# Anatomical survey of the formation of primary xylem and nutrients supply to the reproductive apple bud

**By**

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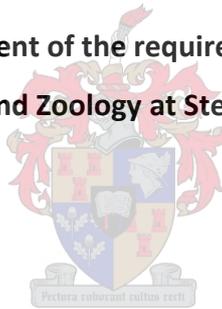
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**DECLARATION**

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously, in the entirety or in part, been submitted at any university for a degree.

March 2017

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# SUMMARY

Apple (*Malus domestica* Borkh.) is a deciduous fruit species, which is subject to dormancy before bud break occurs in spring. Although not noticed externally, anatomical changes occur in the reproductive bud during this dormant period, including formation of the carpels, enlargement of the petals and carpel and anther elongation. In this study, we determined i) the time of primary xylem formation as influenced by cultivar, month and/or climate (expressed by winter chilling) and ii) the distribution and quantification of a selected nutrient calcium (Ca) in, (a) the primary xylem tissue as well as (b) in reserve tissues (spur, leaf and apical meristem).

Four different commercial apple cultivars were selected for this investigation. Golden Delicious and Braeburn represented cultivars with typical Ca deficiency symptoms in the fruit if additional Ca is not applied, whereas Royal Gala and Cripps Pink represented cultivars in which Ca related problems occur less frequently. Golden Delicious and Royal Gala are cultivars associated with high chilling requirements, and Braeburn and Cripps Pink, with medium chilling requirements. Sampling of dormant buds occurred during June, July, August and September in 2012, 2014 and 2015. The different cultivars used for this investigation were cultivated in Stellenbosch and Elgin. Although these regions are both in the Western Cape Province, the Elgin region is an area with a higher chill unit accumulation than Stellenbosch.

Light, fluorescence and scanning electron microscopy imaging techniques were used to study the anatomy of primary xylem development. To differentiate the degree of lignification in primary xylem from secondary xylem, samples were imaged with a Zeiss LSM880 confocal microscope equipped with ZEN 2 software. Scanning electron microscopy images were acquired using a Zeiss Merlin FEG Scanning Electron Microscope (SEM).

After the time (month) of primary xylem establishment in the bud was identified, Ca concentration (%) was quantified in reproductive apple bud tissues of all four cultivars during three seasons (2012, 2014 and 2015). Ca concentration was quantified with Wavelength Dispersive Spectrometer (Oxford Instrument® Wave Dispersive X-ray Spectrometer). Ca was simultaneously quantified with Electron Dispersive X-ray (Oxford Instrument ® Energy Dispersive X-ray).

In Golden Delicious and Braeburn, the process of secondary xylem development commenced earlier than in Cripps Pink and Royal Gala. While this process is slow in Cripps Pink, xylem

development proceeds much faster in Royal Gala and changes from having only primary xylem in July to having secondary xylem a month later. The timing and duration of xylem development in Cripps Pink and Royal Gala were similar in the Stellenbosch and Elgin regions in June, but differed in July and August in all three seasons.

Results clearly show differences in Ca distribution and Ca concentration in the tissues of dormant reproductive apple buds of the four cultivars studied.  High Ca levels were noted in the spur transport tissue of Royal Gala, Cripps Pink and Braeburn during June, with considerably lower levels of Ca in the same region in Golden Delicious. Cripps Pink and Royal Gala have a higher Ca concentration in the xylem in June, followed by an increase in Ca concentration towards September. The reduction in Ca concentration in the spur during the same period may indicate that reserve Ca from the spur is allocated towards the xylem. This needs further investigation.

Although trends were not as clear in Golden Delicious and Braeburn and did not always follow the same pattern, observations in these cultivars differed from those in Cripps Pink and Royal Gala, confirming the higher presence of primary xylem reported previously. Thus, later formation or presence of primary xylem in the dormant bud of these cultivars may partly explain the lower Ca concentrations and difference in Ca distribution between tissues compared to Cripps Pink and Braeburn.

OPSOMMING

Appel (*Mallus domestica* Borkh.) is 'n bladwisselende vrug spesie, wat onderhewig is aan rus voordat knoppe in die lente oopbreek. Alhoewel dit nie ekstern waargeneem word nie, vind anatomiese verandering in die reproduktiewe knop plaas gedurende hierdie rustende periode, insluitend die vorming van vrugblare, vergroting van kroonblare en vrugblaar en meeldraad verlenging. In hierdie studie het ons die volgende bepaal: i) die tyd van primêre xileem vorming soos beïnvloed deur kultivar, maand en/of klimaat (uitgedruk as winter verkoeling) en ii) die verspreiding en kwantifisering 'n geselekteerde voedingstof kalsium (Ca) in (a) die primêre xileemweefsel asook (b) in reserwe weefsels (spoor, blaar en apikale meristeem).

Vier verskillende kommersiele appel kultivars is geselekteer vir hierdie ondersoek. Golden Delicious en Braeburn het kultivars verteenwoording met tipies Ca tekort simptome in vrugte indien addisionele Ca nie toegedien word nie, terwyl Royal Gala en  Cripps Pink kultivars verteenwoordig het waarin Ca verwante probleme minder algemeen voorkom. Golden Delicious en Royal Gala is kultivars geassosieer met 'n hoë verkoeling behoefte, en  Braeburn en Cripps Pink, met medium verkoelings behoeftes. Insameling van rustende knoppe het tydens Junie, Julie, Augustus en September van 2012, 2014 en 2015 plaasgevind. Die verskillende kultivars wat vir hierdie ondersoek gebruik is, is in Stellenbosch en Elgin gekweek. Alhoewel hierdie omgewings beide in die Weskaap Provinsie is, is die Elgin steek 'n area met 'n hoëre verkoelingseenheid akkumulasie as Stellenbosch.

Lig-, fluoressensie- en skandeerelektronmikroskoop beeldvasleggings tegnieke is gebruik om die anatomie van primêre xileem ontwikkeling te bestudeer. Om die graad van lignifikasie van primêre en sekondêre xileem te differensieer, is monsters afgeneem met 'n Zeiss LSM880 konfokale mikroskoop toegerus met ZEN 2 sagteware. Skandeerelektronmikroskoop fotos is verkry deur gebruik te maak van 'n Zeiss Merlin FEG skandeerelektronmikroskoop (SEM).

Nadat die tyd (maand) van primêre xileem vestiging in die knoppe geidentifiseer is, is Ca konsentrasie (%) in reproduktiewe appelknop weefsels vir al vier kultivars tydens drie seisoene (2012, 2014 en 2015) gekwantifiseer. Ca konsentrasie is gekwantifiseer deur Golflengte Versreidende X-straal Spektrometrie (Oxford Instrument® Wave Dispersive X-ray Spectrometer). Ca is gelyktydig gekwantifiseer met Elektron Verspreidings X-strale (Oxford Instrument ® Energy Dispersive X-ray).

In Golden Delicious en Braeburn het die proses van sekondêre xileem ontwikkeling vroeër begin as in Cripps Pink en Royal Gala. Terwyl die proses stadig is in Cripps Pink, het xileem

ontwikkeling baie vinniger plaasgevind in Royal Gala en het verander van slegs die besit van primêre xileem in Julie na die besit van sekondêre xileem 'n maand later. Die tyd en duur van xileemontwikkeling in Cripps Pink en Royal Gala was eenders in die Stellenbosch en Elgin omgewing in Junie, maar het verskil in Julie en Augustus in al drie seisoene.

Resultate wys duidelik verskille in Ca verspreiding en Ca konsentrasie in die weefsels van rustende reproduktiewe appel knoppe van die vier kultivars wat bestudeer is. Hoë Ca vlakke is opgemerk in die spoor vervoerweefsel van Royal Gala, Cripps Pink en Braeburn gedurende Junie, met aansienlik laer vlakke van Ca in dieselfde area in Golden Delicious. Cripps Pink en Royal Gala het hoër Ca konsentrasie in Junie gehad, gevolg deur 'n toename in Ca konsentrasie na September toe. Die reduksie in die Ca konsentrasie in die spoor gedurende dieselfde periode mag aandui dat reserwe Ca van die spoor aan die xileem geallokeer is. Dit benodig verdere ondersoek.

Alhoewel neigings nie duidelik was in Golden Delicious en Braeburn nie, en nie altyd dieslfde patroon gevolg het nie, het waarnemings in hierdie kultivars verskil van dié in Cripps Pink en Royal Gala, wat die hoër teenwoordigheid van primêre xileem wat vroeër gerapporteer is ondersteun. Dus, die later vorming en teenwoordigheid van primêre xileem in die rustende knoppe van hierdie kultivars mag deels die laer Ca konsentrasies en verskille in Ca verspreiding tussen weefsels vergeleke met  Cripps Pink en Braeburn verduidelik.

Dedicated to my mother, Christina Simons and my husband, Isaac and children Micaela and Kelly.

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# CONTENTS

# Chapter 1

## General Introduction

Fruit quality has become very important due to competition within industry and the demand for fruit availability throughout the year. Good quality fruit are determined by parameters such as colour, size, firmness and flavour. All of these parameters are related to an optimum mineral nutrition application, enabling not only the manipulation of reproductive and vegetative development in deciduous fruit trees, but fruit quality as well (Tagliavini and Marangoni 2000).

The management of water and nutrient fertilizer is essential in modern high density orchards (Tagliavini and Marangoni 2000). Mineral nutrition is one of the important factors in fruit tree production, since minerals are responsible for several functions like energy processes, enzyme activation and osmotic regulation of the membranes (Faust 1989; Marschner 1983).

Apple trees require 16 elements for successful completion of their life cycle (Salisbury and Ross 1992). Mineral elements are divided into macro-elements including nitrogen, phosphorus, potassium, calcium, magnesium and sulphur, and micro-elements including iron, manganese, copper, zinc, boron, molybdenum and chloride (Salisbury and Ross 1992).

According to Taiz and Zeiger (1991) ions translocate passively in the xylem vessels to the above-ground parts of the plant. The transportation of nutrients to the leaves is through the transpiration stream, which is influenced by water loss through leaves (transpiration). Most mineral nutrients are rapidly redistributed via the phloem from the leaves to other plant parts such as the growing shoot tips and fruit, which exhibit only minimal transpiration (Tromp 2005).

Phloem contains higher levels of minerals like nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) than calcium (Ca) (Tromp 1979). Such minerals flow into the fruit in high concentrations (Tromp and Oele 1972) late in the season (Lang 1990). In contrast, Ca is not very mobile in the phloem or the symplast (Raven 1977). Wieneke (1969) found the concentration of Ca in phloem sap to be very low. Similarly, Ferguson (1980) found phloem to be unable to provide sufficient concentrations of Ca to different plant parts. Calcium transport is maintained by the xylem from the root to the fruit, however, as the fruit matures,

the xylem in the fruit loses its functionality at a rate that differs between cultivars (Düring *et al*. 1987). According to Lang (1990) the rate of nutrient flow into the fruit generally increases during fruit development. Typically, the K content of mature apples increases most during late fruit development, while apples typically have a high Ca during early fruit development.

It is believed that Ca is mobilized before root growth achieves its full activity. Different reasons have been offered to explain this. According to Mason and Whitfield (1960) the concentration of Ca decreases around the time of bud break. Wieneke (1969) further reported high concentrations of free Ca in the xylem sap early in the season, while Bradfield (1976) and Tromp (1979) both also mentioned higher Ca concentrations in the xylem of apple shoots earlier on during apple development. Xylem vessels have open perforation plates in their end walls, which means that there is low resistance to xylem sap flow in such elements (Nobel, 1999). The relative flow rate of the xylem sap depends on the width (cross-section) of the vessel elements (Zimmermann, 1983), so the rate of flow through the pedicel will depend on the size and number of vessels it contains (Drazeta *et al*. 2004). Calcium moves in the xylem, but is immobile in the phloem stream (Marschner 1983). It reaches all regions of the apple during the first phase of fruit development, without a visible gradient in Ca distribution (Wieneke 1974). As the fruit matures, this even distribution of Ca changes, and Ca becomes less uniformly distributed in the apple.

Calcium is essential for a wide range of metabolic functions in apples. Firstly, it is important in retaining the function and structure of the cell membrane (Rousseau 1972; Zocchi and Mignani 1995), stabilizes cell wall structures and ensures good cell to cell adhesion by the binding action of negative cell surfaces (Zocchi and Mignani 1995). Lastly it fulfils a structural role in the middle lamellae between adjacent cells and plays an important role in cell division (Zocchi and Mignani 1995).

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## Chapter 2

## An anatomical survey of the timing of primary xylem formation in reproductive buds of four different apple cultivars from two climatic regions

### Introduction

### Plant growth

The primary plant body is produced through primary growth, which is regulated through cell division in apical meristems (Myburg and Sederoff 2001). Almost the entire plant body, in most monocots and some eudicots, is the product of primary growth. In woody plants, primary growth produces the innermost layers of xylem of the stem, branches and roots. Along with the phloem tissue, the xylem tissue of young roots and stems is arranged as a ring of separate vascular bundles (Myburg and Sederoff 2001). Primary xylem comprises protoxylem that differentiates first and metaxylem that differentiates later (Myburg and Sederoff 2001). The vascular cambium (lateral meristem) of vascular bundles is located between the primary xylem and phloem in most eudicots and gymnosperm stems. In essence, the vascular cambium may be viewed as an extension of the procambium. The vascular cambium will later give rise to secondary xylem and secondary phloem. Secondary growth occurs in lateral meristems and causes the diameter of stems, branches and roots to increase in all gymnosperms and woody eudicots. Secondary growth results through meristematic activity and cell division in the vascular cambium. At the same time, interfascicular regions between the vascular bundles also become meristematic and connects with the vascular cambium to form a continuous vascular cambium cylinder. Secondary xylem is formed to the inside of the vascular cambium, while secondary phloem is added towards the outside thereof (Cutter *et al*. 1978).

