Understanding mechanisms and identifying markers for the onset of senescent sweetening of potato (*Solanum tuberosum*)

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DECLARATION

I certify that the work contained in this thesis, or any part of it, has not been accepted in substance for any previous degree awarded to me, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations, except where otherwise identified by references and that the contents are not the outcome of any form of research misconduct.

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“Não sou da altura que me vêem, mas sim da altura que meus olhos podem ver.” [1]

~Fernando Pessoa~

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[1] "I’ m not the height they see me, but the height my eyes can see."

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ABSTRACT

Storage potential of processing potatoes is often terminated prematurely by the unpredictable onset of senescent sweetening. The condition, characterised by a rise in reducing sugar content (fructose and glucose), results in darkening of chips and crisps increasing the risk of acrylamide formation through the Maillard reaction. Information is lacking on biochemical mechanisms that trigger senescent sweetening and better predictive methodologies for growers/processors are urgently needed. This study aims to understand mechanism(s) underpinning senescent sweetening and develop predictive tools. Commercial varieties Lady Rosetta ('susceptible' to sweetening) and VR 808 (able to maintain a 'stable' sugar profile), were planted from common seed tubers at 3 UK locations (Norfolk, Shropshire, Yorkshire) during the 1st year, Norfolk (Norwich) during the 2nd year and Yorkshire during the 3rd year of the study. During the first two years Pentland Dell and Russet Burbank, were included in the trial as additional material that provided contrasting profiles of sugar accumulation during storage. During the 3rd year P. Dell tubers chitted (250 days) and non-chitted were added to the trials to determine the influence in aged seed in Senescent Sweetening. Cured and CIPC treated tubers were stored at 10°C (Sutton Bridge Crop Storage Research) and sampled every 6-8 weeks for tuber quality (NRI) for physiological changes during long-term storage at 10°C. Effects of calcium (Ca) in storage potencial were studied in tubers from a fertilization trial, and its effects on dormancy break/sprout growth were tested as well. Duration of storage had the largest impact on sugar accumulation and respiration. Significant varietal differences effected senescent sweetening: respiration and sugar accumulation were higher in L. Rosetta and P. Dell than in VR 808 and R. Burbank. Comparison of fluctuations and the abundance of ROS activity captured by staining help to link possible mechanisms for a decrease in vitamin C during 10°C storage. Quantifying changes in amyloplast integrity, water dikinases activity and ROS activity could help with the development of diagnostic markers for predicting changes in tuber health and be used in the longer term for genetic marker development. It was concluded that Ca regulation had a significant impact in senescent sweetening.

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ABBREVIATIONS

ABA	abscisic acid
ADP	adenosine disphosphate
AMP	adenosine monophosphate
AMY	α-amylases
ANCOVA	analysis of covariance
ANOVA	analyses of variance
AOX	alternative oxidase
APX	ascorbat peroxidase
AsA	ascorbic acid
ATP	adenosine triphosphate
BAM	β-amylases
BE	starch branching enzymes
CAT	catalase
Ca^{2+}	calcium ion
CIPC	chloropropham
CK	cytokinins
CO_2	carbon dioxide
CPD	critical point drying

df	degrees of freedom
DHA	dehydroascorbic acid
dH_2O	deionized water
DM	dry matter
DPE	disproportionating enzymes
DTT	DL-dithiothreitol
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FW	fresh weight
GA	gibberellic acid
GBSS	granule-bound starch synthases
GWD	α-glucan, water dikinase
GPX	glutathione peroxidase
GSH	glutathione
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
HMDS	hexamethyldisilazane
H_2O	water
H_2O_2	hydrogen peroxide
HC	hydrogen cyanamide
$HO^{\bullet}$	hydroxyl radical

HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
ISA	debranching enzymes isomylase
$LaCl_3$	lanthanum (III) chloride heptahydrate
LDA	limit dextrinase
LSD	least significant difference
MDA	malondialdehyde
MDAR	monodehydroascorbate reductase
MH	maleic hydrazide
NO	nitric oxide
O_2	oxygen
O_2^-	superoxide
PAI	physiological age index
PCD	programmed cell death
PHS	α-glucan phosphorylases
P_i	inorganic phosphate
RH	relative humidity
RQ	respiratory quotient
RS	reducing sugars
SBD	starch-binding domain

SDH	succinate dehydrogenase
SEM	scanning electron microscopy
SOD	superoxide dismutase
SRA-buffer	sprout release buffer
SSs	soluble starch synthases
Susy	sucrose synthase
TBA	thiobarbituric acid
TCA	Trichloroacetic acid
UDP	sucrose synthase
UDPGlc	uridine-5-diphosphoglucose
1,4-DMN	1,4-dimethylnapth

Chapter 1. INTRODUCTION

1.1. Background

Potato tubers are the third most important world food crop for human consumption after rice and wheat (https://cipotato.org/crops/potato/, 23-01-2018), and the fifth most important crop produced in the world between 2000 and 2013, after sugar cane, maize, rice and wheat (FAO, 2015). Production is seasonal, with only one crop per season achievable in temperate regions (Jadhav *et al*., 1991a), and requiring a proportion of the crop is stored prior to use (Firman and Allen, 2007; Struik, 2007b). The storage potential of potatoes destined for processing is often terminated prematurely by the unpredictable onset of senescent sweetening which is characterised by a rise in fructose and glucose content in tubers that cannot be mitigated by increasing tuber respiration. For the crisping and chipping industry, accumulation of the reducing sugars glucose and fructose in potatoes must be avoided as it leads to a decline in processing quality (darkening in chips and crisps) and the risk of acrylamide formation as part of the Maillard reaction.

There are various factors that can affect senescence sweetening such as early planting, environmental stress, storage temperatures and cultivar (Knowles et al., 2009). Usually, cultivars with short dormancy tend to be more susceptible to senescent sweetening, although, there are some exceptions such as ‘Maris Piper’ and ‘Record’. When tissue senescence occurs, it is assumed that cellular breakdown is responsible for sweetening of tubers since membrane damage facilitates enzyme access to starch granules speeding up starch breakdown resulting in senescent sweetening (Colgan *et al.*, 2012). It is uncertain whether sweetening is related to genuine senescence, or through mobilisation of sugars due to sprouting cues, in situations where sprout suppressants have prevented visible external sprouting.

There is a lack of information relating to the underlying biochemical mechanism that leads to senescent sweetening and there is an urgent need to develop better predictive methodologies to aid growers and processors.

1.1. Aim and Objectives

The main aims of this research are to understand the mechanism(s) underpinning senescent sweetening and to develop predictive tools of senescent sweetening.

In order to achieve these research aims, several specific objectives have been identified:

1) Determine the effect of growing conditions (planting location, season, agronomic practices and seed physiological age) and tuber maturity at harvest on senescent sweetening.

2) Identify potential management methods that might reduce the impact of senescent sweetening during storage.

3) Determine the pattern of carbohydrate accumulation during storage in varieties with contrasting propensity to senescence sweetening.

4) Seek physiological/ biochemical markers that relate to this pattern of carbohydrate accumulation in storage in contrasting varieties: respiration, tissue integrity (cell wall/ amyloplast), starch phosphorylation by glucan, water dikinase activities (GWDs), levels and distribution of ROS will be investigated.

5) Understand the relationship between calcium status, antioxidant capacity and the degree of oxidative stress of tubers during storage in varieties with contrasting propensity to develop 'Senescent Sweetening'. Specifically, understand the relationship of calcium/calcium oxalate ratio in the context of ascorbate activity and its breakdown products (DHA and oxalate) and the relation between total calcium and bound calcium through the storage season in contrasting varieties. In addition, to study the calcium role on other physiological aspects of tuber health, such as the influence of calcium on signalling/regulation in dormancy break and sprout growth and whether its regulation in meristems can be utilised as a marker for dormancy break and onset of sweetening.

Chapter 2. LITERATURE REVIEW

2.1. Potato

Potato tubers (*Solanum tuberosum*) are a member of the *Solanaceae* family, which includes other food crops such as tomato (*S. lycopersicum*), aubergine (*S. melongena*), cucumber (*S. muricatum*) and chilli pepper (*Capsicum* spp.) (Harris, 1992).

It is now the fourth most important world food crop, surpassed only by wheat, rice, and maize (Messer, 2012), and in 1995 was the first vegetable grown in space (Wheeler, 2009).

Originating from South America (highland tropical areas), potato was introduced into Europe at the end of the 16th century and later distributed throughout the world, due to the colonial expansion of European countries, being a well-established important crop in a wide range of climates, altitudes and soils (Diop and Calverley, 1998).

Besides being a staple food, the potato is grown as a vegetable for table use and after World War II, 75 percent of the potato market was dominated by processed products, especially as frozen or snack foods (Messer, 2012), French fries, dried products and starch production (Kirkman, 2007).

Potato (Figure 2-1) is a dicotyledonous plant growing to a height of between 30 and 100 cm, producing compound leaves, made up of three or four pairs of leaflets, and flowers, grouped in inflorescences near the end of the stem which are gathered in clusters. Flowers can be white, yellow, purple, blue or striped. After pollination potato fruits develop as a green or purplish spherical berry, with a large number of small seeds (Kay and Gooding, 1987). Potato propagation is achieved using a whole or cut tuber (Struik, 2007b), relatively rarely from seed, except for breeding purposes (Cutter, 1992). Although potato is a perennial herb, in agriculture, it is used as an annual crop,

while a significant quantity of it is processed or consumed soon after harvest, most of the tubers are stored prior to use (Firman and Allen, 2007; Struik, 2007b).

The underground part of the potato plant is formed by the roots (numerous, fine, fibrous and adventitious) (Kay and Gooding, 1987) and stolons (underground stems), from where the tubers are formed (Cutter, 1992; Struik, 2007b).

Potato tuber life cycle has been divided into five stages, tuber induction, initiation, enlargement, dormancy and sprouting (Fernie and Willmitzer, 2001).

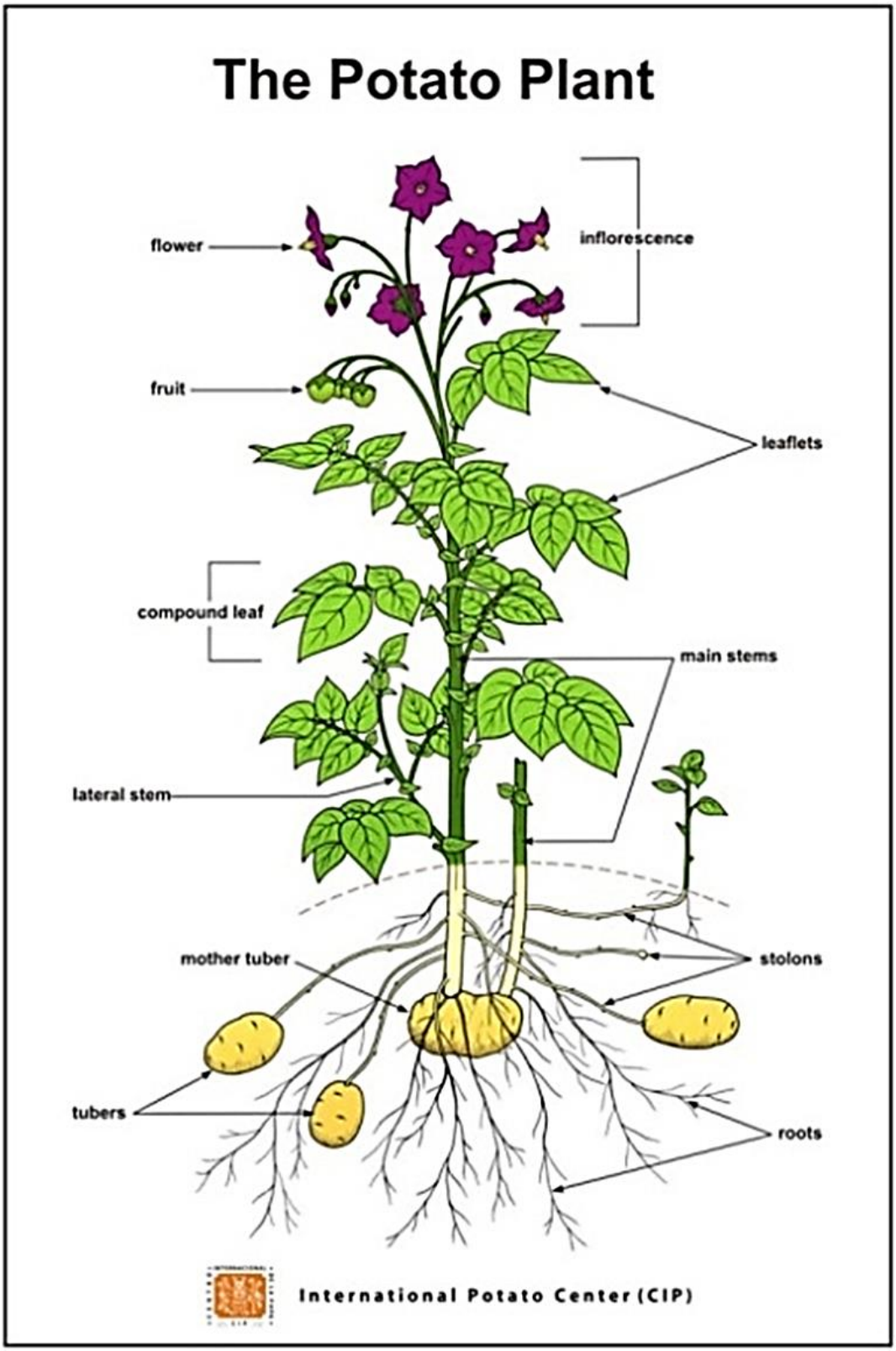

Figure 2-1 - The potato plant (Drawing: © CIP)

2.1.1. Potato composition

Potato tuber composition (Table 2-1) varies with variety, storage conditions, growing season, soil type, preharvest-nutrition and even with the method of analysis (Kadam *et al.*, 1991). Dry matter content is related with starch content and can vary from less than 20% to almost 50% (Bishop *et al.*, 2012).

Starch constitutes between 65% to 80% of the dry weight of the tuber (Diop and Calverley, 1998). Potatoes are also an important source of protein, iron, riboflavin and vitamins, and a major source of ascorbic acid (AsA) (Diop and Calverley, 1998; Bishop *et al.*, 2012). In addition, tubers contain a complex assortment of other low molecular weight compounds such as, polyphenols, flavonols, anthocyanins, phenolic acids, carotenoids, polyamines, glycoalkaloids, tocopherols, calystegines and sesquiterpenes (Navarre *et al.*, 2009).

Ascorbic acid has a major role in detoxifying reactive oxygen species (ROS) in plants (Navarre *et al.*, 2009). Freshly harvested potatoes contain ~30 mg 100 g^{-1} of ascorbic acid (AsA), however, this declines during cooking, processing, or storage (Kadam *et al.*, 1991). The largest decrease in ascorbic acid occurs during the first few weeks of storage, followed by a more gradual decline (Bishop *et al.*, 2012). Both temperature and length of storage period considerably influence the ascorbic acid amount (Kadam *et al.*, 1991).

Potato tubers are an important source of carotenoids such as violaxanthin, antheraxanthin, lutein, zeaxanthin neoxanthin, β-cryptoxanthin, and β,β-carotene (Breithaupt and Bamedi, 2002), these lipophilic compounds are synthesized in plastids from isoprenoids, with the principal function to control the photo and oxidative stress (Navarre *et al.*, 2009). A wide range of mineral elements are present in fruits and vegetables which are classified as major minerals (calcium, potassium, magnesium, sodium, phosphorus, cobalt, manganese, nitrogen and chlorine), and trace minerals (iron, copper, selenium, nickel, lead, sulfur, boron, iodine, silicon and bromine), and potatoes are an important source of some dietary minerals, such as potassium, iron, phosphorus, magnesium, calcium and zinc (Navarre *et al.*, 2009). However, while these minerals are important dietary components, there is evidence that certain

minerals are correlated with after-cooking darkening and acrylamide formation in potato (LeRiche *et al*., 2009; Whittaker *et al.*, 2010). According to Whittaker *et al.* (2010), reducing sugars levels were negatively correlated with potassium (K) and calcium (Ca), and positively correlated with Zinc (Zn) and copper (Cu).

Table 2-1- Average constituents of Potato tubers (source: Diop and Calverley, 1998)

Constituents	Percentage (by weight)
Moisture	50-81
Protein	1.0-2.4
Fat	1.8-6.4
Starch	8-29
Non-starch carbohydrates	0.5-7.5
Reducing sugar	0.5-2.5
Ash	0.9-1.4
Carotene (average)	4 mg 100 g^{-1}
Thiamine	0.10 mg 100 g^{-1}
Riboflavin	0.06 mg 100 g^{-1}
Ascorbic acid	12 mg 100 g^{-1}

2.1.2. Potato varieties

Solanum tuberosum is divided into two subspecies: *andigena* (a diploid adapted to short day conditions and mainly grown in the Andes), and *tuberosum*, a tetraploid, now cultivated around the world, adapted to longer day lengths (FAO, 2008). There are thousands of varieties with different characteristics of size, colour, texture and flavour. In the British potato variety handbook there are more than 200 varieties of potatoes grown as seed in Britain (British Potato Variety Database, 2010).

There are several factors that influence potato production, such as, planting date, soil type and pH, soil moisture and temperature, season, location, soil mineral nutrition, weed, pests and diseases control, time and harvesting method (Smith, 1987a; Sawant *et al.*, 1991).

Potato seasonal harvesting between May to October is dependent on variety and location. Usually potatoes are categorized according to their season, when planted in the winter and ready for harvesting in the spring or early summers are described as ‘earlies’ or ‘new potatoes’. They are potatoes with thinner skin (immature tubers) (Burton, 1989) and with less dry matter than late maturing varieties (Jansky, 2009).

Potatoes harvested in late summer or early autumn when their skins are firm and set are known as ‘maincrop’ (late maturing varieties). The majority of potatoes sold in the UK are maincrop potatoes (mature tubers); available from September to May (Potato Council, 2012) and have a greater capacity for longer storage and for higher-quality processed product (Sawant *et al.*, 1991) due the complete maturation.

Potatoes require an average growth temperature of 10 to 30°C, with optimal production between 18 and 20°C (Burton, 1989). In the UK, most main crop potatoes are planted in April/May.

Senescent sweetening onset differs between potato varieties (Table 2-2), limiting their storage duration. Varieties for crisping have a lower tolerance for reducing sugars (RS) accumulation than potatoes destined for chipping (Figure 2-2) (PotatoPro, 2008).

The varieties used within the senescent sweetening study are Lady Rosetta, Russet Burbank, VR 808 and Pentland Dell. Additional material used in an adjacent investigation on dormancy control and sprout vigour and the influence of calcium on tuber quality relied on varieties: Lady Balfour, Arsenal and Melody.

Product		Maximum %
French Fries		0.5%
Chips		0.2%
Dehydrated Products		0.3%

Figure 2-2- Maximum amount of RS for the main groups of potato products (PotatoPro, 2008)

Table 2-2 - Classification of potato processing varieties by onset of senescent sweetening (Colgan *et al.*, 2012)

Variety	Main market	Development of senescent sweetening	Length of dormancy
Lady Rosetta	Crisp	Early onset	2
Crisps4all	Crisp	Medium onset	3[1]
Hermes	Crisp	Medium onset	3
Pentland Dell	Chip	Medium onset	3
Cabaret	Chip	Late onset	5
Maris Piper	Chip	Late onset	2
Record	Crisp	Late onset	3
Saturna	Crisp	Late onset	4
Verdi	Crisp	Late onset	6
VR808	Crisp	Late onset	4[1]
Lady Claire	Crisp	Very late onset	6
Markies	Chip	Very late onset	5
Russet Burbank	Chip	Very late onset	8

Dormancy (1-9 scale, 9 = Long, NIAB Pocket Guide, 2008, NIAB , Cambridge.
[1]For newer varieties dormancy periods have been estimated.

2.1.2.1. Lady Rosetta

Lady Rosetta is an early maturing cultivar for crisping with high dry matter and low reducing sugar concentration making it ideal for crisp processing either immediately after harvest of after a short period of storage. It is a variety of moderate to high yields of uniform, red skin round tubers, with low out grades and good disease resistance. The tubers have shallow to medium depth of eyes and light yellow flesh with a medium smoothness of skin (British Potato Variety Database, 2010). It is also a variety with early onset to senescent sweetening (NIAB, 2008).

2.1.2.2. Russet Burbank

Russet Burbank is one of the older cultivars, generating a large number of tubers per plant (Thompson, 1987), is a maincrop maturing cultivar with moderate yields and have

very late onset of senescent sweetening (NIAB, 2008). When grown in a moisture stress situation tubers have the tendency to produce misshapen tubers. Tubers are elongated with a characteristic cream skin containing medium to high dry matter leading to good fry colour. Tubers have deep eyes with white flesh and medium smoothness of skin (British Potato Variety Database, 2010).

2.1.2.3. VR 808

VR 808 is a cross between varieties Lady Claire and Atlantic. Lady Claire is a specialist crisping variety with very low and stable reducing sugars, ideal for long-term storage for late crisp production. VR 808 is a maincrop maturing cultivar with round yellow tubers, shallow eyes and light yellow flesh (British Potato Variety Database, 2010) and late onset of senescent sweetening (NIAB, 2008).

2.1.2.4. Pentland Dell

Pentland Dell has a maincrop maturing cultivar with high yields, long dormancy, and moderately high dry matter with a tendency to disintegrate after boiling. Produce oval to long tubers with shallow eyes and cream flesh and smooth cream skin (British Potato Variety Database, 2010). And is a variety with medium onset of senescent sweetening (NIAB, 2008).

2.1.2.5. Lady Balfour

Lady Balfour is a recent variety bred with resistance to Potato Cyst Nematode and lends itself to organic production. It is a maincrop maturing cultivar with very high yielding and exceptional vigour under low fertility conditions. It has an extremely long dormancy (8 in a 1-9 scale) (Greenvale AP, 2012) being suitable for long-term storage without the use of sprout suppressants. Tubers are oval, with medium depth of eyes,

medium smooth partially red coloured skin and white flesh (British Potato Variety Database, 2010; Greenvale AP, 2012).

2.1.2.6. Arsenal

Arsenal is a crisping maincrop variety with medium size round oval tubers of yellow skin colour, light yellow flesh and moderate shallow eyes. It is a variety with good dormancy (6 in a 1-9 scale) and suitable for longer storage at approximately 8ºC. This variety is sensitive to chloropropham (CIPC) damage, and therefor alternative sprout suppressant must be used on this variety (AGRICO UK, 2013).

2.1.2.7. Melody

Melody is a maincrop pre-packing variety with oval, uniform tubers with shallow eyes, light yellow, bright smooth skins and flesh. Is a variety with excellent storage quality (British Potato Variety Database, 2010). This variety has a long to very long dormancy (Netherlands Potato Consultative Foundation, 2011).

2.1.3. The impact of growing location on tuber quality and storage behaviour

Potato yield and tuber composition of potato is not only dependent on the variety but on the soil, climate and agronomic practices during production (Storey and Davies, 1992).

Burton and Wilson (1978) reported the effect of latitude on cultivation and sugar content of the variety Record over three growing seasons; the total content of sugar and the reducing sugar/sucrose ratio was directly correlated with location; the more Northerly sites had higher reducing sugar/sucrose ratio.

Lombardo *et al.* (2013) found that location, especially weather and soil type, had a major influence in determining yield (tuber weight) and nutritional value (mineral composition, AsA and phenolic content) for nine potato cultivars. Payyavula *et al.* (2012) showed that the change in environmental variables, such as differences in day and night temperatures, light intensity, day length, cloud cover, humidity, soil type, time of planting and coastal versus inland locations, all together were sufficient to have a significant impact on the nutritional quality of potatoes. For example, carotenoid profiles were highly influenced by genotype and also location had a correlative link on overall carotenoid content.

In addition to location, seasonal effects on the amylose content of tubers were also observed (Jansky and Fajardo, 2014). Moreover, Simkova *et al.* (2013), found tubers planted in high-altitude sites produced tubers with higher amylose content than the same variteties planted at sea level. However, Fajardo *et al.* (2013) concluded that the effect of location on amylose content was small. Jansky and Fajardo (2014) concluded differences between studies should be expected and were in part, due to the extent of the environmental variation among sites within a study.

2.1.4. Tuber anatomy, induction, initiation and enlargement

Tubers are formed from stolons, and every axillary bud on a potato tuber stem has the potential to develop a new tuber (Ewing and Wareing, 1978; Brown, 2007).

Tuber formation is divided into several distinct processes including stolon formation followed by tuberization of the stolon tip (Kay and Gooding, 1987); where due to rapid cell division and enlargement lateral proliferation of storage tissue occurs (Kadam *et al.*, 1991). Tuber growth depends primarily on the expansion of the internodes present in the apical bud and its structure can be elliptical or spherical depending on the balance between extension and thickness (de Vries 1878 in Cutter, 1992; Fernie and Willmitzer, 2001). Ultimately tuber shape is a varietal characteristic (Cutter, 1992).

Photoperiod is the most important factor that influences tuberization; induction is favoured by long nights (Xu *et al.*, 1998; Fernie and Willmitzer, 2001; Brown, 2007; Dutt *et al.*, 2017). Other environmental and endogenous factors influence tuber induction, such as cool night-time temperatures, low rates of nitrogen fertilization, advanced physiological age of the seed tuber at the point of planting and involvement of plant hormones. Low concentrations of gibberellic acid (GA), high concentrations of cytokinin and jasmonic acid, and a rise in abscisic acid (ABA) concentration along with elevated concentrations of sucrose have been found to promote tuberisation (Xu *et al.*, 1998; Fernie and Willmitzer, 2001; Brown, 2007; Dutt *et al.*, 2017). It was observed that mRNA from GA 20-oxidase ($StGA_{20}ox1$-3) (key regularory enzyme in the GA-biosynthetic pathway) and GA 2-oxidase gene ($StGA_{2}ox1$) were important for the tuberization control through regulation of GA gene regulation in addition, many auxin-related genes (eg. genes involved in auxin transport (PIN gene family) and auxin response factors (ARF)) have different expression levels during tuberization (Dutt *et al.*, 2017).

All the changes that are brought about by the tuber-induction process are regulated at a molecular level, starting a chain of events that effects the morphology and physiology of the plant (Ewing and Struik, 1992; Brown, 2007; Dutt *et al.*, 2017). This will lead to tuber initiation and enlargement, caused by the initial increase in cell division followed by cell enlargement, protein synthesis and starch deposition (Plaisted, 1957; Ewing and Struik, 1992; Fernie and Willmitzer, 2001). Tuberization can be negatively regulated by CONSTANS protein (regulator of photoperiodic mediated plant developmental processes) and positively by the Flowering locus T (FT), an heterodimer of BEL1 (homeobox gene family) and KNOX (knotted-like homeobox) transcription factors (Dutt *et al.*, 2017). Also RNA molecules transported from the leaves (source) to the tuber (sink) had been suggested to be important in tuberization signaling, such as RNA levels of the sucrose transporter 4 (SUT4), mRNA of BEL5 transcription factor and micro-RNA miR172 (Dutt *et al.*, 2017).

Tubers function as a storage reserve for the plant, and are an important source of food starch. During enlargment tubers can store large amounts of starch and significant amounts of protein (see Potato composition section, Table 2-1) however, with time,

changes in tuber physiology and metabolism occurs, and as a typical storage sink, potato tubers decrease their general metabolic activity (Fernie and Willmitzer, 2001).

Tubers (Figure 2-3) have a globose to ellipsoid shape and on the surface have depressions or eyes which result in new shoots (Kirkman, 2007). The mature tuber divides into four principal areas (Figure 2-3) starting from the exterior, periderm, cortex, vascular cylinder perimedullary zone and central pith (Kadam *et al.*, 1991).

The periderm (skin) (Figure 2-3) formation starts soon after stolon tip sweling (Grommers and van der Krogt, 2009) initially forming a layer a few cells thick at the beginning of tuber formation, developing to six to ten suberized cell layers later on in tuber formation (Grommers and van der Krogt, 2009; Kadam *et al.*, 1991)

The major component of tuber tissue are parenchyma cells from the cortex and from the perimedullary zone, which contain starch granules as reserve material (Grommers and van der Krogt, 2009). The central region of the tuber contains the pith, sometimes referred to as the water core, which consists of large parenchyma cells containing less starch than the ones from vascular area and inner-most part of cortex (Kadam *et al.*, 1991; Talburt *et al.*, 1987). According to the work of Karlsson and Eliasson (2004) the dry matter (DM) content can vary between 9-20 % DM in the pith, 28-34 % DM in the cortex and 18-28% DM in the storage parenchyma.

Externally the potato tubers have leaf scars with axillary buds (eyes) (Talburt *et al.*, 1987; Burton, 1989; Kadam *et al.*, 1991) similar to those found on the stem, arranged spirally around the tuber (Talburt *et al.*, 1987; Cutter, 1992). Tuber eyes are composed of at least three buds together with protecting scales, that in normal circumstances, are endodormant (see Dormancy section) at time of harvest (Burton, 1989). The apical bud that develops first, and into the the apical sprout (Talburt *et al.*, 1987).

Initially, apical dominace of the bud end eye prevents sprout growth of other eyes under normal conditions (Talburt *et al.*, 1987). After time, sprouting of lateral buds occurs as apical dominance is lost. Under suitable growing conditions, sprouts develop into new potato plants (Burton, 1989).

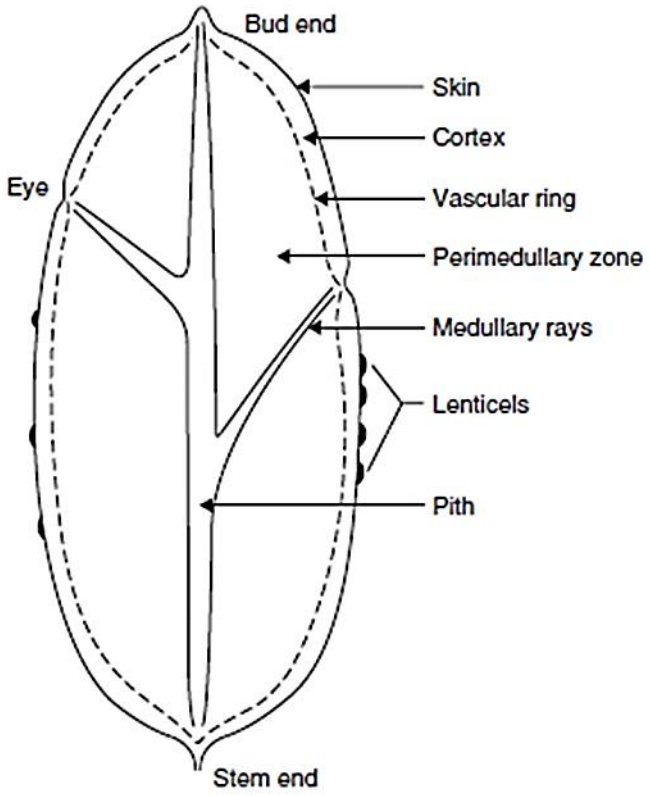

Figure 2-3 - Longitudinal section diagram of a potato tuber (source: Grommers and van der Krogt, 2009).

2.2. Dormancy

Lang *et al.* (1987) defined dormancy as “temporary suspension of visible growth of any plant structure containing a meristem”. In that work, dormancy was divided into three states, paradormancy (regulated by physiological factors outside the affected structure, e.g. apical dominance), endodormancy regulated by physiological factors inside the affected structure, e.g. chilling responses) and ecodormancy (regulated by environmental factors, e.g. water stress, storage temperature (Lang *et al.*, 1987).

Potato tubers are generally dormant at harvest and for some time after (endodormancy) due to endogenous signals (Suttle, 2004; Burton, 1989). Tuber dormancy can be divided into induction, maintenance, and termination (Suttle, 1998),

and is controled by phytohormones (Fernie and Willmitzer, 2001; Weiner *et al.*, 2010) and could be related to changes in carbohydrate metabolism (Aksenova *et al.*, 2013). Dormancy is initially induced at tuber initiation with the symplastic isolation of the apical bud (Viola *et al.*, 2007). Another inducing signal of tuber dormancy is the termination of their supply with metabolites, especially sucrose, from the mother plant. This coincides with vine kill and complete tuber separation from the mother plant after harvest (Aksenova *et al.*, 2013). When dormancy is broken with bromoethane (Alexopoulos *et al.*, 2009) or GA (gibberellic acid) (Alexopoulos *et al.*, 2008) there was an increase in starch breakdown and in the activity of starch metabolism enzymes before the visible sprouting. Viola *et al.* (2007) observed that the symplastic connection bettween the apical bud and the tuber was reestablished in growing buds. Akoumianakis *et al*. (2016) observed a reducing in the activity of glucose-6-phosphate dehydrogenase (G6PDH) and succinate dehydrogenase (SDH) with tuber maturity. G6PDH activity declines over time (von Schaewen *et al.*, 1995) and is essential for the pentose phosphate pathway and has influence in the availability of reduced NADPH (Singh *et al.*, 2012). SDH contributes to mitocondrial ROS production and regulates plant development (Jardim-Messeder *et al.*, 2015) and has been reported to regulate dormancy break in yam tubers (*Dioscorea esculenta* (Lour.) Buurk.). Akoumianakis *et al*. (2016) suggested that changes in SDH activity may influence potato tuber dormancy. In addition the author observed that a decrease in β-amylase activity was related to the onset of dormancy increasing again with sprouting initiation. Changes in carbohydrate metabolism during tuber development due to β-amylase, G6PDH and SDH may be responsible for the induction and duration of tuber dormancy (Akoumianakis *et al.*, 2016). The entry into dormancy is associated with an increase in absidic acid (ABA) and a decrease in GA (Fernie and Willmitzer, 2001; Weiner *et al.*, 2010; Aksenova *et al.*, 2013*)*. It has been established that ABA and ethylene are required for tuber dormancy initiation, however only ABA is needed for the maintenance of tuber dormancy (Suttle, 2004). As the effect of dormancy weakens the concentration of ABA declines and the tuber start to be gradually more sensitivity to exogenous cytokinins (CK) (Suttle, 2004; Nambara and Marion-Poll, 2005; Destefano-Beltran *et al.*, 2006). Leading to the reactivation of meristematic activity (Bromley *et al*., 2014), with an increase in endogenous CK- just before or coinciding with the dormancy termination and starting of sprouting (Suttle, 2004; Nambara and Marion-

Poll, 2005; Destefano-Beltran *et al.*, 2006). StCKP1 (Solanum tuberosum cytokinin riboside phosphorylase) negatively regulates CK in potato tubers by lowering CK activity and can prolongate endodormancy by a chill-reversible mechanism, negatively regulating dormancy (Bromley *et al*., 2014). It was observed that during dormancy there was a decrease in free indole-3-acetic acid (IAA) in buds, the concentration at which it triggers dormancy release was dependent on cultivar and storage temperature, leads to dormancy break (Sorce *et al.*, 2009). Overall, tuber dormancy and its release is regulated by a coupling between a complex of phytohormones interactions over time and changes in metabolism, mainly carbohydrates (Aksenova *et al.*, 2013). However, meristmatic activity is in the end in part controled by the symplastic gating between the apical bud and the tuber, that allows the supply of metabolites to the growing bud (Viola *et al.*, 2007). This dormant period can last from 18 to 33 weeks after harvest, depending on the variety, usually between 20 to 23 weeks (Cutter, 1992; Mani *et al.*, 2014).

Dormancy break is a complex process known to involve several physiological and biochemical changes (Liu *et al.*, 2017). Shoot elongation which is more commonly termed sprouting starts quickly after dormancy break (Suttle, 1996). Dormancy is considered to be a physiological adaptation of the tuber to prevent sprouting during intermittent periods of environmental limitations (Suttle, 2007). In most varieties, immediately after harvest, potato tubers cannot be induced to sprout even under optimum environmental conditions (Cutter, 1992). However, certain diploid Phureja species (*Solanum tuberosum var phureja*) dormancy break can occur in the field.

Tuber maturity at the point of harvest has an important bearing on dormancy, Krijthe (1962) studied the sprouting of seed potatoes and showed that immature tubers have a shorter dormant period and more rapid sprout growth than the mature tubers. Characterisation of tuber maturity is often clouded in confusion due to different types of maturity being referenced (chemical (sucrose content), chronological (days from planting) and physiological).

Others factors that affect the length of the dormant period are soil and weather conditions during growth, timing of foliage removal, if at all before harvesting, degree of tuber damage (bruising) and the storage regime (Burton *et al.*, 1992).

2.3. Storage

Storage in air can last up to 10 months under optimal storage conditions and with repeated application of sprout suppressants (Firman and Allen, 2007; Wustman and Struik, 2007b). The temperature at which tubers are stored is variety dependant, their susceptability to low temperature sweetening, the length of storage required, the options for use of sprout suppressants, incidence of disease, and the destination of the stored crop (ware, processing into chips, crisps, mashed or freeze dried).

Potato storage aims to maintain the quality of the harvested crop providing uniform high quality tuber availability throughout most of the year for table consumption and/or to the processing industry (Smith, 1987b; Jadhav *et al.*, 1991b; Wustman and Struik, 2007b).

Potatoes can be stored for seed or for ware purposes with subsequent utilisation, either for fresh consumption or processed products (Wustman and Struik, 2007b). Storage of seed potatoes centres around delivering tubers sprouted or chitted at planting time, whereas in ware potato storage sprout inhibition is essential to prevent losses (Wustman and Struik, 2007b). While this study concentrates on ware potato storage it is important to take into account the physiological age of seed potatoes at planting as this can subsequently impact on harvest maturity (Groves *et al.*, 2005). The physiological age of seed tubers is important because aging modifies the biochemical, physiological and agronomic traits of both plants and tubers resulting from them (Caldiz *et al.*, 1996). Which in turn influences storage quality and the propensity to undergo senescent sweetening in long-term storage (Groves *et al.*, 2005).

During storage, losses occur due either to physiological breakdown or development of bacterial or fungal disease. Moreover, during prolonged storage potato metabolic activity changes with length of storage and storage temperature, as a consequence respiration rates rise over time and increased water loss and a decline in fresh weight are observed. The extent to which these processes are realised is dependent on the physiological status of tubers entering store, storage temperature the uniformity of air

distribution within the store. More importantly, the amount of moisture loss from the stored crop is directly related to the rate of temperature pull down, the amount of refrigeration required to maintain storage temperature and the efficiency of the cooling system. Ventilation used to refrigerate the storage if not maintained at high relative humidity (RH) will lead to water loss from potato tubers (Smith, 1987b; Wustman and Struik, 2007a). Poorly insulated potato stores require more frequent periods of cooling which leads to more evaporation of moisture from tubers to the cooler evaporative cooling units within the refrigeration system (Wustman and Struik, 2007b).

Good store management should prevent the major types of storage losses, weight losses and losses in quality, due to respiration, sprouting, water evaporating, pests and diseases, changes in the chemical composition and physical properties of the tuber and extreme temperature damage (Smith, 1987b; Jadhav *et al.*, 1991b; Burton *et al.*, 1992; Wustman and Struik, 2007b). These losses are most influenced by storage temperature (Krijthe, 1962), however, while it is possible to provide optimal storage conditions, Burton *et al.* (1992) suggested that "the storage potential of potatoes is already determined before the beginning of storage, by such factors as cultivar; growing techniques; type of soil; weather conditions during growth; diseases before harvesting; maturity of potatoes at the time of harvesting; tuber damage during lifting, transport and filling of the store."

According to Wustman and Struik (2007) it is possible to distinguish seven phases in potato storage:

1. Field drying;
2. Store loading of healthy tubers;
3. Drying at and during store loading;
4. Wound healing after store loading for about 10 days at 15°C and high RH;
5. Cooling to the desired temperature. For the different end uses the optimal temperature ranges are:

    - Seed potatoes: 3−4°C
    - Ware potatoes:

i. Table consumption 4−5°C

ii. French fry production 6−8°C

iii. Crisps production 7−9°C

iv. Flakes, granulates 7−10°C

6. Maintaining the temperature at the desired level;
7. Warming before unloading.

Potatoes can be stored in bulk or in boxes, and during storage fans are used to provide controlled ventilation thoughtout the crop (positive ventilation), and for cooling, as well as to prevent condensation when the air is vented through apertures and re-circulated. Sometimes both refrigeration and heating may be required during storage, depending on the climate (Firman and Allen, 2007).

Ideally, to limit differences in initial storage conditions, stores are loaded within 7-10 days. During the first hours of storage, ventilation assumes an important role in removing the surface moisture from the tubers (Firman and Allen, 2007).

2.4. Sprouting and sprouting control

Sprout inhibition is essential to prevent losses during storage (Wustman and Struik, 2007), not only because the sprout accelerates the rate of moisture loss but because sprouting tubers are not accepted commercially (Schouten, 1988). Sprout inhibition can be induced by physical or chemical damage of the developing buds (Kleinkopf *et al.*, 2003), or in some cases through chemical treatments that affect the mechanism of dormancy break.

The only physical intervention to significantly limit sprouting once dormancy has broken is to keep potatoes below approximately 2^{o}C, which can induce low-temperature sweetening in many varieties, reducing tuber quality, especially if they are destined for processing (Sonnewald, 2001).

The most widely adopted approach is to use chemical sprout suppressants such as CIPC or ethylene (Firman and Allen, 2007). Low temperatures tend to lead to accumulation of reducing sugars (cold-induced sweetening). The rate of sprout growth changes over time, initially slow immediately after the end of dormancy, and as the tubers age sprout rate growth increases to a maximum, usually with just one sprout dominant (apical dominance), inhibiting the sprouting of other buds (Krijthe, 1962). As apical dominance diminishes lateral buds emerge, and when apical dominance is overcome, individual sprouts display multiple branching, with the formation of small micro-tubers (little tuber syndrome) in the final stage (Krijthe, 1962; Daniels-Lake and Prange, 2007; Suttle, 2007).

In ware potatoes, delaying sprouting during storage is favorable, in contrast to seed potatoes where more sprouting sites on the tubers is desired to produce stronger next-generation plants (Sonnewald, 2001). However, management of sprouting is complicated; the onset and vigour of sprouting is affected by cultivar, agronomic influences and storage conditions (Daniels-Lake and Prange, 2007). Several chemical alternatives for sprout inhibition are available on the market. Chloropropham (CIPC), used for more than 40 years (Kleinkopf *et al.*, 2003) and maleic hydrazide (MH), are widely used within the industry and proven to be efficient. However, due to environmental concerns their application is problematic (Sonnewald, 2001). CIPC has been limited and for 2017 total dose rates for crops will be 24 g tonne^{-1} for the fresh market and 36 g tonne^{-1} for processing tubers (http://www.cipccompliant.co.uk/2017/, 26-05-2017). CIPC inhibits sprout development by interfering with cell division during mitosis (Kleinkopf *et al.*, 2003), and in the UK is applied as a hot-fog (http://www.cipccompliant.co.uk/alternative_sprout_suppressants/, 26-05-2017) to bulk potatoes. The process starts after the wound-healing period, since wound-healing requires the production of new cell layers resulting from cell devision, and before dormancy break or sprout growth initiation (Kleinkopf *et al.*, 2003). However, at room temperature CIPC is a solid, and poor distribution of the fog during application can lead to high residues in parts of the crop and stores (http://www.cipccompliant.co.uk/alternative_sprout_suppressants/, 26-05-2017). When potatoes are to be stored for more than 8 months two CIPC applications can be made (Kleinkopf *et al.*, 2003).

MH blocks cell division whereas cell elongation is not affected. This chemical is applied in the field, as a single application during the growing season when at least 80% of the tubers have reached 25 mm diameter and at least 3 weeks before haulm killing (De Blauwer *et al.*, 2011). Whereas MH can replace or reduce CIPC requirement in storage, it is not acceptable in all markets and it has a Maximum Residue Level – MRL of 50 ppm (µg g^{-1}) (http://www.cipccompliant.co.uk/alternative_sprout_suppressants/, 26-05-2017).

Due to the residues on the tuber and persistance in the environment of CIPC and MH, there is a need for alternative strategies for sprout inhibition, Sonnewald (2001) suggests, as an alternative, the possibility of using genetic engineering to produce improved tubers with better post-harvest characteristics and the use of biotechnology to modulate content or responsiveness of phytohormones and alteration of metabolic pathways.

Alternative chemicals have been evaluated as sprout inhibitors, as described in the review on sprout inhibition in storage from Kleinkopf *et al.* (2003). Ethylene gas provides reversible sprout control, and spearmint oil, that can burn back existing sprouts are now availble as commercial alternatives to CIPC.

Other products in the pipeline in the UK market are 1,4-dimethylnapthalene: 1,4-DMN (1,4-SIGHT™), 3-decen-2-one (SmartBlock™), caraway oil: S-carvone, with commercial name Talent™ and clove oil: eugenol (http://www.cipccompliant.co.uk/alternative_sprout_suppressants/, 26-05-2017).

Ethylene is an effective potato tuber sprout inhibitor, but it often darkens fry colour (Daniels-Lake *et al.*, 2005a; Daniels-Lake *et al.*, 2005b). After an initial peak in tuber respiration in response to ethylene, respiration rate declines over the following few days remaining insensitive to additional ethylene exposure (Daniels-Lake *et al.*, 2006 and references therein).

The use of irradiation can be an effective sprout inhibitor; however the consumer perception of this method is currently negative, limiting its usage (Kleinkopf *et al.*, 2003;

Daniels-Lake and Prange, 2007). However, with the threat of food shortages in the future all methods of control that reduce post-harvest losses must be considered.

2.5. Senescent sweetening

Aging and senescence even though distinctly different they overlap in developmental processes. Aging covers the lifetime of the organism, while senescence is the final developmental phase that culminates in the death of the organism (Kumar and Knowles, 1993b). Senescence is a well-defined genetically programmed phase of development while aging is thought to be a more randon process in nature (Nooden, 1988). A recent review written by Penfold and Buchanan-Wollaston (2014) refers to studies in senescing leaves with mutants in genes encoding transcription factors (TFs) such as, plant-specific NAC (for Non Apical Meristem (NAM), ATAF, and CUC) TFs, WRKY TFs, AP2 factors RAV1 and RAP2.4f, MYB, NFY and bZIP that exhibit an altered senescence phenotype, showing that senescence is regulated at least in part at the gene-expression level. However, the signal that initiates senescence is still unknown, and since senescence is induced by diverse developmental and environmental conditions, it is improbable that just one factor is required for senescence initiation (Penfold and Buchanan-Wollaston, 2014).

Even thougth ageing and senescence are distinguishable they share many biochemical pathways (Kumar and Knowles, 1993b).

As explained by Coleman (2000) in his review on physiological aging of potato tubers, there have been many efforts to define the aging process, however, the exact definition is still unclear as many of the molecular mechanisms involved are not fully elucidated.

The rate of physiological aging in potato tubers appears to be cultivar specific, but also individual tuber specific and temperature dependent (Coleman, 2000). In seed tubers, physiological aging is most affected by the cumulative temperature experienced after the end of dormancy during storage, although its effect is moderated by light (if put in

difuse light conditions to chitting) conditions and by genotypic characteristics (Struik, 2007a).

Kumar *et al.* (1999) identified protein modifications related to tuber aging as an increase in protein glycation, oxidation and deamidation/isomerisation/racemisation and suggested that aging was accompanied by increased an respiration rate, oxidative stress, lipid peroxidation and decreased protein content in the tubers. Potato tuber starch is highly phosphorylated (Smith *et al.*, 2005), and even though starch phosphorylation is important for starch metabolism in plants, its mechanisms and implication on starch degradation remained unknown for a long time (Bansal and Das, 2013). Glucan, water dikinase (GWD) is a key enzyme of starch metabolism (Mahlow *et al.*, 2014) because it can catalyze starch phosphorylation in both leaves and different plant storage organs (Bansal and Das, 2013). Glucan, water dikinase (GWD1) phosphorylates glucose residues at the C-6 position and phosphoglucan, water dikinase (GWD3) phosphorylates the glucose residues at the C-3 position in amylopectin chains (Ritte *et al.*, 2006). These enzymes facilitate the breakdown of amylases on the starch granule (Orzechowski *et al.*, 2013) by weakening the amyloplast surface polymers organisation (Smith, 2012). In both leaves and tubers of potato, GWD is enclosed inside the starch granules, as well as associated with the granule surface and solublised within the plastid stroma (Bansal and Das, 2013).

Membrane changes and loss of homeostatic control are viewed as generalised responses to aging. For that reason researchers often support the idea that electrolytic leakage rate may be an effective indicator of physiological aging in potato tubers. Nevertheless, the efficacy of this technique remains uncertain, since leakage responses can be small, and dependent on storage temperature and not consistent among samples (Coleman, 2000 and references therein).

Respiration rate can be used as an indicator of the metabolic activity, and older tubers exhibit an increase in respiratory rates, as well as reduced sprouting vigour, with progressive loss of apical dominance and an increased capacity to produce ATP, when compared with younger tubers (Coleman, 2000 and references therein). Alternative oxidase (AOX) is a non-energy conserving terminal oxidase from the electron transport chain in mitochondria (Vanlerberghe, 2013), in plants its expression is upregulated

when in the presense of stress such aging (May *et al.*, 2015; Rogov and Zvyagilskaya, 2015). When present in plants, AOX activity is associated with a reduced carbon use efficiency, as it is responsible for reducing the respiratory yield of ATP (Vanlerberghe, 2013).

Calcium increases membrane integrity and for that reason is often regarded as an anti-senescence factor (Kumar and Knowles, 1993a). Calcium increases the cell wall rigidity and had an important role in controlling membrane structure and function (Hepler, 2005). Calcium was reported to slow leaf abscission and tissue senescence in bean and maize plants (Poovaiah and Leopold, 1973a; Poovaiah and Leopold, 1973b). In wheat seedlings treated with Ca^{2+} antioxidant enzyme activity was induced leading to a decrease in ROS and lipid peroxidation on the long-term (Agarwal *et al.*, 2005).

However for Dyson and Digby (1975) the effect of calcium on sprout growth in potatoes was clear and that physiological age was a function of calcium metabolism, where a progressive decline in calcium mobility occurs with aging. Sprouting is related to the calcium concentration in the tuber, when more calcium is present sprout elongation is greater. However, in order for the sprout to continue to grow it requires to supply additional calcium (Dekock *et al.*, 1975; Dyson and Digby, 1975; Pang *et al.*, 2007). In grape buds, Pang *et al*. (2007) found that inhibition of dormancy break usingethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or lanthanum (III) chloride heptahydrate ($LaCl_3$) could be reversed by exogenous application of calcium.

Apical dominance in some plants has been closely associated with endogenous plant growth regulators, such as indole-3-acetic acid (IAA), cytokinins (CK) and absisic acid (ABA) (Coleman, 2000 and references therein). However, the role of hormonal regulation in the tuber apical dominance and its subsequent role in programmed cell death remains unclear (Eshel and Teper-Bamnolker, 2012). After harvest, synthesis of endogenous hormones in tubers continue to perform their roles, participating in or even causing physiological events in the tuber during storage. Ceasing their functions only with the death of the tuber, that they may be as well the precursors, since they may affect the rate of aging (Isenberg and Ludford, 1988; Coleman, 2000).

Studies show that ethylene and ABA are associated with the onset and maintenance of tuber dormancy, and that genes associated with the anabolic and catabolic metabolism of ABA are correlated with dormancy in potato meristems and tubers. GAs are involved in sprout growth after dormancy cessation, biologically active CKs increase over time in dormant potato tissues, suggesting a role for this class of hormones in loss of dormancy, and there is evidence that IAA is involved in the control of potato tuber dormancy (Teper-Bamnolker *et al.*, 2012 and references therein; Muthoni *et al.*, 2014).

Teper-Bamnolker *et al.* (2012) proposed as one of the mechanisms of regulating apical dominance is programmed cell death (PCD) in the tuber apical bud meristem, in their research it was suggested that PCD was related with the weakening of the tuber apical dominance.

Studies demonstrated that ethylene plays an important role in PCD during the hypersensitive response following pathogen attack and in maize endosperm PCD (Young *et al.*, 1997; Gunawardena *et al.*, 2001), as well as an important role in tuber dormancy regulation. Endogenous ethylene production increases as tubers initiate sprouting or tubers are exposed to certain dormancy terminating agents that stimulate ethylene production (Suttle, 2009). Short-term exposure (less than 3 days) to exogenous ethylene accelerates tuber dormancy break and tuber sprouting, while long-term or continuous exposure to similar concentrations of ethylene inhibits sprout growth and can result in a significant extension of tuber dormancy. It can result in an undesirable accumulation of reducing sugars (Muthoni *et al.*, 2014) (< 0.15 %FW for crisp and < 0.25 %FW chips production) (OECD, 2015).

Spychalla and Desborough (1990) in their work on oxygen free radicals in plants refer to the work carried out on in *Escherichia coli* where increasing respiratory rates can lead to the increase of O_2^- production. ROS are essential to life and for that reason it is important maintaining the right concentration range, too low or to high ROS levels impair plant growth and development (Mittler, 2017). Increase in stress tolerance is linked with young tissues (Berkowitz *et al.*, 2016). When seeds of *Pisum sativum* L. and *Cicer arietinum* L. were artificially aged there was an accumulation of ROS that led to a loss of viability and vigour in seeds/seedlings (Rughani *et al.*, 2015). According

to the oxidative damage theory of aging, the oxidative and free radical stresses, such as O_2^- production are cumulative over time (Coleman, 2000; Rughani *et al.*, 2015). These products can be neutralized in a decreasing manner during aging by intracellular compartimentalisation, protective enzymes, such as SOD and CAT, and naturally occurring antioxidants (α-tocopherols, AsA). According to this, aging is consider to be mainly caused by exposure to ROS (Coleman, 2000). With aging a process called "autophagy" initiates PCD. Together with senescence control, ROS helps to orchestrate the terminal stages of plant life, and is involved in degradation and recycling of macromolecules, organelles and cytoplasm during PCD. ROS components, such H_2O_2 and O_2^- are key coordinators of senescence and PCD (Nath and Lu, 2015).

Aging tubers start losing their capacity to synthesize protein (Kumar and Knowles, 1993a) and moreover, non enzymatic protein modifications, such as oxidation, glycation and deamination of existing proteins, contributes to increasing the susceptibility of enzymatic inactivation and loss of structural protein integrity (Kumar *et al.*, 1999).

In his review on physiological aging in potatoes, Coleman (2000) refers to the work on specific enzymes involved with physiological aging of potato tubers, a decrease in sprouting capacity was correlated with an increase in peroxidase activity. Van Es and Hartmans (1984) examined starch and sugar content of sprouts as tubers aged and found that carbohydrate translocation was not the limiting factor in sprout growth. Moreover, Mikitzel and Knowles (1989) suggested that efficiency of carbohydrate utilisation was affected by tuber aging and not carbohydrate translocation (Coleman, 2000). Alterations in the concentration of soluble sugars have been shown to affect developmental phase changes in plants, from embryogenesis to senescence (Gibson, 2005).

It was established in studies published in 1938, 1963, 1965 and 1975 that potato tubers sweeten after prolonged storage at higher temperatures (as reviewed by Burton and Wilson, 1978). It is known that in a relatively stress free storage environment, where a tuber begins to approach or surpass 1.0 mg g^{-1} FW of sucrose, this is an indication that tubers are close to the onset of senescence and should be processed quickly

(Sowokinos, 2007a). The process of sugar accumulation due to aging is known as senescent sweetening of tubers, which is an irreversible conversion of starch to sugars (Duplessis *et al.*, 1996). The rate of senescent sweetening onset varies with cultivar (Table 2-2); the higher the storage temperature, the sooner senescent sweetening commences and the faster it develops (Burton and Wilson, 1978; Hertog *et al.*, 1997).

One theory suggests that senescence sweetening is likely to occur due to the progressive degeneration of the amyloplast membranes (Sowokinos *et al.*, 1987), in a range of varieties generally after 5-6 months of storage at 10ºC (Burton, 1989). Loss of amyloplast membrane integrity due to age has been suggested caused by a gradual peroxidation of amyloplast membrane lipids and leads to senescent sweetening in potato tubers (Kumar and Knowles, 1993b).

Understanding the physiological age of the potato tuber is not only useful for the processing industry but also for the seed potato industry, where physiological aged tubers tends to produce weaker plants than those derived from younger seed stock (Colgan *et al.*, 2012).

Physiological age of a tuber is affected by its chronological age and environmental conditions since tuber formation is initiated from the mother plant (van Ittersum, 1992). However, depending on different environmental and management conditions during growth (Van der Zaag and Van Loon, 1987) and storage conditions (Hartmans and Van Loon, 1987), tubers with the same chronological age could have different physiological ages.

Besides the efforts to find the molecular mechanisms involved in the physiological aging of tubers, there have been many attempts to develop an indicator of the physiological age that could quantify and explain cultivar differences in the rate of aging, such as the interaction between cultivar and storage conditions, and the effect of seed age on crop growth and yield (Caldiz *et al.*, 2001). However, until the work of Caldiz *et al.* (2001) there was a lack of any quantative and comparable indicators of the physiological age.

Physiological age index (PAI) includes components of both chronological and physiological age, is simple to measure, non-invasive and requires little capital investement, and can be very useful to quantify physiological age of different cultivars exposed to different environments (Caldiz *et al.*, 2001). This index ranges from 0 (very young seed tubers) to 1 (old seed tubers) and is calculated as PAI = T_1/T_2, where T_1 is the days from T_0 (haulm killing date) to possible planting date, and T_2 is the days from T_0 to the end of the incubation period (time elapsed from sprouting until new tuber formation on the sprouts) (Caldiz *et al.*, 2001).

However, further work on its development is required to allow growers to make decisions on seed purchases or management practices (Caldiz *et al.*, 2001). In later work (Delaplace *et al.*, 2008) the same disadvantage for PAI was reported as the additional time required to measure PAI values. However, the authors propose that PAI could be used as a valid reference frame for biochemical studies of potato tuber aging. These authors sugested that PAI could be used as a parameter to characterise age progression in storage, because PAI showed limited variation and can be measured since the time of haulm killing.

2.6. Senescent sweetening vs low temperature sweetening

Tubers stored at low temperatures tend to accumulate reducing sugars (cold-induced sweetening), reducing tuber quality, especially if destined for the processing industry (Sonnewald, 2001). Reducing sugars accumulate in tuber tissue when tubers are stored below 7ºC, making tubers unacceptable for processing. Typically, tubers are stored between 8-9ºC for the frozen-processing industry, balancing the desirable effects of low-temperature, minimizing pathogen development and prolong dormancy, against maintaing desirable carbohydrate concentration for processing (Driskill *et al.*, 2007).

However, quantitative trait locus (QTL) analyses revealed that factores controlling cold-induced sweetening were located on all potato chromosomes (Menéndez *et al.*, 2002), reducing sugar accumulation in cold-stored tubers is mostly determined by the

vacuolar acid invertase activity (Matsuura-Endo *et al.*, 2004). Vacuolar acid invertase hydrolyzes sucrose into glucose and fructose (Ou *et al.*, 2013). Bhaskar *et al.* (2010) observed a clear correlation between the vacuolar acid invertase *VInv* gene expression and the accumulation of reducing sugars in cold-stored tubers, with the suppression of reducing sugar accumulation after a near 100% silencing of *VInv* gene. Liu *et al.* (2011) showed that the vacuolar invertase gene *StvacINV1* (β-fructofuranosidase) was a key invertase gene in regulating cold-induced sweetening in potato tubers. The lower expression of *StvacINV1* was observed in cold-induced sweetening resistent tubers and a higher expression in sensitive tubers (Liu *et al.*, 2011).

Sucrose content is inversely correlated with acid invertase activity, and when tubers stored at low temperatures undergo a several-fold increase in acid invertase activity resulting in the accumulation of high concentration of reducing sugars (reviewed by Sowokinos, 2001).

Cold-induced sweetening is also regulated by the action of multiple amylases, the amylase gene *StBAM1* may regulate cold-induced sweetening by hydrolysing soluble starch. *StBAM9* gene encodes enzymes capable of releasing soluble glucan by directly attacking starch granules and *StAmy23* gene by degradating cytosolic phytoglycogen. Moreover, *StBAM9* can interacts with other proteins to form protein complexs in order to facilitate starch breakdown (Hou *et al.*, 2017).

In the review by Smith *et al.* (2005) it is suggested that starch degradation both during sprouting and cold-induced sweetening occur within the plastids. However, although there are some studies that reported the disappearance of membranes during the cold-induce sweetening, later reports argue against the loss of amyloplast integrity during cold-induced sweetening, as this process is reversible, prior to tuber senescence (Smith *et al.*, 2005). In low temperature sweetening sugar accumulation during storage decreases with time, and it is suggested to be due to sugar metabolism (Pressey and Shaw, 1966; Dixon and ap Rees, 1980; Colgan *et al.*, 2012), so if the tubers are reconditioned for a sufficient period of time at higher temperatures prior to processing the reducing sugars pool can be reduced, although, the efficacy of reconditioning depends on cultivar and the duration of storage and age of tubers (Knowles *et al.*, 2009).

2.7. Starch

Potato dry matter content is related to starch content, since most of the tuber dry matter consists of starch (Grommers and van der Krogt, 2009). This is important for the processing industry and also for fresh market potatoes, due to its influence on potato texture (Jansky, 2009). However, other structural carbohydrates in the cell wall, and proteins and lipids in the cells all contribute to the overall DM content of tubers (Table 2-10).

Starch distribution in the tuber is uneven, and is correlated with dry matter distribution, with the highest amount in the storage parenchyma, and the lowest in the pith, and longitudinally, increasing from apical end to the stolon end (Storey, 2007).

Starch content is important to the processing industry because its physical, chemical and histological characteristics and concentration are closely related to quality parameters of processed potato products, and because it can influence the operational conditions in the process; low starch and high sugar concentration are associated with poor texture after cooking (Torres and Parreno, 2009).

Dry matter content is mainly determined genetically, but climate, soil and the addition of fertilizer can affect both the growth and dry matter content and distribution within the tuber (Grommers and van der Krogt, 2009).

Starch is the reserve material (Grommers and van der Krogt, 2009) and major component of the potato tuber (Talburt *et al.*, 1987; Kadam *et al.*, 1991; Bertoft and Blennow, 2009) amounting to approximately 65-80% of its dry weight (Talburt *et al.*, 1987).

Starch granules start to be formed when the stolon tip begins to swell during the early stages of tuberization, and its content increases during tuber growth due to the enlargement in size and the increase in cell number of starch granules (Grommers and van der Krogt, 2009).

Starch granules are water-insoluble (Kruger, 1990), with granule sizes ranging from 1 to 110 µm in diameter (Singh *et al.*, 2009) (Plate 2-1). Granules contain two polymers, amylopectin and amylose (Tester *et al.*, 2004; Singh *et al.*, 2009; Meekins *et al.*, 2016), that are deposited as semi-crystalline granules inside the plastids (amyloplast) (Edner *et al.*, 2007). Amylose is a minor, smaller and mostly linear helical polymer made of α-D-glucose units, bonded to each other through (1→4)-α glycosidic bonds while amylopectin has a much larger molecular weight (Bertoft and Blennow, 2009) and is highly branched. Amylopectin units are linked in a linear way with (1→4)-α glycosidic bonds with (1→6)-α branching points bonds occurring every 20-25 glucose units, resulting in a soluble molecule that can be quickly degraded as it has many end points onto which enzymes can attach. In contrast, amylose contains very few (1→6)-α bonds, or even none at all with around 9-20 branching points (3-11 chains per molecule with approximately 200-700 glucose residues). This causes amylose to be hydrolysed more slowly. Amylopectin is 4-5 times the mass of amylose and with relatively short unit chains, around 18 to 25 units long on average, however it can be extended to 19-31 units if high-amylose starches are included (Tester *et al.*, 2004; Bertoft and Blennow, 2009; Pérez *et al.*, 2009; Streb and Zeeman, 2012; Meekins *et al.*, 2016). Usually, amylopectin contributes ≥70% of the dry weight of starch, and is responsible for its semi-crystalline nature (Edner *et al.*, 2007; Bertoft and Blennow, 2009; Streb and Zeeman, 2012; Meekins *et al.*, 2016).

Amylopectin chains can be classified in relation of their lenghts and consequently position within starch granules. Generally, the unit chains of starch are divided into long (30-45 degree of polymerization (DP), very long (>60 DP) and short chains (12-20 DP). Being further divided into A-, B- and C-chains. Where A-chains do not carry any other chains (cannot be substituted by other chains, external chains), B-chains carry one or more chains (substitute with other chains is the grouping of all other chains) and can extend over several clusters of densely packed double helices, and the C-chain just carrying the sole reducing end residue (considered as the ‘root’) in the macromolecules and is considered a special form of B-chain (Bertoft and Blennow, 2009; Pérez et al., 2009; Raguin and Ebenhöh, 2017) (Figure 2-4). Giving rise to the current and widely acepted cluster model. The B-chains can be subdivided according to the number of side chain clusters in which they participate. B1-chains are short B-

chains that with the A-chains form the major group of short chains and participate in one cluster. The long chains are subdivided into B2- and B3-chains extend into two and three clusters respectively, and B4-chains link four or more clusters (Bertoft and Blennow, 2009; Pérez *et al.*, 2009). In native granules, the A and B1 chains are located externaly, forming double helices and crystallites, with a chain length of 12-14 depending on genetic origin. Starchs with “A-type” crystallinity (most cereals) have shorter chain lenghs than “B-type” starches like potato (Tester *et al.*, 2004).

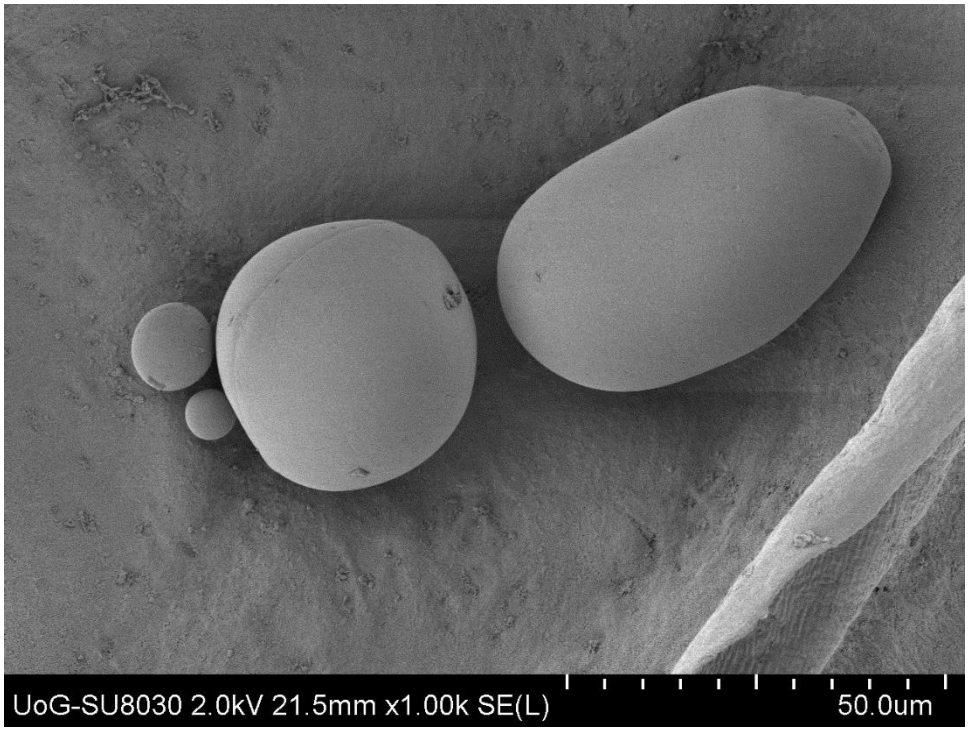

Plate 2-1 - Scanning electron micrograph (SEM) of potato starch

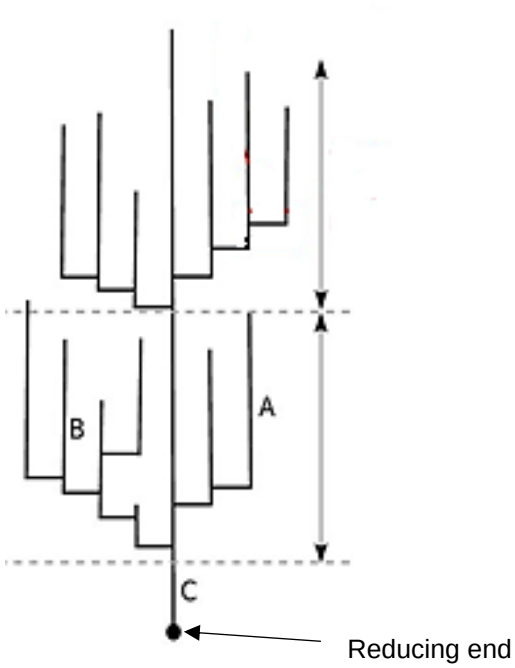

Figure 2-4 – Amylopectin chains A, B and C. A chains are the external chains that do not carry other chains, B chains are a grouping of other chains and C chain is the only chain with a reducing end (Raguin and Ebenhöh, 2017).

Starch granules also contain non-carbohydrate material such as proteins (<0.5% w/w), in the form of enzymes responsible for starch synthesis, lipids (virtually absent in potato), phosphorus in the form of phosphate covalently linked to the amylopectin component, trace amounts of different cations, mostly potassium, conjugated to the phosphate groups (Tester *et al.*, 2004; Bertoft and Blennow, 2009).

Sucrose, is a major direct product of photosynthesis and the main photoassimilate transported from the leaves to the tuber (Sowokinos, 2007b), although in general, there is an considerable delay from the onset of photosynthesis to starch accumulation, since the starch is mobilized and converted into sucrose for export at night (Kruger, 1990; Sowokinos, 2007b). The sucrose conversion to starch through hexose phosphates is the principal flux in potato tuber carbon metabolism (Burton, 1989; Hofius and Börnke, 2007). For long-distance transport of sucrose, from source to sink tissues, apoplastic loading into phloem is the more likely form of shoot to tuber

translocation (Riesmeier *et al.*, 1993; Braun *et al.*, 2014). The sucrose transporter *StSUT1* (Solanum tuberosum sucrose transporter 1) is the main phloem loading transporter in mature potato leaves (Braun *et al.*, 2014; Eggert *et al.*, 2016). Sucrose is then translocated through the vascular system, stem and stolon, being unloaded in the tuber (Sowokinos, 2007b). On reaching potato tubers, starch is synthetized during tuber development and maturation in the amyloplasts (Preiss, 2009) and its deposition is dependent on sucrose transport to the tuber. Therefore, sucrose concentration has a direct linear relationship with the rate of starch synthesis (Avigad, 1982). Starch breakdown products, glucose and fructose are utilised in respiration and sprouting (Kumar *et al.*, 2004).

