combination, of these features allows continuous phloem uptake into the fruit.

Cortical and carpellary vascular systems vary in the dynamics of the xylem breakdown. Dye accumulation was somewhat less disrupted in the carpellary bundles that are seemingly less conductive in regard to their principal function in the fruit (Figs. 4.5 and 4.6). Comparing with primary bundles, it is apparent that dorsal bundles are less affected with a progressive but slower loss of xylem function (Fig. 4.6A) but, similarly, the conductance appears to drop without a lower limit. Changes in D once again occur more rapidly for 'Braeburn' and although statistically significant, cultivar differences were smaller and less regular. Fitted values suggest early (36-42 DAFB) but rather gradual decay in D that takes much longer to reach half its maximum value (142-172 DAFB).

Ventral bundles exhibited distinctively lower D at most stages throughout the growth period (Fig. 4.6B). After the initial rise, xylem functionality declined sharply but proceeded with a rather low, yet steady flow following an exponential decay. Maximum D was reached at about 43 DAFB, than dropped exponentially with a 'half-life' of only 12 days ($t_{½}$). For most of the fruit evaluated, the ventral bundles accumulated substantially less dye and often appeared unstained.

The lower D in the dorsal and ventral bundles could result from naturally reduced dye uptake through the carpellary network, which develops well below the skin and appears less affected by transpiration. Dorsal bundles that follow the outlines of the carpel bases may undergo seasonal changes in xylem function closely related to the growth rates of adjacent tissue. Hence, the xylem in the dorsal bundles may have been stretched beyond

its breaking strain due to a strong increase in the size of the carpellary tissue. Nevertheless, considering that the dorsal bundles branch from the primary bundles at the base of the fruit, the late season decline of flow may indicate blockage beyond the point of separation. If flesh expansion towards the stem-end upsets primary bundle function near the stalk that, in return, could affect the conductance of the branching dorsal bundles.

In contrast, the ventral bundles principally serve the seeds (MacArthur and Wetmore, 1939), which presumably require less nutrient (diminished water influx) than the flesh. This, and the fact that the ventral bundles develop below the carpel bases, may affect dye accumulation in a way that dye spreads slowly throughout the ventral network, which results in consistently lower D when assessed in transverse sections. Simultaneously, the establishment of reproductive structures determines the course of vascular function such that ovule abortion causes degeneration of the supporting ventral bundles (MacArthur and Wetmore, 1939). Considering that seed abortion in developing apples is continuous throughout the season (Lang, unpublished), it seems reasonable that functionality of the ventral bundles is somewhat hampered compared with the other bundles. However, the origin of the functional disturbance at the beginning of the season remains unknown.

The course of xylem dysfunction could be of special importance to the mineral composition of the fruit. A steady decline in xylem conductance under constant fruit transpiration must force a shift in the balance of supply to the fruit of xylem-borne nutrients, notably calcium. Indeed, xylem function decays from relatively early in the season, which mirrors the decline in calcium import (Wilkinson, 1968; Jones *et al.*, 1983). This naturally-occurring mechanism could be principally responsible for the increased phloem inflow to the fruit that maintains growth (Lang, 1990).

Hence, programmed xylem breakdown is likely to reduce apoplastic backflow of solutes (from fruit to the tree), which promotes the partitioning of assimilates to reproductive sinks (Lang and Thorpe, 1989). A rather variable timing of xylem dysfunction can, therefore, create high variability in fruit mineral composition. This could explain the high variability observed in the incidence of calcium-related disorders (Drazeta *et al.*, 2001).

Hence, the earlier start of xylem dysfunction in 'Braeburn' fits with the observation that, of the two examined cultivars, 'Braeburn' is the more susceptible to bitter pit.

4.4.3. Spatial changes in xylem functionality

Significant changes in xylem function were established using contiguous sectioning to evaluate *D* in vascular bundles. The most conspicuous gradient was obtained in the dorsal bundles that on average scored higher *D* values than the other bundle classes and differed between the two cultivars (Fig. 4.7). However, dye concentration declined in nearly all bundles with increasing distance from the stalk end of the fruit suggesting gradual disruption of the flow along the bundles. If the xylem vessels are only partially broken, some dye can still move through the bundle, whereas a complete gap will stop all flow at this point.

The extent of xylem dysfunction signifies the direction and relative intensity of fruit expansion. By cutting 'Gala' through the centre of the fruit and measuring the increase in the 'gape' openings, Opara (1993) showed that the first noticeable growth stress occurred around 60 DAFB and reached maximum just before harvest. Moreover, the extent of gaping was greater in a vertical than a horizontal cut. Thus it may be inferred that internal fruit growth is not uniform in the two directions, leading to an asymmetry of the resulting growth-induced stress. Two possible explanations emerge from this:

- The extent of xylem dysfunction could be greater in the faster-growing regions of the fruit due to the larger strains generated, or
- Tensile stresses created by an expanding fleshy matrix develops evenly along the bundle path so that xylem breakage appears at random as fruit growth continues.

The appearance of multiple breaks along each bundle suggests that bundles are well attached to the expanding flesh tissues. The trend in longitudinal dye accumulation indicates irregular multiple breakages. For this reason, spatial assessment of *D* supplements the data on seasonal xylem functionality although fruit were examined only once, close to harvest maturity, and contains no information on seasonal breakdown in regard to the position within the fruit.

The spatial patterns in xylem function are important to postharvest physiology. The reduction in dye accumulation towards the calyx end of the fruit appears to fit with the reported longitudinal gradient in calcium concentration found in apple fruit (Lewis and Martin, 1973; Lewis, 1980). As the fruit expands, progressive xylem dysfunction is likely to result in a reduction of calcium uptake and displacement within the fruit, leading to the uneven spatial distribution and imbalance with phloem-borne minerals. Consequentially, the distal regions of the fruit are depleted in calcium and are at higher risk of developing calcium-related disorders than the proximal zones. Indeed, bitter pit lesions are commonly observed near the calyx-end of apple fruit (Ferguson and Watkins, 1989). This suggests the localised nature of the disorder and fits with the spatial profile of dye accumulation in the fruit.

4.4.4. Anatomy of xylem breakage

The forms and arrangements of wall thickenings in dysfunctional primary bundles of 'Braeburn' (Fig. 4.9A-D) conform to the pattern of vascular ontogeny in the developing plant body (Esau, 1977). Scattered elements with annular thickening represent the remnants of protoxylem structures that probably cease to have functional importance as soon as organ expansion obliterates their structure. In contrast, spiral depositions dominate the wall thickenings of later formed vessels and appear in two distinctive forms. Deformed and fragmented spiral elements are possibly the earliest metaxylem structure, initiated before protoxylem growth was complete. The common 'uncoiled' appearance of the thickening helices of the broken vessels offers strong evidence of excessive structural damage due to growth-induced strain. The remaining vessels in the xylem strand have less steep helices and appear functional, which suggests that later vessel differentiation has occurred to replace the older, overly stretched and dysfunctional elements.

The mechanical properties of vascular elements make it possible to assign likely changes in the xylem of developing fruit. When a growth-induced strain is applied, cell walls will stretch resulting in the fractional change in length (strain), which is specific for a given structure (Nobel, 1999). The magnitude of breaking strain, therefore, relies on the mechanical properties of the xylem tissue. Lignified tube-like conducting cells of the xylem seem to provide good mechanical support but exhibit rather low elasticity. When

expressed as a percentage of the broken segment length for the peripheral vascular bundles in the grape berry, first breakage occurs close to veraison, with fractional change of *c.* 3% (Düring *et al.*, 1987). This bears close resemblance with calculated breaking strain of *c.* 2-3% for xylem fibers in a variety of woody species (Kollmann and Cote, 1968).

Furthermore, the magnitude of cell wall deformation is proportional to the time of applied stress (Nobel, 1999), indicating that the growth dynamics could principally determine cell functionality in stressed tissue. Indeed, high fruit growth rates decrease calcium supply (Marschner, 1986), and larger fruit are usually more prone to the incidence of bitter pit (Ferguson and Watkins, 1989). Vigorous fruit growth, particularly from the onset of cell expansion is likely to initiate earlier xylem dysfunction, as a result of which, the relationship between xylem conductance and potential fruit size must not be taken at the time of maturity but expressed periodically. In addition, the persistence of xylem conductance depends not only on the active tissue growth but also on the amplitude of the vessel differentiation by the vascular cambium. Only knowledge of the periodicity and intensity of these processes could elucidate actual dynamics of xylem dysfunction.

4.4.5. Growth of the different fruit tissues

Dimensional measurements of ATG and RTG gave a more comprehensive picture of the growth pattern in the two cultivars. It should be noted, however, that the growth of fruit structures was obtained by measuring linear dimensions in a growing spherical organ, which does not capture the exact picture of its volume increments (Bollard, 1970). The exponential phase in ATG was followed by diminishing growth later in the season, which implies a sigmoidal growth pattern as commonly reported in apple (Opara, 2000). In contrast, RTG accounts for growth efficiency of a particular part relative to the growth of the whole, which allows for more equitable inter-tissue comparisons. From the beginning of the growth measurements, the core and cortex showed different relative contributions such that cortex development dominated the change in fruit size (Fig. 4.11), which is in the line with the relative contribution of different tissues to the overall fruit size (Smith, 1950).

The patterns in fruit tissue growth refined the idea of specific structural features leading to analogous physiological responses. Because xylem collapse in the bundles is related to the growth of the fruit, the extent of damage depends upon the rate of tissue enlargement. Although fractional increase in fruit size is mainly driven by cortical enlargement, it is the rate of core growth that governs the intensity of xylem dysfunction in primary bundles. Since fruit development initiates a drift of tissues outwards, growth-induced stretching of primary bundles is exclusively determined by core expansion.