### Xylem anatomy

The function of xylem is to offer mechanical support and to transport water and mineral nutrients from the roots to the rest of the plant body (Myburg and Sederoff 2001). As an adequate supply of water and nutrients is essential for plant growth, xylem is functionally very important. Xylem is a complex tissue that consists of several types of cells including tracheary elements (tracheids and/or vessel elements), xylem parenchyma and sclerenchyma (such as xylem fibres).  Parenchyma cells mostly have a storage function in xylem (Esau *et al*. 1965). Mature xylem parenchyma cells retain functional protoplasm that can store carbohydrates as starch. Another important function of xylem parenchyma is wound healing

by forming callus tissue (wound tissue). Callus is formed by the divisions of parenchyma cells. When callus cells are put under pressure their walls become thickened and lignified, after which they will not be able to regenerate again. Nutrients can move from cell to cell between xylem parenchyma cells, because their primary cell walls are thin and contain many plasmodesmata that extend between cells in areas of primary pit fields (Fahn *et al*. 1982).

Xylem sclerenchyma cells are specialized for mechanical support of xylem. Sclerenchyma typically comprises of fibres, which are long, thin, spindle shaped cells with thick secondary walls. The conducting cells of xylem are collectively known as tracheary elements and consist of two types, the tracheids and vessel elements. The tracheary elements typically develop both a primary and a secondary cell wall. Primary cell walls are formed by a random network of cellulose fibrils, which can stretch, which enabling cell expansion as it grows. Secondary cell walls are deposited on the inner side of the primary wall both during cell elongation and thereafter (Mauseth *et al*. 1988). In the secondary wall the arrangement of the cellulose fibrils strengthens the cell, making it more elastic than the primary wall (Mauseth *et al*. 1988). Primary xylem has primary cell walls and only annular (circular) or helically arranged secondary cell wall deposits. The helix rings may be arranged in a loose or dense manner (Fahn *et al*. 1982). Wall regions between helical wall thickening and pit membranes are not covered by lignified secondary walls, but by a non-cellulosic component of the primary wall (Esau and Charvat1978). Tracheary elements that mature later than the helical ones go through a two-phase wall deposition process (Bierhorst and Zamora 1965). In the first phase the helical framework is formed and during the second phase secondary wall material is deposited between the gyres of the helix. Secondary xylem is characterized by more secondary cell wall deposition, which is typically deposited in a series going from helical to scalariform to reticulate to pitted walls (entire primary wall is covered except for pits). Lignin deposition is dependent on the cell age. With an increase of lignification, resistance to water stress will also increase. Due to the presence of lignified secondary walls, mature tracheal elements are dead cells and have secondary walls that are cross linked with polymers. Tracheids have tapered ends and comparatively thin secondary walls and no perforation plates. Neighbouring tracheids are only connected by pits (Cutter *et al*., 1978). Xylem vessels are stacked one on top of the other with perforation plates in their end walls. The nature of perforation plates can vary, and is formed by the remnants of the degraded end walls. Perforation plates allow for the free, unhindered movement of water from one vessel element to the next. Lateral transport between vessel elements are only possible through pits. Pits occur in pit-pairs in the lateral walls of vessel elements and in the radial walls of tracheids. A

pit pair basically comprises of two neighbouring pits that are perfectly aligned. This allows for the lateral passage of water between tracheal elements (Esau *et al*. 1965).

**Vascular development in reproductive apple buds**

The reproductive bud of an apple is a developing flower and is dependent on the parent plant for growth, but may also be influenced by the environment. During unfavourable environmental conditions the buds will remain dormant, while they will be stimulated to develop under favourable conditions (Bartolini and Giorgelli 1994; Bartolini *et al*. 2006). Dormancy is an important phase in the annual cycle of apple trees, and amongst others prevents external bud growth during the colder autumn and winter periods.  Upon dormancy release the buds start to swell (Lang *et al*. 1987). During dormancy, the trees show no external signs of growth, but there is a degree of continuity in the development of apple flower buds during winter (Zeller 1955, 1960; Reichel 1964; Bergh 1984; Greybe 1997). During this time vascular tissue also progressively differentiates within the buds. Typically, the direction of xylem development extends from the bud axis base to the floral primordia. Xylem finally connects to the young sepals and petals, and later with the filaments and the carpels that form the pistil. So ultimately the vascular tissue forms a continuous network from the bud axis to all the floral whorls (Bartolini and Giorgelli 1994; Bartolini *et al.* 2006). As the flower develops, the procambial tissues mature to primary xylem vessels, and at maturity they comprise of dead cells with lignified walls (Faust *et al*. 1995).


 Xylogenesis is a process through which procambial and cambial initials mature into xylem cells, and can last up to four days in primary xylem. In secondary xylem xylogenesis takes 14 -21 days (Myburg and Sederoff 2001). The phases of xylogenesis include (1) cell division and enlargement, (2) cell wall thickening, (3) lignification and (4) programmed cell death (Myburg and Sederoff 2001).


Dormancy release of apple flower buds relate to stages of differentiation during xylogenesis and to tetrad formation after meiosis in the microspore mother cells of anthers (Bartolini and Giorgelli 1994; Szabo *et al*. 2002; Bartolini *et al*. 2002). Physiological mechanisms and interactions between internal and external factors are responsible for the regulation of these biological processes. Temperature represents an exogenous factor with chilling being a requirement prior to the release of bud dormancy (Perry 1971; Fishman *et al*. 1987).

During early dormancy (around May in the Western Cape of South Africa), ovary growth in reproductive buds occurs mainly through cell multiplication. Cell division gradually ends at the time of anthesis, which is also the time when ovary cell enlargement begins. Cell enlargement ultimately becomes responsible for fruit enlargement (Bergh 1984; Greybe 1997).

## Cultivars

Apple trees can survive the cold winter months due to survival mechanisms such as dormancy. For certain important physiological processes to take place in the dormant bud e,g emerging from dormancy, the tree must be exposed to continuously low temperatures for a certain period of time. Chilling requirements vary between different cultivars and cultivars are classified into low, medium or high chilling requirement categories (Cultivar catalogue, ARC Infruitec-Nietvoorbij).

## Climate

### Chilling accumulation in Elgin vs Stellenbosch

Elgin is classified as a medium chilling area, whereas Stellenbosch is classified as a low chilling area (Lötze *et al*. 1999). This is based on a chill model that quantifies winter temperatures in terms of chill units, which are then used to classify regions into different chilling categories (Linsley-Noakes et al 1994). As apple cultivars differ in their chilling requirement, their phenology, growth and fruit quality may differ if the same cultivar is planted in areas with different chilling accumulation.

### Aim

The aims of this study: i) when primary xylem is established in dormant, reproductive apple buds, ii) if timing differs between the different apple cultivars and iii) if the timing differs between the same cultivar growing in different winter chill regions in the Western Cape Province, South Africa.

**Objectives:**

- Determine when primary xylem is formed during the developmental stages of reproductive apple buds of four apple cultivars
- Identify anatomical differences in xylem cells during the different growth stages of the reproductive buds of four apple cultivars

**Hypothesis**

Primary xylem develops at different stages of dormancy in different apple cultivars, and at different times in the same cultivar grown in two different regions of the Western Cape, South Africa.

**Materials and Methods**

Five dormant reproductive buds were collected from spurs on two-year-old wood of the commercial apple cultivars Royal Gala (RG), Golden Delicious (GD), Cripps Pink (PL) and Braeburn (BBN). This was done at monthly intervals from June to August in 2012 and 2014, and at 14 day intervals for three buds of the same varieties, in 2015. Buds were collected from Elgin/Vyeboom (medium chilling area) and Stellenbosch (low chilling area), Western Cape, South Africa. Due to unfavourable winter chilling conditions, these cultivars all received rest breaking applications at the end of winter to release the buds from dormancy.

**Preparation of specimen for different microscopy techniques**

Buds collected during 2012 and 2014 were fixed in formaldehyde acetic acid (FAA) (90% ethanol, 5% acetic acid, 5% formaldehyde, v:v:v). Then they were dehydrated in an alcohol series, sectioned longitudinally and critical point dried. Dehydrated buds were placed on an aluminium stub with double sided carbon tape and coated with gold for conductivity. Buds were stored in a desiccator until further investigation.

Fresh buds from 2015 were sectioned longitudinally using a razor blade and placed into tissue freezing media (Leica; #14020108926) for 10 minutes at room temperature to infiltrate the sample. Each sample was then centrifuged for 20 seconds to allow air bubbles to exit the sample, before it was frozen in liquid nitrogen cooled n-pentane and stored at -20°C. Sections (10 -12 µm) were obtained with a Leica CM1820 UV cryostat at -20°C, mounted on a glass slide and kept in a fridge (5°C) until further investigation.

**Different microscopy techniques used to identify primary xylem**

**Light Microscopy**

Light microscopy images were taken with a Carl Zeiss Axio Imager.Z2m light microscope with halogen lamp and AxoCam ICc5 camera. To differentiate primary xylem from secondary xylem, samples were imaged with a Carl Zeiss Light Microscope equipped with AxioVision SE64 software. In addition, to differentiate primary xylem from secondary xylem transmitted light with an EC Plan-NEOFLAUR 40x/0.9 Pol 420363-9901 objective was used to distinguish primary cell walls from the more lignified secondary cell walls. Morphological observations were scored according to the stage of development of primary xylem on the different dates for each cultivar and season, indicated as present or not for each image (Table 1).

**Confocal Microscopy**

Unstained flower bud sections were imaged with a Zeiss LSM780 confocal microscope in the Lambda mode for detection of autofluorescence. The samples were excited with a 405 nm laser and detection was via the 32 channel GaAasP detector ranging from 414 nm to 690 nm. Images were acquired either using the EC Plan-Neofluar 10x/0.30 M27 objective, the LD Plan-NEOFLAUR 40x/0.6 Korr M27 objective or the alpha Plan-Apochromatic 100x/1.46 Oil DIC M27 Elyra objective. Tile scans and Z-stacks were acquired to enlarge the field of view.

**Scanning Electron Microscopy**

Additional investigation into the anatomical attributes of dormant reproductive apple buds was performed using Zeiss Merlin FEG Scanning Electron Microscope with a secondary detector for surface imaging. Prior to imaging, samples were sputter coated with gold for conductivity. Morphological observations were scored according to the stage of development of primary xylem on the different dates for each cultivar and season, indicated as present or not for each image (Table 1).

**Results and discussion**

Primary xylem in all cultivars showed a very thin cell wall with flattened annular or helical rings during June in Stellenbosch and Elgin region (Fig. 4). The helical thickening was joined

by a narrow strip to the primary wall (Fig. 4).   As thickening increased the lignification of the cell walls intensified (Fig. 5). Differentiation in cell wall thickening and cell wall lignification is proof of continuous development of the xylem elements during dormancy.

Anatomical observations of dormant reproductive apple buds showed progressive differentiation of the cell wall thickness of primary xylem vessels during June, July and August (Fig 3). Primary xylem gradually transitioned into secondary xylem in Cripps Pink, Royal Gala, Golden Delicious and Braeburn, but the timing of this transition differed between cultivars (Table 1). The development of primary xylem was similar for Golden Delicious and Braeburn, but differed in Royal Gala and Cripps Pink from the low chilling region (Stellenbosch). The transition stage is here defined as the stage in xylem development when primary and secondary xylem is not clearly distinguished. The rings or helices are arranged in a loose manner (Fig. 2). None of the cultivars showed a similar trend in transitional xylem development.  The development of secondary xylem was the same in Royal Gala and Cripps Pink (high and medium chilling cultivars, respectively), but differed between Golden Delicious and Braeburn cultivars that grow in the Elgin region only (medium chilling area).

**Cultivar**

*Cripps Pink*

In mid-June the vessels in the primary xylem (100%) had thin walls, but at the middle of July primary xylem vessels started showing an increase in wall thickness, and the number of primary xylem vessels also increased in all three seasons and both regions (Fig. 3). At the end of June only primary xylem was visible (100%). The transition from primary (50 - 60%) to secondary xylem (40 - 50%) became evident by the end of July for all three seasons in Elgin. However, in Stellenbosch, the transition from primary to secondary xylem only occurred during August and varied between seasons (Table 1). The increased thickness of the helical rings of the secondary walls of xylem vessels at the beginning of August suggests that they had matured to secondary xylem (20 - 50%) in Elgin. Cripps Pink expressed a slow systematic change in differentiation of xylem development from June, July and August during the dormant stage (Table 1).

*Royal Gala*

Xylem development in Royal Gala showed no difference in differentiation of primary xylem between samples from the Stellenbosch and Elgin regions (Table 1) during the three seasons studied. Primary xylem was well established with minimal change in xylem differentiation from June (100%) to end of July (100%).  In the Stellenbosch and Elgin regions primary xylem started to show a steady transition into secondary xylem (20 - 25%) by the beginning of August (Table 1).  In this cultivar, climatic region seems to have no influence on the process of xylem differentiation.

*Golden Delicious*

Primary xylem differentiation in Golden Delicious showed a slow progression during bud dormancy (Table 1). Primary xylem vessels and the transitioned stage of xylem were observed by the end of June (60%).  Xylem transition and secondary xylem (40%) were mainly observed at the beginning of August during the first two seasons, with a similar trend in the third season. The xylem transition stage was present during June, July and August, whereas secondary xylem was only present in the beginning of August (Table 1). In the beginning of August, no primary xylem vessels (0%) in any of the seasons were observed, confirming that xylem differentiation is more rapid in this cultivar than the other three cultivars.