In potato tubers, sucrose is cleaved by sucrose synthase (UDP or Susy) into UDP-glucose and fructose. UDP-glucose is then converted to glucose-1-phosphate (G1P) by UDP-glucose pyrophosphorylase (UGPase). G1P subsequently is transferred to glucose 6-phosphate (G6P) by cytosolic phosphoglucomutase (PGM) which can be imported into the amyloplast by glucose 6-phosphate/phosphate translocator (GPT). In the amyloplast, G6P is then reconverted into G1P by plastidial PGM and as such can serve as a substrate for starch biosynthesis (Figure 2-5) (Van Harsselaar *et al.*, 2017).

In a review by Halford *et al.* (2011), the authors refer to the importance of sucrose for SuSy activity; SuSy gene expression and SuSy activity increases in the presence of elevated sucrose (suggesting the presence of sucrose-inducible SuSy genes in potato). SuSy activity is correlated with starch accumulation and that over-expression of invertase and hexokinase causes a reduction of starch synthesis and over-accumulation of glycolytic intermediates. This suggests that SuSy and invertase activities are not interchangeable, but compartmentalised in some way, as both involve the production of G6P, however, the mechanism behind this is unknown (Halford *et al.*, 2011).

Concerted and controlled action of many enzymes is involved in the metabolism of starch (Kotting *et al.*, 2010). Starch synthesis include soluble (SS) and granule-bound starch synthases (GBSS) for elongation and glucose chains that are then branched by starch branching enzymes (BE) (Smith, 2012); and starch degradation includes the

debranching enzymes isoamylase and limit dextrinase (ISA and LDA), are required to hydrolyse the branch points, α-glucan phosphorylases (PHS), which catalyses the phosphorolysis of G1P from the non-reducing end of chains, disproportionating enzymes (DPE) which catalyses a range of glucanotransferase reactions, α-amylases (AMY), which hydrolyses internal α-1,4 bonds, and β-amylases (BAM), which hydrolyses linear glucans (Streb and Zeeman, 2012).

Starch breakdown is less well understood than starch synthesis (Edner *et al.*, 2007). In germinating cereal seeds, starch degradation process is considered a highly specialized system, involving tissue deterioration and the induction of hydrolytic enzymes (Beck and Ziegler, 1989). However, with tuber and leaf starches, that is not the case. According to Blennow *et al.* (2002) “tuber and leaf starches, contain more starch-bound phosphate, and are degraded without notable changes in cellular structure and there are no reported consistent correlations between amylolytic-enzyme activities and starch degradation”.

In contrast to starch granules of cereal endosperm, that have abundant surface pores, starch granules in potato tubers have few if any pores and are highly resistant to enzymatic attack. This is further evidence to morphological divergence with cereals where loss of internal content occurs before the surface has been attacked, whereas in potato tuber granules, damage appears on the surface as degradation commences. For those reasons, Smith *et al.* (2005) suggested that the enzymes involved in the potato tuber starch granule breakdown may differ from those acting on cereal endosperm. However, the authors indicate a paucity of information and that new studies are required.

Potato tuber starch is highly phosphorylated and that is essential for the starch metabolism (Blennow *et al.*, 2002). Lorberth *et al.* (1998) discovered a 160 kDa protein termed R1, bound to potato starch granules, that when suppressed in potato led to a decrease in the phosphate content and a decrease in the degradability of starch. Ritte *et al.* (2002), verified that R1 protein was a starch-phosphorylating enzyme, and showed that R1 was an α-glucan, water dikinase (GWD) which in an ATP-dependent reaction, phosphorylates starch-like glucans (Equation 1). Later, Kotting *et al.* (2005) identified another starch phosphorylating enzyme, phosphoglucan, water dikinase

(PWD), which was strictly dependent on the pre-phosphorylation of starch by GWD (Equation 2).

Equation 1

α-glucan + ATP + H_2O → α-glucan-P + AMP + P_i (Ritte et al., 2002)

Equation 2

α-glucan-P_n + ATP + H_2O → α-glucan-P_{n+1} +AMP +P_i (Kotting et al., 2005)

In the work of Ritte *et al.* (2006) it was not clear why normal starch degradation depends on two dikinases that act in series and that phosphorylate different positions of glucosyl residues, but the authors believe in the possibility of phosphate esters locally be able to open up the structure of the semicrystalline starch particle, accelerating the attack of degrading enzymes. More recent studies proposed that the glucan dikinases increase starch surface glucan solubility allowing access to individual glucan chains by hydrolytic enzymes (Meekins *et al.*, 2016). The degradation pathway of soluble glucans released from potato tuber starch is still unclear, and while the role of some of the enzymes in the tuber starch degradation pathway have been partially elucidated (Smith *et al.*, 2005; Mikkelsen *et al.*, 2006; Zeeman *et al.*, 2010; Santelia and Zeeman, 2011; Bansal and Das, 2013) more detailed studies are required.

With the degradation of starch the plant obtains monosaccharide sugars that are utilised in respiration, fuelling sprout growth and the next generation in the life cycle of potatoes. As the potato tuber is a storage organ, the degradative and biosynthetic processes are separated in time (Preiss, 2009).

Smith *et al.* (2005) belives that the activities of the enzymes instead of been eliminated in tubers, have been reduced, because in observations made in trangenic plant tubers with reduced activity of PHS that have normal starch content and even sprouts better than normal tubers. Tubers with reduced activities of DPE produce sprouts more slowly than normal tubers, those observations provide little information about the enzymes roles in tuber metabolism. However is not just in potato tubers that there is lack of information in starch metabolism, in Arabidopsis more information is still needed on

the regulation of starch metabolism (Streb and Zeeman, 2012). There is still little understanding of starch degradation in commercial crops, such the ripening of banana fruit and post-harvest deterioration of roots and tubers (Smith, 2012).

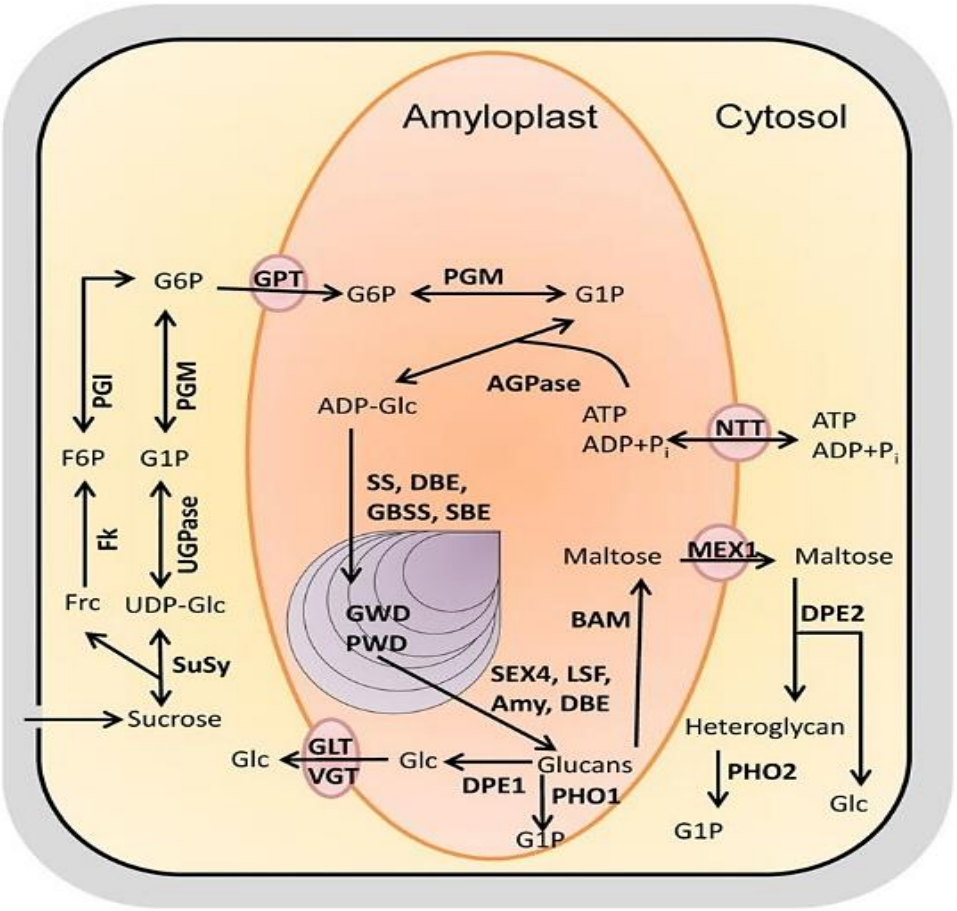

Figure 2-5 – Proposed pathway of starch metabolism in potato tubers with a summary of the enzyme reactions involved in starch-sugar interconversion (source: Van Harsselaar *et al.*, 2017): F6P, fructose-6-phosphate; TP, triose-phosphate; TPT, triose-phosphate/phosphate translocator; Pi, inorganic phosphate; PGI, phosphoglucoisomerase; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; PGM, phosphoglucomutase; SuSy, sucrose synthase; UGPase, UDP-glucose pyrophosphorylase; PPi, inorganic pyrophosphate; AGPase, ADP-glucose pyrophosphorylase; PPase, inorganic pyrophosphatase; SS, starch synthase; GBSS, granule-bound starch synthase; SBE, starch branching enzyme; GWD, glucan, water dikinase; PWD, phosphoglucan, water dikinase; BAM, β-amylase; AMY, α-amylase; SEX4, starch excess 4; LSF, Like starch-excess Four; DPE, disproportionating enzyme; PHO, Alpha-glucan phosphorylase; GPT, glucose 6-phosphate/phosphate translocator, NTT, nucleotide translocator; GLT, glucose transporter; VGT, vacuolar glucose transporter; MEX, maltose transporter; Fk, fructokinase.

2.8. Reducing sugars

When the tuber is mature concentration in free sugars (sucrose) is minimum (Storey, 2007). This state is referred to as chemical maturity, and should be achieved before harvest, at which point excess sucrose will be converted into reducing sugars (RS), that can lead to economical losses due to the unacceptable dark-coloured products after processing (Maillard reaction) (Sowokinos, 2007a).

The principal sugars in potato tubers are the reducing sugars glucose and fructose and the non-reducing disaccharide sucrose, and their concentrations change during crop development and storage (Storey, 2007). High sugar content, specially reducing sugars, is not desirable in potato tubers. Potatoes for the crisp production should not exceed 0.15% FW of reducing sugars and for chip production should not exceed 0.25% FW of reduncing sugars (OECD, 2015).

Glucose and fructose are known as reducing sugars because when in chain form glucose has a free aldehyde group and fructose has a free keto group (Figure 2-6), meaning that both glucose and fructose can act as reducing agents in the Maillard reaction (Halford *et al.*, 2011). Sucrose is not a substract in the Maillard reation, because it is not able to adopt an open chain structure (Figure 2-6) (Halford *et al.*, 2011), however, sucrose can increase the sweet taste of the final product to an undesirable level (Burton *et al.*, 1992).

Whittaker *et al.* (2010) implemented a path analysis model (statistical technique that distinguishes between causation and correlation between variables) to provide additional insight on the interactions between mineral elements, hexose sugars and acrylamide formation. These authors observed that when glucose and fructose were entered separately instead as a sum of total reducing sugars, fructose was preferentially selected by the model as having a positive direct effect on acrylamide formation. However with a lower *P* coefficient than the one for total reducing sugar content. Fructose is the most responsive sugar to changes in storage temperatures (Smith, 1987b). According to the work of Arreguin-Lozano and Bonner (1949) tubers stored at low temperatures are high in fructose and fructose-6-phosphate, and those stored at high temperatures are high in glucose and glucose-6-phosphate. This is

suggested to be due to some varieties, such “Chippewa”, “Kennebec”, “Katahdin” and “R. Burbank”, the ratio glucose to fructose is approximately one at low temperatures and above two at higher temperatures and others, like “White Rose”, just tend to accumulate fructose (Smith, 1987b). Possibly by greater utilisation of glucose at higher temperature.

Considerable variations in sugar concentration may occur at the expense of the starch amount during storage. At harvest time, immature tubers contain significant quantities of free sugars, mainly sucrose, however, as the tubers mature, the amount of sucrose decreases and starch increases Potato tubers destined for processing are stored at higher temperatures (10-13°C), where limited reducing sugar accumulation is observed, unless when tubers are stored for long periods, as respiration utilises excess glucose and fructose (Smith, 1987b). However, storage at high temperatures promotes sprouting, that is associated with carbohydrate mobilization, mainly due to starch hydrolyzation into sucrose. Amyloplast breakdown usually initiates at the basal end of the tuber (Burton *et al.*, 1992), the products from amyloplast breakdown are exported to the cytosol as hexose phosphates (glucose phosphate-phosphate translocator), or as free sugars (glucose and/or maltose transporters), where they are converted in to sucrose via sucrose phospate synthase (reviewed by Malone *et al.*, 2006). Sucrose formed in the parenchyma cells, is then translocated (via phloem) to the tuber apical region and emerging sprout, where it is hydrolysed, via the vacuolar-enzyme acid invertase, at least in part, into glucose and fructose, and utilized in cell growth, development and respiration (Burton *et al.*, 1992; Hajirezaei *et al.*, 2003; Sonnewald and Sonnewald, 2014).

Due to the demand of sucrose in the developing sprouts, the concentration of soluble sugar decreases in storage parenchyma cells, which may serve as signal to trigger starch breakdown into assimilates for sprout growth (Hajirezaei *et al.*, 2003). Hajirezaei *et al.* (2003) concluded that “low sucrose concentrations trigger starch mobilization in stored potato tubers”, however, a sucrose-specific sensor has not yet been discovered (Wind *et al.*, 2010). Sugar levels in plants fluctuated not only due to photosynthetic efficiency but as well on energy and growth requirements (Ljung *et al.*, 2015), with sucrose acting as a long-distance signal in promoting root growth in *Arabidopsis*

(Kircher and Schopfer, 2012) and bud dormancy and apical dominance in *Pisum sativum* cv. Torsdag (Mason *et al.*, 2014). However, more work is still needed as the role of sugars in the regulation of hormone synthesis, as well as hormone signalling have yet to be elucidated (Ljung *et al.*, 2015).

According to Burton *et al.* (1992), three different mechanisms take place simultaneously, and should be distinguished: sugar accumulation at low temperature, sugar formation accompanying the sprouting process (both mechanisms referred above), and sugar formation due to senescent sweetening, as explained under section 2.5 Senescent Sweetening.

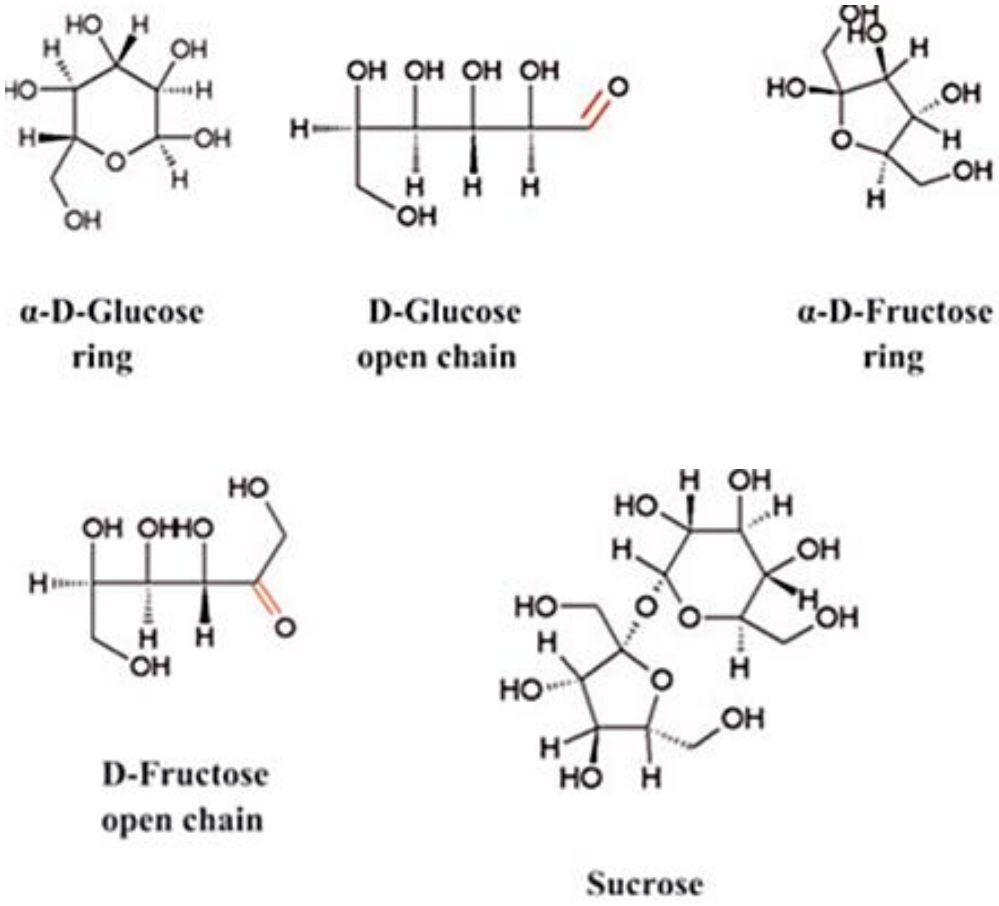

Figure 2-6 - Structures of glucose and fructose (ring and open chain form) and the disaccharide sucrose. The carbonyl groups of glucose and fructose are shown in red (source: Halford *et al.*, 2011).

2.9. The Maillard reaction

The Maillard reaction, sometimes described as exogenous glycation (Haase, 2007), consists of a series of non-enzymatic reactions between sugars and amino groups (principally free amino acids and in particular asparginine) and was first described by Louis Camille Maillard in 1912 (see Haase, 2007; Halford et al., 2011).

This reaction occurs mainly in cooked foods prepared by frying, baking and roasting. It is promoted by high temperature and low moisture content, and requires the presence of reducing sugars (Halford *et al.*, 2011), in the case of potato, glucose and fructose (Haase, 2007).

The Maillard reaction is subdivided into three major stages, the first stage starts with the condensation of the carbonyl (C-O) group with an amino compound producing a Schiff base (Haase, 2007; Halford *et al.*, 2011). Where the sugar is an aldose it will form an Amadori rearrangement product, if the sugar is a ketose, it will form a related Heyns rearrangement product (Haase, 2007; Halford *et al.*, 2011).

In the second stage, the Amadori and Heyns rearrangement products undergo deamination and decarboxylation reactions through the Strecker degradation process, an important reaction for flavour generation (Haase, 2007). The products from these two phases are colourless or pale yellow (Haase, 2007).

During the final stage further condensation reactions between carbonyls and amines occur producing coloured high molecular mass products known as melanoidins (advanced glycation end products - AGEs) (Haase, 2007).

The formation of Maillard reaction products depends on the superficial reducing sugar content (Pedreschi, 2007), and is influenced by the temperature and the duration of the heating process (Haase, 2007).

Although many of these products contribute to the flavour, colour and aroma of food, some are undesirable. Exogenous glycations and AGEs are important contributors to inflammation and disease states (Haase, 2007). A very important undesirable product from the Maillard reaction is acrylamide, a known carcinogenic substance in mammals and probably humans, as well as neurotoxin and mutagen (Pedreschi, 2007). Acrylamide is mostly formed due to the reaction of the amino acid aspargine, in combination with reducing sugars and early Maillard reaction products (Pedreschi, 2007) (Figure 2-7).

French fries and crisps are in the group of foods with possibly the highest concentration of acrylamide, 424 μg^{-1} kg and 1739 μg^{-1} kg respectively (Pedreschi, 2009), caused in part by the high levels of free asparagine present in potato tuber (Zyzak *et al.*, 2003). However, asparagine content, and potato tubers suitability for processing, is not only influenced by cultivar and storage conditions, but by differences in location, fertilisation and processing (Pedreschi, 2009 and references therein).

As detailed earlier, fry colour is a result of the Maillard reaction, which depends on the quantity of superficial reducing sugars and the temperature of frying oil and frying time, so as the frying temperature increases from 150 to 190°C, the final product (french fries and chips) becomes more red and darker (Pedreschi, 2009).

Until now, several methods to reduce acrylamide formation have been studied (blanching, soaking, acid addition), however, colour, flavour and taste of fried products are dependant on the Maillard reaction, and in the case of fried potatoes products, as stated by Pedresch (2009) “the major challenge is to produce fried potatoes with low acrylamide content without affecting their traditional flavour, taste, and other sensorial atributes”.

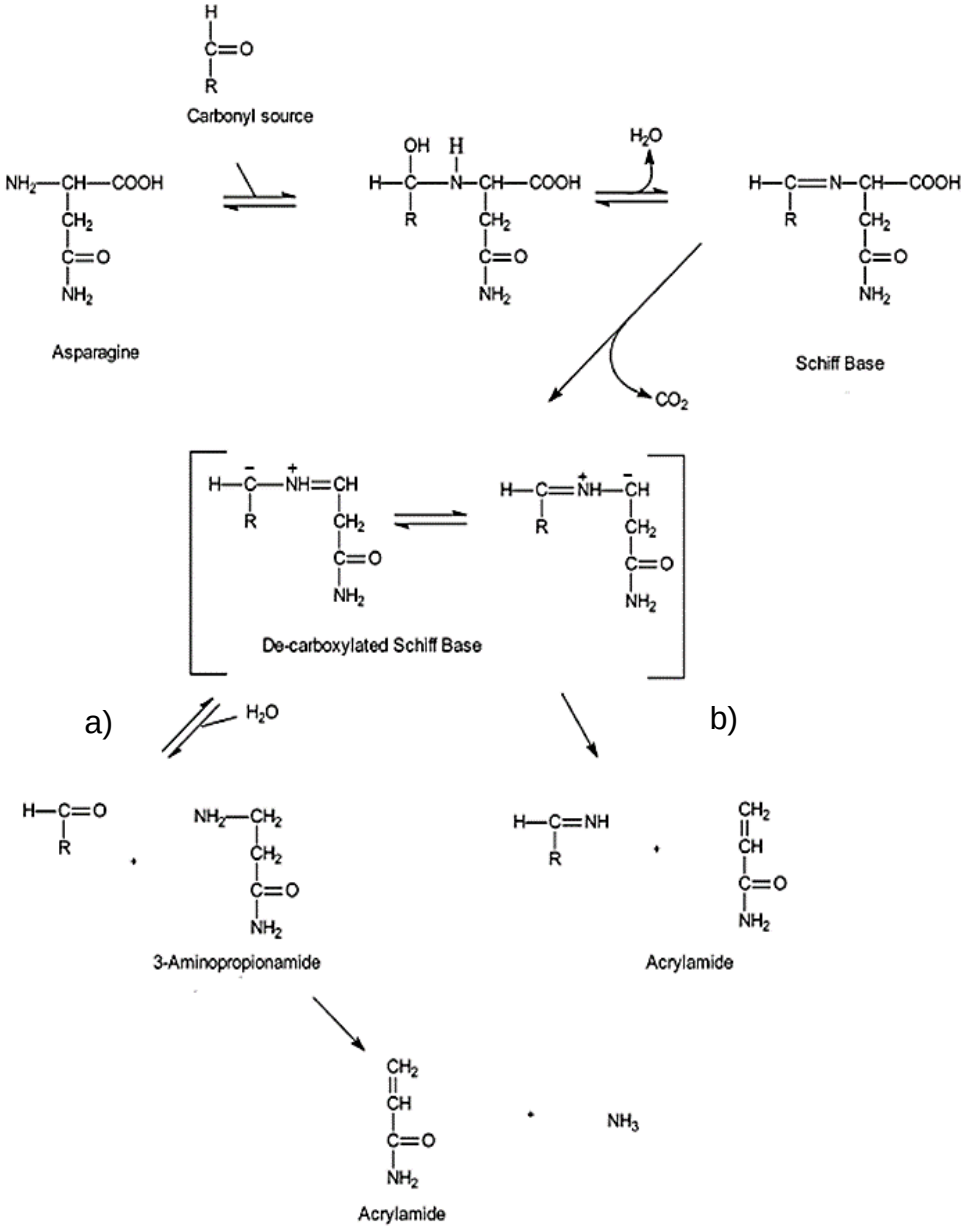

Figure 2-7 - Mechanism of acrylamide formation in heated foods proposed by Zyzak *et al.* (2003). The α-amino group of free asparagine reacts with a carbonyl source that will form a Schiff base. When heated, the Schiff base decarboxylates, forming a product that can react one of two ways: a) hydrolysing to form 3-aminopropionamide that can further degrade into acrylamide when eliminated the ammonia when heated; b) or it can decompose directly to form acrylamide eliminating an imine (Zyzak et al., 2003).

2.10. Tuber Respiration

Respiration is the oxidative breakdown of the more complex substrates normally present in the cells, such as starch, sugars, and organic acids, into smaller molecules (CO_2 and H_2O), with the production of energy and other molecules that can be used by the cell for synthetic reactions (Burton *et al.*, 1992; Wills *et al.*, 2007).

The primary purpose of respiration is the regeneration of the supply of adenosine triphosphate (ATP) (Burton *et al.*, 1992). Small amounts of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$)) are also produced throughout the pentose phosphate pathway (Hodson and Bryant, 2012).

When in the presence of oxygen, respiration is considered aerobic (Wills *et al.*, 2007) with the overal purpose to regenerate ATP from ADP (adenosine disphosphate) and P_i (inorganic phosphate) with the release of CO_2 and H_2O. If the substrate is hexose sugar, then the overral equation is written as in below (3) (Burton *et al.*, 1992).

Equation 3

$$C_6H_{12}O_6 + 6\ O_2 + 38\ ADP + 38\ Pi \rightarrow 6\ CO_2 + 44\ H_2O + 38\ ATP$$

The overall respiratory pathway occurs in three interdependent reactions, glycolysis (also known as the Embden-Meyerhof-Parnas pathway, EMP), the tri-carboxylic acid (TAC, also known as the Krebs cycle, or the citric acid cycle), and the electron transport chain (Hopkins and Hüner, 2004; van Dongen *et al.*, 2011; Hodson and Bryant, 2012).

Glycolysis takes place in the cytosol and is the first stage of respiratory carbon metabolism where initially hexose substrates are converted to pyruvate (Hopkins and Hüner, 2004; Hodson and Bryant, 2012). During the first set of reactions of glycolysis glucose and fructose, derived from storage carbohydrate, are converted to triose-phosphate (triose-P) via the hexose-phosphate pool, and then triose-P is converted to pyruvate, the end product of glycolysis (Figure 2-8 a) (Hopkins and Hüner, 2004). Under aerobic conditions pyruvate is transported into the mitochondria for entry into the TCA cycle (Figure 2-9). In the absence of oxygen as terminal electron acceptor,

mitochondrial respiration will stop and metabolism will shift over to fermentation (Figure 2-8 b) (Hopkins and Hüner, 2004; Hodson and Bryant, 2012).

The second stage of respiration is the complete oxidation of pyruvate to CO_2 and H_2O through several oxidative steps that take place in the TCA cycle (Figure 2-9) (Hopkins and Hüner, 2004; Hodson and Bryant, 2012).

The TCA cycle enzymes are predominantly located in the mitochondrial matrix (less succinic dehydrogenase, reaction 7 from Figure 2-9) and includes four oxidative reactions; three that yield NADH (nicotinamide adenine dinucleotid) and one that yields $FADH_2$ (flavin adenine dinucleotide) (Hopkins and Hüner, 2004; Hodson and Bryant, 2012). During the TCA cycle one molecule of ATP is generated and the oxaloacetate is regenerated, which is available to recycle (Hopkins and Hüner, 2004; Hodson and Bryant, 2012).

The NADH and $FADH_2$ produced during the TCA cycle are oxidased via the electron transport chain in the mitochondrial inner membrane. The chain consists of four multi-protein complexes in a series linked by mobile electron carriers. The multi-protein complexes are arranged so that the passage of electrons from NADH dehydrogenase to the final electron acceptor, cytochrome oxidase, is accompanied by passage of protons across the membrane into the inter-membrane space, building a proton gradient across the membrane, used to drive ATP synthesis (Hopkins and Hüner, 2004; Hodson and Bryant, 2012). In the final complex, cytochrome oxidase, the electrons are transfer to molecular oxygen forming water (Hopkins and Hüner, 2004).

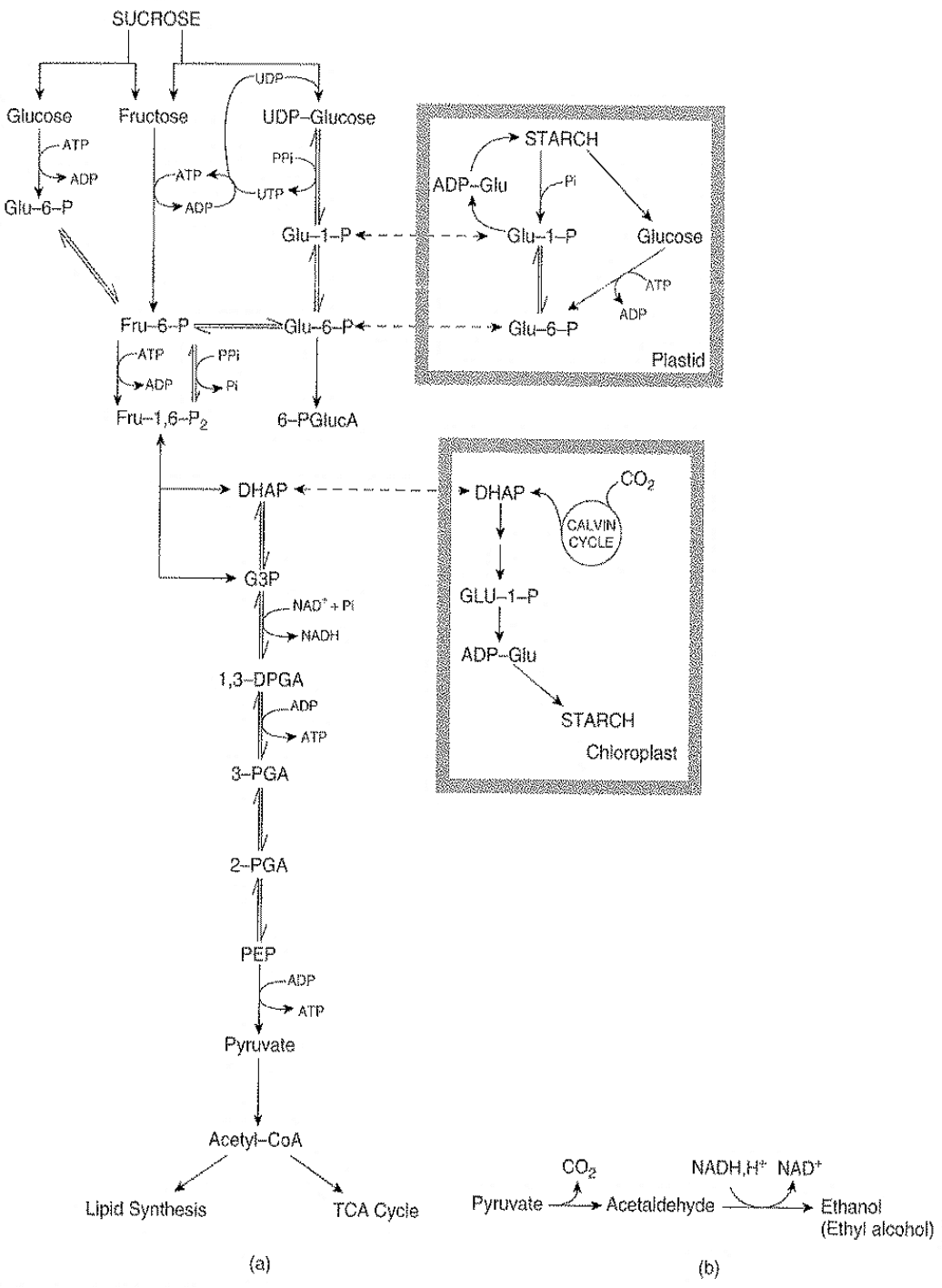

Figure 2-8 – Glycolysis or the EMP pathway (a) and recycling of NADPH by fermentation under anaerobic conditions (b) (taken from Hodson and Bryant, 2012).

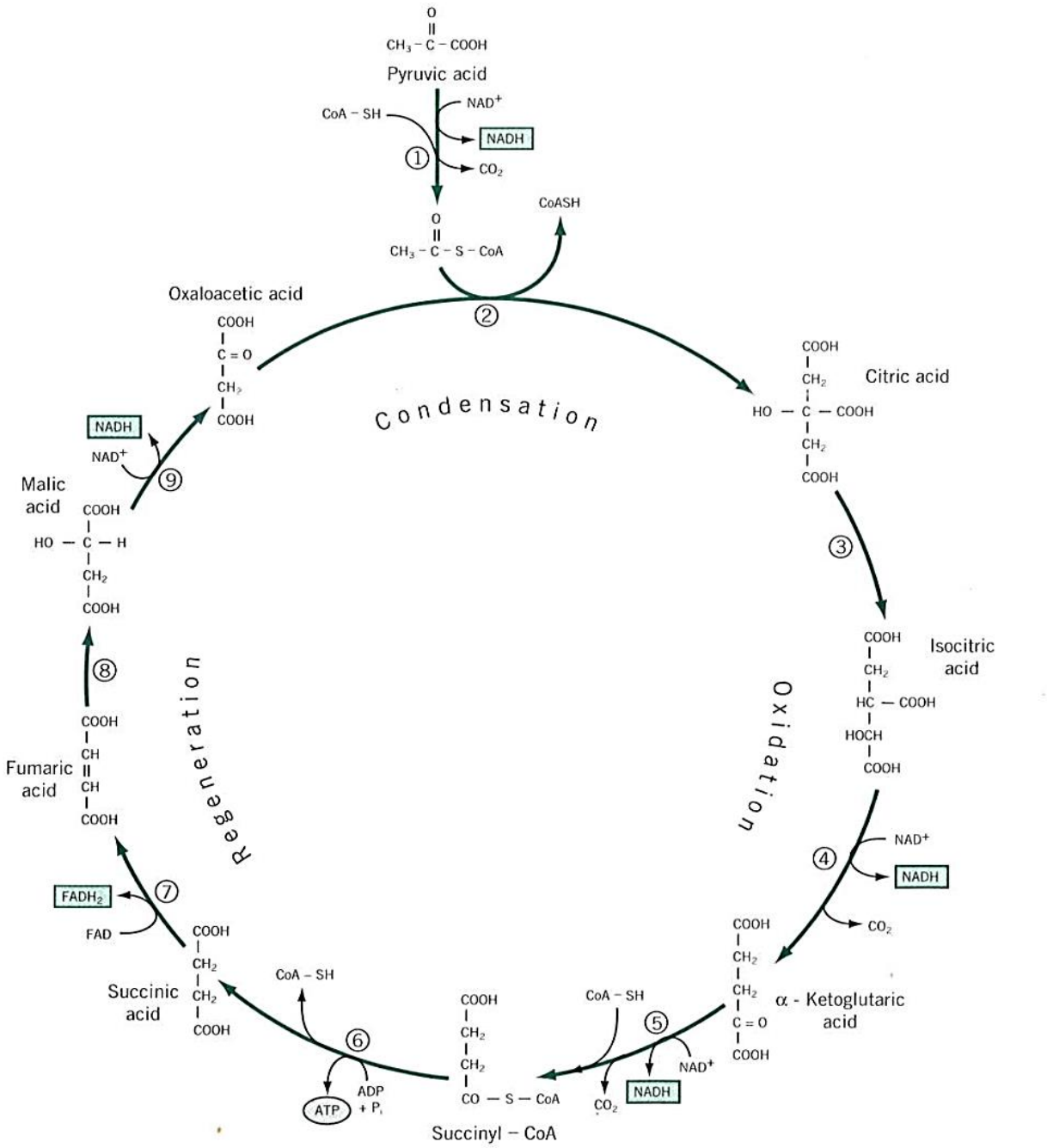

Figure 2-9 - The tricarboxylic acid (TCA) or Krebs cycle (taken from Hopkins and Hüner, 2004).

The respiration rate is a good indicator of tissue metabolic activity and is explain by the concept of respiratory quotient (RQ), being an useful guide for predicting the potential storage life of the tuber (Wills *et al.*, 2007). With under normal aerobic respiration the CO_2 production is equivalent to consumption of O_2 (RQ=1) (Equation 4) (Burton, 1989). However, Burton (1974) reported RQ values of 0.8-0.9 in the early storage period when the tubers were still dormant, and RQ=1.3 later when tubers were sprouting (Burton *et al.*, 1992). According to Isherwood and Burton (1975) senescent potato tubers have a higher production of CO_2 when compared with the O_2 consumption. Accurate measurements of RQ have in the past been difficult to determine due in the most part to sampling methods and sensitivity of O_2 and CO_2 detectors. Older tubers characteristically have a higher respiration rate (Kumar and Knowles, 1996a; Kumar and Knowles, 1996b) and tuber respiration rate seems to be the "pacemaker" of aging in potato tubers (Blauer *et al.*, 2013a). According to the work of Copp *et al.* (2000) the onset of the decline in chip colour quality correlated with the increase in the respiration rate of the stored tubers.

Equation 4

$$RQ = \frac{volume\ formed\ (CO_2\ h^{-1})}{volume\ absorbed\ (O_2\ h^{-1})} = 1$$

Tuber respiration rate during storage is influenced by morphological characteristics (the skin and lenticels are very important for the O_2 permeability), maturity, dormancy, physiological age, cultivar, inhibitory or stimulatory chemicals compounds present, handling and healing wounds; and conditions of storage, such as temperature and the O_2 and CO_2 concentrations in the storage atmosphere (Burton *et al.*, 1992).

The ability of a tuber to respire aerobically depends on the O_2 supply to the respiring tissue, in potato tubers this is limited by the periderm, which acts as a gaseous diffusion barrier. The majority of the gaseous diffusion occurs through the lenticels (about 100 per tuber). The resistance to gas diffusion in potato tubers is not limited by the lenticels

but by the intercellular spaces immediately underlying them, meaning that the O_2 and CO_2 concentrations in the tissues are equilibrated with the ambient atmosphere (Burton, 1978 in Burton *et al.*, 1992; Abdul-Baki and Solomos, 1994).

Thus, in potato tubers, O_2 diffusion occurs through the skin lenticels followed by diffusion in the intercellular spaces where eventually respiration takes place in the cytoplasm-mitochondria, respiratory CO_2 follows the reverse path (Woolley, 1962; Banks and Kays, 1988; Ho *et al.*, 2010). However, cellular structure of potato tuber tissue is not homogeneous and varies depending on variety (Konstankiewicz *et al.*, 2002; Gancarz *et al.*, 2014). The major component of tuber tissue are parenchyma cells from the cortex and from the perimedullary zone, which contain starch granules as reserve material (Grommers and van der Krogt, 2009).

Parenchyma cells of the perimedullar area are bigger and store more starch than the ones from medullary zone (pith) (Reeve *et al.*, 1969; Kadam *et al.*, 1991; Konstankiewicz *et al.*, 2002; Sadowska *et al.*, 2008; Gancarz *et al.*, 2014).

Devaux (1891) demonstrated that intercellular spaces in thick plant tissues are interconnected and air-filled. The microscale topology of cells and intercellular spaces determined to a large extent gas transport in the tissue, therefore, plant tissue cannot be considered as a real continuum material (Verboven *et al.*, 2008; Ho *et al.*, 2011). It was observed that the gas-filled intercellular spaces are inerconnected with narrow capillary tubes (Woolley, 1962), therefore, as the cell becomes more hydrated additional resistence to gas diffusion might be observed. In senescent tissues the intercellular spaces may became filled with cellular solution, impeding O_2 movement, resulting in anaerobic conditions within the tissue (Burton *et al.*, 1992).

It was observed that CO_2 diffusivity of the tissue was much higher than O_2 diffusivity. It was hypothesized that CO_2, besides the gas phase transport, could also be transported in the water phase from cell to cell, due to its higher solubility in the solution phase, while O_2 is mainly transported through the gas phase (Ho *et al.*, 2007; Ho *et al.*, 2011). Ho *et al.* (2011) found that compact cell clusters reduce the gas-liquid exchange surface and could increase the barrier to gas diffusion. In Pyrus pears, it was observed that O_2 and CO_2 diffusivity was lower at the skin and that along the equatorial radial

direction the diffusivity of both gases was almost constant in the cortex tissue and high in the core of the pear. As well, gas diffusivity was higher in the vertical axis when compared with the diffusivity along the radial direction (Ho et al., 2006a; Ho et al., 2006b). With CO_2 diffusivity in potatoes, Abdul-Baki and Solomos (1994) showed that the peel of R. Burbank had lower CO_2 diffusivity when compared to the flesh. In a recent study on the blackheart disorder in potato Maris Piper variety, it was suggested that the discontinuity of the gas-filled intercellular spaces may be the major factor leading to the large variation in O_2 diffusivities between flesh and heart tuber tissue (Kiaitsi, 2015).

For the determination of respiration rate the consumption of O_2 is compared with the production of CO_2, and if the respiration rate is measured during all the storage phases, a respiratory pattern for the storage period can be observed (Wills *et al.*, 2007). Respiration rate of the stored crop is a useful indicator to determine the volume night-time air required to ventilate the storage facility to mantain temperatures of bulk stored potatoes and thus avoid excessive accumulation of CO_2 (Bethke and Busse, 2010). Ethylene within the storage atmosphere, at very high concentrations of 0.1% v/v in the air, was effective in increasing the respiration rate at 15°C, mainly later in the tuber storage life (Burton *et al.*, 1992 and references therein). Besides external sources of ethylene potato tubers produce small quantities of ethylene (0.0008 to 0.015 $\mu L\ kg^{-1}\ h^{-1}$ from intact tubers) and an increase of 2- to 25-fold was observed during sprouting and stress (Daniels-Lake *et al.*, 2005b). Exposure to ethylene increases tuber respiration rate and accelerates the conversion of starch to sugars causing a dose-dependent effect in the darkening of potato fry colour (Daniels-Lake *et al.*, 2005a and references therein). Additional studies by Daniels-Lake *et al.* (2005b) found the effects of elevated CO_2 concentrations, reduced O_2 concentrations and ethylene gas on the fry colour and sugar content in the variety Russet Burbank. The authors found that tubers exposed to both elevated CO_2 concentrations and ethylene were darker and with higher reducing sugar concentrations, not only than the controls, but those treated just with ethylene, suggesting a synergistic negative effect of trace ethylene and elevated CO_2 on fry colour.

2.11. Reactive oxygen species (ROS) role in tissue senescence

Aerobic organisms have molecular dioxygen (O_2) as the terminal electron acceptor; its reduction is responsible for the production of highly reactive intermediates, known as reactive oxygen species (ROS) (Gechev *et al.*, 2006).

ROS in plants cause oxidative stress damage, due to biotic and abiotic stress conditions (Arora *et al.*, 2002; Krishnamurthy and Rathinasabapathi, 2013), such as drought, temperature, nutritional limitations, air pollutants, plant pathogen interactions and chemicals e.g. paraquat (Iannelli *et al.*, 1999; Arora *et al.*, 2002; Krishnamurthy and Rathinasabapathi, 2013).

The role of ROS has diversified beyond the initial host defence interactions to act more widely as key signalling molecules in many biological systems, including the induction of programmed cell death, apical dominance and senescence (Hancock *et al.*, 2001; Gechev *et al.*, 2006; Mittler, 2017). In plants, ROS functions and phytohormones are interconnected in a signalling network, with 'cross-talk' between hormones, inducing a regulatory, repair or death response (Foyer *et al.*, 2017).

However, little is still known about the ROS signaling pathways (Noctor and Foyer, 2016). ROS are believed to affect gene expression, in plants, ROS-induced genes have been identified for receptor kinase, annexin and peroxisome biogenesis, as well inducing signaling cascades that impact on gene expression of proteins with antioxidant functions or associated with defence response or other stresses, as well some genes coded for proteins with signaling functions (Apel and Hirt, 2004).

ROS-related signaling is integrated with many signal transduction pathways, interacting with different molecules such nitric oxide (NO), calcium, phytohormones and sugars. This suggests ROS signalling has a central role in the interface between metabolism and developmental or environmental responses within plants (Foyer *et al.*, 2017).

A wide range of genes are regulated by H_2O_2 (Hancock *et al.*, 2001), in yeast cells that are exposed to H_2O_2, there is a response in genes denoted as common environmental

stress response (CESR). These CESR-induced genes have a range of roles such as carbohydrate and metabolite transport; and CESR-repressed genes are involved in energy consumption (Apel and Hirt, 2004). In suspension cultures of *Arabidopsis thaliana*, H_2O_2 induced the expression of genes for glutathione S-transferase, phenylalanine ammonium lyase and plant gp91-*phox* homologue expression (Hancock *et al.*, 2001). In *Arabidopsis,* ROS species induce the expression of genes encoding kinase, a senescence-related and DNA damage repair protein (Hancock *et al.*, 2001). ROS may influence signaling pathways by oxidizing their components, or even changes in gene expression by targeting and modifying transcription factors activity (Apel and Hirt, 2004). However, more information about H_2O_2 concentrations and distribution of it in different compartments in plants are still needed (Noctor and Foyer, 2016).

In plants, ROS are continuously produced in a controlled manner within chloroplasts, mitochondria and peroxisomes. However, when ROS species are produced in an uncontrolled manner they result in cellular damage, and therefore removal of ROS, via scavenging mechanisms (enzymatic and non-enzymatic) occur in cells (Hancock *et al.*, 2001).

ROS is produced by several enzymatic activities; of these NADPH oxidases are the most studied (Mittler *et al.*, 2004). The first ROS species formed after O_2 reduction is superoxide (O_2^-) and in most biological systems O_2^- is promptly converted into the more stable hydrogen peroxide (H_2O_2), by superoxide dismutase (SOD) (Figure 2-10andFigure 2-11.a) (Gechev *et al.*, 2006). O_2^- and H_2O_2 react together in the presence of metal ions, by a mechanism known as a Haber-Weiss reaction, producing the highly toxic hydroxyl radical ($HO^{\bullet}$) (Figure 2-10) (Mittler *et al.*, 2004; Gechev *et al.*, 2006).

There are two types of mechanisms for ROS scavenging, non-enzymatic and enzymatic mechanisms (Figure 2-11) (Apel and Hirt, 2004; Gill and Tuteja, 2010; Sharma *et al.*, 2012). Cells are constantly producing ROS as a by product of aerobic metabolism and removing it by antioxidative mechanisms to prevent oxidative stress (Mittler, 2017). Non-enzymatic antioxidants include ascorbate and glutathione (GSH), tocopherol, flavonoids, alkaloids and carotenoids and enzymatic mechanisms include SOD, the only plant enzyme able to scaveng O_2^-, ascorbate peroxidase (APX),

glutathione peroxidase (GPX) and caetalase (CAT) (Apel and Hirt, 2004; Gechev *et al.*, 2006).

This series of antioxidative systems in conjunction with ROS signalling reactions regulate the cell response to a specific stimulus and this determined by the fine balance between ROS production for signalling, the rate of ROS diffusion and reactivity, and the cells capacity for scavenging ROS in the different cellular compartments and the integration of all those signals (Mittler, 2017).

Developmental stage (age) of the plant (Petrov *et al.*, 2015) also influences ROS synthesis and action. From a certain age, there is a decrease in the photosynthetic activity with a progressive increase in oxidative stress, which promote senescence, due to metabolic alterations related to age, such as reduction in AsA concentration and antioxidant enzymes (Petrov *et al.*, 2015).

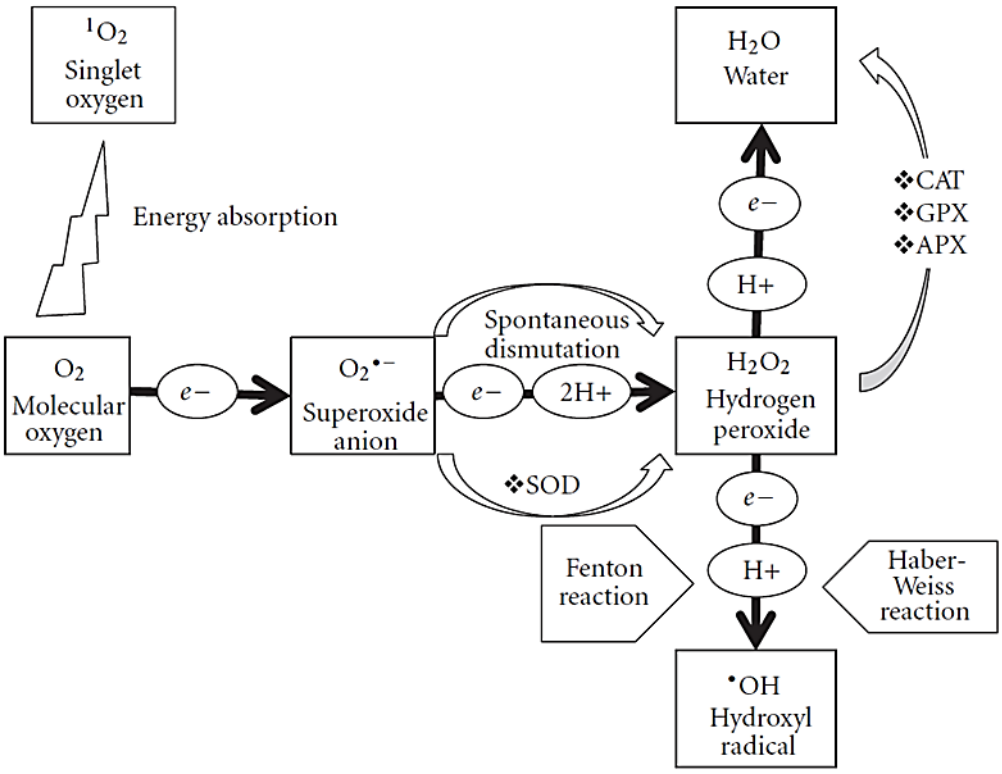

Figure 2-10 - ROS generation in plants (Sharma *et al.*, 2012).

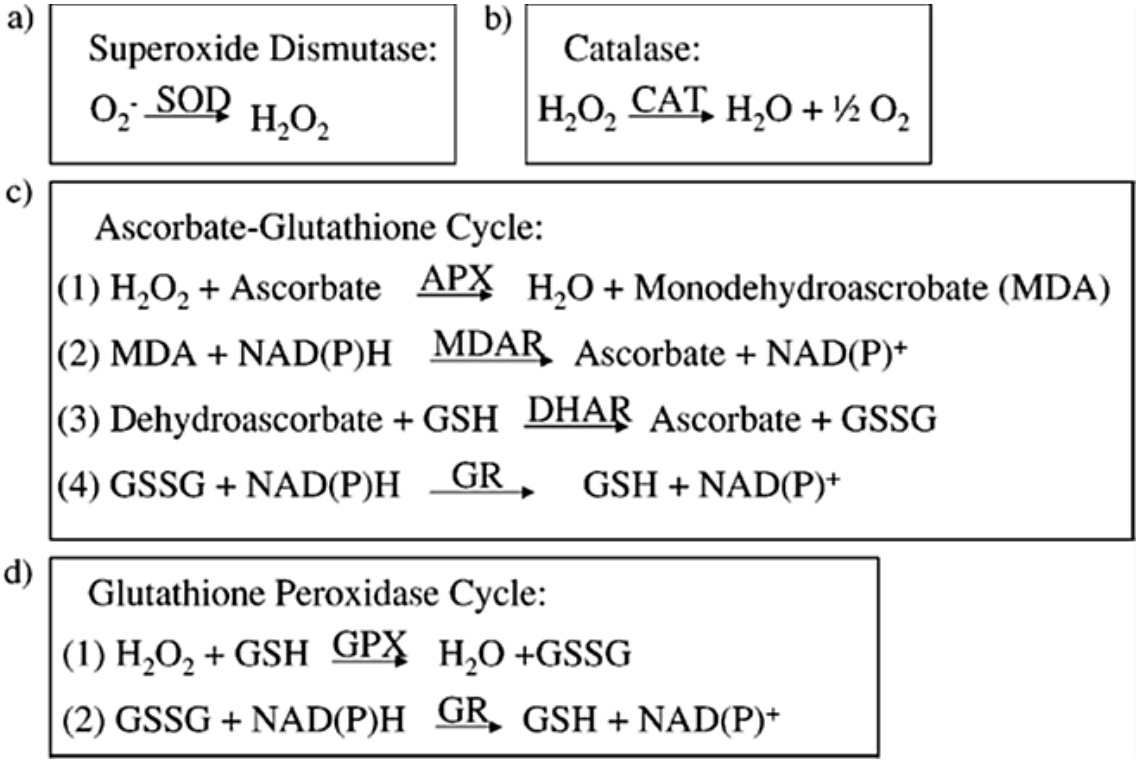

Figure 2-11 - Principle methods for ROS scavenging (Apel and Hirt, 2004)

2.12. Calcium signalling/regulation in dormancy breaking and sprout growth

Calcium (Ca^{2+}) is essential in growth and development of plants (Hepler, 2005) and has important roles in: cell division (important in the formation of the mitotic spindle and middle lamella); membrane integrity and function; and as secondary messenger in various metabolic processes (seed development, stomata, response to abiotic stresses and pathogens attack) (reviewed by Yencho *et al.*, 2008; Palta, 2010).

It is interesting to notice that a single messenger like Ca^{2+} can express information for specific responces to an extensive variety of different stimuli, for exemple, an elevation in Ca^{2+} in stomatal guard cells can lead to stomatal inhibition of opening (promotion of

closure) (reviewed by Sanders *et al.*, 2002), in the presence of auxin, or closing in the presence of absisic acid (reviewed by Sanders *et al.*, 2002; de Freitas *et al.*, 2014).

Cells access calcium in two ways, finite stores located in intracellular organelles and a more substantial pool of extracellular Ca^{2+} (Peppiatt *et al.*, 2004). Symptoms of Ca^{2+} deficiency disorders are characterized by cell plasmolysis and water-soaked appearance of blosson-end tissues leading to the eventual cell death as tissue becomes dark brown in fruits (de Freitas *et al.*, 2010). In potato tubers it is belived that Brown Center disorder (brown tissue discoloration with necrotic lesions in the central pith) may be caused during the pre-harvest period due to stressful growth conditions, such as cool temperature soils (<10 – 15°C) and nutrient deficiency mainly calcium (Ca^{2+}) and potassium (K) (Davies, 1998; Bussan, 2007; Sowokinos, 2007a; Palta, 2010).

Ca^{2+} increases membrane integrity and for that reason is often regarded as an anti-senescence factor (Kumar and Knowles, 1993a), low Ca^{2+} concentrations in the membranes leads to leaky membranes resulting in loss of cellular salts and organic compounds, and if not reversed can lead to cell death (reviewed by Palta, 2010).

As well, Poovaiah and Leopold (1973a; 1973b) have observed that Ca^{2+} enhanced the ability of cytokinin to retard leaf senescence and leaf abscission. In cut flowers, Ca^{2+} could be involved in senescence delay by acting as a second messenger in the signalling pathway leading to the induction of SOD, CAT and APX, leading to decrease in lipid peroxidation and increasing membrane stability, and being a component of cell membranes and walls, strengthen those structures delaying membrane deterioration and senescence (Sairam *et al.*, 2011).

Nevertheless, for Dyson and Digby (1975) a major influence of Ca^{2+} on the life cycle of the tuber was the rate of sprouting. A direct relationship between the Ca^{2+} concentration in the tuber and the rate sprout elongation was found, with higher Ca^{2+} content encouraging sprout growth, although, it was necessary to supply the growing sprout with additional Ca^{2+} in order for the sprout to continue to grow (Dekock *et al.*, 1975; Dyson and Digby, 1975).

Ca^{2+} is reported to facilitate sugar unloading in cells; in apple sorbitol is the main product of photosynthesis, and apples with low Ca^{2+} content are at greater risk of sorbitol accumulation within the apoplast and interstial air spaces causing a glassy appearance referred to as water-core (reviewed by Colgan *et al.*, 2012). In melon a similar water-core disorder was associated with low Ca^{2+} concentration, that increase the polygalacturonase activity and anticipate the ethylene production peak in melons grown with Ca^{2+} -deficient solutions (Serrano *et al.*, 2002).

Hepler (2005) in a review about Ca^{2+} refers the work of Dickinson (1967) that showed pollen tubes changed in their permeability in response to low Ca^{2+} concentration, including a significant release of carbohydrates into the medium.

Ca^{2+} is affected by other mineral nutrients, magnesium and potassium can displace calcium from the pectin matrix between cell walls weakening cell to cell cohesion and accelerating tissue senescence rates, also when conjugated with oxalate ions it can lead to a loss of cell wall strength, being implicated in increasing in the flexibility of cell walls (McNeil *et al.*, 1984). Oxalate is formed through the oxidation of AsA that results initially in the formation of monodehydroascorbate radical, that then forms ascorbate and dehydroascorbic acid (DHA), which is unstable above pH 7 (reviewed by Smirnoff, 1996). DHA broken down could generate wall oxalate that can then influence free Ca^{2+} concentration (Parsons *et al.*, 2011).

Palta (2010) refers to previous studies on the existence of functional roots on the tuber and at the tuber-stolon junction and that these roots were able to supply water to the tuber whereas the main root system supplied water to the leaves of the plant. Since Ca^{2+} moves in the plant only through xylem (Ho and White, 2005; White, 2001), increasing Ca^{2+} supply to the main root system did not lead a proportional increase in the Ca^{2+} concentration of the tuber tissue. However, Ca^{2+} was applied to the tuber and stolon areas resulted in an increase in Ca^{2+} concentration in the tuber peel and medullary tissue. Therefore tuber Ca^{2+} content can be increased through precision placement (fertiliser arround the tubers) of Ca^{2+} in the tuber and stolon areas of the tuber (Palta, 2010).

2.13. Key points

- At harvest time tubers are generally dormant (endodormancy). The dormant period is affected by tuber maturity at harvest and dormancy break is associated with the start of sprouting.
- Starch breakdown is less well understood than starch synthesis (lack of consistent correlations between amylolytic-enzyme activities and starch degradation in tuber and leaf starches).
- Sugar can accumulate in potato tubers due to low temperature storage, the sprouting process and senescent sweetening.
- Sugar concentrations in tubers change during crop development and storage, from very low concentrations, when the tuber is mature, to higher levels with time in storage.
- Potato tubers for processing in crisps and chips should be stored between 10-13°C to the reducing sugar accumulation be lower.
- The principal sugars in potato tubers are the reducing sugars (glucose and fructose) and the non-reducing disaccharide sucrose. Of these sucrose is the only one that does not contribute to the Maillard reaction, which is responsible for the dark colour in the fry products.
- The onset of the change in chip colour quality coincides with the increase in the respiration rate of the stored tubers.
- The exact definition of physiological age of potato tubers (affected by its chronological age and environmental conditions), still have many molecular mechanisms involved that need elucidation; tubers with the same chronological age (time from tuber initiation) could have different physiological ages.
- Aging is accompanied by increased respiration rate, oxidative stress, lipid peroxidation and decreased protein content in the tubers.
- Senescent sweetening of tubers is the process of sugar accumulation due to aging, is irreversible and its onset differs between varieties. This contrasts with cold-induced sweetening, that is a reversible process.
- One theory is that senescence sweetening is likely to occur due to the progressive degeneration of the amyloplast membranes.

Chapter 3. ASSESSMENT OF PHYSIOLOGICAL CHANGES OF TUBERS DURING LONG-TERM STORAGE

3.1. Introduction

The research was centred around the study of Lady Rosetta, a variety 'susceptible' to sweetening, and VR 808, considered to maintain 'stable' sugar profiles, with both of them used in the crisp industry (NIAB Pocket Guide, 2008, NIAB, Cambridge in Colgan *et al.*, 2012). As additional material, that provided contrasting profiles of sugar accumulation during storage, Pentland Dell (susceptible to sweetening) and Russet Burbank (stable sugar profile), both used for chip production (NIAB Pocket Guide, 2008, NIAB, Cambridge in Colgan *et al.*, 2012), were included in the experiment.

Monitoring changes in tuber respiration, biomechanical and biochemical properties were used to chart changes in the aging of potatoes during storage that may help understand processes leading to senescent sweetening.

This experiment also focussed on the role of calcium in the stability of cell membranes and its potential role in maintaining the integrity of amyloplast membrane. Important in maintaining the stability of cell membranes are the Reactive Oxygen Species (ROS), signaling compounds (H_2O_2, O_2) formed by the incomplete reduction of oxygen. ROS stimulates cell membrane damage during aging and activates the programmed cell death (Daudi and O'Brian, 2012) response, increasing cell leakage and possibly contributing to increased sugar accumulation in older tubers.

Moreover, calcium's role in other processes linked to the quality of tubers in store such as its role in dormancy breaking and sprouting was also investigated as these processes have a direct bearing on tuber quality.

Besides calcium, a wide range of mineral elements are present in the tuber, which are classified as major minerals (calcium, potassium, magnesium, sodium, phosphorus, cobalt, manganese, nitrogen and chlorine), and trace minerals (iron, copper, selenium, nickel, lead, sulphur, boron, iodine, silicon and bromine) (Navarre *et al.*, 2009). Some of which according to Whittaker *et al.* (2010), may be correlated with reducing sugars levels.

3.2. Material and Methods

For the 1st year (2013/14) of this study, differences between geographical locations of planting sites were determined by, sampling tubers of VR 808 and L. Rosetta from PepsiCo trials planted in 2013 in Norfolk, Shropshire and Yorkshire. Tubers from each site were harvested based on crop maturity over a period of 2 weeks from each location and sent to Sutton Bridge Crop Storage Research (SBCSR) where they were cured. Tubers were treated with CIPC after curing and again at the end of January and mid of April 2014, mimicking commercial practice to prevent sprout growth (Appendix I). Tubers were stored at 10°C (SBCSR) and sampled for tuber quality at Natural Resources Institute (NRI), 1st sampling point was in December 2013 and last sampling point in July 2014 (Appendix II).

In year 2 (2014/15) tubers of VR 808 and L. Rosetta were harvested from a PepsiCo trial site in Norfolk. Tubers were harvested over a 3 week period and sent to SBCR where they were cured and CIPC treatments applied. Tubers were stored at 10°C (SBCSR) and sampled for tuber quality at NRI, 1st sampling time in November 2014 and last sampling time in July 2015 (Appendix III). P. Dell and R. Burbank were additional material included in the trial that provided contrasting profiles of sugar accumulation during storage.

The final year, (2015/16) a study charting changes in the physiology of VR 808 and L. Rosetta from PepsiCo (Yorkshire) during storage was undertaken. In the final year, a study on physiological aging of seed potato was investigated to determine the effect of

chronological age of crop on the propensity to develop senescent sweetening during storage. Physiological aged seed (*chitted* seed) of P. Dell tubers from a McCain *chitting* trial (0˚C days and 250˚C days) grown in Shropshire were used. After harvest, tubers were sent to SBCR where they were cured and CIPC treatments applied. Tubers were stored at 10°C (SBCSR) and sampled for tuber quality at NRI, 1st sampling time in December 2015 and last sampling time in June 2016 (Appendix IV).

Samples of tuber cortex were taken from opposite eighths (ends), sections related to the periderm, cortex, vascular ring and outer core, and from the middle of the tuber (inner core – medulla or pith) using a cork borer size Nº 5 (10 mm) to capture the maximum range in sugars across the tuber. In the 1st year each replicate consisted of cores combined from 3 tubers, with three replicates per variety for each sampling occasion. In the 2nd and 3rd year replicates consisted of a composite of 4 tubers, with five replicates per variety for each sampling occasion. In both years 2 types of tissue were selected from each tuber: samples from apical and stolon end (opposite ends - periderm, cortex, vascular ring and outer core) were combined to provide an average of the extremes in sugar and mineral profiles and for assement of amyloplast membrane integrity across the tuber and an additional sample was taken from mid section (inner core – medulla or pith) of the tuber, with five replicates per variety for each sampling occasion.

For vitamin C determination (AsA + DHA) samples were taken from opposite ends of potato using a cork borer (size Nº 5). Each replicate consisted of a composite of 4 sampler tubers from apical and stolon end (opposite ends) were combined to provide an average of the extremes in AsA, DHA and total vitamin C profiles across the tuber, with 5 replicates per variety for each sampling occasion.

All the samples were snap frozen in liquid nitrogen before storage at -80°C and subject to 48 hours freeze drying (Supermodulyo 12 K Edwards High Vacuum Internationa) before grinding to a fine powder in a pestle and mortar.

3.2.1. Assessment of sugar accumulation during storage

Sugars were extracted and analysed using an adaptation to the method used in Giné Bordonaba and Terry (2010) and Glowacz *et al.* (2015). Sugars were extracted from powdered potato samples (0.2 g) with 2 mL of 80:20 (ethanol:water (v/v)) for 2 hours at 70°C in a shaking water bath. Samples were centrifuged at 10,000 g for 5 minutes, the supernatant was filtered through a 0.45 µm PTFE syringe filter.

5 µL samples were injected onto an HPLC column (Agilent Zorbax Carbohydrate150 mm x 4.6 mm x 5 µm column) maintained at 30°C using 75% acetonitrile running at 2 mL min^{-1} as the mobile phase. Sugars were detected using a refractive index detector (Agilent 1200 refractive index detector). Data was analysed by using data system EZChrom 3.3 (Agilent).

3.2.2. Assessment of tuber respiration and ethylene production and tuber sprout growth during storage

For the 3 years of study, each sample was divided into two replicates (10 potatoes per replicate). For each replicate, the state of dormancy and later sprout growth was assessed before tubers were weighed and placed in a 5.5 L lock-tight plastic box 'static' enclosed environment for 3 hours at 10°C. Thereafter, the concentration of CO_2 and O_2 was measured using a Dual Gas Analyser-containing an infra-red gas analyser and an electrochemical oxygen cell (ICA, Kent, UK) (Plate 3-1).

The concentration of ethylene in the headspace was determined by using a gas chromatograph (ATI-Unicam 610 series) fitted with a flame ionization detector set at 250°C and a 1 m long, 6 mm OD glass column packed with 100/120 mesh alumina maintained at 130°C. The production rate of ethylene was expressed as nL $g^{-1}h^{-1}$ and carbon dioxide and oxygen as mL $g^{-1}h^{-1}$.

Tuber sprout measurements during the 1st year were made using a RS Baty caliper, Stock no. 613-959, 0-150 mm (RS Components Corporation, Switzerland) and during

the 2nd and 3rd year using a Traceable™ Digital Caliper, 0-150 mm (Fisher Scientific, UK).

Plate 3-1 - Measuring CO_2 and O_2 from the 5.5 L air tight plastic box

3.2.3. Assessment of ascorbic acid accumulation during storage

Ascorbic acid (AsA) accumulation during storage was monitored during the 2nd and 3rd years of the study. Ascorbic acid and dihydroascorbic acid (DHA) were extracted and analysed using an adaptation to the method of Glowacz *et al.* (2014).

Freeze dried potato powder (2.5 g) was placed in a 15 mL centrifuge tube to which 10 mL of 6% meta-phosphoric acid (HPO_3) was added. Samples were immediately homogenized using a vortex. The extracts were centrifuged at 3220 g for 40 minutes at 4°C. Sampler of 1.5 mL of the supernatant was then collected in microcentrifuge tubes and centrifuged at 9391 g for 5 minutes.

Following filtration using a 0.45 µm PTFE syringe filter, 500 µL were transferred into high-performance liquid chromatography (HPLC) vials for AsA determination. Further 500 µL were transferred to new 1.5 mL Eppendorf tubes and mixed thoroughly using a vortex with an equal volume of 1% DL-dithiothreitol (DTT). The DTT was used to

reduced the DHA to AsA, allowing total AsA content to be determined (Washko *et al.*, 1992).

The DTT solution samples were left for 40 min at room temperature and then centrifuged at 9391 g for 5 min. Samples were filtered with a 0.45 µm PTFE syringe filter and transferred into HPLC vials for total ascorbic acid (AsA+DHA) determination.

Samples (5 µL) were analysed using an Agilent 1200 series HPLC (Agilent, Stockport, Cheshire, UK) with a Agilent Zorbax SB-Aq 250 mm x 4.6 mm x 5 µm column at a flow rate of 1 mL min^{-1}. The mobile phase consisted of 20 mM phosphate buffer (2.76 g monosodium phosphate per litre of deonised water, at pH 2.0) with 1% acetonitrile.

Ascorbic acid was detected using a UV at 243 nm detector (Agilent). Data was analysed by using data system Agilent EZChrom Elite version 3.3.

3.2.4. Determination and detection of reactive oxygen species (ROS)

ROS stimulates cell membrane damage during aging and activates programmed cell death response (Daudi and O'Brian, 2012), increasing cell leakage. PCD may contribute to increased sugar accumulation in older tubers through enhanced breakdown of the amyloplast membrane.

Reactive oxygen species (ROS) high reactivity and relative instability makes detection and measurement difficult. Indirect or semi quantitative methods for ROS detection measuring the end products of which reactions are often used for the basis of detection (Thannickal and Fanburg, 2000; Jambunathan, 2010).

Staining and quantification of ROS protocols were developed for superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in segments of tubers during storage during the 2nd and 3rd year of this study. O_2^- was detected by the formation of a purple/blue precipitation on addition of nitroblue tetrazolium (NBT), and when diaminobenzidine tetrahydrochloride (DAB) is used as a substrate it reacts with H_2O_2 forming a brown product from polymerization (Jambunathan, 2010).

3.2.4.1. Detection of hydrogen peroxide by 3,3'-diaminobenzidine (DAB) staining

The *in situ* detection of hydrogen peroxide (H_2O_2) (one of several reactive oxygen species) was performed on four tubers per variety using 3,3'-diaminobenzidine (DAB) adaptating the method of Daudi and O'brian (2012). DAB oxidization via hydrogen peroxide occurs in the presence of heam peroxidases generating a dark brown precipitate (Daudi and O'Brian, 2012).

In 50 mL flask, 50 mg DAB was diluted with 45 mL of deionized H_2O to a final concentration of 1 mg^{-1}mL DAB the pH was adjusted to 3.0 by addition of 1 M of HCl under constant stirring, and to prevent light degradation the flask was covered with aluminium foil. The reaction was activated by the addition of 2.5 mL of 1 mM K_2HPO_4 to DAB solution (pH ~ 6.5). Four tubers per variety were stained and two control tuber slices were included where no DAB solution was applied. Tuber slices (3-4 mm in thickness) were prepared with a mandoline slicer and placed in separate Petri dishes. Samples were bathed in the DAB solution and gently vacuum infiltrated for 10 to 15 minutes in a desiccator, and left covered to prevent ingress of light for 4 hours. Control samples were infiltrated with water. Samples were washed with sterile water, placed in a light box and the extent of staining was captured by digital photography.

The extent of precipitate formation was captured using Image J and the intensity of red, green and blue (RGB) pixels was quantified by the method from Skipper (2010), using scripts developed in R.

RGB values for the white background in each tuber slice was used to normalise each tuber slice using an elliptical tool. Using the polygon drawing tool, it was possible to trace around the circumference of the tuber slices image to facilitate the processing of colour. The RGB values for the tuber slices were determined using the colour histogram function. Scripts developed in R generated normalised red, green and blue values from the pixels in each image (Skipper, 2010).