The dissimilar spatial layout of the primary bundles observed between 'Granny Smith' and 'Braeburn' conforms with the fact that the cross-sectional outline of apples is one of the structural features used for pomological description (Bultitude, 1983). In mature 'Granny Smith', primary bundles form a somewhat angular pattern (with petal bundles outlying the arrangement of sepal bundles), whereas in 'Braeburn' the layout of primary bundles is ring-shaped. This is in line with conspicuous cultivar differences in the mean ratio of the radial distance to primary bundles (Fig. 4.12), and coincides with the prolonged functionality of sepal bundles in 'Granny Smith'.

This observation implicates a 'protective' role of the locule. Slower growth of a locule is likely to reduce early season motion of sepal bundles that are positioned closer to the locule. This can also restrict the influence of core enlargement by producing fewer cell layers of the pith. Hence, the magnitude of the drift of the sepal bundles is not fully attained at the time that locule growth ceases, since the core continues to grow through the increase of the pith (Fig. 4.10). As a result, the difference in drift of sepal bundles appears by the latter half of the season and increases towards fruit maturity (Fig. 4.13). However, no degree of protection applies to the petal bundles that are fully exposed to the rapid flesh growth.

These morphological and physiological aspects of tissue growth emphasise the significance of variations in the internal structure of apple fruit. Since the pattern of fruit growth is a peculiar cultivar feature (Pratt, 1988), particular structural attributes could determine functional response of the whole fruit. Moreover, the extent of xylem breakdown as the season progresses is of considerable relevance to the nutrition of the expanding fruit. It was observed that some earlier-maturing cultivars grew more rapidly

(Tukey and Young, 1943), which could lead to premature xylem dysfunction and change the balance of minerals in the developing fruit. The importance of growth dynamics is further strengthened by the fact that calcium concentration peaked early in the season (Jones *et al.*, 1983), when the rate of growth was notably slower (Percy *et al.*, 1996). Consequentially, the outcome of increased growth rates over the brief period of time (i.e. early in the season) could result in greater calcium deficiency.

4.5. CONCLUSIONS

The phenomenon of progressive xylem dysfunction in apple fruit was studied by assessing the intensity of dye infusion in the bundles. The structural nature of the changes in function of the xylem was assessed using a microscopic procedure. Fruit growth dynamics were also analysed and critical morphological features were identified. The literature clearly supports the pattern of xylem dysfunction, the observation of physical damage to the xylem vasculature and features of the gross development of fruit structure.

This study has shown that the xylem vasculature of apple fruit suffers progressive dysfunction during the growing season. Visual signs of physical disruption of the bundles seem to explain this dysfunction. Dysfunction was greatest in the primary bundles suggesting a likely impact on fruit mineral uptake. Interestingly, in the two cultivars studied, dysfunction occurred earlier and was more pronounced in the one with the heightened susceptibility to bitter pit. Spatial patterns of dye infusion indicate a marked reduction in function of the xylem towards the calyx-end of the fruit. This fits with the observation that calcium (mobile only in the xylem) is least concentrated at this end of the fruit. The observation supports the view that progressive xylem breakdown underlies and is the principal determinant of fruit mineral status and distribution.

Frequent sampling of fruit during the season has been used to evaluate internal tissue growth and the corresponding changes in the spatial arrangement of the primary bundles within the flesh. It was found that fruit grew mainly through an increase in the volume of

the cortical tissue while the extent of difference in the layout of the primary bundles may account for the different degrees of dysfunction between cultivars.

In conclusion, the entire work contained in this chapter suggests that growth-induced strains damage the vessels and impact negatively on xylem inflow resulting in changes in mineral composition as the fruit matures. There is a need to further elucidate these processes in relation to fruit growth dynamics during the season. It is hoped that the results and discussion presented will contribute to a more comprehensive understanding of developmental changes in xylem function of the fruit. In this way, the apple industry might be able to minimise the impact of early vigorous growth in apples (i.e. through better cultivar selection and through appropriately adjusted crop load) so as to reduce the occurrence of calcium-related disorders.

Chapter 5. Air and cell mapping of apple fruit

5.1. INTRODUCTION

An apple fruit is composed of many different tissues but the greatest volume comprises the flesh (Smith, 1950). This is made of parenchyma permeated with vasculature and intercellular spaces (Esau, 1977). The fractional air volume increases during fruit growth so as to occupy a considerable proportion of the flesh volume by harvest (Bain and Robertson, 1951; Skene, 1966; Westwood *et al.*, 1967; Soudain and Phan Phuc, 1979; Goffinet and Maloney, 1987; Harker and Ferguson, 1988; Yamaki and Ino, 1992; Ruess and Stösser, 1993). The increase in air space during development is accompanied by a proportional decline in fruit density; the density of the fruit cells themselves remains roughly constant (Bain and Robertson, 1951; Skene, 1966; Westwood *et al.*, 1967). Furthermore, the volume of the intercellular air spaces continues to expand during storage (Soudain and Phan Phuc, 1979; Hatfield and Knee, 1988; Harker and Hallett, 1992; Ruess and Stösser, 1993).

The fraction of intercellular air in the flesh differs between the cultivars (Reeve, 1953; Baumann and Henze, 1983; Vincent, 1989; Ruess and Stösser, 1993), while larger fruit of the same cultivar have a higher proportion of intercellular air than smaller fruit (Reeve, 1953; Ruess and Stösser, 1993; Volz *et al.*, 2002). However, the significance of air volume on fruit size is a long-standing matter of controversy of whether the late fruit growth was due mainly to an increase in the size of intercellular spaces (Tetley, 1930; MacArthur and Wetmore, 1941; Reeve, 1953) or not (Skene, 1966; Goffinet *et al.*, 1995).

Intercellular air volume is also an important factor in understanding the postharvest quality of apple fruit. Fruit with greater fractional air volumes have been shown to be softer (Hatfield and Knee, 1988; Yearsley *et al.*, 1997a, 1997b; Volz *et al.*, 2002), or more mealy (Harker and Hallett, 1992) and to have greater internal gas diffusion rates (Rajapakse *et al.*, 1990).

A range of techniques has been developed to measure the intercellular air in apple fruit. One is based on weighing the amount of water that replaces an equal amount of air removed by a vacuum from the submerged tissue. A number of studies have used this principle with a range of sampling methods, vacuum pressures, treatment durations and infiltration media (Reeve, 1953; Harker and Ferguson, 1988; Vincent, 1989; Rajapakse *et al.*, 1990; Yamaki and Ino, 1992; Yearsley *et al.*, 1996). In contrast, Calbo and Sommer (1987) estimated intercellular air volume using a gasometric technique that measured the amount of air extracted from samples when subjected to vacuum. An alternative way to calculate air fraction has been to assess the weight and volume of a whole fruit when submerged in water (Bain and Robertson, 1951; Skene, 1966; Westwood *et al.*, 1967; Baumann and Henze, 1983; Hatfield and Knee, 1988; Harker and Hallett, 1992). Other authors have taken a stereological approach to estimate air volume in the tissue (Soudain and Phan Phuc, 1979; Ruess and Stösser, 1993; Goffinet *et al.*, 1995).

The intent of this study was to develop methods through which to measure internal air in fruit flesh and to elucidate tissue structure at the cellular level. 'Braeburn' was used for this study as it is known to have a relatively dense and firm flesh (Dadzie, 1992; Yearsley *et al.*, 1997a; 1997b), poor flesh gas-diffusivity (Rajapakse *et al.*, 1990) and low skin gas-permeance (Rajapakse *et al.*, 1990; Dadzie, 1992). All these features could lead to increased susceptibility to tissue browning and damage.

5.2. MATERIALS AND METHODS

5.2.1. Plant material

The study used mature trees of 'Braeburn' apples grown at the Massey University Fruit Crops Unit, Palmerston North, New Zealand. Trees were planted on MM 106 rootstock and managed according to standard commercial practice. Fruit were picked at late harvest and selected to be without obvious blemishes. To minimise variation, fruit of similar size were chosen (*c.* 200-250 g). They were stored in a cool-store (0.5°C, *c.* 95% RH) for up to 5 months pending analysis. Day-to-day storage was in a cold room (2°C, no humidity control).

5.2.2. Air volume measurement

The intercellular air volume of freshly excised blocks of apple flesh was measured using methods based upon Archimedes' principle. The technique employs two experimental arrangements involving 1 mg sensitivity electronic balances.

The first arrangement measures sample volume by recording the buoyant upthrust experienced by a sample when it is fully immersed in water (Fig. 5.1A). To do this, a small block of flesh was impaled on the tip of a fine (0.24 mm diameter) steel entomological pin and this attached to a magnet mounted on a light stand that rested on the bench. The use of a pair of plastic (non magnetic) forceps simplified sample handling. The components were so positioned that when attached to the magnet the sample was held just beneath the surface of water that was contained in a small (50 cm^3) plastic container that rested on the pan of a pre-tared electronic balance. The buoyant upthrust experienced by the sample appears to the balance as an equal and opposite downthrust and is expressed as an apparent increase in the weight of the water in the container. Because the density of water at 20°C is 0.99823 gcm^{-3}, the balance reading (g) can be directly interpreted as a sample volume (V) (cm^3). With a 1 mg sensitivity electronic balance the system yields sample-volume estimates to the nearest 1 µl. A wetting agent (0.1% Silwet L-77) was added to the water to help eliminate entrainment of air bubbles as the sample was immersed. The force of the meniscus acting upon the pin as it broke the surface (about 1 mg) was minimised both by the use of a fine pin and by the wetting agent. This surface tension induced error was not only small but it was also systematic and appeared equally in all measurements so tended to cancel.