*Braeburn*

Xylem differentiation in Braeburn showed no distinct differences during the months of June and July for either of the first two seasons (40%) or the last season (25%) (Table 1). Throughout the months of June and July xylem vessel elements had cell walls of comparable thickness, and they all had similar helical ring thickenings on their primary walls that remained equally spaced apart throughout the study period (Fig. 4). At the beginning of August, the xylem transition stage became evident with between 75 and 80% transition xylem (all three seasons). There was no clear time demarcation of when primary xylem turned to secondary xylem in this cultivar. In August, the primary xylem cell wall showed a slight increase in cell wall thickness and spaces between helical rings decreased, but showed mainly transition stage of primary to secondary xylem (Fig. 4). The xylem differentiation was slower in this cultivar than in the other cultivars studied, with no clear differentiation of secondary xylem even in August.

In the cultivars Golden Delicious and Braeburn xylem developed slightly later, with only 40% of primary xylem in Braeburn and 60% of primary xylem in Golden Delicious visible during June. In July 2012, 60% of Braeburn and Golden Delicious showed primary xylem transition to secondary xylem. In July 2014, 80% of primary xylem transition to secondary xylem in Golden Delicious with helical rings increased slightly in thickness. The primary xylem included a few vessels with annular or helical rings arranged loosely during June and July. The helical rings started to increase in thickness and the spaces between the rings became narrower at the beginning of August.

*Climatic regions*

*Elgin*

Cripps Pink, Royal Gala, Braeburn and Golden Delicious differed from one another in terms of xylem maturation patterns during the seasons studied. Braeburn showed no distinct period of transition from primary xylem to secondary xylem.  Golden Delicious was the only cultivar where the primary xylem was replaced by transition stage and secondary xylem in the month of August. Cripps Pink displayed the most steady xylem maturation of all the cultivars during the same season. Royal Gala showed the least xylem transition stage, whereas primary xylem was the most prevalent in this cultivar of all cultivars studied.

As far as region was concerned, in Elgin (higher chilling area) primary xylem development occurred early (June) in Royal Gala and Cripps Pink and was confirmed anatomically by a few vessels with loose annular or helical rings. The helical rings were flattened, with wide openings between the rings. During July, as primary xylem continued to differentiate, the number of vessels increased. The helical rings became thickened to the extent that the gaps between the rings decreased in size. At the beginning of August there were signs that the primary xylem started to transition to secondary xylem. The helical rings became much thicker and the number of vessels increased.

*Stellenbosch*

Xylem vessel differentiation in Cripps Pink and Royal Gala was slower and more systematic during the dormant season. Royal Gala and Cripps Pink showed a similar pattern in xylem differentiation throughout the season.

In the Elgin region, primary xylem started to show a steady transition into secondary xylem (40%) by the end of July. In the Stellenbosch region, primary xylem started to show a steady transition into secondary xylem by the beginning of August (20%). This indicated that climatic region has an influence on the progress of xylem differentiation in this cultivar.

The transition of primary into secondary xylem in Cripps Pink, Royal Gala, Golden Delicious and Braeburn differed between cultivars (Table 1). The development of primary xylem was similar for Royal Gala and Cripps Pink, 100% in June, and differed from that of Braeburn (25 - 40%) and Golden Delicious (50 – 60%) in June, which indicated that it could have occurred earlier in the last two cultivars. The early development of the primary xylem in Royal Gala and Cripps Pink was similar in both climatic areas – indicating that areas play a secondary role to cultivar in xylem development in the dormant, reproductive apple bud.

This information has not been published before and thus could not be compared to existing literature.

**Microscopy technique**

**Sample preparation**

The sample preparation technique used with buds collected in 2012 and 2014 limited the investigation with the different microscopy techniques to observe the xylem developmental stages adequately at the tissue level. The wax embedding technique was unsuccessful due to the woodiness of the tissues. Wax infiltration was limited and samples crumbled during the process of microtomy. In 2015, additional samples were collected to test the new protocol developed to investigate the xylem developmental stages. The results with the new protocol were very successful and xylem developmental stages were observed with three different microscopy techniques. High-quality images were obtained by Light microscopy, Scanning Electron microscopy and Confocal microscopy techniques allowing tissue identification and recognition of subcellular structures. Despite the large sizes of the samples (approx. 5 mm in diameter), sufficient structure preservation could be obtained and maintained throughout the cryogenic workflow by shock freezing. Sample structure preservation was good during all the different microscopy techniques, especially during the Scanning Electron microscopy investigation, which requires dehydrated samples.

Sample preparation played a crucial role in the study of primary xylem anatomy. The final quality of the different types of micrographs obtained was contingent on the success of the sample preparation. Sample surface quality and physical texture determined the accuracy of the anatomical observation. With light microscopy the primary xylem was visible and identifiable based on annular or helical secondary wall thickenings. The rings or helices were arranged in either a loose or dense manner. As vessel maturation continued, it became difficult with the light micrographs to clearly distinguish between primary xylem and secondary xylem. However, the confocal micrographs were used to distinguish the degree of cell wall lignification. In the confocal images the extent of wall lignification was evident. Scanning electron microscope (SEM) micrographs clearly showed the thickness of the xylem wall and the spaces between the vessels were easily visible. The primary xylem wall was easily identifiable between the openings between the rings. Secondary cell wall showed a combination of more than one form of thickening and it was easily visible with the SEM. SEM micrographs of the secondary walls revealed the range of variation in patterns of cell wall thickening, which made it a more reliable technique to distinguish between primary and secondary xylem.

The different microscopy techniques used in this study helped tremendously in gaining detailed information about the anatomical development of primary xylem. Light microscopy micrographs revealed morphological characteristics of the vessels (Fig. 3). The technique revealed the number of vessels present, but did not specifically distinguish the primary xylem from the secondary xylem. The confocal micrographs (Fig. 4) provide additional opportunities to identify cell wall lignification intensity. Lignin has a broad fluorescence emission range and can be excited with both UV and visible light (Donaldson *et al.* 2013). Under UV and visible light excitation plant cell walls exhibit auto-fluorescence resulting from lignin. The lifetimes and intensity show the distribution of fluorophores (a single type of fluorescent molecule) with different lifetimes in the cell walls. The lifetimes in the middle lamella layer are somewhat shorter (blue) than those of secondary walls (green). The degree of cell wall thickening is expressed in the intensity of lignification of the secondary wall. Detailed information about the differentiation in cell wall thickening was gained with the aid of the scanning electron micrographs (Fig. 5).

**Conclusions**

In this study we were able to determine the best microscopy techniques for identifying xylem development in a dormant, reproductive apple bud – using micrographs of the SEM. This aided us towards showing that timing of primary xylem formation in four different apple cultivars varied between cultivars and not, in the case of Cripps Pink and Royal Gala, between low and medium chilling regions. Cripps Pink and Royal Gala showed full primary xylem development (100 %) during June, contrasting with lower primary xylem development in June for Golden Delicious (50 – 60 %) and Braeburn (25 – 40%), as well as earlier secondary xylem development in July compared to the first two mentioned cultivars. This may indicate that primary xylem development occurred before June for Golden Delicious and Braeburn. This information is perceived as being novel and has not been reported before.

The presence and development of the xylem may prove to be valuable information in the field of horticultural science when transport of mineral nutrients like calcium, which are mainly transported in the xylem, are investigated in the dormant, reproductive bud development.

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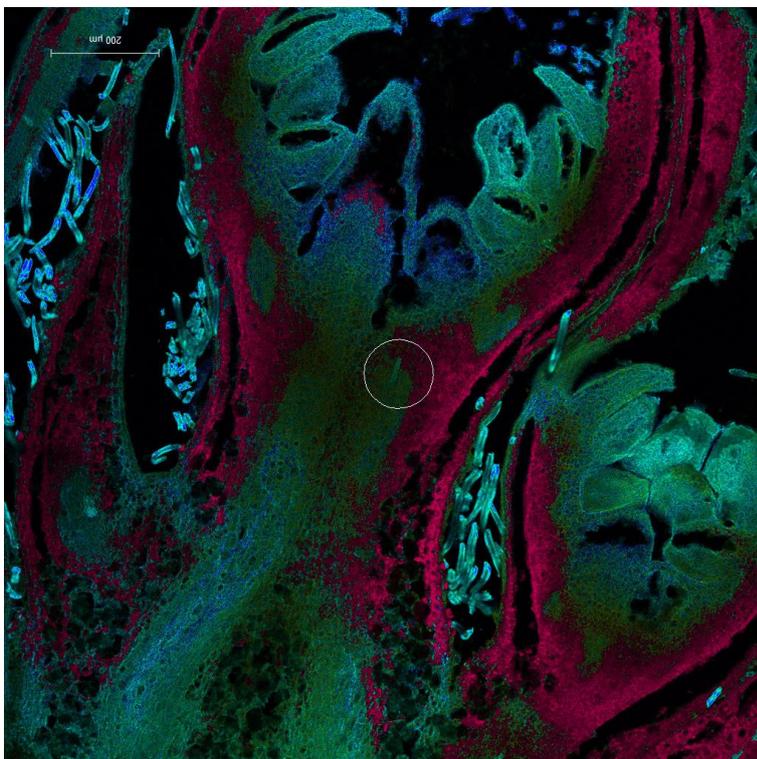
Fig 1: Confocal microscopy image indicating the area of interest where primary xylem development was investigated.
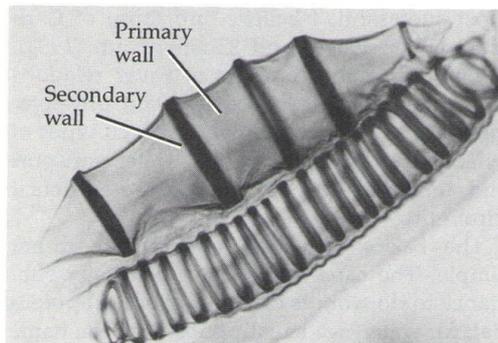
Fig 2: Text book image indicating the primary xylem anatomical features used in light microscopy (Mauseth, J. 1988).
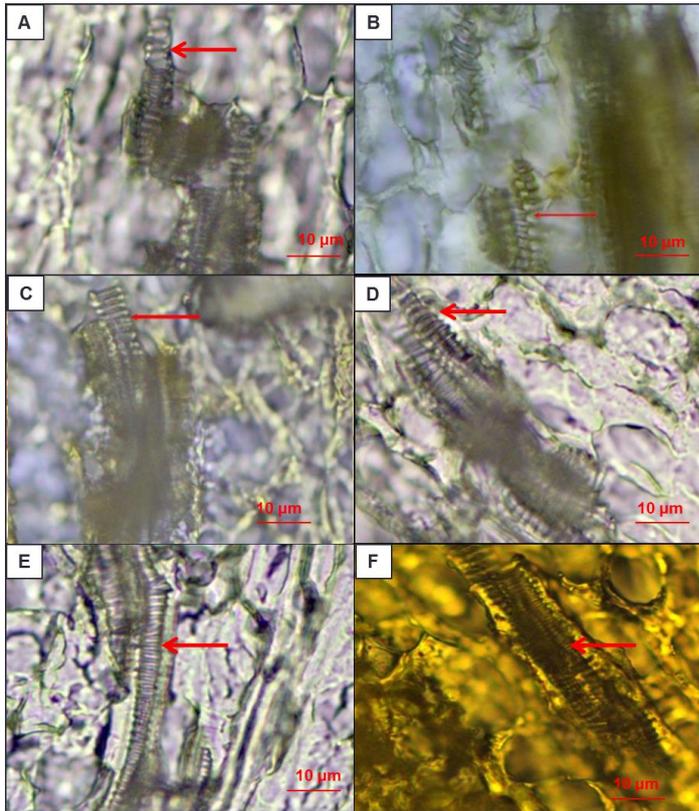


