Final Report

Understanding mechanisms and identifying markers for the onset of senescent sweetening

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1. SUMMARY

1.1. Aim

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 accumulation of carbohydrate content with an increase in respiration, and changes in tissue integrity (cell wall/ amyloplast), starch phosphorylation by glucan, water dikinase activities (GWDs), levels and distribution of ROS.

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.

6) Specifically, to understand the relationship between the bioavailability of calcium and ascorbate activity during changes in tuber maturity. 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.

1.2. Methodology

The research was centred around the study of Lady Rosetta, a variety 'susceptible' to sweetening, and VR 808, considered to maintain 'stable' sugar profiles, both of them are 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), used for chip production (NIAB Pocket Guide, 2008, NIAB, Cambridge in Colgan *et al.*, 2012), were included in the trial.

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

This project 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 to maintain 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 a programmed cell death (Daudi and O'Brian, 2012) response in the cells, 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 break 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.

1.2.1. Assessment of physiological changes of tubers during long-term storage

During the 1st year of the study, in order to determine potential differences between planting sites, Lady Rosetta and VR 808 tubers were planted from common seed tubers at 3 locations (Norwich, Shropshire and Yorkshire).

After finding that planting location had no significant effect on respiration and sugars, Lady Rosetta and VR 808 tubers were planted from common seed tubers in a single site close to Norwich (Norfolk) during the 2nd year and at a site in Yorkshire during the 3rd year of the project.

Pentland Dell and Russet Burbank were included in the trial in years 1 and 2, as additional material that provided contrasting profiles of sugar accumulation during storage.

During the 3rd year Pentland Dell tubers that had previously been chitted by McCain (250ºC days) and non-chitted seed were included in the trials to find the influence in aged seed and physiological maturity of harvest crop on Senescent Sweetening. The chitting treatment consist of holding seed tubers for a period of time at cool temperatures in the light so that at the plantation time seed had, in this case, 250°C days of chitting (McKeown, 1994).

All varieties were grown and harvested under commercial practice. Tubers were transported to Sutton Bridge Crop Research Station (SBCSR) where curing and CIPC treatments were applied. Tubers were stored at 10°C (SBCSR) and sampled every 6- 8 weeks for tuber quality (Natural Resources Institute, University of Greenwich).

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.

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

Reactive oxygen species (ROS) high reactivity and relative instability make them very difficult to detect or even measure, thus detection of ROS are made by indirect and semi quantitative methods, by measuring the end products that are formed when they react with certain substances (Thannickal and Fanburg, 2000, Jambunathan, 2010).

Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) activity during storage are forms of ROS that were study on the stored tubers during the 2nd and 3rd year of this study. O_2^- is detected by the formation of a purple/blue precipitation in a final endpoint reaction when nitroblue tetrazolium (NBT) is applied, and where Diaminobenzidine tetrahydrochloride (DAB) is incorporated as a substrate it reacts with H_2O_2 forming a brown polymerization product (Jambunathan, 2010).

1.2.1.2. Assessment of amyloplast and cellular changings during storage using scanning electron microscope (SEM)

In order to determine changes in amyloplast structure scanning electronic microscope (SEM) techniques were used. During the 1st year of this study 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 a series of ethanol (10-100%) solutions, 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 was observed in all the four methods used for sample preparation, however, samples obtain by CPD, HMDS and dehydration by freeze-drying were more porous turning the sample sometimes instable under the beam. For that reason,

powdered potato obtained by freeze drying the tissue was the method chosen to further analyse the amyloplast changes during the storage during the 2nd and 3rd year of this study. Moreover, freeze drying afforded the benefit of increasing the number of available amyloplasts that could be assessed than in the other 3 methods since the amyloplasts are all outside the potato cells. To ensure fractures of amyloplast membranes were not artefacts of the freeze drying dehydration process used (powder freeze dry tissue), additional samples sampled at the beginning, middle and end of the 2nd year storage season samples were subject to dehydration by CPD.

1.2.1.3. Determination of mineral accumulation on tubers

Minerals are important dietary components, and 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). For that reason mineral content was determined during the 2nd year of this work.

1.2.1.4. Investigation of the effect of calcium in the storage potential of potato tubers

Calcium ions (Ca^{2+}) are essential in growth and development in plants (Hepler, 2005). Ca^{2+} is vital for cell wall strengthening and cell-cell adhesion (Bush *et al.*, 2001; Marry *et al.*, 2006), for that reason is often regarded as an anti-senescence factor (Kumar and Knowles, 1993a). When in low concentrations in the membranes can leads to membrane leakage resulting in loss of cellular salts and organic compounds, and when not reversed can lead 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 can be beneficial such in reducing incidents of internal russet spot, sprout subapical necrosis and soft rot during storage (Kratzke and Palta, 1986). In order to test the effect of calcium in the storage potential of potato tubers and its influence on senescent sweetening of stored tubers, a series of experiments where conducted where calcium was applied to potato plants during the growing season

1.2.1.5. Investigation of the effect of calcium in dormancy and sprouting

Dormancy break is quickly followed by sprout elongation (Suttle, 1996). Dyson and Digby (1975) found that sprouting was related to the calcium concentration in the tuber, when more calcium was present the sprout elongation was greater. The start of sprouting was used as a way to visualize the end of dormancy in potato tubers in a series of experiments using an excised bud biossays system using a number of calcium and calcium chealtors/inhibitors (Lanthanum (III) chloride heptahydrate ($LaCl_3$) and the calcium chelator Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)) were tested on dormancy break and sprout vigour.

1.2.1.6. Variety comparison in terms of gene expression using real time quantitative reverse transcription polymerase chain reaction (RT qPCR)

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 granule surface polymers organization, facilitating access to degrading enzymes to bind (Smith, 2012). Glucan, water dikinase (GWD1), 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).

GWD3 (PWD) acts downstream of GWD1, since it phosphorylates the substracts previously phosphorylated by GWD1 (Hejazi *et al.*, 2009; Kotting *et al.*, 2005). 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).

Using real-time qPCR (RT qPCR) changes in StGWD1 and StGWD3 transcrip levels in tuber of VR 808 and L. Rosetta were studied.