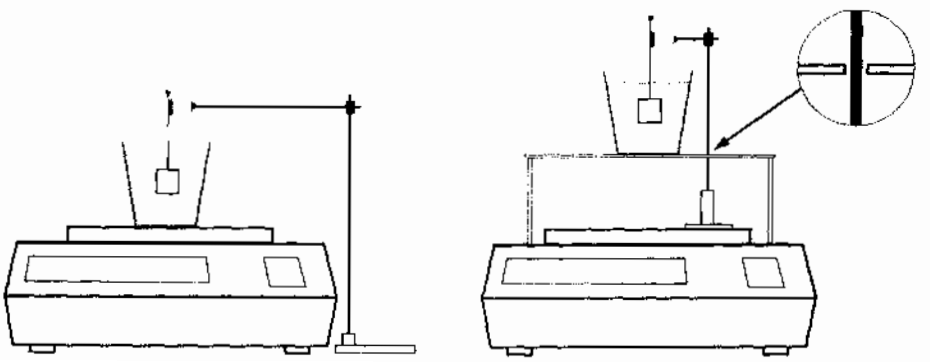
A second balance arrangement was used to measure sample air volume. This involves a subtle variation of the first (Fig. 5.1B). Here, the small container instead rested on a light 'bridge' mounted on the body of a similar pre-tared balance in such a way that the container was supported just *above* the pan. A similar magnet was mounted on a wire support and a light stand and this assembly rested on the balance pan. By attaching the pin holding the flesh sample to the magnet, the sample was again held just beneath the water. Using this arrangement, the balance recorded the weight of the sample while under water. Prior to air extraction the recorded weight was usually negative because apple flesh floats. By weighing the sample just before (W_1) and just after (W_2) the removal of

tissue air under vacuum it is possible to determine the volume of air extracted as an apparent increase in sample weight ($W_\Delta = W_2 - W_1$). Again, because the density of water is very close to unity, the difference in balance readings (g) can be interpreted directly as the volume of air removed by vacuum extraction (cm^3). A 1 mg sensitivity balance measures air volumes to the nearest 1 µl.

Air extraction (after the first weighing and before the second) was accomplished by transferring the sample still with its pin to a 50 cm^3 plastic vial of water containing 0.1% Silwet L-77. A small sinker weight made of a spiral of wire solder was threaded onto the pin to hold the sample beneath the water. While under water in this vial, the sample was exposed for 60 s to reduced atmospheric pressure causing the intercellular air to expand and to bubble out through the cut tissue surfaces. The vacuum generated by a vacuum pump in the presence of water at 20 °C is approximately 18 mm Hg (the vapour pressure of water at this temperature). This causes the intercellular air, initially at the atmospheric pressure (760 mm Hg), to expand its initial volume so that most of it bubbles out. At this low pressure a small residue of air remains within the tissue (about 18×100/760 or 2.3% of the initial amount) but the vast majority (i.e. 97.6%) is removed. This small error is systematic and can either be ignored or allowed for in calculation if the water temperature is approximately known. Following release of the vacuum, the sample was held under the water for a further 60 s. This caused the residue of internal air to contract and the internal spaces (previously air-filled) to became occupied with water (now at atmospheric pressure).

The fractional air content (A) of the sample (%) is, therefore, given as the difference between the two under-water weights expressed as a percentage of the sample volume

$$A = (W_\Delta / V)\,100 \qquad (1)$$



A. Measuring sample volume by displacement

B. Measuring sample weight under water

Fig. 5.1. Experimental arrangements for measuring fractional air volume. (A) Sample volume was first recorded by measuring the upthrust on the sample when fully immersed (equal to the weight of displaced water and expressed as an equal and opposite downthrust on the balance). (B) Sample air content was measured by weighing the same sample under water before and after vacuum air extraction.

5.2.3. Timings

A preliminary study showed that 60 s of air extraction (under vacuum) followed by 60 s of water replacement (at atmospheric pressure) was sufficient for most of the air in a 3×12×12 mm tissue block to be extracted and replaced by water. It was desirable, however, to determine *optimal* timings so some further study was required. For this, equatorial discs (15 –20 mm thick) were taken from several fruit. For excision of flesh samples, we used Leica Model 818 disposable microtome blades to minimise tissue damage close to the cut surfaces. The discs were cut so as to produce pairs of blocks 12×12×r mm with their long axes (r) aligned with the fruit radius. One of each block pair was used as control (with fixed air-extraction and water-replacement timings) while the other was exposed to a range of timings. This plan allowed data to be normalised (i.e. to remove errors due to fruit:fruit and radial variability) by taking the ratio of air volume estimates for a pair of matched blocks (treatment/control).

To determine the optimum time for water replacement, the control blocks were exposed to 120 s of vacuum extraction and to 480 s of water replacement. Both these timings

were judged to be longer than the times required for completion. To determine the optimal water-replacement timing, extraction times for the treatment blocks were fixed at 120 s (like the controls) while their changing weights (as they took up water) were recorded every 30 s until a steady state was achieved (<480 s). Five replicate pairs of blocks of three sizes (r = 3, 6 and 12 mm) were used to assess the influence of sample size on water-replacement times.

Once the optimum water-replacement time had been found (300 s for a 12×12×12 mm block), five replicate pairs of blocks were used to determine the optimal vacuum extraction time. Tested times were 5, 15, 30, 60, 120, 240 and 480 s. The control blocks were all extracted for 480 s.

5.2.4. Spatial distribution of intercellular spaces

Fruit were cut at the equator to excise transverse discs about 15 - 20 mm thick. Radial transects (12×12×r mm) were cut from these discs. Transects extended for the full distance (*c.* 30 mm) from the skin to the fruit axis and included the petal bundle located between a pair of locules (Fig. 5.2A). To facilitate vacuum infiltration, the skin and the cartilaginous walls of the locules were shaved off very thinly. This radial transect was then sliced (blade aligned parallel to the skin) so as to create from it ten ≅ 3 mm thick blocks. Even spacing of cuts was facilitated by making these cuts while the block rested atop a 1 mm grid. Each block was measured carefully and each series of 10 blocks provided the samples for measuring air volume change along a fruit radius.

Tangential variation in air distribution around the fruit was examined using a single fruit with five symmetrically excised transects. This provided 50 blocks (five replicates for each tissue depth). To investigate the radial disposition of tissue air we tested ten fruit. One transect was taken from each fruit giving 100 blocks (ten replicates for each tissue depth). The volumes of intercellular air were combined across replicates for each tissue depth so as to yield a mean fractional air content (%) along the radii. Each transect contained eight rectangular 3×12×12 mm blocks (B_1 to B_8) plus two trapezoidal blocks close to the fruit axis (B_9 and B_{10}). These blocks were also 3 mm thick but their shape was dictated by the proximity of the locules (Fig. 5.2A).

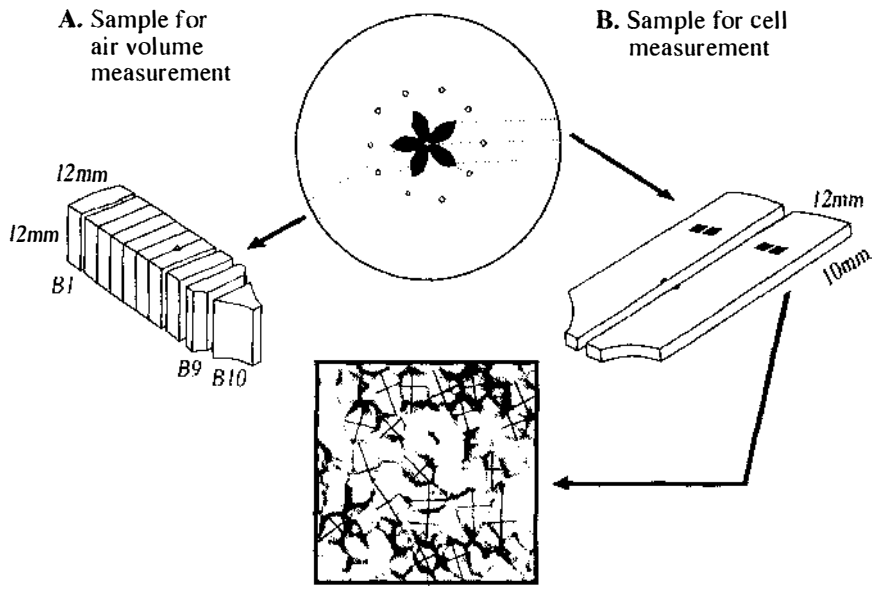


Fig. 5.2. Tissue sampling for air volume and cell measurements. A flesh disc about 15 - 20 mm thick was cut from the fruit equator. (A) Each of ten transects (12×12×*r* mm) was subsequently cut into ten blocks for air volume measurement (note the trapezoidal shape of the two inner blocks, B_9 and B_{10}). (B) Five pairs of thin transects were also taken from one fruit and stained to reveal the walls of cells just beneath the surface (entire cells were visible in 2-dimensional view). The two orthogonal diameters of 400 well-defined cells were randomly measured from two fields in the middle of the cortex for each transect.

5.2.5. Cell measurements

5.2.5.1. ***Staining.*** Fruit were cut transversely to form five pairs of flat equatorial blocks (Fig. 5.2B) and these rinsed briefly with water to remove cell-content residues from the cut surfaces. For ferric tannate staining (Goffinet *et al.*, 1995), the transects were infused for three minutes in 5% aqueous tannic acid (this infiltrates the cell walls), then briefly rinsed with water to prevent extra-cellular depositions of precipitate. Next, the tissue was flooded with 1% aqueous iron alum (ferric ammonium sulphate) for 60 s. This reacts with tannic acid to form a black tannic-iron precipitate within the cell wall matrix (Austin, pers. comm.). Lastly, blocks were rinsed to remove surplus precipitate and analysed immediately to minimise possible dimensional change due to osmotic water uptake.

5.2.5.2. *Cell dimensions.* Cell dimensions were measured using a video-microscope system (a Wild M3Z dissecting microscope coupled to a JVC TK-1280E video camera and this connected to a PC running *Video Master*™ software). Transects were mounted in a petri dish under water to create an optically flat meniscus that minimised light scattering from the irregular block surface. To stop transects from moving they were lightly impaled on a thin spike attached to the bottom of the dish. This arrangement presented a sharp image of whole cortical cells from just beneath the cut surface onto a 17" flat VDU screen at ×195 magnification. The software allowed measurement, using the mouse, of a live (unfrozen) image and direct data acquisition to disc. This greatly facilitated decision-making because it was possible to adjust the focus slightly as each measurement was being made.