To evaluate the stain due to DAB and NBT techniques the red, green and blue values (RGB) needed to be transformed into a single value. Using the formulas for RGB % for

“Dark brown / #654321 hex color” (39.6%, 26.3%, 12.9%) and “Dark violet / #6317a9 hex color” (38.8%, 9%, 66.3%) from ColorHexa website (2012 - 2017), red, green and blue values were transformed into brown for DAB staining and purple (dark violet) for NBT staining. RGB values are encoded as 8-bit integers, which range from 0 to 255. In order to normalise the values, brown and purple were divided by 255. Finally, to the normalized values of the treated slices (stained) the values of the control slices (unstained) were subtracted to remove general colour changes that developed in untreated tubers over time.

3.2.4.2. Detection of superoxide by Nitroblue tetrazolium chloride (NBT) staining

The histochemical staining for superoxide (O_2^-) was made by staining with Nitroblue tetrazolium chloride (NBT) using an adaptation of the method of Jambunathan (2010). Four tubers per variety were used, two of which were used as control as well (no NBT solution was applied). Tuber slices (3-4 mm in thickness) were prepared with a madoline slicer and placed in separate Petri dishes.

A NBT 0.1% (w/v) staining solution in 10 mM of sodium azide (NaN_3) was dissolved in 50 mM of potassium phosphate (KH_2PO_4)

Potato slices were infiltrated with the NaN_3 / NBT solution for 10 minutes followed by incubation for 2 hours at room temperature, the extent of staining was captured by digital photography.

To view and process the RGB values for the captured images from the staining used the same methodology described in section (3.2.4.1).

3.2.5. Assessment of tuber texture during storage

Texture was measure only in the 1st year. Three tubers per variety were selected for texture analysis using the wedge fracture test developed by Vincent *et al.* (1991) which reproduces a relatively simple, and accurate method to mimick the action of the incisors in the propagation of the crack formation in food.

A 10 mm section along the mid-part of each tuber (Plate 3-2) was scored with a double bladed knife, with blades set 10 mm apart, before being cut with a single blade-knife. A second 10 mm slice (chip section) was excised from either tuber end, a further perpendicular 10 mm section was cut creating a 1 cm^3 of tissue, to ensure the orientation of tissue was kept constant during analysis, cubes of tissue were marked by removing a slither from the corner of the cube representing the outer end of the potato. Due to the radial-orientation of the cells within the tuber - accurate wedge fracture analysis texture requires the same orientation of the tissue during analysis (Plate 3-3).

Wedge fracture tests were carried out using a Lloyd Instruments model LRX-plus with a 50 N load cell and running with R control software v3.23. A wedge (30° included angle) was driven at 33.3 x 10^{-3} mm sec^{-1} (2 mm min^{-1}) into 10 mm cubes of tuber tissue orientated radially (Plate 3-3, Plate 3-4 and Plate 3-5). Wedge movement was halted when a crack could be seen ahead of the wedge tip (after reaching the peak load) and then the total crack length was measured. The wedge was then withdrawn from the sample at the same speed so that the energy still stored in the sample could be subtracted from the total energy. Peak load, load and distance at the start of crack propagation were determined from the load-distance (from top of sample) curves (Figure 3-1). In addition, work of fracture was calculated as (area under force-distance curve) / (total crack length * sample width) (Vincent et al., 1991).
The load-distance curve (Figure 3-1) for each tissue was re-displayed to identify the point of first failure and the start of crack propagation (using the cursor) so that the relevant loads and distances could be recorded. Rcontrol was set up to produce a results table and a conversion file in “Lotus” format for each sample. These files were

used to create “Excel” spreadsheets, measured crack lengths were entered and work of fracture calculated.

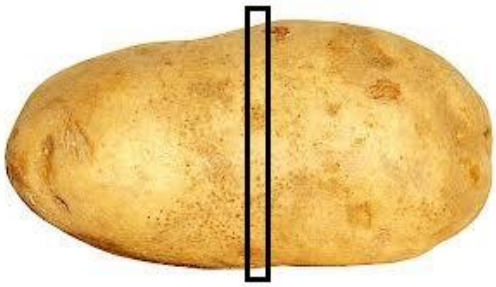

Plate 3-2 - Section where a slice of 10 mm width was cut along the middle part of the tuber.

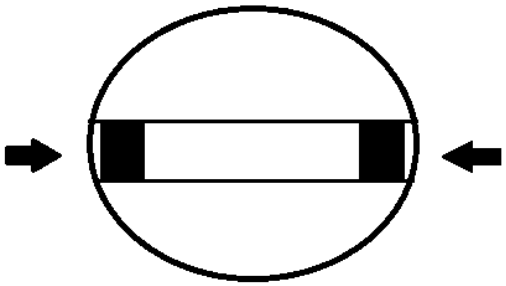

Plate 3-3 - Chip shape section with two black squares representing the two 10 mm cubes from the end of chip section. The arrow indicates the direction and orientation of the wedge during the wedge fracture test.

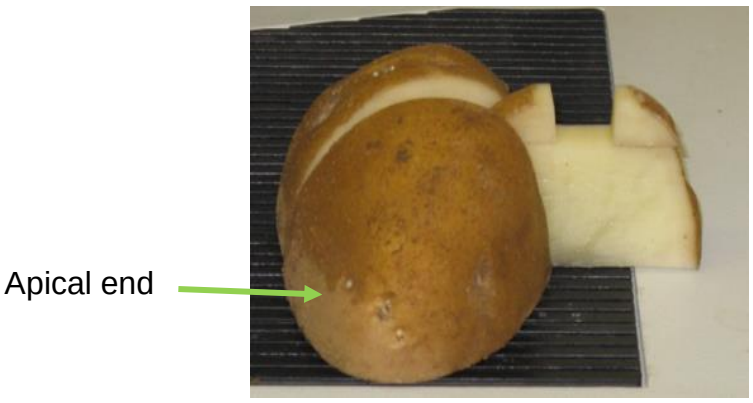

Plate 3-4 - Cuttings in the tuber

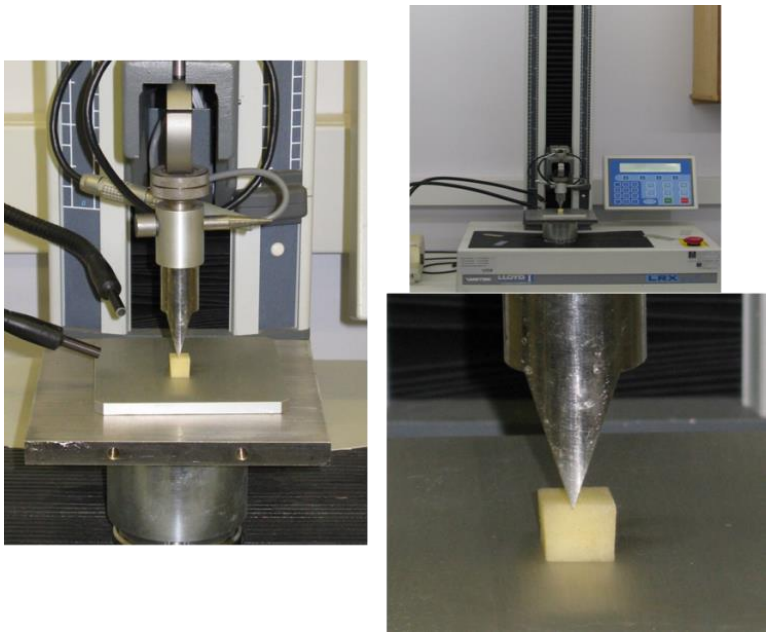

Plate 3-5 - Lloyd LRX-plus texture analyser with 10 mm cubes of tuber tissue orientated radially

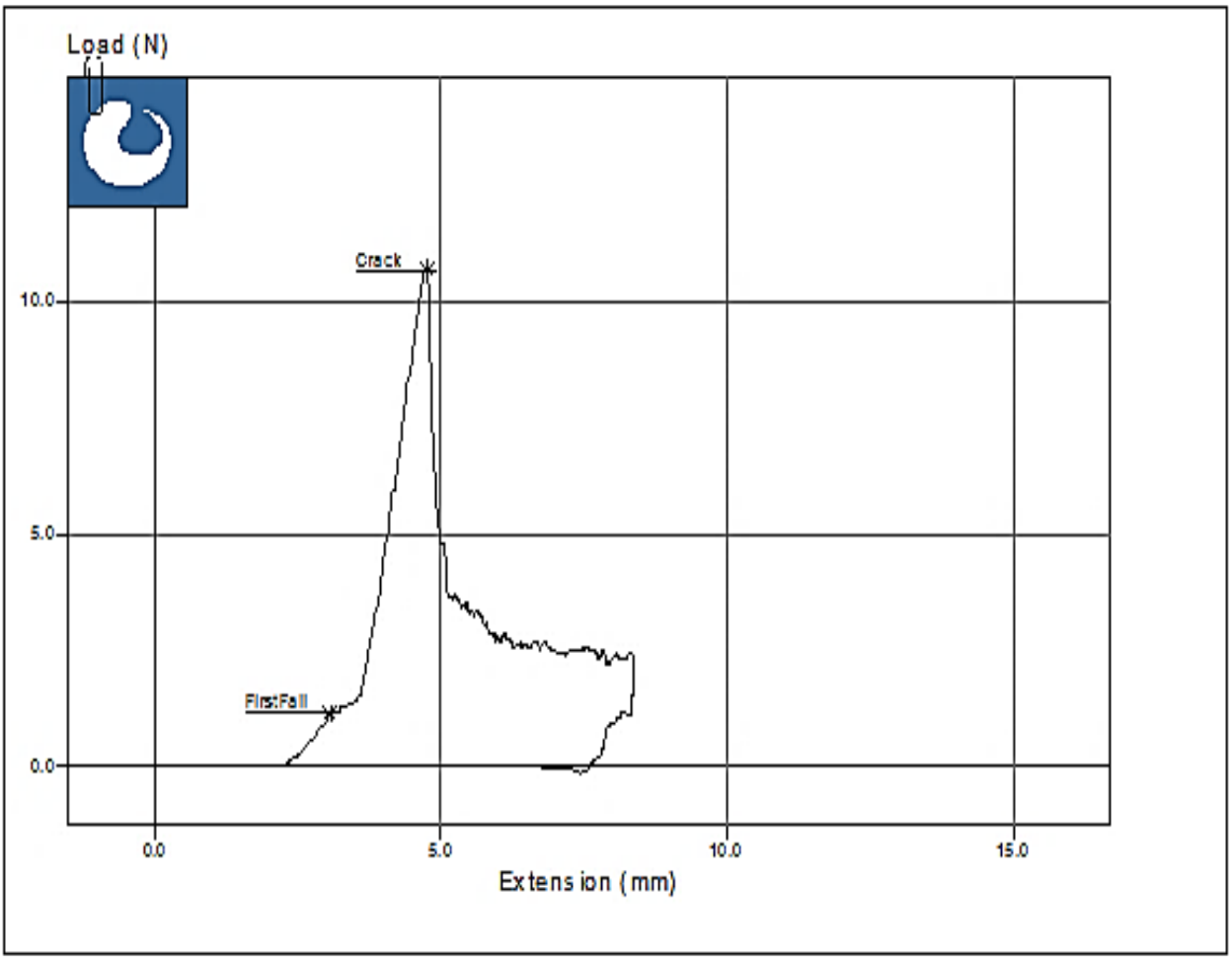

Figure 3-1 – Example of a graph of the load-distance curve produced by Lloyd LRX-plus texture analyser in potato. The load-distance curve for each tissue was re-displayed to identify the point of first failure ("First Fall") and the start of crack propagation ("Crack") (using the cursor) so that the relevant loads and distances could be recorded to produce a results table for each sample.

3.2.6. Assessment of amyloplast changes during storage using scanning electron microscopy (SEM)

In order to determine changes in amyloplast structure scanning electronic microscopy (SEM) techniques was used. During the 1st year preliminary investigations into the best technique for sample preparation were undertaken. Four methods of sample fixation/drying for SEM analyse, were investigated; three of the methods started with glutaraldehyde fixation followed by chemically drying samples through an ethanol series (10-100% (v/v)), followed by three different methods of dehydration (Critical point drying (CPC), hexamethyldisilazane (HMDS) and freeze dry) and in the fourth method used powder freeze dried tissue.

For this study, identical amyloplast preservation results were observed in all the four methods used for sample preparation, however, samples obtain by CPD, HMDS and dehydration by freeze-drying were more porous sometimes turning the sample unstable under the electron beam. For this reason, powder obtained by freeze drying the tissue was the method chosen to analyse changes in amyloplast integrity during the storage during the 2nd and 3rd year of this study. This method allowed analysis of greater number of amyloplasts than in the other 3 methods since the amyloplasts were visible outside the cell wall structure.

To ensure no artefacts in the preparation of freeze dried material were interfering with the interpretation of the amyloplast structure at the beginning, middle and end of the 2nd year storage season samples were dehydrated by CPD as well.

3.2.6.1. Fixation with glutaraldehyde and ethanol

Fine slices of tuber (±1 mm thickness) were prepared with a mandoline slicer followed by resizing of material from the central region of the slice ending up with a 1 cm^2 of material used for SEM fixation.

Tissue sections were placed immediately in 2 mL of sodium phosphate (25 mM, pH 7). Tissue fixation was performed using an adaptation of the glutaraldehyde fixation method describe by Talbot and White (2013).

After sodium phosphate buffer removal, a further 2 mL of 3% [v/v] glutaraldehyde solution (prepared in 25 mM sodium phosphate buffer (pH 7)), was added to tissue sections followed by vacuum infiltration (2 hours) until the tissue sank.

Following infiltration, the solution was removed and an additional 2 mL of 3% [v/v] glutaraldehyde was added and left overnight at 4°C.

Tissue were washed 3 x 10 mins in 25 mM sodium phosphate buffer (pH 7), followed by rinsing with distilled water and dehydrated through an ethanol series in 10% increments, starting at 10%, each step lasting 30 min. Once in 100% dry ethanol, samples were exposed to 2 x 30 min changes in 100% dry ethanol, and left in 100% dry ethanol at 4°C, over night until dehydration step in the next day.

3.2.6.1.1. Dehydration using critical point drying (CPD)

Critical point drying (CPC) was performed using a K850 Critical Point Drier (Quorum Technologies Ltd, Ashford, Kent, UK) according to the K850 Critical Point Drier Instruction Manual (Quorumtech, 2012). Table 3-1 provides a summary of the steps performed.

Table 3-1 – K850 Critical Point Drier operation summary (Source: Quorumtech, 2012)

PROCEDURE		TEMPERATURE/TIME PRESSURE
1	Dehydrate specimens Acetone or Ethanol	
2	Ensure all valves are closed.	
3	Pre-cool chamber using cool valve (Blue). Avoid excessive flow and freezing up.	+5°C - 4 Minutes
4	Load specimens and chamber insert ensure thumb-screws correctly tightened. Check chamber is depressurised.	
5	Fill chamber, with meniscus to centre of view window using inlet valve (Green) all other valves closed.	1 Minute
6	Soak (with stirrer if required).	+5°C - 3 Minutes
7	Purge chamber, maintaining meniscus at least mid way on view window using slow, steady flow using balance of inlet valve (Green) and exhaust valve (Black).	1 Minute
8	Soak (with stirrer if required). If necessary - pre-cool chamber using cool valve (Blue). All other valves closed.	+5°C - 3 Minutes
9	(Repeat 7). Checking with filter paper at outlet of exhaust at rear of instrument for damp patch indicating solvent exchange condition.	1 Minute
10	Fill Chamber to centre of chamber to **the Top of the upper red line** Close all valves. Switch on heater. Allow stable conditions.	+35°C - 35 Minutes 1250psi.
11	De-pressurise system: (a) Exhaust valve (Black) - Non delicate or; (b) Bleed valve (Red) - Delicate	100psi/Minute +35°C 10 Minutes 1000cm^3/Minute +35°C 20 Minutes
12	Repeat run. Pre cooling.	8 Minutes +35°C TO 5°C
13	Shut down instrument. (a) Close CO_2 cylinder valve. (b) Open exhaust valve (Black). (c) Open cool valve (Blue). (d) Switch off mains electricity.	

3.2.6.1.2. Dehydration using Hexamethyldisilazane (HMDS)

Hexamethyldisilazane (HMDS), an alternative method to CPD, using an adaptation of the methods from Fischer *et al.* (2012) and Hutchinson (2010).

After the ethanol dehydration series samples were subject to a mixture of 1:1 of HMDS:ethanol for 10 minutes, followed by 2 x 10 minutes in 100% HMDS solution.

3.2.6.1.3. Dehydration by freeze drying

After the ethanol dehydration series (see 3.2.6.1) samples were transferred to the freeze dryer (Supermodulyo 12k freeze dryer, Edwards High Vacuum international, Crawley, West Sussex, England) for 90 minutes.

3.2.6.2. Freeze drying of tissue samples

Samples of tuber cortex were taken from opposite eighths of potatoes using a cork borer (size Nº 5) and from the middle section of the tuber. Each replicate consisted of a composite of 5 tubers; 2 types of tissue were selected from each tuber: samples from apical and stolen end (opposite eighths) were combined to provide an average of variation of the extreme amyloplast profiles across the tuber and an additional sample was taken from mid section of the tuber. Five replicates were used for each variety.

Before grinding to a fine powder in a pestle and mortar samples were frozen immediately at -80°C and subject to 48 hours freeze drying as above (3.2.1.).

3.2.7. Determination of mineral accumulation in tubers

Minerals are important dietary component, certain minerals (P, S, Ca, Cu, Mg, Zn and K) are correlated with after-cooking darkening and acrylamide formation in potato (LeRiche *et al.*, 2009; Whittaker *et al.*, 2010). For that reason, mineral content was determined during the 2nd year of this work.

3.2.7.1. Mineral analyses

Mineral analysis was performed by “Inductivity Coupled Plasma, Optical Emission Spectrometer” (ICP-OES) and “Atomic Absorption Spectrometer (AAS), in collaboration with School of Science (University of Greenwich / Medway).

Samples of tuber cortex were taken from opposite eighths of potato using a cork borer (size Nº 5). Each replicate consisted of a composite of 5 tubers; 2 types of tissue were selected from each tuber: samples from apical and stolen end (opposite eigths) were combined to provide an average of the extremes in mineral profiles across the tuber and an additional sample was taken from mid section of the tuber, with five replicates per variety for each sampling occasion.

Samples were snap frozen in liquid nitrogen before storage at -80°C and subject to 48 hours freeze drying before grinding to a fine powder with a pestle and mortar. 0.25 g of sample was added to 5 mL concentrated nitric acid (HNO_3 70%) and 0.25 mL hydrogen peroxide (H_2O_2) was added. Samples were digested in a microwave accelerated reaction system (CEM MARS 6 240/50, Matthews, Inc, USA). The programme was set to a maximum pressure of 400 psi, 1200 W of power for 20 minutes and maximum temperature 190°C to ensure maximum digestion, and hydrolysis of organic materials, in order to facilitate samples analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES). The digested samples were diluted to 50 mL with deionised water before proceeding with the analysis.

ICP-OES (PerkinElmer, UK) was calibrated using seven multi-element calibration solutions. ICP-MS standards for calcium (Ca), potassium (K), magnesium (Mg), iron (Fe), copper (Cu), phosphorus (P) and zinc (Zn) were prepared by diluting HNO_3 5% in 50 mL plastic volumetric flasks providing a serial dilution range of standards from 0.05 µL L^{-1}, 0.25 µL L^{-1}, 0.5 µL L^{-1}, 0.75 µL L^{-1}, 1 µL L^{-1}, 1.25 µL L^{-1} and 1.5 µL L^{-1} (for Ca, Mg, Fe, Zn, Cu and P), and dilutions from 1.5 µL L^{-1}, 2.5 µL L^{-1}, 3.5 µL L^{-1}, 4.5 µL L^{-1}, 5.5 µL L^{-1}, 6.5 µL L^{-1}, 7.5 µL L^{-1} and 8.5 µL L^{-1} (for K). A calibration curve was constructed over a range of seven and eight concentrations, for K determination, using ICP-OES software. A threshold of 0.99 is required for a coefficient correlation of calibration curves for each element (Mindak *et al.*, 2006). Mineral analysis data was

subject to correction factors to take into account samples weights and dilutions. The final mineral content was calculated as mg^{-1}100g dry weight (DW).

3.2.7.2. Calcium oxalate extraction

For cold oxalate extraction, the method of Al-Wahsh *et al.* (2012) modified by Mirzaee (2015) was used to measure total oxalate content of potato tuber samples, total Ca^{2+} was measured by ICP-OES.

Samples of tuber cortex were taken from opposite eighths of potato using a cork borer (size Nº 5). Each replicate consisted of a composite of 5 tubers; 2 types of tissue were selected from each tuber: samples from apical and stolen end (opposite eigths) were combined to provide an average of the extremes in calcium oxalate ($Ca(COO)_2$) profiles across the tuber and an additional sample was taken from mid section of the tuber, with five replicates per variety for each sampling occasion.

Samples were snap frozen in liquid nitrogen before storage at -80°C and subject to 48 hours freeze drying before grinding to a fine powder with a pestle and mortar.

In 15 mL centrifuge tubes, 1 g of each sample was weighed and 5 mL of 2N HCl added, then centrifuged at 3381 g for 10 minutes. The supernatant was transferred to 25 mL volumetric flasks. This process was repeated two more times by adding 5 mL of 2N HCl to the pellet to extract the remaining oxalate. The final volume of supernatant from the three successive extractions was diluted to 25 mL with distilled deionised water. Oxalate extracts were filtered with 20 mL syringes through 0.45 µm filters.
Before analysing for extracted oxalate, the AAS (Thermo Fisher Scientific ICE 3300, US) was calibrated with a range of eight calcium calibration solutions prepared using a calcium standard (Inorganic Ventures, US) diluted by 5% HNO_3 in 50 mL plastic volumetric flasks from 1000 µL L^{-1} to a final concentration range 1, 2, 3, 4, 5, 6, 8 and 1 mL L^{-1}.
Samples were aspirated under ASS transforming solutions into an aerosol; absorption spectrometry determined the concentration of the calcium in the sample as calcium oxalate.

The amount of calcium extracted from oxalate ($Ca(COO)_2$) measured by this technique was subtracted from total calcium (Ca_{total}) obtained with the ICP-OES to find the amount of bound calcium (Ca_{bound}) and the bound calcium as a percentage of total calcium in each sample according to the following Equation 5:

Equation 5

$$1 - \frac{Ca_{total} - Ca_{bound}}{Ca_{total}} * 100$$

3.2.8. Data Analyses

Data analysis and graphical outputs were performed using Microsoft Excel 2013 and Rstudio (R Core Team, 2014).

ANOVA (analyses of variance) was used to perform an analysis of the relative contributions from explained and unexplained sources of variance in a continuous response variable. Significant effects were tested with the F statistic, which assumes random sampling of independent replicates, homogeneous within-sample variances, and a normal distribution of the residual error variation around sample means (Doncaster and Davey, 2007). ANOVA was carried out to determine whether there were significant differences between samples with one up to four factors (ROS), one up to two factors (AsA, DHA and total vitamin C) and one up to three factors (rest of the experiments) using Rstudio (R Core Team, 2014).

To determine significantly differences between means Tukey's HSD (honest significant difference) test (TukeyHSD) was carried out using the “agricolae” package from Rstudio (R Core Team, 2014), following "A statistical analysis tool for agricultural research", National Engineering University (UNI), Lima-Peru (https://CRAN.R-project.org/package=agricolae, 27-04-2017).

Correlations were determined using Pearson tests ($p<0.05$) were performed using GGally package from Rstudio (R Core Team, 2014). GGally package is an extension package "ggplot2" (a system to create graphs), (https://CRAN.R-project.org/package=GGally, 27-04-2017). Correlation coefficients from Lindley and Scott (1995). Differences in the outputs between the 3 years of data were analysed, with correlations between changes in physiological factors, between VR 808 and L. Rosetta over the 3 consecutive years, while comparison of P. Dell and R. Burbank were over 2 consecutive years analyse, while a single year's data exits for the analysis of data for the effect of chitting on post-harvest quality.

3.3. Results

3.3.1. Assessment of tuber respiration, ethylene production, sprout growth and sugar accumulation during storage

Overall effect of varieties

Over the 3 years of this study an increase in %FW of reducing sugars and sucrose was observed during storage across all varieties. As sugars increased respiration rates of tubers increased.

There was a significant effect of the variety in the %FW of reducing sugars (sum of fructose and glucose) and individually with fructose, glucose and sucrose during the time of this study ($p<0.001$). The sugar profiles over time were significantly different between varieties. P. Dell accumulated the highest concentration of reducing sugars (0.043 %FW), followed by R. Burbank (0.020 %FW) and L. Rosetta (0.013 %FW) while VR 808 (0.003 %FW), accumulated the least sugars. P. Dell was the variety with the highest concentration of fructose and glucose and VR 808 with the lowest. All the varieties were significantly different from each other according the Tukey HSD test ($HSD_{0.05} = 0.001$ for %FW of fructose and $HSD_{0.05} = 0.001$ for %FW of glucose).

While the ranking in sucrose accumulation did not follow the same order, P. Dell (0.025 %FW) was the variety with the highest accumulation, followed by L. Rosetta (0.020

%FW), and with VR 808 (0.012 %FW) and R. Burbank (0.012% FW) having the lowest sucrose content.

P. Dell and L. Rosetta tubers had the highest O_2 consumption during storage, while VR 808 had the lowest O_2 consumption. Similarly, with CO_2 production P. Dell had the highest production and VR 808 with the lowest.

No varietal effect on ethylene production was observed during storage, L. Rosetta had a 3 year mean of 0.04 nL g^{-1} h^{-1}, VR 808 had a 3 year mean of 0.03 nL g^{-1} h^{-1}, P. Dell and R. Burbank had a 2 year mean of 0.02 nL g^{-1} h^{-1} ($HSD_{0.05}$ = 0.024).

As expected sprout growth increased during storage ($p < 0.001$), growth rates were interrupted by the application of CIPC. There was significant variety effect in the sprout growth rate ($p < 0.001$). L. Rosetta had a significantly higher rate of sprout growth compared to the other varieties.

Overall effects of seasons

Variability in reducing sugars was seen between years with the highest concentration found in year 2 for L. Rosetta, in the final year the lowest concentration was found ($p < 0.001$). While sucrose content remained stable. The opposite situation was observed with VR 808 variety, reducing sugars content remained stable, while sucrose content varied ($p < 0.001$). In L. Rosetta O_2 consumption rates over the 3 years were stable. However, for VR 808 O_2 consumption and the CO_2 production for both varieties, varied significantly between years ($p < 0.001$). It was notice that the increase in respiration rates accompanied the increase in sugars accumulation.

For all the varieties, ethylene production ($p < 0.001$) and sprout growth ($p < 0.001$) varied between seasons. For ethylene production, on average, 1st year had the highest ethylene production (0.06 nL g^{-1} h^{-1}). Significantly different from ($p < 0.05$) the 2nd (0.01 nL g^{-1} h^{-1}) and 3rd (0.03n L g^{-1} h^{-1}) years ($HSD_{0.05}$ = 0.021). Sprout growth appeared to be affected by the CIPC treatments efficacy. Across all varieties variation in sprout growth was observed between storage seasons and between varieties ($p < 0.001$), in VR 808 and L. Rosetta with higher sprout growth in the 3rd year and lower in the 1st year. And in P. Dell and R. Burbank with higher sprout growth in the 2nd year.

Interaction of varieties, location and seasons

The interaction between planting location and the accumulation of reducing sugars for VR 808 and L. Rosetta in (2013/14) was significant for L. Rosetta ($p < 0.05$) but not for VR 808. Lady Rosetta grown in Shropshire (0.0132 %FW of RS) and Norfolk (0.0127 %FW of RS) was significantly higher than Yorkshire grown material (0.011 %FW of RS), ($HSD_{0.05} = 0.0019$). However, for sucrose planting site had effect on VR 808 ($p < 0.05$) but not for L. Rosetta. Yorkshire grown VR 808 (0.010 %FW sucrose) was significant lower from Norfolk (0.012 %FW of sucrose), while Shropshire grown material (0.011 %FW of sucrose) was not significant different from either ($HSD_{0.05} = 0.002$).

For individual fructose and glucose content, planting site did not had effect on VR 808. However, in L. Rosetta %FW of glucose ($p < 0.001$) was affected by growing location, Yorkshire grown material (0.006 and 0.005 %FW of fructose and glucose, respectively) was significant greater than Shropshire (0.006 and 0.007 %FW of fructose and glucose, respectively) and Norfolk (0.006 and 0.007%FW of fructose and glucose, respectively, $HSD_{0.05} = 0.001$).

Respiration rates of tubers grown at different locations were not affected by planting location for VR 808. However, for L. Rosetta, planting site had a significant effect on CO_2 production ($p < 0.05$). Shropshire (1.1 mL g^{-1} h^{-1}) was significant different lower than Norfolk (1.3 mL g^{-1} h^{-1}), but Yorkshire (1.2 mL g^{-1} h^{-1}) was not significant different from both ($HSD_{0.05} = 0.186$).

Planting site had a significant effect on ethylene production in VR 808 ($p < 0.01$) and no significant effect on L. Rosetta. Norfolk (0.082 nL g^{-1} h^{-1}) was significant higher from Shropshire (0.035 nL g^{-1} h^{-1}) and Yorkshire (0.045 nL g^{-1} h^{-1}, $HSD_{0.05} = 0.025$).

Even though tubers were treated with CIPC, residual sprouting was influenced by planting site in L. Rosetta ($p < 0.01$), growth in Shropshire (0.709 mm) was significant higher than in Norfolk (0.559 mm), and Yorkshire (0.596 mm) ($HSD_{0.05} = 0.122$), where there was no significant effect in VR 808.

Changes in tuber physiology during storage

Storage season of December 2013 to July 2014

Respiration rate in 2013/14 increased during storage with a rise in CO_2 production and O_2 consumption concomitantly with an increase in fructose and glucose (%FW) content of tubers ($p < 0.001$) for L. Rosetta, P. Dell and VR 808 and this was observed alongside rise in sucrose. While CO_2 production for R. Burbank increased alongside a rise in fructose ($p < 0.05$) and % FW of sucrose ($p < 0.001$). Length of storage had significant effect of ethylene production ($p < 0.001$) for VR 808 but no significant effect for L. Rosetta, P. Dell and R. Burbank (Appendix VI).

VR 808 tubers retained a low sugar profile for over 7 months of storage (May) (Appendix II) where fructose, glucose and sucrose remained low for over 7 months of storage at 10°C, thereafter sugar profiles had a slight increased (Table 3-2). A temporary dip in respiration rates were observed prior to the extended rise in respiration rate which denotes the onset of sugar accumulation (Figure 3-2 and Table 3-3) creating a U shape curve in both O_2 consumption and CO_2 production. However, the intensity and timing of the rise in O_2 consumption and CO_2 production, and therefore the shape of the curves was site dependent.

L. Rosetta tends to have shorter storage-life, with the rise in glucose developing after 4 months (Appendix II) of storage at 10°C (March) (Figure 3-3 and Table 3-2). A similar, U shape curve in both O_2 consumption and CO_2 production was observed (Figure 3-3). Again, the timing of the rise and rate in O_2 consumption and CO_2 production was site dependent; with tubers harvested from the Norfolk site showing earlier signs of heightened O_2 consumption (Table 3-3). Sucrose and fructose accumulated at a slower rate, then respiration with an increase from base line measurements recorded after 7 months (May 2014) (Figure 3-3 and Table 3-2).

The relationship with changes in tuber respiration and changes in the reducing sugar and sucrose profile was dependant on variety. A significant increase in fructose and glucose (Figure 3-4) was observed from May (> 7 months in storage) (Appendix III) in

P. Dell (Table 3-2) while sucrose started to increase from January (> 3 months storage) (Figure 3-4 and Table 3-2). At the same time a rise in O_2 consumption was observed until the end of the storage season (U shape curve). In January (> 3 months in storage) CO_2 production started to increase. However, there was no such difference between December (2 months is storage) and January CO_2 production levels as the ones observe for O_2 consumption (Table 3-3).

In R. Burbank, no significant difference between the length storage months was observed for the total %FW of reducing sugars as well for glucose accumulation (Figure 3-5 and Table 3-2). However, there was a significant difference between months for sucrose (Figure 3-5) and fructose accumulation (Table 3-2). In this variety, the levels of sucrose started to increase from January (> 3 months in storage) (Appendix III) and fructose levels from May (> 7 months in storage) (Table 3-2). In R. Burbank, no U shape curve was observed for O_2 consumption and CO_2 production. Instead a gradual increase in the respiration rates during storage (Table 3-3) was observed.

Table 3-2 - %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) by sampling month for the 1st year (2013/14).

		Means			
Variety	**Month**	**RS**	**Sucrose**	**Fructose**	**Glucose**
L. Rosetta	**December**	0.001^{d}	0.009^{c}	0.0005^{c}	0.001^{d}
	January	0.002^{d}	0.010^{c}	0.001^{c}	0.001^{d}
	March	0.007^{c}	0.015^{c}	0.002^{c}	0.004^{c}
	May	0.022^{b}	0.024^{b}	0.011^{b}	0.011^{b}
	July	0.030^{a}	0.034^{a}	0.016^{a}	0.014^{a}
	***HSD*$_{0.05}$**	*0.003*	*0.009*	*0.002*	*0.002*
VR 808	**December**	0.001^{c}	0.009^{c}	0.0004^{c}	0.001^{b}
	January	0.001^{c}	0.006^{c}	0.0002^{c}	0.001^{b}
	March	0.002^{bc}	0.007^{c}	0.0003^{c}	0.002^{ab}
	May	0.004^{b}	0.014^{b}	0.002^{b}	0.002^{a}
	July	0.008^{a}	0.019^{a}	0.004^{a}	0.003^{a}
	***HSD*$_{0.05}$**	*0.002*	*0.003*	*0.001*	*0.002*
P. Dell	**December**	0.021^{c}	0.010^{c}	0.009^{c}	0.012^{b}
	January	0.018^{c}	0.015^{b}	0.008^{c}	0.010^{b}
	May	0.056^{b}	0.029^{a}	0.024^{b}	0.031^{a}
	July	0.071^{a}	0.033^{a}	0.034^{a}	0.037^{a}
	***HSD*$_{0.05}$**	*0.014*	*0.004*	*0.006*	*0.008*
R. Burbank	**December**	0.026^{a}	0.009^{c}	0.011^{ab}	0.016^{a}
	January	0.022^{a}	0.011^{bc}	0.008^{b}	0.014^{a}
	May	0.025^{a}	0.012^{ab}	0.010^{ab}	0.014^{a}
	July	0.030^{a}	0.014^{a}	0.013^{a}	0.017^{a}
	***HSD*$_{0.05}$**	*0.009*	*0.002*	*0.004*	*0.005*

Mean values with different letters are significantly different according to the Tukey HSD test.

Table 3-3 - O_2 consumption (O_2 , mL $g^{-1}h^{-1}$), CO_2 production (CO_2, mL $g^{-1}h^{-1}$), ethylene production (Ethylene, nL $g^{-1}h^{-1}$) and sprout growth (sprout, mm) by sampling month for the 1st year (2013/14).

		Means			
Variety	**Month**	**O_2**	**CO_2**	**Ethylene**	**Sprout**
L. Rosetta	**December**	2.28^{b}	0.95^{cd}	0.06^{a}	0^{c}
	January	1.98^{b}	0.97^{c}	0.10^{a}	0.87^{a}
	March	2.60^{b}	0.70^{d}	0.04^{a}	0.78^{ab}
	May	3.79^{ab}	1.38^{b}	0.07^{a}	0.68^{b}
	July	5.36^{a}	3.09^{a}	0.01^{a}	0.75^{ab}
	$HSD_{0.05}$	*2.13*	*0.31*	*0.11*	*0.12*
VR 808	**December**	3.09^{ab}	0.83^{c}	0.04^{b}	0^{c}
	January	2.26^{bc}	0.98^{c}	0.12^{a}	0.74^{a}
	March	1.97^{c}	0.59^{d}	0.04^{b}	0.65^{ab}
	May	3.75^{a}	1.73^{b}	0.03^{b}	0.10^{c}
	July	3.96^{a}	2.19^{a}	0.02^{b}	0.62^{b}
	$HSD_{0.05}$	*1.16*	*0.27*	*0.04*	*0.07*
P. Dell	**December**	4.15^{ab}	0.76^{a}	0.03^{a}	0^{c}
	January	2.53^{b}	0.83^{a}	0.14^{a}	0.92^{a}
	May	4.41^{ab}	2.33^{a}	0.04^{a}	0.29^{b}
	July	5.05^{a}	2.59^{a}	0.01^{a}	0.66^{a}
	$HSD_{0.05}$	*2.18*	*1.92*	*0.66*	*0.27*
R. Burbank	**December**	2.16^{a}	0.77^{b}	0.03^{a}	0^{b}
	January	3.29^{a}	0.88^{b}	0.09^{a}	0.60^{a}
	May	3.78^{a}	1.97^{a}	0.03^{a}	0.62^{a}
	July	3.97^{a}	2.45^{a}	0.01^{a}	0.64^{a}
	$HSD_{0.05}$	*10.24*	*1.07*	*0.35*	*0.22*

Mean values with different letters are significantly different according to the Tukey HSD test.

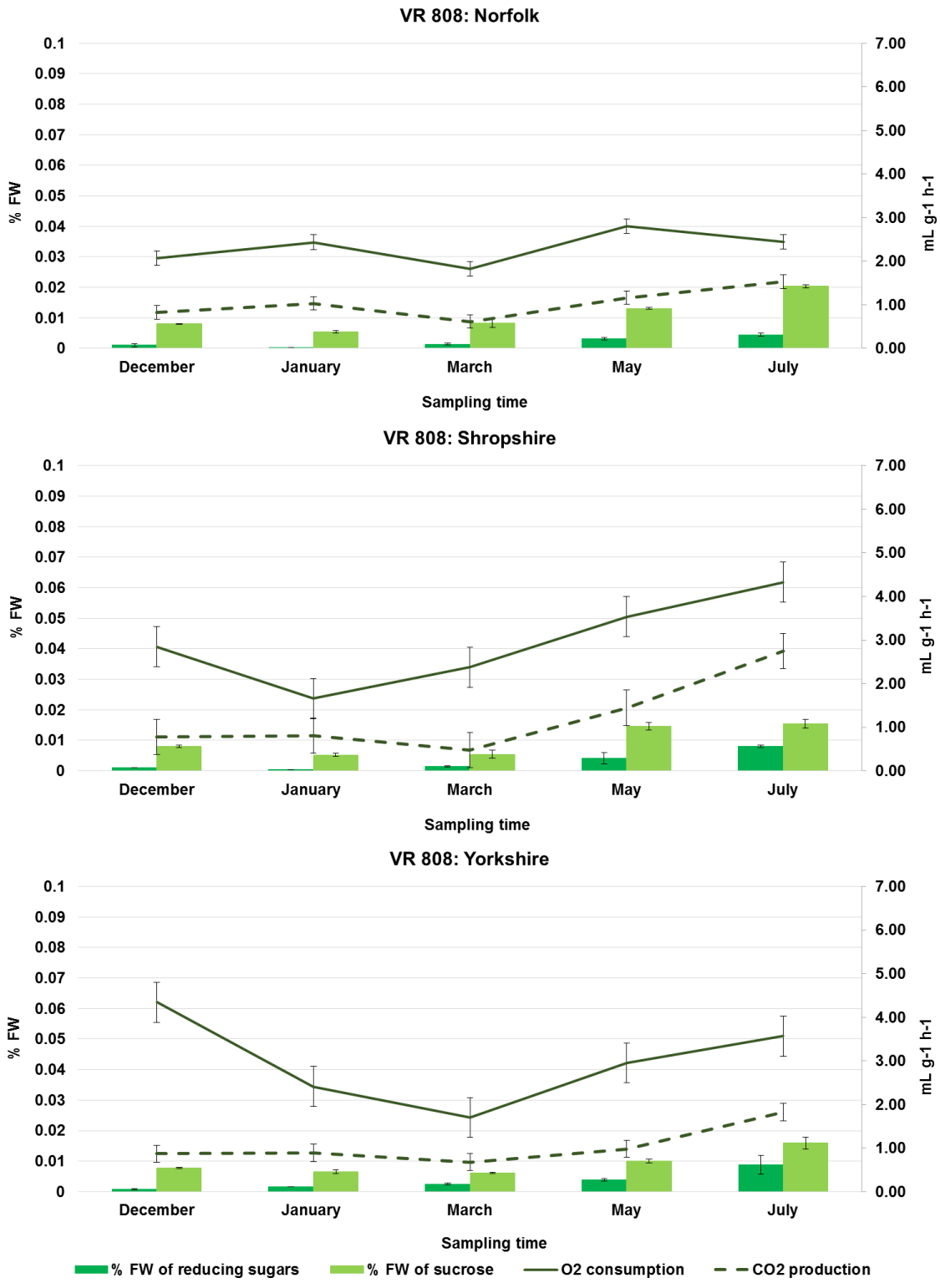

Figure 3-2 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 1st year of the study (season 2013/14) from VR 808, with SE bars. For stats see Table 3-2 and Table 3-3.

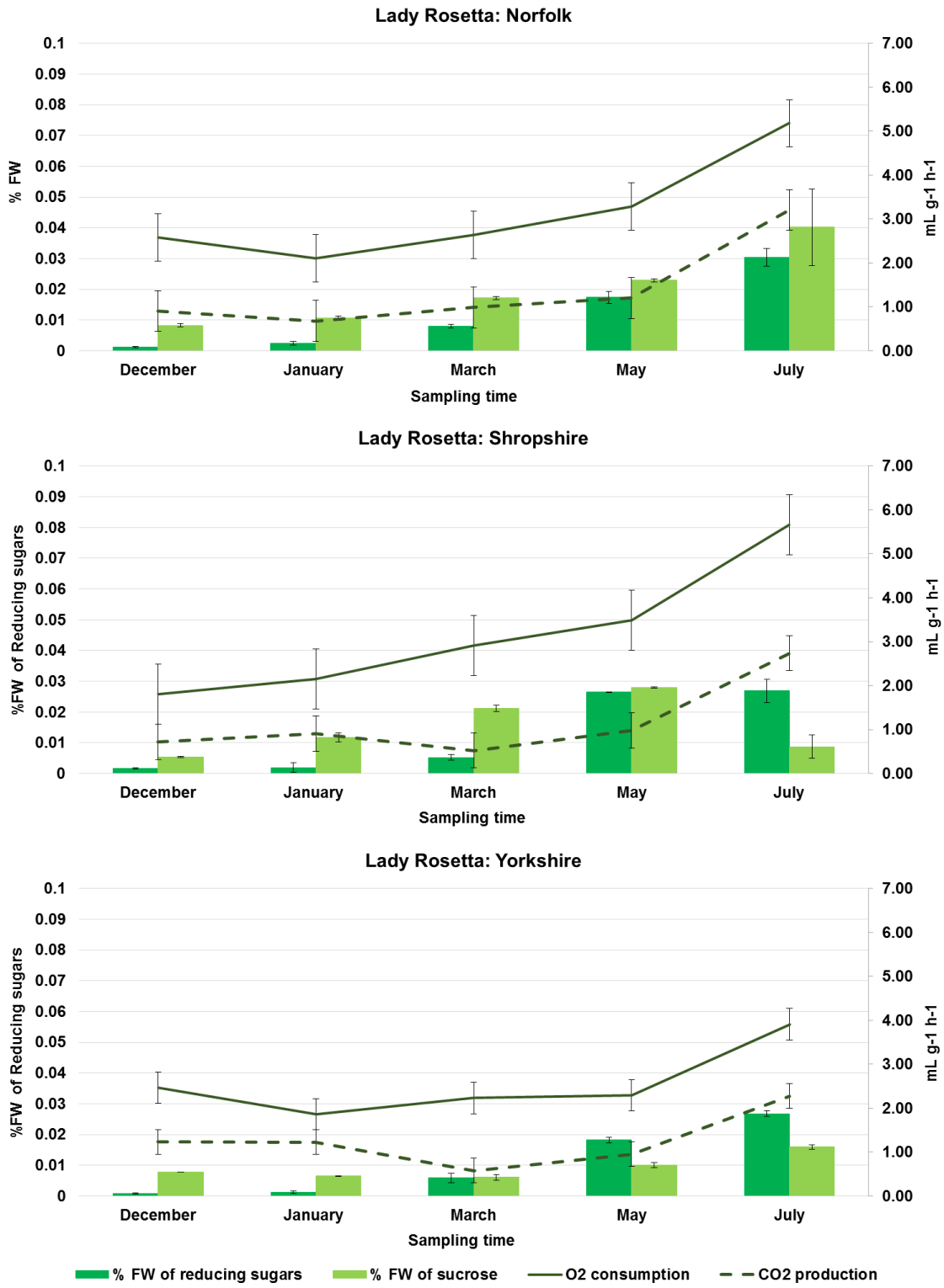

Figure 3-3 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 1st year of the study (season 2013/14) from L. Rosetta, with SE bars. For stats see Table 3-2 and Table 3-3.

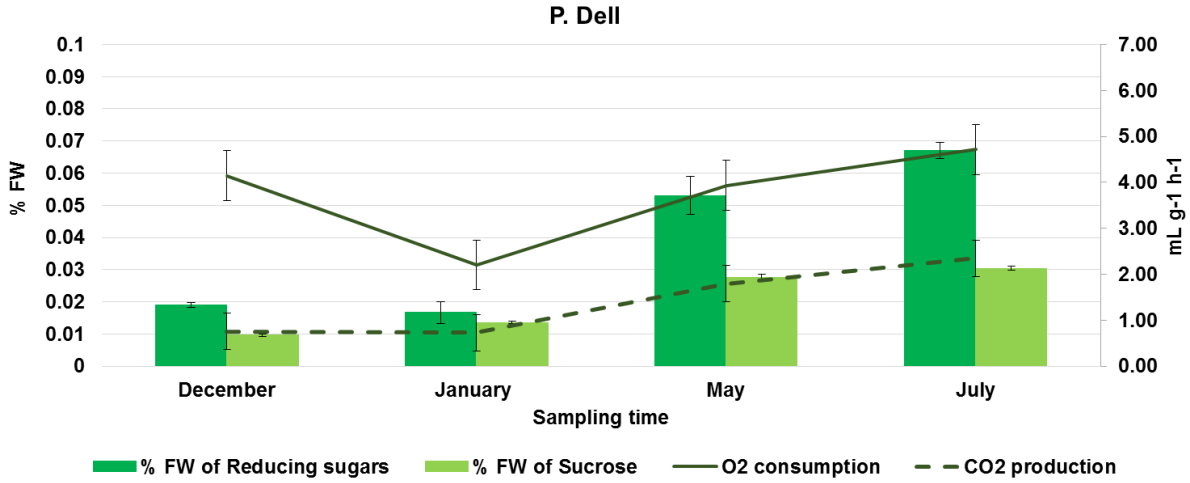

Figure 3-4 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 1st year of the study (season 2013/14) for P. Dell, with SE bars. For stats see Table 3-2 and Table 3-3.

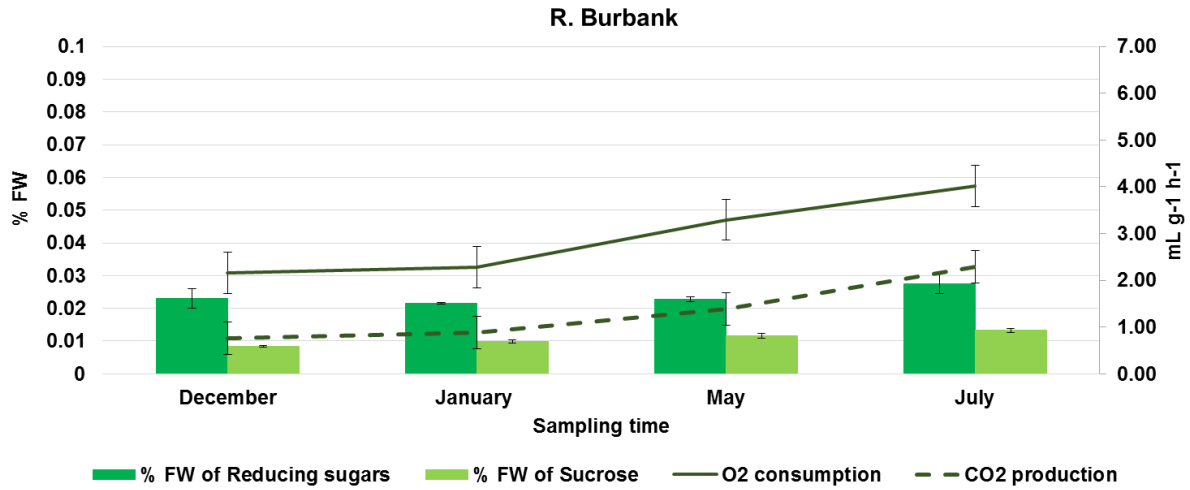

Figure 3-5 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 1st year of the study (season 2013/14) for R. Burbank, with SE bars. For stats see Table 3-2 and Table 3-3.

Storage season November 2014 to July 15

Across all varieties, a general trend in sugar and respiration rates were observed during storage, with a reduction in reducing sugars and sucrose from harvest over the first 5 months of storage (Appendix IV) mirrored by a reduction in respiration rates. Thereafter, sugars rise and respiration rate increased. The magnitude of these changes was variety dependant.

The length of apical sprouts correlates with increases in respiration rates and a rise in glucose, fructose and sucrose during storage and while tubers were treated with CIPC, sprout regrowth were linked to increases in respiration for all the varieties (Table 3-4 and Table 3-5). P. Dell was the only variety where ethylene production increased during storage time (Table 3-5). Patterns of O_2 consumption and CO_2 production were variety dependant.

During this season, the reducing sugar content of VR 808 was very low (0.002 % FW) at harvest and remained low during all but the final sampling point (June), where small rise in glucose content was recorded, however, this was below the standard commercial threshold of 0.2% FW (Figure 3-6).

O_2 consumption for VR 808 was lower than the other varieties and remained low during storage (Figure 3.10 and Table 3-4). Sucrose content dipped from 0.015% FW at harvest to <0.01% FW for the first 4-5 months during storage followed by a slow increase in sucrose from April through to July. A small rise in CO_2 production was observed from March onwards and remained at a similar rate thereafter (Figure 3-6 and Table 3-4).

In contrast, to VR 808 reducing sugar content of L. Rosetta was higher (0.009 % FW) at harvest (November) and increased significantly in tubers from April (7 months storage) through to July. An increase in reducing sugars was mirrored with an increase in respiration rate which was significantly higher than tubers of VR 808. CO_2 production at harvest was 2 mL g^{-1} h^{-1} and reached 5.5 mL g^{-1} h^{-1} by the end of almost 10 month's storage at 10°C. O_2 consumption followed a similar patter but was significantly lower

than CO_2 production. Changes in reducing sugar content was concomitant with an increase in reducing sugars accumulation and sprout growth (Table 3-4 and Table 3-5).

CIPC application (Appendix I) at harvest and again in the last application in March caused a reduction in demand for reducing sugars and a temporary decline in respiration rate (Figure 3-7 and Table 3-4) probably correlated with sprout growth (Table 3-5).

Sucrose content in L. Rosetta at harvest was similar (0.016 %FW) to VR 808, however, in L. Rosetta no decline in sucrose was observed during the early stages of storage and rose significantly from April (7 months) (Appendix IV) onwards (Figure 3-7 and Table 3-4).

P. Dell reducing sugar and sucrose content was significantly higher that L. Rosetta and VR 808 at harvest. A decline in sucrose and reducing sugars was observed during the initial 4 months of storage, and then significant increase in reducing sugars in April samples (6 months) was observed, rising above 0.2 %FW, peaking in June and July at 0.09 %FW. Sucrose content also showed a similar profile but concentrations were lower with maximum sucrose content (0.045 % FW) observed in June and July (Figure 3-8). CO_2 production was higher than L. Rosetta and VR 808 at harvest (3.5 mL g^{-1} h^{-1}) (Table 3-5), there was a decrease in both in April, and remained constant during the first 5 months of storage followed by a dip before respiration rates increased from April (6 months) onwards which corresponded to an increase in total %FW of reducing sugars and sucrose (Figure 3-8).

R. Burbank exhibited a stable reducing sugar profile during storage with reducing sugars remaining below 0.2 %FW (Figure 3.13). Unlike VR 808, CO_2 production was higher at harvest 3.5 mL g^{-1} h^{-1} before dipping to 2.5 ml g^{-1} h^{-1} after 6 months' storage (April) in line with a small reduction in reducing sugars and sucrose, thereafter a rise in CO_2 production was observed rising to 4.5 ml g^{-1} h^{-1} by 8 months while reducing sugars and sucrose remained <0.02 %FW. O_2 consumption remained significantly below CO_2 output for the first 6 months of storage before rising in line with sugars but at lower rates than CO_2.

Table 3-4 - %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) by sampling month for the 2nd year (2014/15)

		Means			
Variety	**Month**	**RS**	**Sucrose**	**Fructose**	**Glucose**
L. Rosetta	**November**	0.009^{cd}	0.016^{d}	0.008^{b}	0.001^{c}
	January	0.010^{cd}	0.012^{d}	0.010^{b}	0.001^{c}
	March	0.003^{d}	0.015^{d}	0.002^{c}	0.001^{c}
	April	0.013^{bc}	0.029^{b}	0.010^{b}	0.003^{c}
	May	0.019^{b}	0.024^{c}	0.011^{b}	0.007^{b}
	June	0.045^{a}	0.037^{a}	0.024^{a}	0.021^{a}
	July	0.040^{a}	0.039^{a}	0.022^{a}	0.018^{a}
	HSD$_{0.05}$	*0.008*	*0.004*	*0.005*	*0.004*
VR 808	**November**	0.003^{b}	0.016^{b}	0.002^{a}	0.002^{ab}
	January	0.006^{ab}	0.005^{d}	0.004^{a}	0.002^{ab}
	March	0.003^{b}	0.006^{d}	0.002^{a}	0.001^{ab}
	April	0.002^{b}	0.012^{c}	0.002^{a}	0.001^{b}
	May	0.002^{b}	0.012^{c}	0.002^{a}	0.001^{ab}
	June	0.004^{ab}	0.019^{a}	0.002^{a}	0.002^{ab}
	July	0.008^{a}	0.018^{ab}	0.005^{a}	0.003^{a}
	HSD$_{0.05}$	*0.004*	*0.003*	*0.004*	*0.002*
P. Dell	**November**	0.031^{c}	0.022^{c}	0.018^{c}	0.013^{c}
	January	0.007^{d}	0.011^{d}	0.006^{b}	0.001^{d}
	March	0.008^{d}	0.014^{d}	0.007^{b}	0.001^{d}
	April	0.033^{c}	0.037^{b}	0.021^{c}	0.012^{c}
	May	0.055^{b}	0.034^{b}	0.031^{b}	0.024^{b}
	June	0.089^{a}	0.047^{a}	0.044^{a}	0.045^{a}
	July	0.091^{a}	0.048^{a}	0.045^{a}	0.047^{a}
	HSD$_{0.05}$	*0.014*	*0.007*	*0.009*	*0.006*
R. Burbank	**November**	0.008^{bc}	0.017^{a}	0.005^{ab}	0.002^{cd}
	January	0.008^{bc}	0.012^{c}	0.006^{ab}	0.002^{cd}
	March	0.006^{c}	0.008^{e}	0.004^{ab}	0.001^{d}
	April	0.011^{ab}	0.007^{e}	0.007^{ab}	0.004^{b}
	May	0.007^{bc}	0.011^{d}	0.004^{b}	0.003^{bc}
	June	0.013^{a}	0.015^{b}	0.007^{ab}	0.007^{a}
	July	0.014^{a}	0.014^{bc}	0.007^{a}	0.007^{a}
	HSD$_{0.05}$	*0.004*	*0.002*	*0.001*	*0.001*

Mean values with different letters are significantly different according to the Tukey HSD test.

Table 3-5 - O_2 consumption (O_2 , mL $g^{-1}h^{-1}$), CO_2 production (CO_2, mL $g^{-1}h^{-1}$), ethylene production (Ethylene, nL $g^{-1}h^{-1}$) and sprout growth (sprout, mm) by sampling month for the 2nd year (2014/15)

		Means			
Variety	**Month**	**O_2**	**CO_2**	**Ethylene**	**Sprout**
L. Rosetta	**November**	1.93^{c}	0.59^{d}	0.02^{a}	2.48^{a}
	January	2.70^{bc}	1.11^{c}	0.01^{a}	1.46^{ab}
	March	3.74^{ab}	2.13^{b}	0.005^{a}	1.55^{ab}
	April	2.79^{bc}	1.00^{cd}	0.017^{a}	0.92^{b}
	May	3.75^{ab}	1.76^{b}	0.002^{a}	2.60^{a}
	June	4.58^{a}	2.26^{b}	0.02^{a}	2.00^{ab}
	July	5.36^{a}	3.98^{a}	0.01^{a}	1.998^{ab}
	$HSD_{0.05}$	*1.79*	*0.52*	*0.03*	*1.50*
VR 808	**November**	1.55^{a}	0.52^{c}	0.01^{a}	0^{d}
	January	1.92^{a}	0.92^{bc}	0.01^{a}	0.60^{c}
	March	2.16^{a}	1.40^{ab}	0.01^{a}	0.66^{bc}
	April	1.86^{a}	0.84^{bc}	0.01^{a}	0.65^{bc}
	May	2.27^{a}	1.28^{ab}	5.00E-04^{a}	0.77^{ab}
	June	2.17^{a}	1.13^{abc}	0.02^{a}	0.90^{a}
	July	2.52^{a}	1.84^{a}	0.004^{a}	0.86^{a}
	$HSD_{0.05}$	*1.17*	*0.75*	*0.03*	*0.14*
P. Dell	**November**	3.55^{abc}	2.02^{bc}	0.01^{ab}	0^{d}
	January	3.28^{bc}	1.91^{bcd}	0.01^{ab}	0.60^{c}
	March	3.57^{abc}	2.20^{bc}	0.02^{a}	0.61^{d}
	April	2.54^{c}	1.27^{d}	0.01^{ab}	0.67^{bc}
	May	2.84^{c}	1.71^{cd}	0^{b}	0.86^{b}
	June	4.17^{ab}	2.48^{b}	0.002^{ab}	1.23^{a}
	July	4.81^{a}	3.40^{a}	0.01^{ab}	1.14^{a}
	$HSD_{0.05}$	*1.32*	*0.74*	*0.02*	*0.25*
R. Burbank	**November**	3.19^{bc}	1.10^{c}	0.03^{a}	0^{c}
	January	2.68^{bc}	1.15^{c}	0.004^{a}	0.10^{c}
	March	2.27^{bc}	1.09^{c}	0.004^{a}	0.53^{b}
	April	2.16^{c}	1.24^{bc}	0.01^{a}	0.53^{b}
	May	2.61^{bc}	1.52^{bc}	0.02^{a}	0.60^{b}
	June	3.61^{ab}	1.97^{b}	0.01^{a}	1.07^{a}
	July	4.64^{a}	3.13^{a}	0.004^{a}	1.23^{a}
	$HSD_{0.05}$	*1.30*	*0.74*	*0.03*	*0.34*

Mean values with different letters are significantly different according to the Tukey HSD test.

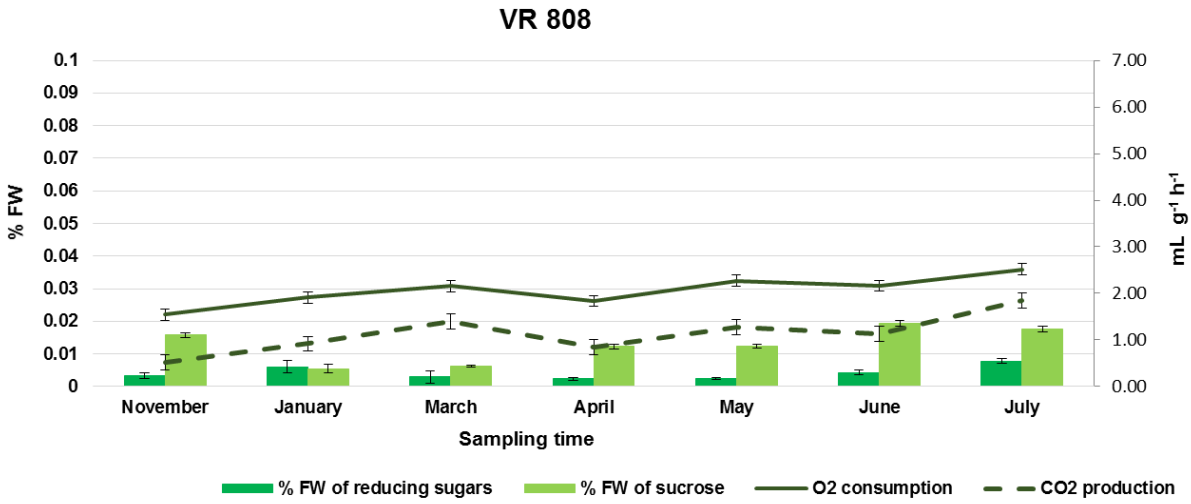

Figure 3-6 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 2nd year of the study (season 2014/15) for VR 808, with SE bars. For stats see Table 3-4 and Table 3-5.

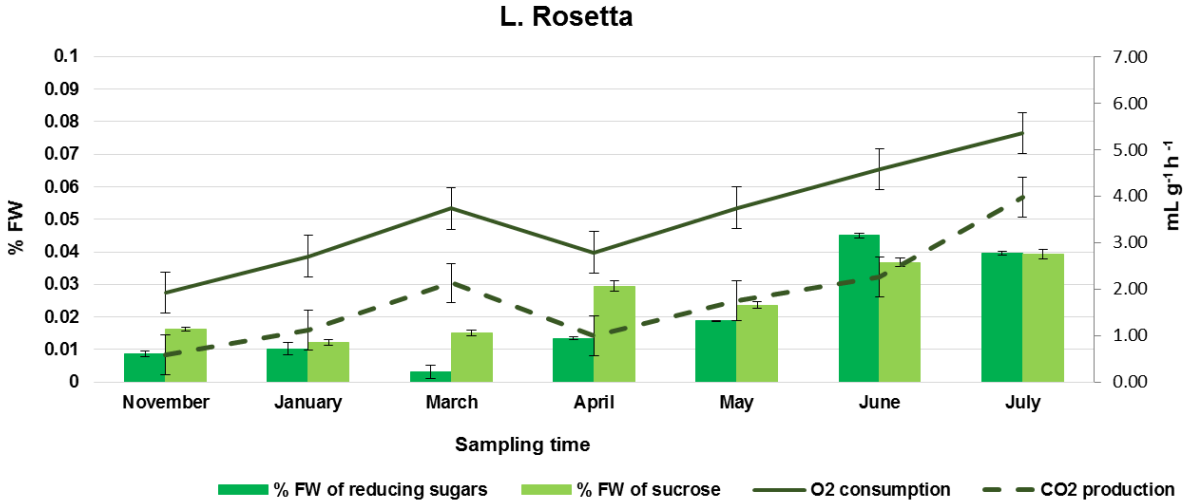

Figure 3-7 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 2nd year of the study (season 2014/15) for L. Rosetta, with SE bars. For stats see Table 3-4 and Table 3-5.

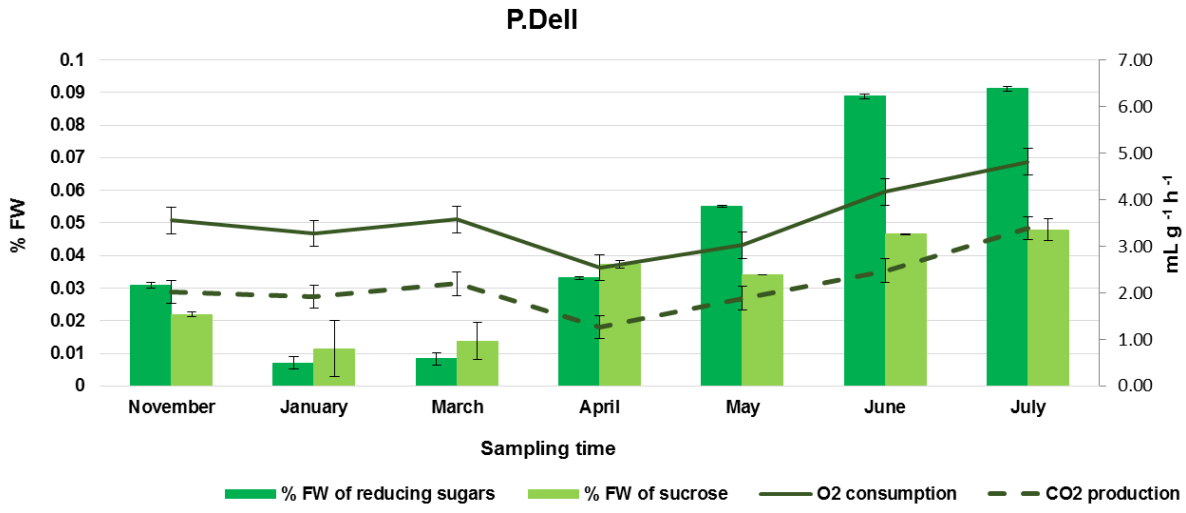

Figure 3-8 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 2nd year of the study (season 2014/15) for P. Dell, with SE bars. For stats see Table 3-4 and Table 3-5.

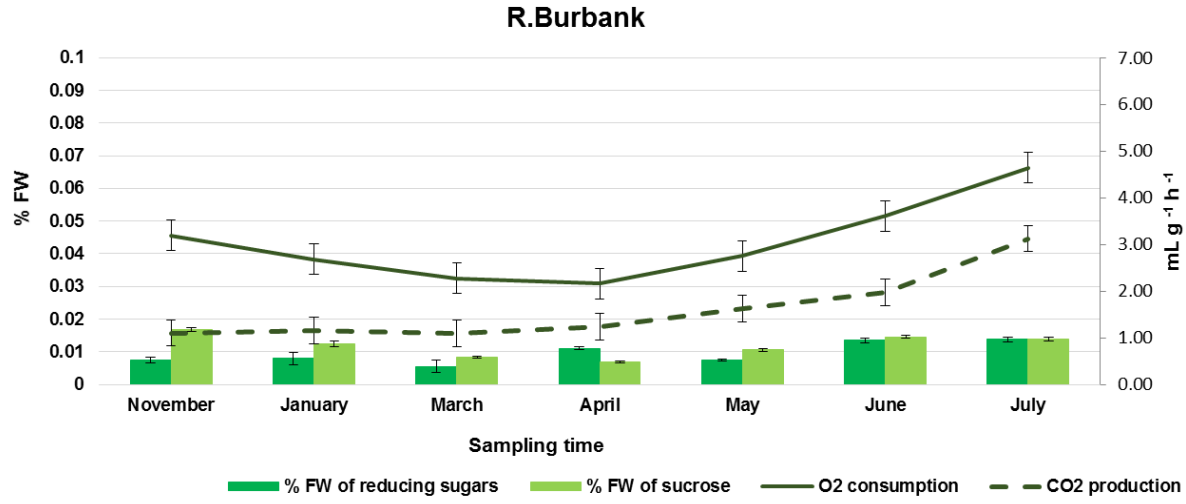

Figure 3-9 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 2nd year of the study (season 2014/15) for R. Burbank, with SE bars. For stats see Table 3-4 and Table 3-5.

Storage season December 2015 to June 2016

During the last season of this study, ethylene production in VR 808 and L. Rosetta, was not significant (both varieties) and moreover, changes in O_2 consumption for VR 808 did not change over the storage season (Appendix VII).

Reducing sugars in VR 808 remained low (0.003 %FW) and declined to lower concentrations after 4-6 months' storage (March-April, Appendix V) (0.001 %FW) before rising at the end of storage (0.007 %FW). Reducing sugars remained well below commercial thresholds. Unlike the previous year sucrose content rose significantly to 0.025 %FW after almost 4 months' storage (March) and increased until the end of storage to 0.04 %FW.

Similar to the season 2013/14, O_2 consumption over the 8 month storage season for VR 808 displayed a U shaped pattern (Figure 3-10).

The decrease in O_2 consumption mid-season correlated with a decrease in fructose and glucose (Figure 3-10, Table 3-6 and Table 3-7), with O_2 consumption rising again from April.

Sucrose content in tubers declined during the first half of storage dipping to its lowest concentration in January before rising again thereafter (Figure 3-10, Table 3-6 and Table 3-7). Changes in sucrose content in tubers during storage led to corresponding changes in respiration. The dip in sucrose in January resulted in a decline in CO_2 production when sucrose increased higher CO_2 production ensued (Figure 3-10, Table 3-6 and Table 3-7).

Reducing sugar profiles in VR 808 were very stable during storage with a rise in fructose, glucose and sucrose accumulation after more than 7 months of storage (Table 3-6). There was not an expected equal rate of O_2 consumption compared with CO_2 production, when fructose and glucose content decreased during storage (Figure 3-10).

In L. Rosetta sucrose accumulation corresponded to a reduction in fructose content and a rise in sucrose, which may suggest that the pool of glucose and fructose are

being fully utilised during respiration (Figure 3-11). Interesting at the same time in March there was an increase in glucose accumulation (Table 3-6). This season was the one with the lowest %FW of reducing sugars for L. Rosetta, however still higher than VR 808 (0.024 %FW and 0.022 %FW, respectively, $HSD_{0.05}$ = 0.003).

Table 3-6 - %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) by sampling month for the 3rd year (2015/16)

		Means			
Variety	**Month**	**RS**	**Sucrose**	**Fructose**	**Glucose**
L. Rosetta	**December**	0.002^{b}	0.010^{b}	0.001^{b}	0.001^{c}
	January	0.005^{b}	0.008^{b}	0.003^{b}	0.002^{bc}
	March	0.004^{b}	0.031^{a}	0.0003^{b}	0.004^{b}
	April	0.005^{b}	0.032^{a}	0.002^{b}	0.002^{bc}
	June	0.017^{a}	0.038^{a}	0.009^{a}	0.008^{a}
	$HSD_{0.05}$	*0.004*	*0.008*	*0.003*	*0.003*
VR 808	**December**	0.003^{b}	0.011^{c}	0.002^{ab}	0.001^{b}
	January	0.003^{b}	0.006^{c}	0.002^{ab}	0.001^{ab}
	March	0.001^{b}	0.025^{b}	0.0001^{b}	0.001^{b}
	April	0.0005^{b}	0.027^{b}	5.78E-05^{b}	0.0004^{b}
	June	0.007^{a}	0.041^{a}	0.02^{a}	0.003^{a}
	$HSD_{0.05}$	*0.004*	*0.013*	*0.002*	*0.002*

Mean values with different letters are significantly different according to the Tukey HSD test.