**Fig 3**: Light microscope images of longitudinal sections of xylem development in different apple cultivars. (A) Primary xylem in Royal Gala, June, Stellenbosch, showing xylem with annular wall thickening. (B) Primary xylem in Braeburn, June, Elgin, showing rings or helices arranged loosely. (C) Primary transition to secondary xylem in Cripps Pink, July, Stellenbosch, showing helical thickening where more than one helix is present. (D) Primary transition to secondary xylem in Royal Gala, July, Elgin. (E) Secondary xylem in Golden Delicious, August, Elgin, showing more dense helical thickening. (F)  Secondary xylem in Cripps Pink, August, Elgin, showing the helical bands become joined in certain areas to form a more dense helical thickening.
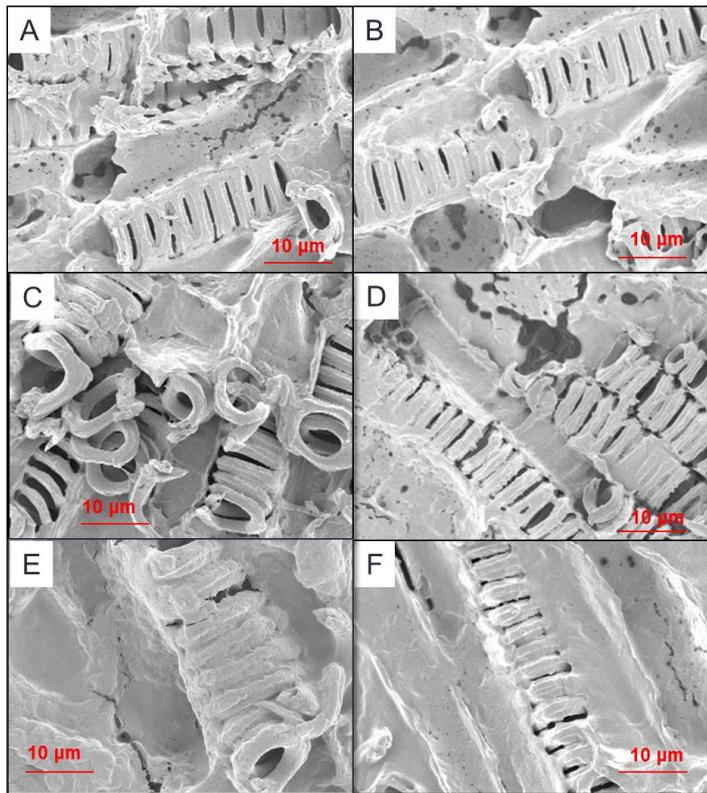
**Fig 4**: Scanning electron microscope images of xylem development in different apple cultivars. (A) Primary xylem in Cripps Pink, June, Stellenbosch. (B) Primary xylem in Braeburn, June, Elgin. (C) Primary xylem transition to secondary in Royal Gala, July, Elgin. (D) Primary xylem transition to secondary xylem in Braeburn, July, Elgin. (E) Secondary xylem in Golden Delicious, August, Elgin. (F) Secondary xylem in Cripps Pink, August, Elgin.
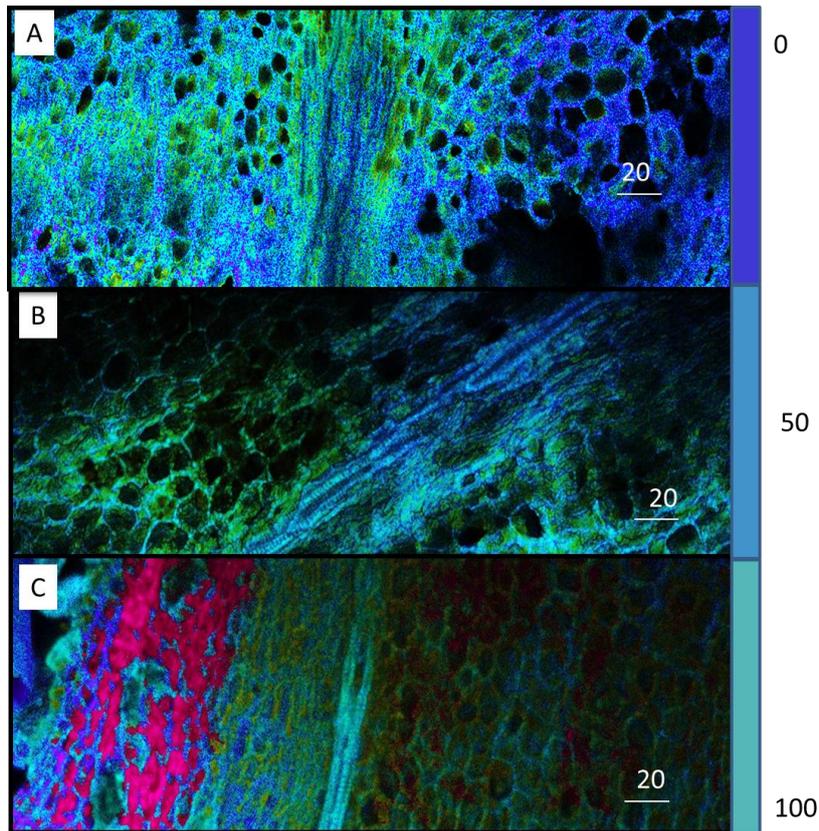
**Fig 5**: Confocal microscopy images showing the degree of lignification in xylem cell walls in different apple cultivars. The plant cell walls fluoresce as a result of UV and visible light excitation due to the presence of lignin. The intensity of primary xylem cell wall (dark blue), transition cell wall (blue green) and secondary cell wall (green) are spatially resolved in dark blue, blue green and green colours (Donaldson *et al*. 2013). The red represents chloroplasts. (A) A lesser degree of lignification (dark blue) in xylem wall indicating primary xylem in Braeburn from Elgin in June. (B) Transitional xylem stage in Cripps Pink in July from the Welgevallen region indicating a slight intensification in degree of lignification (blue green) in primary xylem walls. (C) Secondary xylem in Royal Gala in August in the Elgin region with stronger lignified (green) cell walls.

Table 1: Timing of visual xylem formation in reproductive apple buds of different cultivars during three seasons using light, confocal and SEM microscopy images. P-primary xylem visible, T-primary transition to secondary, S-secondary xylem. Data is presented as percentage buds showing the phase of development out of 5 (first 2 seasons) or 3 (last season) potential buds.

| Year | | 2012 | | | 2012 | | | 2012 | | |
|------|--|------|--|--|------|--|--|------|--|--|
| Month | | Jun | Jun | Jun | July | July | July | Aug | Aug | Aug |
| Xylem development stage % | | P | T | S | P % | T | S | P | T | S |
| **Region** | **Cultivar** | | | | | | | | | |
| Elgin | Cripps Pink | 100 | 0 | 0 | 60 | 40 | 0 | 40 | 40 | 20 |
| Elgin | Royal Gala | 100 | 0 | 0 | 100 | 0 | 0 | 20 | 20 | 60 |
| Elgin | Braeburn | 40 | 60 | 0 | 40 | 60 | 0 | 20 | 80 | 0 |
| Elgin | Golden Delicious | 60 | 40 | 0 | 40 | 60 | 0 | 0 | 60 | 40 |
| Stellenbosch | Cripps Pink | 100 | 0 | 0 | 100 | 0 | 0 | 20 | 20 | 60 |
| Stellenbosch | Royal Gala | 100 | 0 | 0 | 100 | 0 | 0 | 20 | 20 | 60 |
| **Year** | | **2014** | | | **2014** | | | **2014** | | |
| **Month** | | Jun | Jun | Jun | July | July | July | Aug | Aug | Aug |
| **Xylem development stage** | | P | T | S | P | T % | S | P | T | S |
| Elgin | Cripps Pink | 100 | 0 | 0 | 60 | 40 | 0 | 40 | 20 | 20 |
| Elgin | Royal Gala | 100 | 0 | 0 | 100 | 0 | 0 | 40 | 0 | 60 |
| Elgin | Braeburn | 40 | 60 | 0 | 40 | 60 | 0 | 20 | 80 | 0 |
| Elgin | Golden Delicious | 60 | 40 | 0 | 20 | 80 | 0 | 0 | 60 | 40 |
| Stellenbosch | Cripps Pink | 100 | 0 | 0 | 100 | 0 | 0 | 20 | 20 | 60 |
| Stellenbosch | Royal Gala | 100 | 0 | 0 | 100 | 0 | 0 | 20 | 20 | 60 |
| **Year** | | **2015** | | | **2015** | | | **2015** | | |
| **Month** | | Jun | Jun | Jun | July | July | July | Aug | Aug | Aug |
| **Xylem development stage** | | P | T | S | P | T % | S | P | T | S |
| Elgin | Cripps Pink | 100 | 0 | 0 | 50 | 50 | 0 | 25 | 50 | 25 |
| Elgin | Royal Gala | 100 | 0 | 0 | 100 | 0 | 0 | 20 | 20 | 60 |

| Elgin | Braeburn | 25 | 75 | 0 | 25 | 75 | 0 | 25 | 75 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|
| Elgin | Golden Delicious | 50 | 50 | 0 | 25 | 75 | 0 | 0 | 50 | 50 |
| Stellenbosch | Cripps Pink | 100 | 0 | 0 | 75 | 25 | 0 | 25 | 25 | 50 |
| Stellenbosch | Royal Gala | 100 | 0 | 0 | 100 | 0 | 0 | 25 | 25 | 50 |

# Chapter 3

## Selective macro nutrient element dynamics of reproductive apple bud tissues during dormancy

### Introduction

Management of water and nutrient fertilizer is essential in modern high density plantations to enable the manipulation of reproductive and vegetative development as well as fruit quality in deciduous fruit trees (Tagliavini and Marangoni 2000). Once the primary xylem is established in the apple reproductive bud, the concentration of macro-nutrients increases. According to Lang (1989) the rate of nutrient flow into the fruit generally increases during fruit development. At harvest the concentration of minerals supplied by the phloem and xylem is regulated by the balance between phloem and xylem at this late stage of development, and not by the balance between these two tissue types during early stages of fruit development. Effectively this means that a mineral such as potassium will accumulate in the fruit during late fruit development, while a xylem borne mineral such as calcium will have been acquired during early fruit development. Mineral nutrition is one of the important factors in fruit tree production, since minerals are responsible for several functions such as energy processes, enzyme activation and osmotic regulation of the membranes (Faust 1989; Marschner 1993). Therefore, it is important to determine the mineral requirements of trees and at what phenological stage a certain element is taken up in so that the correct nutrients can be applied at a time that will benefit the physiological processes taking place in the tree at such time. If nutrients are applied at the wrong time it may affect the phenological and physiological plant processes negatively (Stassen *et al*. 1981b; Faust 1989). Of all the nutrients, nitrogen is the most critical and must be managed carefully, as it determines the balance between reproductive and vegetative growth.

Apple trees require 16 elements for successful completion of their life cycle (Salisbury and Ross 1992). Mineral elements are divided into macro-elements including nitrogen, phosphorus, potassium, calcium, magnesium and sulphur, and micro-elements including iron, manganese, copper, zinc, boron, molybdenum and chloride (Salisbury and Ross 1992). Although micro-elements are taken up by plant roots through mass flow and diffusion (Salisbury and Ross 1992), they are relatively immobile once they are incorporated into the soils (Tisdale et al. 1985).

Most of the nutrients lost from the soil are due to fruit harvesting, therefore the need to replace nutrients is largely a function of crop yield. In more sandy and gravel soil types nutrient losses by leaching can also take place under conditions of high rainfall or irrigation (Kotze 2001). Furthermore, a certain amount of nutrients are removed by pruning and a certain amount is fixed as part of the permanent structure of the tree (Stassen 1987; Stassen and North 2005). According to Stassen *et al*. (1997b), nitrogen, phosphorus and potassium are translocated back from the leaves to the permanent parts before leaf drop, but immobile calcium and to an extent moderately mobile magnesium, are not redistributed or translocated from the leaves to the permanent parts.

Phosphorus (P)

According to Faust (1989), P is accumulated in the permanent structures and translocated during the times when demand is higher than uptake. Phosphorus uptake is relatively low and starts increasing three weeks before bud break in apple trees and decrease in the permanent parts especially the roots. Therefore, P is redistributed from permanent parts to support developing organs such as leaves, fruit and new growth. One of the main functions of P is that it serves as one of the building blocks of both DNA and RNA, and in addition it helps to maintain the firmness of many fruits. At a physiological level, P plays a critical role in energy transfer and the regulation of many enzymes. P is mostly required at stages of meristematic activity when roots and shoots emerge, particularly at planting (Neilsen and Neilsen 2003). It is found that annual P uptake can be high early in the season when cell division takes place in developing leaves, shoots and fruitlets.

Potassium (K)

Potassium content increases from three weeks after bud break until harvest in apple trees (Stassen 1987). According to Stassen *et al*. (1983), K is accumulated mostly in permanent structures where it is later used for bud development as well as new growth.

Potassium improves fruit quality with respect to fruit size, taste (acidity), aroma, colour and the reduced risk of low-temperature breakdown (Marcelle 1995). Potassium deficiency is harmful to the physiological functions as well as fruit development, while excess K stimulates a number of physiological disorders (Shear & Faust, 1980; Stassen *et al*., 1999). K is mobile in phloem, resulting in a good supply of K into fleshy fruit. Tromp (1981) found that the uptake of K is linearly related to the metabolic activity.

Magnesium (Mg)

Magnesium accumulates in the leaves and in case of need, it is translocated from older leaves to younger leaves (Faust 1089). According to Terblanche (1972), Mg moves from the permanent parts to the leaves where it is lost after leaf drop.

The requirement and quantities of uptake of Mg and Ca by fruit trees are rather similar (Faust 1989). Given that Mg is very mobile in the plant, it can easily be moved from old leaves to younger once. Interestingly, there is an inverse correlation between Mg and Ca in fruit, such that when the concentration of the one mineral is high, the concentration of the other is low (Faust, 1989). Excess Mg prevents Ca uptake and therefore the correct quantities must be given to reduce Ca related disorders (Shear and Faust 1980). A deficiency in Mg inhibits $CO_2$ assimilation, reduces photosynthesis and is detrimental to metabolic processes (Shear and Faust 1980; Marschner 1993; Stassen *et al*. 1999).

Calcium (Ca)

Different seasonal patterns of Ca uptake have been observed in apples. There is a large inflow of Ca in the four to six week period of cell division following flowering (Wilkinson 1968), but also low but steady uptake throughout the growing season (Faust 1989). Calcium accumulation in the fruit takes place in the first part of fruit growth and starts decreasing with fruit maturation (Faust 1989). Calcium content of the whole tree remains constant from harvest until the beginning of leaf drop. This indicates that a small amount is removed at harvest, but it does not have an effect on the content of the whole tree. Three to nine weeks after harvest, Ca accumulation increases in the roots. The Ca lost through leaf drop reduces the total tree Ca content. According to Terblanche (1972), more Ca is lost from the tree than what was in the leaves before leaf drop. Therefore, he suggests that Ca moves from the permanent parts before leaf drop and is lost through leaf drop. The most important function of Ca is the maintenance of cell integrity, and is thus very important in the determination of the quality of fruit. When the Ca concentrations are high enough, it enhances the firmness of apples (Conway *et al*. 2002). Calcium is also involved in cell division, cell elongation, germination and pollen tube growth (Mengel and Kirkby 1982). It forms an important component of cell walls, where it helps binding pectin-protein complexes, and as such help to make both cell walls and cell membranes stable and strong (Zocchi and Mignani 1995; Bramlage *et al*. 1980; Jackson 2003). Calcium is not very mobile in phloem (Zocchi and Mignani, 1995), so it is believed to be transported via xylem sap (Vang-Petersen, 1980;

Jackson, 2003). Factors that influence the movement of Ca through xylem include exchange adsorption to xylem walls, the force of transpiration flux (Bangerth 1979; Banuelos *et al*. 1987; Faust 1989; Jackson 2003) and how functional fruit xylem is at different stages of development (Dichio *et al*., 2003).