For each block, cell dimensions (2 orthogonal diameters) were obtained for forty cells from two adjacent fields in the middle of the cortex approximately 10 mm in from the skin (Fig. 5.2B). The first diameter measurement (d_1) was always made in line with the long axis of the cell with the second measurement (d_2) at right angles to this. For later calculations, the geometrical mean of these diameters (D) was calculated as

$$D = (d_1\ d_2)^{0.5} \qquad (2)$$

5.2.6. Adjustment for air loss

When a block of apple flesh is immersed in water there is a tendency for water to invade the air spaces near the cut surface. Air displaced in this manner at the initial weighing (W_1) is not recorded by this method. This air loss causes, therefore, underestimation of air content. The magnitude of the error will depend on the surface area to volume ratio of the sample (and thus on its size and shape). This loss can be quantified, and corrected for, by considering it as equal to a loss of air in a superficial layer of thickness (T) all around the sample.

The value taken by T will likely be similar in the tangential directions (t) relating to the 2 transverse and 2 radial-longitudinal faces of a tissue block but may be greater in the radial direction (t_x) relating to the 2 tangential block faces. This is because apple parenchyma is highly anisotropic (Vincent, 1989), with a network of elongated air spaces radiating out from the centre (Reeve, 1953; Khan and Vincent, 1990).

It is unlikely that on sample immersion, water will invade an air space where the cut aperture to this is smaller than its maximum diameter. For this to happen would require an increase in the area of invading meniscus and thus the input of energy (e.g. from a vacuum pump). It is more likely that only those cut air spaces whose diameters *decrease* with depth will be invaded. This insight leads to the hypothesis that in the two tangential directions, t will take a value equal to about ¼ of the average cell diameter. Recall that, and with random sectioning, the air spaces between close-packed spheroidal cells will be so cut that either: the aperture falls *before* the space's mid-point (in this case no air is lost), or it falls *after* the mid-point (in this case water enters freely and all air is displaced). Air spaces, having similar maximum dimensions to the spheriodal cells that form their boundaries, and these two scenarios occurring with the similar likelihood, it is likely that air loss will occur to a depth of a ¼ cell diameter. The conspicuous radial air channels of some cultivars may offer freer access to water invasion in the radial direction. In this case the effective thickness from which air is lost (t_x) may be greater in this direction.

To check these predictions, pairs of 12×12×12 mm blocks of cortical tissue (cut from 4 – 16 mm below the skin), were taken from a number of apples. The air volume of one block was measured intact while the air volume of the other was measured (by summing) after it had been cut up into a number of slices. Sixteen such pairs of blocks were measured. In six cases the sliced block was cut into two 6 mm slices, in four into three 4 mm slices, in three into four 3 mm slices, in two into six 2 mm slices, and in one into twelve 1 mm slices. For each pair, the sum of the air volumes in the slices (ΣV) was compared with the value for the paired intact block (V) where, because of the block 'edge' effect, $V > \Sigma V$. A model was then used to evaluate a correction factor (F) that takes as input the shape, size and orientation of the block where

$$V_{\text{corrected}} = V_{\text{measured}}\ F \qquad (3)$$

The error is expected to be inversely related to the size of the sample. The model was fitted by least squares using PROC NLIN (SAS, 2000).

5.3. RESULTS

5.3.1. Timings

The Figure 5.3 shows a plot of the relative air volume evaluated for blocks of different sizes and times. Data were normalised by dividing the air content value obtained for the matching block pair that had been exposed for times judged to be excessive (*viz* 120 s for extraction and 480 s for water replacement). This creates the property that results in an asymptote to unity.

The largest blocks (12×12×12 mm) required 300 s for water replacement and this was longer than for the smaller ones. Thus 6×12×12 mm blocks required 120 s and 3×12×12 mm blocks required just 30 s (see Fig. 5.3A). Air extraction, however, even from the largest (12×12×12 mm) blocks was completed within about 60 s (see Fig. 5.3B).

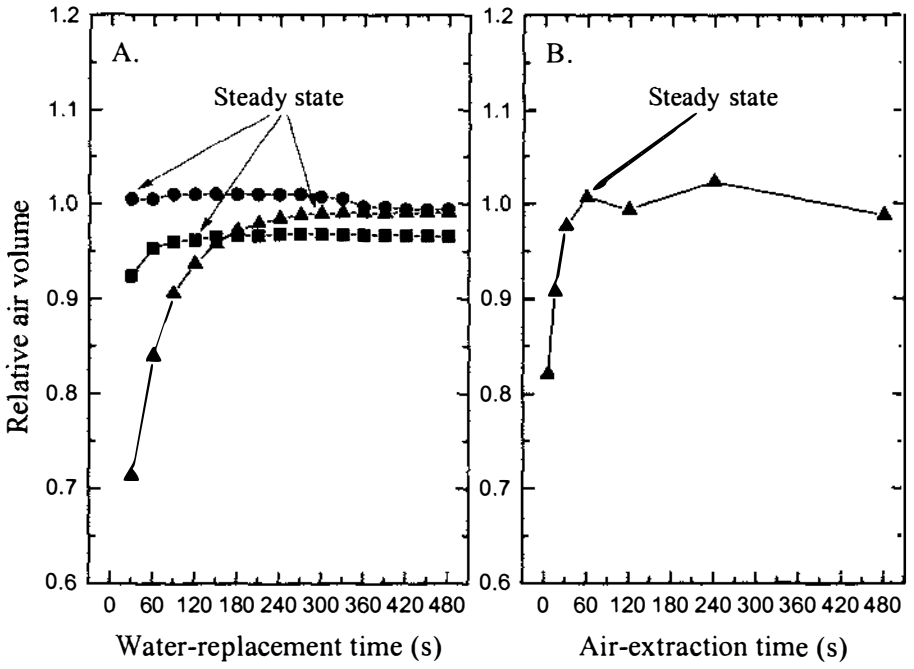


Fig. 5.3. Relative air volume during water replacement (A) and air extraction (B). The points are mean values (n = 10) for the block sizes 3×12×12 mm (●), 6×12×12 mm (■) and 12×12×12 mm (▲).

5.3.2. Cell measurements

Significant differences existed between the two cell diameter measurements. For cells taken from the mid-cortex, the cell aspect ratio of the long to the short cell diameter averaged 1.44 (the mean cell length was 294 μm and the mean cell width was 205 μm). The geometrical mean diameter was thus calculated as 245 ± 2 μm, which yields a mean cell volume of 0.0077 mm^3.

In 'Braeburn' the parenchyma cells of the outer cortex are large, thin-walled, irregular spheroids. They are usually slightly elongated along one axis and they have facetted surfaces (Fig. 5.4A). Also, at the tissue level, the alignment of their long axes appears random (i.e. their alignment was *not* related to the fruit's major axes). Intercellular air spaces were conspicuous and each cell could be seen to make contact with the walls of a number of adjacent cells. Although cell sizes and shapes were relatively uniform across the cortex (data not shown) it appears that cell orientation and cell aspect ratio do change towards the centre of the fruit. Parenchyma cells of the pith were packed more closely (Fig. 5.4B), especially near to structures such as the locules and vascular bundles. Here, cells were grouped into compact radiating tiers and were elongated so as to be almost cylindrical in shape.

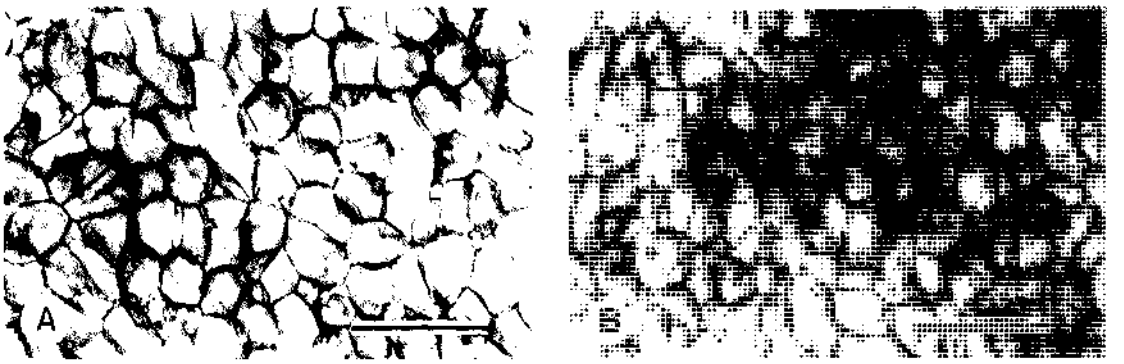


Fig. 5.4. Micrographs of the stained flesh of 'Braeburn' fruit. (A) In the mid-cortex, whole cells appear as irregular spheroids, randomly arranged and loosely packed. (B) In the mid-pith, the cells are more elongated, radially aligned and more closely packed. The bars represent lengths of 500 μm.

5.3.3. Adjustment for air loss

The prediction was that there would be a small loss of intercellular air from the edges of a flesh sample as it is immersed in water. This means that, when measuring the air content of a 12 by 12 by x mm block, one is actually only measuring the air contained in a block that is 12-2t by 12-2t by x-2t_x mm (Fig. 5.6). Consider the ratio (y) of the air volume in a sliced 12 ×12×12 mm block with that in an intact block, which will be independent of t (the thickness from which air is lost from the transverse and longitudinal block faces). This ratio is given by

$$y = \frac{12}{12-2t_{12}} \frac{x-2t_x}{x} \tag{4}$$

where t_x and t_{12} are the thickness from which air is lost from the tangential faces of blocks having radial dimensions of x mm and 12 mm respectively.

First, consider the possibility that $t_x = t_{12}$. Here, the effective thickness from which air is lost from the tangential faces will be the same for all slice dimensions x. If all the data are grouped together and t_x is estimated using a least squares regression, the value $t_x = 0.154$ is obtained. This fixed T value does *not* fit the ratio data well (Fig. 5.5, dashed line). The idea of t_x being a constant is therefore untenable.