Table 3-7 - O_2 consumption (O_2, mL $g^{-1}h^{-1}$), CO_2 production (CO_2, mL $g^{-1}h^{-1}$), ethylene production (Ethylene, nL $g^{-1}h^{-1}$) and sprout growth (sprout, mm) by sampling month for the 3rd year (2015/16)

		Means			
Variety	**Month**	**O_2**	**CO_2**	**Ethylene**	**Sprout**
L. Rosetta	**December**	2.72[b]	1.36[b]	5.00E-04[a]	0.70[c]
	January	2.38[b]	1.43[b]	0.05[a]	3.56[a]
	March	2.76[b]	2.43[a]	0.08[a]	2.59[ab]
	April	3.12[b]	2.70[a]	0.03[a]	4.11[a]
	June	4.16[a]	2.52[a]	0.01[a]	1.41[bc]
	$HSD_{0.05}$	*5.67*	*0.59*	*0.09*	*1.58*
VR 808	**December**	3.23[a]	1.94[ab]	0.01[a]	0.12[c]
	January	2.35[a]	1.29[b]	0.04[a]	1.52[ab]
	March	1.35[a]	2.52[a]	0.02[a]	1.99[a]
	April	1.63[a]	2.35[a]	0.03[a]	1.22[ab]
	June	2.66[a]	2.09[ab]	0.005[a]	0.85[bc]
	$HSD_{0.05}$	*3.59*	*0.85*	*0.05*	*0.95*

Mean values with different letters are significantly different according to the Tukey HSD test.

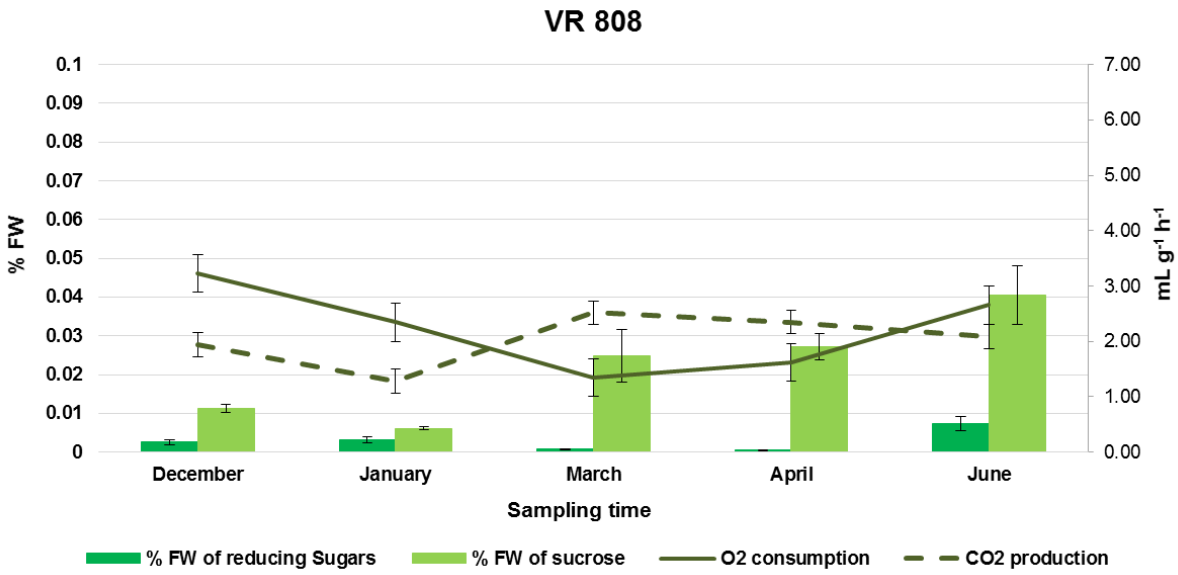

Figure 3-10 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 3rd year of the study (season 2015/16) for VR 808, with SE bars. For stats see Table 3-6 and Table 3-7.

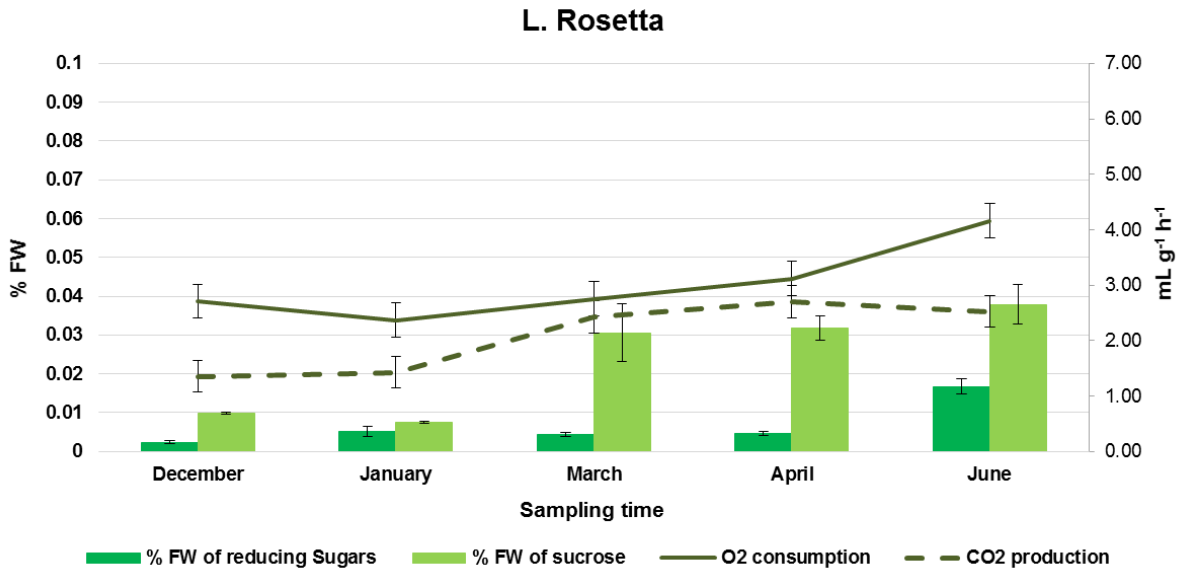

Figure 3-11 - %FW of reducing sugars, %FW of sucrose, consumption of O_2 (mL O_2 $g^{-1}h^{-1}$) and production of CO_2 (mL CO_2 $g^{-1}h^{-1}$) of the 3rd year of the study (season 2015/16) for L. Rosetta, with SE bars. For stats see Table 3-6 and Table 3-7.

Sugar accumulation within the tuber

Significant differences between position of the samples taken within the tuber (ends and middle part of the tuber) during the 1st and 3rd year of the study, for VR 808 was only observed for sucrose accumulation ($p < 0.001$). Sucrose accumulation was higher at the ends than in the middle during the 1st year (Table 3-8). Opposite occur during the 3rd year, with the middle part accumulating more sucrose than the ends (Table 3-8). During the 2nd year the position of the samples within the tuber was significant for fructose ($p < 0.001$) and sucrose ($p < 0.01$) accumulation, with middle part having the higher sucrose and fructose accumulation (Table 3-8).

For the other varieties, similar variability in sugar profiles across the tuber were apparent between seasons. For L. Rosetta fructose and glucose content was higher in the middle of the tuber (Table 3-8). In contrast sucrose accumulated more on the outer ends where the opposite ends sections were taken in the 1st year. However, in later years of the study sucrose was higher in the middle of the tuber (Table 3-8).

The sugar content in P. Dell, was dependant on the position of the samples taken within the tuber. In particular fructose content was higher in the centre of the tuber (Table 3-8). In year 2 a higher concentration of sucrose, fructose and glucose was recorded in the middle pith of the tuber (Table 3-8).

For R. Burbank, the position of the samples within the tuber was significant for the total %FW of reducing sugars from 1st year, sucrose accumulation from 2nd year and glucose accumulation for both years. At the ends of the tuber was the higher total %FW of reducing sugars, sucrose, fructose and glucose for the 1st year. Opposite for the 2nd year, exception for %FW of fructose (Table 3-8).

Table 3-8 – %FW of reducing sugars (RS), %FW of sucrose (Suc), %FW of fructose (Fru) and %FW of glucose (Glu) for VR 808 and L. Rosetta over 3 years of data, and 2 years of data for P. Dell and R. Burbank.

	VR 808								
	1st year			2nd year			3rd year		
	Ends	**Middle**	**$HSD_{0.05}$**	**Ends**	**Middle**	**$HSD_{0.05}$**	**Ends**	**Middle**	**$HSD_{0.05}$**
RS	0.003^{a}	0.003^{a}	0.001	0.003^{b}	0.006^{a}	0.002***	0.003^{a}	0.003^{a}	0.002
Suc	0.012^{a}	0.010^{b}	0.001***	0.012^{b}	0.014^{a}	0.001**	0.014^{b}	0.030^{a}	0.006***
Fru	0.001^{a}	0.001^{a}	0.0004	0.001^{b}	0.004^{a}	0.001***	0.002^{a}	0.001^{a}	0.001
Glu	0.001^{a}	0.002^{a}	0.001	0.001^{a}	0.002^{a}	0.001	0.001^{a}	0.002^{a}	0.001
	L. Rosetta								
	1st year			2nd year			3rd year		
	Ends	**Middle**	**$HSD_{0.05}$**	**Ends**	**Middle**	**$HSD_{0.05}$**	**Ends**	**Middle**	**$HSD_{0.05}$**
RS	0.011^{b}	0.013^{a}	0.002*	0.017^{b}	0.022^{a}	0.003***	0.006^{a}	0.007^{a}	0.002
Suc	0.020^{a}	0.016^{b}	0.003**	0.023^{b}	0.026^{a}	0.001***	0.015^{b}	0.032^{a}	0.004***
Fru	0.006^{a}	0.006^{a}	0.001	0.010b	0.014a	0.002***	0.003^{b}	0.004^{a}	0.001*
Glu	0.005^{b}	0.007^{a}	0.001**	0.007^{a}	0.008^{a}	0.001	0.003^{a}	0.004^{a}	0.001
	P. Dell					
	1st year			2nd year		
	Ends	**Middle**	**$HSD_{0.05}$**	**Ends**	**Middle**	**$HSD_{0.05}$**
RS	0.038^{a}	0.045^{a}	0.007	0.035^{b}	0.059^{a}	0.005***
Suc	0.022^{a}	0.021^{a}	0.002	0.028^{b}	0.035^{a}	0.002***
Fru	0.017^{b}	0.020^{a}	0.003*	0.018^{b}	0.033^{a}	0.003***
Glu	0.021^{a}	0.024^{a}	0.004	0.017^{b}	0.026^{a}	0.002***
	R. Burbank					
	1st year			2nd year		
	Ends	**Middle**	**$HSD_{0.05}$**	**Ends**	**Middle**	**$HSD_{0.05}$**
RS	0.029^{a}	0.023^{b}	0.005*	0.009^{a}	0.010^{a}	0.001
Suc	0.012^{a}	0.011^{a}	0.001	0.011^{b}	0.013^{a}	0.001***
Fru	0.012^{a}	0.010^{a}	0.002	0.006^{a}	0.006^{a}	0.001
Glu	0.017^{a}	0.013^{b}	0.003*	0.003b	0.005a	0.001***

*Mean values with different letters were significantly different according to Tukey HSD test. Level of significance in HSD column (p<0.001 " ***" p<0.01 "**" p<0.05 "*").*

Correlations between the different physiological factors

As expected in all the cases total %FW of reducing sugars was positively correlated with fructose and glucose accumulation (Figure 3-12 and Figure 3-13). In the varieties, VR 808, L. Rosetta and P. Dell reducing sugars were positively correlated with sucrose accumulation, length of storage and respiration based on O_2 consumption. In these varieties sucrose accumulation was positively correlated with storage duration (Figure 3-12 and Figure 3-13).

In all the varieties CO_2 production increased over the storage period and positively correlated with the number of days in storage. Respiration rates based on O_2 consumption were positively correlated with reducing sugars and with sucrose content (Figure 3-12 and Figure 3-13).

The increase in O_2 consumption rates over time was positively correlated in L. Rosetta and R. Burbank (Figure 3-12 and Figure 3-13), while O_2 consumption rates were more related to the rate of sprout growth in P. Dell and R. Burbank rather than the duration of storage (Figure 3-13). Which suggests that rate of sprouting and effectiveness of CIPC application are just as important factors to consider along with the age of tuber in terms of factors controlling respiration rates and thus sugar accumulation.

CO_2 production was positively correlated with residual sprout growth for VR 808, L. Rosetta and R. Burbank (Figure 3-12 and Figure 3-13), and O_2 consumption for R. Burbank (positively) and a negative relationship between O_2 consumption and VR 808 (Figure 3-12 and Figure 3-13). These relationships with sprout growth are tentative due to previous CIPC application.

In the varieties VR 808, L. Rosetta and P. Dell glucose and fructose accumulation were positively correlated with the length of storage and respiration rates (CO_2 production and O_2 consumption), and sucrose accumulation (Figure 3-12 and Figure 3-13).

Ethylene production in tubers was very low during storage and it is difficult to link small changes in production rates with physiological function. However, a significant negative correlation with sucrose accumulation for VR 808 was observed (Figure 3-12) caused by a decrease in ethylene later in storage (Figure 3-12). R. Burbank was the

only variety that had a significantly positive correlation between ethylene production and fructose and glucose accumulation (Figure 3-13). Interestingly, this variety is considered very responsive to ethylene (Haines *et al.*, 2003).

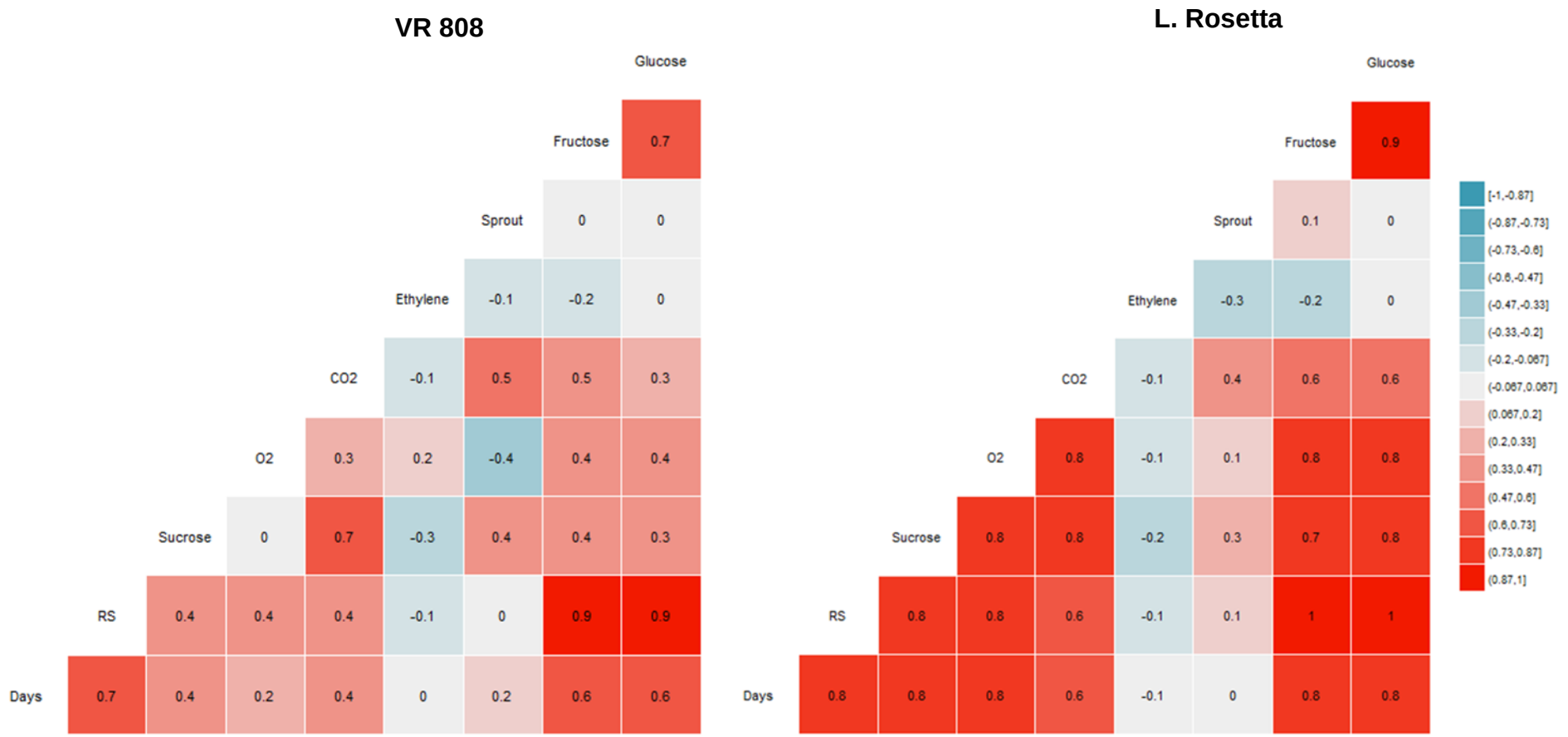

Figure 3-12 - Correlation between days in storage (Days), %FW of reducing sugars (RS), %FW of sucrose (Sucrose), O_2 consumption (O_2), CO_2 production (CO_2), Ethylene production (Ethylene), sprout growth (Sprout), %FW of fructose and %FW of glucose for the variety VR 808 and L. Rosetta (df = 25). Significant Pearson correlation coefficients from 0.32 to 1 and from -0.32 to -1 ($p < 0.05$).

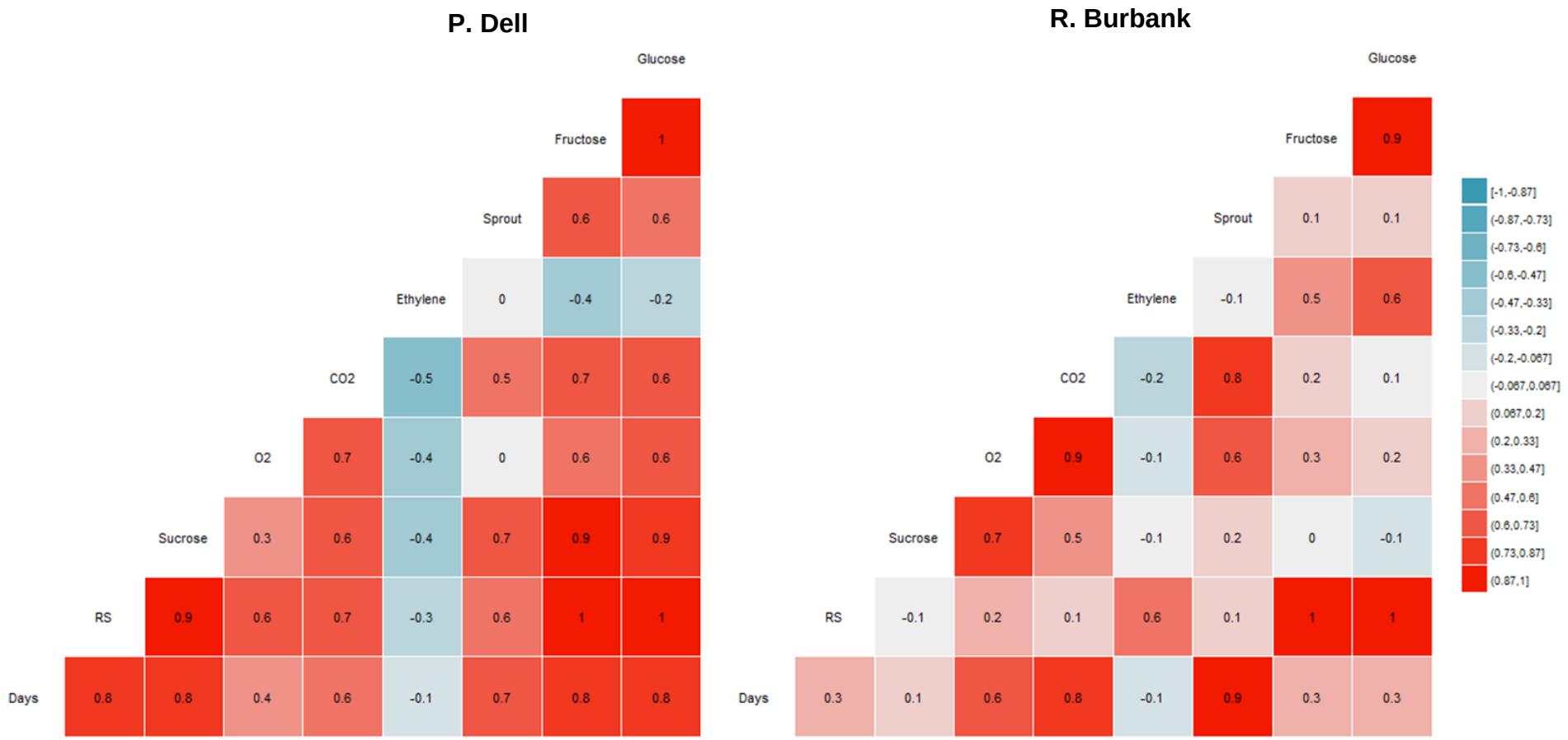

Figure 3-13 - Correlation between days in storage (Days), %FW of reducing sugars (RS), %FW of sucrose (Sucrose), O_2 consumption (O_2), CO_2 production (CO_2), Ethylene production (Ethylene), sprout growth (Sprout), %FW of fructose and %FW of glucose for the variety P. Dell and R. Burbank (df = 9). Significant Pearson correlation coefficients from 0.52 to 1 and from -0.52 to -1 ($p < 0.05$).

The effect of physiological aging (chitting) of seed potatoes on senescent sweetening

While physiological aging (chitting) of seed prior to planting is known to hasten the chemical maturity of tubers at harvest; only a slight increase in reducing sugar content at harvest was observed in tubers harvested from aged (250°C) seed and thereafter no differences were seen.

Respiration and sprouting rate for chitted and non-chitted seed were similar. There was no significant difference between tubers generated from chitted and non-chitted seed ($p > 0.05$). In both cases there was a significant increase in ($p < 0.001$) sucrose, fructose, glucose, O_2 consumption, CO_2 production and ethylene production (Figure 3-18, Figure 3-19, Table 3-9 and Table 3-10).

O_2 consumption describes a U shape for both treatments and CO_2 production an inverted U shape (Figure 3-15). The peak in CO_2 production in March (>3 months in storage at 10°C) corresponded to the start in the sucrose and fructose accumulation in both treatments (Table 3-9 and Table 3-10). The lowest O_2 consumption rate at 0°C corresponded with the start in the sucrose and fructose accumulation rise (March) and in 250°C that lowest peak is 48 days (January) (Table 3-9 and Table 3-10).

For both treatments from the chitting experiment, position of the samples taken within the tuber was not significant only for glucose accumulation (Table 3-11). The middle region of the tuber has the higher total %FW of reducing sugars, sucrose and fructose (Table 3-11).

Total %FW of reducing sugars and sucrose content increased over storage leading to a strong positive correlation (Figure 3-16). While increases in sugars were seen over time in both chitted and non-chitted stock multiple correlation analysis found that in tubers from chitted seed the rate of increase over time reached significance (Figure 3-16). In general, in this experiment, increase in residual sprout growth led to a reduction of sugars and this correlation was significant in tubers from chitted seed (Figure 3-16).

Table 3-9 - %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) by sampling month for the chitting experiment.

	RS	Sucrose	Fructose	Glucose
December	0.013^{b}	0.010^{c}	0.003^{c}	0.010^{b}
January	0.012^{b}	0.014^{c}	0.004^{c}	0.008^{b}
March	0.013^{b}	0.024^{b}	0.005^{bc}	0.008^{b}
April	0.017^{b}	0.030^{ab}	0.007^{b}	0.010^{b}
June	0.040^{a}	0.032^{a}	0.018^{a}	0.023^{a}
HSD$_{0.05}$	0.006	0.006	0.003	0.004

Mean values with different letters are significantly different according to the Tukey HSD test.

Table 3-10 - O_2 consumption (O_2, mL $g^{-1}h^{-1}$), CO_2 production (CO_2, mL $g^{-1}h^{-1}$), ethylene production (Ethylene, nL $g^{-1}h^{-1}$) and sprout growth (sprout, mm) by sampling month for the chitting experiment.

	O_2	CO_2	Ethylene	Sprout
December	2.544^{a}	1.178^{c}	0.035^{bc}	7.232^{a}
January	1.710^{b}	1.146^{c}	0.092^{a}	5.227^{ab}
March	1.679^{b}	2.969^{a}	0.053^{b}	3.710^{bc}
April	2.282^{a}	2.36^{b}	0.019^{bc}	2.858^{bc}
June	2.226^{ab}	1.318^{c}	0.009^{c}	1.762^{c}
HSD$_{0.05}$	0.549	0.437	0.037	2.653

Mean values with different letters are significantly different according to the Tukey HSD test.

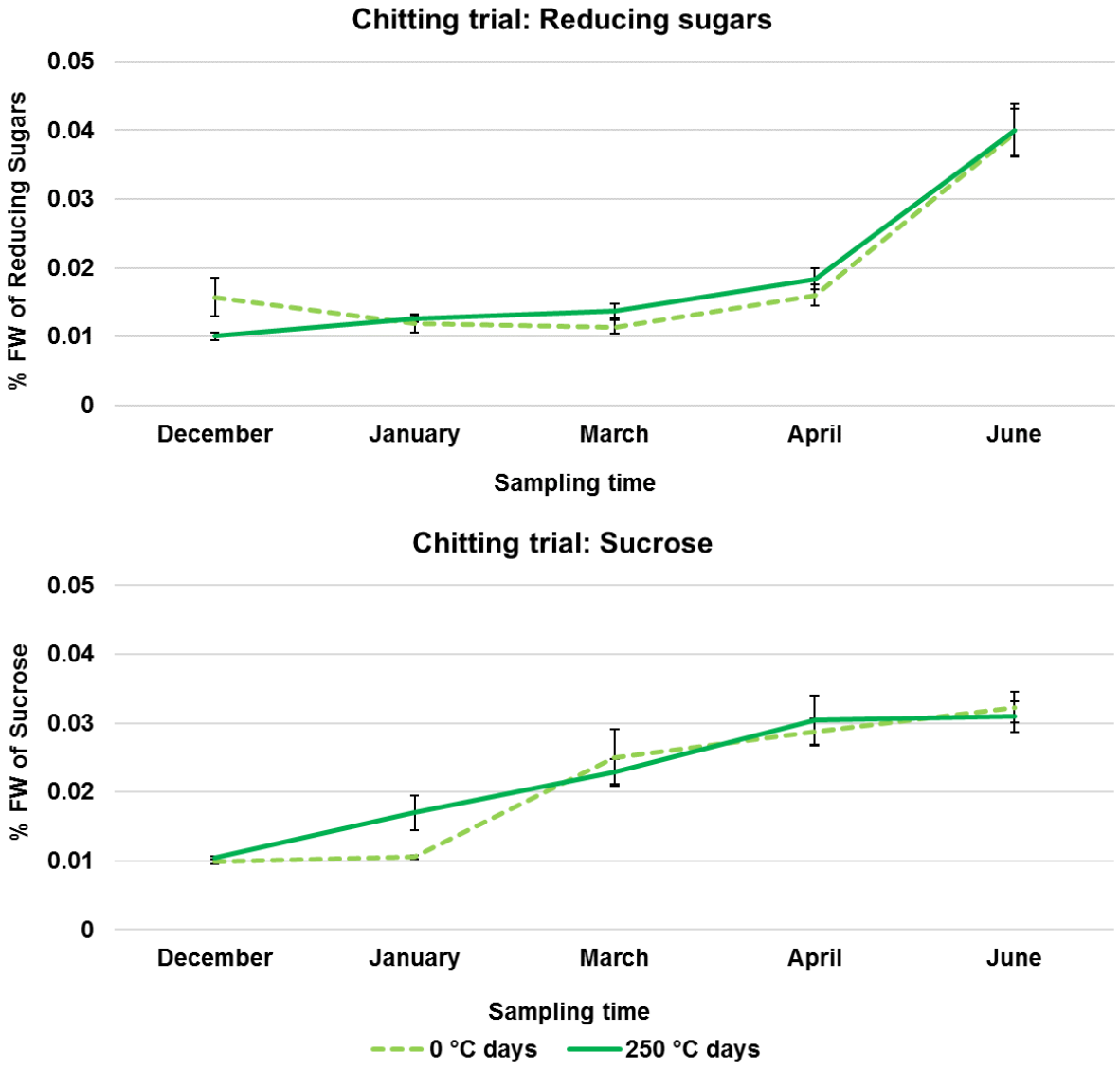

Figure 3-14 - %FW of Reducing sugars and %FW of Sucrose for P. Dell from the chitting experiment, with SE bars. For stats see Table 3-9 and Table 3-10.

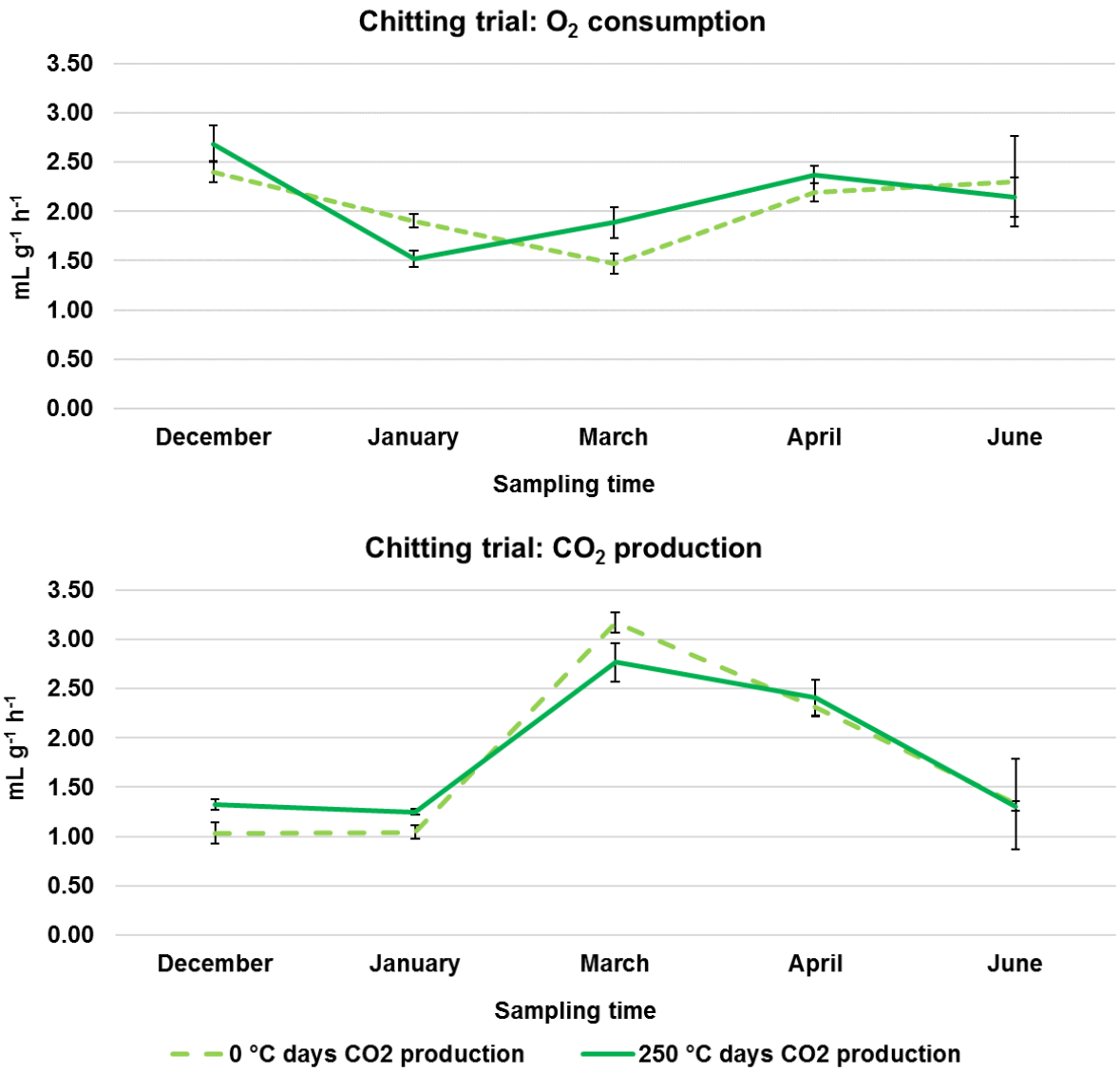

Figure 3-15 - Consumption of O_2 (mL $g^{-1}h^{-1}$) and CO_2 (mL $g^{-1}h^{-1}$) production for P. Dell from the chitting experiment, with SE bars. For stats see Table 3-9 and Table 3-10.

Table 3-11 - %FW of reducing sugars (RS), %FW of sucrose (Suc), %FW of fructose (Fru) and %FW of glucose (Glu) for the chitting experiment.

	0°C days				250°C days			
	Ends	**Middle**	**$HSD_{0.05}$**	**p-value**	**Ends**	**Middle**	**$HSD_{0.05}$**	**p-value**
RS	0.017^{b}	0.021^{a}	0.003	0.013	0.016^{b}	0.021^{a}	0.002	$9.30e^{-16}$
Suc	0.017^{b}	0.025^{a}	0.003	$8.69e^{-7}$	0.018^{b}	0.026^{a}	0.004	$4.95e^{-5}$
Fru	0.005^{b}	0.009^{a}	0.001	$7.85e^{-13}$	0.006^{b}	0.010^{a}	0.001	$1.60e^{-11}$
Glu	0.012^{a}	0.012^{a}	0.003	0.81	0.011^{a}	0.012^{a}	0.001	0.16

Mean values with different letters were significantly different according to Tukey HSD test.

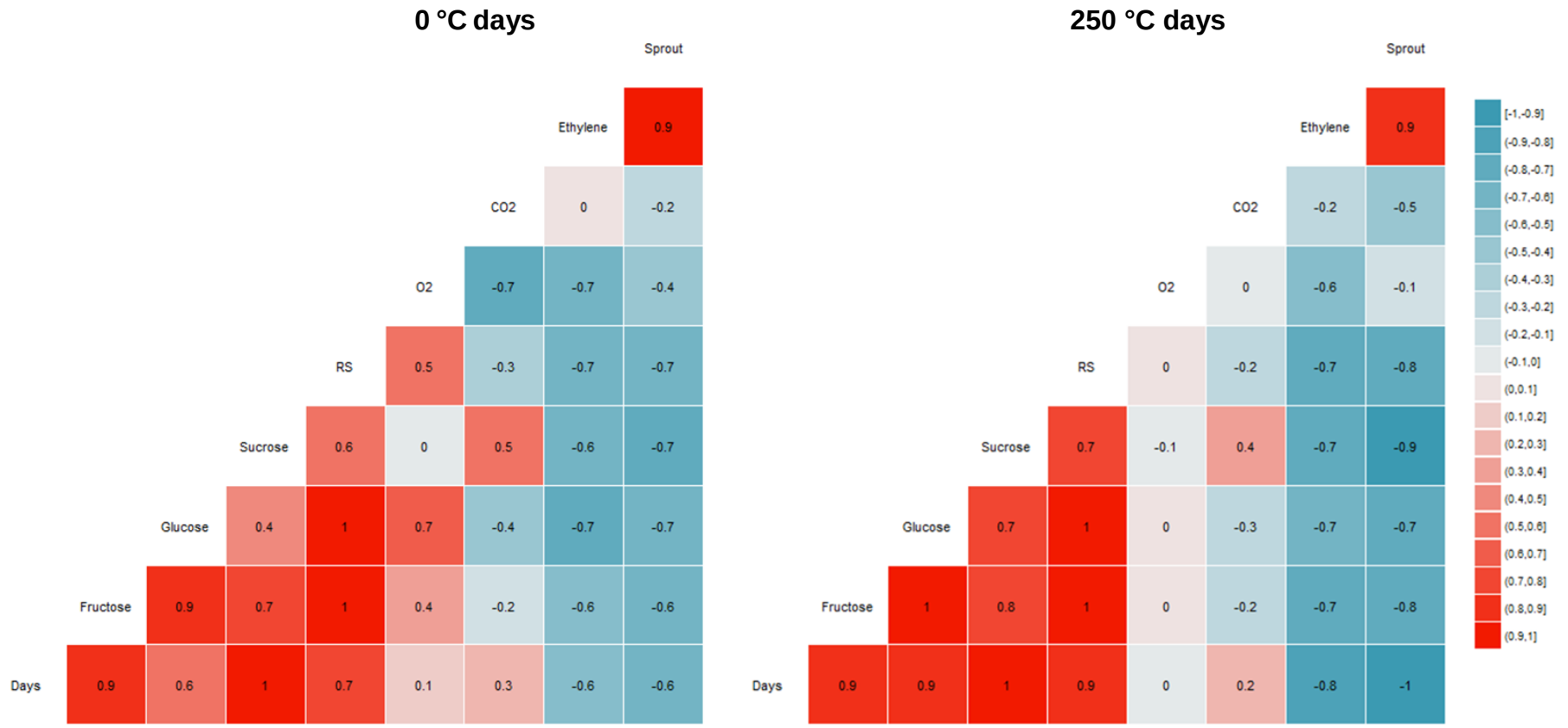

Figure 3-16 - Correlation between days in storage (Days), %FW of fructose, %FW of glucose, %FW of sucrose (Sucrose), %FW of reducing sugars (RS), O_2 consumption (O_2), CO_2 production (CO_2), Ethylene production (Ethylene) and sprout growth (Sprout) for 0 and 250°C days from the chitting experiment (df = 3). Significant Pearson correlation coefficients from 0.81 to 1 and from -0.81 to -1 ($p < 0.05$).

3.3.2. Assessment of ascorbic acid accumulation during storage

For the season 2014/15, analysis of ascorbic acid (AsA), dehydroascorbic acid (DHA) and total vitamin C (sum of AsA with DHA) from samples taken every six weeks over the storage season showed varietal differences ($p < 0.001$) in potato tubers from VR 808, L. Rosetta, P. Dell and R. Burbank. In this season, L. Rosetta had the highest AsA accumulation averaging 4.9 mg 100 g^{-1} FW and P. Dell and VR 808 the lowest value. DHA profiles recorded with R. Burbank the highest ranked variety, followed by P. Dell, L. Rosetta and VR 808 (Table 3-12).

In both years, L. Rosetta retained more AsA for the following season (2015/16) yielded significantly higher AsA and DHA concentration compared to the previous year's data and differences ($p < 0.001$) between varieties and seasons were found (Table 3-12).

In both seasons (2014-2016) L. Rosetta contained a significantly higher AsA content than VR 808. AsA and DHA content was 2-3 fold higher in potato samples analysed in 2015/16 season. However, the ratio of AsA/DHA for L. Rosetta and VR 808 were similar across seasons (Table 3-12).

In both seasons the concentration of AsA and DHA was highest at the start of harvest and declined during storage. Changes in AsA/DHA ratio were observed during storage for P. Dell, R. Burbank and L. Rosetta from season 2015/16 (data not shown). AsA/DHA ratios for VR 808 and L. Rosetta for season 2014/15 (data not shown) had a peak in April, and VR 808 for season 2015/16 (data not shown) had an increase in December and April that was not statistically significant.

Table 3-12 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for seasons 2014/15 and 2015/16.

	Season 2014/15		
	AsA	**DHA**	**VIT C**
L. Rosetta	4.9[a]	3.5[b]	8.4[b]
VR 808	3.6[c]	3.4[b]	7.0[c]
P. Dell	3.6[c]	4.7[a]	8.3[b]
R. Burbank	4.3[b]	5.3[a]	9.5[a]
HSD$_{0.05}$	*0.5*	*0.8*	*0.7*
	Season 2015/16		
	AsA	**DHA**	**VIT C**
L. Rosetta	16.0[a]	10.1[a]	26.1[a]
VR 808	10.5[b]	6.5[b]	16.3[b]
HSD$_{0.05}$	*2.0*	*1.6*	*1.2*

Mean values with different letters were significantly different according to Tukey HSD test.

Table 3-13 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for L. Rosetta and VR 808 for season 2014/15.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vit C**
L. Rosetta	**November**	6.6^{a}	5.6^{a}	12.2^{a}
	January	4.4^{ab}	5.6^{a}	10.0^{b}
	March	3.7^{b}	4.1^{a}	7.8^{c}
	April	5.3^{ab}	0.6^{b}	4.7^{d}
	May	5.5^{a}	3.6^{a}	9.1^{bc}
	June	5.2^{a}	3.8^{a}	7.1^{c}
	July	2.4^{a}	2.2^{ab}	7.5^{c}
	p-value	*< 0.01*	*< 0.001*	*< 0.001*
	$HSD_{0.05}$	*4.5*	*3.4*	*2.0*
VR 808	**November**	6.7^{a}	4.6^{b}	11.2^{a}
	January	3.3^{bc}	3.3^{bc}	6.5^{c}
	March	2.8^{cd}	3.4^{bc}	6.3^{c}
	April	3.6^{bc}	0.2^{d}	3.4^{d}
	May	2.1^{d}	7.0^{a}	9.2^{b}
	June	2.7^{cd}	3.6^{bc}	6.3^{c}
	July	4.0^{b}	2.2^{c}	6.1^{c}
	p-value	*< 0.001*	*< 0.001*	*< 0.001*
	$HSD_{0.05}$	*1.2*	*1.6*	*1.5*

Mean values with different letters were significantly different according to Tukey HSD test.

Table 3-14 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for L. Rosetta and VR 808 for season 2015/16.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vit C**
L. Rosetta	**December**	49.3^{a}	10.6^{b}	59.9^{ab}
	January	35.7^{ab}	26.8^{a}	62.5^{a}
	March	28.0^{bc}	25.5^{a}	53.5^{b}
	April	24.6^{bc}	19.0^{ab}	43.6^{c}
	June	20.8^{c}	14.6^{ab}	35.4^{d}
	p-value	*< 0.001*	*< 0.05*	*< 0.001*
	$HSD_{0.05}$	*13.9*	*14.1*	*14.1*
VR 808	**December**	37.2^{a}	5.9^{b}	36.0^{a}
	January	20.5^{ab}	12.0^{ab}	32.4^{ab}
	March	13.1^{b}	17.4^{a}	30.5^{ab}
	April	17.4^{b}	4.7^{b}	22.1^{b}
	June	12.0^{b}	14^{ab}	26.0^{ab}
	p-value	*< 0.01*	*< 0.01*	*< 0.05*
	$HSD_{0.05}$	*17.7*	*10.8*	*11.2*

Mean values with different letters were significantly different according to Tukey HSD test.

For VR 808 the highest AsA content over the two seasons study was recorded at harvest and declined during storage (10°C) (Table 3-13 and Table 3-14). DHA profile followed a similar pattern to AsA content in season 2014/15 with a rise in DHA during late (May) storage. In the 2015/16 season AsA content in VR 808 was significantly higher (35 mg 100 g^{-1}) and declined to (12 mg 100g^{-1}) after 7 months storage at 10°C (Table 3-14). Unlike the previous year, DHA content at harvest was significantly lower than AsA content at 5.9 mg 100 g^{-1} then rose to a peak of 17 mg 100 g^{-1} after 4 months storage in March at 10°C (Figure 3-17, Table 3-13 and Table 3-14).

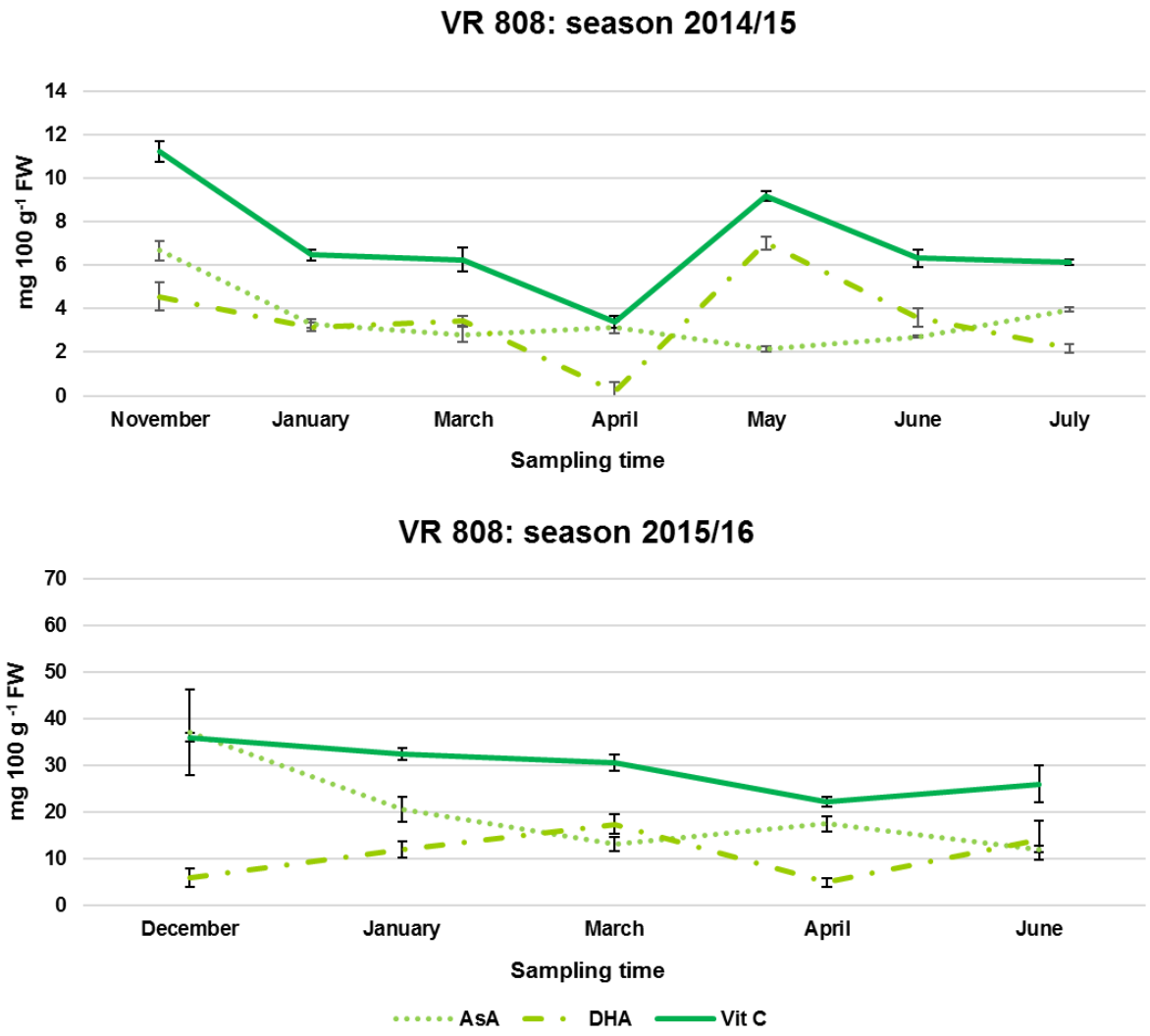

Figure 3-17 - Accumulation of AsA (mg 100 g^{-1} FW), DHA (mg 100 g^{-1} FW) and total vitamin C (Vit C) (mg 100 g^{-1} FW) for seasons 2014/15 and 2015/16 for VR 808, with SE bars. For stats see Table 3-13 and Table 3-14.

The AsA concentration of the senescent sweetening sensitive variety L. Rosetta was similar (6.6 mg $100g^{-1}$) to VR 808 at harvest in season 2014/15 and AsA concentration remained between 3.3 – 6.6 mg $100g^{-1}$ throughout storage (Figure 3-18 and Table

3-13). DHA content declined during storage to a low after 5 months storage at 10°C followed by a rise thereafter. The following year, the AsA content at harvest was significantly higher (49.3 mg 100^{-1}) than the previous year and higher than the sweetening resistant VR 808. During storage, AsA declined to 20.6 mg $100g^{-1}$ (Figure 3-18 and Table 3-14). The DHA content of L. Rosetta at harvest was 10.6 mg $100g^{-1}$ which was higher than VR 808. DHA content increased during first month of storage then mirrored AsA with decline during prolonged storage at 10°C. In this season, for this variety, the beginning of the storage had the higher AsA/DHA ratio (data not shown).

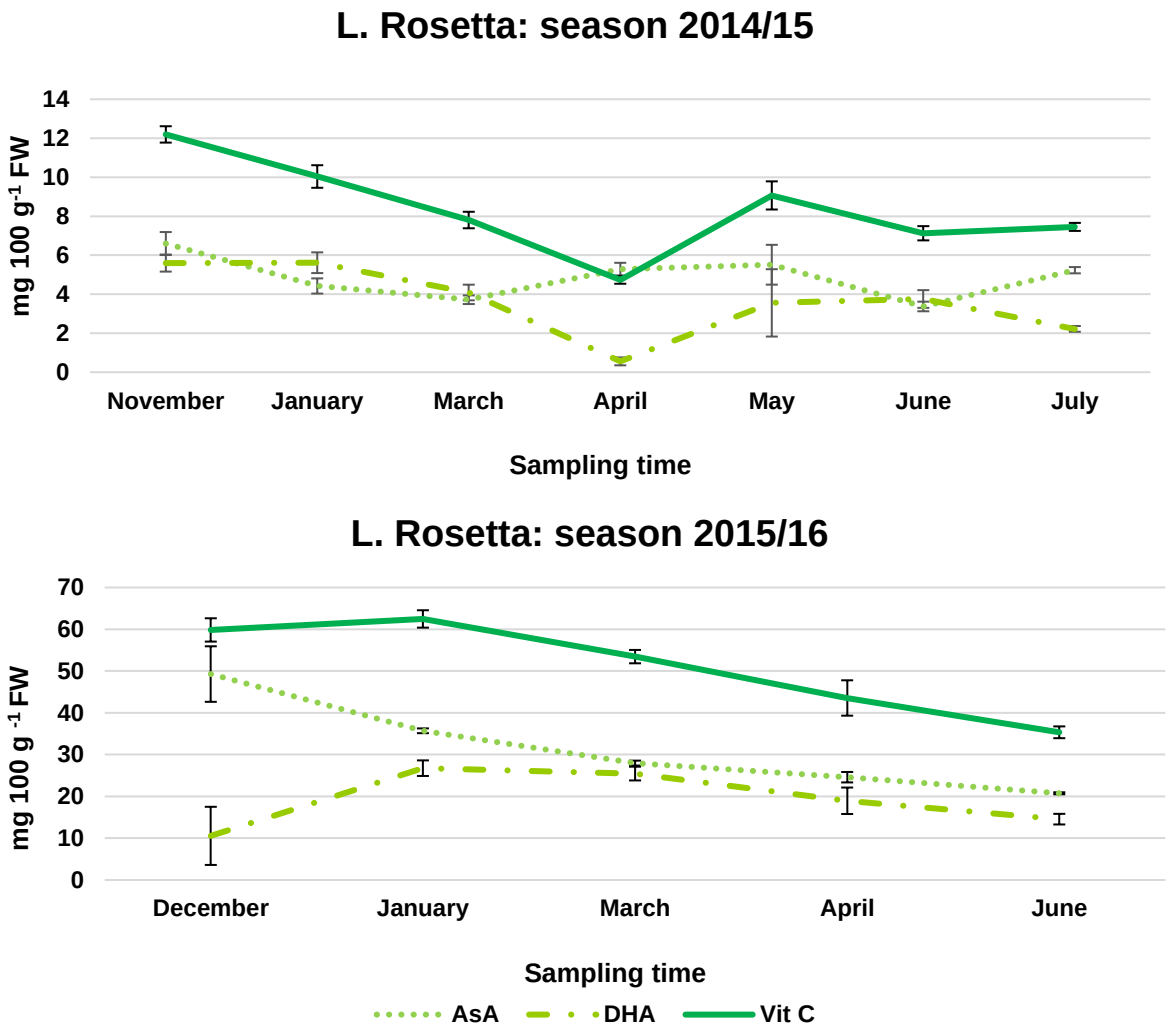

Figure 3-18 - Accumulation of AsA (mg 100 g^{-1} FW), DHA (mg 100 g^{-1} FW) and total vitamin C (Vit C) (mg 100 g^{-1} FW) for seasons 2015/16 for L. Rosetta, with SE bars. For stats see Table 3-13 and Table 3-14.

AsA and DHA content of P. Dell in season 2014/15 was 5.4 mg 100 g^{-1} at the beginning of the storage and thereafter declined during the first 4 months of storage at 10°C to 2.2 mg 100 g^{-1} while DHA content remained constant (Figure 3-19 and Table 3-15). After 6 months storage (May), AsA/DHA ratio was at its highest value (data not shown),

at the same time the AsA had risen and DHA declined to a concentration of 3.7 mg 100 g^{-1} in July (Figure 3-19 and Table 3-15).

A decline in AsA and DHA ($p < 0.001$) was observed as well in R. Burbank with an intermittent peak in DHA content in after 6 months storage (Figure 3-19 and Table 3-15). Such peaks and troughs in AsA and DHA are seen in other varieties and may be associated with sprouting or late season CIPC application.

Table 3-15 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for P. Dell and R. Burbank for season 2014/15.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vit C**
P. Dell	**November**	5.4^{a}	6.2^{a}	11.6^{a}
	January	3.1^{bc}	5.7^{a}	8.7^{ab}
	March	2.2^{c}	4.3^{ab}	6.5^{b}
	April	2.2^{c}	6.4^{a}	8.6^{ab}
	May	4.9^{a}	2.5^{b}	7.4^{b}
	June	3.1^{bc}	4.0^{ab}	7.2^{b}
	July	4.2^{ab}	3.7^{ab}	8.0^{b}
	p-value	*< 0.001*	*< 0.01*	*< 0.001*
	$HSD_{0.05}$	*1,5*	*2.9*	*3.1*
R. Burbank	**November**	5.5^{a}	7.4^{b}	12.8^{a}
	January	4.2^{abc}	6.3^{b}	10.4^{b}
	March	3.1^{bc}	3.6^{c}	6.7^{d}
	April	2.9^{c}	10.0^{a}	13.0^{a}
	May	4.7^{a}	3.6^{c}	8.3^{c}
	June	4.7^{a}	2.6^{c}	7.3^{cd}
	July	4.6^{ab}	3.6^{c}	8.2^{cd}
	p-value	*< 0.001*	*< 0.001*	*< 0.001*
	$HSD_{0.05}$	*1.6*	*1.7*	*1.4*

Mean values with different letters were significantly different according to Tukey HSD test.

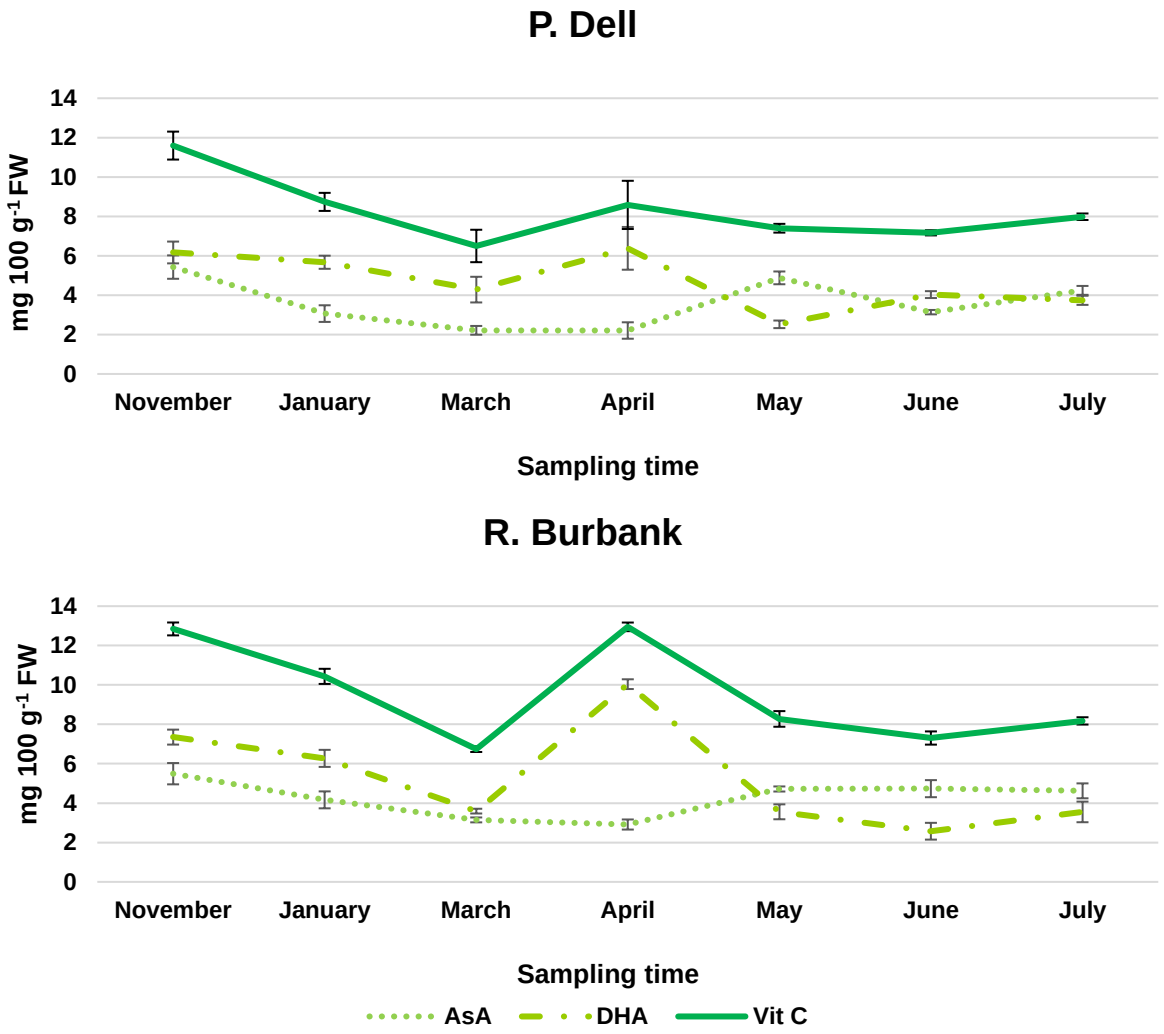

Figure 3-19 - Accumulation of AsA (mg 100 g^{-1} FW), DHA (mg 100 g^{-1} FW) and total vitamin C (Vit C) (mg 100 g^{-1} FW) for season2014/15 for P. Dell and R. Burbank, with SE bars. For stats see Table 3-15.

The effect of physiological aging (chitting) of seed potatoes on AsA accumulation

Physiologically aging seed tubers prior to planting had significant effect on AsA accumulation ($p < 0.001$) and vitamin C accumulation ($p < 0.001$). Non-aged seed tubers (0°C days) had higher AsA accumulation than aged seed tubers (250°C days), and higher total vitamin C accumulation (Table 3-16).

In both cases, storage length was significant for AsA accumulation ($p < 0.001$), DHA accumulation ($p < 0.001$) and total vitamin C accumulation (Table 3-17).

AsA content of was highest in tubers sampled after a month's storage (December) for chitted (250ºC) an non-chitted (0°C) tubers (Figure 3-20 and Table 3-17), then a unexpected drop in AsA content occurred during the following month (January) (0°C and 250°C days) and April (250°C days). A decrease in the AsA accumulation was mirrored in an increase in DHA content (Figure 3-20). The lowest AsA/DHA ratios were observed in January and April (Figure 3-20 and Table 3-17).

Table 3-16 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for the chitting experiment.

	AsA	**DHA**	**VIT C**
0°C	22.4[a]	15.1[a]	37.3[a]
250°C	19.8[b]	14.0[a]	33.9[b]
HSD$_{0.05}$	*1.3*	*1.8*	*1.9*

Mean values with different letters were significantly different according to Tukey HSD test.

Table 3-17 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for the chitting experiment.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vit C**
0°C	**December**	31.5^{a}	8.4^{c}	38.9^{a}
	January	14.6^{d}	23.7^{a}	38.3^{a}
	March	24.3^{b}	13.5^{bc}	37.7^{ab}
	April	20.0^{c}	20.1^{ab}	40.1^{a}
	June	21.5^{bc}	9.9^{c}	31.4^{b}
	p-value	*< 0.001*	*< 0.001*	*< 0.01*
	$HSD_{0.05}$	*4.1*	*6.7*	*6.5*
250°C	**December**	27.4^{a}	10.5^{c}	37.9^{a}
	January	13.3^{c}	25.4^{a}	38.7^{a}
	March	21.5^{b}	8.8^{c}	30.3^{bc}
	April	16.1^{c}	18.6^{b}	34.7^{ab}
	June	20.8^{b}	6.8^{c}	27.6^{c}
	p-value	*< 0.001*	*< 0.001*	*< 0.001*
	$HSD_{0.05}$	*4.1*	*4.3*	*4.1*

Mean values with different letters were significantly different according to Tukey HSD test.

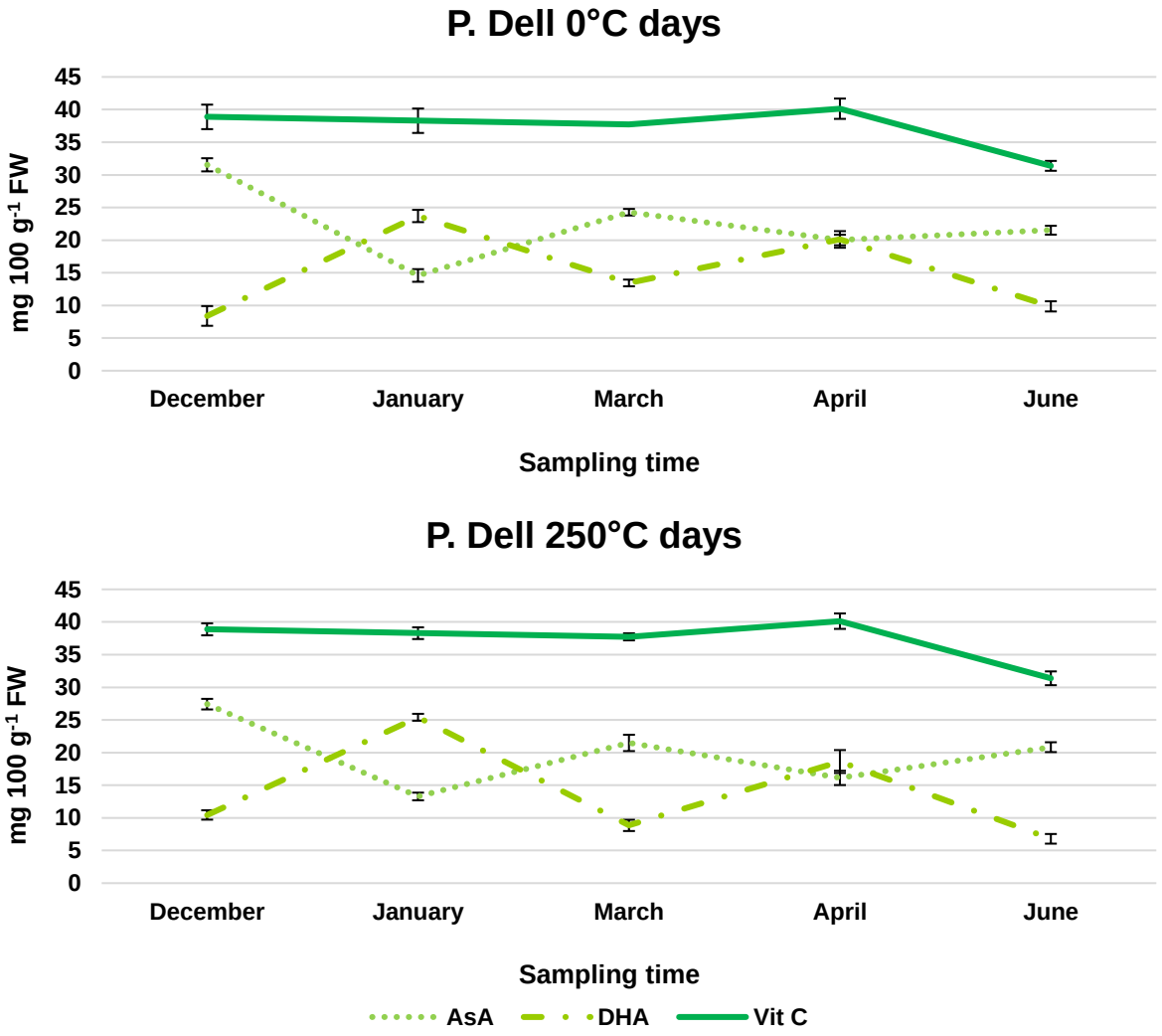

Figure 3-20 - Accumulation of AsA (mg 100 g^{-1} FW), DHA (mg 100 g^{-1} FW) and total vitamin C (Vit C) (mg 100 g^{-1} FW) for the season 2015/16 for P. Dell, 0°C days and 250°C days (chitting experiment), with SE bars. For stats see Table 3-17.

3.3.3. Determination and detection of reactive oxygen species (ROS)

Superoxide was detected by the formation of a purple/blue precipitation in the presence of nitroblue tetrazolium (NBT), and the reaction of hydrogen peroxide with diaminobenzidine tetrahydrochloride (DAB) resulting in the formation of a brown polymerization product.

An example of hydrogen peroxide (H_2O_2) tissue staining for season 2014/15 can be seen in Plate 3-6, where a brown polymerization was formed around, but not exclusively, the vascular tissue in the peripheral outer cortex. The inner cortex of the tuber, which remained unstained suggesting low or no H_2O_2 activity.
In contrast, as an example for superoxide tissue staining (O_2^-) (2014/15) depicted as a purple/blue precipitate was more prevalent in the middle cortex shown in Plate 3-7 seen in the interior of the sample.

The presence of H_2O_2 and O_2^- increased with successive sampling with darker stained tissue in later samplings. A similar pattern of H_2O_2 and O_2^- activity was observed during the third year of experiments.

Plate 3-6 - DAB staining (H_2O_2 tissue staining) on L. Rosetta, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

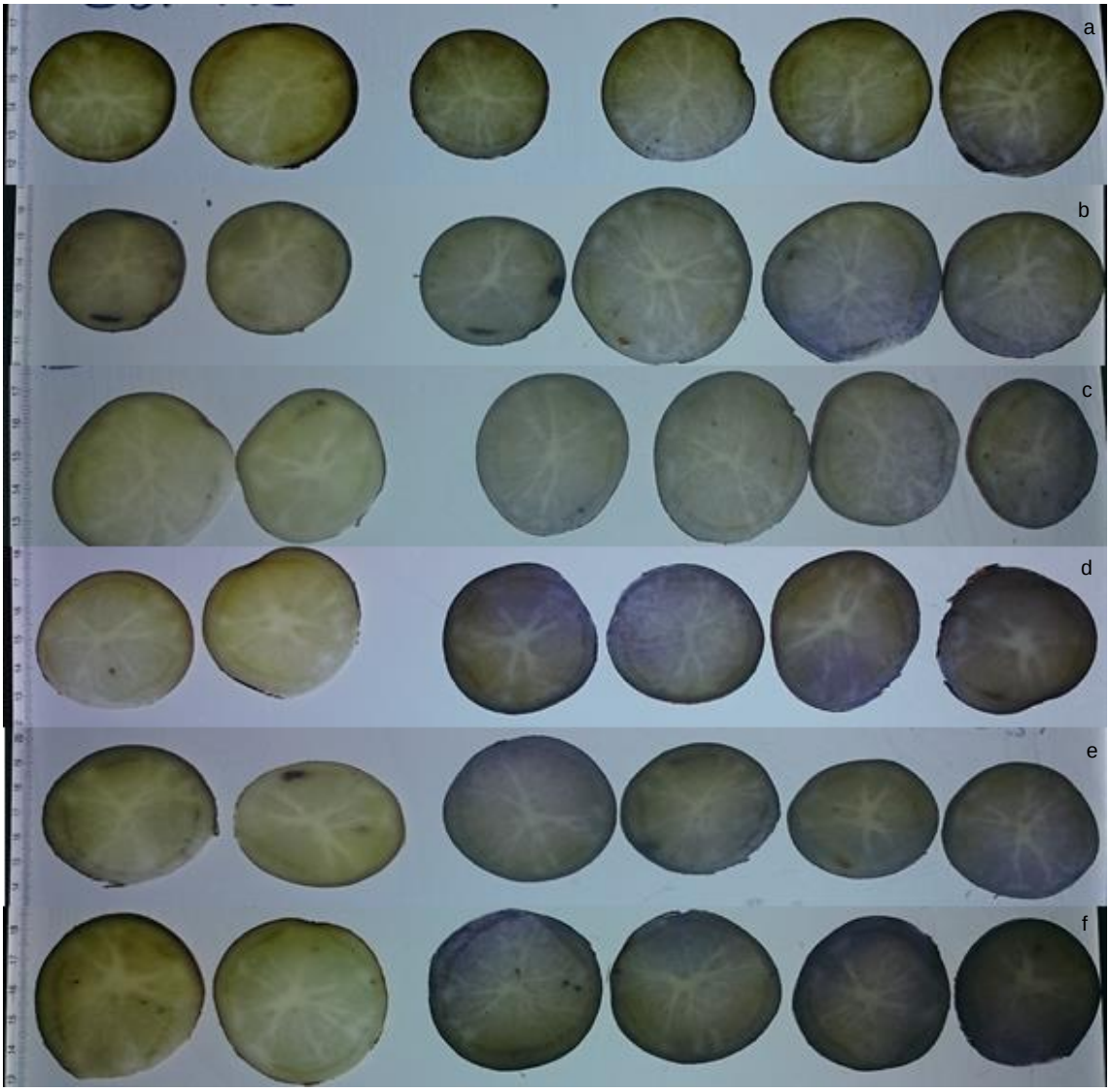

Plate 3-7 - NBT staining (O_2^- tissue staining) on L. Rosetta, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

Development of purple and brown precipitates within tissue from potato slices from DAB and NBT staining was analysed using ImageJ. R scripts were used to quantify the proportion of red, green and blue pixels in each image. From these readings, the amount of brown (DAB tissue staining of H_2O_2) and purple (NBT tissue staining of O_2^-) colour in the image were calculated. Negative values for purple and brown colour were observed where on occasion unstained control slices developed a degree of discolouration on cutting.

A large difference between seasons in ROS detection was observed, but it was not easily compare between season variation due to the difference sampling points within the season (in 2014/15 measurements started in January and in 2015/16 measurements started in December).

Between varieties a variation in the rate of H_2O_2 ($p < 0.001$) and O_2^- ($p < 0.05$) was observed when data was averaged across all sampling points in 2014/15. In general, an increase in H_2O_2 and O_2^- content was recorded with the duration of storage at 10°C.

In year 1 (2014/15) significant variation between the extent of the content of H_2O_2 and O_2^- (purple and brown colouration) was observed between all four varieties ($p < 0.001$ and $p < 0.05$, respectively). VR 808 was the variety with the lowest H_2O_2 content. However, there was no significant difference between varieties for H_2O_2 content. Changes in H_2O_2 and O_2^- content were seen during the storage season (Table 3-18).