The concentration of minerals such as N, P, K and Mg in phloem is typically much higher than that of Ca, which is not mobile in phloem or the simplast (Raven 1977). Such minerals are mainly transported into the fruit later in fruit development, much closer to the time of fruit maturity (Tromp and Oele 1972; Lang 1990). Wieneke (1969) confirmed Ca to be virtually absent from the phloem sap, and Ferguson (1980) agreed that phloem could not provide sufficient Ca to all the parts of the plant where it is required. Based on these findings it is clear that Ca must be transported by the xylem from the root to the fruit. There is a decline in Ca concentration in the bark at about the time of bud break (Mason and Whitfield, 1960). Wieneke (1969) further reported high concentrations of free Ca in the xylem sap of apple trees at the beginning or early in the season. Bradfield (1976) and Tromp (1979) both also mentioned higher Ca concentrations in the xylem of apple shoots early on during apple development.  It is likely that extra Ca is mobilized to young apples as insoluble Ca oxalate, but once starch formation initiates (Liegel and Buchloh 1976) during June or July, the Ca oxalate may be remobilized.

**The role of tracheal sap in apple tree nutrition**

Apples have two distinct vascular bundle systems, the cortical and the carpellary.  There are ten primary bundles associated with the cortical system, while there are ten ventral and five dorsal primary bundles in the carpellary system and these fifteen bundles are joined to each other (MacArthur and Wetmore 1939; MacDaniels 1940). Xylem and phloem comprise the transport tissues within vascular bundles.

Xylem vessels are very rigid and inelastic in fruit (Drazeta *et al*. 2004). As the fruit expands and develop xylem vessels tend to break down and become dysfunctional (Drazeta *et al.* 2004; Lang and Ryan 1994). This happens early in the season for apple cultivars such as Braeburn and Golden Delicious, while it occurs later in a cultivar such as Cripps Pink (personal communication, E Lötze, SU).

Open perforation plates between individual vessel elements reduce resistance to water flow through vessels (Nobel 1999). How much xylem sap flows through the pedicel is determined by how many and how large the vessels in the petiole are (Drazeta 2003).

In this paper, we aim to determine the Ca, Mg, K and P concentration in the apple bud during dormancy and quantify changes in these concentrations over time.

**Hypothesis**

All macro-nutrient element concentrations increase in reproductive apple buds of Cripps Pink and Braeburn during dormancy.

**Material and Methods**

Two commercial apple cultivars, Cripps Pink and Braeburn, were selected to determine changes in Ca, Mg, K and P concentrations in dormant reproductive buds through the dormant season (June to September). Sampling of dormant buds occurred between June to September during 2012 and 2014. Macro-nutrient concentration levels in the whole bud were quantified during this period.

Five buds per sampling date were prepared for imaging and analysis of macro-nutrients. Longitudinal sectioned buds were dehydrated through an ethanol series, sectioned with a sharp Minora razor blade and then critical-point-dried using liquid carbon dioxide to ensure that the cell structure stays intact. Samples were stored in an evacuated desiccator with silica-gel to ensure that they stayed dry. Double sided carbon type was used to mount the sample onto an aluminium stub then sputter coated with gold to ensure good conductivity. Backscatter surface images and analysis of macro-nutrients were studied using a Scanning Electron Microscope (Zeiss EVO® MA15). Calcium elemental phase mapping was quantified with the wavelength dispersive spectrometry (WDS) detector (Oxford Instrument® Wave Dispersive X-ray Spectrometer) and the Mg, P, K, Ca levels were analysed using the electron dispersive detector (EDX) using Oxford INCA software. Beam settings during analysis were 20 kV and 11 nA I-Probe, with a working distance of 8.5 mm and a specimen current of -20 nA. Analyses were done in area mode, with process time four and 10 live time seconds. Pure Cobalt was used periodically to correct for detector drift. The analysis performed for Ca was 30 seconds peak to 15 seconds background counting times. The Positron Emission Tomography (PET) crystal was used for Ca analysis. The detection limits for EDX was 0.1 weight% and WDS was 0.01 weight%.

**Elemental mapping**

The spatial distribution of a specific element can be revealed by recording a 'map' of the intensity of its characteristic X-rays while the beam is scanned in a raster of the whole bud. X-

ray maps are produced by recording the number of X-ray photons for a fixed time at each point in the scanned area and storing the data in the memory of the computer. A visible image is generated by converting this number into brightness on a screen. In elemental mapping, bright intensities are related to high concentrations of elements, whereas dark intensities are related to low concentrations of elements in all standard images. In addition, because the human eye can only distinguish between 16 different 'grey levels' in a monochrome image (Reed 2005), it seemed plausible to replace the grey-scale level with 'false' colours. The 'rainbow' scale (violet, blue, green, yellow and red) was applied to monochrome images, to confidently identify several elements within the selected samples.

Several elemental maps were generated from the two different cultivars. However, only four elemental maps were selected to be presented here (Fig 1-4) to provide a general observation and description of each representative cultivar within the Elgin region. The elemental maps were mainly used for phase nutrient identification and also to compare nutrient concentration within the buds.

Analyses of the data were performed using Excel 2010 software to determine significant differences in nutrient concentrations between Cripps Pink and Braeburn.

## Results

### Elemental maps

In Cripps Pink Ca X-ray elemental maps from June to September showed high intensities of Ca in parenchyma tissues and lower intensities in vascular tissues (Fig 2). Potassium X-ray elemental maps showed K sporadically spread during the two seasons in the bud of Cripps Pink. X-ray elemental maps for Mg and P were low in intensities during the two seasons.

The backscatter electron (BSE) image and four electron maps highlighted the spatial distribution of each element (Mg, P, K and Ca) across longitudinal sections of Braeburn (Fig 3 and 4) and the spatial distribution for Ca in Cripps Pink buds (Fig 2). Calcium elemental maps for June of Braeburn (Fig 3) showed that Ca is highly distributed in parenchyma tissues and very low in the vascular bundle region. The relative intensity of P X-rays within the elemental map showed mainly in the parenchyma tissue at the lower region of the Braeburn bud. Potassium is sporadically spread within the bud. Magnesium elemental maps showed low intensities of X-rays in the Braeburn bud during both seasons.

*Mineral nutrient concentrations*

P, K, Mg and Ca distribution in whole dormant bud of two apple cultivars was analysed over two seasons 2012 and 2014. Cripps Pink showed a high Ca concentration during June (Fig 5) in both seasons. From July up to September, the Ca concentration in Cripps Pink decreased steeply from approx. 5 to less than 4. Potassium concentration in Cripps Pink in 2012 and 2014 increased from June (0.8; 0.9) towards August (1.4; 1.0) and decreased thereafter in September (0.6; 0.7). P increased from June towards August (0.1-0.2), before declining in September 2012 (0.1) (Fig 5). In 2014, P was relatively stable from June to August (0.2 − 0.1) before the increase in September (0.2). Mg increased from June to September (0.16 − 0.23) during 2012. In 2014, Mg was higher (0.25 − 0.26) but similar for all months (Fig 5).

Ca concentration is the lowest (1.0; 1.7) during June in Braeburn (Fig 5) and showed a steady increase from until September (1.6; 2.2) for both seasons. Potassium showed a very small increase from June to September (0.2 − 0.3) during 2012. During 2014 the increase was slightly higher from June to August (0.24 − 0.4) and continue stable in September (0.4). P values varied and similar, lower values (0.47) was found in June and August 2012, with higher values (0.54; 0.46) in September (Fig 5). In 2014, P was relative stable from June to August (0.41 − 0.41) before increasing in September (0.46). Mg showed a declining trend from June to September for both years. However, values differ and in 2012 June started with 0.28 and declined to 0.15 in comparison with the initial value of 0.16 in June 2014 that declined to 0.10 in September.

**Discussion**

Results clearly showed a difference between Cripps Pink and Braeburn in Ca, K, P and Mg distribution as well as the concentration of Ca, K, P and Mg

The parenchyma tissue maintained a high Ca concentration during the entire dormant season. The tissues that spread into the leaf areas also contained high Ca concentrations. The vascular tissues that spread from the spur and continued into the developing flower region had the lowest Ca concentrations. Phosphorous was concentrated in the parenchyma tissues in areas where Ca is least concentrated. Magnesium concentration was stable throughout the dormant season in the different tissues in the bud. Potassium accumulated in higher quantities in the parenchyma tissues and in small amounts in the vascular tissues.

Actual mineral nutrient concentrations of the bud differed between cultivars in terms of trends as well as actual values of the four elements quantified. The Ca concentration of Cripps Pink

was overall higher than that of Braeburn, from June to September and for both seasons. This confirms previous reports on fruit Ca concentrations of these cultivars and thus indicated that already on reproductive bud level, cultivars can be classified as potential Ca deficient cultivars or not. Cripps Pink Ca concentrations declined during dormancy whereas the Ca concentration of Braeburn was more even with a slight increase towards September.

K concentration in Cripps Pink was similarly notably higher in all months and for both seasons compared to Braeburn. Again concentrations for Braeburn was more similar between months with a slight increase in August and September of 2014, In contrast, concentrations increased from June to August and then declined sharply in September for both months. The increase in K in Cripps Pink is expected with the decrease in Ca levels as reported in literature in fruit as well, with less of an interaction between elements in the case of Braeburn where no clear trends were observed.

In the case of P, Braeburn concentrations were much higher than those of Cripps Pink during the whole period and for both months. No clear trends were observed.  Mg showed an increasing trend towards September in Cripps Pink for 2012 and confirmed the antagonistic relationship with Ca which is well known. However, this was not observed in 2014 when concentrations were still higher than those for Braeburn, but with a more even trend. Braeburn Mg concentrations tended to decrease from June to September in both seasons and could be related to an extent to a slight increase in Ca concentration.

Quantification of the practical value of the outcomes of this paper lies outside of the scope of the study. Nevertheless, this information has never been published before and clearly indicates noticeable changes in the concentrations of P, K, Mg and Ca even on reproductive bud level during dormancy. Results illustrated interactions between these elements at this phenological stage and clearly showed differences between apple cultivars in this respect. This newly acquired knowledge should be of great interest in the field of horticultural science for further investigation.

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**Figures**
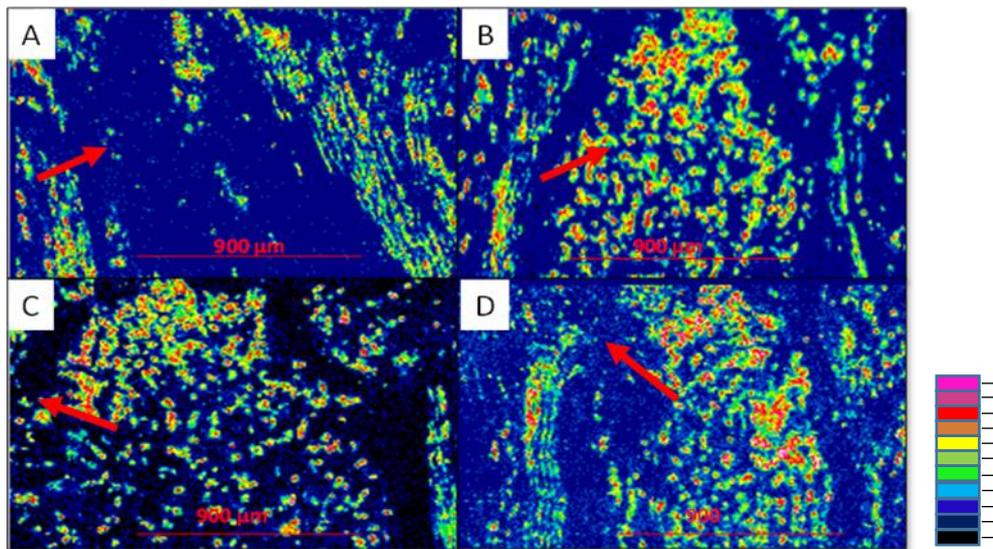


**Fig 1**: Calcium elemental map of Braeburn over four months. (A), Ca map for June with irregular Ca distribution scattered in the vascular region (B), Ca map for July showing a high Ca distribution in the parenchyma cell and very low Ca distribution in the vascular region and (C) Ca map for August showing a slight increase in Ca in the vascular region. (D) Ca map for September showing an increase in Ca distribution in the vascular region (higher than in August).
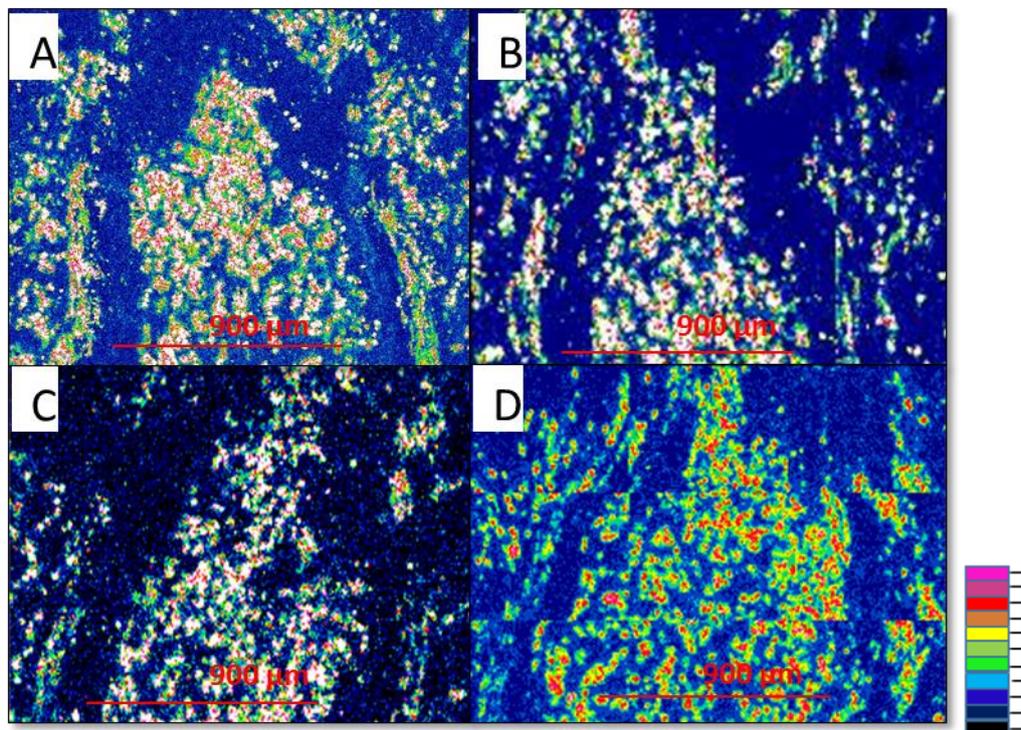