A more reasonable approach is to assume that t_x is relatively constant for large slice thickness, but as the slice dimension x, in the radial direction, reduces to zero, so does t_x. One way to describe such a relationship is by

$$t_x = t_{max}(1-\exp(-kx)) \tag{5}$$

where t_{max} and k are the parameters to be fitted. The ratio y is therefore given by

$$y = \frac{12}{12-2t_{max}(1-\exp(-12k))} \frac{x-2t_{max}(1-\exp(-kx))}{x} \tag{6}$$

Fitting this equation to all 16 data points using a least squares regression gives t_{max} = 0.292 mm and k = 0.544 mm^{-1}. This fits the data well (Fig. 5.5, solid line). The calculated effective surface layer thickness ranges from 0.123 mm for a 1 mm slice to 0.281 mm for a 6 mm slice (Table 5.1), and the latter is close to the maximum value t_{max} = 0.292 mm. Hence, to allow for air loss from a surface on immersion in water, the air content

measured in a rectangular 12 by 12 by x mm blocks (e.g. B_1 to B_8 in Fig. 2) should be corrected using F

$$F = \left[\frac{12}{12-2t} \frac{12}{12-2t} \frac{x}{x-2t_x} \right] \tag{7}$$

where t is the effective thickness from which air is lost in the tangential direction (equivalent to about ¼ of a cell diameter as predicted), and t_x can be calculated for any radial thickness from equation (5) and amounts to 0.235 mm for a 3 mm slice (equivalent to about one cell diameter).

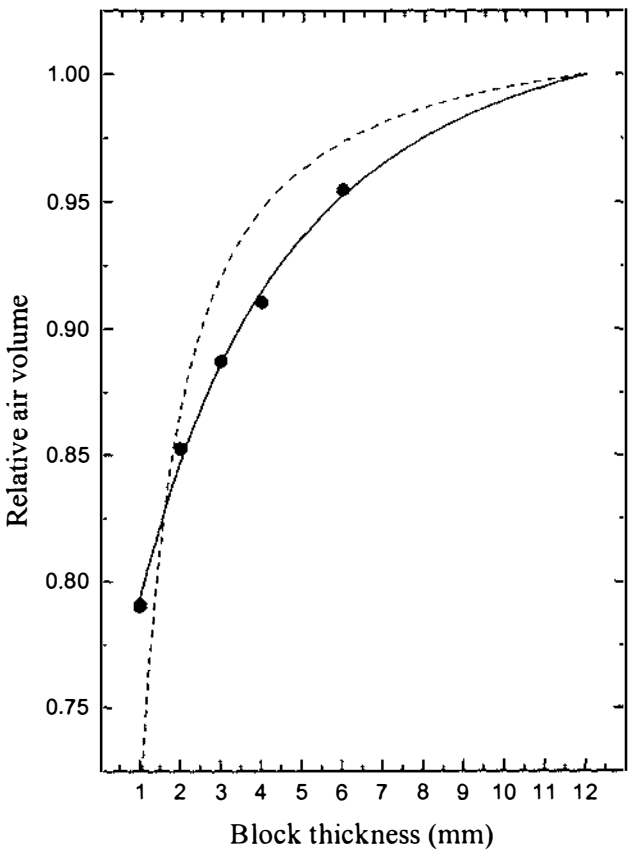


Fig. 5.5. Average scores for relative air volume in blocks of different radial dimensions. Each mean value (●) represents normalised measurements for each block size. The lines were fitted using T value fixed (dashed line) and T value varying with block thickness (solid line).

Table 5.1. Air loss in the radial direction (t_x) due to water invasion calculated using Eq. 4. An effective surface layer thickness from which air is lost due to water invasion is dependent on the dimension of the block (in the radial direction).

Block thickness (x) (mm)	Effective surface layer thickness (t_x) (mm)
1	0.123
2	0.194
3	0.235
4	0.259
6	0.281

The blocks bordering the locules (B_9 and B_{10}) are more difficult as they are 'trapezoidal' in shape. For simplicity, a similar approach can be applied, taking off a thickness t_x in the radial direction and t in the tangential direction (Fig. 5.6). Given the minimum (a) and maximum (b) widths of the trapezoid and the height (x), then assuming symmetry the correction factor becomes

$$F_{\text{trapezoid}} = \left[\frac{\frac{1}{2}(a+b)}{\frac{1}{2}(a+b) - 2t[\sin(\theta)]^{-1}} \frac{12}{12-2t} \frac{x}{x-2t_x} \right] \quad (8)$$

where $\tan(\theta) = \dfrac{2x}{b-a}$.

The adjustment factors for the rectangular block samples can be calculated as 1.21 (B_1 – B_8), and for two trapezoids as 1.22 (B_9) and 1.23 (B_{10}). Although these factors are not very different, it is clear that air loss will be proportionately higher in the smaller blocks.

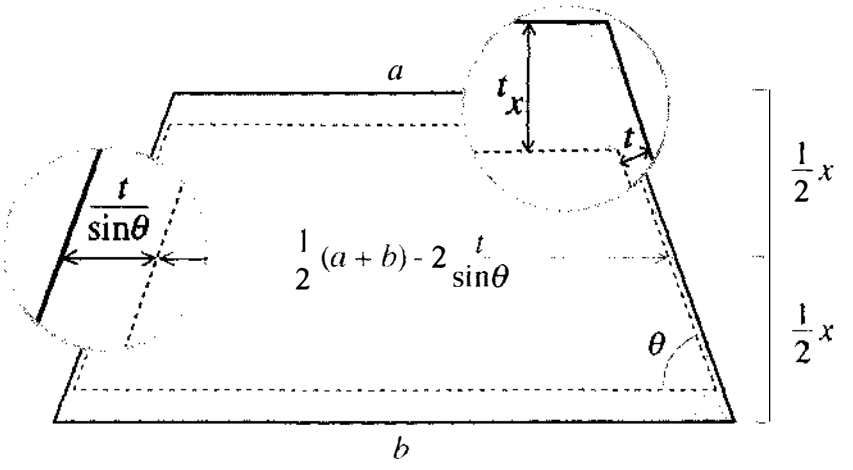


Fig. 5.6. The calculated adjustment for a 'trapezoidal' sample (B_9 and B_{10}). The shaded area represents the effective surface layer thickness from which air is lost due to water invasion. The adjustment for air loss is calculated from the difference in the volumes of the inner and outer trapezoids based on average dimensions of a, b and x measured in a number of samples.

5.3.4. Spatial distribution of intercellular spaces

When air distribution was measured tangentially around the fruit, no significant asymmetry was found ($p = 0.545$). This removed the requirement to adopt any special criterion when deciding along which radius to sample. The results show clearly that fractional air content reduces strongly as one moves from the skin towards the fruit axis ($p \leq 0.001$).

With the data now adjusted for the small air losses suffered on water immersion, a more accurate picture of radial air distribution in the fruit emerges (Fig. 5.7). The air content change follows a distinct pattern in the cortex and this pattern is recapitulated in the pith - these two concentric fleshy tissues originate from different parts of the flower (Esau, 1977). In the cortex, the air fraction first increases from a subepidermal level of about 18%, to reach a peak of about 23% in the layer just beneath. It then falls steadily to about 12% at the core line. Here, at the boundary between cortex and pith, a sharp discontinuity occurs with the air fraction rising suddenly to about 15% before falling steadily again to about 7% at the centre of the fruit.

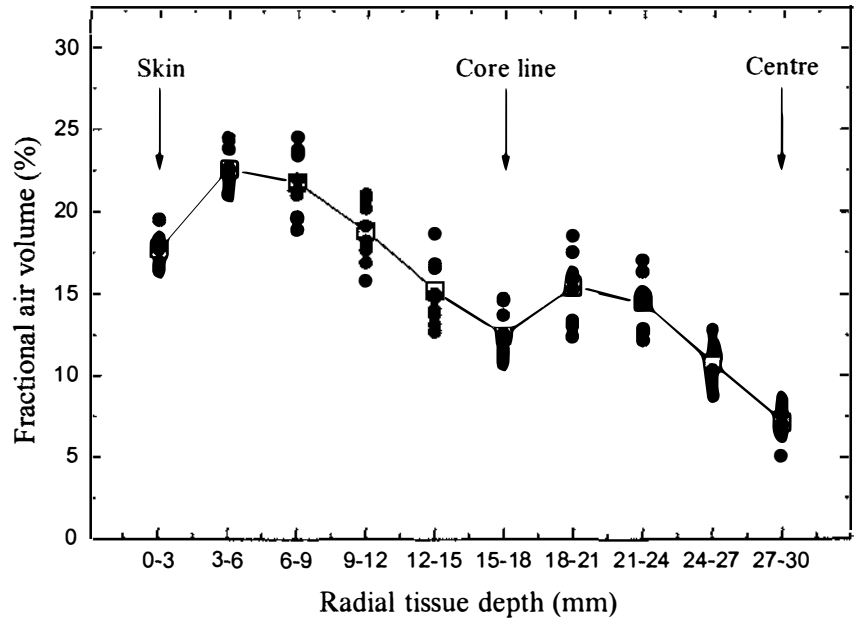


Fig. 5.7. Changes in fractional air content along a radius in 'Braeburn' fruit. Ten tissue blocks ($B_1 - B_{10}$) were measured sequentially between the fruit surface (0-3 mm) and the centre (27-30 mm). Mean values (□) are calculated from 10 measured values (●).

5.4. DISCUSSION

5.4.1. Timings

The magnitude of convective fluid flow through the air spaces of fruit flesh tissue will depend on the viscosity of the fluid, the cross-sectional area of the channels and the length and tortuousity of the path (the last three properties being dependant on cell and tissue structure and on the size of the sample).