During 2015/16 analysis was restricted to VR 808 and L. Rosetta and no difference between varieties in H_2O_2 or O_2^- content was recorded (Table 3-18). During storage, an increase in H_2O_2 but not in O_2^- content ($p < 0.001$) was observed in both varieties.

Table 3-18 - H_2O_2 and O_2^- content for seasons 2014/15 and 2015/16.

	Season 2014/15	
	H_2O_2	**O_2^-**
L. Rosetta	40.0[a]	38.9[a]
VR 808	27.2[a]	-0.9[b]
P. Dell	39.3[a]	48.6[a]
R. Burbank	27.0[a]	28.8[a]
HSD$_{0.05}$	*15.4*	*35.1*
	Season 2015/16	
	H_2O_2	**O_2^-**
L. Rosetta	38.3[a]	17.3[a]
VR 808	30.5[a]	9.2[b]
HSD$_{0.05}$	*14.1*	*24.2*

Mean values with different letters were significantly different according to Tukey HSD test. Content of O_2^- and H_2O_2 are expressed in terms of a numerical value to describe intensity of the purple colouration produced by NBT staining and brown colouration produced by DAB staining respectively.

O_2^- and H_2O_2 content in VR 808 showed different patterns in activity between the two years of observations. The low level of ROS-staining observed in the first 2-3 months of storage, was masked by general discolouration of tuber slices in untreated control samples making early detection difficult. Subtracting background colours of the control slices against the brown and purple colours in the ROS stained tissue often led to negative readings at this stage of the storage season.

In 2014/15 season activity of ROS was low for the first 5 months of storage at 10°C but a rapid increase in activity was seen after (Figure 3-21). When ROS activity was plotted against AsA and DHA content, DHA content raised soon after ROS (Figure 3-21).

A much intense staining pattern for ROS content was observed in VR 808, in 2015/16 from harvest and during storage, and an increase in H_2O_2 content was observed soon after harvest ($p < 0.001$) while a smaller rise in O_2^- occurred but failed to reach

statistical significance (Figure 3-21). H_2O_2 and O_2^- content depicted by staining patterns decreased after the early rise returning to content levels seen at or soon after harvest (Figure 3-21).

There was a poor relationship between H_2O_2 and O_2^- content and the presence of AsA and DHA accumulation. In general, it is expected as AsA declines the amount of DHA increases however, in these experiments where AsA concentration declined within 2 months of harvest at the point where ROS content increased, no increase in DHA concentration was observed (Figure 3-21).

In year 1 (2014/15), for L. Rosetta, content of H_2O_2 and O_2^- was low during early storage (4 months), increasing from March (5 months) until July (7 months storage) (Figure 3-24). By the end of the storage season, an increase in O_2^- content occurred concurrently with an increase in AsA and a decrease in DHA (Figure 3-24). However, higher and earlier H_2O_2 and O_2^- content was observed in year 2 (2015/16). Changes in the individual content of H_2O_2 and O_2^- during this season were similar during storage with mirrored fluctuations in content (Figure 3-25).

In P. Dell and R. Burbank, O_2^- and H_2O_2 content increased with the length of storage (Figure 3-27). Changes in the content of O_2^- and H_2O_2 were similar. Especially in P. Dell, the increase in ROS corresponded with an increase in AsA and a decrease in DHA (Figure 3-27).

Multiple level correlation outputs between H_2O_2 and O_2^- with AsA/DHA and sugar profiles can be viewed in Figures 3-23, 3-25, 3-26 and 3-29. A significant correlation between H_2O_2 and O_2^- content depicted through staining profiles and sucrose content in VR 808, L. Rosetta and P. Dell in season 2014/15. In that same season reducing sugars content positively correlated with H_2O_2 and O_2^- activity in L. Rosetta and P. Dell (Figure 3-25 and Figure 3-28). For R. Burbank even though H_2O_2 and O_2^- content positively correlated with reducing sugars but not sucrose (Figure 3-29). R. Burbank was the only variety where an increasing ratio of AsA/DHA was correlated with higher H_2O_2 content (Figure 3-29). P. Dell and R. Burbank, O_2^- and H_2O_2 content increased with the length of storage and glucose content (Figure 3-28 and Figure 3-29). However,

just in P. Dell that O_2^- and H_2O_2 content positively correlated with fructose content (Figure 3-25).

In season 2015/16 the relationship between ROS content and sugar were opposite to previous years of study in VR 808 and L. Rosetta (Figure 3-23 and Figure 3-26), with a negative correlation between a decreasing H_2O_2 content and an increase in sucrose content. In VR 808 a decrease O_2^- content was correlated with a rise in sucrose content.

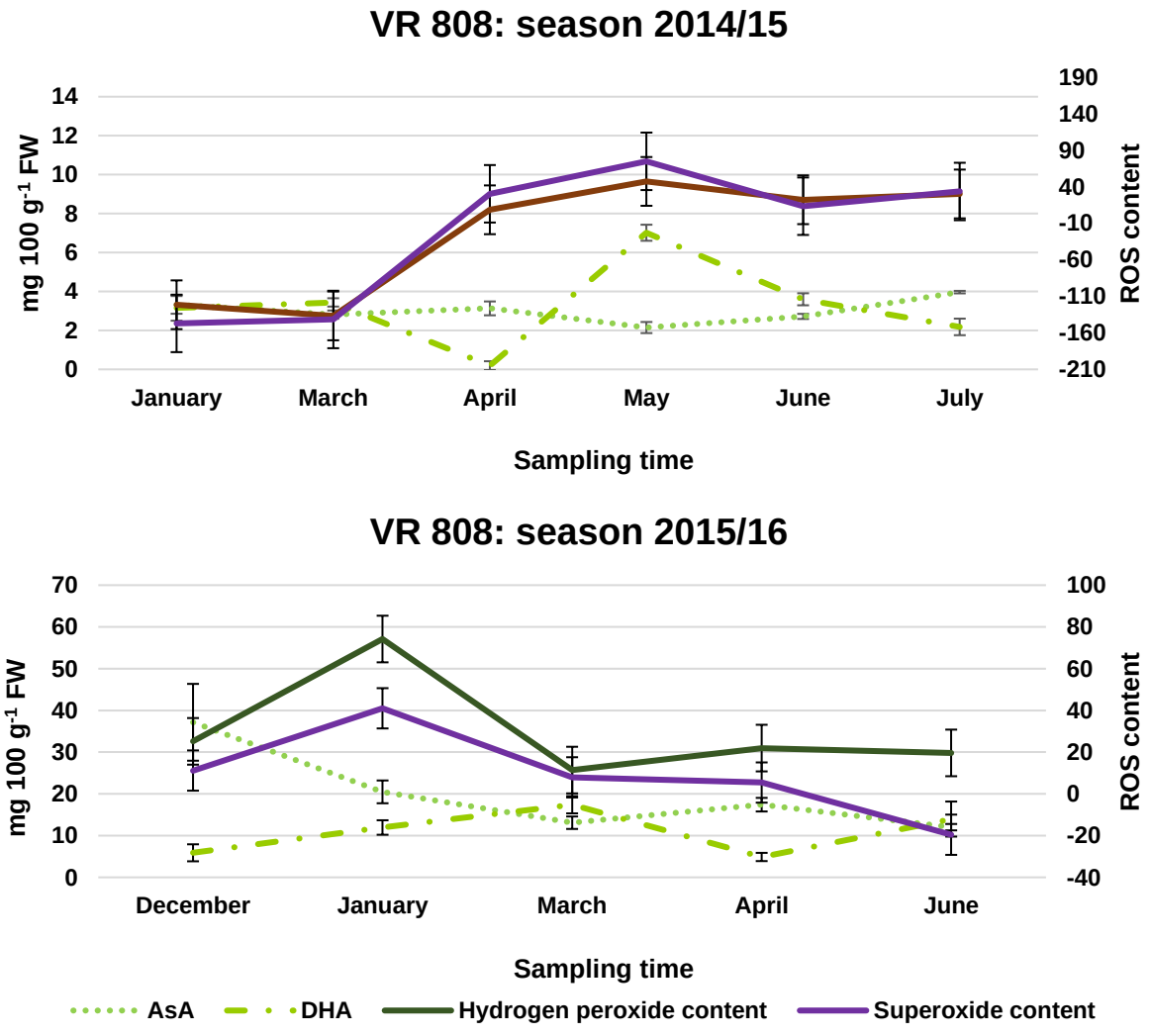

Figure 3-21 – Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) content in relation to AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) concentration for VR 808 tubers from seasons 2014/15 and 2015/16.

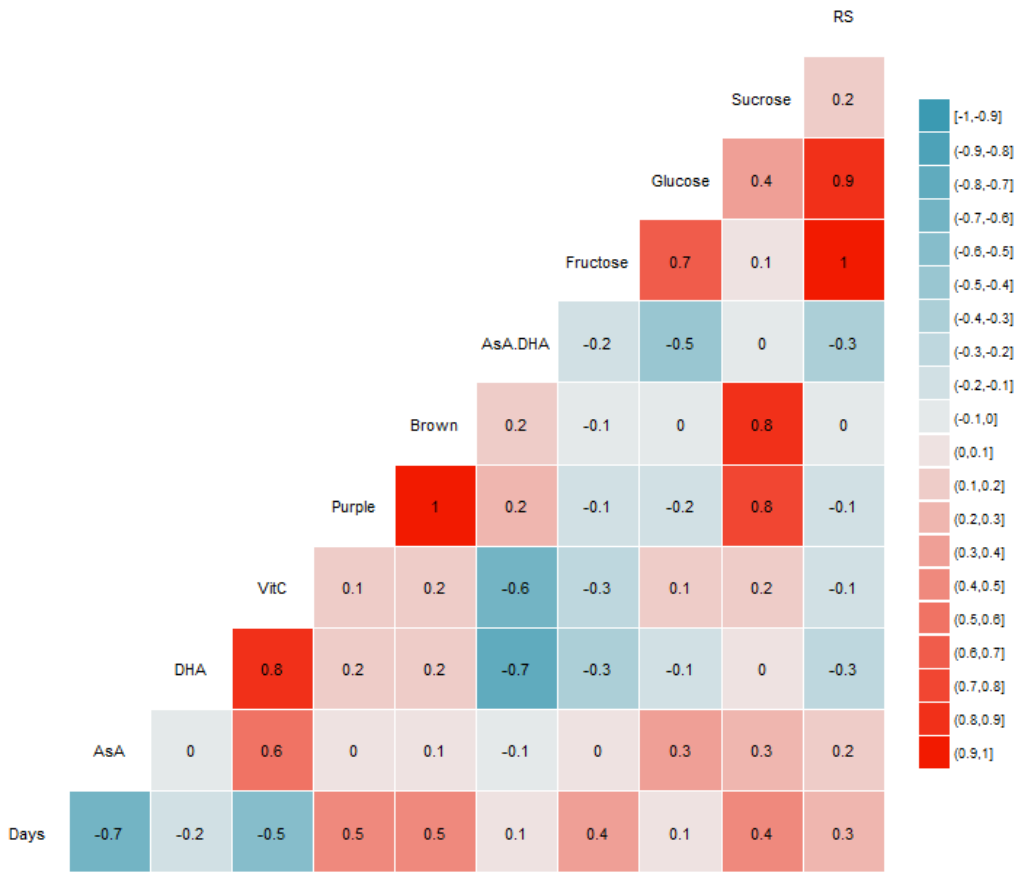

Figure 3-22 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), $O_2^{\cdot-}$ content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for VR 808 (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

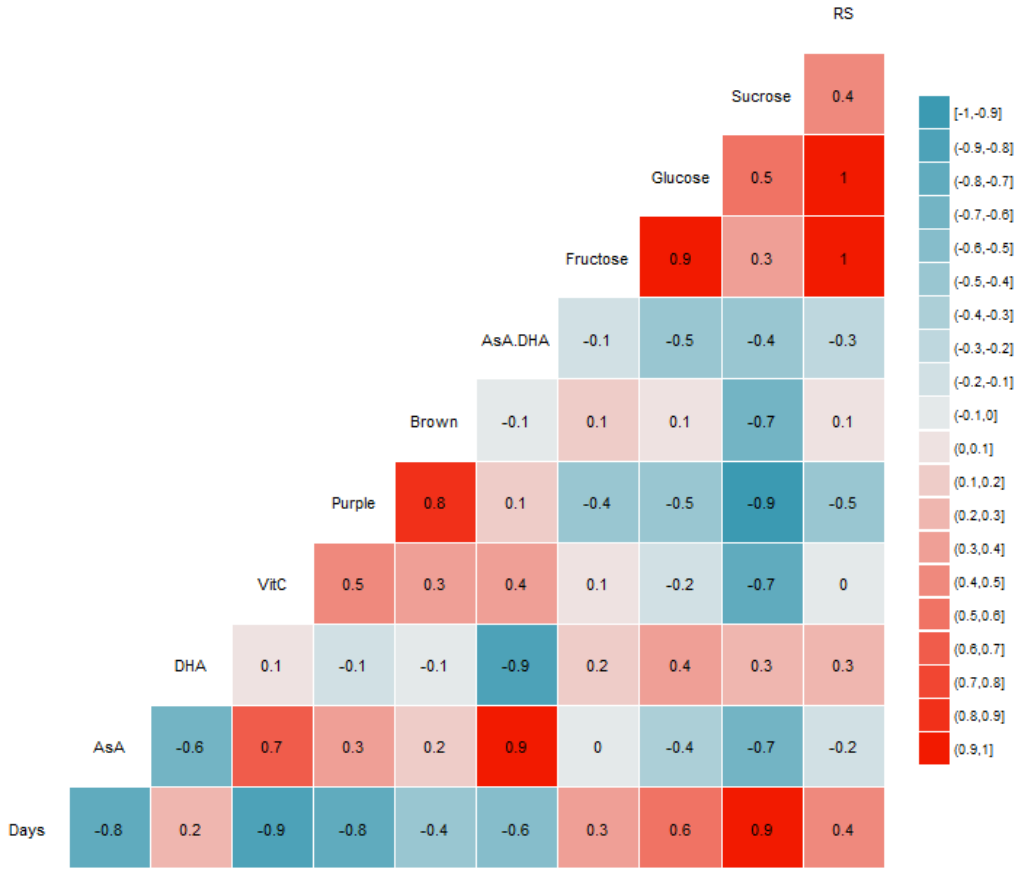

Figure 3-23 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for VR 808 (2015/16) (df = 4). Significant Pearson correlation coefficients from 0.73 to 1 and from -0.73 to -1 ($p < 0.05$).

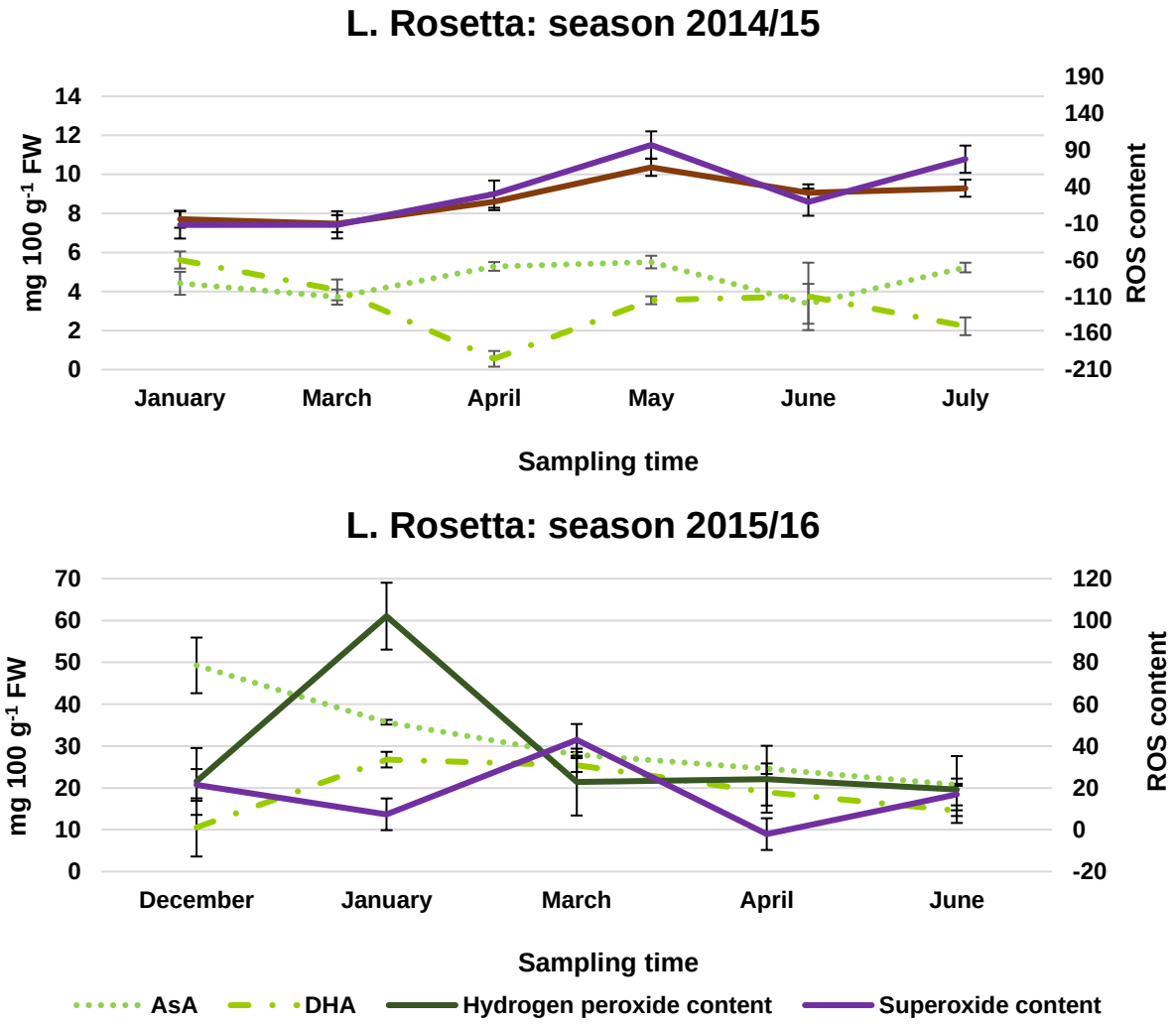

Figure 3-24 – Superoxide (O_2^-) and hydrogen content (H_2O_2) content in relation to AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) concentration for L. Rosetta tubers from seasons 2014/15 and 2015/16.

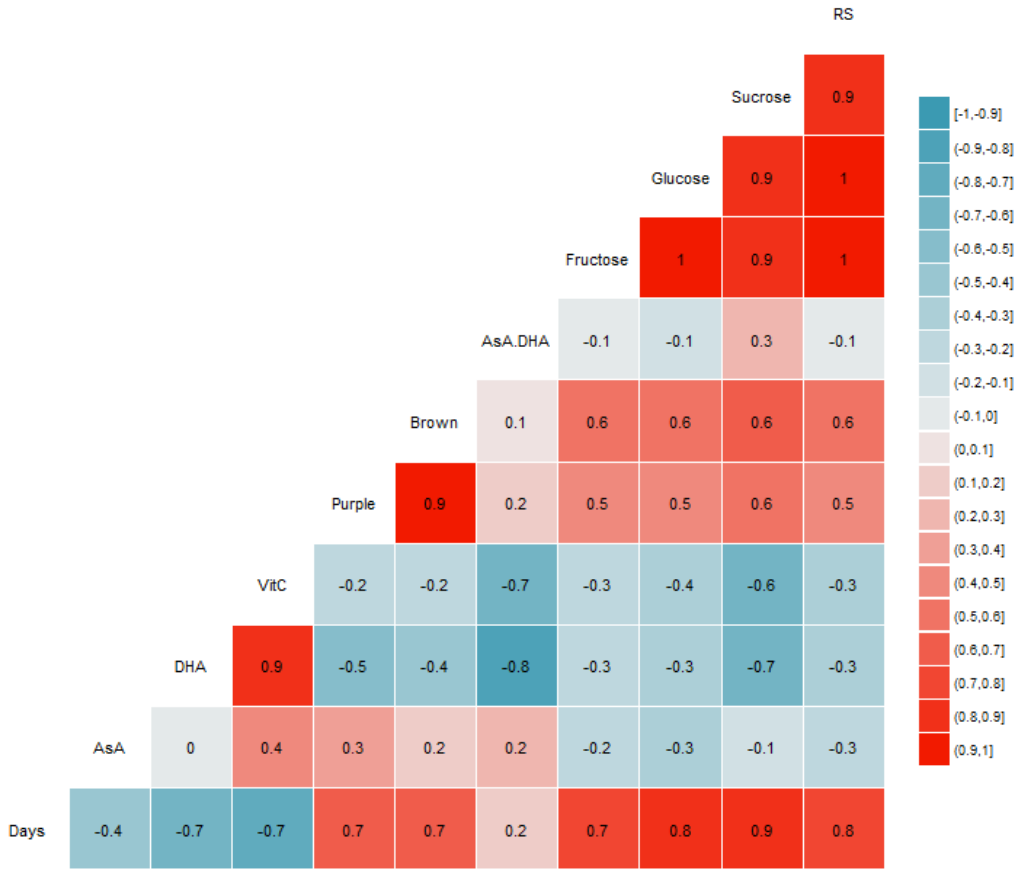

Figure 3-25 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for L. Rosetta (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

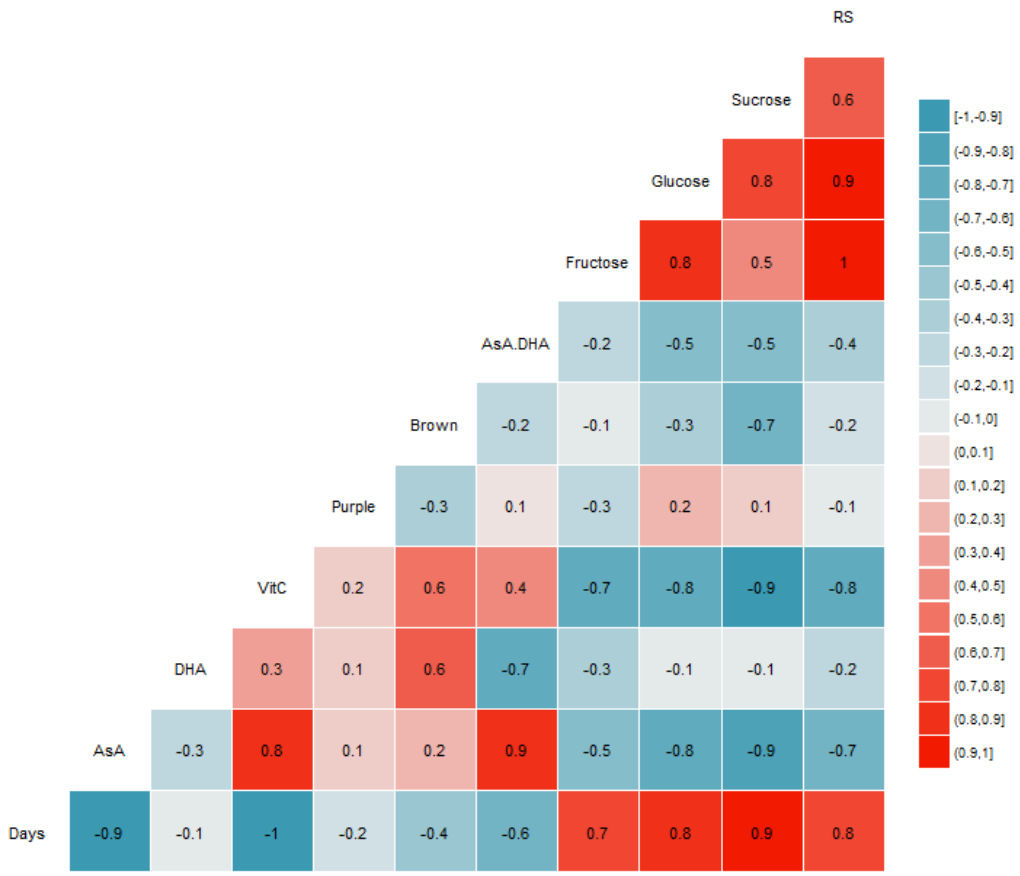

Figure 3-26 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for L. Rosetta (2015/16) (df = 4). Significant Pearson correlation coefficients from 0.73 to 1 and from -0.73 to -1 ($p < 0.05$).

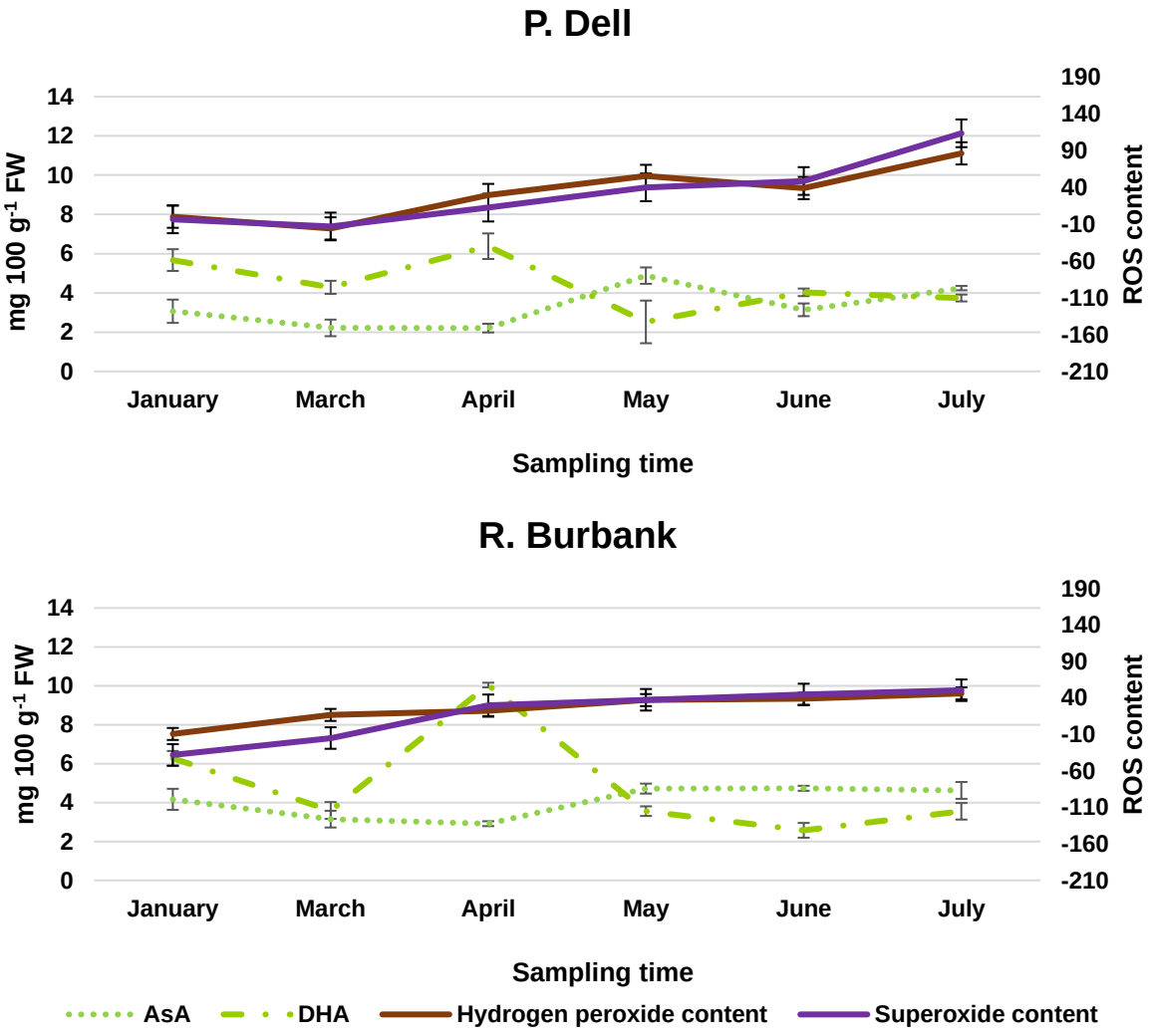

Figure 3-27 – Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) content in relation to AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) concentration for P. Dell and R. Burbank tubers from season 2014/15.

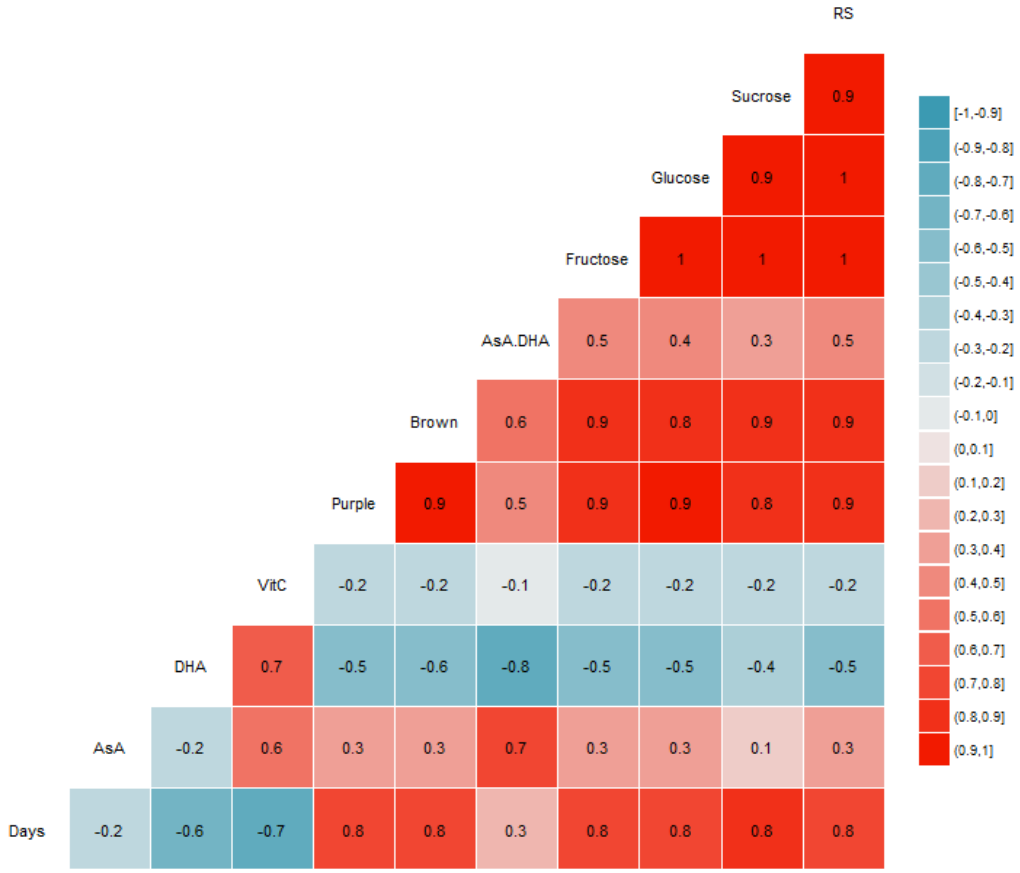

Figure 3-28 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for P. Dell (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

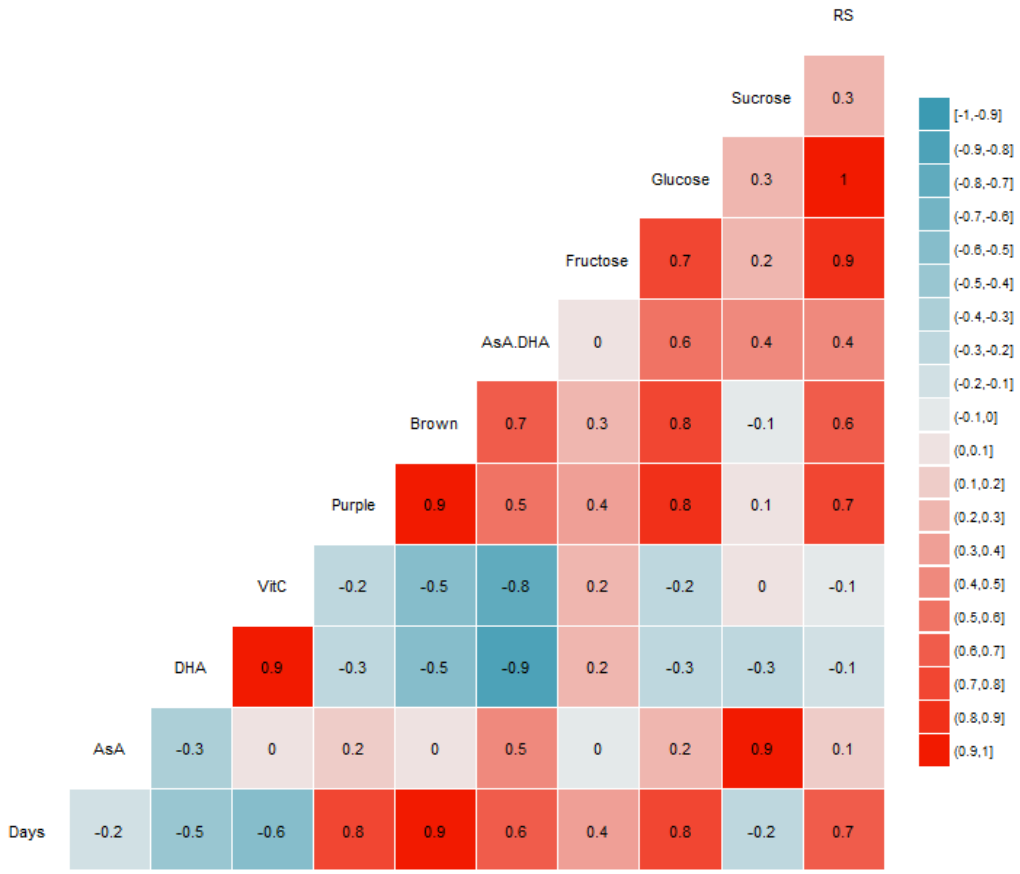

Figure 3-29 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for R. Burbank (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

The effect of physiological aging (chitting) of seed potatoes on ROS accumulation

Physiologically aging of seed tubers (chitted) prior to planting influenced O_2^- content ($p < 0.01$), but not H_2O_2 content in tubers harvested from chitted plants. An overall increase in H_2O_2 content ($p < 0.001$) was observed across treatments after 2 months (January) of storage and then declined afterwards (Figure 3-30-c).

Multiple correlation analysis of ROS, antioxidant content and sugars performed against tubers from plants grown from chitted and non-chitted seed showed a decrease in H_2O_2 content over time. In non-physiologically aged seed tubers (non-chitted) an increase in DHA accumulation, and all the sugars occurs with a rise in H_2O_2 content (Figure 3-31).

For physiologically aged seed tubers H_2O_2 content decreased over the storage period while fructose and glucose increased. O_2^- content was negatively correlated with DHA concentration and positively correlated with sucrose accumulation (Figure 3-32).

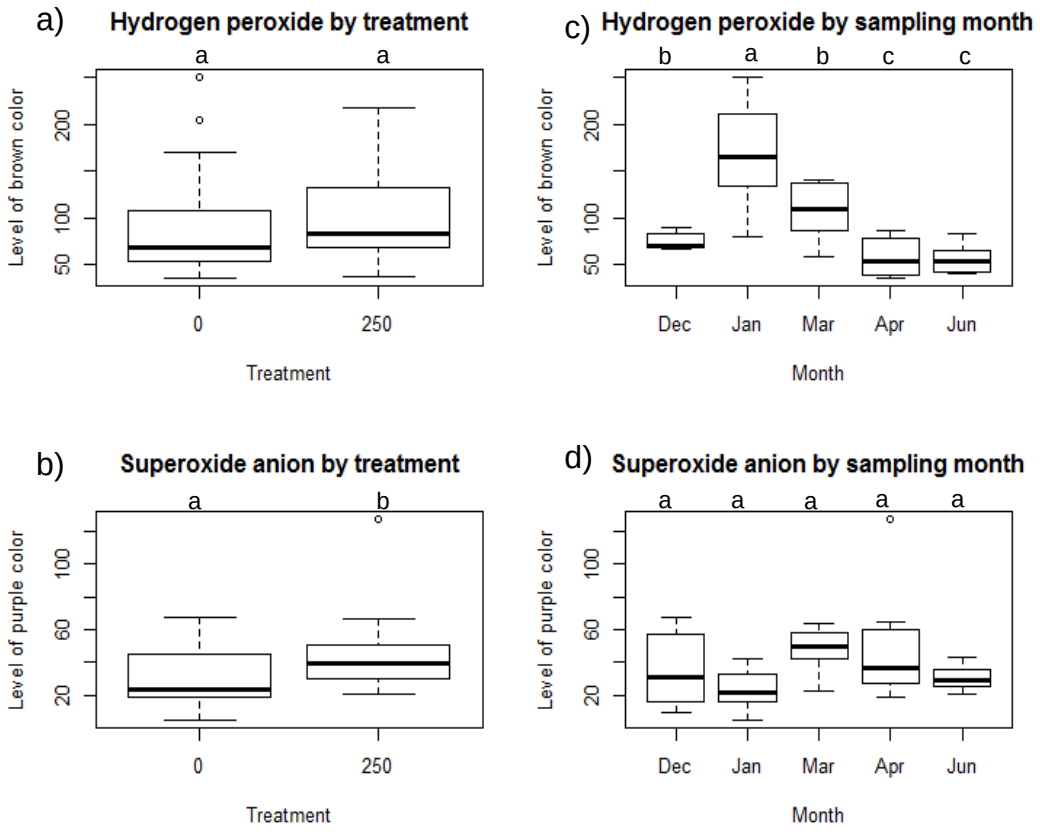

Figure 3-30 – Hydrogen peroxide (H_2O_2) activity by treatment (0°C days and 250°C days) (a) and by sampling month (b), and superoxide anion (O_2^-) levels by treatment (0°C days and 250°C days) (c) and by sampling month (d), for P. Dell from the chitting experiment. Mean values with different letters were significantly different (a) $HSD_{0.05}$ = 17.42, b) $HSD_{0.05}$ = 13.06, c) $HSD_{0.05}$ = 39.13 and d) $HSD_{0.05}$ = 29.33). The median is shown as a thicker dark line.

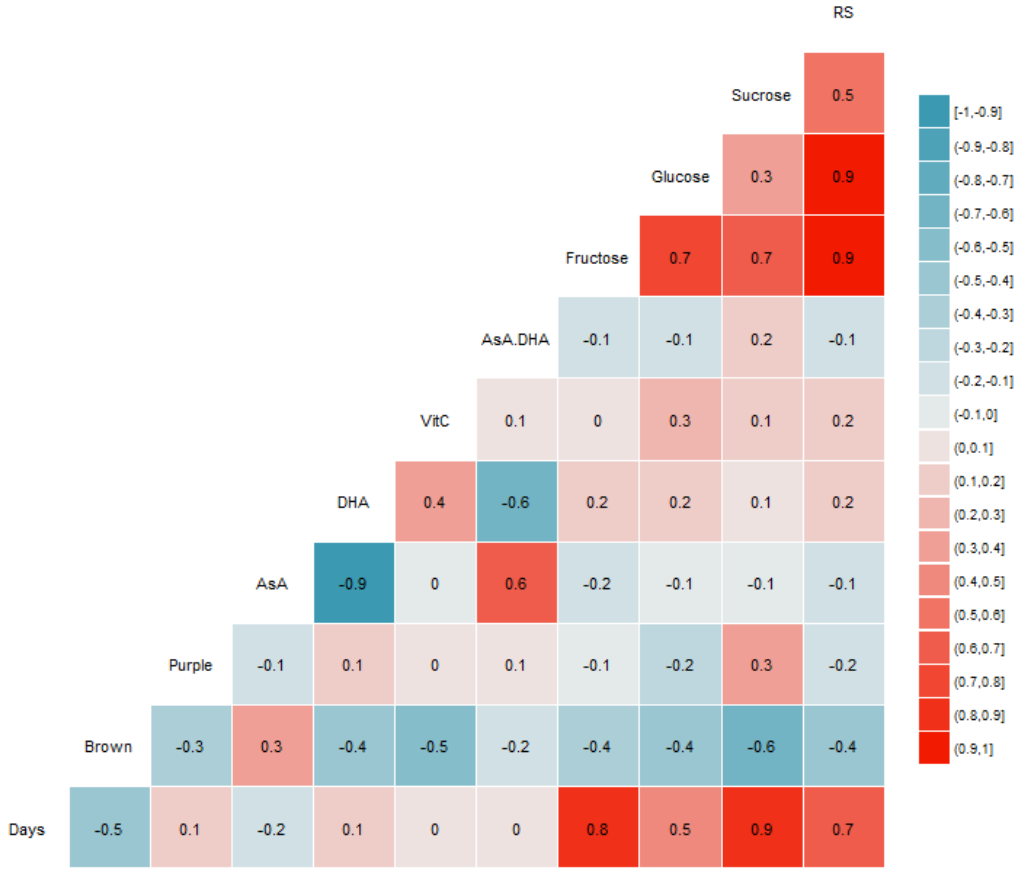

Figure 3-31 – Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for 0°C days from the chitting experiment (df = 18). Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$).

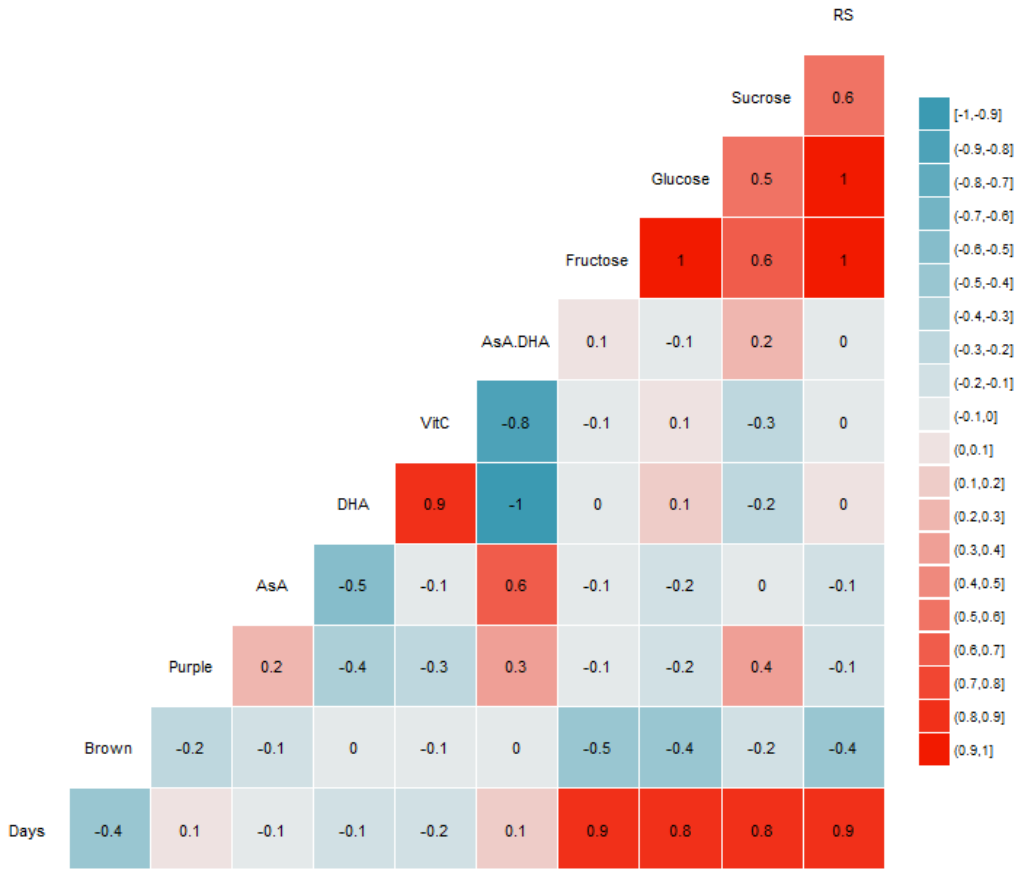

Figure 3-32 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for 250°C days from the chitting experiment (df = 18). Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$).

3.3.4. Assessment of tuber texture during storage

Wend fracture analysis provides a more discriminating method for determining of cell wall strength than analysis by penetrometer, or instron, where multiple forces of compression tear and shear make up the displacement of the probe.

In general, there was an increase in resistance to cell fracture as tubers aged due to water loss leading to increased elasticity of tissue (Figure 3-33 and Figure 3-34). P. Dell (Figure 3-39) required the most energy to induce cell fracture due to increased deformation of tissue (Plate 3-8), with tubers sampled at the end of storage compared to samples taken at harvest.

Location of cultivation had no effect on firmness for VR 808 (Figure 3-36) but changes in texture were seen during storage with increasing amounts of energy required to generate a crack over time ($p < 0.001$). Overall VR 808 required less energy to propagate cell fractures than other varieties (Figure 3-39).

The biomechanical properties of L. Rosetta were affected by planting location ($p < 0.001$). L. Rosetta from Norfolk requires greater energy to generate fracture than tubers grown from Shropshire and Yorkshire (Figure 3-36). With increasing storage duration, there was an increased in resistance to fracture (Figure 3-33).

The energy required to generate cracking in P. Dell and R. Burbank tubers increases with the length of storage ($p < 0.01$ and $p < 0.001$, respectively) (Figure 3-34). Resistance to cell fracture increase over a 7-8 months period during storage at 10°C and then decreased by the final sampling at 9-10 months (July).

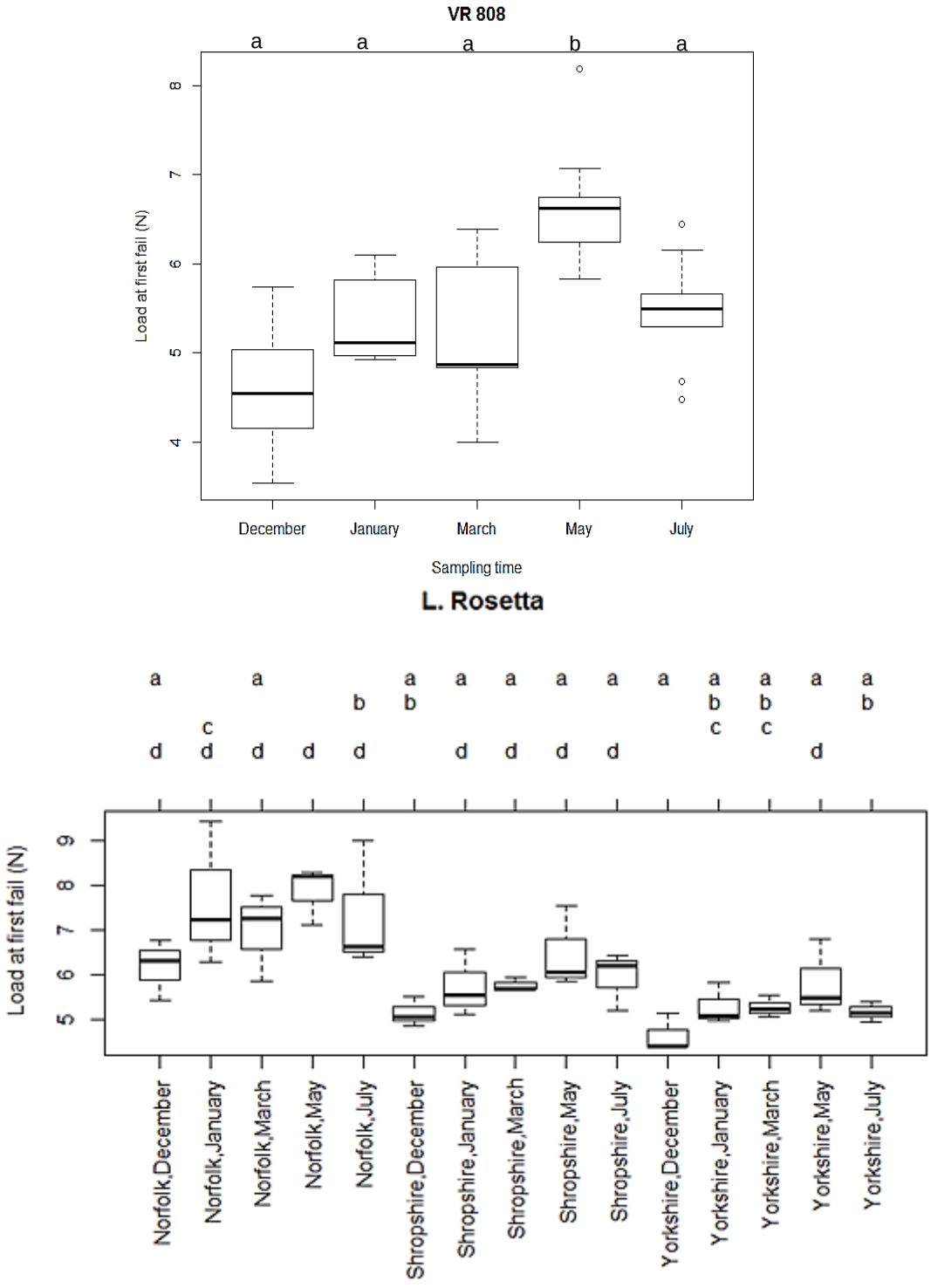

Figure 3-33 - Comparison of the variation in firmness (load at first fail (N)) per sampling occasion for season 2013/14 for VR 808 and L. Rosetta. Mean values with different letters are significantly different ($HSD_{0.05}$ VR 808 = 0.99 and $HSD_{0.05}$ L. Rosetta= 1.1). The median is shown as a thicker dark line.

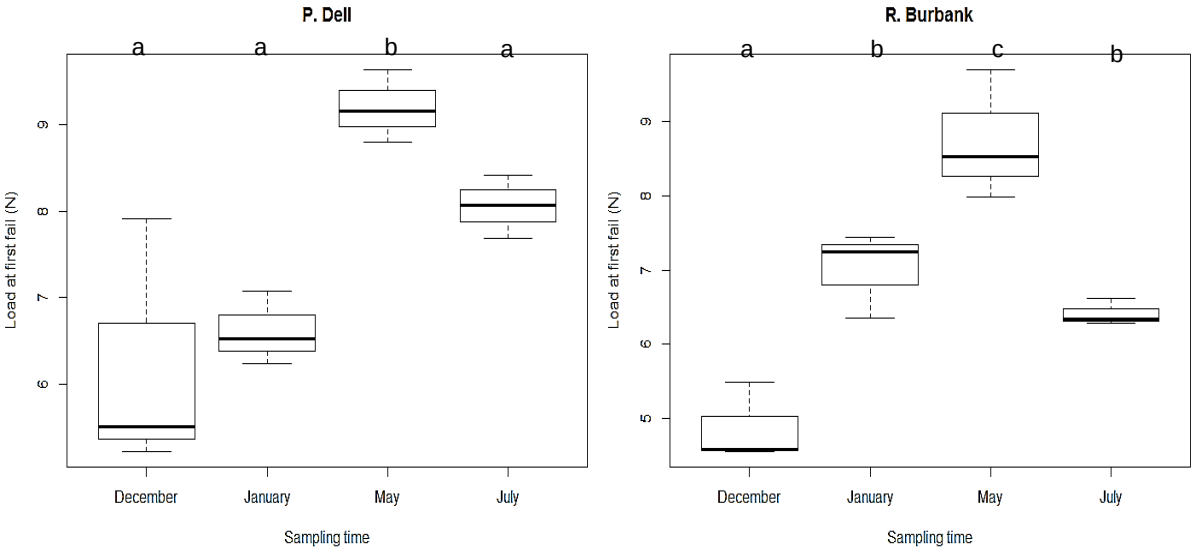

Figure 3-34 - Comparison of the variation in firmness (load at first fail (N)) per sampling occasion for season 2013/14 for ($HSD_{0.05}$ P. Dell = 2.13 and $HSD_{0.05}$ R. Burbank = 1.55). The median is shown as a thicker dark line.

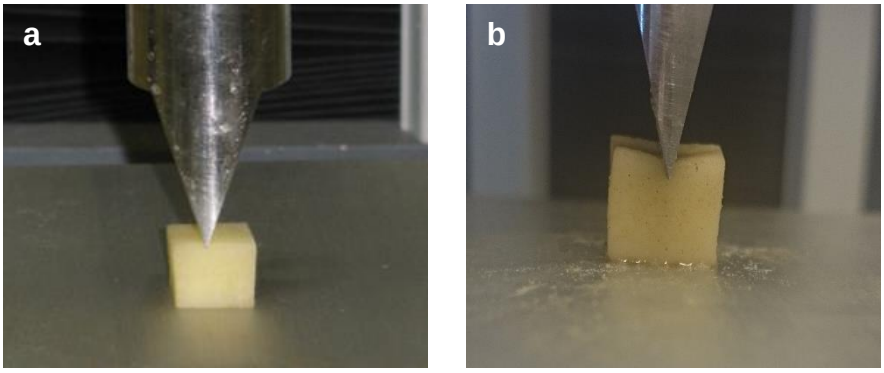

Plate 3-8 – VR 808 sample in Lloyd LRX-plus texture analyser from December 2013 (a) and May 2014 (b)

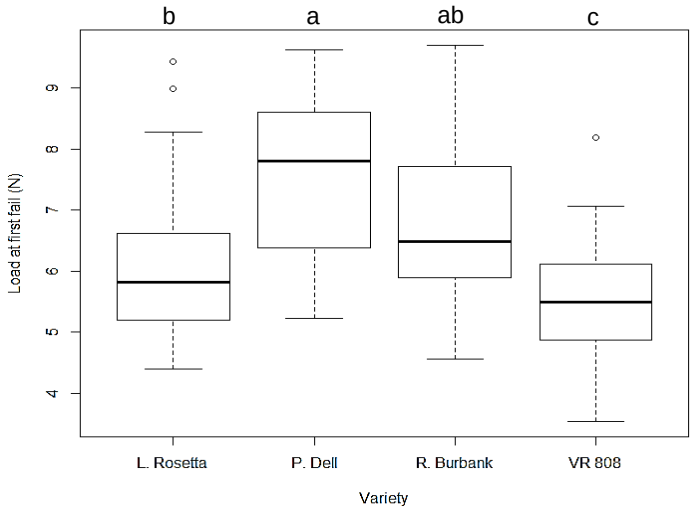

Figure 3-35 - Comparison of the variation in firmness (load at first fail (N)) for the 4 varieties used during season 2013/14. Mean values with different letters are significantly different ($HSD_{0.05} = 0.76$). The median is shown as a thicker dark line.

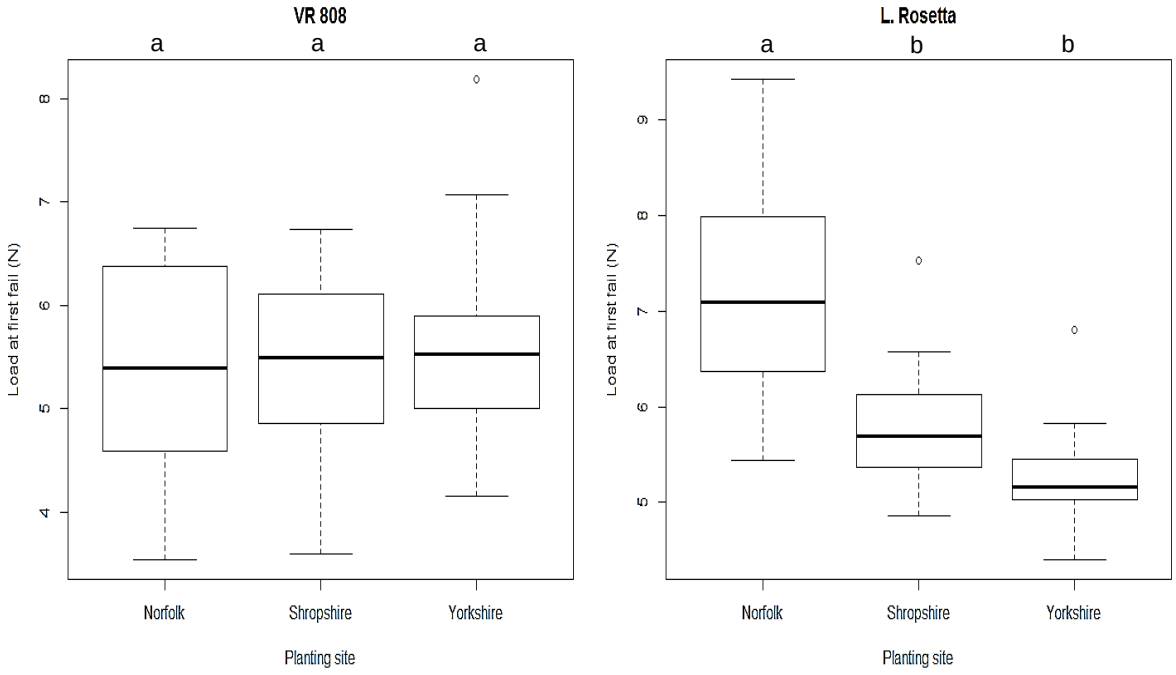

Figure 3-36 - Comparison of the variation in firmness (load at first fail (N)) for the 3 planting sites of season 2013/14 for VR 808 and L. Rosetta. Mean values with different letters are significantly different ($HSD_{0.05}$ VR 808 = 0.65 and $HSD_{0.05}$ L. Rosetta = 0.72). The median is shown as a thicker dark line.

3.3.5. Assessment of amyloplast and cellular changings during storage using scanning electron microscopy (SEM)

During the 1st year of this study (2013/14) analysis of freeze dried powdered samples of VR 808 tubers was performed with SEM in an attempt to observe changes in the amyloplast surface during the storage season.

The appearance of surface fractures on amyloplasts first observed at the end of storage (9 months at 10°C).

Over the next two years of this study (2014/15 and 2015/16) a more detailed study was extended to further include L. Rosetta, R. Burbank and P. Dell.

A comparison of preservation techniques found that sample drying through critical point drying (CPD), chemical drying (HSMS) and freeze drying proved to be equally effective in maintaining amyloplast integrity within freeze dry powder (Plate 3-9). However, the benefit of using freeze dried powders was that more amyloplasts were visible for analysis, as cell wall structure had been broken down to reveal amyloplasts. Moreover, under the SEM beam powder freeze dried samples were more stable under the electron beam compared to samples subject to CPD, HMDS or freeze dry tissue because these processes increased sample porosity making them more unstable.

Qualitative SEM analysis, of amyloplasts at the start of the storage season in November 2014/15 (1,5 month (L. Rosetta and VR 808) and <1 month (P. Dell and R. Burbank) in storage at 10°C), found the amyloplast surface free of fractures. The first signs in changes in amyloplast membrane integrity were the appearance of surface fractures. Fractures started to appear in January (4 months) (Plate 3-10 and Appendix VIII) in samples of L. Rosetta (Appendix IX) and VR 808 (Plate 3-10), but such symptoms were delayed until March (5 months) for P. Dell (Appendix X) and R. Burbank (Appendix XI), until the end of the storage season (10 months for L. Rosetta and VR 808, and 9 months for P. Dell and R. Burbank) (Plate 3-10).

The timiming of onset of fractures was dependant on variety and on position of the samples taken within the tuber. In general, the first signs of amyloplast cracking

observed for VR 808 were from samples taken from opposite eighths (ends) sections related to the periderm, cortex, vascular ring and outer core after 121 days in storage (January) whereas cracking of amyloplasts taken from middle of the tuber (inner core – medulla or pith) was first observed later after 170 days of storage (March, Appendix VIII). In contrast, the other varieties fractures in the amyloplast were found simultanously in sections of tuber taken from the inner core and from the opposite eighths of the tuber. In L. Rosetta the onset of cracking of amyloplasts was first observed after 121 days in storage (January), while for P. Dell and R. Burbank changes in amyloplast integrity were observed 141 (March) days in storage.

To cross check the development of fractures was not an artefact of the freeze drying process, additional samples underwent critical point drying (CPD) at the beginning, middle and end of the storage season (Plate 3-11), the development of fractures appears to be independent of the method of sample preparation.

In the second year (2015/16) SEM analysis was restricted to VR 808 and L. Rosetta and unlike the previous year, fractures of the amyloplast were observed at harvest (1 month and half) and continued to develop during storage (Plate 3-12 and Plate 3-13). Of the two varieties under observation, VR 808 had a greater incidence of fractures on the surface of the amyloplast. The depth and frequency of fractures appeared to increase- although no quantitative method for assessing these attributes were developed during the thesis. No difference between the onset of fractures was observed between sampling positions within the tuber for either variety.

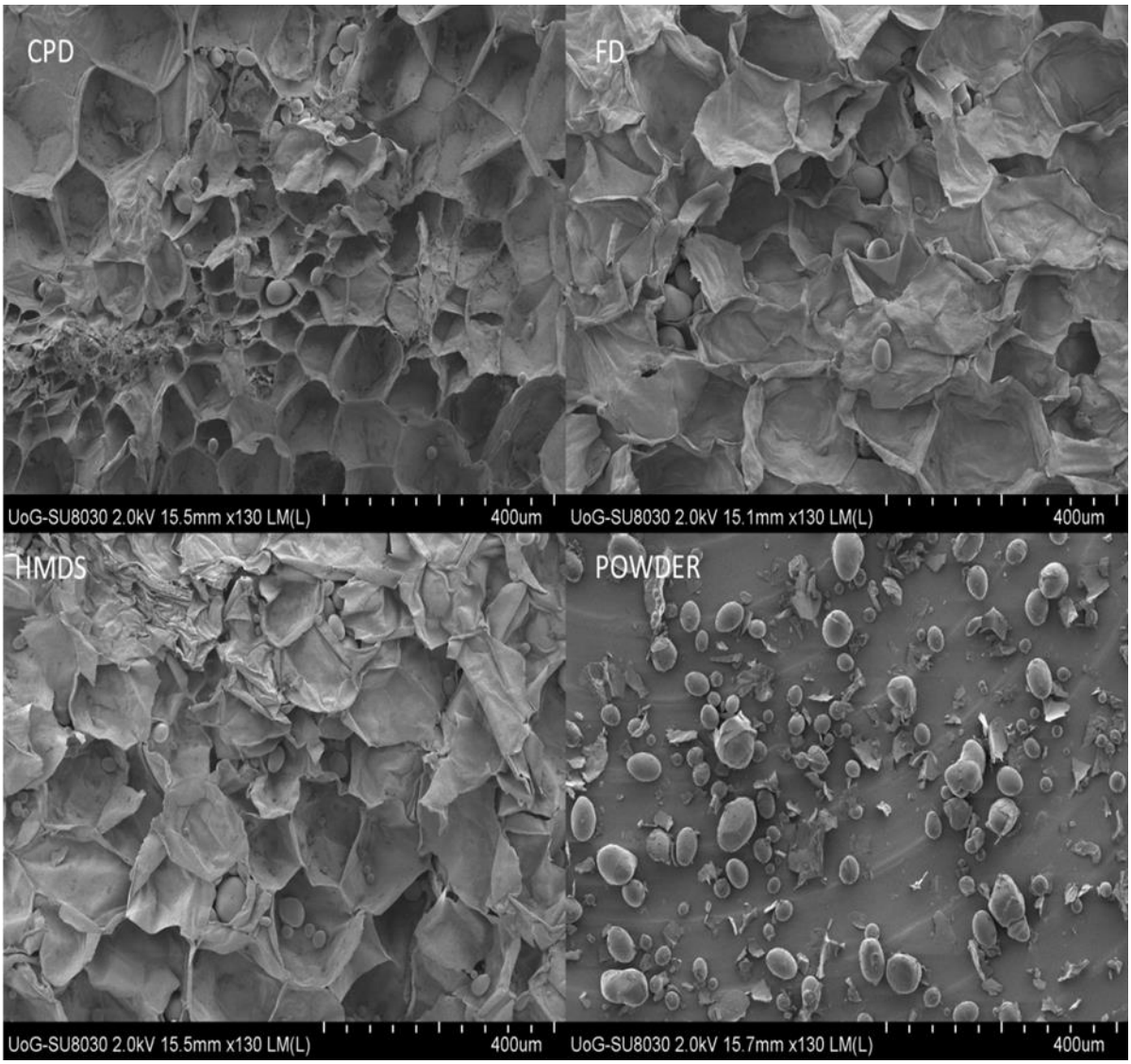

Plate 3-9 - SEM images obtained from the different methods of fixation/dehydration of samples (CPD, Critical Point Dry; FD, Dehydration by freeze drying; HMDS, hexamethyldisilazane, and powder, powder freeze dry tissue)

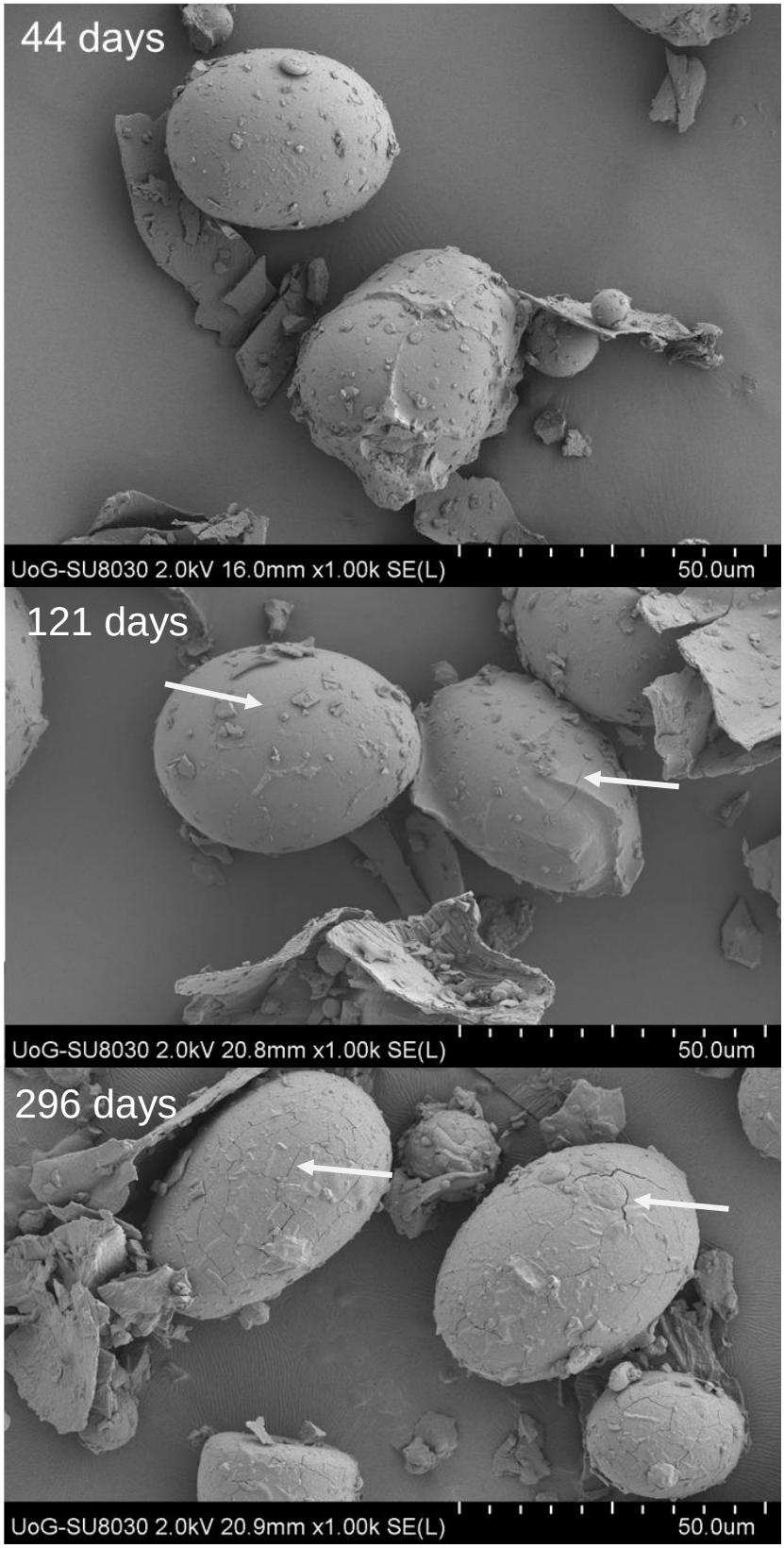

Plate 3-10 - SEM images from tuber ends section of VR 808 with 44 days (November 2014), 121 days (January 2015, 1st visible fractures) and 296 days (July 2015) of storage. White arrows point to fractures.

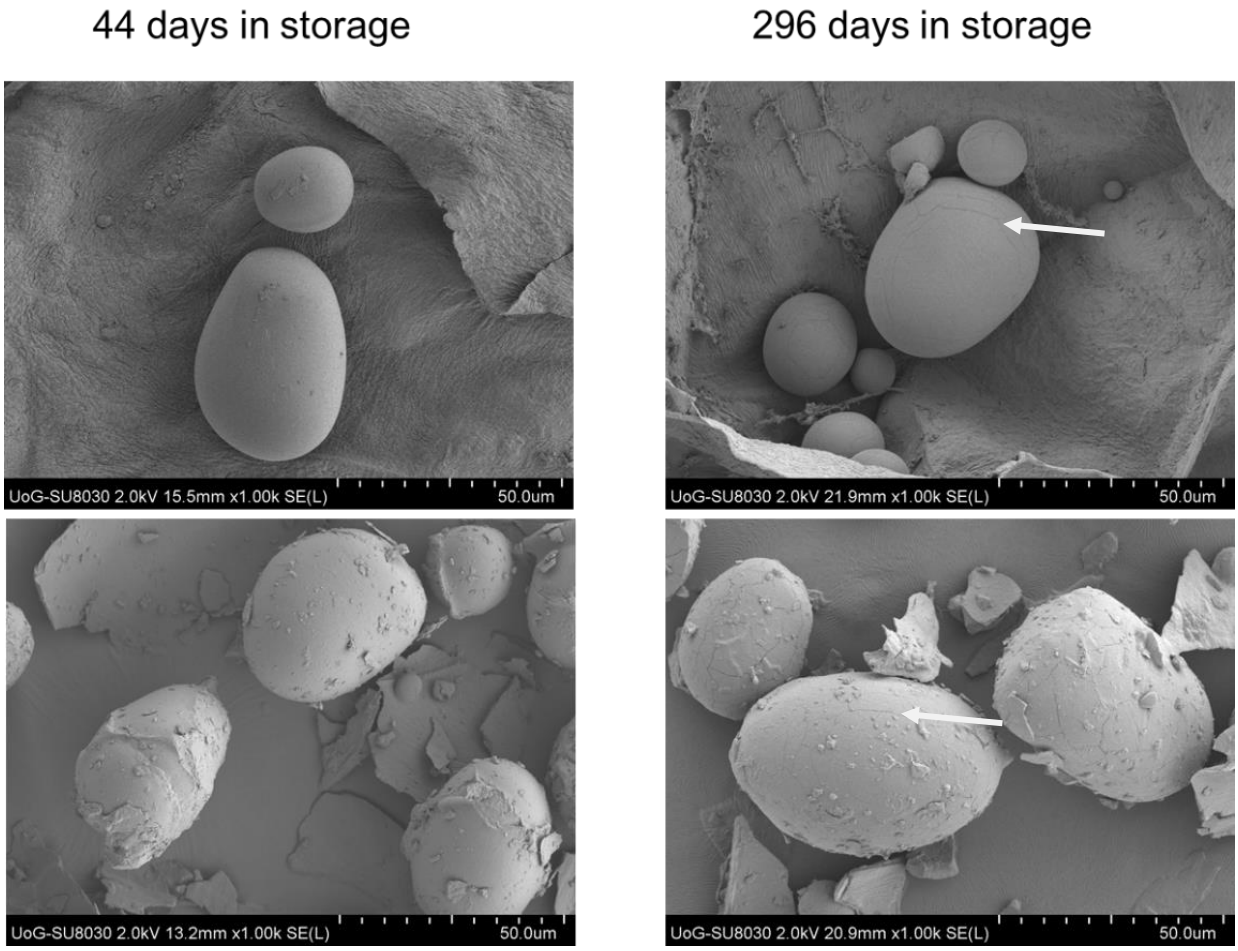

Plate 3-11 - Samples from VR 808 (storage season 2014/15), top images obtained with CPD and down images obtained with powder freeze dry tissue. White arrows point to fractures.