1.2.1.7. Reactive Oxygen Species (ROS) generation and tuber physiological changes

Methyl viologen dichloride hydrate (Paraquat) is known to be responsible for oxidative stress in plants (Iannelli *et al.*, 1999), and was used to artificially induce ROS activity in cores and slices of VR 808 tubers. The *in situ* detection of hydrogen peroxide was made by staining with 3,3'-diaminobenzidine (DAB) using an adaptation of the method of Daudi and O'Brian (2012). And superoxide was detected by staining with Nitroblue tetrazolium chloride (NBT) using an adaptation from the method of Jambunathan (2010).

Three concentrations of paraquat (1 µM, 2 µM and 5 µM) were applied to tissues to optimise ROS production detected by staining. The highest dose tested (5 µM) induced the most ROS. To investigate whether ROS can be artifically reduced by the appliction of ‘anti-senescence’ products tubers previously treated with pararquat were subsequently treated a comercial preparartion Alethea (Plant Impact , UK) (Wargent *et al.*, 2013).

1.3. Key findings

- Varietal differences in senescent sweetening observed previously were confirmed.
- An increase in respiration is associated with a rise in senescent sweetening.
- Increased antioxidant capacity, reduces ROS.
- Studies on tissue stress during storage found differential expression of ROS activity within different cell types within tissues.
- Loss of tuber turgor increases resistance to cell fracture and slicing during storage.
- The time taken for physical changes in amyloplast membrane integrity observed through SEM analysis were variety and season dependent.
- There was a putative relationship between StGWD expression and sweetening.
- There was seasonal variation in senescent sweetening symptoms
- The relationship between Ca and senescent sweetening are not fully understood. Further study is required to investigate the relationships between [Ca] and [K], and the ratio of [Ca]: [Mg+K]. Moreover, the bioavailability of free calcium needs to be more clearly defined using better analytical techniques as the ratio $[Ca^{2+}]_{bound}/[Ca^{2+}]_{total}$ may be used as a marker for the onset of senescent sweetening. Increasing Ca content of tubers may delay senescent sweetening. As well it could be possible that the more Ca the less accumulation of sucrose.
- Ca^{2+} is necessary/speeds up dormancy break. The bioavailability of Ca in the tuber bud, influences 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 is reduced and thus extending dormancy.
- Tubers from physiologically aged potato seed tubers (chitted seed) did not impact on changes in sugars accumulation, respiration rate, sprout growth or ethylene production. Chitted seed could have an adverse effect in the storage potential of the stored crop, once tubers from chitted seed accumulated less AsA and had higher O_2^- levels.
- During this study comparison of fluctuations and the abundance of ROS activity captured by staining may help to tie in possible mechanisms for decrease in AsA content during 10°C storage.
- Generation of ROS in tuber tissue using paraquat and to reverse its effects using a plant health activator comercially know as “Alethea” provided insights into how ROS activity influenced starch break down.

1.4. Practical recommendations

It is essential that when storing potato tubers the concentrations of CO_2 and O_2 in storage should be tracked. Storage potential of the tubers start in the field, so besides ensuring tubers are harvested at optimum physiological maturity, it is important as well to pay attention to the mineral nutrition status of the tubers. Ca, K and Mg appear to have a great importance in the onset of senescent

sweetening. Managing Ca 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 and tuber quality.

Quantifying changes in amyloplast integrity and ROS activity will go some way towards developing diagnostic markers for predicting changes in tuber health and in the longer term provide targets for genetic marker development, aiding marker assisted breeding programmes.

2. INTRODUCTION

Aging and senescence even though distinctly different, 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 less controlled process. (Nooden, 1988). A recent review by Penfold and Buchanan-Wollaston (2014) refers to studies in senescing leaves which denotes 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 though ageing and senescence are distinguishable they share similarities at the biochemical level (Kumar and Knowles, 1993b). Kumar *et al.* (1999) identified protein modifications related to tuber aging and suggested that aging is accompanied by increased respiration rate, oxidative stress, lipid peroxidation and decreased protein content in the tubers. 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 efficient 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 is an excellent 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).

Calcium increases membrane integrity and for that reason is often regarded as an anti-senescence factor (Kumar and Knowles, 1993a). However for Dyson and Digby (1975) the effect of calcium on sprout growth in potatoes was clear and that physiological age is a function of calcium metabolism,

where a progressive decline in calcium mobility occurs. Sprouting is partly-related to the calcium concentration on tuber, when more calcium is present the sprout elongation is greater, however, in order for the sprout to continue to grow it is necessary to supply the growing sprout with additional calcium (Dekock *et al.*, 1975; Dyson and Digby, 1975).

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 could lead to the increase of super oxide radicles (O_2^-) production. According to the oxidative damage theory of aging, the oxidative and free radical stresses, such as O_2^- production are cumulative over time. These products can be neutralized in a decreasing manner during aging by intracellular compartmentalisation, protective enzymes, such as superoxide dismutase (SOD) and catalase (CAT), and naturally occurring antioxidants (vitamin E and C). According to this, aging is consider to be mainly caused by exposure to Reactive Oxygen Species (ROS) (Coleman, 2000). In his review on physiological aging in potatoes Coleman (2000) refers to the work on specific enzymes that have been examined for evidence in correlations with physiological aging of potato tubers, such as decrease in sprouting capacity was correlated with an increase in peroxidase activity. An example is the work of Hartmans and Van Es from 1984 where they examined starch and sugar content of sprouts as tubers aged and found that carbohydrate translocation was not the limiting factor in sprout growth, and the work of Mikitzel and Knowles from 1989 where it is suggested that efficiency of carbohydrate utilization was affected by tuber aging but not the carbohydrate translocation (Coleman, 2000).

Alterations in the levels of soluble sugars have been shown to affect developmental phase changes in plants, from embryogenesis to senescence (Gibson, 2005). The process of sugars 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 variety, the higher the storage temperature, the sooner senescent sweetening commences and the faster it develops (Hertog *et al.*, 1997; Burton and Wilson, 1978). One theory is 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). As tubers age, gradual peroxidation of amyloplast membrane lipids leads to age-induced loss in amyloplast membrane integrity (Kumar and Knowles, 1993b). A senescent amyloplast membrane will affect the compartmentalization provided by the organelle affecting the transport of different effectors (such as Pi, G6P) and intermediaries of starch metabolism (O'Donoghue *et al.*, 1995).