**Fig 2**: Ca elemental map for Cripps Pink over four months. A and C show the same concentration of Ca during June (A) and August (C), whereas B and D show the same concentration of Ca during July (B) and September (D).
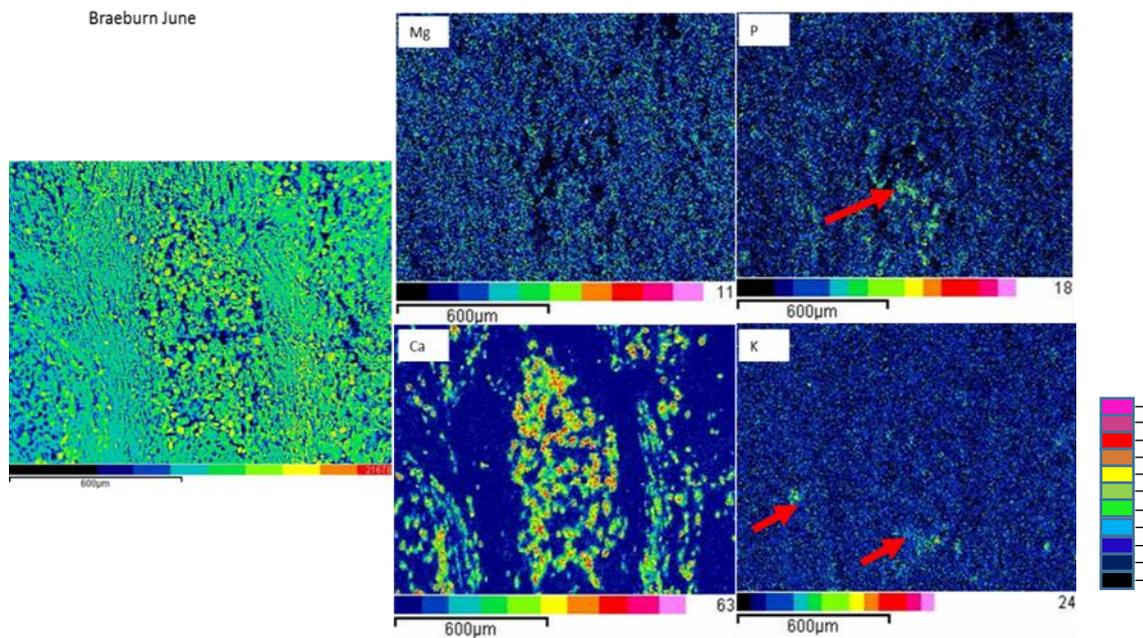
**(a)**            **(b)**

**Fig 3**: Elemental map for Braeburn from the Elgin region during June. (a) BSE image of Braeburn during July. (b) X-ray maps for Mg, P, Ca and K. Ca showed the highest concentration in the whole bud. P concentration is mainly in the parenchyma cells and K is scattered randomly around the bud. Mg is the lowest in concentration.
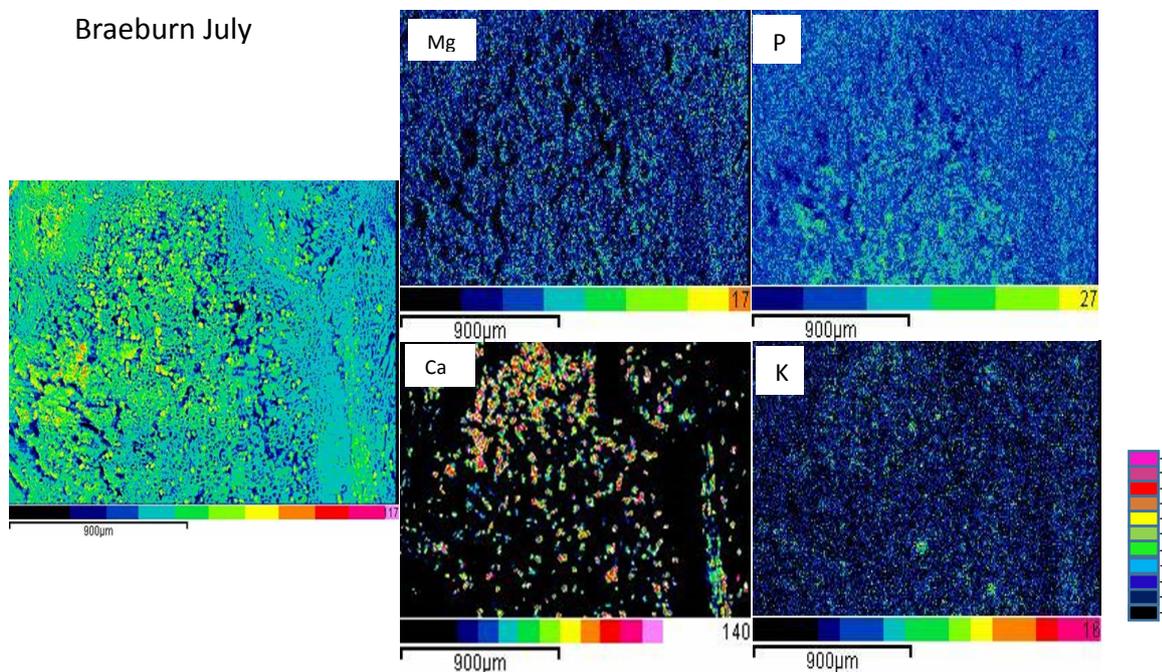


**(a)**            **(b)**

**Fig 4:** Series of coloured elemental maps showing the distribution of key elements for Braeburn from the Elgin region during July. (a) BSE image of Braeburn during July. (b) X-ray maps for Mg, P, Ca and K. Ca showed the highest concentration in the whole bud. P is

mainly concentrated in the lower part of the parenchyma cells in the bud and K is scattered randomly around in the bud. Mg showed the lowest concentration compared to Ca, P and K.
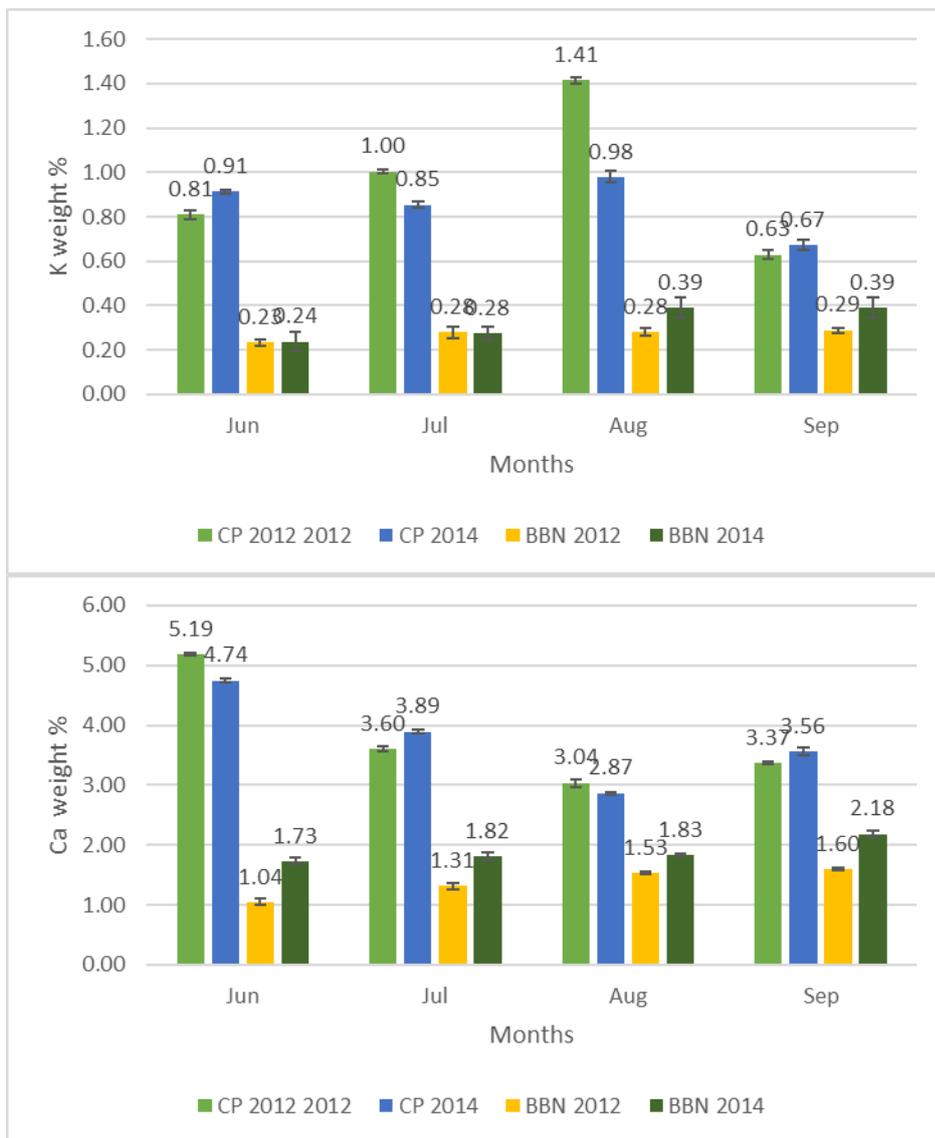
**Fig 5**: Wavelength and Electron Dispersive X-ray surface analyses obtained, selected to cover the whole bud. Macronutrients P, Mg, K and Ca acquired indicate the distribution expressed in weight % during June, July, August and September in Cripps Pink (CP) and Braeburn (BBN) in the Elgin region.

**Chapter 4**

**Dynamics of nutrient allocation from reserve tissues, using calcium as a model in
dormant, reproductive apple buds of four apple cultivars**

**Introduction**

During dormancy, cell division occurs continuously and most of the total of approximately 25
cell divisions occurs before bud break (Bergh 1984; Greybe 1997). Ca stabilizes cell wall
structures and ensures good cell to cell adhesion by the binding action of negative cell
surfaces (Zocchi and Mignani 1995). It also fulfils a structural role in the middle lamellae
between adjacent cells and plays an important role in cell division (Zocchi and Mignani
1995).  This implies that developing buds requires Ca for normal cell wall and cell membrane
development and function (Zocchi and Mignani 1995).

According to Ferguson (1980), there is a peak in the concentration of Ca in the xylem of kiwi
fruit just before bud break, indicating that xylem formation has been completed. However,
there is little information about the exact timing of the formation in the xylem in dormant,
reproductive apple buds and the possible role it may play in supplying Ca to the developing
bud before bud break occurs.

Translocation of ions in the xylem vessels to the above-ground parts is passive (Taiz and
Zeiger 1991). Nutrients are transported to the leaves through the transpiration stream, which
is influenced by water loss through the leaves (transpiration) (Taiz and Zeiger 1991; Salisbury
and Ross 1992).

At the beginning of the growing season, large amounts of nutrients are remobilized from
reserve tissues in the tree towards developing shoots and flowers. Remobilisation from the
reserves supplies developing shoots with Ca before root uptake has started (Ferguson 1980).
Mason and Whitfield (1960) also reported that Ca is mobilized before roots are fully active,
and motivated this by the observed decline in the concentration of Ca in the bark at around
bud break (Mason and Whitfield 1960). Ferguson (1980) reported an increase in the
concentrations of potassium (K), magnesium (Mg), phosphorus (P) and Ca in shoot sap just
before emergence of the first leaves in kiwi fruit. Ca concentrations reached a sharp peak in
the xylem sap about one week after bud break and this early remobilization can supply 25%
of the Ca contained in the new growth in apples (Bradfield 1976; Terblanche *et al*. 1979a;
Tromp 1979; Himelrick and McDuffie 1983).

About 40% of the Ca in an apple shoot is located in the bark, with smaller amounts occurring in the wood (Ferguson and Turner 1981). About 50% of this Ca becomes fixed as Ca oxalate and will not be remobilized to the fruit in the short term (Ferguson and Turner 1981). Most of the Ca in new growth will therefore derive from remobilized Ca in smaller amounts from reserve Ca in the wood (Ferguson and Turner 1981). Some of the free Ca stored in the bark can be remobilized towards the wood in the long term; however, Ca associated with the bark will not be transported directly to the new growth (Ferguson and Turner 1981).

According to Mason and Whitfield (1960) the concentration of Ca decreases in the bark at about the time of bud break (Mason and Whitfield 1960). Weineke (1969) further reported high concentrations of free Ca in the xylem sap of apple trees early in the season, Wieneke (1969), Bradfield (1976) and Tromp (1979) all mentioned higher Ca concentrations in the xylem of apple shoots early on during apple development. All of these arguments support the notion that Ca is mobilized before roots become fully active.

The aim of this paper was to quantify Ca located in the spur, leaf primordia and primary xylem of the dormant reproductive apple bud over four months (June to September) and investigate the change of these Ca concentrations over time and between tissues. As reserve Ca is transported towards the bud at the end of dormancy (winter/August) for utilization from spring (September), the hypothesis was that an increase in Ca concentration in the xylem from Jun towards September should be mirrored by a decrease in the Ca concentration of reserve tissues (spur and/or leaf) during the same time if these tissues are indeed the source of reserve Ca for the dormant bud.