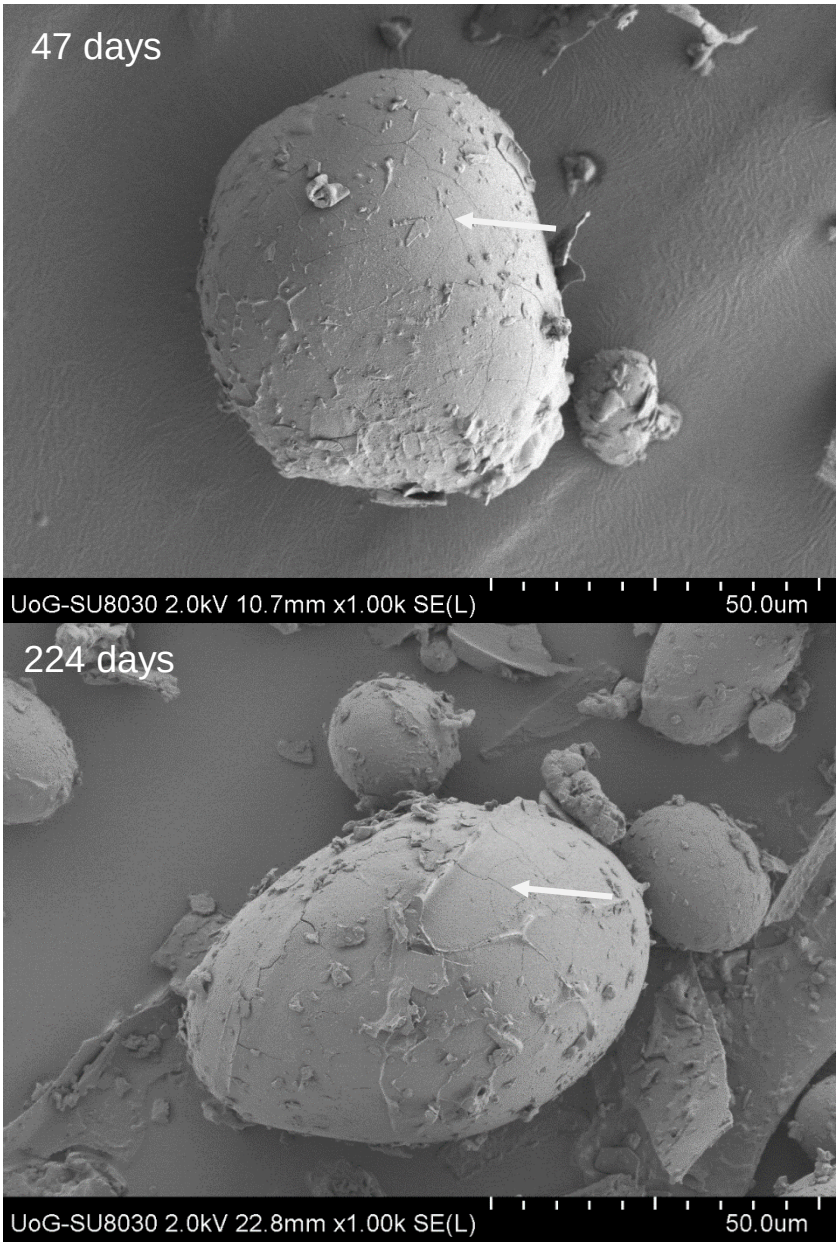

Plate 3-12 - SEM image from tuber ends section of VR 808 with 47 days (November 2015, with visible fractures) and 224 days (June 2016, with deeper fractures) of storage. White arrows point to fractures.

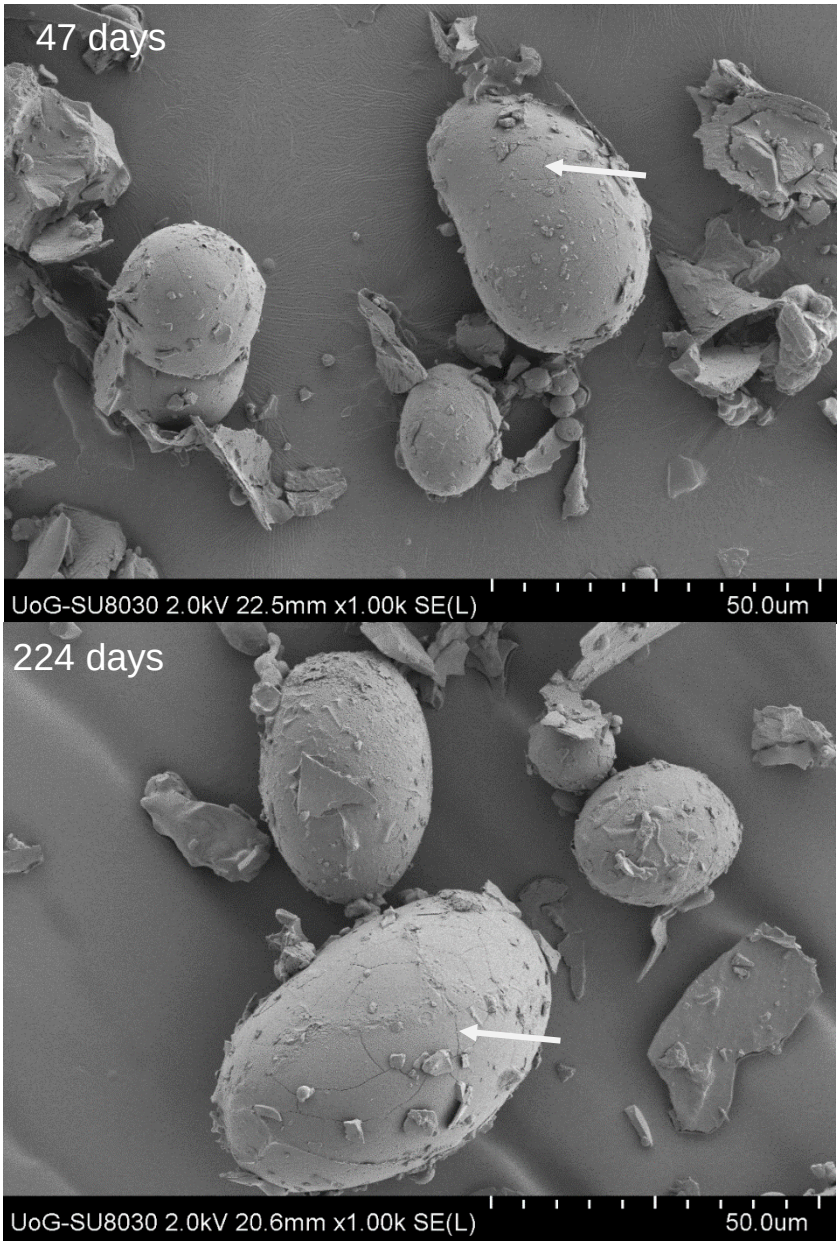

Plate 3-13 - SEM image from tuber ends section of L. Rosetta with 47 days (November 2015, with visible fractures) and 224 days (June 2016, with deeper fractures) of storage. White arrows point to fractures.

3.3.6. Determination of mineral accumulation on tubers

A comparison of calcium content of the combined opposite eights sections (periderm, cortex, vascular ring and outer core) versus the middle part (inner core – medulla or pith) of potato tubers was undertaken for VR 808, L. Rosetta, P. Dell and R. Burbank, at two time points November and end of the storage in July.

In general, mineral profiles between varieties were similar and differences found could not be attributed to an increased propensity of L. Rosetta and P. Dell to sweeten earlier than VR 808 or R. Burbank. Calcium analysis is presented as total soluble calcium (Ca_{total}) and the proportion that was bound/conjugated to oxalate and other compounds is expressed as Ca_{bound}.

P. Dell was found to have the highest concentration of total calcium (37.1 mg $100g^{-1}$) when averaged across the two samples taken from the ends and mid-sections of the tuber.

P. Dell and L. Rosetta had the greatest proportion of bound/conjugated calcium (4.1 and 3.9%, respectively) (Table 3-19).

Other mineral elements such as Mg and K can compete and displace calcium from binding sites and influence the overall calcium activity even when the calcium content is high. Therefore, the presence of high concentrations of either of these elements can influence storage potential of crops.

The increased propensity of P. Dell and L. Rosetta to have poor storage characteristics could not be fully explained by the availability of calcium antagonists. While, P. Dell, tubers accumulated greater amounts of K (1226 $mg100g^{-1}$) the lowest content was found in L. Rosetta (979 $mg100g^{-1}$However, L. Rosetta (54 $mg100g^{-1}$) and P. Dell (53 $mg100g^{-1}$) were higher in Mg (Table 3-19).

In other stored crops, such as apple the ratio of K/Ca or (K+Mg)/Ca can provide an indication of storage potential. L. Rosetta and VR 808 had the higher (K+Mg)/Ca ratios again suggesting these parameters do not provide a clear indicator of storage potential at harvest.

While other mineral elements do not have reported roles in retaining the storage potential of potatoes, many act as electron donors (Fe^{2+}, Cu^{2+}) in many biochemical reactions, while the availability of phosphate (phosphate starvation) can influence many phosphorylation events. Analysis of these microelements did not show any particular trends. VR 808 had higher Cu content, and together with L. Rosetta were high in Fe^{2+} while P. Dell contained more P (51.6 $mg100g^{-1}$) (Table 3-19).

For all the varieties Ca_{total} and Ca_{bound} content in the ends of the tuber were significantly different from the inner core of the tuber (Table 3-20) suggesting that availability of calcium may change across the tuber. Comparison of nutrients sampled at the opposite ends and the inner core exemplified an interesting distribution of minerals. Calcium was more abundant at the outer regions of potato, reflecting possibly more cell wall bound calcium in regions of greater cell density. While more soluble K and Mg were higher in the sections samples in the middle of the tuber in VR 808 and L. Rosetta. The opposite was observed in P. Dell and R. Burbank (Table 3-20). No clear relationship between mineral distribution and the propensity to sweeten could be concluded from the data, with the exception of K which was significantly higher in the middle cortex samples of P. Dell and may reflect a greater degree of antagonism against calcium in this region.

Analysis of samples were taken at the beginning (November) and the end of storage (July) in order to see if changes in the availability and distribution were apparent. While it was not expected that overall concentrations of nutrients should change, increased binding of calcium may lead to a reduction in calcium bioavailability. Results in Table 3-21 show no increase in calcium binding, and on a couple of occasions the amount of bound calcium decreased (L. Rosetta and R. Burbank). No significant trend in other minerals between sweetening and their distribution was observed (Table 3-21).

For each variety correlations between of Ca_{total}, Ca_{bound}, Cu, Fe, K, Mg, P, Zn, $\%Ca_{bound}$, (K+Mg)/Ca, %FW of sucrose and %FW of reducing sugars was performed. In both sweetening resistant varieties (VR 808 and R. Burbank), %FW of sucrose was negatively correlated with Fe and Ca_{total}, and negatively correlated with $\%Ca_{bound}$ in both sensitive varieties (L. Rosetta and P. Dell) (Figure 3-37 and Figure 3-38). Just in

R. Burbank was observed a negative correlation between %FW of sucrose and %FW of reducing sugars (Figure 3-38).

Table 3-19 – Ca_{total}, Ca_{bound}, $\%Ca_{bound}$, K, Mg, (K+Mg)/Ca, Cu, Fe, P and Zn for VR 808, L. Rosetta, P. Dell and R. Burbank.

	Ca_{total} mg 100 g^{-1} FW	**Ca_{bound} mg 100 g^{-1} FW**	**% Ca_{bound}**	**K mg 100 g^{-1} FW**	**Mg mg 100 g^{-1} FW**	**K+Mg / Ca**	**Cu mg 100 g^{-1} FW**	**Fe mg 100 g^{-1} FW**	**P mg 100 g^{-1} FW**	**Zn mg 100 g^{-1} FW**
VR 808	26.84^{c}	1.03^{a}	3.32^{b}	1133^{b}	49.14^{b}	48.15^{a}	0.60^{a}	0.65^{a}	41.77^{b}	0.87^{a}
L. Rosetta	21.03^{d}	0.86^{b}	3.85^{ab}	978.6^{c}	54.28^{a}	52.26^{a}	0.46^{b}	0.63^{a}	37.89^{b}	0.82^{a}
P. Dell	37.12^{a}	1.06^{a}	4.07^{a}	1226^{a}	53.03^{a}	35.89^{c}	0.42^{b}	0.43^{c}	51.60^{a}	0.79^{a}
R. Burbank	31.18^{b}	0.85^{b}	2.67^{c}	1096^{b}	46.96^{b}	42.62^{b}	0.47^{b}	0.50^{b}	42.64^{b}	0.87^{a}
$HSD_{0.05}$	*3.44*	*0.10*	*0.55*	*73.15*	*2.96*	*4.14*	*0.08*	*0.07*	*6.08*	*0.18*

Mean values with different letters are significantly different according to Tukey HSD test.

Table 3-20 – Ca_{total}, Ca_{bound}, $\%Ca_{bound}$, K, Mg, (K+Mg)/Ca, Cu, Fe, P and Zn by part analysed for VR 808, L. Rosetta, P. Dell and R. Burbank.

	Part analysed	Ca_{total} mg 100 g^{-1} FW	Ca_{bound} mg 100 g^{-1} FW	% Ca_{bound}	K mg 100 g^{-1} FW	Mg mg 100 g^{-1} FW	K+Mg / Ca	Cu mg 100 g^{-1} FW	Fe mg 100 g^{-1} FW	P mg 100 g^{-1} FW	Zn mg 100 g^{-1} FW
VR 808	Ends	32.21[a]	1.15[a]	3.25[a]	877.2[b]	45.33[b]	28.77[b]	0.42[b]	0.84[a]	35.51[b]	0.75[b]
	Middle	21.48[b]	0.91[b]	3.38[a]	1389[a]	52.95[a]	67.54[a]	0.78[a]	0.46[b]	48.03[a]	0.99[a]
	HSD$_{0.05}$	*1.74*	*0.12*	*0.6*	*80.33*	*2.6*	*5.75*	*0.08*	*0.07*	*3.1*	*3*
L. Rosetta	Ends	24.27[a]	0.93[a]	3.32[b]	782.6[b]	48.37[b]	34.55[b]	0.37[b]	0.82[a]	27.49[b]	0.69[b]
	Middle	17.79[b]	0.79[b]	4.38[a]	1175[a]	60.19[a]	69.96[a]	0.55[a]	0.44[b]	48.28[a]	0.95[a]
	HSD$_{0.05}$	*1.78*	*0.13*	*0.82*	*61.11*	*3.41*	*4.68*	*0.12*	*0.06*	*11.8*	*0.09*
P. Dell	Ends	40.42[a]	1.21[a]	3.70[b]	928.3[b]	47.86[b]	24.21[b]	0.33[b]	0.53[a]	40.12[b]	0.68[b]
	Middle	33.82[b]	0.90[b]	4.44[a]	1523[a]	58.20[a]	47.57[a]	0.52[a]	0.33[b]	63.07[a]	0.90[a]
	HSD$_{0.05}$	*4.09*	*0.11*	*0.68*	*102.38*	*2.71*	*2.68*	*0.07*	*0.04*	*4.32*	*0.06*
R. Burbank	Ends	41.02[a]	0.98[a]	2.70[a]	991.1[b]	45.36[a]	26.06[b]	0.41[b]	0.60[a]	36.95[b]	0.90[a]
	Middle	21.35[b]	0.72[b]	2.63[b]	1200[a]	48.56[a]	59.19[a]	0.53[a]	0.41[b]	48.33[a]	0.85[a]
	HSD$_{0.05}$	*6.18*	*0.11*	*0.29*	*83.52*	*4.43*	*5.15*	*0.09*	*0.12*	*4.84*	*0.39*

Mean values with different letters are significantly different according to Tukey HSD test.

Table 3-21 – Ca_{total}, Ca_{bound}, $\%Ca_{bound}$, K, Mg, (K+Mg)/Ca, Cu, Fe, P and Zn by sampling month for VR 808, L. Rosetta, P. Dell and R. Burbank.

	Month analysed	Ca_{total} mg 100 g^{-1} FW	Ca_{bound} mg 100 g^{-1} FW	% Ca_{bound}	K mg 100 g^{-1} FW	Mg mg 100 g^{-1} FW	K+Mg / Ca	Cu mg 100 g^{-1} FW	Fe mg 100 g^{-1} FW	P mg 100 g^{-1} FW	Zn mg 100 g^{-1} FW
VR 808	November	27.12[a]	1.02[a]	2.68[b]	1124[a]	47.69[b]	47.49[a]	0.72[a]	0.95[a]	41.97[a]	0.86[a]
	July	26.56[a]	1.04[a]	3.95[b]	1142[a]	50.59[a]	48.81[a]	0.48[b]	0.36[b]	41.57[a]	0.87[a]
	HSD$_{0.05}$	*1.74*	*0.12*	*0.6*	*80.33*	*2.6*	*5.75*	*0.08*	*0.07*	*3.10*	*3.00*
L. Rosetta	November	19.82[b]	0.91[a]	4.69[a]	982.8[a]	52.85[a]	55.09[a]	0.54[a]	0.70[a]	40.97[a]	0.79[a]
	July	22.25[a]	0.81[b]	3.01[b]	974.4[a]	55.72[a]	49.43[b]	0.38[b]	0.56[b]	34.8[b]	0.85[a]
	HSD$_{0.05}$	*1.78*	*0.13*	*0.82*	*61.11*	*3.41*	*4.68*	*0.12*	*0.06*	*11.8*	*0.09*
P. Dell	November	35.86[a]	1.02[b]	4.67[a]	1238[a]	52.05[b]	38.03[a]	0.47[a]	0.45[a]	49.38[b]	0.76[a]
	July	38.38[a]	1.09[a]	3.47[b]	1214[a]	54.01[a]	33.75[b]	0.38[b]	0.40[b]	53.81[a]	0.82[a]
	HSD$_{0.05}$	*4.09*	*0.11*	*0.68*	*102.38*	*2.71*	*2.68*	*0.07*	*0.04*	*4.32*	*0.06*
R. Burbank	November	28.7[a]	1.00[a]	2.82[a]	1110[a]	46.32[a]	46.53[a]	0.38[b]	0.45[b]	42.54[a]	0.73[a]
	July	33.66[a]	0.70[b]	2.51[b]	1082[b]	47.60[a]	38.72[b]	0.55[a]	0.56[a]	42.74[a]	1.02[a]
	HSD$_{0.05}$	*6.18*	*0.11*	*0.29*	*83.52*	*4.43*	*5.15*	*0.09*	*0.12*	*4.84*	*0.39*

Mean values with different letters are significantly different according to Tukey HSD test.

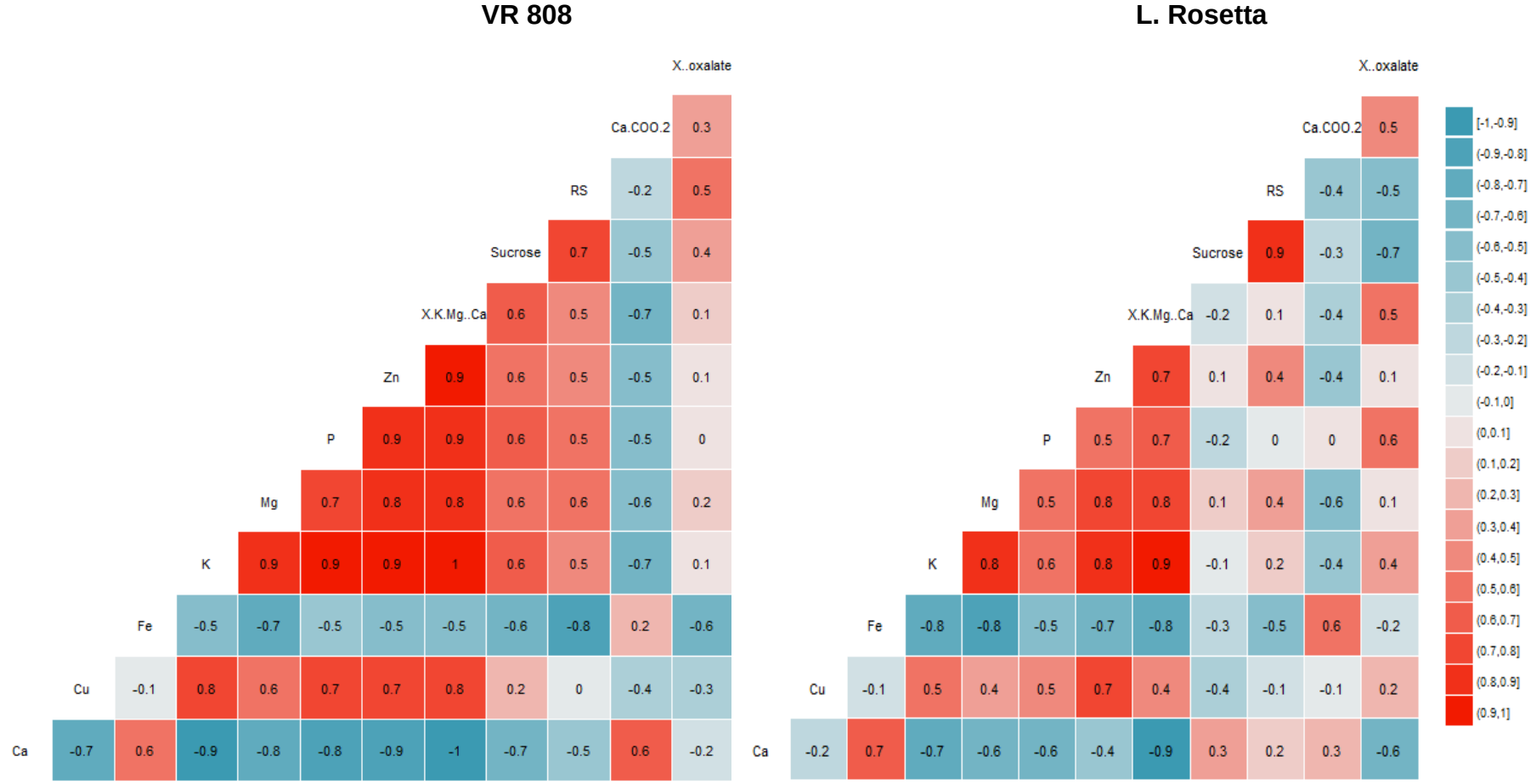

Figure 3-37 – Correlation between Ca_{total} (Ca), Cu, Fe, K, Mg, P, Zn, (K+Mg)/Ca (X.K.Mg..Ca), sucrose, reducing sugars (RS), Ca_{bound} (Ca.COO.2) and $\%Ca_{bound}$ (X..oxalate), during the storage season 2014/15 for the varieties VR 808 and L. Rosetta. Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$, df = 18).

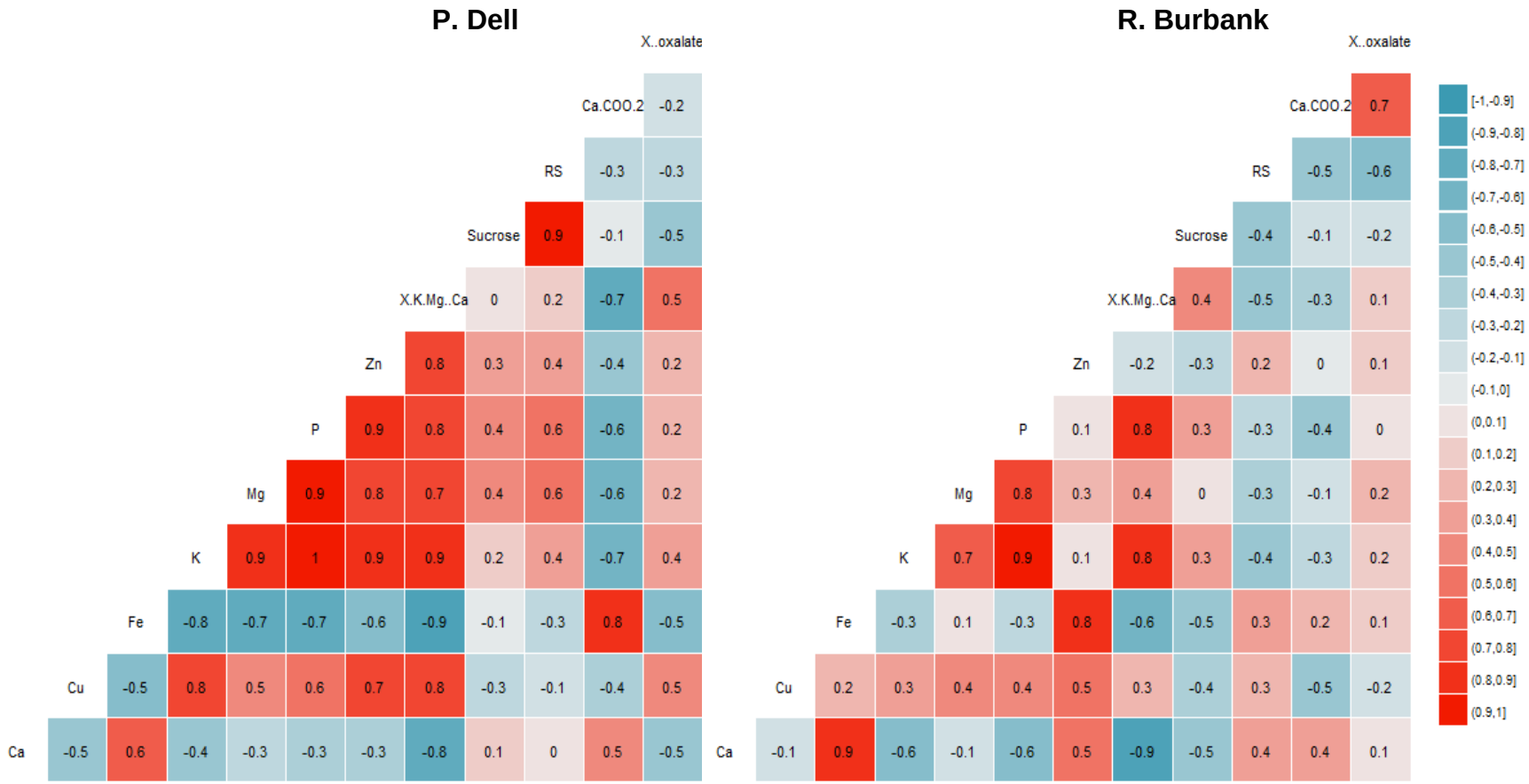

Figure 3-38 - Correlation between Ca_{total} (Ca), Cu, Fe, K, Mg, P, Zn, (K+Mg)/Ca (X.K.Mg..Ca), sucrose, reducing sugars (RS), Ca_{bound} (Ca.COO.2) and $\%Ca_{bound}$ (X..oxalate), during the storage season 2014/15 for the varieties P. Dell and R. Burbank. Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$, df = 18)

3.4. Discussion

Sugar accumulation and respiration during storage

The principal sugars in potato tubers are the reducing sugars glucose and fructose and the non-reducing disaccharide sucrose, and their concentrations change during crop development and storage (Sowokinos, 2001; Kumar *et al.*, 2004; Storey, 2007). During sprouting cellular metabolism changes from net synthesis of reserve compounds to net degradation, starch and protein breakdown overcomes their synthesis leading to the formation of soluble sugars and amino acids (Hajirezaei *et al.*, 2003).

The influence of planting locations for VR 808 and L. Rosetta during the 1st year of this study was generally small. Kumar *et al.* (2004) reviewed about factors affecting sugar content of potatoes and tuber maturity, such as genotype, environmental conditions and cultural practices during growth, and several post-harvest factors including storage. Kumar *et al.* (2004) concluded that sugar was most affected by fertilization, temperature and soil moisture. In this study, even though tubers were grown in distinct geographic locations climates effect had little impact on storage quality.

In general, P. Dell, had the highest reducing sugars and sucrose accumulation over the storage period and VR 808 the lowest. VR 808 is a recently released variety exclusively owned by PepsiCo used for crisp production, and has many of the agronomic traits such as high yielding and a low sugar profile during storage. In contrast, P. Dell is a more traditional variety used for chip production, which accumulates reducing sugars more rapidly than the other varieties in this study.

Previous assessment of varieties propensity to develop sweetening during storage (Table 2-2, (NIAB, 2008)) ranks P. Dell as having a moderate capacity (3) for developing sweeting, while L. Rosetta, is considered more sensitive to senescent sweetening.

Crisping varieties, L. Rosetta and VR 808, have a lower tolerance for reducing sugars accumulation than potatoes destined for chipping, eg. P. Dell and R. Burbank (OECD,

2015) as the degree of acceptability to discolouration is slightly lower in chipping varieties.

This study confirmed R. Burbank, characteristic of very late onset of senescence sweetening in contrast to L. Rosetta which shows early onset (NIAB, 2008).

In general changes in sugar profiles was influenced by sprouting and the effectiveness of sprout suppression. Where dormancy break and sprout growth is controlled by early CIPC application reducing sugar content is lowered in all varities.

Later in storage when sprouts start to re-emerge a second peak in reducing sugars occurs at the time when with the remergence of meristematic activity initiates the breakdown of starch to sugars. While the increase in availability of sugars is required to fuel sprout growth, reapplication of CIPC cuts off demand for sugars leading to a second rise in reducing sugars. The extent of the second increase in reducing sugar content was variety dependant. Hence the double peak in sugar accumulation is the combination of CIPC application and the onset of senescent sweetening. Hertog *et al.* (1997) reported that senescent sweetening was only observed where sprout suppression iwas applied which this data supports. Sprouting is associated with carbohydrate mobilization, mainly due to starch hydrolysation into sucrose (Burton *et al.*, 1992) and CIPC application has reduced sprouting and temporarily halted the accumulation of reducing sugars.

After 2-3 months of storage (January), all the varieties had broken ecodormancy and were sprouting, which led to a concurrent decrease in the concentration of reducing sugars and sucrose. During sprouting a demand for sucrose in the developing sprouts, causes a decrease in soluble sugar content in storage parenchyma. Such a depletion of soluble sugar within the storage parenchyma serves as signal to trigger starch breakdown into assimilates for sprout development (Hajirezaei *et al.*, 2003).

Across all varieties there was a point when O_2 consumption and CO_2 production decreased; the extent of this decline depended on the season. At this point the demand for sugars in respiration or sprout growth was low; leading to an accumulation within the tuber and restimulating respiration.

In these experiments differences in the propensity to accumulate sugars was dependant on variety. Halford *et al.* (2012) highlighted that long-term storage increased acrylamide formation with some varieties more susceptable to accumulating acrylamide precursors. The results of this study conclude with Halford *et al.* (2012) in that concentrations of reducing sugars increased in most varieties with time in storage. With aging there is a loss in membrane integrity caused by free radicals leading to increase utilisation of ATP and loss of energy, with increasing respiratory rates (Coleman, 2000).

Hence L. Rosetta, which commercially is stored until January, and P. Dell, until the end of February, started to present symptoms of senescence sweetening in March (1st year) and April (2nd year) while the long-storage varieties R. Burbank and VR 808 maintained stable sugars until June. These data is in agreement with Halford *et al.* (2012), where L. Rosetta and P. Dell sugar concentrations decreased between November and December and increased from March to July as senescent sweetening increases. During the 3rd year L. Rosetta had a lower sucrose and reducing sugars compared to previous years.

In R. Burbank an increase in the respiration rate late in storage (May), led to a decrease in the reducing sugars content. For R. Burbank and VR 808 reducing sugar content increased at the end of the storage and the ability to preserve a low sugar profiles was due to there efficiency in respiring the sugars.

Over time the concentration of reducing sugars increased in proportion with sucrose content, due to starch break down content during storage (Smith, 1987b; Hertog *et al.*, 1997; Kumar *et al.*, 2004).

In most cases sucrose content was higher than fructose and glucose content with the exception of P. Dell and R. Burbank (1st year). Sugar accumulation during storage is variety specific (Kumar *et al.*, 2004), and the timing of sweetening is dependent on the variety, duration of storage and storage temperature. In the 3rd year for both L. Rosetta and VR 808, sucrose content was double that of reducing sugars by the end of the storage season. Wills *et al.* (2007) concluded that respiration rate was an excellent indicator of the metabolic activity and an useful guide for predicting the storage life of

tubers. Increasing respiratory rates will lead to an increase of O_2^- production and according to the oxidative damage theory of aging, the oxidative and free radical stresses are cumulative over time (Spychalla and Desborough, 1990). When stored for long periods at high temperature (>4ºC) there is an increase in basal metabolism that can possibly accelerate tuber aging (Kumar and Knowles, 1996a; Blauer *et al.*, 2013a).

L. Rosetta and P. Dell had higher respiration rates and were the varieties with the highest O_2 consumption and CO_2 production. However, at the same time they were the varieties with the highest reducing sugars and sucrose accumulation from all the four varieties from this study, suggesting a possible lack of utilisation of sugars in respiration or that high respiration may signal greater starch breakdown. An increase in respiration rate was also noted by Kumar and Knowles (1996a; 1996b) who suggested tuber respiration rate was the "pacemaker" of aging in potato tubers (Blauer *et al.*, 2013a). All the four varieties had a different propensity to develop senescent sweetening (L. Rosetta: early, P. Dell: medium, R. Burbank very late onset, and VR 808 late) (NIAB, 2008).

NADPH oxidase, known as respiratory burst oxidase homologues, catalyzes the production of H_2O_2 and O_2^-, and gradually increases until reaching a maximum which coincide with dormancy break (Liu *et al.*, 2017). A relationship between the onset of senescent sweetening and the rise in ROS is established during this thesis. A rise in H_2O_2 and O_2^- content was observed in P. Dell and R. Burbank and was positively correlated with length of storage leading to visible darkening of staining patterns for H_2O_2 and O_2^- during storage. The content of H_2O_2 and O_2^- during storage was related with the pattern in respiration rate, and with an increase in H_2O_2 and O_2^- content. The relative rates of ROS content varied between years and a consequence of the combination of changes in respiration efficiency during storage, and tissues ability to quench free radicals and ROS, in particular O_2^- through antioxidant systems (protective enzymes, such SOD, APX, GPX and CAT, and non-enzymatic low molecular weight metabolites such AsA, GSH, α-tocopherol, carotenoids and flavonoids) (Keunen *et al.*, 2013). Changes in the ability of ROS scavenging could be related to the increase in respiration rate leading to an increase in O_2^- production (Coleman, 2000), and elevated respiration rates related to aging in tubers (Blauer *et al.*, 2013a). With tuber aging there

is a reduction in AsA levels and antioxidant enzymes with a progressive increase in oxidative stress (Petrov *et al.*, 2015).

Sprouting

The application of CIPC during storage prevented the full interaction of dormancy break/sprout growth and respiration rate from being observed. It is uncertain whether changes in respiration rate was initiated by sprout growth or by the physiology of the whole tuber. However, a positive correlation between CO_2 production and sprout growth for all varieties was observed. Copp *et al.* (2000) concluded that sprouts by themselves do not change the rate of tuber respiration, but rather it is the consequence of physiological changes in the tuber that are responsible for sprouting. Therefore, the increase in sprout growth with the rise in respiration rate is a consequence of tuber aging rather than the specific rate of sprouting. When the suppressive effect of CIPC declines sprouting resumes as the tubers age and with an increase in respiration rate.

Amyloplast changes, texture and sweetening

Tuber texture

Potato texture is mainly associated with the properties of starch, that are affected by physiological processes (Abbasi *et al.*, 2015). Cell wall and middle lamella of the tissue are affected by loss of turgor pressure and other biochemical reactions (interactions between loss of turgor, cell wall plasticity, and cell reorientation during preloading) (Brusewitz *et al.*, 1989; Scanlon *et al.*, 1996; Alvarez *et al.*, 2000). Pectins, celluloses and hemicelluloses are mainly responsible for the rigid structure of the raw potato, playing a major role in intracellular adhesion and contributing to the mechanical strenght of the cell wall (Abu-Ghannam and Crowley, 2006). Characteristics of parenchyma cells and the extent and strength of adhesion areas between adjacent cells determine the fundamentals for firmness and juiceness in the case of fleshy fruits (Paniagua *et al.*, 2014). Softening in raw potato can be caused by the degradation of the cell walls (Solomon and Jindal, 2005) through the breakdown of hemicelluloses or loss of insoluble pectins from the middle lamella will lead to a reduction in cell to cell cohesion (Sharma *et al.*, 1959). Turgor pressure loss is usually observed during fruit

ripening (Paniagua *et al.*, 2014), in potato tubers water loss is a major contributer to changes in texture over time (Scanlon *et al.*, 1996; Alvarez *et al.*, 2000). During fruit ripening cell turgor can be influenced by transpirational water loss through the cuticle and due to cell wall mechanical properties modifications (Paniagua *et al.*, 2014). Potatoes moderate respiration rates coupled with a large volume to surface area ratio and a thin cuticle make them prone to significant water loss during storage (Hardenburg *et al.*, 1986). Moreover, this is compounded in a commercial setting with inefficient refrigeration systems and poor store insulation increasing the potential for moisture loss.

These factors led to an increase in the elasticity of tuber tissue during storage and the requirement for more force (N) to fracture tubers tissues. Softness of the tuber tissues after storage was observed by Sharma *et al.* (1959) and Solomon and Jindal (2005).

The use of the wedge fracture test was designed to mimmick humans perception of food texture and in the way it fractures, particularly at the first bite. The fracture wedge test determines the true material property of food (fracture toughness) and also imitates the incisors bite. The method discriminates between “crunchy” and “soggy” (elasticity) materials (Vincent *et al.*, 1991).

The dynamics of fracture analyses are dependent on the mechanical properties of the tissue under test. Vincent *et al.* (1991) reported the “harder” the specimen the greater the force required to fracture tissue. Uncooked potatoes provided an atypical matrix as tissue become more elastic over time and require more force to generate fractures caused by the loss of turgor during storage, increasing the resistance as the blade penetrated the tissue (Plate 3-8). The lack of cell wall softening, or loss of cell to cell cohesion underlies the tubers role as a storage organ, unlike many fleshy fruits that require transformation from hard-unripe to a soft and succulent tissue.

Amyloplast integrity

Membrane changes and loss of homeostatic control are regarded as generalised reactions to aging (Coleman, 2000). There is some debate as to the integrity of amyloplasts during different forms of sugar storage accumulation. Smith *et al.* (2005)

suggests that starch degradation during sprouting and cold-induced sweetening occur within plastids. However, some report the disappearence of membranes during the cold-induce sweetening (Ohad *et al.*, 1971), while others argue against the loss of amyloplast integrity during cold-induced sweetening (Isherwood, 1973; Isherwood, 1976; Sowokinos *et al*., 1985; Deiting *et al.*, 1998). As the process of sweetening is reversible, prior to tuber senescence it suggests that cellular compartmentalisation has not been lost. Nevertheless tuber ability to respire excess sugars may be independent of amyloplast membrane integrity. Senescence sweetening was reported as the progressive degeneration of the amyloplast membranes (Sowokinos *et al.*, 1987), in a range of varieties generally after 5-6 months of storage at 10°C (Burton, 1989).

SEM analysis suggests the initial loss of amyloplast membrane integrity through the appearance of small fractures after 4 months storage. In L. Rosetta first fractures coincided, with an increase in respiration rate after 4 months storage.

Thereafter, the concentration of sucrose, glucose and fructose started increased in conjunction with an increase in the degree of surface fractures and a subsequent rise in respiration and ROS. An increase in the respiration rates due to starch degradation (from reducing sugars accumulation) generates ROS, which with a decrease in antioxidant capacity with tuber aging results in oxidative stress, lipid peroxidation and membrane damage (Zommick *et al.*, 2013). The accumulation of sugars over time may be related to the kinetics of respiration efficiency, and requires more detailed analysis in future.

A similar pattern in amyloplast deterioration, respiration rate and sugar accumulation was observed with P. Dell, with a rise in respiration rate and the appearance of the first fractures on the amyloplast membrane observed after 5 months of storage in air at 10°C, with a concurrent decrease in reducing sugars accumulation. The decrease in sugars started as early as January. It is assumed that this decrease is the result of utilisation through respiration. Sugars in P. Dell increased after 6 months storage at a time when ROS activity began to increase. Higher ROS activity may be indicative of general cellular dysfunction or ROS signalling may lead to the stimulation of the Krebs cycle or possible uncoupling of components of the respiration cycles. According to Kumar and Knowles (1996b) older tubers have higher respiration rates than younger

tubers, and higher respiration rates tend to generate more ROS, further contributing to oxidative deterioration and tuber decline, as well for the expression of genes related with senescence (Hancock *et al.*, 2001). In this study, the rise in respiration rate could not fully utilise the pool of reducing sugars for L. Rosetta and P. Dell, possibly associated with ageing affecting the efficiency of carbohydrate methabolism. Mikitzel and Knowles (1990) suggested that ageing affects the efficiency of carbohydrate utilisation with higher rates of sucrose hydrolysis in older tubers being responsible for limiting the carbohydrate translocation to developing sprouts. L. Rosetta, commercially is stored until end of February, so the presence of fractures in the amyloplast and a rise in reducing sugars could be expected after 3-4 months of storage at 10°C.

The non-synchronous changes in the amyloplast fracturing between the middle cortex tissue and the outer parenchyma in VR 808 suggest that changes in the maturation or physiological stress occurs more rapidily in the central middle cortex than the outer cortex. Moreover there are multiple mechanisms controlling starch breakdown and sugar metabolism. VR 808 is retained for extended periods, likewise, changes in amyloplast structure are not correlated with increased sugar accumulation in this variety.

The rate of amyloplast degradation in tubers appears to be variety dependant, in general plastids are particularlly sensitive to early-stage senescence breakdown. The loss of chloroplast integrity was reported as the first senescent event in leaf cells, and other cellular constituents required for nutrient recycling process as such nucleus are the last to senesce (Gan and Amasino, 1997). In leaves, the absence of external stimuli to induce senescence, such shade or leaf age, has the major influence in initiation of senescence, with the decline in photosynthesis varying among species (Gan and Amasino, 1997).

Age-induced loss in potato amyloplast membrane integrity may be the result of gradual peroxidation of membrane lipids, which is a major cause of senescence sweetening of potato tubers (Kumar and Knowles, 1993b).

Loss of amyloplast membrane integrity leads to a loss of cellular compartmentalisation and affects the transport of different effectors (such as P_i, G6P) and intermediaries of starch metabolism (O'Donoghue *et al.*, 1995).

Electrolytic leakage may indicate physiological aging in potato tubers, but the efficacy of measurement remains variable, since leakage responses are often small, and dependent on storage temperature and are not consistent among samples (Coleman, 2000 and references therein) whereas analysis of amyloplast integrity by SEM provides a more robust technique.

In the final year of study fractures first appeared at harvest. Weather conditions or agronomic factors during the growing season are known to impart stresses that manifest in the tubers during storage (Kumar et al., 2004). For example in VR 808 accelerated fracturing of amyloplast was correlated with increased sucrose, and higher tuber respiration, but led to lower reducing sugar content achieved either through reduced hydroylsis of sucrose or increased respiration.

Ascorbic acid, ROS and sweetening

AsA has a major role in detoxifying ROS in plants (Navarre *et al.*, 2009). According to the oxidative damage theory of aging, ROS products accumulate over time and are neutralised in a decreasing manner during aging by intracellular compartmentalisation, protective enzymes, and naturally occurring antioxidants (Coleman, 2000).

ROS content is highly regulated to allow ROS driven redox changes to act as secondary messengers (Noctor and Foyer, 2016). Plants complement of multiple antioxidant systems, including AsA, GSH, carotenes, carotenoids, tocopherols and polyphenols, help to counteract the rise in ROS. AsA concentration surpasses the concentration of other antioxidants, and thus the capacity of AsA to supress ROS activity is of particular importance (Gallie, 2013).

AsA content of potatoes at harvest is ~30 mg 100 g^{-1}, and declines during, processing, or storage (Kadam *et al.*, 1991). This concurs with this study where AsA content was highest at harvest. Previous studies (Bishop *et al.*, 2012) report the largest decrease in AsA happens during the first few weeks of storage, followed by a more gradual

decline, with both temperature and length of storage period considerably influencing the AsA content (Kadam *et al.*, 1991).

AsA content in this study was variety dependent, and in accordance with previous studies (Hamouz *et al.*, 2007; Blauer *et al.*, 2013b; Kulen *et al.*, 2013; Valcarcel *et al.*, 2015). Laing *et al.* (2015) reported AsA variations was due to a mutation affecting the functionality of a conserved non-canonical upstream open reading frame in the long 5' untranslated region of GGP-L-galactose phosphorylase (a major control enzyme in the AsA biosynthesis pathway (Bulley *et al.*, 2012)) that takes place at the post-transcriptional level, or due to other factors that interact with AsA.

Considerable variation in AsA and DHA content was measured across the two years of analysis (2014/15 and 2015/16) for VR 808 and L. Rosetta (Table 3-12). The difference could not be assigned to variance in HPLC analyses between years, as cross validation of samples by HPLC from both years was performed.

AsA synthesis and catalysis are affected by ROS activity (Fry, 1998; Lisko et al., 2014). L. Rosetta accumulated more AsA but started to senescent sweetening earlier. Lisko *et al.* (2014) suggested that AsA synthesis was stimulated by stress responses, so varieties with a tendency to senesce early accumulate more AsA in order to scavenge the ROS generated through senescence. Therefore, the proportion AsA/DHA ratio provides an indication of the activity of AsA than overall concentration of more AsA.

Variation between AsA and DHA content over prolonged storage at 10°C occurred and the magnitude of the difference in AsA/DHA ratios was dependant on variety; comparison of fluctuations and the abundance of ROS captured by staining may help to tie in possible mechanisms for decrease in AsA/DHA (Section 4.2).

In all the varieties and in both seasons, the ratio of AsA/DHA favoured AsA accumulation after harvest. During year 2, DHA increased above AsA concentrations after around 4-6 months of storage at 10°C (January to March for L. Rosetta and VR 808 and January to April for P. Dell and R. Burbank); at a time when H_2O_2 and O_2^- radicals were less active, as determined from the lower ROS staining patterns in tuber slices.

Similarly, when H_2O_2 and O_2^- levels increased, a concurrent decrease in AsA was observed alongside an increase in DHA. In plants, AsA interacts enzymatically and non-enzymatically with ROS, and is able to terminate a radical chain reaction by disproportionation to non-toxic, non-radical products such as DHA (Davey *et al.*, 2000; Gallie, 2013).

ROS content in VR 808 observed through DAB staining was significantly lower than other varieties. In both seasons, a gradual but small increase in H_2O_2 with DAB staining was observed. The decreased ROS content may contribute to a lower degree of senescent sweetening and this variety may have inherently lower ROS accumulation. During senescence, plants lose their antioxidant capacity leading to an increased release of ROS (reviewed by Barth *et al.*, 2006). In potato tubers the rise in ROS has been linked with the release of dormancy (Bajji *et al.*, 2007; Liu *et al.*, 2017). A variety more resistant to senescence will preserve its antioxidant capacity for longer.

O_2^- with NBT staining in VR 808 was difficult to visualise. The lack of ROS may be a consequence of tubers lower respiration rates; one of the metabolic processes responsible for ROS production (Lisko *et al.*, 2014) and in general O_2^- is short-lived and highly reactive been rapidly converted into H_2O_2 (Hancock, 2017).

Ascorbic acid oxidation cycle leads to the intermediate formation of monodehydroascorbate (MDHA), that then forms AsA and DHA (unstable above pH 7) (reviewed by Smirnoff, 1996). DHA is recycled to AsA by dehydroascorbate reductase (DHAR), which uses glutathione (GSH) as reductant. Under certain conditions DHA can undergo irreversible hydrolysis to 2,3-diketogulonic acid (2,3-DKG), which cannot be reconverted to AsA (Gallie, 2013).

The ascorbate-glutathione (Asc-GSH) cycle, includes a number of enzyme intermediates including MDAR (monodehydroascorbate reductase), DHAR, and glutathione reductase (GR), these have an important recycling role regenerating AsA and GSH through oxidation in situations of oxidative stress (Gallie, 2013). The ascorbate pool is highly reduced (favouring AsA) under optimal conditions, but as the oxidative load increases a shift towards a more oxidized state (DHA) is achieved (Foyer and Noctor, 2011).

During storage, changes in the overall concentrations of AsA and DHA and ratio of AsA/DHA were observed, with DHA exceeding AsA under conditions of oxidative stress, but by the end of tuber storage DHA concentration decreased below AsA content due to the regeneration of AsA.

Tuber slices stained for ROS showed differential expression of H_2O_2 or O_2^-. Differential formation of brown polymers (DAB staining) or purple/blue precipitates (NBT staining) across the tuber transect indicating that H_2O_2 activity was mostly associated with vascular tissue, while O_2^- activity was restricted to the middle cortex (peri-medulla). This pattern of activity suggests different tissue types undergo different forms of stress response.

H_2O_2 content in vascular tissue may suggest a signaling role, based on the review of Thannickal and Fanburg (2000) ROS's role in cell signaling indicated site-specific reactive species, and the rates of synthesis were important factors in the determination of the physiological actions and effects of ROS in cell signaling.

Precursor synthesis of AsA may influence overall abundance of AsA content; hydrolysation of sucrose into hexoses, fructose and glucose can serve as a substrate for AsA biosynthesis (Wheeler *et al.*, 1998). Dipping tomatoes in 5% sucrose doubled AsA content (Badejo *et al.*, 2011). Potato vars. P. Dell, L. Rosetta and VR 808 accumulated more sucrose and AsA.

Seasonal influence on sucrose concentration in tubers also affected AsA concentration, tubers harvested in the 3rd year were higher in sucrose and AsA. In the final year, a higher sucrose content led to lower ROS content in VR 808.

Sucrose concentrations within tubers was dependant on tissue location, initially formed in parenchyma cells, it is translocated (via phloem) to the tuber apical region and the emerging sprouts, where upon it is hydrolysed, into glucose and fructose (Burton *et al.*, 1992; Hajirezaei *et al.*, 2003; Sonnewald and Sonnewald, 2014). The relationship between sucrose and glucose and fructose content remains complex with the dynamics of starch breakdown and glucose and fructose utilisation under multiple feedback mechanisms, reducing sugars fuel respiration activity providing energy for

dormancy break/sprout growth, which in turn generates ROS but also provides the building blocks for AsA synthesis.

Mineral accumulation and sweetening

A wide range of mineral elements are present in plant organs and tissues which are classified as major minerals (calcium, potassium, magnesium, sodium, phosphorus, cobalt, manganese, nitrogen and chlorine), and trace minerals (iron, copper, selenium, nickel, lead, sulfur, boron, iodine, silicon and bromine). Potatoes are an important source of some dietary minerals, such as potassium, iron, phosphorus, magnesium, calcium and zinc (Navarre *et al.*, 2009).

Overall mineral concentration between different varieties L. Rosetta, P. Dell, R. Burbank and VR 808 were observed and more importantly distribution across the tuber was variable and concurs with the results of LeRiche *et al.* (2009) and Subramanian *et al.* (2011) where calcium concentration decreased from rose end to apical end. Changes in mineral content with sampling time are less well understood. As minerals are not metabolized, apparent changes in the mineral concentration of the tuber during storage maybe due to inherent variabibilty between tubers, sampling error, or even a redistribution of the minerals between centre of the tuber and peripheral regions.

There is evidence that certain minerals (P, S, Ca, Cu, Mg, Zn and K) are correlated with after-cooking darkening and acrylamide formation in potato (LeRiche *et al.*, 2009; Whittaker *et al.*, 2010).

According to Whittaker *et al.* (2010), concentration of reducing sugars increased in response to lower K and Ca concentration, and positively correlated with Zn and Cu. In this study however, was found other correlations between varieties, and in the most part in contrast to those reported by Whittaker *et al.* (2010) with the $\%Ca_{bound}$ having a significant influence on sugars. As well in this study significant correlation values for reducing sugars concentration and K, Ca, Zn and Cu were not observed in all varieties. Variation in mineral concentration in potato tubers between varieties and planting location have been reported (Tack, 2014). The study of Whittaker *et al.* (2010) used *cvs* Arinda, Rossa di Cetica and Sieglinde cultivated in Italy and so direct comparison

with the varieties used in this study is limited. Geographical location may influenced nutrient profiles, due to differences in soil composition - in the second year of study VR 808 and L. Rosetta were grown at a common site.

Ca is reported to facilitate sugar unloading in apple cells; where calcium deficiency leads to accumulation of photosynthate (sorbitol) in the air spaces a disorder termed water-core (reviewed by Colgan *et al.*, 2012). In melon a similar water-core disorder is also associated with low Ca concentration, leading to an increase in polygalacturonase activity (Serrano *et al.*, 2002). However in potato the main photosynthate is sucrose, whether Ca influences in sucrose and unloading in cells is unclear. In this study the two varieties with the lowest sucrose accumulation (VR 808 and R. Burbank) had the highest calcium concentration (Ca_{total}).

Ca binding is affected by Mg and K that are reported to displace Ca from the pectin matrix between cell walls weakening cell to cell cohesion and accelerating tissue senescence rates (reviewed by Colgan *et al.*, 2012). Although negative correlation between Ca_{total} and K and Ca_{total} and Mg existed across varieties, the (K+Mg)/Ca ratio had higher influence on L. Rosetta and VR 808 than in R. Burbank and P. Dell.

The fluxes of free calcium in the cytosol and/or active cellular organelles are more important with respect to changes in metabolism, growth and development, than the total Ca content (Berridge *et al.*, 2003). The biggest pool of Ca in plant tissue is in the cell wall (reviewed by Aghdam *et al.*, 2012). In this study, bound calcium as a percentage of total calcium (% Ca_{bound}) was highest at harvest (November) for L. Rosetta, P. Dell and R. Burbank. Ca binding to cell walls and within the pectin matrix between cells is degraded by the action of cell wall degrading enzymes that increase after harvest (reviewed by Aghdam *et al.*, 2012). Since Ca^{2+} contributes to cell wall structure by cross-linking pectins and regulates membrane permeability (Ho and White, 2005), low Ca concentrations in membranes increases leakiness resulting in loss of cellular salts and organic compounds, and if not reversed can lead to cell death (reviewed by Palta, 2010). A decline in Ca^{2+} can influence the regulation of cell turgor via chloride chanels in vacuole membrane and thus reduce cell turgor (Stow, 1989; Mansfield *et al.*, 1990).

Comparer to P. Dell and L. Rosetta, R. Burbank has a very late onset of development of senescent sweetening. So, by the end of storage the proportion of Ca_{bound} has decreased, and this may be due to variety senesce via an increase in the proportion of soluble calcium as a result of cell walls breakdown - releasing pectin-bound calcium. For the longer dormant varieties, R. Burbank and VR 808, the relationship was less clear.

The methodology used (Al-Wahsh et al., 2012, modified by Mirzaee, 2015; 2015) to distinguish bound (Ca_{bound}) versus soluble calcium and assumed all bound calcium was in the form of calcium oxalate. However, conjugation to phosphate, phytates and pectins and xyloglucan molecules will also influence the proportion of soluble calcium solubility (Franceschi and Nakata, 2005; Galon *et al.*, 2010; Hashimoto and Kudla, 2011; Virdi *et al.*, 2015).

The proportion of bound calcium (% Ca_{bound}) was significantly correlated (positively) with the (K+Mg)/Ca ratio in L. Rosetta and P. Dell, while, VR 808 and R. Burbank the (K+Mg)/Ca ratio was positively correlated with %FW of sucrose. Calcium homeostasis is tightly controlled within plants; vacuole calcium is 10-100 fold higher than the cytoplasm (White and Broadley, 2003), while the cell wall contains approximately 60% of the total calcium, so gross measurement of calcium fails to reflect the complex balance in calcium across tissues and organelles.

P. Dell with its high Ca_{total} content did not avoid the early onset of senescent sweetening and thus led to a significant correlation (negative) between %Ca_{bound} and Ca_{total}.

Ca availability in plants is tightly regulated with reversible and irreversible inactivation via conjugation to calmodulin protein complexes and oxalates and phosphates (Franceschi and Nakata, 2005; Galon *et al.*, 2010; Hashimoto and Kudla, 2011; Virdi *et al.*, 2015). Inactivation via conjugation with oxalate ions can lead to cell wall strength loss (Fidler *et al.*, 1973), being as well implicated in increasing in the flexibility of cell walls, required for shoots and roots expansion (McNeil *et al.*, 1984). L. Rosetta and P. Dell were the varieties that terminated dormancy first, with the start of sprout growth (after 44 and 92 days in storage, respectively). Sprouting and physiological age of potato tubers are in part a function of calcium regulation and are related to the calcium

availability in the tuber (Dyson and Digby, 1975). Oxalate is formed through the oxidation of AsA that results initially in the formation of an MDHA radical, that is unstable above pH 7. It is still not clear if the actual precursor of oxalate is ascorbate or DHA, but it is suggested that DHA could generate cell wall oxalate that can then influence free calcium concentration (reviewed by Smirnoff, 1996).

From this results it is possible to assume that relations between Ca and K, Ca and Mg and Ca and ratio of bound:total Ca could be used as a practical marker for the onset of senescent sweetening. Changes in the relative Ca concentrations are more important than threshold concentrations, moreover, the ratio of calcium with other mineral antagonists (K or Mg) may provide a more meaningful estimate of the influence of calcium's bioavailability. However, increasing calcium content in tubers by pre- or post-harvest application be beneficial by increasing the cell membrane integrity and stability delaying senescence (Sairam *et al.*, 2011). But calcium's restricted mobility in the xylem (White, 2001; Atkinson, 2014) are likely to make it difficult to enhance tuber calcium concentration.

Effect of chronological age of crop on the propensity to develop senescent sweetening

The physiological age of seed tubers influences the rate of plant emergence and stem number and therby affects the agronomic traits of the mother plant and subsequent tuber formation and maturation (Caldiz et al., 1996).

Caldiz *et al.* (1986) demonstrated that physiologically aged tubers accumulated higher total and reducing sugars once tubers had sprouted, suggesting the rise in sugars were associated with senescence. However, in this chitting experiment, physiological aging P. Dell seeds prior to planting did not influence chemical maturity (% glucose, fructose or sucrose), or respiration rate, at harvest or during storage.

Groves *et al.* (2005) reported chitting had an adverse effects on processing quality during storage while some seed-tuber cvs were more responsive to physiological aging (Struik *et al.*, 2006). Sampling profiles have a significant impact on the interpretation

of sugar analysis and make it hard to interpret the results of others. In this study the middle cortex tissue accumulated more reducing sugars and sucrose.

Chitted tubers had a lower accumulation of AsA and higher accumulation of H_2O_2 and O_2^-. As discussed previously, AsA interacts enzymatically and non-enzymatically with ROS, by disproportionation to terminate a radical chain reaction in non-toxic and non-radical products such as DHA (Davey *et al.*, 2000; Gallie, 2013). Chitting tubers is a way to enhance tuber maturity at harvest, that in general adversely affects processing quality during storage, specialy by the end of the storage season due to the start of senescence sweetening (Groves *et al.*, 2005) This could be due to the fact that the onset of the senescence process in plant storage tissue is followed by a noticeable increase in the H_2O_2 content (Van Es and Hartmans, 1987).

Chapter 4. INVESTIGATION OF THE EFFECT OF CALCIUM ON THE STORAGE POTENTIAL OF POTATO TUBERS

4.1. Introduction

Calcium ions (Ca^{2+}) are essential in the growth and development of plants (Hepler, 2005). Ca^{2+} is vital for cell wall strengthening and cell-cell adhesion (Bush *et al.*, 2001; Marry *et al.*, 2006), and is regarded as an anti-senescence factor (Kumar and Knowles, 1993a). Low concentrations in the membranes results in leakage and loss of cellular salts and organic compounds, leading to cell death (reviewed by Palta, 2010). However, potato tubers have much lower Ca^{2+} concentrations than the above-ground vegetative portion of the plant (Kratzke and Palta, 1986). Potato tuber nutrient status at tuber initiation is essential to subsequent tuber quality (Olsen *et al.*, 1996). Increasing calcium concentration in potato tubers has been shown to be beneficial, by reducing the incidence of internal rust spot, sprout subapical necrosis and soft rot, during storage (Kratzke and Palta, 1986).

4.2. Material and Methods

The influence of calcium fertilisation on senescent sweetening was explored using P. Dell tubers (from AHDB-Potato-1100004 Storage Fellowship trial managed by McCains in the 2015/16 season), where randomised plots were fertilised with calcium (Tropicote, 380 Kg ha^{-1}) or grown without calcium fertilization. Tropicote is a calcium nitrate product for field application from YaraLiva composed of 15.5% N and 19% Ca (http://www.yara.co.uk/crop-nutrition/fertiliser/calcium-nitrate/0146-yaraliva-tropicote/, 04-05-2017).Tubers were, hand lifted on the 21st October 2015.

Determination of sugars (see section for method 3.2.1) and AsA (see section for method 3.2.3), ROS determination (see section for method 3.2.4) and mineral analyses (ground freeze-dried samples were sent to an accredited laboratory, Yara Analytical

Services, York, UK) were performed. Sugars were also measured at vine kill (9th October 2015). Scanning electron microscopy (SEM), performed as in section 3.2.6, was used to determine if differences could be detected in amyloplast membranes structure. Data analyses was performed as in section 3.2.8.

4.3. Results

Sugar accumulation

Tropicote treatments had no significant effect on sugar accumulation (%FW of reducing sugars, fructose, glucose and sucrose). Total %FW of reducing sugars increased with time in storage, from vine kill until January, however there were no significant differences between sampling occasions (Table 4-1).

There was a significant difference in concentration of reducing sugars across the tuber ($p < 0.01$); the middle cortex had a higher accumulation of reducing sugars than the ends (Table 4-2). Fructose increased after 3 months storage at 10°C (Table 4-1). Fructose and sucrose accumulation was higher in the middle cortex of the tuber than the ends (Table 4-2).

Table 4-1 - %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) during storage of P. Dell tubers in the fertilization experiment.

	RS	Sucrose	Fructose	Glucose
October	0.007^{c}	0.013^{b}	0.003^{c}	0.004^{c}
December	0.007^{c}	0.013^{b}	0.003^{c}	0.004^{c}
January	0.006^{c}	0.008^{b}	0.003^{c}	0.003^{c}
May	0.014^{b}	0.032^{b}	0.008^{b}	0.006^{b}
June	0.035^{a}	0.036^{a}	0.019^{a}	0.017^{a}
HSD$_{0.05}$	*0.003*	*0.007*	*0.001*	*0.002*

Mean values with different letters are significantly different according to the Tukey HSD test.

Table 4-2 - %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) by tuber position in P. Dell tubers analysed for the fertilization experiment.

	RS	Sucrose	Fructose	Glucose
Ends	0.013^{c}	0.019^{b}	0.006^{b}	0.007^{a}
Middle	0.014^{a}	0.022^{a}	0.008^{a}	0.006^{a}
HSD$_{0.05}$	*0.001*	*0.003*	*0.001*	*0.001*

Mean values with different letters are significantly different according to the Tukey HSD test.

Ascorbic acid accumulation

Tubers that received calcium fertilization (Tropicote) were higher in AsA content ($p<0.01$) and lower in DHA ($p<0.01$), therefore having a higher AsA/DHA ratio, while total vitamin C (AsA + DHA) was not affected by calcium treatments (Table 4-3).

In both treated and untreated tubers the content of AsA increased while DHA decreased over storage at 10°C with the highest ratio of AsA/DHA recorded after 7 months storage (June) (Table 4-3).

AsA concentration was more uniform over storage time for Tropicote treatment than for control, and DHA accumulation was more uniform over storage time for control tubers (Table 4-3).

Table 4-3 – Accumulation of AsA (mg 100 g^{-1} FW), DHA (mg 100 g^{-1} FW) and total vitamin C (Vit C, mg 100 g^{-1} FW) for the calcium fertilization experiment.

	AsA	DHA	Vit C
Tropicote			
December	18.17^{b}	13.19^{a}	31.35^{ab}
January	20.53^{ab}	ND	10.95^{c}
May	21.17^{a}	11.36^{ab}	32.53^{a}
June	21.01^{a}	7.74^{b}	28.75^{b}
HSD$_{0.05}$	*2.76*	*4.03*	*2.62*
***p*- value**	*<0.05*	*<0.001*	*<0.001*
Control			
December	14.78^{b}	18.04^{a}	32.82^{a}
January	14.24^{b}	6.98^{b}	20.22^{a}
May	23.09^{a}	5.02^{b}	23.03^{a}
June	20.70^{a}	9.01^{ab}	29.76^{a}
HSD$_{0.05}$	*4.44*	*10.95*	*15.30*
***p*- value**	*<0.001*	*<0.05*	*>0.05*
Mean$_{Tropicote}$	20.11^{a}	10.76^{b}	30.88^{a}
Mean$_{Control}$	17.92^{b}	13.03^{a}	30.95^{a}
HSD$_{0.05}$	*1.57*	*1.65*	*1.28*
p-value	*<0.01*	*<0.01*	*>0.05*

Mean values with different letters are significantly different according to Tukey HSD test. ND = not detected.

Mineral accumulation

There were no significant differences between treatments for Ca, N, K, P and Zn accumulation as well for the (K+Mg)/Ca and N/Ca ratios (Table 4-4). Control tubers had significantly higher accumulation of Mg and B than the ones fertilized with Tropicote (Table 4-4).

Table 4-4 – Mineral concentrations in P. Dell potato tubers from the calcium fertilization experiment, sampled in December 2015 (beginning of storage season).

	Ca mg $100g^{-1}$ FW	**N mg $100g^{-1}$ FW**	**N/Ca**	**K mg $100g^{-1}$ FW**	**Mg mg $100g^{-1}$ FW**	**K+Mg Ca**	**P mg $100g^{-1}$ FW**	**B mg $100g^{-1}$ FW**	**Zn mg $100g^{-1}$ FW**
Tropicote	58.71[a]	1447[a]	24.65[a]	1644[a]	85.81[b]	29.46[a]	157[a]	0.60[b]	1.63[a]
Control	66.09[a]	1462[a]	22.12[a]	1815[a]	93.21[a]	28.87[a]	168.5[a]	0.66[a]	1.73[a]
$HSD_{0.05}$	8.77	138.20	2.81	183.85	6.85	3.95	15.62	0.05	0.17

Mean values with different letters are significantly different according to Tukey HSD test.

ROS determination

Calcium treatment significantly reduced ($p < 0.05$) H_2O_2 content visualized through DAB staining in P. Dell tubers during storage (Table 4-5). However, there was no significant effect on O_2^- content.

H_2O_2 content decreased in calcium-treated tubers during storage while an increase in H_2O_2 content was seen after 6 months storage at 10°C in untreated tubers (Figures 4-1 and Figure 4-2). Conversely calcium-treated tubers were higher in O_2^- during the first 6 months of storage, after which decreased (Figure 4-1).

H_2O_2 and O_2^- content increased as DHA accumulated and AsA declined in untreated tubers (Figure 4-2). In Calcium-treated tubers, higher concentrations of AsA were related to lower H_2O_2 content (Figure 4-1).

Lower H_2O_2 content was correlated with higher fructose and glucose accumulation in calcium-treated tubers (data not shown).

Table 4-5 - Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) determined in P. Dell tubers from the fertilization experiment, by month and treatment, and by treatment.

	O_2^-	H_2O_2
Tropicote		
December	24.88[a]	56.70[a]
January	28.51[a]	37.06[ab]
May	45.50[a]	31.26[ab]
June	-3.61[a]	2.42[b]
***HSD*$_{0.05}$**	*52.78*	*0.05*
***p*- value**	*>0.05*	*<0.05*
Control		
December	38.10[ab]	50.15[ab]
January	51.59[a]	59.47[ab]
May	-10.56[b]	18.08[b]
June	50.03[a]	74.16[a]
***HSD*$_{0.05}$**	*56.90*	*51.06*
***p*- value**	*<0.05*	*<0.05*
***Mean*$_{Tropicote}$**	32.29[a]	50.47[a]
***Mean*$_{Control}$**	23.82[a]	31.86[b]
***HSD*$_{0.05}$**	*20.13*	*18.41*
p-value	*>0.05*	*<0.01*

Content of O_2^- and H_2O_2 are expressed in terms of a numerical value to describe intensity of the purple colouration produced by NBT staining and brown colouration produced by DAB staining respectively.

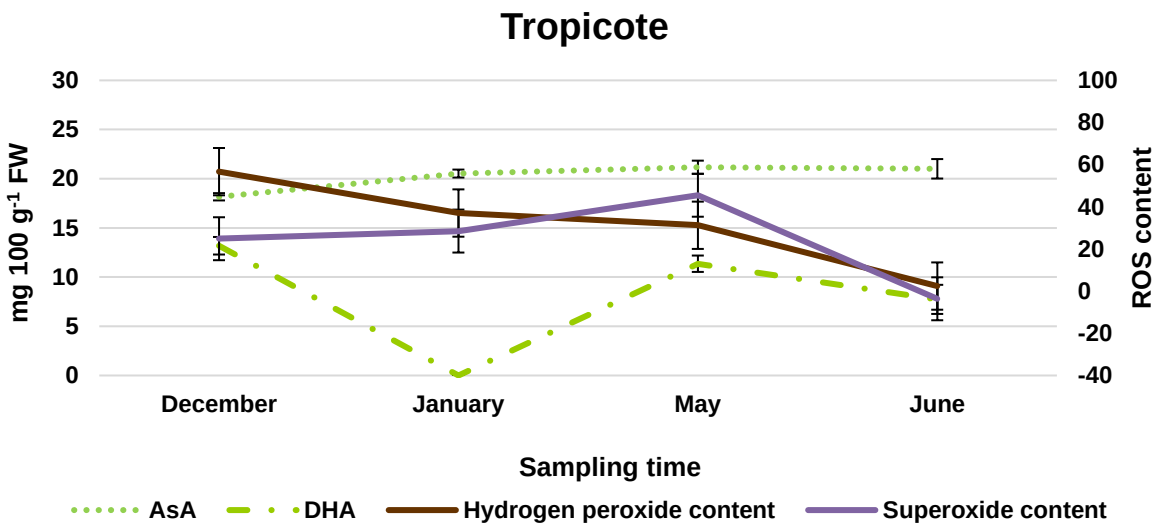

Figure 4-1 - Hydrogen peroxide content (H_2O_2), superoxide content (O_2^-), AsA and DHA concentration during storage at 10°C for P. Dell tubers treated with calcium fertilization (Tropicote), with SE bars. For stats see Table 4-3 and Table 4-5.