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.

3. MATERIALS AND METHODS

For the 1st year (2013/14) of this study, differences between planting sites were determined by, sampling tubers of VR 808 and L. Rosetta from a PepsiCo trial 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 at the end of January and mid of April 2014 (8.1 Appendix I), mimicking commercial practice to prevent sprout growth. Tubers were stored at 10°C (SBCSR) and sampled as in the 8.2 Appendix II and 8.3 Appendix III for tuber quality at Natural Resources Institute (NRI), 1st sampling point was in December 2013 and last sampling point in July 2014.

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 (8.1 Appendix I). Tubers were stored at 10°C (SBCSR) and sampled as in the 8.4 Appendix IV for tuber quality at NRI, 1st sampling time in November 2014 and last sampling time in July 2015. Pentland Dell and Russet 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) were used. After harvest, tubers were sent to SBCR where they were cured and CIPC treatments applied (8.1 Appendix I). Tubers were stored at 10 °C (SBCSR) and sampled as in the 8.5 Appendix V for tuber quality at NRI, 1st sampling time in December 2015 and last sampling time in June 2016.

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) 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 stolen end (opposite eigths - 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 were just used samples taken from opposite eighths of potato using a cork borer (size Nº 5). Each replicate consisted of a composite of 5 tubers from apical and stolen end (opposite eigths) were combined to provide an average of the extremes in AsA, DHA and total vitamin C profiles across the tuber, with five 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 KEdwards High Vacuum Internationa) before grinding to a fine powder in a pestle and mortar.

3.1. 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) 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. Assessment of tuber respiration and ethylene production 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 10ºC. 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).

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 250ºC and a 1 m long, 6 mm OD glass column packed with 100/120 mesh alumina maintained at 130ºC. The production rate of ethylene was expressed as nL $g^{-1}h^{-1}$ and carbon dioxide ml CO_2 $g^{-1}h^{-1}$.

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).

3.3. Assessment of tuber texture during storage

Texture were measure just in the 1st year. Three tubers per variety were selected for texture analysis using the wedge fracture test developed by Vincent *et al.* (1991) or measuring the food texture, that is relatively simple, and that reproduces accurately 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 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 edge, a further perpendicular 10 mm section was cut creating a $1cm^3$ of tissue, to ensure the orientation of tissue is kept constant during analysis cubes of tissue were marked by removing a slither from the corner of the cube representing the outer edge 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.

Wedge fracture tests were carried out using a Lloyd Instruments model LRX-plus with a 50N load cell and running with Rcontrol software v3.23. A wedge (30º included angle) was driven at 33.3×10^{-3} mm sec^{-1} (2 mm min^{-1}) into 10 mm cubes of tuber tissue orientated radially. Wedge movement was halted when a crack could be seen ahead of the wedge tip (after reaching the peak load) and 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. In addition, work of fracture was calculated as (area under force-distance curve) / (total crack length * sample width).

The load-distance curve 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.

3.4. Assessment of ascorbic acid accumulation during storage

Ascorbic acid accumulation during storage was monitored during the 2nd and 3rd years of the study. Ascorbic acid (AsA) and dihydroascorbic acid (DHA) were extracted and analysed using an adaptation to the method used in 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 4000 rpm for 40 minutes at 4^{o}C. 1.5 mL of the supernatant was then collected in *Eppendorf* tubes and centrifuged at 10000 rpm 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% 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 10000 rpm 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 250mm x 4.6mm x 5µm 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.

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

ROS stimulates cell membrane damage during aging and activates a programmed cell death (Daudi and O'Brian, 2012) response in the cells, 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^- is detected by the formation of a purple/blue precipitation when nitroblue tetrazolium (NBT) reacts with it, and when Diaminobenzidine tetrahydrochloride (DAB) is used as substrate it reacts with H_2O_2 forming a brown polymerization (Jambunathan, 2010).

3.5.1. **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 haem peroxidases generating a dark brown precipitate (Daudi and O'Brian, 2012).

In 50 mL flask, 50 mg DAB was diluted with 45 mL of sterile H_2O to a final concentration of 1 mg/mL DAB the pH was reduced to 3.0 by addition of 1 M of HCl under constant stirring, to prevent light degredation 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 and two of (no DAB solution was applied). Tuber slices (3-4 mm in thickness) were prepared with a madoline 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, blue and green (RBG) pixels was quantified by the method from Skipper (2010), using scripts developed in R were used.

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

To evaluate the stain due to DAB and NBT staining techniques the red, blue and green values (RGB) needed to be transformed into just one value. Using the formulas for RGB percent 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, blue and green 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 obtained, 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. In that way the part of the treated slice that was without stain was excluded leaving just the value of the stain.

3.5.2. **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 from the method of Jambunathan (2010). Four tubers per variety

was 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 azime (NaN_3) and 50mM of potassium phosphate (KH_2PO_4) was prepared.

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 capture images from the staining it was used the same method described in the previews section.

3.6. Assessment of amyloplast and cellular changings during storage using scanning electron microscope (SEM)

In order to determine changes in amyloplast structure scanning electronic microscope (SEM) techniques were used. During the 1st year of this study 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 a series of ethanol (10-100%) solutions, 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 was observed in all the four methods used for sample preparation, however, samples obtain by CPD, HMDS and dehydration by freeze-drying were more porous turning the sample sometimes instable under the beam. For that reason, powder obtained by freeze drying the tissue was the method choose to further analyse the amyloplast changes during the storage during the 2nd and 3rd year of this study. This method is as well easier to analyse more amyloplasts than in the other 3 methods since the amyloplasts are all outside the potato cells.

To be sure that what was observed were not due to the dehydration process used (powder freeze dry tissue), at the beginning, middle and end of the 2nd year storage season samples were dehydrated by CPD as well.

3.6.1. 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 place immediately in 2 mL of sodium phosphate (25 mM, pH=7). Tissue fixation was performed using an adaptation of the glutaraldehyde fixation describe in 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 h) until the tissue sank.