## Material and methods

Four different commercial apple cultivars were selected to determine changes in Ca concentrations in three different regions (leaf, spur and the region below the flower-(hereafter referred to primary xylem) of reproductive apple buds.  Royal Gala is an early season cultivar with high inherent Ca concentration in the fruit, while Golden Delicious and Braeburn represented cultivars harvested mid season and with a low inherent Ca concentration in the fruit, whereas Cripps Pink is harvested late in the season and has a high inherent Ca concentration in the fruit.  Sampling of dormant buds occurred between June and September during 2012 and 2014. Ca levels in different regions (leaves, spur, primary xylem) were quantified during this period (Fig 1).

Five buds per sampling date were prepared for imaging and analysis of macro-nutrients. Buds were dehydrated through an ethanol series, sectioned with a sharp Minora razor blade and then critical-point-dried using liquid carbon dioxide to ensure that the cell structure stays intact. Samples were stored in an evacuated desiccator with silica-gel to ensure that the samples stayed dry. They were then mounted onto an aluminium stub with double sided carbon tape and coated with a thin layer of gold to ensure good conductivity.

Bud anatomy and analysis of macro-nutrients were accomplished using a Scanning Electron Microscope (Zeiss EVO® MA15) at the SEM Unit of the Central Analytical Facility at Stellenbosch University. Ca concentration was quantified with the wavelength dispersive spectrometry (WDS) detector (Oxford Instrument® Wave Dispersive X-ray Spectrometer). Beam conditions during the analysis were at 20 kV and approximately 11 nA I-Probe, with working distance of 8.5 mm and specimen beam current of approximately -20 nA. Analyses were conducted in area mode, with process time four and 10 live time seconds. Pure Cobalt was used periodically to correct for detector drift. The detector limits for the WDS is 0.01 weight%. The analyses were performed with WDS for Ca (30 to 15 seconds peak to background counting times). Calcium was analysed with Positron Emission Tomography (PET) crystal (Fig 2).

**Results**

*Ca distribution between three tissues*

The Ca distribution (weight %) between the three tissues (leaf, spur and xylem), calculated as percentage, is presented per cultivar and per season, in Figures 3 to 6. The spur (S) represented the tissue with highest Ca distribution (approx. 60%), followed by the leaf (L) and the xylem (X) with a much lower and smaller Ca distribution (approx. 20% in Braeburn, Fig. 3). This was consistent for both seasons. There was a slight increase in Ca concentration from June to September in the xylem for both seasons. This seemed to coincide with a slight decline in September in both the Ca concentrations from the leaf and spur.

Golden Delicious showed a similar trend with the highest Ca distribution in the spur (approx. 60%) and lower, equal percentage in the xylem and leaf (approx. 20%) (Fig 4). There was a more prominent decreasing trend in Ca concentration in the xylem from June to September during both seasons, followed by a slight increase in the leaf Ca concentration. The Ca concentration in the spur increased sharply during 2012 (50 to 60%), but was more stable in

2014 (59%). The initial (June) Ca concentration in the xylem in 2012 was higher (31%) than in 2014 (21%).

Distribution of Ca was still higher in the spur (> 46%) than the other tissues (12 − 38%), but now the Ca concentration in the xylem increased substantially from June to September for both seasons for Cripps Pink (Fig 5). This coincided with a substantial decrease in the Ca concentration of the spur. During 2012 the Ca concentration of the leaf also declined from 23% to 15% and there was a slight decrease in the leaf in 2014 from 16% to 12%.

Royal Gala Ca distribution showed the lowest average values for spur (approx. 41%) in this study with different trends between seasons. In 2012, Ca concentrations were stable from June to September, before declining in September. In 2014 Ca concentrations increased slightly from 41% to 45% before declining to 42% in September. Leaf Ca concentrations were also the lowest of all cultivars in 2012 (approx. 10%), with the leaf Ca concentrations in 2014 being similar (approx. 25%) to those of Golden Delicious (approx. 23%) in 2014. During 2012 the leaf Ca concentrations were similar from June to September, whereas during 2014, there was a slight decline from June (29%) to September (23%). Xylem Ca concentrations were the highest of all cultivars, with 2012 also being higher than 2014. The increase in xylem Ca concentration from June to September 2012 coincided with the decline in spur Ca concentrations from 47% to 29%. In 2014, the Ca concentration showed an increase from June to July, followed by a decrease in August and an increase in September and this seemed to be mirrored by the spur.

In Figs 7 − 10 the actual Ca concentrations (weight %) is illustrated for the same cultivars, seasons and tissues. The highest Ca concentrations (weight %) in Braeburn were found in the spur, followed by xylem and lastly leaf in both seasons. During 2012 there was an increasing trend in all tissues from June to September. During 2014, xylem Ca concentration increased from June to September while Ca concentration in the leaf decreased during the same period and the Ca concentration in the leaf varied, but started (20.3%) and ended (2.07%) with a similar concentration.

In both seasons, the highest Ca concentrations (weight %) in Golden Delicious were found in the spur, followed by xylem and leaf that did not differ. During 2012, both xylem and leaf showed an increase from June to July, then a decrease in August and an increase again in September. During 2014, the Ca concentration increased from June to September in both the leaf and the xylem.

In both seasons, the spur Ca concentration of Cripps Pink declined from June to September. Actual Ca concentrations were higher during 2014 than in 2012. In contrast, the Ca concentration of xylem increased during the same period, for both seasons, with actual Ca concentrations being higher in 2012 than 2014. Leaf Ca concentrations were similar for both seasons, but increased from June to July, decreased in August and an increase again in September, reaching similar concentrations as it started with in June.  In 2014, leaf concentrations declined from June to September.

Ca concentrations of Royal Gala declined sharply from June to September in both seasons in the spur. Actual Ca concentration was higher in 2012 than 2014, with an expectant concentration of 9.22% in June 2012. Ca concentrations of both the xylem and leaf were much lower and also declined from June to September, with xylem in September 2012 showing an increase from August.

Ca concentrations of Royal Gala declined sharply from June to September in both seasons in the spur. Actual Ca concentration was higher in 2012 than 2014, with an expectant concentration of 9.22% in June 2012. Ca concentrations of both the xylem and leaf were much lower and also declined from June to September, with the xylem in September 2012 showing an increase from August.


**Discussion**

Results of Chapter 1 showed that primary xylem differentiation was initiated and established in June for cultivars Cripps' Pink and Royal Gala, but later (not defined) in Braeburn and Golden Delicious. This may indicate earlier transport of mobile nutrient mineral elements that are dependent on the xylem, like Ca, to the bud. If this is indeed the case, the quantification of Ca in the dormant bud in this trial should confirm higher Ca concentrations in the buds of Cripps Pink and Royal Gala than Braeburn and Golden Delicious.

According to Ca distribution between the three tissues investigated, the highest Ca presentation was found in the spur of all cultivars during both seasons – indicating the important role of the spur as reserve Ca tissue during dormancy. Royal Gala buds showed the lowest S distribution during dormancy, with less than 50%, compared to the other cultivars ranging from 50 – 60%. This resulted in the highest Ca distribution in the xylem for Royal Gala, ranging between 30% and 60%, compared to the other cultivars (12 – 38%). The distribution in the leaf differed between seasons and ranged between 12% and 29%. This

indicates that there are differences between cultivars with regards to Ca distribution between the spur, xylem and leaf. Royal Gala has the lowest distribution of Ca in the spur and the highest in the xylem, compared to Braeburn, Cripps Pink and Golden Delicious. Both Cripps Pink and Royal Gala showed a declining trend in Ca distribution of the spur and leaf from June to September and an increasing trend in xylem in 2012, as well as in 2014 for Cripps Pink. Royal Gala also showed a decline in leaf distribution in 2014, but the distribution of Ca in xylem and spur fluctuated. This indicates that in the two cultivars with early primary xylem formation (June), Ca is distributed from spur and leaf (storage organs for Ca) towards xylem, resulting in a higher distribution of Ca in the xylem in September.

The Ca distribution trends in Braeburn and Golden Delicious were more variable. Spur Ca distribution in Braeburn first declined, but then increased in September during both seasons, whereas Golden Delicious showed the opposite trend in 2012 and very little change in 2014. In Braeburn, the Ca distribution increased in the xylem for both seasons, similar to the trend in Cripps Pink and Royal Gala, in contrast with the decline in Golden Delicious. The leaf Ca distribution of Braeburn followed the same declining trend in 2014 as that of Cripps Pink and Royal Gala, whereas Golden Delicious only showed a declining trend in 2012.

With regards to the actual Ca concentration in the different tissues, higher concentrations occurred in the spur in Cripps Pink and Royal Gala in both seasons, compared to the other cultivars, whereas higher Ca concentrations occurred in the xylem and leaf tissues of Braeburn and Golden Delicious. This quantifies differences in Ca accumulation between cultivars at this phenological stage. The Ca concentration in the spur increased from June to September in Golden Delicious and Braeburn (2012), in contrast to the decreasing trend in Cripps Pink and Royal Gala. The higher initial Ca concentrations, as well as an increase from June to September observed in Cripps Pink and Royal Gala, may again indicate the possible role of the presence of primary xylem already in June, compared to lower Ca concentrations and later development of the primary xylem in Braeburn and Golden Delicious.

In conclusion, results clearly showed a difference between the four different cultivars in Ca distribution as well as Ca concentration in the tissues of dormant, reproductive apple buds. Indications are that early primary xylem formation in Cripps Pink and Royal Gala results in a higher Ca concentration in the xylem in June, followed by an increase in Ca concentration towards September. The reduction in Ca concentration in the spur during the same period may indicate that reserve Ca from the spur is allocated towards the xylem. This needs further investigation.

Although trends were not as clear in Golden Delicious and Braeburn and did not always follows the same pattern, trends in these cultivars differed from those observed in Cripps Pink and Royal Gala and confirmed the effect of the later development of the primary xylem reported previously. Thus, later formation or presence of primary xylem in the dormant bud of these cultivars may partly explain the lower Ca concentrations and difference in Ca distribution between tissues compared to Cripps Pink and Braeburn.

**Acknowledgements**

**Figures**
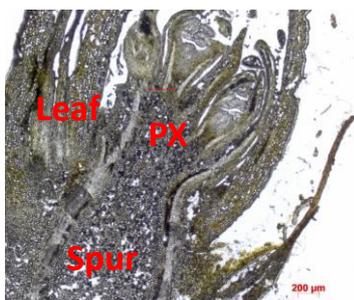


Fig 1 Identification of tissues in the dormant, reproductive apple bud used for Ca concentration quantification.
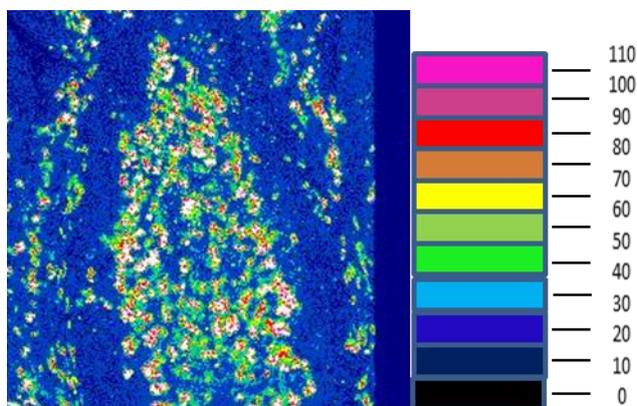


Fig 2 Royal Gala Ca map for the different tissues in September (700micon)
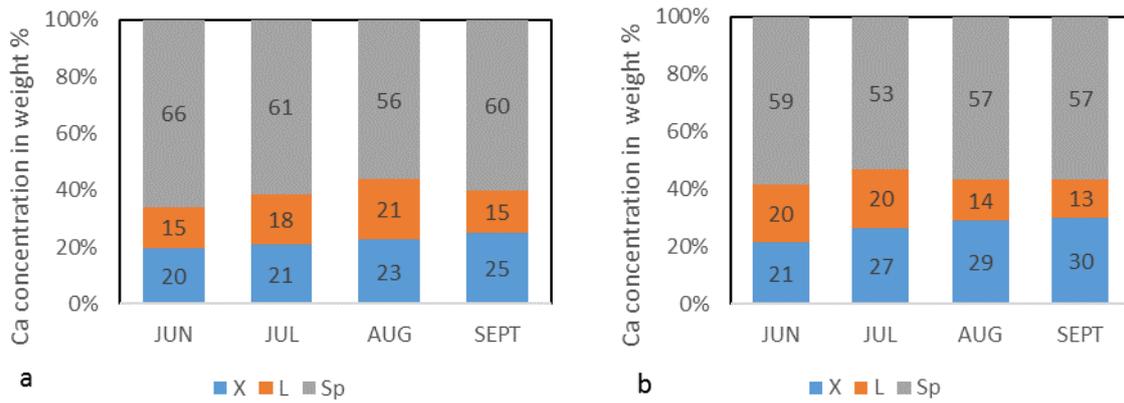
Fig 3 Relationship between the Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) presented as 100% for Braeburn for 2012 (a) and 2014 (b).
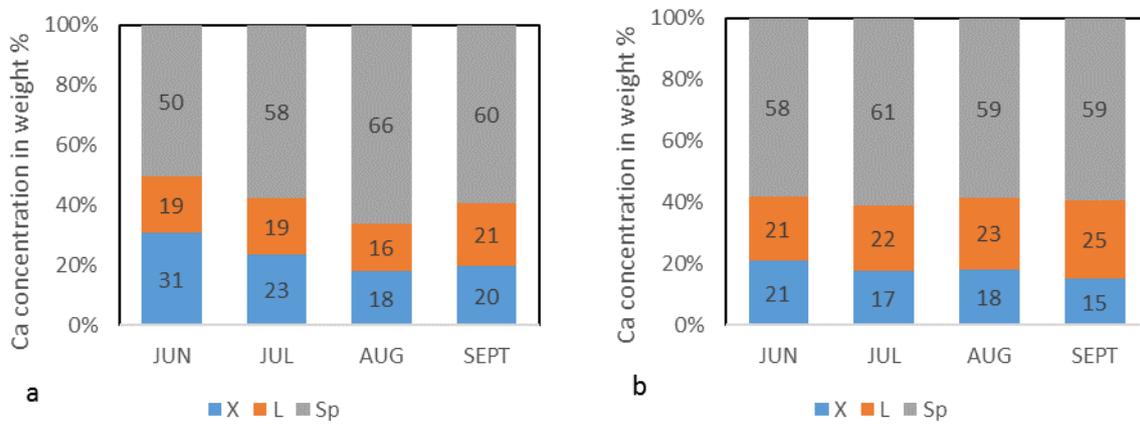