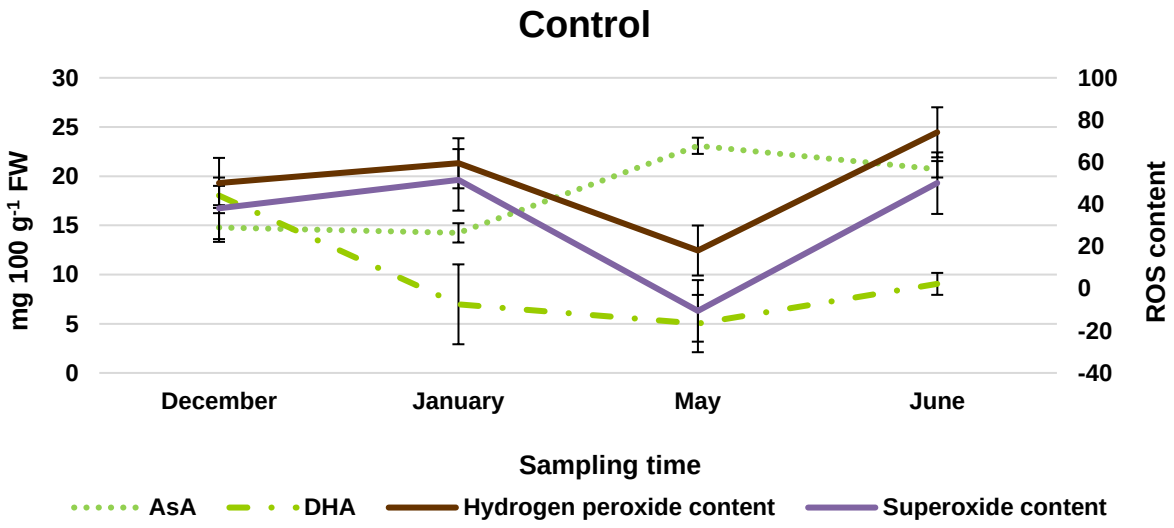

Figure 4-2 - Hydrogen peroxide content (H_2O_2), superoxide content (O_2^-), AsA and DHA concentration during storage at 10°C for P. Dell tubers treated without calcium fertilization (Control), with SE bars. For stats see Table 4-3 and Table 4-5.

Assessment of amyloplast and cellular changes due to calcium fertilization using scanning electron microscopy (SEM)

The first samples for SEM analysis were prepared after 2 months storage at 10°C. Untreated control tubers presented the first signs of surface fractures on the amyloplast (Plate 4-1). However, at this point calcium treated tubers showed no signs of cracking on the amyloplast (Plate 4-2); in these tubers fractures were first visible after 3 months storage at 10°C. After 8 months storage at 10°C control and calcium-treated tubers exhibited very few fractures, on the amyloplast surface.

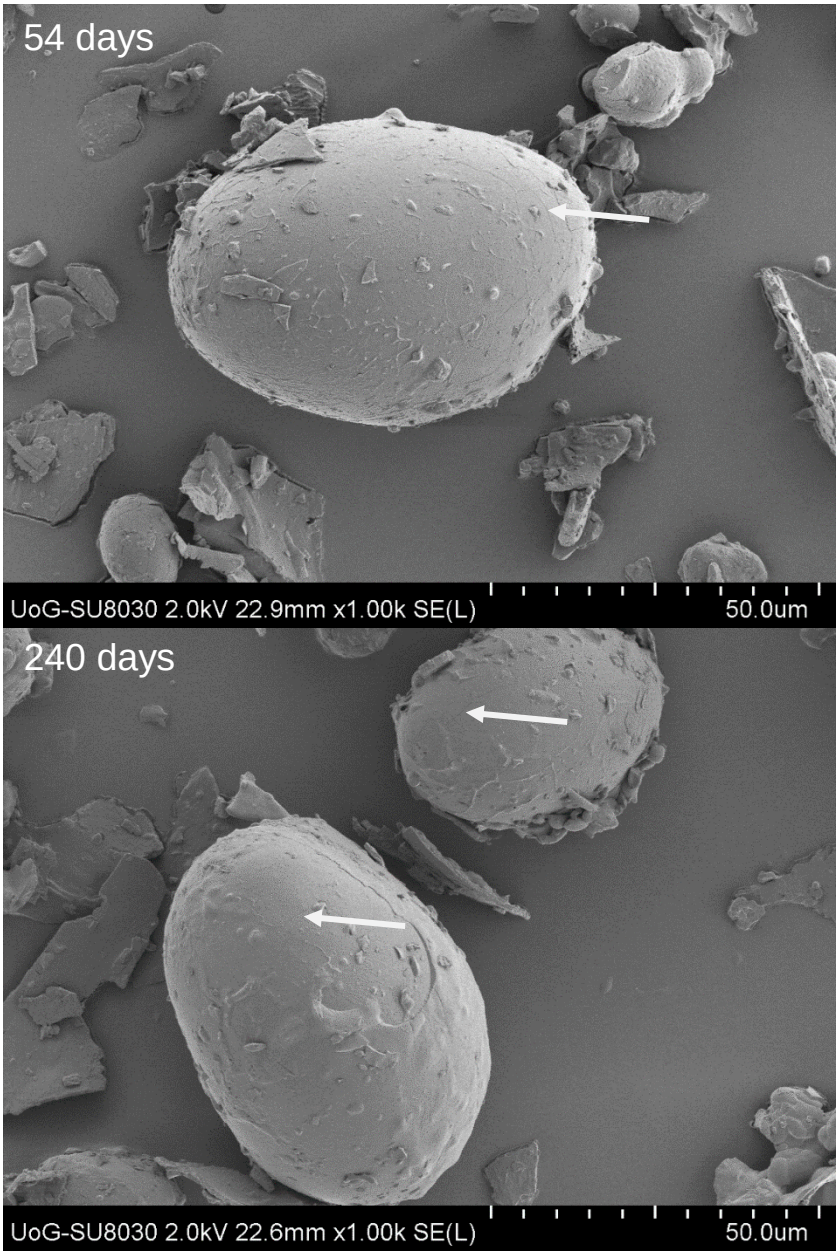

Plate 4-1 - SEM image from the tuber ends section of P. Dell (control) with 54 days (December 2015) and 240 days (June 2016) of storage with fractures on amyloplast surface. White arrows point to fractures.

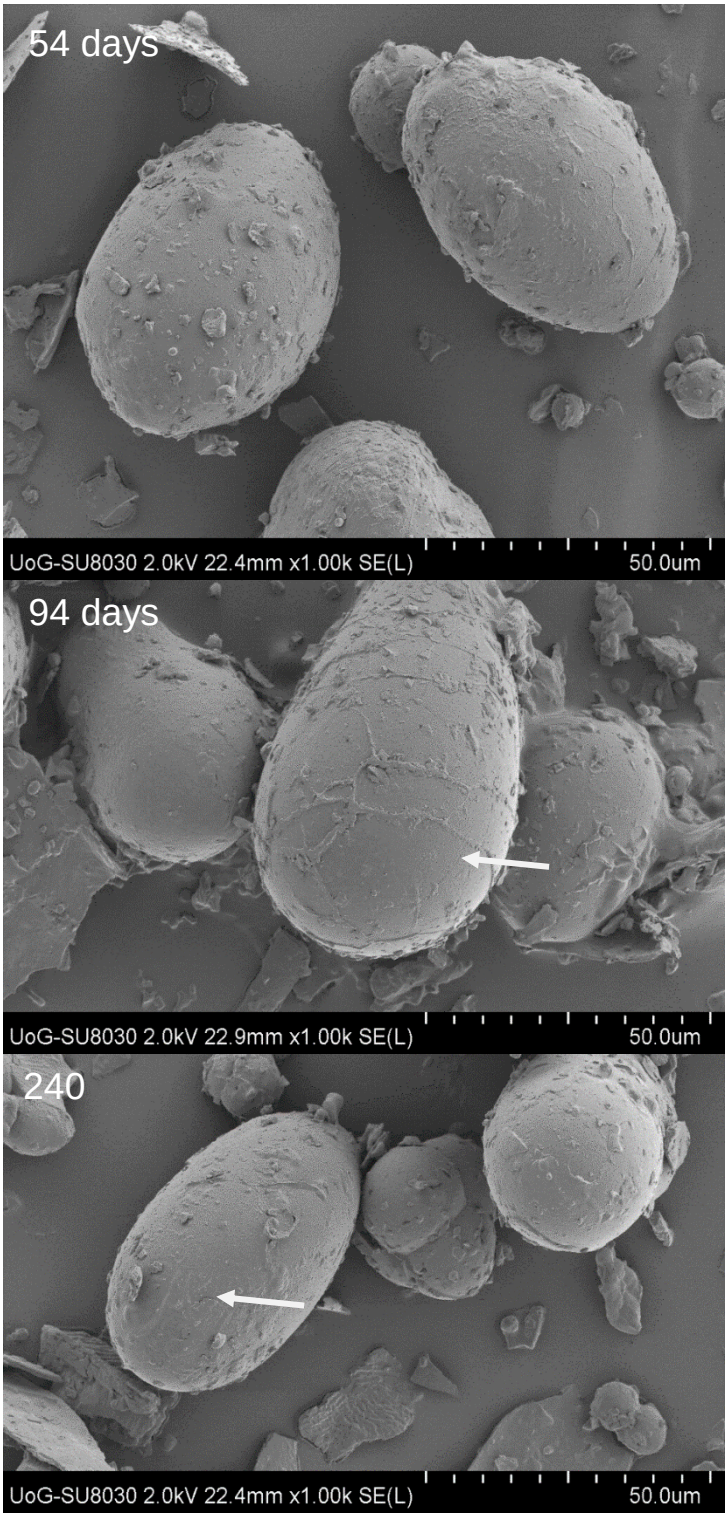

Plate 4-2 - SEM image from the tuber ends section of P. Dell with calcium fertilization (Tropicote) with 54 days (December 2015, no visible fractures), 94 days (January 2016, with 1st visible fractures) and 240 days (June 2016) of storage with few visible fractures on the surface of the amyloplast. White arrows point to fractures.

4.4. Discussion

Mineral content in potato tubers is influenced by the soil properties as well by their interaction with the plant, with the plant adjusting its metabolism to the mineral supply (White *et al.*, 2009). According to LeRiche *et al.* (2009) calcium is negatively correlated with discolouration developing during the post-cooking period in potatoes. Under abiotic and biotic stresses calcium plays an important role in tuber quality and plant growth, since it is able to stabilise phospholipids within cell membranes and strengthen cell walls (Palta, 2010).

There was no significant difference in sugar accumulation between tubers fertilized with calcium (Tropicote) and controls. While, calcium-treated tubers were higher in AsA and lower DHA, and O_2^- and H_2O_2 than control tubers, these may be precursors to events that lead to enhanced starch breakdown, the magnitude of these events was not sufficient to alter sugar accumulation.

Further large scale trials on calcium are included in the AHDB (1100004 Storage Fellowship). Nevertheless, calcium supplementation to potato plants under heat or frost stress can mitigate its effects (Palta, 2010).

Plant resistance to stress is highly correlated with the the oxidative/reductive status of AsA/DHA (Li *et al.*, 2010). APX catalizes the reduction of H_2O_2 in the AsA-GSH cycle into water with AsA serving as electron donor. An oxidative/reductive environment can be maintained by the AsA-GSH cycle throught AsA/DHA regulation, GSH/GSSG and NAD(P)H/NAD(P) inter-conversion (Aghdam *et al.*, 2012).

Until the end of the storage the sugars levels increased with senescence sweetening, however the levels of DHA increased only in control tubers. Studies show that resistence to high or low temperatures in grape leaves is accompained by increase in AsA but not in malondialdehyde (MDA) and that high content of GSH and AsA in maturing senescence fruits could reduce the accumulation of ROS (reviewed by *Li et al.*, 2010).

Calcium-treated (Tropicote) tubers had a higher AsA/DHA ratio than untreated tubers. Changes in AsA/DHA and GSH/GSSG are more important than as AsA or GSH content alone for cell resistance to ROS (Kocsy *et al.*, 2001; Wang and Li, 2006).

Calcium is identified as an anti-senescence factor capable of maitaining membrane integrity (Kumar and Knowles, 1993a). Calcium treatment of tubers delayed the onset of amyloplast cracking by around 40 days. Agarwal *et al.* (2005) found calcium treated wheat seedlings expressed a transient increase in H_2O_2, inducing antioxidant enzyme activity leading to a decrease in ROS and lipid peroxidation. Further work is needed to disentangle the relationship between calcium concentration in tubers and the antioxidant potential and sugar accumulation.

Is important to determine the amount of free calcium available. Cell wall oxalate content does influence free calcium content, and is formed through the oxidation of AsA (reviewed by Smirnoff, 1996). The content of soluble versus non-soluble calcium was determined following the method of Al-Wahsh *et al.* (2012) modified by Mirzaee (2015), which provides an estimate of free calcium and that bound as calcium oxalate. In control tubers AsA accumulation increased with sucrose accumulation. The fact that there was no significant difference in sugar accumulation between treatments could be because in unfertilized tubers sucrose hydrolysation into hexoses (fructose and glucose) were used to produce AsA (Wheeler *et al.*, 1998), that was mostly oxidized into DHA to protect them from the higher ROS levels, when compared to fertilized tubers.

Chapter 5. ASSESSMENT OF CALCIUM EFFECT IN DORMANCY AND SPROUTING

5.1. Introduction

The role calcium plays as a secondary messenger in dormancy was tested as part of this thesis as recommendations to improve calcium concentration in tubers to improve senescent-related sweetening may have deterimental effects on dormancy.

Cells have access to calcium in two ways, finite stores located in intracellular organelles and a more substantial pool of extracellular Ca^{2+} (Peppiatt *et al.*, 2004).

Pang *et al*. (2007) reported that calcium supplementation to dormant grape buds enhanced dormancy break, while addition of calcium inhibitors including a plasma membrane calcium channel blocker lanthanum (III) chloride heptahydrate ($LaCl_3$) and the extracellular free calcium chelator ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) extended dormancy of grape buds. These same buds treated later with of Ca^{2+} (10 mM), exhibited a 80-100% recovery in bud-break. Dormancy break was quickly followed by sprout elongation (Suttle, 1996) and increasing calcium supplemenation increases sprouting (Dyson and Digby, 1975).

A series of experiments were conducted to validate different calcium inhibition mechanisms using an excised bud biossay system with calcium, the calcium inhibitor, $LaCl_3$ and the calcium chelator EGTA on dormancy break and sprout vigour.

Initial experiments evaluted concentration effects for all these chemicals on apical and lateral buds from the mid-whorl and stolon end to determine the influence of apical dominance on dormancy break found in whole tubers.

5.2. Material and Methods

5.2.1. Assessment of Ca^{2+}, $LaCl_3$ and EGTA concentration effects on tuber dormancy break and sprouting

Potato varieties Lady Balfour, Arsenal and Melody selected from organic production, with no previous sprout control applied were selected. Individual buds were excised from either the apical, mid-whorl or the stolon end of the tuber (Plate 5-1) by removing a clyindrical plug surrounding buds using a cork borer n^{o}.4 (8.75 mm). In order to minimise the influence of apical dominace on bud location and increase the independence of treatment effects on buds from different locations across the tuber, apical, mid-whorl bud and stolon end buds were excised from different tubers. Therefore no two buds were harvested from the same tuber. Plugs were trimmed to 5-6 mm. Solutions (20, 30 and 40 mM) of Ca^{2+}, EGTA and $LaCl_3$ were prepared in deionized water (dH_2O) in conjunction with a dH_2O control.

The buds were washed three times with SRA-buffer (20 mM MES potassium salt, 300 mM mannitol, 5 mM AsA, pH 6,5) with gentle agitation (Cheema, 2010). Buds were dried and transferred to tissue culture plates and incubated for 5 minutes in the calcium/inhibition solution followed by gently blotting with sterile filter paper. Treated buds were randomized in sterile culture tissues plates containing filter paper soaked with dH_2O (Plate 5-2). Plates were covered with aluminium foil anddark incubated at 20°C. Six buds per location (apical, mid-whorl, stolon) with two replicates per treatment were prepared. Dormancy assessment and bud/sprout growth commenced 2 days after treatment.

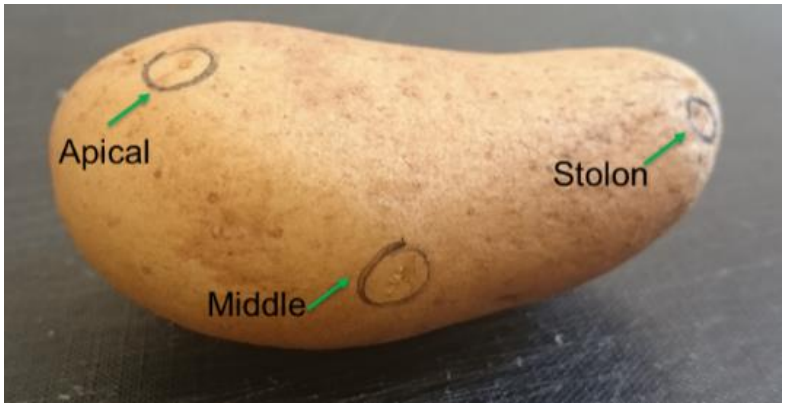

Plate 5-1 - Tuber with apical, middle and stolon buds

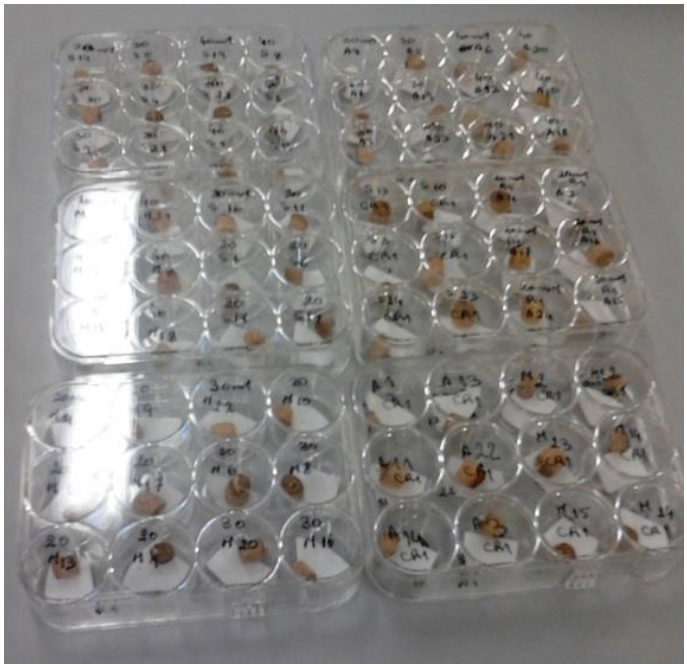

Plate 5-2 - Tuber buds placed in culture tissues plates containing filter paper soaked with dH_2O

5.2.2. Assessment of the influence of calcium on dormancy and sprout growth in selected varieties

The effect of calcium in promoting dormancy break/sprout growth was tested on cultivars Arsenal and Melody stored without sprout supressants. Buds were removed from the apical and stolon end of the tuber.

Solutions of 30 mM Ca^{2+} ($CaCl_2$); 30 mM EGTA and 30 mM $LaCl_3$ were prepared in dH_2O and the control was dH_2O.

The buds were washed three times each for 15 minutes with SRA-buffer (20 mM MES potassium salt, 300 mM mannitol, 5 mM AsA, pH 6,5) with gentle agitation. as decribed in section 5.2.1. Measurements were undertaken after 3 to 4 days and then every 2 days.

5.2.3. Assessment of the influence of calmodulim blockers on dormancy and sprout growth

The effect of calmodulim blockers in regulating dormancy break/sprout growth was tested in Lady Balfour. For these experiments calmidazolium chloride (CC), an inhibitor of calmodulin-regulated enzymes, and the calmodulin antagonist N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (Sigma-Aldrich) were used.

Cylindrical cores of tuber cortex containing either apical or stolon end buds were excised from individual tubers (section 5.2.1). Solutions of 1 mM, 500 µM and 100 µM of CC and W7, were prepared in dH_2O from stock solutions prepared in 100% ethanol. Additionally, 30 mM Ca^{2+} ($CaCl_2$); EGTA and $LaCl_3$ were prepared in dH_2O and the control was dH_2O. Assays were incubated in the dark at 20°C.

During initial trials treatements with different concentrations of W7 showed no significant difference in dormancy break or for sprout growth. The sprout growth for 1 mM CC was not significantly different from 100 µM and 500 µM. Therefore for further

experiments at 10°C solutions of 1 mM CC, 1 mM W7, 30 mM of Ca^{2+} ($CaCl_2$); 30 mM of EGTA and 30 mM of $LaCl_3$ were used.

The buds were washed three times each for 15 minutes with SRA-buffer with gentle agitation. Buds were dried in sterile paper and transferred to culture tissues plates and incubated for 5 minutes in the respective solution. After 5 minutes the buds were handled and assessed as decribed in section 5.2.1. Measurements were undertaken after 4 days and then every 2 days.

5.2.4. Data Analyses

Data was graphed using Rstudio and Microsoft Excel 2013. Dormancy break and sprout growth data was subject to ANOVA performed with R (R Core Team, 2014). Tukey's HSD (honest significant difference) test (TukeyHSD) was carried out using the "agricolae" package, followed by all pair-wise comparisons from Rstudio (R Core Team, 2014).

The Tukey test was carried out on a series of growth models for comparing the rate of dormancy break and sprout growth were investigated; using "multcomp" and "dae" packages from R (R Core Team, 2014). The "Multcomp" package allows multiple comparison procedures targeting a simultaneous inference (Bretz *et al.*, 2011). While "dae" function was used for ANOVA determination (https://CRAN.R-project.org/package=dae, 27-04-2017).

The "multicomp" package is used in survival models and simultaneous tests and provides confidence intervals for general linear hypotheses (Hothorn *et al.*, 2016). Together with the "survival" package it was possible to predict dormancy duration in the presence of calcium, calcium inhibitors and calmodulim blockers. A Weibull distribution analysis (generated using paramameters estimated from "survReg" function (from "survival" package in Rstudio)) was used to estimate the number of days each treatment extended dormancy. The Weibull distribution describes populations where its individuals can exist in either of two states, such as dormant versus non-

dormant and has been used previously to estimate cumulative rates of germination (Brown and Mayer, 1988). The “survReg” function is used to fit a parametric survival regression model (https://stat.ethz.ch/R-manual/R-devel/library/survival/html/survreg.html, 27-04-2017). Since calcium inibitors/blokers influence tuber tissue structure, tuber plugs could not last long (+-10 days); prediction models were used and then tested against final survival data.

5.3. Results

5.3.1. Assessment of Ca^{2+}, $LaCl_3$ and EGTA on sprout growth and dormancy break

Control buds and buds incubated with Ca^{2+} remained viable for up to 22 days compared to buds treated with EGTA (9 days) and $LaCl_3$ (11 days). However control buds started to dissicate and deteriorate much faster than buds incubated with Ca^{2+}.

Buds incubated in Ca^{2+} initiated rapid shoot length compared to buds incubated in deionized water (control) (Figure 5-1). Buds excised from the stolon end of the tuber were much slower to break dormancy and grow, because they were still under the effects of apical dominance, and as a consequence the addition of calcium increased the rate of dormancy break and sprout growth.

In buds subject to Ca^{2+} treatment the growth rate of buds were dependant on the position taken from the tuber. Apical buds had a significantly ($p < 0.001$) greater lenghts than lateral buds selected from either mid- or stolen end of the tuber. Apical buds length increased more rapidly than lateral buds with an increase in sprout vigour by the 8^{th} day of incubation (1.05 mm), lateral buds from mid-whorl 11^{th} day (1.44 mm), and stolen buds by the 13^{th} day (1.63 mm).

Growth rates of lateral buds from the stolon end were stimulated by the addition of 40 mM of Ca^{2+} (Table 5-1), however, in general, increasing calcium above 30 mM of Ca^{2+} suppressed length of apical and mid-whorl lateral buds with growth rates similar to the

control. Higher Ca^{2+} in the cytosol has been reported to be toxic for phosphate-based energy systems (reviewed by Virdi *et al.*, 2015).

Buds treated with calcium antagonists EGTA and $LaCl_3$ had a slower rate of bud emergence and sprout length (Figure 5-2 and Figure 5-3). The application of the calcium chelator EGTA and the $LaCl_3$ channel blocker delayed dormancy break and reduced sprout length in a dose responsive manner ($p < 0.001$). While EGTA application inhibited dormancy break, increasing EGTA concentrations above 20 mM did not extend dormancy in excised buds (Table 5-1). A similar effect was observed in $LaCl_3$ mid-whorl and stolon buds (Table 5-1). Interestingly buds excised from the mid-whorl did not respond to EGTA treatments (Table 5-1). When $LaCL_3$ was applied its effects were observed across buds excised from all locations, which was in contrast to of Ca^{2+} and EGTA application.

In general, 30 mM of Ca^{2+} was the treatment with the that produced the greater shoot growthcompared to 0 mM of Ca^{2+}. Given that 30 mM of EGTA and $LaCl_3$ (0.12 mm) were not significant different from 20 mM and 40 mM, for the next part of this experiment it was decided to use 30 mM of Ca^{2+}, EGTA and $LaCL_3$.

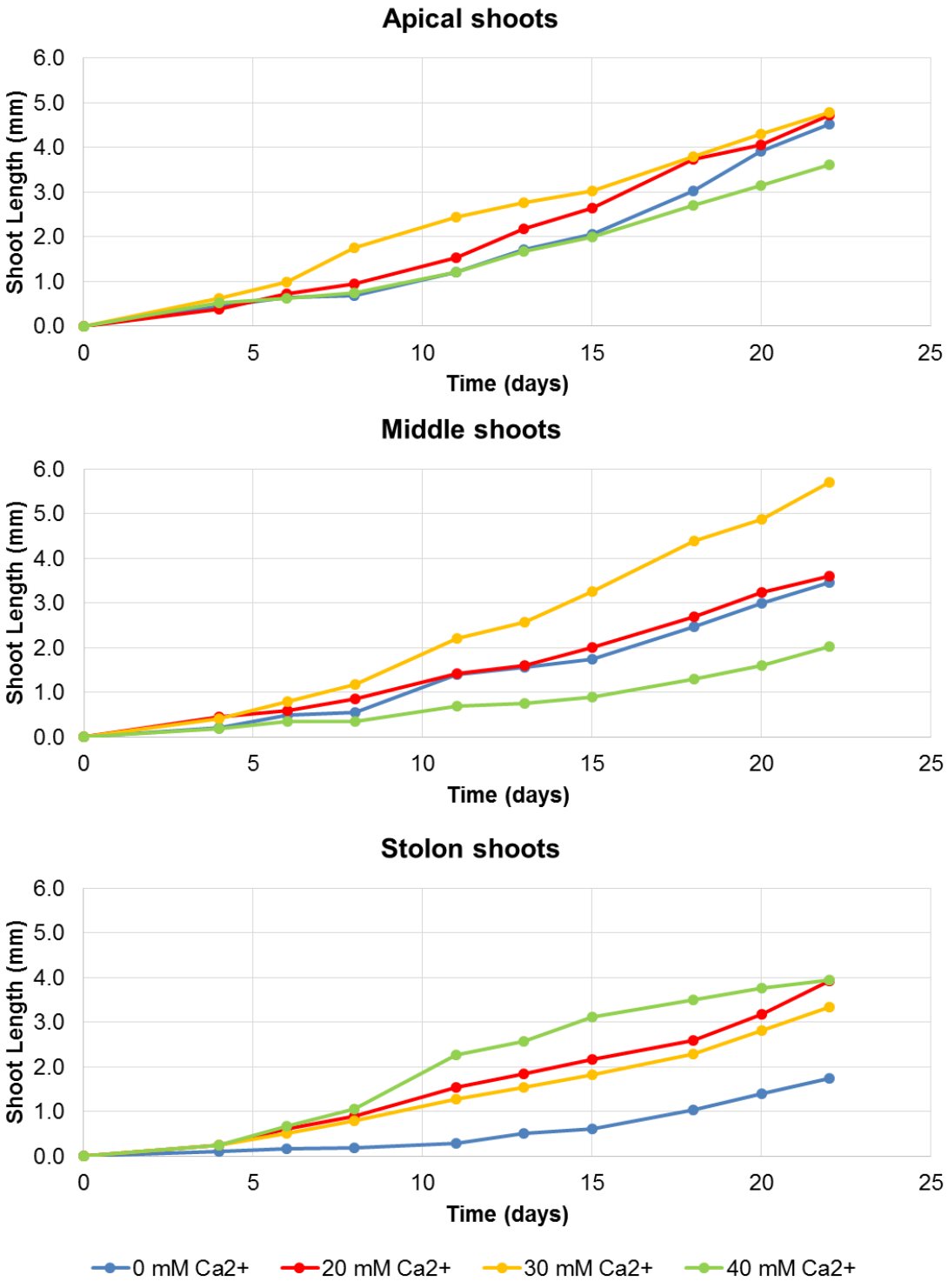

Figure 5-1 - Shoot length (mm) after the application of different concentrations of calcium chloride (Ca^{2+}) to apical, middle and stolon excised buds from Lady Balfour tubers

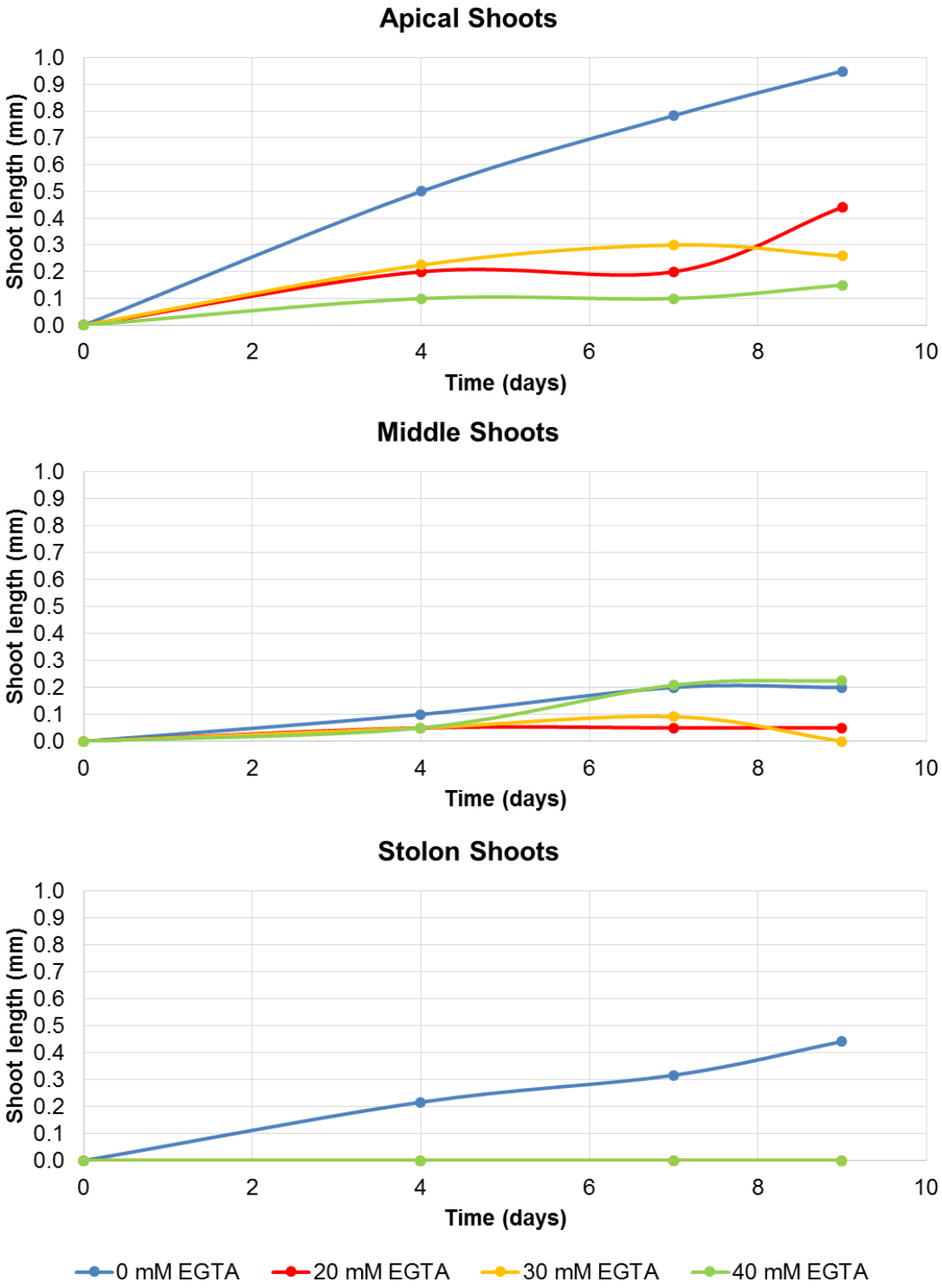

Figure 5-2 - Shoot length (mm) after the application of different concentrations of Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) to apical, middle and stolon excised buds from Lady Balfour tubers

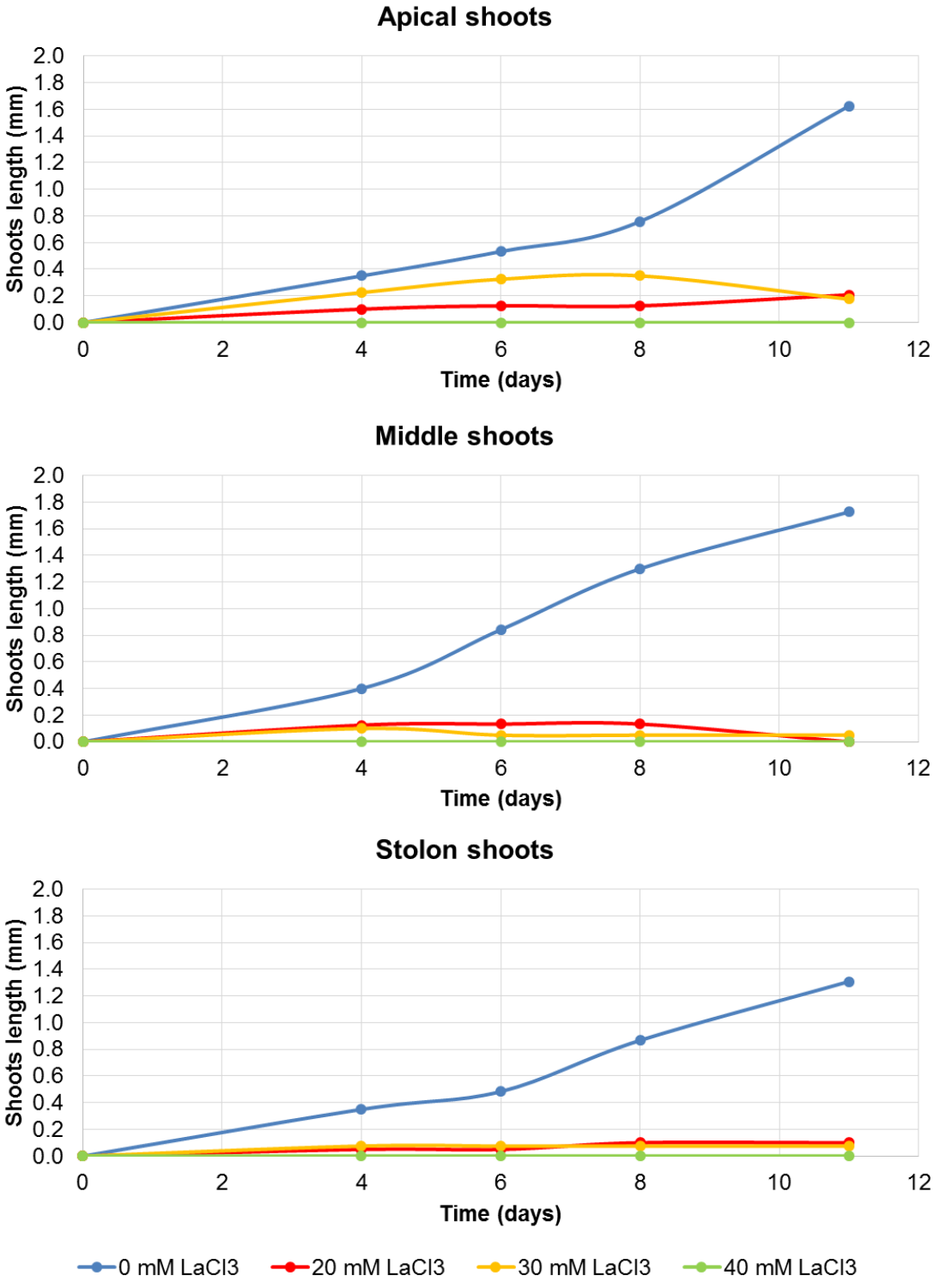

Figure 5-3 - Shoot length (mm) after the application of different concentrations of Lanthanum (III) chloride heptahydrate ($LaCl_3$) to apical, middle and stolon excised buds from Lady Balfour tubers

Table 5-1 – Shoot length (mm) from buds excised from different regions of potato tubers and treated with Ca^{2+}, EGTA and $LaCl_3$.

mM	Apical	Middle	Stolon
0 Ca^{2+}	1.84[b]	1.49[b]	0.61[b]
20 Ca^{2+}	2.10[ab]	1.66[b]	1.71[a]
30 Ca^{2+}	2.47[a]	1.54[a]	1.47[a]
40 Ca^{2+}	1.63[b]	0.82[c]	2.12[a]
$HSD_{0.05}$	0.53	0.44	0.70
0 EGTA	0.56[a]	0.13[a]	0.25[a]
20 EGTA	0.21[b]	0.04[a]	0[b]
30 EGTA	0.20[b]	0.04[a]	0[b]
40 EGTA	0.09[b]	0.13[a]	0[b]
$HSD_{0.05}$	0.26	0.16	0.03
0 $LaCl_3$	0.66[a]	0.86[a]	0.60[a]
20 $LaCl_3$	0.13[bc]	0.09[b]	0.06[b]
30 $LaCl_3$	0.24[b]	0.05[b]	0.08[b]
40 $LaCl_3$	0[c]	0[b]	0[b]
$HSD_{0.05}$	0.22	0.33	0.11

Mean values with different letters are significantly different according to Tukey HSD test.

5.3.2. Assessment of the influence of calcium and calcium inhibitors on dormancy and sprout growth

To determine further the interaction of treatments on dormancy break and sprout growth and the bud location in other varieties, assays were repeated using buds excised from varieties Melody and Arsenal.

A comparison of buds excised from the apical and stolon end of tuber, was made in terms of response to 30 mM Ca^{2+}, 30 mM EGTA, 30 mM $LaCL_3$. A Weibull distribution analysis (generated using paramameters estimated from survReg in R) was used to

estimate the number of days each treatment extended dormancy (Figure 5-4 and Figure 5-5).

A significant effect of treatment ($p < 0.001$) on dormancy extension was observed, tubers treated with EGTA or $LaCl_3$ remained dormant for longer than untreated controls (Figure 5-4 and Figure 5-5). Varietal effects were observed in response to treatments ($p < 0 .05$) and further interactions between variety and bud location ($p < 0.001$) were observed between the two varieties under test in terms of dormancy. Buds excised from Arsenal remained dormant for longer when treated with EGTA. Conversley, buds of Melody treated with $LaCl_3$ excised from the stolen remained dormant for longer than apical buds (Figure 5-4 and Figure 5-5).

Treatment and variety had significant effect ($p < 0.05$) on shoot growth (mm/day) as well the interations between variety and bud location ($p < 0.001$). Apical buds from Arsenal tended to grow slower when treated with EGTA and $LaCl_3$ as did stolon buds from Melody variety. In these experiments application of 30 mM Ca^{2+} had no significant effect in sprout growth which was in contrast to early experiments using Lady Balfour (Figure 5-6 and Figure 5-7).

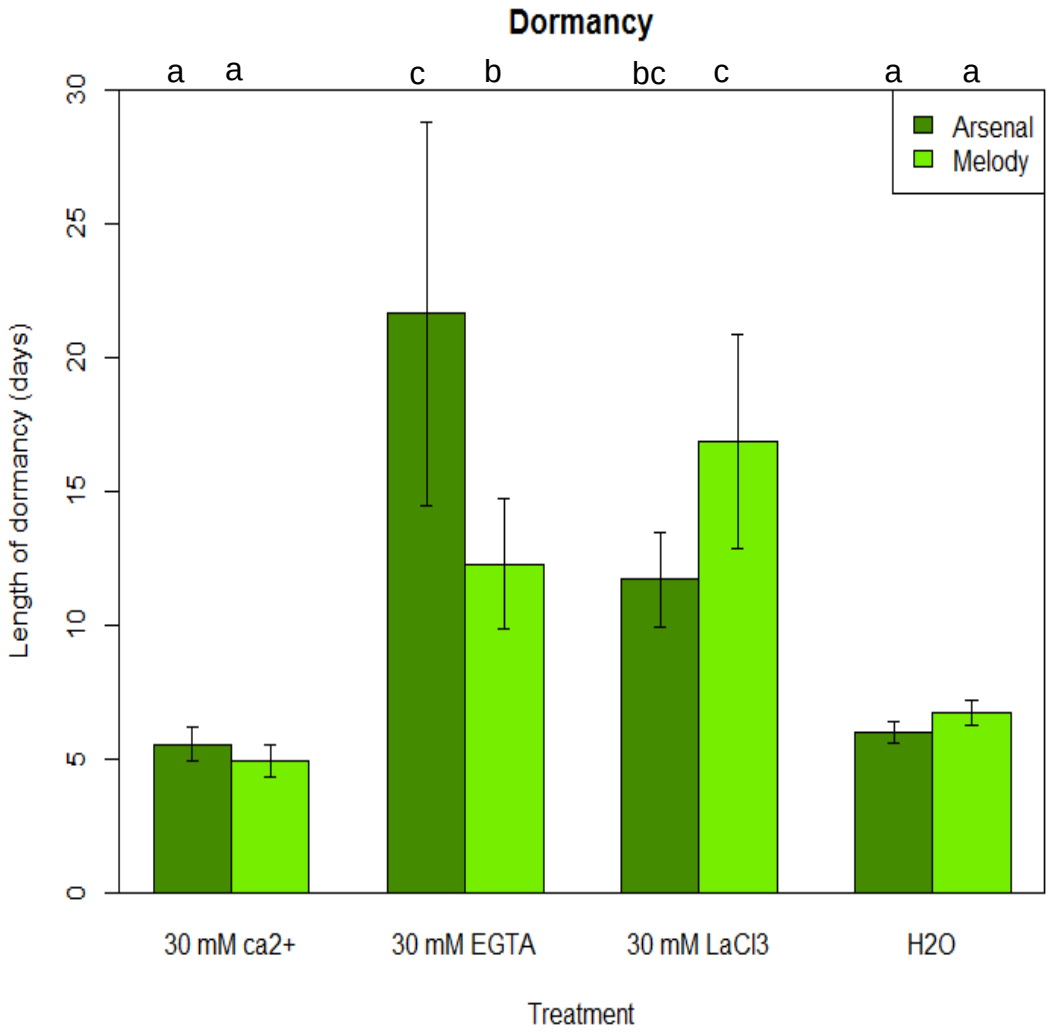

Figure 5-4 – Dormancy prediction based on Weibull distribution for the different varieties with different treatments. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package “multcomp”), with SE bars.

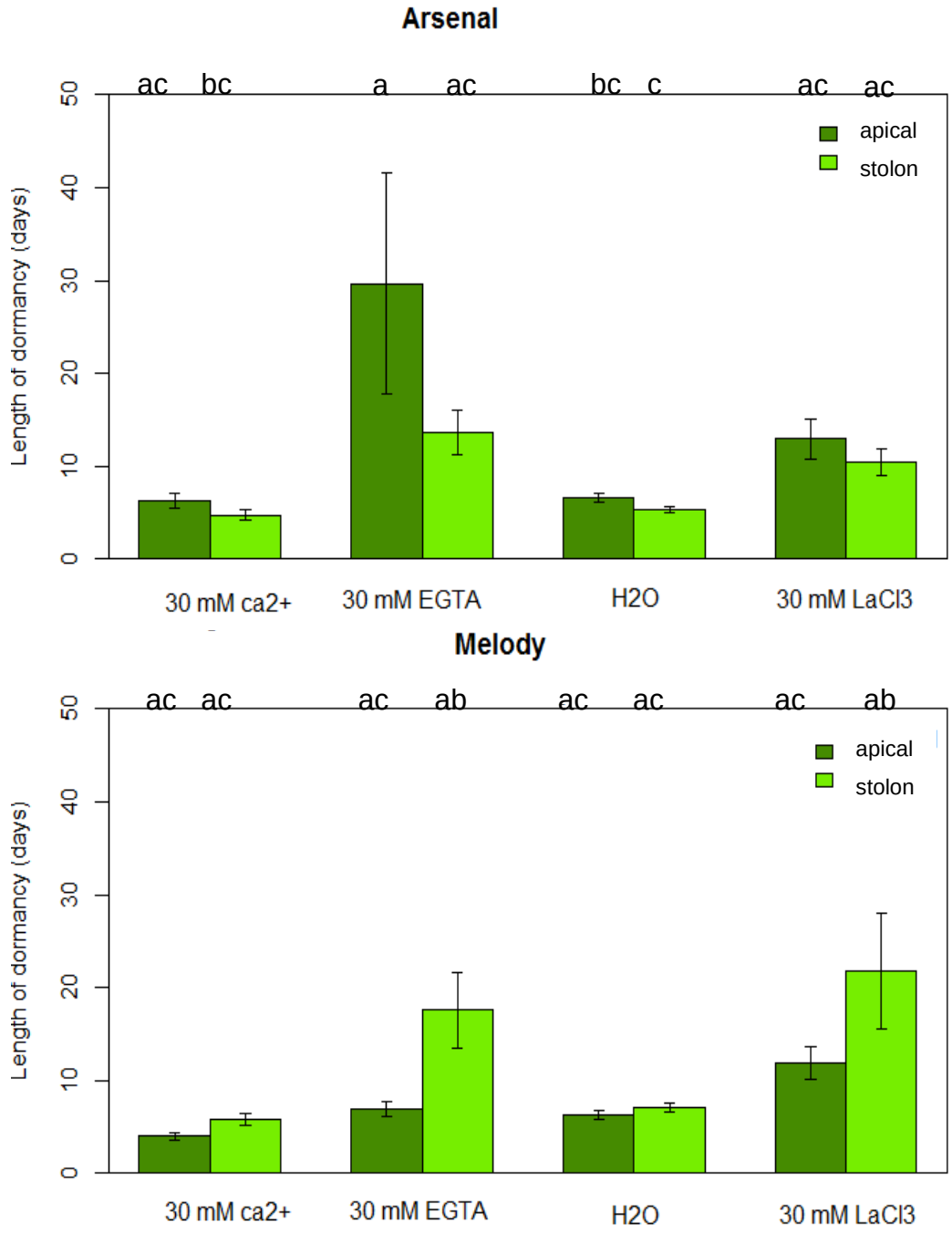

Figure 5-5 - Dormancy prediction on Weibull distribution for the different varieties with different treatments by tuber site. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package “multcomp”), with SE bars.

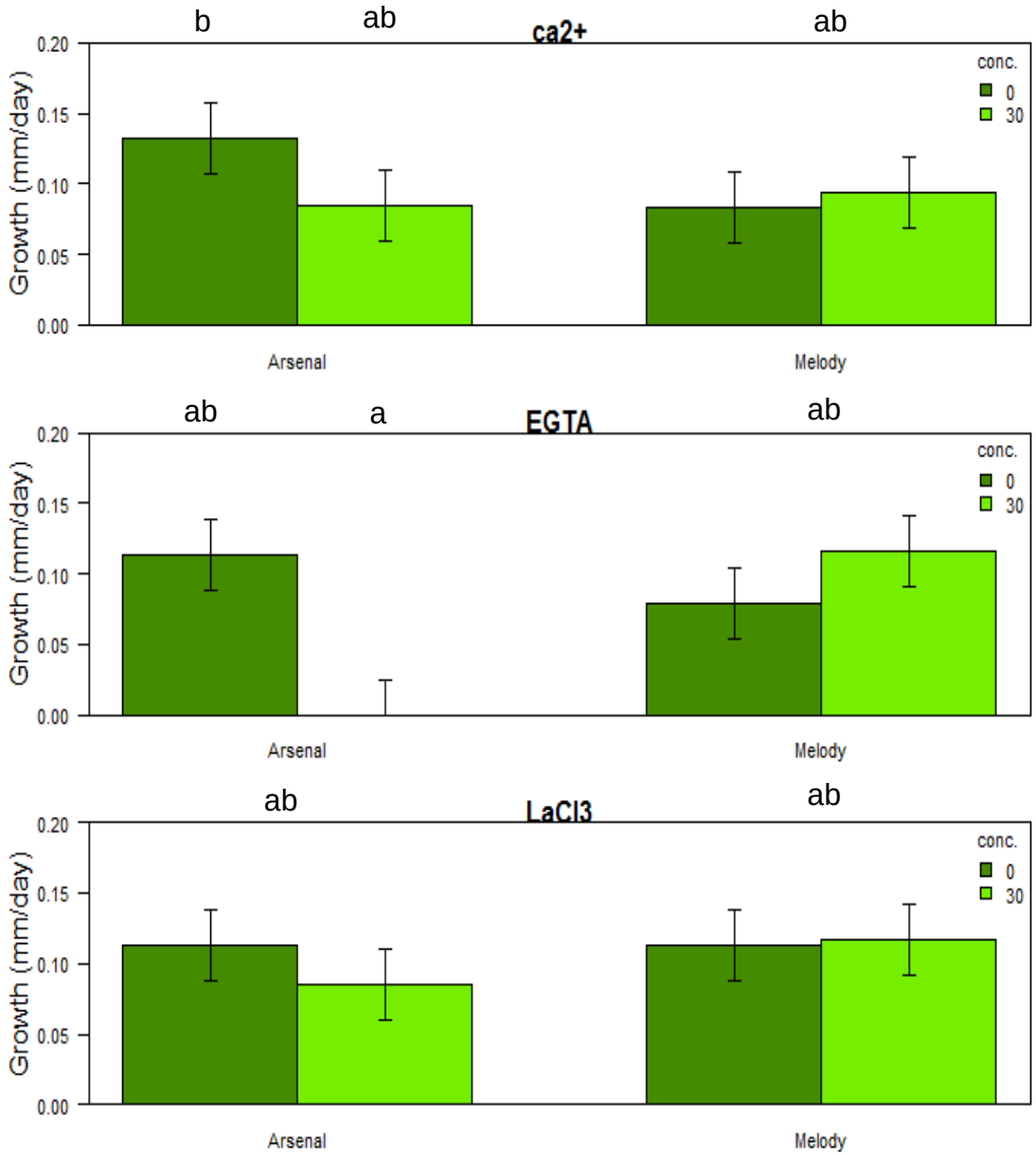

Figure 5-6 – Shoot growth (mm/day) for each variety and treatment for apical site. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package “multcomp”), with SE bars.

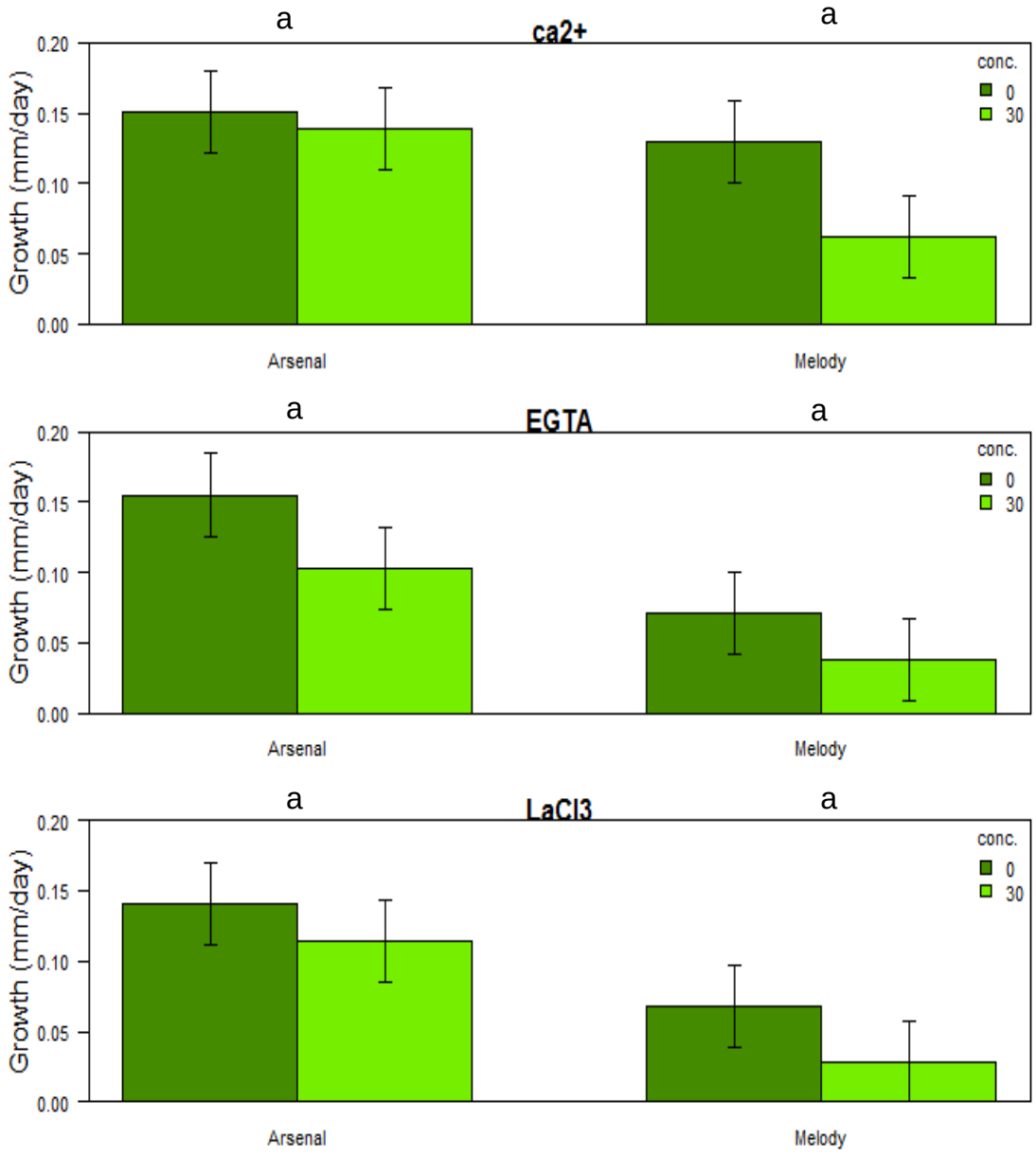

Figure 5-7 – Shoot growth (mm/day) for each variety and treatment for stolen site. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package “multcomp”), with SE bars.

5.3.3. Assessment of the influence of calmodulim blockers on dormancy and sprout growth

Calmodulin blockers accelerated sprout growth and dormancy break ($p < 0.001$), however the effect was temperature dependent.

Incubating buds in 500 µM of calmidazolium chloride at 20°C increased sprout growth significantly, while blocking calcium receptors with 30 mM $LaCl_3$ resulted in the lowest sprout growths (Table 5-2). Buds treated with $LaCl_3$ stayed dormant for longer (Table 5-3). Application of a second calmodulin inhibitor W7 failed to effect dormancy break or sprout growth.

Repeat experiments carried out at 10°C were restricted to 1 mM of calmidazolium chloride and 1 mM of W7, the interaction between location of excised buds and treatment effects was significant for sprout growth and dormancy break ($p < 0.001$).

$LaCl_3$-treated buds had the lowest sprout number, while sprout growth in calmidazolium chloride, was lower than untreated control buds incubated at 10°C (Table 5-4). For this incubation temperature, buds treatead with $LaCl_3$ were dormant than control buds (Table 5-5). Dormancy duration was not significantly different between buds treated with Ca^{2+} and the ones treatead with W7. Nevertheless, calmidazolium chloride show significant dormancy duration from the Ca^{2+} buds (Table 5-5).

When comparing both incubation temperatures (10°C and 20°C) sprout growth was significant higher at 20°C than 10°C ($p < 0.001$). With sprout growth at 20°C (0.04 mm d^{-1}) compared to 10°C (0.03 mm d^{-1}) ($HSD_{0.05} = 0.005$).

Buds kept at 10°C were expected to stayed dormant for longer (Figure 5-8). On average, dormancy break was just expected to happen by the 10^{th} day for buds keept at 10°C and by the 5^{th} day for the ones kept at 20°C ($HSD_{0.05} = 0.5$).

Table 5-2 – Sprout length (mm) for buds incubated at 20°C in different concentrations of calmidazolium chloride (CC), W7, EGTA, $LaCl_3$, Ca^{2+} and control (dH_2O).

	CC	W7	EGTA	$LaCl_3$	Ca^{2+}	Control
100 µM	1.6^{bc}	1.7^{abc}	-	-	-	-
500 µM	2.0^{a}	1.8^{abc}	-	-	-	-
1 mM	1.8^{abc}	1.6^{bc}	-	-	-	-
30 mM	-	-	2.0^{ab}	0.2^{d}	1.6^{c}	-
0 mM	-	-	-	-	-	1.7^{bc}

Means with the same letter are not significantly different according to Tukey HSD test ($HSD_{0.05} = 0.4$).

Table 5-3 – Number of days to dormancy break (predicted by Weibull distribution) for buds treated with calmodulin blockers calmidazolium chloride (CC) and W7, EGTA, $LaCl_3$, Ca^{2+} and control (dH_2O) incubated at 20°C.

	CC	W7	EGTA	$LaCl_3$	Ca^{2+}	Control
100 µM	4^{b}	4.1^{b}	-	-	-	-
500 µM	4.3^{b}	4^{b}	-	-	-	-
1 mM	4^{b}	4.2^{b}	-	-	-	-
30 mM	-	-	4^{b}	10.6^{a}	4^{b}	-
0 mM	-	-	-	-	-	4^{b}

Means with the same letter are not significantly different according to Tukey HSD test ($HSD_{0.05} = 0.9$).

Table 5-4 – Sprout growth (mm) for buds incubated at 10°C with calmidazolium chloride (CC), W7, EGTA, $LaCl_3$, Ca^{2+} and control (dH_2O).

mM	**CC**	**W7**	**EGTA**	**$LaCl_3$**	**Ca^{2+}**	**Control**
1	0.5^{b}	0.4^{b}	-	-	-	-
30	-	-	0.4^{b}	0.06^{d}	0.2^{c}	-
0	-	-	-	-	-	0.7^{a}

Means with the same letter are not significantly different according to Tukey HSD test ($HSD_{0.05} = 0.1$).

Table 5-5 – Days for dormancy break (predicted by Weibull distribution) for buds incubated at 10°C with calmidazolium chloride (CC), W7, EGTA, $LaCl_3$, Ca^{2+} and control (dH_2O).

mM	**CC**	**W7**	**EGTA**	**$LaCl_3$**	**Ca^{2+}**	**Control**
1	8.6^{c}	9.4^{bc}	-	-	-	-
30	-	-	9.5^{bc}	14.2^{a}	11.0^{b}	-
0	-	-	-	-	-	6.7^{d}

Means with the same letter are not significantly different according to Tukey HSD test ($HSD_{0.05} = 1.8$).

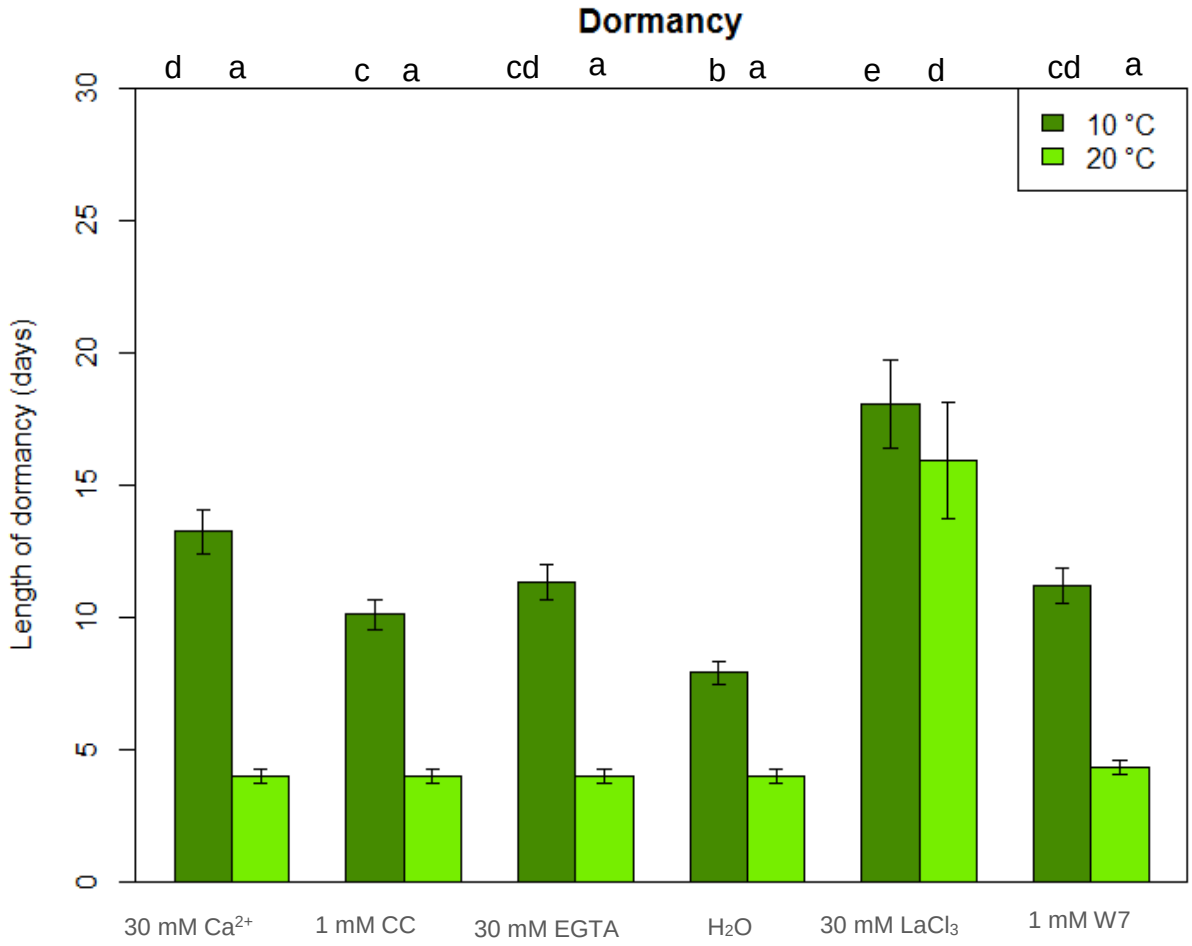

Figure 5-8 - Dormancy prediction by Weibull distribution for Lady Balfour for 10°C and 20°C with different treatments. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package “multcomp”). Error bars indicate SE.

5.4. Discussion

Assessment of Ca^{2+}, $LaCl_3$ and EGTA concentration effects on tuber dormancy break and sprouting

The location of buds taken across the tuber had a significant effect on the response to calcium treatments; buds excised from the stolon end of the tuber responded more positively to calcium, and were retarded to a greater extent with EGTA compared to apical buds. The degree of apical dominance will influence the rate of dormancy (Eshel and Teper-Bamnolker, 2012).

The action of the calcium receptor inhibitor $LaCl_3$ was not site specific and suppressed sprout growth across all tubers. Due to the nature of $LaCl_3$ action, general senescence of the tissue subtending the bud may have caused lack of sprout growth rather than an effect on the mechanism of cell elongation. Earlier trials to break dormancy chemically with bromoethane, prior to treatment with calcium inhibitors did not effect the influence the interaction between treatments and sprout location.

The role of calcium receptor inhibition by $LaCl_3$ is unclear. Whether calcium signalling is involved in the suppression of dormancy break through apical dominace requires further investigation. Alternatively receptor inhibition may have a more general supression effect on cellular activity, and enhanced senescence that has a knock on effect, preventing bud emergence and apical dominance.

Assessment of the influence of calcium on dormancy and sprout growth

The lack of dormancy break in buds treated with calcium blockers led to no significant differences between treatments. However, the use of the Weibull model to predict the onset of dormancy break, with the limited data available gave a prediction of the result, and from this model buds treated with 30 mM EGTA or $LaCl_3$ were predicted to delay the onset of dormancy in vars Arsenal and Melody.

Nevertheless, the degree of predictive dormancy extension response of varieties varied between treatments with Arsenal remaining dormant for longer in the presence of 30 mM of EGTA treatment. In contrast, dormancy break in Melody buds treated with

$LaCl_3$ was slower than buds from variety Arsenal. Rappaport *et al.* (1965) reported a variation in total sprouting among excised buds, that appeared to be correlated with the rate of sprouting of the population of tubers from where the buds were excised. Suggesting that the influence of endo- and eco-dormancy are retained in the excised state. Confirmation of this differential response is required on a larger sample size, on freshly harvested samples to ensure calcium inhibition is breaking tuber endodormacy. Excision of buds from dormant potato tubers initiates quickly the sequence of events that lead to sprouting (Rappaport *et al.*, 1965). Improved diagnosis of the initial breaking of dormancy would facillitate more accurate assessment of calcium's role in dormancy break. Currently the degree of dormancy is measured on the basis of visible bud movement, while initial meristem activation and reconnection with tissues subtending the bud may occur before any visible increase in bud movement was observed.

Assessment of calcium's role in subsequent shoot growth found a strong varietal response. The model for dormancy, predicts the number of days buds show no shoot movement and number of shoots with shoot movement, this may have led to a small bias in the observation between varieties. More extensive trials in the future are needed to confirm the differential response.

The rate of sprout rate is strongly dependent on variety (Daniels-Lake and Prange, 2007) and is not necessarily related to the length of dormancy, although recent studies on dormancy break in a diploid population (6h01a) suggest that sprout vigour in longer dormant progeny decreased with length of dormancy (Colgan pers com.). The varietal response to EGTA and $LaCl_3$ could be attributed to sensitivity or differences in uptake and movement within the bud and subtending tissue. The mode of action of these two chemicals differs; EGTA a chelator of extracellurar free Ca^{2+} and $LaCl_3$ a blocker of plasma membrane calcium channels (Pang *et al*., 2007). Data suggests that by lowering calcium concentration in tuber buds, by the addition of a calcium chelator or a plasma membrane calcium channel blocker, the buds dormant longer. Unexpectedly, the growth rate of buds treated with calcium (30 mM of Ca^{2+}) was similar to untreated buds. The dormancy model predicted that calcium has no effect on bud break or shoot extension for the varieties Melody and Arsenel, but in earlier experiments with Lady

Balfour calcium was seen to have a clear positive response on incrementing shoot length. Interesting, in a experiment with biofortified tubers, tubers that were fortified with 32 µL Ca^{2+} had the higher sprout growth comparing to tubers fortified with calcium ferrite (24 µL Ca^{2+}) and control (0 µL Ca^{2+}) (data not shown). However, in the case of tubers fortified with calcium ferrite the lower sprout growth shown could have been a consequence of raised iron content. This idea is supported by parallel experiments at Nottingham-Trent University high iron concentration suppressed sprouting (Ms Karen Davis pers. com.). The age of tubers may have some bearing on response to calcium. The tubers of varieties Melody and Arsenal, were less than 2 months from harvesting, whereas Lady Balfour had been stored for 4 months, without sprout suppressers at the point of testing.

It is possible to infer that calcium is required for the initiation of sprouting (dormancy break?) and shoot growth, because when calcium is not available, due to a calcium chelator or a plasma membrane calcium channel blocker, sprout and shoot growth tends to be delayed. Earlier studies by Pang *et al*. (2007) found that inhibition of dormancy break in grape buds by EGTA or $LaCl_3$ could be reversed by exogenous application of calcium.

Further experiments are required to test post-calcium treatment in potato buds treated previously with EGTA and $LaCl_3$. Preliminary data (data not shown) showed that re adding calcium to buds can overcome the effects of buds treated with calcium blockers and chelators, however, more tests are needed.

Additional work is required to determine the role of calcium in true dormancy versus its effect on shoot growth, since calcium is involved in many cellular processes (Snedden and Fromm, 2001; Zhang and Lu, 2003; Peppiatt *et al.*, 2004; Hepler, 2005).

Assessment of the influence of calmodulim blockers and storage temperature on dormancy and sprout growth

The role of the calcium binding protein calmodulin (CaM) in dormancy break was investigated using calmodulin blockers W7 and calmidazolim chloride. CaM regulates

Ca^{2+} intracellular levels by modulating Ca^{2+} - ATPases activity (reviewed by Virdi *et al.*, 2015).

Tuber buds were very sensitive to $LaCl_3$ application but less so to EGTA. Timing of application in relation to tuber age appears to influence the reponse of buds to calcium and its antagonists, with older tubers less responsive.

Removal of extracellurar free calcium by EGTA has less influence on [Ca^{2+}] than $LaCl_3$. Suggesting intracellular [Ca^{2+}] is more important for regulating dormancy break and sprouting restricting Ca^{2+} movement from the cytosol into the intercellular compartiments. While Ca^{2+}-permeable ion channels in plasma membranes facillitate movement of Ca^{2+} entry across plasma membranes, $[Ca^{2+}]_{cyt}$ in the cytoplasm is mantained at low concentrations (reviewed by Virdi *et al.*, 2015). Removal of $[Ca^{2+}]_{cyt}$ to the apoplast or lumen of intracellular organelles, such as the vacuole or the endoplasmatic reticulum is regulated by Ca^{2+}-ATPases and H^+/Ca^{2+}-antiporters (reviewed by White and Broadley, 2003). Such movements of Ca^{2+} generates changes in Ca^{2+} cytoplasmic concentrations initiating cellular responses to a diverse range of developmental and environmental signals (reviewed by White and Broadley, 2003). Application here of $LaCl_3$ to buds blocks Ca^{2+}-permeable ion channels that appears to prevent Ca^{2+} signal changes in cytosolic calcium responsible for the initiation of sprouting and end of dormancy in tubers.