Following infiltration, solution was removed and 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.

3.6.2. **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). In Table 1 is a summary of the steps performed.

Table 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.	

3.6.3. **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.

3.6.4. **Dehydration by freeze drying**

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

3.6.5. **Freeze drying of tissue samples**

Samples of tuber cortex were taken from opposite eighths of potatoes using a cork borer and from the middle section of the tuber (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 amyloplast profiles across the tuber and an additional sample was taken from mid section of the tuber.

Samples were frozen immediately at -80°C before subject to 48 hours freeze drying as above.

3.7. Determination of mineral accumulation in tubers

Minerals are an 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.

3.7.1. **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 extemes 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 as maximum pressure 400 psi, power 1200 W for 20 minutes and maximum temperature 190°C were selected for digesting dissolving and hydrolysing organic materials and preparing samples for analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES). The digested samples were diluted to 50 mL with deionised water before preceding 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 50 ppb, 250 ppb, 500 ppb, 750 ppb, 1000 ppb, 1250 ppb and 1500 ppb (for Ca, Mg, Fe, Zn, Cu and P), and dilutions from 1500 ppb, 2500 ppb, 3500 ppb, 4500 ppb, 5500 ppb, 6500 ppb, 7500 ppb and 8500 ppb (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 dilutions. The final mineral content was calculated as mg/100g dry weight (DW).

3.7.2. **Calcium oxalate extraction**

For cold oxalate extraction it was used the method from Al-Wahsh *et al.* (2012) modified by Mirzaee (2015) for measuring total oxalate of potato tuber samples with their total Ca^{2+} 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 extemes 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 4200 rpm for 10 minutes. The supernatant was transferred to 25 mL volumetric flasks. This process repeated two more times by adding 5 mL of 2N HCl to the remaining pellets 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 Atomic Absorption Spectrometer (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 ppm to a final concentration range 1, 2, 3, 4, 5, 6, 8 and 10 ppm.

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 value 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 this expressed as a percentage of total calcium in each sample according to the following Equation 1:

Equation 1

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

3.8. Investigation of the effect of calcium in the storage potential of potato tubers

In order to test the influence of calcium fertilisation in Senescent Sweetening of stored tubers, and taking advantage of the starter of the AHDB storage fellowship (Storage Fellowship - Sustaining expertise in potato post-harvest physiology; 1100004 Storage Fellowship) in the 2015/16 season, during the 3rd year of this study it was used Pentland Dell tubers from the AHDB storage fellowship from plots fertilised with calcium (Tropicote, 380 Kg/ha) and without calcium fertilisation. Tropicote is a calcium nitrate product for field application from YaraLiva composed by 15.5% N and 19% Ca (http://www.yara.co.uk/crop-nutrition/fertiliser/calcium-nitrate/0146-yaraliva-tropicote/, 04-05-2017).

For this study it was used for the replications 1 and 3 for the Tropicote and untreated treatments from the AHDB storage fellowship for P. Dell variety, hand lifted on the 21st October 2015.

Determination of sugars, ascorbic acid, ROS determination performed as above and mineral analyses (grind freeze dry samples were sent to an accredited laboratory, Yara analytical services, York, UK) were performed. Scanning electron microscope (SEM), was used to see if it was possible to see any differences in the amyloplast membrane between treatments. Sugars were measured as well at vine kill (9th October 2015).

3.9. Investigation of the effect of calcium in dormancy and sprouting

3.9.1. Assessment of Ca2+, LaCl3 and EGTA concentrations

The potato variety Lady Balfour selected from organic production was chosen for these experiment. Individual buds were excised from either the apical, mid-whorl or the stolon end of the tuber (Plate 3-1) by removing a plug using a cork borer nº.4 (0.5 cm^2), and then trimming the plug to 5-6 mm length. Initially, the same tuber was used to harvest buds from the three regions, the cork borer was sterilised in ethhanol between excisions. Solutions (20, 30 and 40 mM) of Ca^{2+}, EGTA and $LaCl_3$ were prepared in deionized water (dH_2O) in conjunction with a water control. All solutions were prepared immediately before use.

The buds were washed three times during 15 minutes with SRA-buffer (20 mM Potassium salt, 300 mM Mannitol, 5mM Ascorbic acid, pH 6,5) with gentle agitation (Cheema, 2010). Buds were dried in sterile paper and transferred to culture tissues plates (12 numerated wells) and incubate for 5 minutes in the respective solution followed gently blotting with sterile filter paper. Treated buds were

randomized in sterile culture tissues plates (12 numerated wells) containing filter paper soaked with dH_2O (Plate 3-2). Culture tissues plates were cover with aluminium foil and keep in dark at 20ºC. 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.

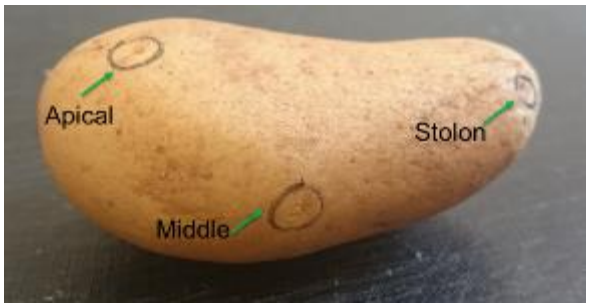


Plate 3-1 - Tuber with apical, middle and stolon buds

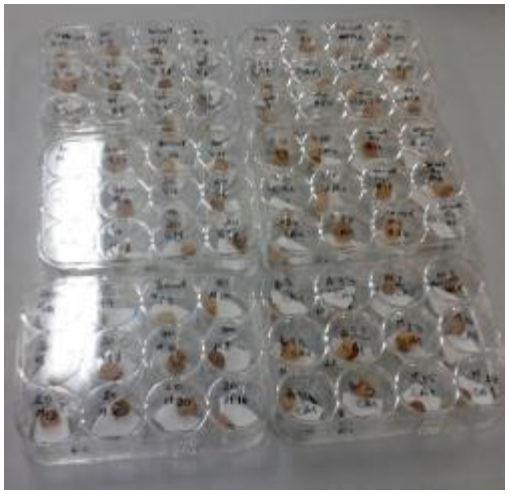


Plate 3-2 - Tuber buds placed in culture tissues plates containing filter paper soaked with dH2O

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

The effect of calcium in promoting dormancy break/sprout growth was tested in an extende range of varieties. Lady Balfour previously cultivated under organic production was paired with varieties Arsenal and Melody kept without sprout supressants application. Buds were removed from the apical and stolen end of the tuber with a cylindrical shape as in previews section.