The air measurement method described here involves the removal of air from the flesh sample and its replacement by water. It is known that the flow of a fluid is inversely proportional to its viscosity - the viscosity of air at 20°C being 1.813×10^{-5} Pa s and this is much lower than the value for water of 1.002×10^{-3} Pa s (Nobel, 1999). Hence, air will experience much less resistance to its outflow than will water to its inflow, which is

reflected in the distinct differences in time required for air extraction and for water replacement (compare Figs. 5.3A and 5.3B). The same effect is also evident from Vincent (1989) who observed a markedly slower fluid infiltration. This can be also attributed to the fact that he used 5% aqueous mannitol, which is more viscous than water.

Moreover, an increase in the size of the sample will have an influence on the time taken for each of these flows to run to completion. For example, every increase of block size will *increase* the volume of air that must flow out (or of water that must flow in) while it will *reduce* the steepness of the pressure gradients driving these flows. Again this effect is reflected in the equilibration times of Fig. 5.3A with larger blocks requiring longer timings.

5.4.2. Cell measurements

5.4.2.1. *Staining*. The staining method proved simple and well suited for the staining of mature apple parenchyma containing large cells. Entry into the cell walls was fast and enabled a relatively good definition of individual cells. The tannic-iron also penetrated deeper into the tissue and stained some cell walls beneath the surface layer (Fig. 5.4). The contrast improved if stained samples were left in a refrigerator for 1 – 2 days.

5.4.2.2. *Cell dimensions*. This study has found that the mean cell diameter of 'Braeburn' (*c.* 250 μm in the mid-cortex) is in the close agreement with values reported by Tukey and Young (1943), Bain and Robertson (1951), Khan and Vincent (1990) and Harker *et al.* (1997), but slightly higher than those of Reeve (1953). From this information, it is possible to arrive at a reasonably good estimate of the number of cortical cells in an apple fruit. Cell number can be calculated using the mean cell diameter value to arrive at a value for mean cortical cell volume (using $4/3\pi r^3$). Cortical cell number is then calculated as the ratio of this volume to the fruit's cell-fraction volume (i.e. the total volume of the fruit minus the volume of the contained air). Cell number estimates arrived at in this manner are especially useful where it is desired to record changes in cortical cell number in response to some treatment (e.g. to a range of imposed crop loads). Hence, the various

systematic errors contained in the underlying assumptions (e.g. the contribution to fruit weight made by the skin or core tissues has not been allowed for) will tend to cancel.

5.4.2.3. ***Cell shape and cell packing.*** Information on cell shape and cell packing provide an important tool for understanding the textural and physiological properties of fruit flesh. Cells taken from the mid-cortex of 'Braeburn' are prolate spheroids having an aspect ratio close to 1.5 (their long diameter is about half as great as their short diameter). Furthermore, the flesh cells gradually increase in length and also become more aligned towards the interior of the fruit (e.g. compare Figs. 5.4A and 5.4B). This conforms to the pattern seen in a number of other apple cultivars (Bain and Robertson, 1951; Reeve, 1953; Khan and Vincent, 1990; Ruess and Stösser, 1993). It would seem that changes in cell shape and packing cannot be explained simply on the basis of mechanical factors (e.g. forces) acting on the cells – the physical environment in which the cells develop is unlikely to be all that different in the different regions within the fruit. Instead, other factors (e.g. gradients in hormone concentration along which the cells align their long axes) could be involved.

These changes result in the numerous air spaces between the cells that became increasingly organised deeper into the flesh so as to form distinct radial air channels closer to the centre of the fruit (Reeve, 1953; Vincent, 1989; Khan and Vincent, 1990). The channels have little lateral contact with one another and communicate effectively only in the inner parenchyma (Vincent, 1989). Their presence strongly contributes to the anisotropy and heterogeneity of apple flesh tissue (Vincent, 1989; Khan and Vincent, 1990). For example, when compressed radially, the tissue tends to fail in sympathy with the air channels (Khan and Vincent, 1993). Since flesh textural attributes are very important determinants of fruit quality and the different apple cultivars vary widely in texture (Janick *et al.*, 1996), it is likely that differences in the size, orientation and distribution of the air channels will make an important contribution to the perceived textural properties of the fruit.

It is also likely that the flesh of an apple is under pressure while the skin is in tension (Vincent, 1992) and that these opposing forces operate to form the structural unit of the whole. The relatively large proportion of air and the relatively rounded shape of the flesh

cells suggest, however, that this 'tissue pressure' (the force imposed by the tension in the skin that tends to press the cells against one another) is quite gentle. The degree of faceting in the fully turgid tissue will, therefore, depend upon the balance between cell turgor and tissue pressure. Given a certain tissue pressure, a higher cell turgor will tend to increase tissue air volume, and create more rounded cells with smaller facet areas. Higher internal air volumes will also lead to better internal gas exchange.

5.4.3. Adjustment for air loss

An air:water meniscus is in tension and thus will always tend to minimise its area in order to minimise surface energy. This means that when the cut surface of a block of apple flesh tissue is immersed in water, water will tend to invade any air-filled cavities in the irregular surface but only where to do so involves a *reduction* in the meniscus area. Correspondingly, water will tend *not* to displace air from cavities whose shape is such that where to do so the meniscus area is required to increase. In practice, only air spaces lying close to the surface are invaded whereas the majority of the air is contained deep within the tissue and thus is measured.

For water to invade the deeper compartments, energy must be input (in this study it was contributed by a vacuum pump). It can be predicted from a simple geometrical consideration that, before the vacuum is applied, air spaces lying between regularly packed spheroidal cells will, in general, not be invaded beyond a depth of about a ¼ cell diameter. However, the radial air channels should allow water to penetrate deeper in this direction. If this increased depth of penetration approaches half the sample's dimension (in the radial direction) there is the possibility that some of the internal air will be displaced even from the deepest regions of the block.

The model proposed, therefore, accounts not only for all the air displaced around the edges of the block but also from air spaces beneath the cut surfaces that are open to water invasion in the radial direction. Thus for the range of samples (1 to 6 mm in the radial direction), t_x is observed to change gradually with slice thickness (Table 5.1). In a 1 mm slice, air loss occurs from around 25% of the radial thickness, whereas for a 6 mm slice, loss occurs from less than 10% of the radial thickness. Not surprisingly, therefore, measurement error is inversely related to block size such that 1 mm thick slices account

for only 0.79 of the total air volume, while for 6 mm slices the figure is 0.95 (Fig. 5.5.). Consistent with the degree of spatial resolution required, when using this technique for air content measurement, block sizes should be maximised in order to minimise the impact of air loss on the result.

5.4.4. Spatial distribution of intercellular spaces

In 'Braeburn', a progressive decrease in the volume of intercellular air occurs along a radius (Fig. 5.7). The decrease is not uniform though, and the transect contains two peaks - one in the cortex and another in the pith with the highest fractional air volumes being recorded just inside the bounding tissues (*viz* the skin and the core line). Other authors have investigated air space distribution in apple. Reeve (1953) and Vincent (1989) reported a gradient in the volume of the intercellular spaces along a radius. Their results were obtained by vacuum infiltration of rather large samples that gave a result that referred to the whole of the outer or inner flesh. Soudain and Phan Phuc (1979) and Ruess and Stösser (1993) also observed a general decline in the intercellular air content in a radial direction although the latter obtained a distinct bell-shaped air distribution. Their results were based on stereological estimates from 2-dimensional sections. In this study, a more direct measurement method has been employed, using a contiguous sampling of still-turgid tissues with adjustment for air loss around the sample edges.

The pattern of change in air content, with its sharp discontinuity at the core line (Fig. 5.7), is mirrored by changes in both tissue organisation and cell packing. This leads to the idea that the fruit can usefully be considered as comprising two fleshy parts in line with the traditional botanical perspective (Esau, 1977). The cells at the core line are distinctively smaller, are more elongated and have thicker walls than the other parenchyma cells (MacDaniels, 1940). This phenomenon is expressed to such a degree that the core-line cells form a visible 'sheet' between the outer cortex and the inner pith (Fig. 1.2). Furthermore, some apples possess a 'cleavage plane' at the core line associated with a sharp increase in intercellular spaces (MacDaniels, 1940). Various degrees of demarcation between the two fleshy tissues may have functional implications in terms of the physiology of gas transfer.

A question here is whether these factors could impact on the storage quality in 'Braeburn'. Several lines of evidence support this idea. Steep gradients in the concentration of gases were found between the centre and the skin in 'Braeburn' (Rajapakse *et al.*, 1990; Dadzie, 1992) suggesting a heterogeneity of internal atmospheres and thus a degree of gaseous compartmentation. Gas movement will experience a greater resistance to diffusion in a tight-packed tissue, with low air content such as the fruit of 'Braeburn' (Rajapakse *et al.*, 1990; Yearsley *et al.*, 1997a; 1997b). Whether gas diffusion occurs mainly through the air channels (Solomos, 1987) and/or in a combination with fluid/solid matrix of the flesh (Rajapakse *et al.*, 1990), a region of reduced intercellular air content will act to impede gaseous diffusion. A partial diffusion barrier at the core line could further restrict gas exchange and lead to increased CO_2 and lower O_2 in the inner parenchyma.

The internal concentration of respiratory gases is also dependent on the permeance of the skin. The skin acts as the primary barrier to gas diffusion in apple fruit having a 10 to 20-fold higher resistance than the flesh (Solomos, 1987). 'Braeburn' is known to exhibit high skin resistance (Rajapakse *et al.*, 1990; Dadzie, 1992), high internal partial pressure of CO_2 (Dadzie, 1992; Yearsley *et al.*, 1997b) and low respiration rates (Rajapakse *et al.*, 1990; Yearsley *et al.*, 1997a) relative to other cultivars. Hence, when the skin is intact and the tissue is not water soaked, diffusional gas flow seems to be dominated by the resistance of the skin.