CaM is present in the apoplast, cytosol, endoplasmatic reticulum and nucleus of plant cells, and can bind with different proteins responsible for different physiological processes, such as $[Ca^{2+}]_{cyt}$ homeostasis. When CaM binds to Ca^{2+} it initiates the cellular perception and transduction of a $[Ca^{2+}]_{cyt}$ signal (reviewed by White and Broadley, 2003). Buds treated with CaM blockers showed sprout growth rates similar to buds treated with Ca^{2+}. CaM blockers shown to trigger an increase in cytosolic $[Ca^{2+}]_{cyt}$ content triggering the Ca^{2+} response (Kaplan *et al.*, 2006). The differential response in bud break and sprout growth observed between two types of calmodulin inhibitors may either reflect different modes of action or preferential uptake. In the case of dormancy break the profile of buds treated with W7 dormancy break were similar to those treated with Ca^{2+}, while buds treated with calmidazolium chloride remained dormant for longer. Calmidazolium chloride is an inhibitor of calmodulin-regulated

enzymes (Kitagawa and Yoshizaki, 1998), while W7 besides inhibiting of calmodulin-regulated enzymes acts as a calmodulin antagonist (Kiselev *et al.*, 2013). Kaplan *et al.* (2006) observed that CaM antagonists can trigger a similar response in time and rate to Ca^{2+} cytosolic however not identical. According to those authors, this disparity could be due to differences in cell permeability or due to the target specificity of the different compounds.

In poplar (*Populus deltoides* Bartr. ex Marsh) a high degree of dormancy was reached when the $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuclie}$ decreased, causing an accumulation of Ca^{2+} in intercellular spaces and in cell walls, suggesting a dynamic flux in Ca^{2+} distribution across the cell during bud dormancy (Jian *et al.*, 1997). Blocking Ca^{2+}-permeable ion channels and preventing transport of Ca^{2+} to the cytosol may induce pertubations of calcium across the cell that might lead to an extension of G-phase arrest during the cell cycle and thus extend dormancy even when stored at higher storage temperatures.

Low temperatures significantly limit sprout growth once dormancy has broken (Sonnewald, 2001). In this experiment storage at higher temperatures led to rapid dormancy break and higher rates of sprout growth. Differential responses to blocking Ca^{2+}-permeable ion channels in terms of sprouting and increased dormancy release were observed. Incubation at 20°C resulted in all the treatments triggering dormancy synchronistically with the exception of $LaCl_3$ treatment where dormancy was extended. Storage at 10°C led to a greater range of treatment differences.

Chapter 6. VARIETY COMPARISON IN TERMS OF GENE EXPRESSION USING REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT qPCR)

6.1. Introduction

Several factors can affect the rate of starch degradation in plants, one of which is the starch phosphorylation by glucan, water dikinase activities (GWDs) (Orzechowski *et al., 2013)*. Starch phosphorylation can weaken the organisation of the starch granule surface polymers, facilitating access to degrading enzymes (Smith, 2012). Glucan, water dikinase (GWD1), also known as R1 protein in *S. tuberosum* and SEX1 in *A. thaliana*, phosphorylates glucose residues at the C-6 position and phosphoglucan, water dikinase (PWD/GWD3) phosphorylates the glucose residues at the C-3 position in amylopectin chains (Ritte *et al.*, 2006). Highly ordered insoluble starch is phosphorylated by GWD1. The glucan phosphorylation at the C-6 position results in a shift of the phosphoglucans state, which is less ordered but still insoluble, being an appropriate substrate for C-3 phosphorylation by GWD3, after which the phosphoglucan finally becomes soluble (Ritte *et al.*, 2006; Fettke *et al.*, 2009; Smith, 2012).

GWD3 (PWD) acts downstream of GWD1, since it phosphorylates the substrates previously phosphorylated by GWD1 (Kotting *et al.*, 2005; Hejazi *et al.*, 2009). Similar to the GWD3 in *Arabidopsis*, Mikkelsen *et al.* (2005) suggested that there was at least one additional GWD homologue in *S. tuberosum* that was independent of the redox potential, StGWD3, as named by Orzechowski *et al.* (2013), existing in a fully reduced and active form (Mikkelsen *et al.*, 2005).

Real-time qPCR (RT qPCR) is highly sensitive allowing quantification of transcripts and small changes in gene expression. In addition it is easy to perform, with necessary

accuracy and produces reliable and rapid quantification of results (Pfaffl, 2001). RT qPCR was used to analyse the changes in StGWD1 and StGWD3 transcript levels relative to the expression of the housekeeping gene (elongation factor 1-α (EF1-α)) during the 3rd year of tuber storage for VR 808 and L. Rosetta.

6.2. Material and methods

6.2.1. RNA extraction

Five tubers of each variety were used for each sampling time (December 2015, January, March, April and June 2016). Tissue from opposite eighths of cortex was snap frozen in liquid nitrogen individualy and stored at -80ºC followed by freeze drying and grinding in liquid nitrogen using a mortar and pestle. Freeze dried tissue was kept at -80ºC until RNA extraction.

RNA extraction was performed using an adaptation of the method by Otti (2016). Approximately 50 mg of freeze dried potato tuber tissue sample was mixed with 1 mL of cetyl trimethyl ammonium bromide (CTAB) extraction buffer ((2% w/v), 3 M NaCl, 20 mM EDTA and 100 mM Tris-HCl (pH 8.0)) pre-heated in a water bath for 10 minutes at 65ºC, after addition of 1% (v/v) 2-mercaptoethanol. Approximately, 800 µL of sample mix was transferred to a 2 mL tube with an equal volume (800 µL) of phenol: chloroform: isoamyl alcohol (25:24:1) and the mixture centrifuged at 15871 g for 10 minutes. The upper aqueous phase was transferred to a new 1.5 mL tube and an equal volume of absolute ethanol was added and mixed by gently pipetting up and down. The solution was transferred to an RNeasy® Mini spin column from QIAGEN's RNeasy® Plant Mini Kit (Qiagen, UK) (pink column) and centrifuged for 15 seconds at 15871 g, discarding the flow-through. The subsequent steps of the extraction protocol were carried out according to QIAGEN's RNeasy® Plant Mini Kit protocol (Qiagen, UK). Genomic DNA was removed using DNase I, RNase-free (Thermo Scientific, UK) according to the manufacture instructions.

RNA concentration was measured using a NanoDrop 2000 (Thermo Scientific, UK), before and after the genomic DNA removal, by placing a 1 µL drop of RNA solution in the sample port of the analyser and measuring as ng/µL. Absorbance at A_{260} (nucleic acid) and A_{280} (protein) was estimated along with the ratio A_{260}/A_{280}. A ratio of 1.7 to 2.0 indicates RNA preparations free of contaminating proteins**.**

The quality of the RNA extracted was assessed by gel electrophoresis. 1% [w/v] agarose gel in 0.5×TBE (Tris Borate EDTA) buffer (Brody and Kern, 2004). The gel was stained with 10 µL of SYBR® Safe DNA Gel Stain (ThermoFisher Scientific, UK). Samples (8 µL of RNA and 3 µL of gel loading dye orange (New England Biolabs, USA)) were loaded into lanes. The agarose gel was run at 110 mA (milliamps) for 20 minutes in 0.5% TBE, allowing sufficient time for the major RNA sub-fragments (18 S, 25 S and 5S) to be separated. Gels were visualized using a gel imaging suite (Syngene G: Box, UK).

6.2.2. Real time reverse transcription analysis of mRNA

As a qualitative method to detect gene expression, RT qPCR requires the creation of cDNA (complimentary DNA) transcripts from the RNA. One µg of RNA was used as a starting material for reverse transcription into cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN, UK), according to the manufacturer's instructions. cDNA was stored at -20°C or used directly for RT qPCR reactions.

The primer sets used were designed by Orzechowski *et al.* (2013), StGWD3-F (CAATAGCTATGCGTCAGAAGTG), StGWD3-R (GCTTTGCATTCCTCGGGCTTC), StGWD1-F (CCCACGATCTTAGTAGCAAA) and StGWD1-R (TTAGCTCCAACCATTTCACT). The elongation factor 1-α (EF1-α) was chosen as housekeeping gene and was used using the primers designed by Nicot *et al.* (2005), EF1-α-F (ATTGGAAACGGATATGCTCCA) and EF1-α-R (TCCTTACCTGAACGCCTGTCA). Primers (Sigma-Aldrich) were re-suspended using nuclease free water (Sigma-Aldrich, UK) to form a stock solution of 100 µM. A 10 µM working primer concentration was prepared from the stock solution.

QuantiTect® SYBR® Green PCR kit (Qiagen, UK), was used for qPCR with a 5 µL template (cDNA sample) added to each well of a Hard-Shell® 96-Well Semi-Skirted PCR Plate (Bio-Rad, USA) followed by the addition of 15 µL PCR Mix, prepared according to manufacturer's instructions. Each biological replicate (n=5) was replicated 3 times on each 96 well qPCR plate. Housekeeping primers were tested alongside genes under investigation, control wells containing water (background control) and those where the template was replaced with water (negative control). Plates were sealed with Microseal® 'B' seal (Bio-Rad, UK).

RT qPCR was performed in a Bio-Rad CFX96TM Real-Time System (Bio-Rad, UK) according to the manufacturer's instructions. PCR conditions were as follows: 95^{o}C for 15 minutes, followed by 40 cycles of 94^{o}C for 15 seconds, 58^{o}C for 30 seconds and 72^{o}C of 30 seconds.

RT qPCR data expressed as CT threshold values were exported to MS Excel. Data was subject to normalisation using the $2^{-\Delta\Delta CT}$ method revised by Livak and Schmittgen (2001). Thresholds for up regulation were set as $2^{-\Delta\Delta CT} \geq 2$ and down regulation when $2^{-\Delta\Delta CT} < 1$.

An ANOVA was carried out to determine whether there were significant differences between samples with two up to three factors using Rstudio (R Core Team, 2014).

6.3. Results

High quality tuber RNA was obtained with the A_{260}/A_{280} ratio ranging from 2.04 to 2.37, indicating the absence of dissolved impurities (protein contamination) (Gasic *et al.*, 2004; Bansal and Das, 2013), with yields ranging from 46 to 74.6 ng μL^{-1}. The RNA integrity was assessed by the presence of distinct 18 S and 25 S ribosomal RNA bands visualized on a 1% agarose gel between 1.0 and 2.0 Kb in size (https://www.neb.com/products/n3232-1-kb-dna-ladder) (Appendix XII and Appendix XIII).

There was a significant change in StGWD1 and StGWD3 expression in L. Rosetta tubers during storage (10°C) when comparing to the housekeeping gene (EF1-α) ($p < 0.001$), but this was not observed in the sweetening resistant VR 808 ($p > 0.05$). When comparing to EF1-α, over time in store (10°C) L. Rosetta gene expression profiles of StGWD1 showed 3 fold increase in expression, while a slight increase in StGWD3 gene expression (0.89 to 0.97 fold) by the end of the storage, coinciding with the increase in sugar accumulation (Figure 6-1). In VR 808 a 1.5 fold reduction in StGWD1 expression and 2.7 fold reduction in StGWD3 gene expression was observed between the beginning and end of storage, in relation to EF1-α. Expression patterns were variable between sampling occasions after an initial increase in expression of StGWD3 transcripts after 6 weeks storage followed by a 4 fold decrease just after 10 weeks storage at 10°C corresponding to a decrease in sucrose accumulation (Figure 6-1).

Gene expression profiles of StGWD1 and StGWD3 in L. Rosetta were lower than VR 808 during the initial 6 week period of the storage (10°C). However, by the end of the storage (7.5 months) StGWD1 expression increased more than 2.5 fold in L. Rosetta whereas StGWD3 in VR 808 over 1.4 fold (Figure 6-1).

When comparing to EF1-α, in L. Rosetta StGWD1 and StGWD3 expression was down regulated at the beginning of the storage at 10°C, and up regulated by the end of the storage season (7.5 months) for StGWD1. However, in this variety StGWD3 exhibits the tendency to up regulated after the beginning of storage (10°C). In VR 808, down regulation was just observed in StGWD1 after 7.5 months in storage (10°C) (Figure 6-2).

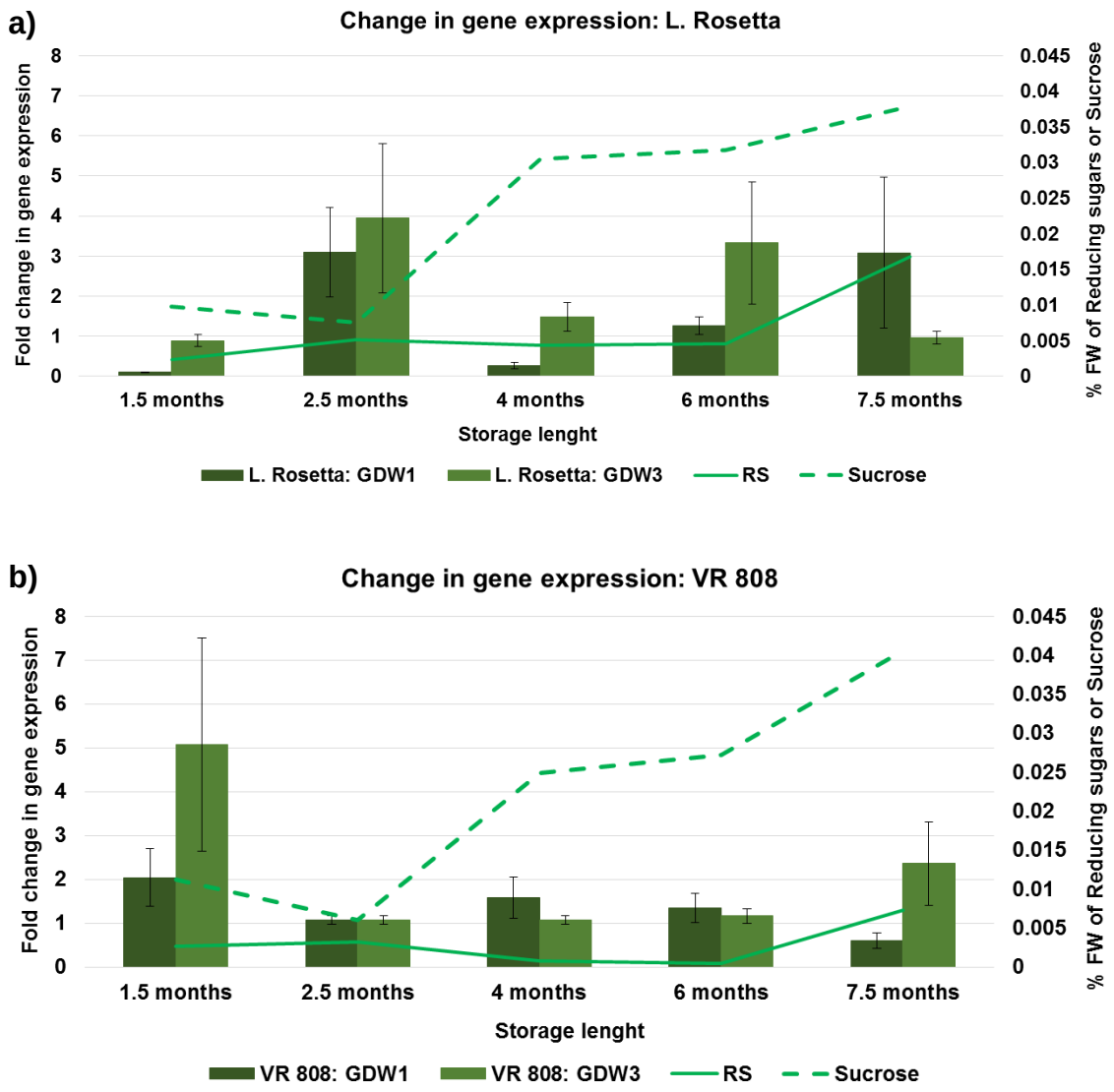

Figure 6-1 - Change in StGWD1 and StGDW3 gene expression in comparison to %FW of reducing sugars and %FW of sucrose during storage at 10°C in L. Rosetta (a) and VR 808 (b) during season 2015/16 with SE bars.

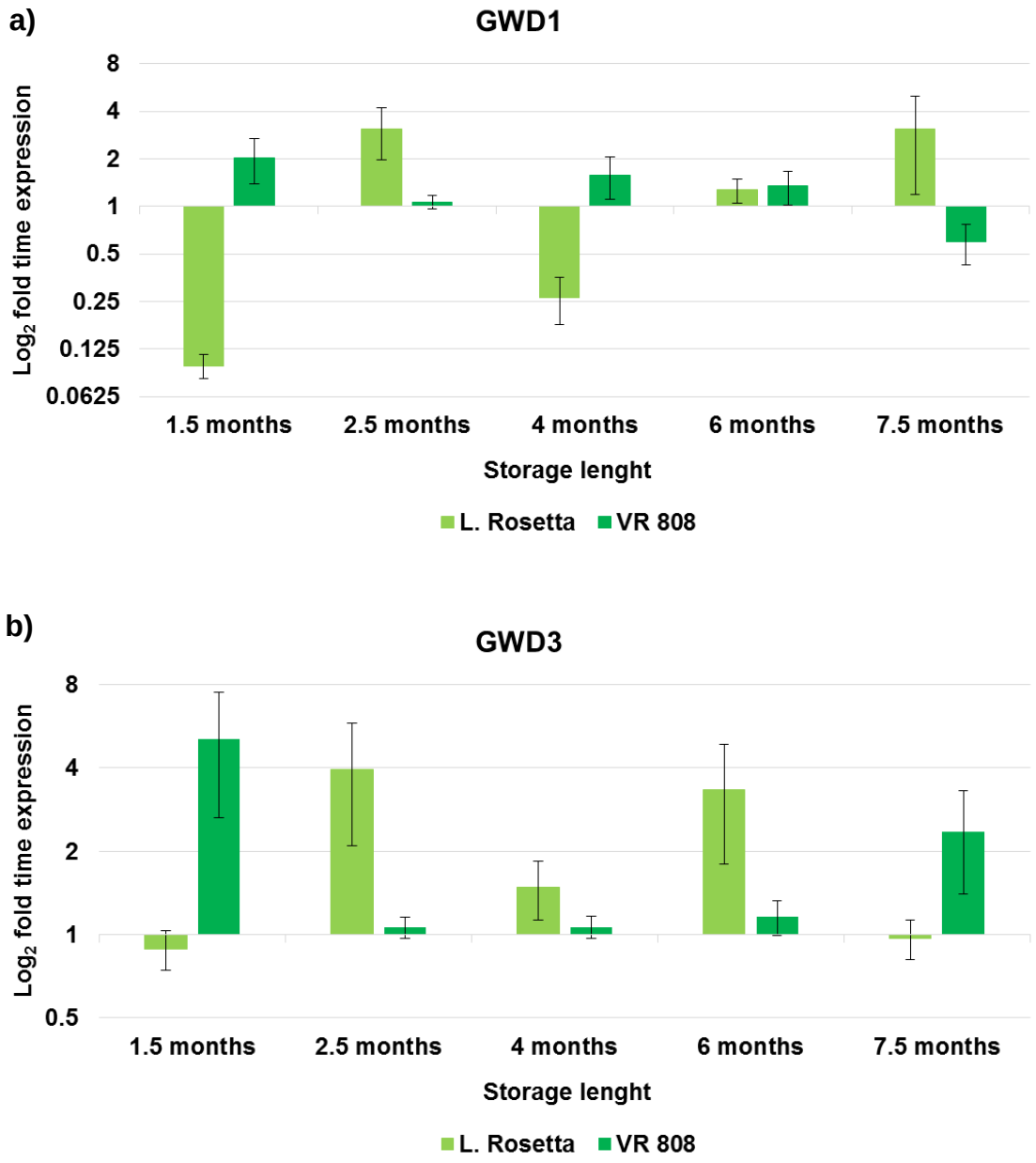

Figure 6-2 – Log_2 fold time expression ($2^{-\Delta\Delta CT}$) of StGWD1 (a) and StGWD3 (b) for L. Rosetta and VR 808, with SE bars. Up regulation when $2^{-\Delta\Delta CT} > 2$ and down regulation when $2^{-\Delta\Delta CT} < 1$.

6.4. Discussion

Starch phosphorylation is a key determinant in the regulation of starch metabolism in plants (Bansal and Das, 2013). GWD catalyze starch phosphorylation in both leaves and different plant storage organs (Bansal and Das, 2013; Mahlow *et al.*, 2014) opening up the surface of the starch granule to attack by amylases (Orzechowski *et al.*, 2013). In both leaves and tubers of potato GWD can exist in several states, either enclosed inside the starch granules or associated with the granule surface or soluble in the plastid stroma (Bansal and Das, 2013).

Two methods of quantification in RT qPCR could be used, absolute quantification based on an internal or an external calibration curve, or relative quantification based on the relative expression of a target gene versus a reference gene (Livak and Schmittgen, 2001; Pfaffl, 2001). In this study the $2^{-\Delta\Delta CT}$ was used to chart changes in expression in StGWD1 and StGWD3 gene expression, so the relative expression ratio to the reference gene was obtained (Livak and Schmittgen, 2001; Pfaffl, 2001).

GWD1 activity is regulated by changes in the cellular redox potential, and is inactivated when fully oxidised (Mikkelsen *et al*., 2005). Increasing ROS content may interfere with the action of GWD1 (Mikkelsen *et al*., 2005). However, while increased transcripts levels were observed during storage the increase in O_2^- and H_2O_2 may decrease effectiveness of newly sythesised phosphorylating enzymes. Without directly measuring StGWD1 or StGWD3 enzyme activity it is not possible to assume an increase in StGWD1 and StGWD3 transcripts leads to enhance starch membrane breakdown. Differences in expression between varieties could have been due to the allelic differences in genes between varieties and/or varying maturation levels of the potato tubers (Bansal and Das, 2013).

Expression of StGWD1 decreases with a lowering in the concentration of sucrose while StGWD3 expression increases with increasing reducing sugars accumulation. So changes in expression may be controlled by the pool of available sugars which are in constant flux, through changes in the rate of starch breakdown and sugar mobilisation. Results of starch-binding domain (SBD) homology modelling confirm the assumption of differences between the two potato dikinases in SBD-localised (Orzechowski *et al.*,

2013). The activity of GWD1 and GWD3 are interrelated (Mahlow *et al.*, 2014), with GWD3 (PWD) activity higher in GWD1 deficient lines, suggesting a compensatory response.

When looking at the StGWD3 expression and reducing sugar accumulation of L. Rosetta at the end of the storage there was a reduction in StGWD3 expression even though the accumulation of both sucrose and reducing sugars were the higher of the season. According to Orzechowski *et al.* (2013) StGWD3 is key in the storage starch decomposition but in sprouting tubers is less involved in the late reserve release.

The difference in the timing of breaking of dormancy between L. Rosetta and VR 808 will have a significant effect on changes in StGWD1 and StGWD3 expression as sucrose reserves will be metabolised to fuel sprout growth. The changes in expression levels of these two genes are governed by a number of physiological processes, and although such activities as sprouting lead to an increase in respiration, the rate at which both processes are occuring may be asynchronous leading to fluctuations in the pools of reducing sugars and sucrose. Hence, intermittent expression of StGWD1 and StGWD3 is most likely to occur during storage.

Gene expression was only assessed for a single season, and there is no way of determining whether this was representative of the normal conditions. The assumption is that GWD facilitated digestion of starch would be visualised as fractures through SEM. This is consistent with the findings of Mahlow *et al.* (2014) who compared starch from Arabidopsis leaves from GWD-deficient and wild type plants and observed that the main differences were at the surface of amyloplasts due to phosphorylation of glucans. SEM analysis was carried out over more than one season. This season was unusual in that visible fractures on the amyloplast surface were observed soon after harvest with VR 808 having the greater incidence of fractures in contrast to other seasons. This supports the observations made here with the observation that in this season VR 808 having a higher StGWD3 expression at the beginning and end of storage (Plate 3-12 and Plate 3-13).

The differences in the StGWD1 and StGWD3 gene expression between varieties could be one of the factors responsible for the differences in the sugar metabolism between

L. Rosetta and VR 808. Similar to what is observed with the sugar accumulation, during the storage at 10°C it is possible to see variations in the regulation of StGWD1 and StGWD3 in L. Rosetta, and however in VR 808 those are just visible in the beginning and at the end of the storage season.

Chapter 7. REACTIVE OXYGEN SPECIES (ROS) GENERATION AND TUBER PHYSIOLOGICAL CHANGES

7.1. Introduction

This experiment was to determine the biochemical response of potato tissues to oxidative stress, and thereby determine how senescent sweetening is related to stress responses, a series of trials were carried out on potato tissue using chemical treatments known to induce oxidative stress and to relieve oxidative stress respectively.

Methyl viologen dichloride hydrate (Paraquat) is known to induce oxidative stress in plants (Iannelli *et al.*, 1999). Paraquat accepts and donates electrons to oxygen, forming O_2^- (Bowler *et al.*, 1991), for that reason it was used in cores and slices of VR 808 potato tubers harvested in 2016 to induce stress and to generate ROS.

Subsequently a new plant health activator technology, commercially know as “Alethea” (Plant Impact Plc, Hertfordshire, UK) was used. “Alethea” has been shown to facilitate the recovery of induced abiotic stress (Wargent *et al.*, 2013) in tomato plants. The objective was to use “Alethea” to reduce the amount of ROS produced and to determine whether this led to a decrease in sugar accumulation. If successful in the future this kind of technology could be used in potato tubers to extend tuber storage.

This chapter reports experiments carried out first to optimise the concentrations of paraquat and “Alethea” for treatment of potato tissues, in terms of ROS response, followed by an assessment of the effect on sugar concentrations.

7.2. Material and Methods

For this experiment, VR 808 tubers harvested on the 4th October 2016 were used, courtesy of Sutton Bridge Crop Storage Research Station. Tubers were stored at 10°C and used 106 days (January 2017) after harvest.

This experiment was divided into two parts; firstly, to determine the appropriate concentration of paraquat and secondly to test the effect of using a new plant health activator technology, commercially know as “Alethea” (Plant Impact Plc, Hertfordshire, UK) in the recovery of induced abiotic stress due to paraquat (see Appendix XIV for Alethea formulation).

The method was adapted from Kraus *et al.* (1995), Seppänen *et al.* (2003) and Wargent *et al.* (2013), using 4 replicate samples each composed of middle and core slices from 5 tuber slices placed in a Petri dish and cores placed in plastic tubes and the various treatments added accordingly.

Paraquat was dissolved in 600 mM glycerol in 10 mMKH_2PO_4 buffer (to prevent desiccation of the slices/cores overnight) with 0.1% (v/v) of Tween 20, at 3 different concentrations; 1 µM, 2 µM and 5 µM. Glycerol buffer with Tween 20 was used as the control. Slices were then put in Petri dishes for ROS staining and cores in plastic flasks for sugar extraction and AsA and DHA determination. Both slices in Petri dishes and cores in plastic flasks were vacuum infiltrated for 15 minutes and then left to incubate overnight (18 hours) under light conditions. Samples for sugar extraction and AsA and DHA determination were washed with dH_2O prior to freezing at -80^oC (to reduce contamination with paraquat, samples were not snap frozen in liquid nitrogen before storage at -80^oC). Samples in the Petri dishes were washed with dH_2O prior to ROS staining which was performed and analysed as in sections 3.2.4.1 and 3.2.4.2.

The 2nd part of this experiment was carried out over three days, on the 1st day “Alethea” was dissolved in 600 mM glycerol in 10 mMKH_2PO_4 buffer (to prevent desiccation of the slices/cores overnight) at two concentrations; 10:1 (v/v) and 50:1 (v/v). 600 mM glycerol in 10 mM KH_2PO_4 buffer was used for the control. Similar to the paraquat part of the experiment slices were put in Petri dishes for ROS staining and cores in plastic

flasks for sugar extraction and AsA and DHA determination. Both were vacuum infiltrated for 15 minutes and then left to incubate for 24 hours. The next day samples were washed with 600 mM glycerol in 10 mMKH_2PO_4 buffer with 0.1% (v/v) of Tween 20. After this, half of the samples (slices and cores) were put in 5 µM paraquat solution and the other half in 600 mM glycerol in 10 mMKH_2PO_4 buffer with 0.1% (v/v) of Tween 20 (control). Samples were vacuum infiltrated for 15 minutes and left to incubate overnight (18 hours) under light conditions. On the 3rd day samples were washed with dH_2O and ROS staining (see sections 3.2.4.1 and 3.2.4.2.) of the tuber slices was carried out while the cores were frozen at -80^{o}C for subsequent sugar extraction and AsA and DHA determination.

Sugar extraction and AsA and DHA determination were performed and analysed as described in section 3.2.1 and 3.2.3, respectively. Determination of O_2^- and H_2O_2 content where performed as described in section 3.2.4.

7.3. Results

7.3.1. Optimizing methyl viologen dichloride hydrate (Paraquat) treatments

None of the paraquat treatments (0, 1, 2 or 5 µM) had a significant effect ($p > 0.05$) on O_2^- content as determined by purple colouration of staining. However, a significant ($p < 0.001$) change in H_2O_2 content as detected by brown colouration was observed.

For the H_2O_2 content, there was no significant difference between 0 µM and 1 µM of paraquat. However, 2 µM and 5 µM were significant different from 0 µM and 1 µM (Table 7-1).

There was no significant effect of treatment on sugar accumulation (fructose, glucose and sucrose accumulation) or on AsA and DHA accumulation ($p > 0.05$) (Table 7-1).

Due to these results, it was decided that the next part of this experiment would focus on 0 and 5 µM paraquat.

Table 7-1 – H_2O_2 content (DAB staining), O_2^- content (NBT staining), sucrose (Suc, %FW), reducing sugars (RS, %FW), AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) accumulation observed in potato slices following overnight treatment with different concentrations of paraquat.

	H_2O_2	O_2^-	Suc	RS	AsA	DHA
1 µM	119.9[a]	43.42[a]	0.016[a]	0.001[a]	0.34[a]	0.11[a]
2 µM	24.41[b]	88.12[a]	0.015[a]	0.001[a]	0.35[a]	0.02[a]
5 µM	-34.37[b]	78.01[a]	0.016[a]	0.001[a]	0.38[a]	0.13[a]
0 µM	153.7[a]	87.19[a]	0.015[a]	0.001[a]	0.36[a]	0.20[a]
HSD$_{0.05}$	*92.85*	*73.35*	*0.005*	0.001	*0.07*	*0.34*
P-value	*<0.001*	*0.32*	*0.34*	*0.90*	0.34[a]	0.11[a]

Each value is the mean of slices from four tubers. Means with the same letter are not significantly different according to Tukey HSD test. Content of O_2^- and H_2O_2 are expressed in terms of a numerical value to describe intensity of the purple colouration produced by NBT staining and brown colouration produced by DAB staining respectively.

7.3.2. Optimizing "Alethea" treatments for reducing induced oxidative stress and impact on reducing sugar, AsA and DHA accumulation

Sugar accumulation

For this experiment fructose concentration were so low that the HPLC was not able to detect them. Concentration of paraquat (0 and 5 µM) had no significant effect ($p > 0.05$) on sugar accumulation (total %FW of reducing sugars and sucrose accumulation). However, % of Alethea (from 0 to 10 %) had a significant effect on both %FW of reducing sugars ($p < 0.05$) and %FW of sucrose ($p < 0.001$). Even though concentration of paraquat alone did not affect the sugar accumulation, its relation with % of Alethea used had a significant effect on %FW of reducing sugars ($p < 0.05$) (Table 7-2).

Treatments with Alethea were responsible for the lowest sucrose accumulation and can be correlated with a reduce in the accumulation of reducing sugars when tubers were treated with paraquat (Table 7-2).

Table 7-2 – %FW of sucrose (Suc) and %FW of reducing sugars (RS) for paraquat and Alethea treatment.

	Suc	**RS**
5 µM paraquat + 0% Alethea	0.014[a]	0.0015[a]
5 µM paraquat + 5% Alethea	0.002[b]	0.0005[b]
5 µM paraquat + 10% Alethea	0.002[b]	0.0004[b]
0 µM paraquat + 0% Alethea	0.011[a]	0.0007[ab]
0 µM paraquat + 5% Alethea	0.003[b]	0.0008[ab]
0 µM paraquat + 10% Alethea	0.003[b]	0.0006[ab]
HSD$_{0.05}$	*0.005*	*0.0009*
p-value (paraquat)	*0.85*	*0.86*
p-value (Alethea)	*<0.001*	*0.04*
p-value (paraquat:Alethea)	*0.19*	*0.04*

Means with the same letter are not significantly different according to Tukey HSD test.

AsA accumulation

Concentration of paraquat (0 and 5 µM) had no significant effect ($p > 0.05$) but % of Alethea (from 0 to 10%) had a significant effect on AsA accumulation ($p < 0.001$). Similar to the %FW of reducing sugars, even though concentration of paraquat alone did not affect the AsA accumulation, its relation with % of Alethea used had a significant effect on AsA accumulation ($p < 0.05$). Treating with 5 or 10% of Alethea was significant different from the Alethea control (0% of Alethea) (Table 7-3).

Tuber cores treated with 0 µM of paraquat and 0% of Alethea had the highest AsA concentration and higher AsA/DHA ratio, followed by cores treated with 5 µM of

paraquat and 0% of Alethea. In the rest of the treatments AsA was not detected (Table 7-3).

Similar to AsA accumulation, % of Alethea was significant for DHA accumulation ($p < 0.001$) and concentration of paraquat had no significant effect ($p > 0.05$) on it. For DHA there was no significant effect of the relation of % of Alethea and concentration of paraquat ($p > 0.05$). Alethea control (0 % of Alethea) was the treatment with the higher DHA accumulation.

Both concentration of paraquat and Alethea ($p < 0.01$) as well as their interaction ($p < 0.05$) were significant for total AsA accumulation. Lowest concentration (0 µM) of paraquat was related to a greater accumulation of total AsA than the higher concentration. The treatment of 0 µM of paraquat and 0% of Alethea was the one with the highest total AsA accumulation, not significant different from the treatment of 0 µM of paraquat and 5% of Alethea.

Table 7-3 – Ascorbic acid (mg of AsA 100 g^{-1} FW) and dehydroascorbic acid (mg of DHA 100 g^{-1} FW) accumulation, H_2O_2 and O_2^- content measured in potato tuber tissues treated with different concentrations of paraquat and Alethea.

	AsA	**DHA**	**H_2O_2**	**O_2^-**
5 µM paraquat + 0% Alethea	0.16^{b}	0.06^{a}	43.3^{bc}	-148.2^{b}
5 µM paraquat + 5% Alethea	0^{c}	0.05^{a}	237.8^{a}	-145.9^{b}
5 µM paraquat + 10% Alethea	0^{c}	0.03^{a}	12.9^{c}	-126.9^{ab}
0 µM paraquat + 0% Alethea	0.19^{a}	0.04^{a}	34.2^{c}	-34.2^{ab}
0 µM paraquat + 5% Alethea	0^{c}	0.12^{a}	226.1^{ab}	67.0^{a}
0 µM paraquat + 10% Alethea	0^{c}	0.06^{a}	27.4^{c}	-113.0^{ab}
$HSD_{0.05}$	*0.03*	*0.11*	*1.75.36*	*237.56*
p-value	*<0.001*	*0.08*	*< 0.001*	*<0.05*

Means with the same letter are not significantly different according to Tukey HSD test. Content of O_2^- and H_2O_2 are expressed in terms of a numerical value to describe intensity of the purple colouration produced by NBT staining and brown colouration produced by DAB staining respectively.

ROS determination

Paraquat and Alethea concentration had significant effect on H_2O_2 content (brown colour) and on O_2^- content (purple colour) (Table 7-3). There was no significant difference between 0, 5 and 10% of Alethea for O_2^- content, however 5 % of Alethea had higher H_2O_2 content than 0 and 10% of Alethea (Table 7-3).

In treatments where paraquat was combined with Alethea application no statistical differences were observed in O_2^- content between treatments with and without application of Alethea. However, when applying 10% Alethea after an application of 5 µM of paraquat the H_2O_2 content was reduced compared to the rest of the treatments (Table 7-3).

Paraquat treatments were negatively correlated with O_2^- content and Alethea was positively correlated with H_2O_2 content and negatively correlated with AsA, DHA and sucrose accumulation (Figure 7-1).

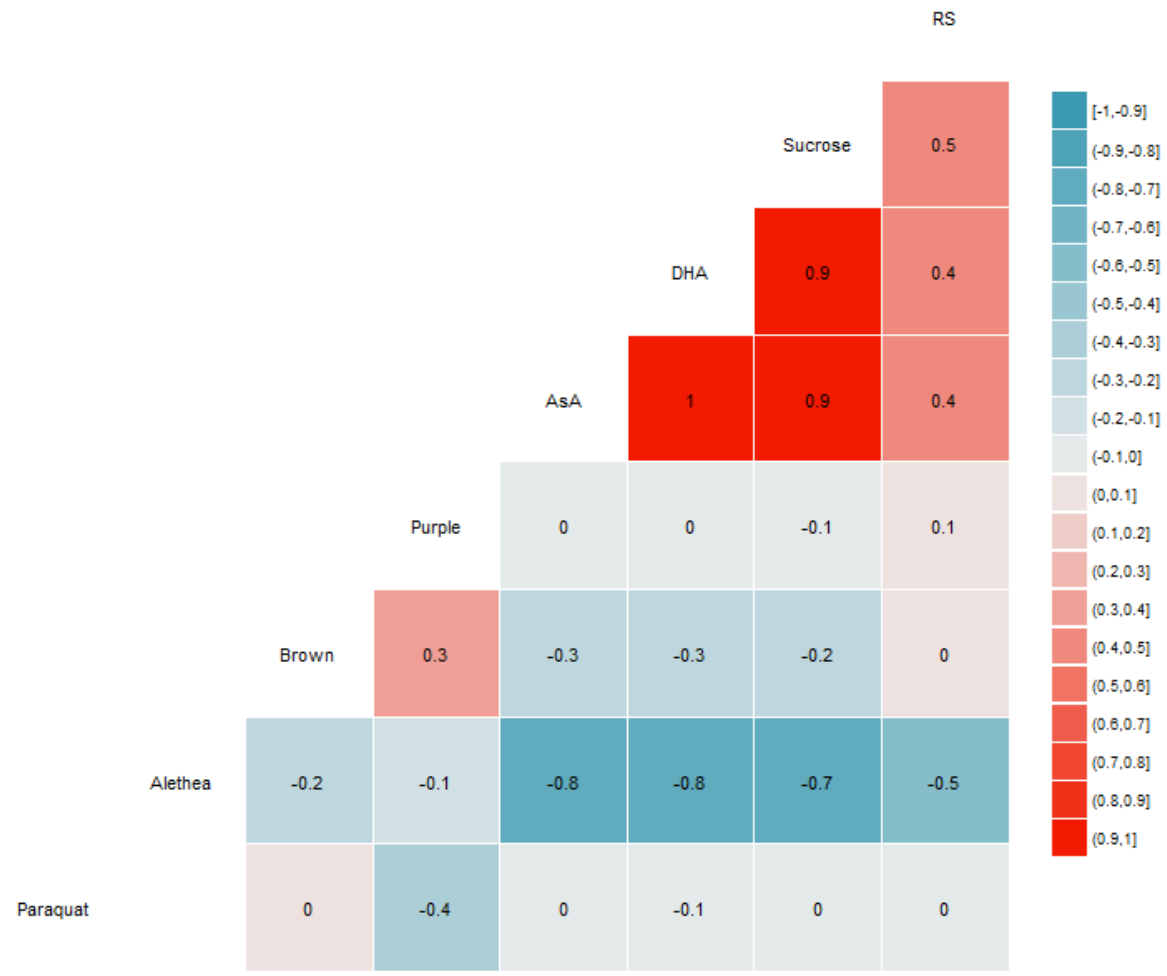

Figure 7-1 - Correlation between paraquat, Alethea, H_2O_2 content (Brown), O_2^- content (Purple), ascorbic acid content (AsA), dehydroascorbic acid content (DHA), %FW of sucrose (Sucrose), %FW of reducing sugars (RS) for VR 808 tubers harvested on October 2016, treated with 0 µM and 5 µM of paraquat, and 10:1 Alethea (100 mL L^{-1}), 50:1 Alethea (50 mL L^{-1}) and 0:1 Alethea (0 mL L^{-1}). Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$, $df = 18$).

7.3. Discussion

ROS and sugar signalling are kept at a delicate balance in plants (Deryabin *et al.*, 2007). In response to abiotic stresses plants tend to accumulate intracellularly low-molecular soluble sugars (sucrose, fructose, glucose, mannose and mannitol)

(Deryabin *et al.*, 2007). On the one hand higher sugar accumulation can be responsible for the oxidative burst in plants exposed to environmental stresses, but on the other hand sucrose might serve as a substrate or signal for stress-induced alterations (reviewed by Van den Ende and Valluru, 2009). This balance is observed in this experiment, the higher accumulation of glucose and sucrose was observed in the treatment with paraquat and no Alethea, and the lower levels of this sugars in the treatments with Alethea. Pre-treatments of Alethea were able to significantly reduce the glucose and sucrose accumulation in tuber cores treated with paraquat. Raffinose family oligasaccharides (RFO) sugars (water-soluble carbohydrates derived from sucrose) and galactinol can lead to an increase in oxidative stress tolerance when plants are treated with paraquat (reviewed by Van den Ende and Valluru, 2009).

Although a higher ratio of AsA/DHA was expected in Alethea treated samples due to facilitated recovery of induced abiotic stress (Wargent *et al.*, 2013), no AsA was detected in the samples treated with Alethea. When the Alethea treated samples were prepared for AsA analysis, it was noted that the samples were very difficult to push through the syringe filter. This was not observed for any other samples analysed within this research programme. This could be symptomatic of some physical change in the sample that could have interfered with the chemical analysis since Alethea in its composition contains iron (see Appendix XIV). Iron ions can react with O_2^- and H_2O_2 (Haber-Weiss reaction or the Fenton reaction) producing the highly toxic hydroxyl radical ($HO^\bullet$) (Mittler *et al.*, 2004; Gechev *et al.*, 2006). ROS scavenging enzymes together with AsA and GSH are used by plant cells to detoxifying O_2^- and H_2O_2, together with the sequestering of metal ions by ferritin and other metal-binding proteins preventing the $HO^\bullet$ formation (Mittler et al., 2004), which are probably causing the oxidation of AsA. Environmental conditions and exogenous formulations can change the oxidation/reduction status of plants (Li *et al.*, 2010). One of the key compounds of Alethea is sodium benzoate (SB), a carboxylic acid precursor of salicylic acid (SA) (Wargent *et al.*, 2013). SA is an important regulating and signal transducing subtract in response to environmental stresses. From a study with “Cara Cara” navel orange it was suggested that a SA pre-treatment was beneficial in maintaining antioxidant activity during storage (reviewed by Li *et al.*, 2010).

Alethea is a product with a very dark green coloration, so in treatments where Alethea was applied tuber slices acquired a greenish coloration, so this factor could have had an effect on the purple and brown values obtained with the ROS staining. The difference in O_2^- content between samples treated with paraquat alone and paraquat plus Alethea could have been because of the greenish colouration after Alethea application. Hence the purple colour is composed by 66.3% of green colour and brown colour just 12.9% (ColorHexa, 2012 - 2017), the bigger influence on O_2^- detection by staining technique.

Chapter 8. CONCLUSIONS AND FUTURE WORK

Although the aim of this research was to understand the mechanism(s) underpinning senescent sweetening and to develop predictive tools of senescent sweetening, it was necessary to confirm varietal differences in senescent sweetening. These differences were confirmed with analysis of sugars, sprout growth and respiration rates during storage. In addition, ROS content, AsA and DHA concentration, amyloplast integrity, tuber texture and tuber mineral concentration were used to help to establish differences in senescent sweetening onset between varieties. Parallel experiments were conducted to determine the influence of ROS content, starch phosphorylation by glucan, water dikinase activities, and the relationship between calcium status, antioxidant capacity and the degree of oxidative stress of tubers during storage.

Assessment of physiological changes of tubers during long-term storage

As expected duration of storage had the largest impact on sugar accumulation and respiration. Varietal differences were significant; senescent sweetening, respiration and sugar accumulation were higher in L. Rosetta and P. Dell than in VR 808 and R. Burbank. In this study, P. Dell accumulated the highest sugar concentrations and VR 808 the lowest. L. Rosetta and P. Dell exhibited senescent sweetening in March/April, VR 808 and R. Burbank showed no sweetening until after May. With time the concentration of reducing sugars increased in relation to the proportion of sucrose, suggesting a possible lack of utilisation of reducing sugars in respiration.

In conclusion, genetic effects were more significant than environmental effects on sugar accumulation. In this study the effect of growing location was variety dependent; hence the effect of location was not always significant nor consistent for each variety.

The relationship between antioxidant capacity, ROS and senescent sweetening.

A relationship between the onset of senescent sweetening and an increase in ROS was shown. Senescent sweetening resistant variety VR 808, exhibited a delayed rise in ROS accumulation. Varieties more resistant to senescence were able to preserve

their antioxidant capacity for longer, because with senescence there was a loss in antioxidant capacity leading to an increase of ROS.

During storage, variation in the concentrations of AsA and DHA were observed, AsA accumulation decreased with the length of storage. Varietal differences in AsA accumulation were observed. The variability observed was related to ROS accumulation during storage. DHA content was greater than that of AsA under conditions of oxidative stress (higher content of H_2O_2 and O_2^-), while at the end of storage AsA concentration was higher ; this is suggested to occur through regeneration of AsA.

There was an increase in O_2^- concentration in the medullar region which contrasted with an increase in H_2O_2 activity in the cortex, suggesting specific regions of the tuber are subjected to different forms of oxidative stress.

Loss of tuber turgor is related with changes in texture during storage.

Changes in texture were observed over time in all varieties. There was an increase in resistance to fracture as moisture loss and degradation of the cell walls occured during storage which led to increased sponginess of tuber cortex tissue.

Amyloplast integrity

The time scale for observing changes in amyloplast membrane integrity were variety and season dependent. SEM images obtain from preliminary studies with VR 808 from 2013/14 season and SEM images from VR 808, L. Rosetta, P. Dell and R. Burbank varieties from the 2014/15 season showed initial loss of amyloplast membrane integrity via appearance of fractures from 5-8 months in storage. This suggestsa relationship between loss of amyloplast integrity and senescence sweetening. However, for unknown reasons, in 2015/16 season fractures in the amyloplast surface were visible from the beginning of storage for VR 808 and L. Rosetta. For this reason this method of assessing tuber quality, should be used with caution.

There was a possible relationship between StGWD expression and sweetening.

Changes in StGWD1 and StGWD3 gene expression between varieties was either due to the genotype and/or varying maturation levels of tubers. It appears that StGWD1 expression decreases with a reduction in sucrose accumulation and StGWD3 expression increases with reducing sugar accumulation.

Calcium and other minerals.

The relationship between Ca and senescent sweetening is complex. However, the conclusion is that with further study the relationships between Ca and K, Ca and Mg and Ca and ratio of bound:total Ca could be used as a marker for the better onset of senescent sweetening. Increasing Ca content of tubers may delay senescent sweetening, and the observation was that the higher the concentration of Ca the lower accumulation of sucrose.

Uniformity of tissue sampling can influence mineral analysis results and increased weight loss (tissue water loss) over time will influence concentrations, moreover, redistribution of the minerals between middle (inner core – medulla or pith) and outer regions (sections related to the periderm, cortex, vascular ring and outer core) of the tuber have been reported to influence mineral concentrations in other crops such as apple.

Ca fertilization may retain tuber quality, although managing uptake into the tuber rather than the above plant parts is challenging, but precision application to a zone around the stolon/tuber root hairs may improve calcium accumulation in the tuber.

Ca fertilization appears to maintain tuber quality by maintaining AsA concentrations and reduction of AsA to DHA, and possibly limiting the activity of ROS activity. Ca appears to protect the amyloplats membranes, by delaying their degradation.

Ca regulation is an important component in dormancy release. This was exemplified by the addition of calmodulin blockers that enhanced the rate of dormancy break, while, EGTA (calcium chelator) and $LaCl_3$ (Ca channel blocker) delayed dormancy break. The bioavilability of Ca in the tuber bud, influenced the rate of dormancy release; application of Ca chelator or a plasma membrane Ca channel blocker delayed sprout

and shoot growth. If the Ca^{2+}-permeable ion channels are blocked, transport of Ca^{2+} to the cytosol can be reduced and thus extending dormancy.

Effects of chitting

Tubers from physiologically aged seed potatos (*chitted* seed) did not give show any important changes in sugars accumulation, respiration rate, sprout growth, or ethylene production compared to non chitted seed tubers.

However, for tubers from chitted seed there was a correlation between % FW of reducing sugars and storage length. Chitted seed showed an adverse effect on storage potential, because they accumulated less AsA and had higher production of O_2^-.

Developing a model to examine the role of stress in senescent sweetening.

Comparison of fluctuations and the abundance of ROS content were captured by tissue staining and provide a possible mechanisms for the observed decrease in AsA concentration during storage at 10°C. Further targeted analysis of ROS activity using fluorescent bioassay did not provide a more quantitative method for describing overall changes in ROS activity over time.

Generation of ROS in tuber tissue using paraquat and its reversal using a plant health activator comercially know as “Alethea” provided new insights into how ROS activity influenced starch breakdown. Pre-treatments of “Alethea” were able to significant reduce the glucose and sucrose accumulation in tuber cores treated with paraquat, most likely as the sugars were utilised for ROS scavenging.

Implications of this work

The main contributions of this work are a better understanding of the biochemical changes in potatoes during storage which influence tuber storage potential, and which could aid the development of biochemical markers to be used to improve store management and the scheduling of the potato crop to the processing industry. In the short term, optimising/decreasing storage costs and wastage is a key driver for the potato industry. In the longer term, a deeper understanding of the process(es)

underlying senescent sweetening will enable strategies to mitigate the problem and provide markers for variety/trait selective breeding programmes.

Future work

Investigating different ROS detection methodologies to acquire improved quantification, in different anatomical sections of tuber would be helpful. Such a method based on the enzymes activity such as that described by Meloni *et al.* (2003), or based on the fluorescence approach used by Hideg *et al.* (2002), or quantifying O_2^- and H_2O_2 production rates reported by Liu *et al.* (2017). Increasing replications of Ca fertilized tubers and use of tubers treated with Ca nano-particles could provide a greater in depth study of the role of calcium uptake in maintaining tuber quality. Opportunistically, some of the findings from this PhD are now being explored in a continuing AHDB funded project (Storage Fellowship - Sustaining expertise in potato post-harvest physiology). Further work to follow-up the StGWD1 and StGWD3 activity changes during storage will help to elucidate the role of these two genes on the breakdown of starch membranes. Moreover, there will be an opportunity to follow-up the Ca effect on dormancy break/sprout growth in further experiments.

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APPENDICES

Appendix I Storage date and CIPC application dates for all the varieties and all seasons

Variety	Season	Storage date	CIPC 1st application	CIPC 2nd application	CIPC 3rd application
VR 808 (Norfolk)	2013/14	10/10/13	21/10/13	21/01/14	17/04/14
VR 808 (Shropshire)	2013/14	23/09/13	02/10/13	21/01/14	17/04/14
VR 808 (Yorkshire)	2013/14	26/09/13	07/10/13	21/01/14	17/04/14
L. Rosetta (Norfolk)	2013/14	10/10/13	21/10/13	21/01/14	17/04/14
L. Rosetta (Shropshire)	2013/14	23/09/13	02/10/13	21/01/14	17/04/14
L. Rosetta (Yorkshire)	2013/14	26/09/13	07/10/13	21/01/14	17/04/14
R. Burbank	2013/14	11/10/13	21/10/13	21/01/14	17/04/14
P. Dell	2013/14	11/10/13	21/10/13	21/01/14	17/04/14
VR 808	2014/15	30/09/14	16/10/14	30/01/15	31/03/15
L. Rosetta	2014/15	30/09/14	16/10/14	30/01/15	31/03/15
R. Burbank	2014/15	29/10/14	06/11/14	30/01/15	31/03/15
P. Dell	2014/15	29/10/14	06/11/14	30/01/15	31/03/15
VR 808	2015/16	16/10/15	02/12/15	19/01/16	27/04/16
L. Rosetta	2015/16	16/10/15	02/12/15	19/01/16	27/04/16
P. Dell	2015/16	05/11/16	02/12/15	19/01/16	27/04/16

Appendix II Days of storage for each sampling time in the varieties VR 808 and L. Rosetta from the 3 sites for 2013/2014 (1st year)

Site	Sampling time	Days in storage
Norfolk	December	57
Norfolk	January	110
Norfolk	March	158
Norfolk	May	230
Norfolk	July	278
Shropshire	December	74
Shropshire	January	127
Shropshire	March	175
Shropshire	May	247
Shropshire	July	295
Yorkshire	December	77
Yorkshire	January	130
Yorkshire	March	178
Yorkshire	May	250
Yorkshire	July	298

Appendix III Days of storage for each sampling time in the varieties R. Burbank and P. Dell for 2013/2014 (1st year)

Variety	Sampling time	Days in storage
R. Burbank	December	56
R. Burbank	January	109
R. Burbank	May	229
R. Burbank	July	277
P. Dell	December	56
P. Dell	January	109
P. Dell	May	229
P. Dell	July	277

Appendix IV Days of storage for each sampling time for all the varieties for 2014/2015 (2nd year)

Sampling time	VR 808 and L. Rosetta	P. Dell and R. Burbank
November	44	15
January	121	92
March	170	141
April	212	183
May	232	203
June	273	244
July	296	267

Appendix V Days of storage for each sampling time for all the varieties for 2015/2016 (3rd year)

Sampling time	VR 808 and L. Rosetta	P. Dell
December	47	28
January	77	58
March	125	106
April	173	154
June	224	205

Appendix VI F value and p value for the 1st year (2013/14) and 2nd year (2014/15) by sampling month for VR 808, L. Rosetta, P. Dell and R. Burbank

	VR 808		L. Rosetta		P. Dell		R. Burbank	
	F value	**p value**	**F value**	**p value**	**F value**	**p value**	**F value**	**p value**
				Season 2013/14				
O2	12.925	0.000899 ***	8.24	0.00444 **	27.29	0.0356 *	0.7	0.634
CO2	120.226	8.36e-08 ***	182.061	1.34e-08 ***	24.44	0.0396 *	56.85	0.0173 *
Sprout	167.737	<2e-16 ***	54.718	< 2e-16 ***	30.62	2.14e-12 ***	28.06	1.06e-11 ***
Ethylene	23.99	8.09e-05 ***	2.067	0.168	1.008	0.533	1.257	0.472
RS	22.865	9.55e-09 ***	324.19	< 2e-16 ***	60.613	5.89e-09 ***	2.165	0.13202
Suc	73.484	4.62e-15 ***	24.4	4.63e-09 ***	114.504	5.04e-11 ***	14.425	8.2e-05 ***
Fruc	48.062	1.26e-12 ***	260.723	< 2e-16 ***	77.979	9.14e-10 ***	3.292	0.04776 *
Glu	8.496	0.000104 ***	218.236	< 2e-16 ***	46.444	4.03e-08 ***	1.27	0.318338
				Season 2014/15				
O2	2.325	0.147	13.66	0.00149 **	10.07	0.00145 **	9.675	0.000515 ***
CO2	10.15	0.00369 **	148.4	5.12e-07 ***	23.83	4.84e-05 ***	20.82	1.09e-05 ***
Sprout	75.75	<2e-16 ***	2.802	0.0124 *	47.71	<2e-16 ***	31.82	<2e-16 ***
Ethylene	0.88	0.554	1.795	0.231	4.063	0.0299 *	2.215	0.114
RS	4.188	0.001578 **	81.876	< 2e-16 ***	111.74	< 2e-16 ***	11.557	3.14e-08 ***
Suc	49.404	< 2e-16 ***	135.150	< 2e-16 ***	97.156	< 2e-16 ***	71.137	< 2e-16 ***
Fruc	2.449	0.036258 *	54.414	< 2e-16 ***	53.92	< 2e-16 ***	2.540	0.0310 *
Glu	3.485	0.00552 **	99.633	<2e-16 ***	163.96	< 2e-16 ***	42.692	< 2e-16 ***

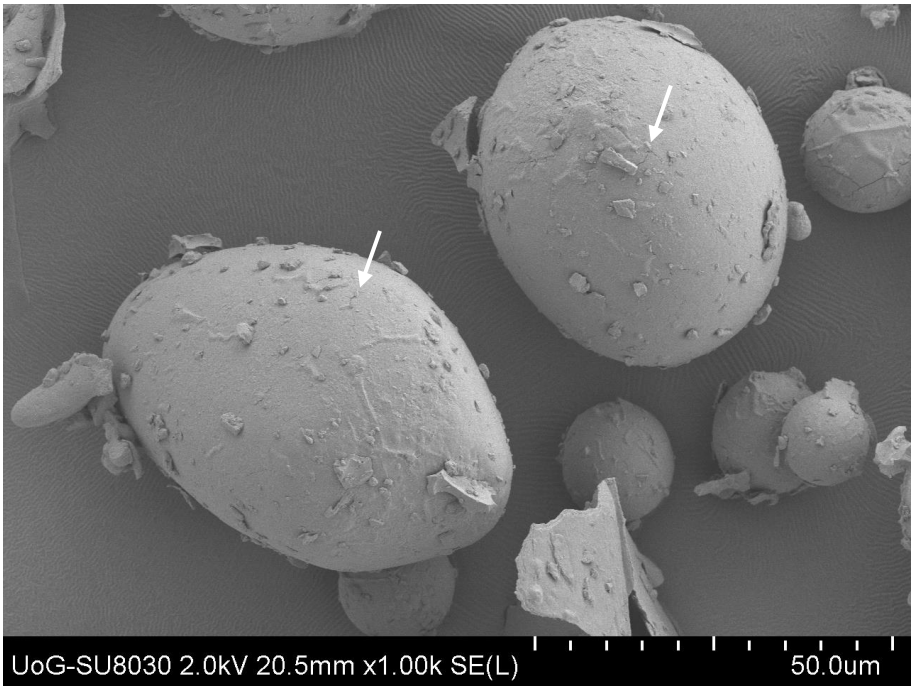
VR 808 and L. Rosetta: 4 df (2013/15) and 6 df (2014/15), P. Dell and R. Burbank: 3 df (2013/14) 6 df (2014/15)

Appendix VII F value and p value for the 3rd year (2015/16) by sampling month for VR 808 and L. Rosetta

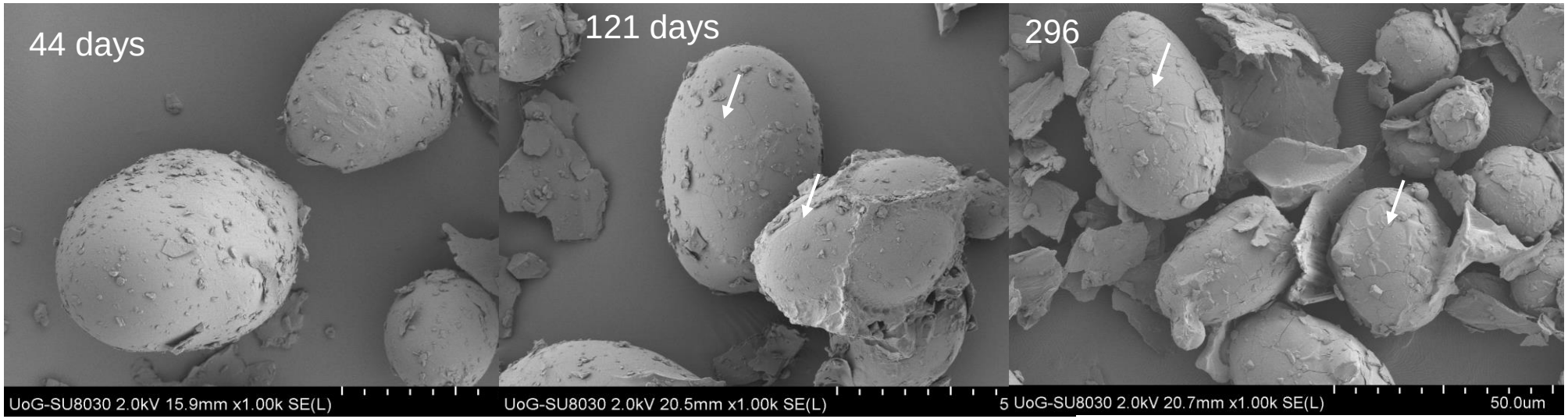
	VR 808		L. Rosetta	
	F value	**p value**	**F value**	**p value**
O2	1.453	0.341	21.91	0.00228 **
CO2	10.04	0.0132 *	38.07	0.000616 ***
Sprout	8.412	3.94e-06 ***	12.43	1.02e-08 ***
Ethylene	2.872	0.139	4.475	0.0658
RS	9.082	2.57e-05 ***	124.580	< 2e-16 ***
Suc	18.969	8.14e-09 ***	17.391	2.48e-10 ***
Fruc	7.449	0.000139 ***	131.568	< 2e-16 ***
Glu	6.033	0.000677 ***	47.714	< 2e-16 ***

VR 808 and L. Rosetta: 7 df

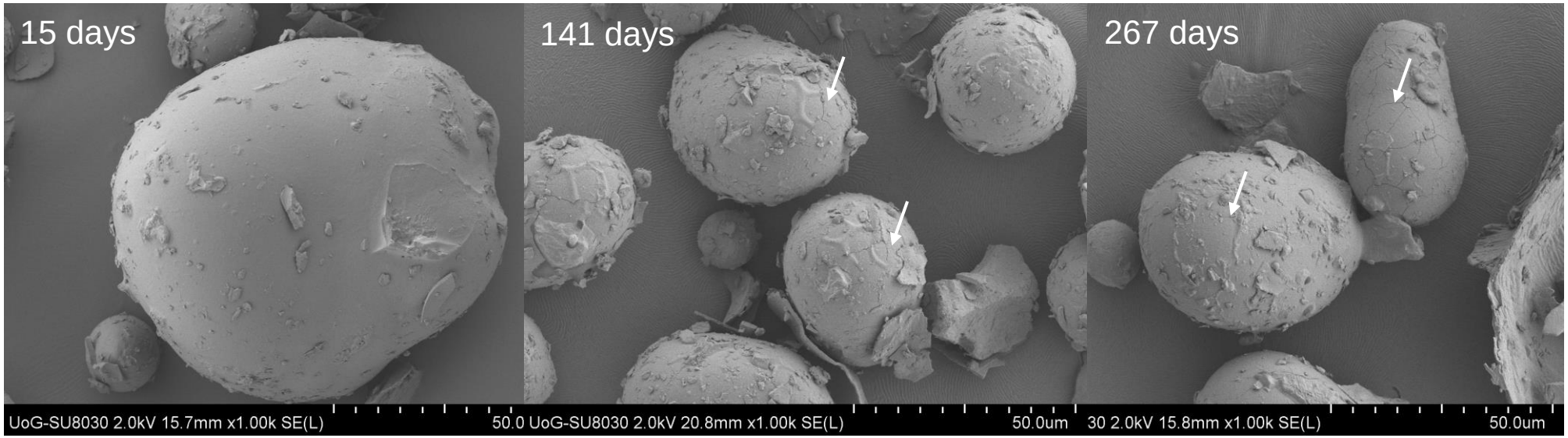
Appendix VIII **SEM image from middle section of VR 808 with 170 days of storage with 1st visible fractures on the surface of the amyloplast (March 2015). White arrows point to fractures.**



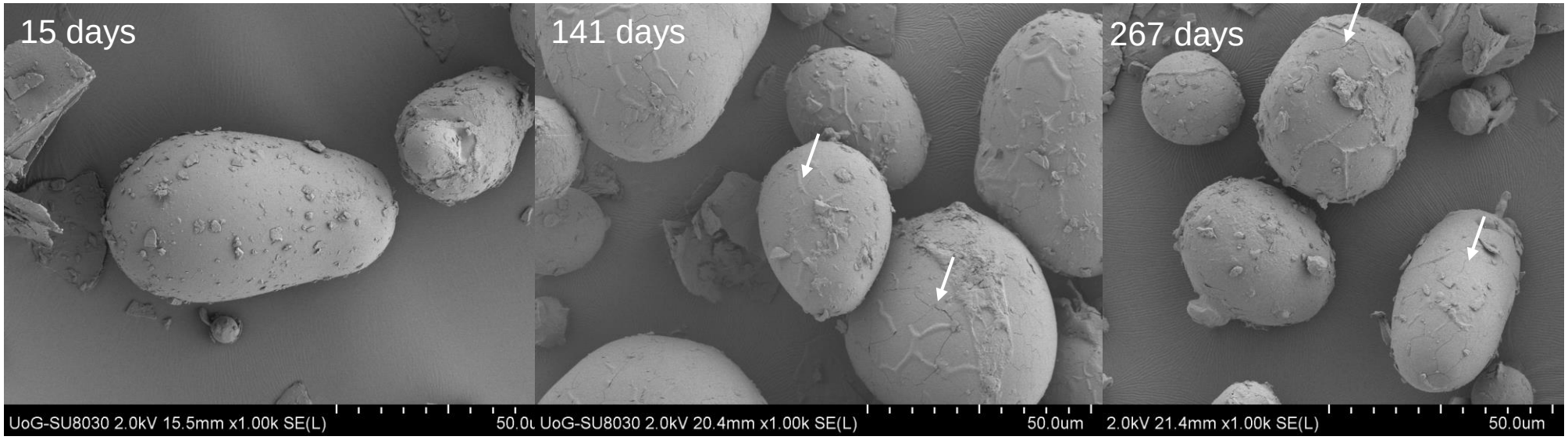
Appendix IX SEM image from the ends section of L. Rosetta with 44 days (November 2014), 121 days (January 2015) and 296 days (July 2015) of storage. White arrows point to fractures.



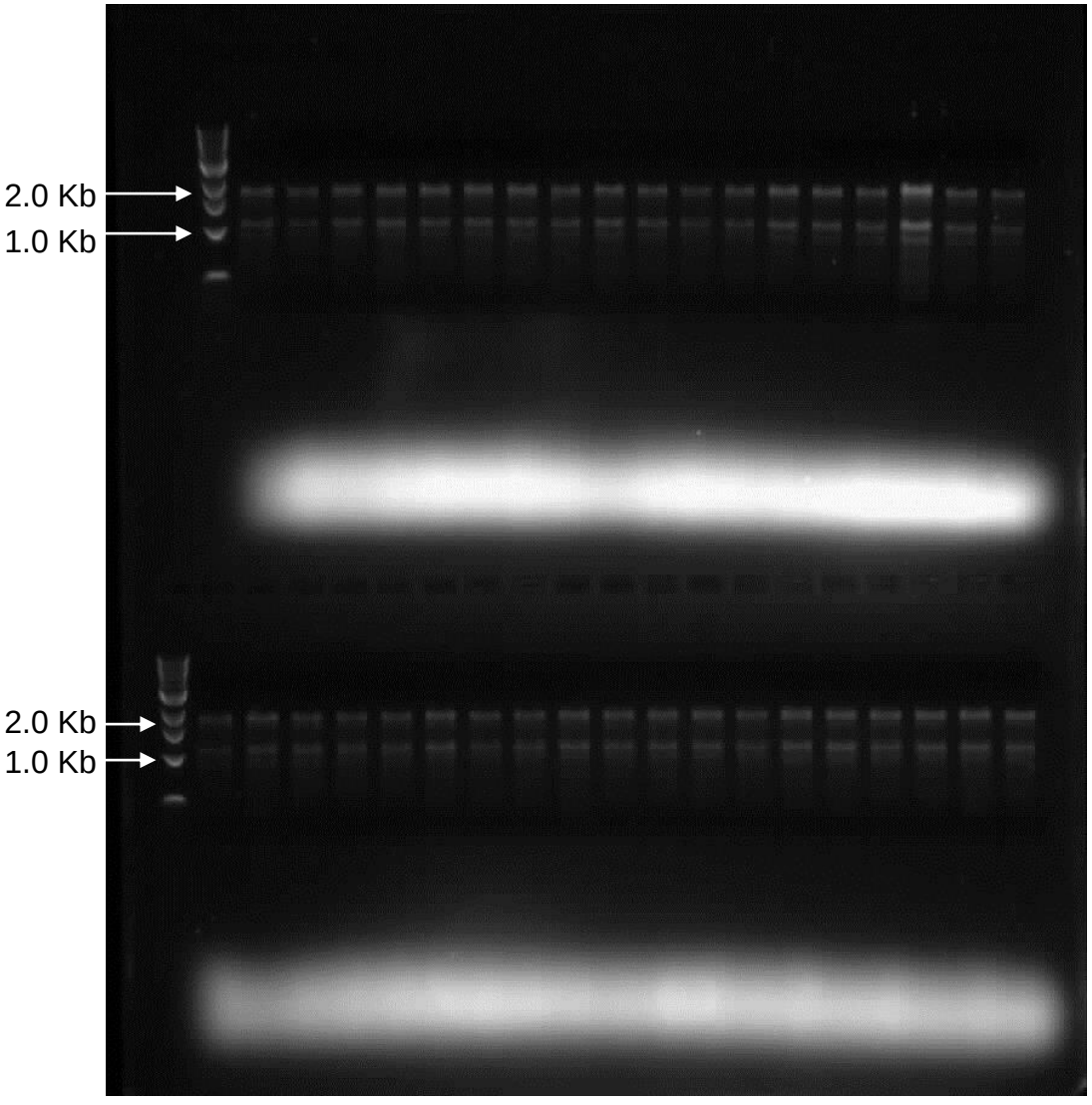
Appendix X SEM image from the ends section of P. Dell with 15 days (November 2014), 141 days (March 2015) and 267 days (July 2015) of storage. White arrows point to fractures.



Appendix XI SEM image from the ends section of R. Burbank with 15 days (November 2014), 141 days (March 2015) and 267 days (July 2015) of storage. White arrows point to fractures.



Appendix XII **RNA integrity in 1% agarose gel electrophoresis: 1st row: 1st well 1Kb ladder (NEB), 2nd to 6th well VR 808 from December 2015, 7th to 11th well VR 808 from June 2016, 12th to 16th well L. Rosetta from December 2015, 17th to 18th L. Rosetta from June 2016; 2nd row: 1st well 1Kb ladder (NEB), 2nd to 3rd well L. Rosetta from June 2016, 4th to 8th well VR 808 from January 2016, 9th to 13th VR 808 from March 2016, 14th to 18th L. Rosetta from January 2016, 19th to 20th L. Rosetta from March 2016**



Appendix XIII **RNA integrity in 1% agarose gel electrophoresis: a) 1st well 1Kb ladder (NEB), 2nd to 4th well L. Rosetta from March 2016; b) 1st to 4th well VR 808 from April 2016, 5th to 8th well L. Rosetta from April 2016, 9th well 1Kb ladder (NEB).**