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

The buds were washed three times during 15 minutes with SRA-buffer (20 mM Potassium salt, 300 mM Mannitol, 5mM Ascorbic acid, pH 6,5) 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 bud dishes and buds were handled and assessed as decribed previews. Measurements were undertaken after 3 to 4 days and then every 2 days.

3.9.3. Assessment of the influence of calmodulin blockers in dormancy and sprout growth

The effect of calmodulin blockers in promoting dormancy break/sprout growth was tested in Lady Balfour cultivated under organic production for 2 different temperatures, 10°C and 20°C. For this experiments was used calmidazolium chloride, an inhibitor of calmodulin-regulated enzymes, and the calmodulin antagonist N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (both from Sigma-Aldrich).

Buds were removed from the apical and stolon end of the tuber with a cylindrical shape as in previews sections. Solutions of 1 mM, 500 µM and 100 µM of calmidazolium chloride and of W7, were prepared in dH_2O from stock solutions prepared in 100 % ethanol. Solutions of 30 mM of Ca^{2+} ($CaCl_2$); 30 mM of EGTA and 30 mM of $LaCl_3$ were prepared in dH_2O and the control was dH_2O. All this solutions were used at 20 °C to test as well which was the best concentration of calmidazolium chloride and W7 to use in the next part of the experiment.

Once there was no significant difference between treatment for dormancy break and between treatments for sprout growth for W7. And the sprout growth for 1 mM of calmidazolium chloride was not significant different from the one from 100 µM and 500 µM. For that reason, in experiments at 10°C it was just prepared solutions of 1 mM of calmidazolium chloride, 1 mM W7, 30 mM of Ca^{2+} ($CaCl_2$); 30 mM of EGTA and 30 mM of $LaCl_3$ and the control was dH_2O.

The buds were washed three times during 15 minutes with SRA-buffer (20 mM Potassium salt, 300 mM Mannitol, 5mM Ascorbic acid, pH 6,5) 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 bud dishes and buds were handled and assessed as decribed previews. Measurements were undertaken after 4 days and then every 2 days.

3.10. Variety comparison in terms of gene expression using real time Reverse Transcription Polymerase Chain Reaction (RT qPCR)

3.10.1. RNA extraction

Five tubers of each variety were used in each sampling time (December 2015, January, March, April and June 2016). Each tuber were snap frozen in liquid nitrogen individually and after kept at -80^{o}C until been freeze dry and grind in liquid nitrogen using porcelain mortar and pestle. Freeze dry tissue was kept at -80^{o}C until RNA extraction.

RNA extraction was made using an adaptation of the method used by Otti (2016), as follows. Approximately 50 mg of freeze dry potato tuber tissue samples was mixed with 1 mL of cetyl trimethyl ammonium bromide (CTAB) extraction buffer ((2% w/v), 3 M NaCl, 20mM EDTA and 100mM Tris-HCl (pH 8.0)) preheated in a water bath for 10 minutes at 65ºC, after addition of 1.0% (v/v) 2-mercaptoethanol. Approximately 800 µL of sample mix was transferred to a 2 mL *Eppendorf* tube and an equal volume (800 µL) of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the mixture centrifuged at 13,000 rpm for 10 minutes. The top aqueous phase was transferred to a new 1.5 mL *Eppendorf* tube and an equal volume of absolute ethanol was added and mixture was gently pipetted up and down to mix. Up to 700 µL of the mixture was then transferred to an RNeasy® Mini spin column from QIAGEN's RNeasy® Plant Mini Kit (Qiagen, UK) (pink column) and centrifuged for 15 seconds at 13,000 rpm. The flow-through was discarded. 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 the manufacture instructions.

The concentration of extracted RNA was measured by spectrophotometer NanoDrop 2000 (Thermo Scientific, UK), before and after the genomic DNA removal, by placing 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 was prepared by the addition of 1 g agarose+100ml 0.5×TBE (Tris Borate EDTA) buffer 5% (Brody and Kern, 2004) and put into the microwave oven to dissolve. The gel was stained with 10 µL of SYBR® Safe DNA Gel Stain (ThermoFisher Scientific, UK) to 100 mL agarose solution after removing the flask from the microwave oven. The flask was gently swirl to mix the solution and poured into the gel tray after cooling. A comb was immersed in the solution and let to solidify, to form the wells to load the sample. Samples were prepared with 8 µL of RNA (after genomic DNA removal) and 3 µL of gel loading dye orange (New England Biolabs, USA). The agarose gel was immersed in 0.5 x TBE gel buffer and loaded with samples. The agarose gel was run at 110 mA (milliamps) for 20 minutes, allowing sufficient time for the major RNA sub-fragments (18 S, 25 S and 5S) to be separated. Finally, the agarose gel was placed in a gel imaging suite (Syngene G: Box, UK) and it was used the software (Synoptics Group, UK) to capture the image.

3.10.2. **Real time reverse transcription analysis of mRNA**

RT qPCR is the most sensitive and flexible of the quantification methods for gene expression (Wang and Brown, 1999). However, as a qualitative method to detect gene expression requires the creation of cDNA (complementary DNA) transcripts from the RNA. 1 µg of RNA was used as a starting material for reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN,

UK), according 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). As housekeeping gene it was used the elongation factor 1-α (EF1-α) using the primers designed by Nicot *et al.* (2005), EF1-α-F (ATTGGAAACGGATATGCTCCA) and EF1-α-R (TCCTTACCTGAACGCCTGTCA). Primers were supplied by Sigma-Aldrich as freeze-dried powders and 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.