However, if the tissues suffer a partial loss of membrane functionality such that the air spaces become flooded with water, capillarity dictates that this extracellular fluid will migrate towards the tissue regions containing the smallest pores. These lie around the core line and near the centre of the fruit (see Fig. 5.7). Flooding of air spaces will effectively seal off the inner tissues to respiratory gas exchange (diffusion in a liquid phase is 10^4 times slower than that in a gas phase, Nobel, 1999). In 'Braeburn' this would cause a sharp rise in the internal partial pressure of CO_2 and a lowering in that of O_2, which could lead to tissue breakdown.

A number of studies refer to CO_2-related disorders in 'Braeburn' although the mechanism by which CO_2 causes damage remains unclear (Watkins *et al.*, 1997; Elgar *et al.*, 1998). Internal injury in 'Braeburn' is seen as randomly distributed discrete lesions that enlarge and merge in storage (Clark and Burmeister, 1999). It was also shown that severe watercore may lead to cavity development and mealiness after visible symptoms have disappeared (Clark and Richardson, 1999).

These observations are consistent with a model in which the tight-packed internal structure of 'Braeburn' (particularly at the core line) reduces O_2 and CO_2 diffusion resulting in a special susceptibility to internal injury. It is reported that a number of pre-harvest factors contribute to physiological changes in the fruit that predispose it to injury (Ferguson *et al.*, 1999). It is suggested that the link may in fact be mediated through patterns of flesh structure (*viz.* air-space distribution) that are modified by factors in the pre-harvest environment.

5.5. CONCLUSIONS

The study was undertaken to develop methods through which to determine patterns of intercellular air content and of cell shape, size, number, and packing within the flesh tissue as a whole. A gravimetric technique based on Archimedes' principle was developed and refined through which accurate assessments of air content can be made in plant tissues.

In 'Braeburn', the intercellular air content of the flesh shows a systematic decline between the fruit surface and the centre with a marked discontinuity at the core line. This discontinuity effectively separates the fruit into two concentric parts. The radial decline in air content is accompanied by an increase in the degree of cellular organisation towards the centre. Quite steep gradients in air content underline the importance of location of flesh samples for air content assessment. They also render relatively meaningless the assumption that an air content measurement made at one point can be taken to refer to the whole fruit.

Chapter 6. General discussion and conclusions

The preceding parts of this thesis contain a number of experimental studies that explore various aspects of developmental fruit physiology. Research carried out included studies on the dynamics of initiation and functionality of fruit and stalk xylem, seed effects on fruit shape and fruit minerals and air and cell alignments in mature fruit. The work describes and illustrates the relationships between structure and function of the tissues with special emphasis on the mechanisms underlying physiological responses of the fruit.

The objective of this chapter is to provide a final discussion from the results of the experimental studies and reviewed literature conceptualised in the model that describes the interactions between the elements that contribute to the development of fruit quality (Fig. 1.3) and thus the management policies for the crop.

6.1. GENERAL DISCUSSION

6.1.1. Seed as a source of developmental stimuli

The causative relationship between seed set and shape defects is well established (Heinicke, 1917; Latimer, 1931, 1937; Roberts, 1946; Way, 1978; Brault and de Oliveira, 1995; Brookfield *et al.*, 1996) but lacks an intensive evaluation in regard to the morphology of the apple fruit. By sectioning the whole fruit on the basis of extrapolated carpellary partitioning (Fig. 2.2), fruit asymmetry, size and seed distribution were assessed with a three-dimensional perspective. The fruit tends to grow lopsided when seeds confined to a particular part fail to develop (Fig. 2.4). The growth behaviour, therefore, appears to be modular where each carpel exerts a somewhat autonomous effect on fruit growth and, at least partially, develops as a unit. The dynamics of fusion between the carpels comprising an inferior ovary conform to this outcome. Delayed fusion along the carpellary margins (Esau, 1977) indicate that, at least in the early stages of growth, carpels are physically separated and thus are likely to exhibit a physiological autonomy within the assemblage (fruit).

Several important implications emerge from these premises. Seed and fruit development are related, such that seed aberrations parallel that of fruit shape. Seed development appears to be a prerequisite for fruit development and a likely source of developmental stimuli that radiate towards the skin. Fruit development, therefore, occurs through the combined growth of the carpels that strongly influence the growth of the extracarpellary tissue in a centrifugal direction (first-order effect) and to a lesser extent laterally (second-order effect), (Fig. 2.6). Hence, the overall growth of the apple fruit appears to be determined by the dynamics of ovary growth, which in a biological sense represents the 'true' fruit of a *pome* (Abercrombie *et al.*, 1981).

The localised trend in fruit asymmetry was also related to the spatially confined mineral composition. The larger sectors of lopsided fruit tended to have lower mineral concentrations but only that of the calcium was significantly affected (Figs. 2.7 and 2.8). This is in line with the expected influence of an increased growth rate that could lead to a dilution of the mineral concentration within the rapidly expanding tissues of the fruit (Ferguson and Watkins, 1989). The ratio of calcium to magnesium and potassium in the larger sectors thus changed unfavourably with respect to calcium although no symptoms of bitter pit were noticed in the stored apples assessed in this study.

This implies that better seed set will on average lower the calcium concentration by promoting tissue growth, which contradicts the claim that an increase in seed number will increase fruit calcium in the population as a whole (Tomala and Dilley, 1989; 1990; Bramlage *et al.*, 1990; Broom *et al.*, 1998). If seeds can directly influence fruit calcium this effect should be independent of environmental conditions. In addition, more seeds should stimulate auxin export from the fruit (Bangerth *et al.*, 1989; Callejas and Bangerth, 1997; Bangerth, 2000), which Banuelos *et al.* (1987) considered promoted calcium import by the fruit. However, where seed set has been affected by pollination, the relationship with fruit calcium tended to be masked by tree effects (Brookfield *et al.*, 1996; Volz *et al.*, 1996b).

The relationship between seed set and the calcium concentration of the fruit is clearly complex and requires a better understanding of the impacts of a range of factors. It is common for an over-simplistic description to lead to a contradiction. However, it is

possible to create a more sophisticated explanation of the 'seed-set:fruit calcium-concentration' relationship (Fig. 6.1).

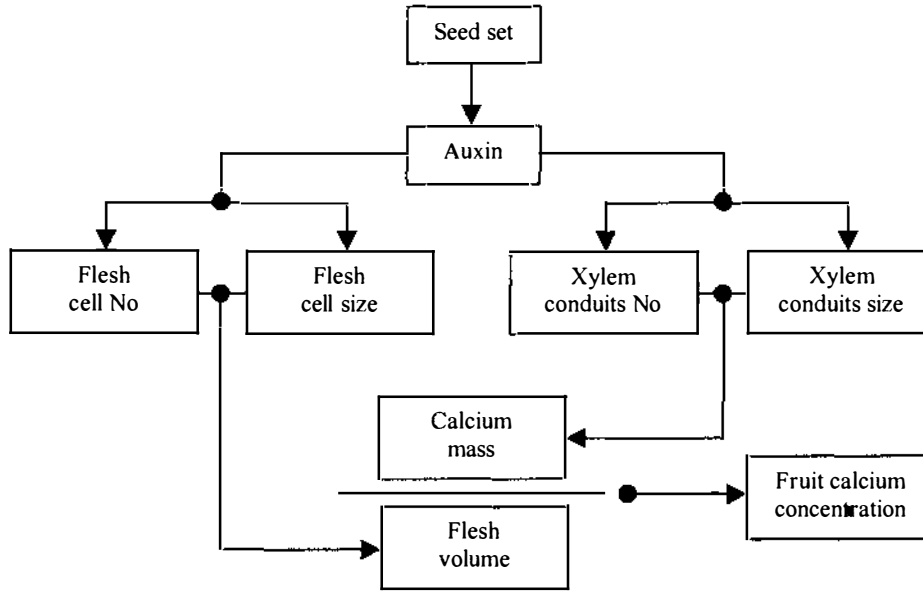


Fig. 6.1. Causative links between seed set and fruit calcium concentration (the mass of calcium in a particular volume of flesh).

The sequence in Fig. 6.1 describes the dependence of fruit calcium concentration on the parallel developments of fruit vasculature and of flesh volume growth. Both pathways appear to be regulated by seed-derived auxin. However, both pathways will also likely be influenced by a host of 'external' factors such as genotype, crop load, temperature, light, and water availability. Where the rate of a process depends on many factors, its sensitivity to change in any one factor will depend upon the extent to which this factor is in deficit. This concept of 'limiting factors' was first enunciated by Liebig (1841) and Blackman (1905) and is discussed in Salisbury and Ross (1992).

The concept of 'limiting factors' suggests that the relative strengths of the two auxin effects will depend upon the extent to which auxin is limiting for each pathway compared with the other potentially limiting factors. For example, auxin will have its dominant effect on fruit calcium concentration *via* calcium mass (the numerator, and governed by the right-hand pathway in Fig. 6.1) when the other factors affecting calcium mass are in

relative excess and auxin is limiting. Here more seeds (auxin) will increase calcium mass. If, instead, one of the other factors is in deficit (i.e. is more limiting than auxin), increasing seed number will *not* have much of an effect on calcium mass. The same arguments can be erected for flesh-volume (the denominator and left-hand pathway).

Because the physiology of the two pathways are quite different, and are affected differently by the external factors, this simple model can explain many paradoxical results. Changing seed set could increase, decrease, or have no significant effect upon, fruit calcium concentration.

6.1.2. Nature of the developmental stimulus

The presence of seeds provides the necessary stimulus for fruit growth including mineral accumulation (Chapter 2). However, determining whether seeds act as source or sink of such stimuli will establish a key link in the model of fruit quality development (Fig. 1.3).

Exogenous applications of the auxin transport inhibitor (NPA) altered vessel differentiation in the stalk while controlling the extent of premature abscission, seed and fruit development (Chapter 3). This supports the hypothesis that auxin emanating from the fruit is the principal developmental stimulus. The timing and dynamics of vessel differentiation suggest a pattern of auxin efflux that is closely related to the stage of flower/fruit growth (Fig. 3.5). In addition, the changes in vessel formation along the stalk (Fig. 3.7) and the adverse response to auxin inhibition in terms of seed and fruit size (Tables 3.3 and 3.4) indicate a possible negative feedback regulation of auxin biosynthesis in the fruit.