Fig 4 Relationship between the Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) presented as 100% for Golden Delicious for 2012 (a) and 2014 (b).
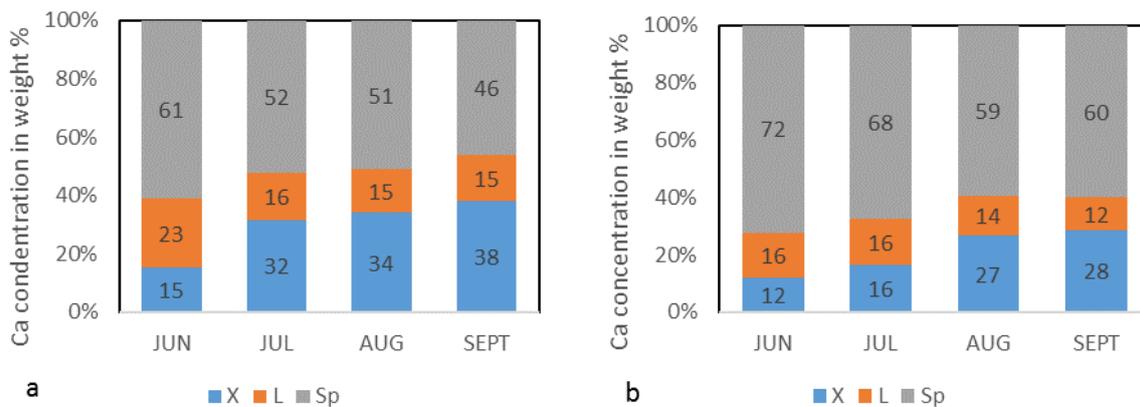


Fig 5 Relationship between the Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) presented as 100% for Cripps Pink for 2012 (a) and 2014 (b).
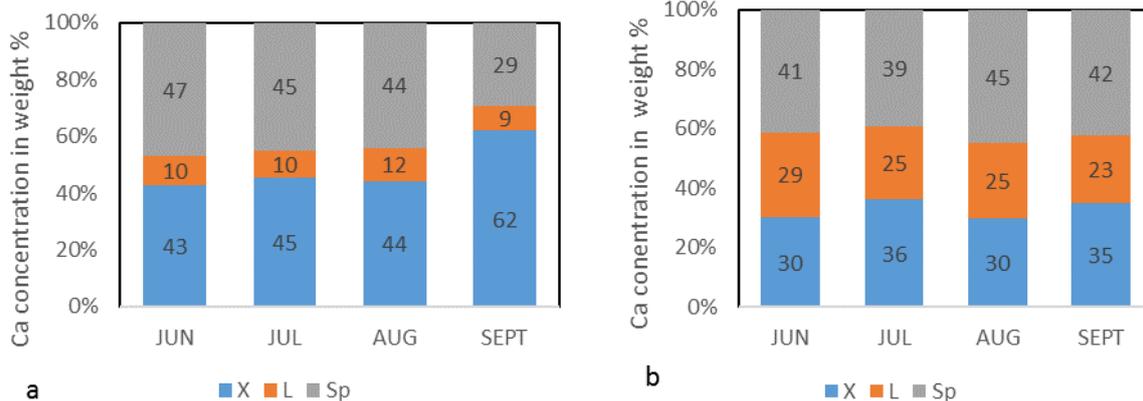
Fig 6 Relationship between the Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) presented as 100% for Royal Gala for 2012 (a) and 2014 (b).
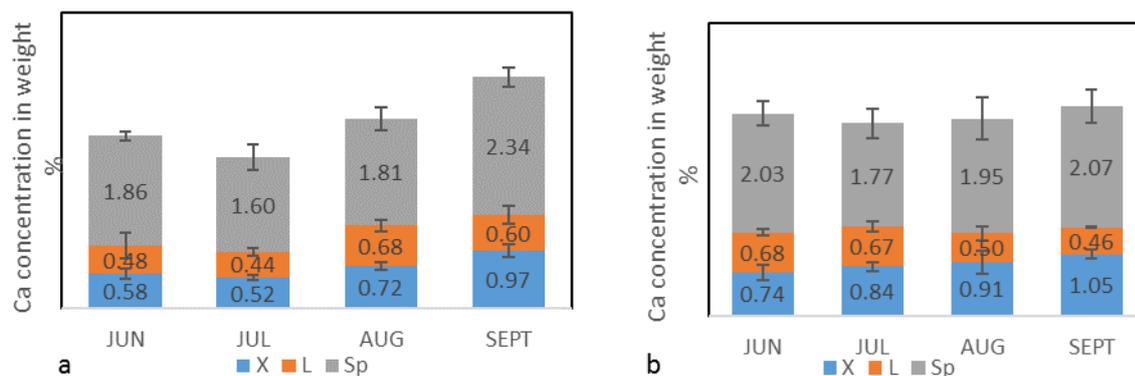


Fig 7 Actual Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) for Braeburn for 2012 (a) and 2014 (b).
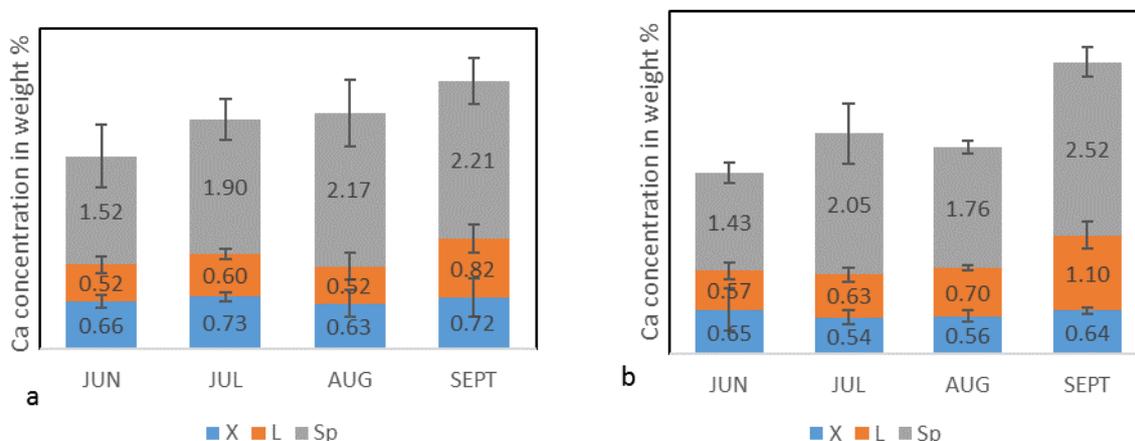


Fig 8 Actual Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) for Golden Delicious for 2012 (a) and 2014 (b).
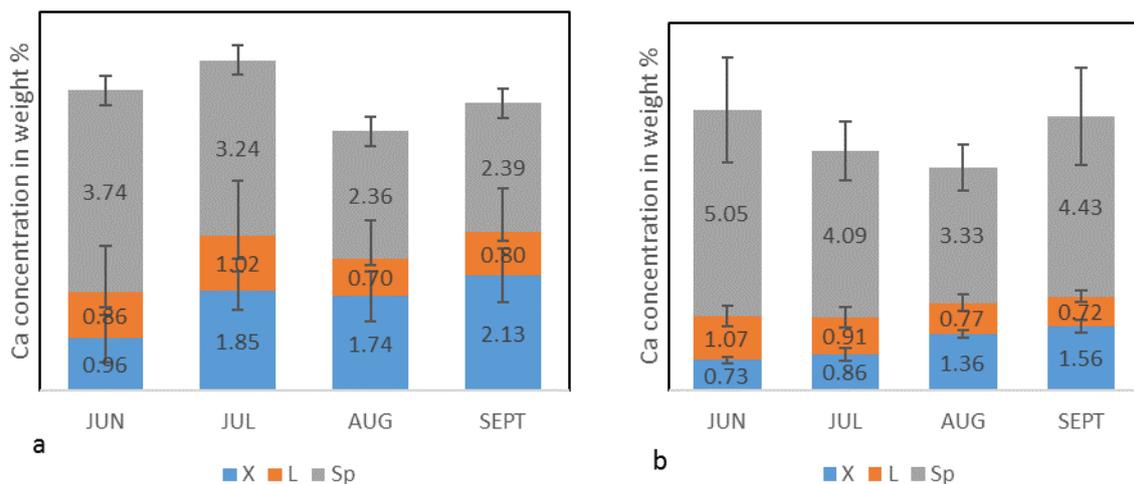
Fig 9 Actual Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) for Cripps Pink for 2012 (a) and 2014 (b).
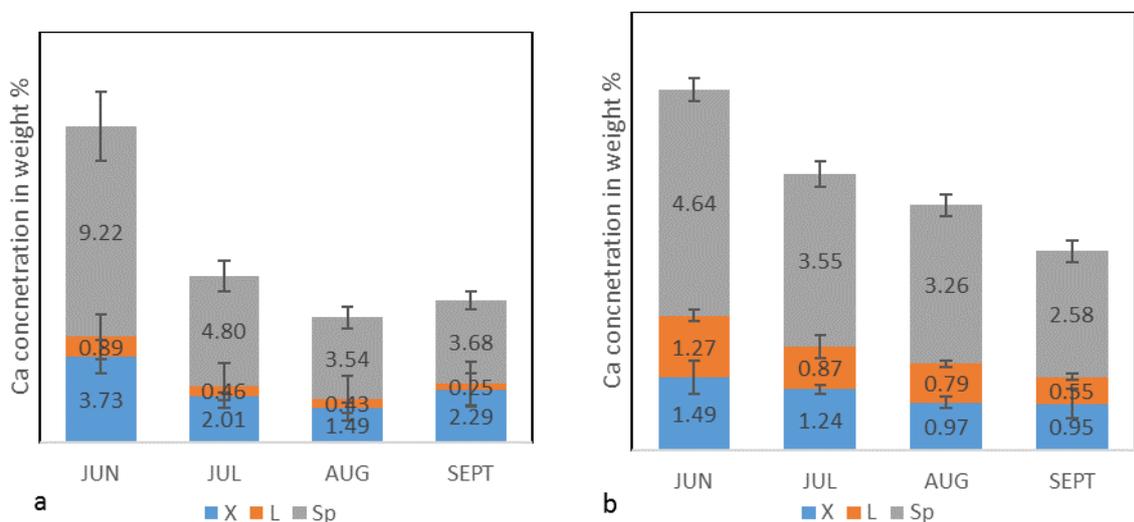


Fig 10 Actual Ca concentration (weight %) of primary xylem (X), leaf (L) and spur (S) for Royal Gala for 2012 (a) and 2014 (b).

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# Chapter 5

## General Conclusions

During this study different microscopy techniques were utilized, but the SEM proved to be the best technique for the anatomical identification of the different cell types in xylem tissue. The SEM micrographs provided details of structural and developmental stages in xylem development. The micrographs showed convincing evidence of the integration of different types of cell wall thickening with regards to features like annular thickening and helical thickening.  This provided evidence that continuous growth in the dormant reproductive apple bud takes place during dormancy.

All four cultivars had primary xylem in June, suggesting that primary xylem development started before June. The exact timing of xylem development thus needs to be investigated further.  This is important, because the time of establishment of the primary xylem and the distribution of nutrients (Ca, K, P and Mg) during dormancy is valuable to horticultural science, especially the distribution of Ca that is mainly transported in the xylem.

Results of this study clearly showed differences in the distribution of nutrients in the four cultivars studied. The highest Ca concentration was found in parenchyma cells, with the lowest Ca concentration in the vascular tissues throughout the entire dormant period. The Ca concentration of Cripps Pink was higher in June than during the rest of the dormant period. This suggests early xylem formation before June, followed by a slight increase in Ca concentration towards September. The reduction in Ca concentration may indicate that Ca is allocated towards the continuous developing tissues in the reproductive dormant apple bud. This needs further investigation. While Ca concentration increased over the dormant period, K concentrations displayed a reverse trend during the same period for Cripps Pink. This suggests that a higher K concentration is required during the later stages of development, while later Ca concentration will be relatively low for this cultivar. In Braeburn, both Ca and K displayed a comparable trend in concentration over the same period. In Braeburn the Ca/K concentration followed a linear pattern, with the Ca concentration relatively higher than K. This corresponds with the xylem developmental stages, where the primary xylem continuously matures to secondary xylem from June to August. The nutrient patterns in Cripps Pink and Braeburn showed significant varietal differences, which may explain their deferring susceptibilities to Ca deficiency.

The spur, leaf and primary xylem tissues indicated variation in Ca concentration with no comparisons between cultivars, but the proportion of the Ca content was similar for all four cultivars between the different tissues. This suggests that the spur may have a Ca release mechanism that meets the demand for cell wall development during xylem development from June to September. This would suggest that Ca is released only on demand during the spur/leaf/primary xylem tissue developmental stages.