For the PCR reactions it was used the QuantiTect® SYBR® Grenn PCR kit (Qiagen, UK), 5 µL template (cDNA sample) was pipetted into individual wells of a Hard-Shell® 96-Well Semi-Skirted PCR Plates (Bio-Rad, USA) and mixed with 15 µL PCR Mix, prepared as manufacturer's instructions. Each sample was replicated 3 times on each 96 well qPCR plate. House-keeping 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: PCR initial heat activation at 95ºC for 15 minutes, followed by 40 cycles of 94ºC of 15 seconds (denaturation), 58ºC of 30 seconds (annealing) and 72ºC of 30 seconds (extension).

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). It will be considered up regulation when $2^{-\Delta\Delta CT} \geq 2$ 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).

3.11. Reactive Oxygen Species (ROS) generation and tuber physiological changes

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

This experiment was divided in two parts; one to determine the best concentration of paraquat and the other part to test the effect of used a new plant health activator tecnology, comercially know as

"Alethea" (Plant Impact Plc, Hertfordshire, UK) in the recovery of induced abiotic stress due to paraquat (see 8.26 Appendix XXVI for Alethea formulation).

It was used an adaptation from the methods from Kraus *et al.* (1995), Seppänen *et al.* (2003) and Wargent *et al.*(2013). It was made 4 replicates with 5 tubers each, compose of middle slices and cores from each. Slices will be put in a Petri dish and cores will be placed in plastic tubes with different treatments.

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. There were made 3 different concentrations, 1 µM, 2 µM and 5 µM. Glycerol buffer with Tween 20 was used as control. Slices were put in Petri dishes for ROS staining and cores in plastic flasks to sugar and vitamin C extraction. Both slices in Petri dishes and cores in plastic flasks were vacuum infiltrated for 15 minutes and after left to incubate overnight (18 hours) under light conditions. In the next day samples for sugars/ vitamin C extraction were washed with dH_2O prior to freeze the samples 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 ROS staining that was performed and analysed as in previous sections above.

The 2nd part of this experiment was made in 3 days, in the 1st day "Alethea" was dissolved in 600 mM glycerol in 10 mMKH_2PO_4 buffer (to prevent desiccation of the slices/cores overnight). For control it was used 600 mM glycerol in 10 mMKH_2PO_4 buffer. There were made 2 concentrations of "Alethea", 10:1 (v/v) and 50:1 (v/v). Similar to the paraquat part of the experiment slices were put in Petri dishes for ROS staining and cores in plastic flasks for sugars and vitamin C extraction. Both were vacuum infiltrated for 15 minutes and after left to incubate for 24 hours. In 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 half of the samples (slices and cores) were put in 5 µM paraquat solution or in 600 mM glycerol in 10 mMKH_2PO_4 buffer with 0.1% (v/v) of Tween 20 (this will be used as control). Samples were vacuum infiltrated for 15 minutes and left to incubate overnight (18 hours) under light conditions. In the 3rd day samples were washed with dH_2O and it was performed the ROS staining of the tuber slices and the cores were frozen at -80^oC.

Sugars and vitamin C extraction and determination of O_2^- and H_2O_2 levels were performed and analysed as described previously. Scanning electron microscope (SEM), was used to see if it was possible to see any differences in the amyloplast membrane between treatments.

3.12. Data Analyses

Data plotting was made using Microsoft Excel 2013 and Rstudio. All data was performed with R (R Core Team, 2014).

Using ANOVA (analyses of variance) it was possible 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 which means were significantly different from each other a Tukey's HSD (honest significant difference) test (TukeyHSD) was carried out using the “agricolae” package from Rstudio (R Core Team, 2014). The idea for this package was first presented in the thesis "A statistical analysis tool for agricultural research" to obtain the degree of Master on science, National Engineering University (UNI), Lima-Peru. This package is especially useful for agricultural and plant breeding experiments (https://CRAN.R-project.org/package=agricolae, 27-04-2017).

Correlations were determined using Person 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), by adding several functions to reduce the complexity of combining geometric objects with transformed data. Some of these functions include a pairwise plot matrix, a two group pairwise plot matrix, a parallel coordinates plot, a survival plot, and several functions to plot networks (https://CRAN.R-project.org/package=GGally, 27-04-2017). Correlation coefficients from Lindley and Scott (1995).

Even though there were differences between years, for the study of the correlation between different physiological factors, the 3 years of data for VR 808 and L. Rosetta, and the 2 years of data for P. Dell and R. Burbank were analysed as one. Just for the chitting trial, which the objective was to see the differences between the 2 treatments, 0 °C days and 250 °C days were correlated separately, nevertheless losing degrees of freedom leading to the need of a higher correlation coefficient to be significant ($p < 0.05$).

Visual assessment of the obtain images by SEM were made to perceive if there were visible changes on the amyloplast surface during storage and when those changes start to arise.

Tukey test was carried 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). 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 calmodulin 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.

4. RESULTS

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

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 (Figure 4-1-I). P. Dell was the variety with the highest concentration of fructose and glucose and VR 808 with the lowest. All the varieties were significant different from each other according the TukeyHSD 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 higher 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 (Figure 4-1-II).

P. Dell and L. Rosetta tubers had the highest O_2 consumption during storage, while VR 808 had the lowest O_2 consumption (Figure 4-2-I). Similarly, with CO_2 production P. Dell had the highest production and VR 808 with the lowest (Figure 4-2-II).

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 stronger rate of sprout growth compared to the other varieties.

4.1.1. 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 ($p < 0.001$, Figure 4-3-I). While sucrose content remained stable (Figure 4-3-II). The opposite situation was observed with VR 808 variety, reducing sugars content remained stable (Figure 4-3-III), while sucrose content varied ($p < 0.001$, Figure 4-3-IV). In L. Rosetta O_2 consumption rates over the 3 years were stable (Figure 4-4-I-A and I-B). However, for VR 808 O_2 consumption and the CO_2 production for both varieties, varied

significantly between years ($p < 0.001$, Figure 4-4-I-B, II-A and II-B). 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, in 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 growths appeared to be affect 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 in the lower in the 1st year. And in P. Dell and R. Burbank with higher sprout growth in the 2nd year.