Clearly, auxin efflux stimulates vascular differentiation in the stalk, which supports the increasing nutritional demands of the rapidly growing fruit tissues. Considering that the relative strength of the auxin efflux governs the dominance of the fruit (Gruber and Bangerth, 1990), it seems likely that the rate of descending vessel differentiation will physically limit the rate of ascending sap flow. As a consequence, the relative strength of auxin efflux may not just determine the sink capacity of the fruit according to its dominance hierarchy but also promote the flow capacity of its xylem. Furthermore, the region of lowest conductance across the abscission zone (Fig. 3.2) dominates convective

flow in the vessels and creates the weakest point of fruit attachment to the tree that enables efficient fruit shedding.

6.1.3. Character of fruit tissue growth as a determinant of fruit quality development

Spatial variations in the mineral status of lopsided fruit (Figs. 2.7 and 2.8) highlight the link between mineral uptake and fruit growth. Dye infusion of the fruit revealed a progressive breakdown in xylem flow, more pronounced in the primary bundles, the principal suppliers of the fruit flesh (Figs. 4.5 and 4.6). A microscopic assessment showed that the mode of dysfunction is due to the physical damage of the xylem structure in the bundle (Fig. 4.9), similar to that observed in some other fruit crop species (Findlay *et al.*, 1987; Düring *et al.*, 1987; Wolswinkel *et al.*, 1999). The suggestion that expansion of the fruit induces breakage of the vessels implies that fruit growth rates govern the patterns of change in the hydraulic properties of the fruit xylem and thus can affect fruit mineral composition.

Several lines of evidence support this suggestion. Because the xylem tissue is the principal pathway for calcium transport (Marschner, 1986) any changes in the functionality of the xylem will affect calcium accumulation in the fruit. Indeed, the xylem dysfunction in primary bundles (Fig. 4.5) can be positively correlated with the decline in fruit calcium import shown by Wilkinson (1968) and Jones *et al.* (1983). Furthermore, the sudden drop in fruit calcium concentration in apples coincides with the onset of rapid fruit growth (Jones *et al.*, 1983).

Because the pattern of fruit growth is specific for a given cultivar (Pratt, 1988), the dynamics of fruit xylem dysfunction appear cultivar-dependent. Significant differences in the pattern of xylem flow were observed between cultivars that vary in their susceptibility to the calcium-related disorder, bitter-pit (Fig. 4.5; Lang, 1990; Lang and Ryan, 1994). In addition, the decline in dye import towards the calyx end of the fruit (Fig. 4.7) again positively correlates with the more pronounced calcium deficiency and bitter pit frequency in the distal part of the fruit (Ferguson and Watkins, 1989).

However, the ring of ten primary bundles appears to lie along the dorsal margins of the core (Fig. 1.2). While fruit growth will cause the bundles to drift outwards, only the growth of the core can affect the primary bundles. Hence, cultivar differences in the dynamics of xylem dysfunction depend upon the growth of the carpellary tissues. Both examined cultivars ('Braeburn' and 'Granny Smith') showed similar tissue growth patterns (Figs. 4.10 and 4.11) but the relative increase in the distance between the locule tips and the aligned sepal bundles differed significantly (Fig. 4.13). The proximity of the sepal bundles to the locules coincides with a prolonged functionality of these bundles during the later stages of fruit growth (Fig. 4.14). This suggests a 'protective' role of the locules whose slower growth will slow the drift of a bundle in close proximity and thus prolong the functionality of its xylem.

It has been shown in apple fruit that the direction of xylem movement reverses diurnally such that sap flow is from fruit to tree during the day and from tree to fruit at night (Lang, 1990; Lang and Volz, 1998). Recently, it has also been found that the concentration of osmotic materials (sugars, minerals etc) in the cell-wall free space rises as apple mature (Lang and Diack, unpublished). This rise is suspected to follow some level of dysfunction of the cell membranes (Lang and Düring, 1991). It follows that the outflowing xylem sap will be rich in these osmotically active materials - the xylem sap being continuous with the cell-wall free space (apoplast). Hence, xylem dysfunction can be seen as minimising the loss of these materials *from* the fruit but at the expense of reduced import of xylem borne minerals *to* the fruit.

Two consequences arise from these changes. First, phloem sap inflow to the fruit will be stimulated. The increased osmotic concentration of the fruit apoplast (Lang and Diack, unpublished) will reduce the turgor in the terminal sieve tubes strongly promoting phloem sap inflow to the fruit (Lang *et al.*, 1986, Lang and Thorpe, 1989, Lang and Düring, 1991) and will explain the observed increase in phloem import (Lang, 1990). Second, the likelihood of calcium deficiency disorders will be increased. Phloem immobile minerals like calcium (Marschner, 1986) will tend to be excluded from the fruit giving rise to relative deficiency and thus increased occurrence of physiological disorders like bitter pit. The view is, therefore, taken that dysfunction of the xylem and cell membrane discussed above is an integral part of normal fruit development.

6.1.4. Tissue air and cell alignments and fruit storage quality

It is likely that the textural properties of an aggregated tissue will arise from the character of its components such that specific changes in texture will be founded in the structure and individual properties of the elements of which it is composed. The dynamics of apple parenchyma development can create distinctly different textural properties in the fruit, some of which are peculiar to particular cultivars (Janick *et al.*, 1996). Anisotropy can also increase complexity by creating textural properties that vary with direction (Vincent, 1989; Khan and Vincent, 1993).

Changes in cell shape and packing along the radius (Fig. 5.4) conform to a general pattern of there being increased tissue organisation with increasing depth beneath the skin (Bain and Robertson, 1951; Reeve, 1953; Khan and Vincent, 1990; Ruess and Stösser, 1993). Also, the radial gradient in air content (with a sharp discontinuity at the core line) was measured with high accuracy and by contiguous sampling (Fig. 5.7). These various patterns evidence the distinctive disposition of the intercellular air network in 'Braeburn'.

The nature of tissue air distribution in 'Braeburn' might be related to its susceptibility to internal storage disorders. The tight-packed internal structure (Dadzie, 1992; Yearsley *et al.*, 1997a; 1997b) and potential diffusion barrier at the core line is likely to impact on gas transfer within the fruit. Furthermore, the appearance of free water in the intercellular spaces (e.g. due to partial loss of membrane functionality) will further impede gas diffusion. These features are likely to reduce the ability of respiratory gases to move freely across the flesh. Together with a low skin permeance to gases (Rajapakse *et al.*, 1990; Dadzie, 1992) these flesh properties will determine the storage behaviour of 'Braeburn'.

6.2. GENERAL CONCLUSIONS

In conclusion, some aspects that ultimately affect quality development in apple fruit have been studied and the causative relationships between structure and function for some of its components assessed. The literature clearly points out the reasons why some fruit fail to meet particular quality criteria but often provides no more than an overly simplified relationship between a likely cause and its effect on quality.

The conceptual model (Fig. 1.3) is based on the premise that the seed represents the primary reproductive organ and is the principal source of developmental stimuli for the surrounding fruit tissues. It is apparent from the model that fruit quality development is controlled by a complex of synchronised physiological responses interacting within and across the paths of the major quality attributes. Hence, the assessment of physiological properties can frequently include properties, processes or factors from other developmental paths. This makes the model of quality development in apple fruit generally applicable to other fruit crop species.

In summary, the studies reported in this thesis provide an assessment of the mechanisms that control some aspects of quality development in the apple fruit. These are:

- That the modular growth of the syncarpous ovary affects shape and spatial mineral distribution in the fruit;
- That auxin emanating from the fruit controls vessel differentiation, abscission, seed and fruit development;
- That it is the structure and character of tissue growth that controls the progressive xylem dysfunction in the fruit; and
- That it is the structure of the flesh that determines the nature of air distribution and thus the storage behaviour of the fruit.

Hence, identifying the mechanisms underlying apple fruit development has been valuable in conceptualising the mechanistic links between structure, function and quality. The implications of these findings in a wider developmental context are considerable.

From a biological standpoint, the fruit is the structure produced to protect and disperse seeds and thus ensure the propagation of the species. Hence, the role that fruit play in plant ontogeny determines the nature of its development. Some defects of the fruit that we define as 'quality defects' are, in fact, an integral part of the developmental process.

The dependence of fruit development on seed development conforms to the principal role of seeds in plant organ hierarchy. The peculiar character of apple fruit asymmetry is the consequence of modular growth of 'lopseeded' syncarpous ovary that presumably provides developmental stimuli for all fruit tissues (including fruit stalk). Textural and physiological properties of fruit flesh will, therefore, serve to determine an organoleptic perception of the fruit. In addition, the progressive xylem dysfunction is programmed to minimise daily losses of solutes from the growing flesh (through diurnal cycling), and so to increase assimilate partitioning in favour of the developing fruit. The increased assimilate content of the fruit enhances its attractiveness to dispersal agents. However, inter-linked processes that serve to adjust for high sink demands are not without cost. The bias of assimilate partitioning at the expense of xylem sap flow will presumably affect mineral composition (notably calcium) and storage potential of the fruit. This naturally-occurring mechanism is, therefore, the putative link to the occurrence of calcium-related disorders that also affect other fruit crop species, such as pear, avocado, mango, tomato, watermelons, cabbage, carrots, celery and capsicum.

Hence, it is hoped that this thesis will improve the understanding of the physiological processes investigated here and will help initiate new approaches to optimise crop production. The techniques described here can be used to further understanding of developmental processes in other fruit crop species. However, due to the morphological diversity of fruit, the interactions between the processes of fruit development should be assessed within the limits of fruit structure. In general, apple can be used as a model fruit to illustrate patterns in quality development but more specifically, development of a particular quality attribute should be aligned with the relationships between structure and function of its fruit tissues. For this reason, an integrative structure:function approach to the processes of fruit tissue development will lead to better strategies to remedy the negative effects of some developmental patterns in fruit.

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