4.1.2. 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 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 was not effected by planting location for VR 808. However, for L. Rosetta, planting site had significant effect on CO_2 production ($p < 0.05$). Shropshire (1.112 mL g^{-1} h^{-1}) was significant different lower than Norfolk (1.316 mL g^{-1} h^{-1}), but Yorkshire (1.211 mL g^{-1} h^{-1}) was not significant different from both ($HSD_{0.05} = 0.186$).

Planting site had 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.

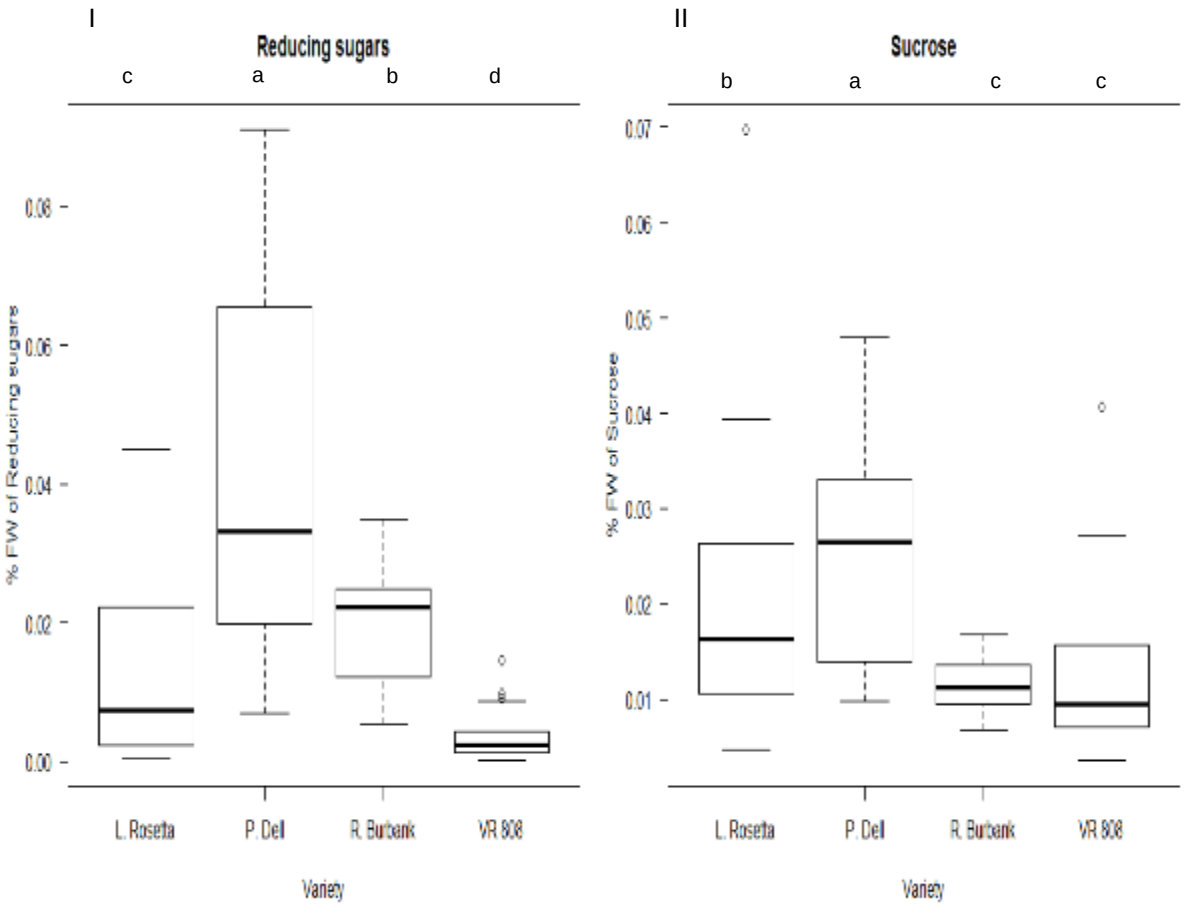


Figure 4-1 - *Comparison of %FW of reducing sugars (I) and %FW of sucrose (II) for the 4 varieties over 3 years of data for L. Rosetta and VR 808 and 2 years of data for P. Dell and R. Burbank. Mean values with different letters were significantly different ($HSD_{0.05}$ = 0.002 for % FW of reducing sugars and $HSD_{0.05}$ = 0.003 for % FW of Sucrose). The median is shown as a thick darker line.*

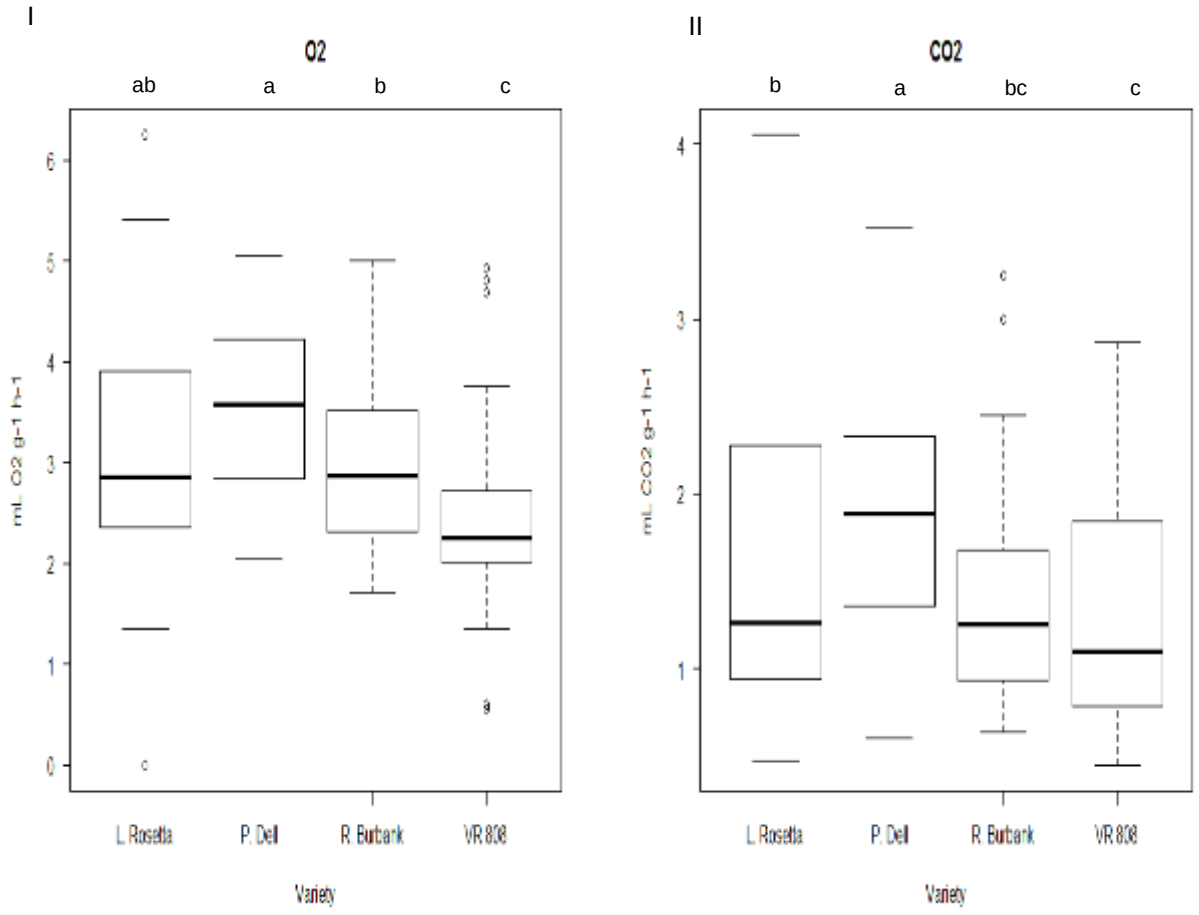


Figure 4-2 - *Comparison of O_2 consumption (I) and CO_2 production (II) for the 4 varieties over 3 years of data for L. Rosetta and VR 808 and 2 years of data for P. Dell and R. Burbank. Mean values with different letters were significantly different ($HSD_{0.05}$ = 0.447 for O_2 consumption and $HSD_{0.05}$ = 0.162 for CO_2 production). The median is shown as a thick darker line.*

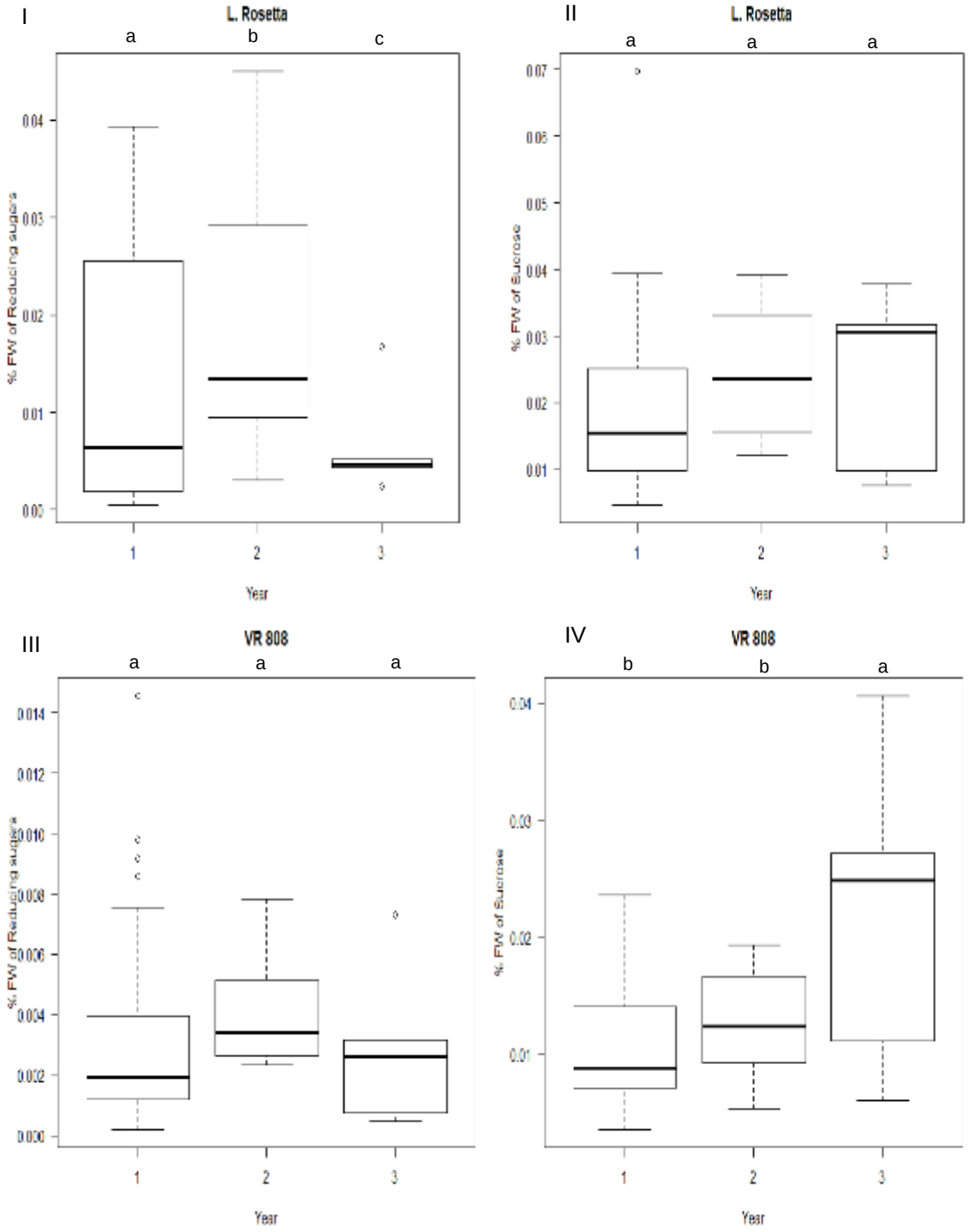


Figure 4-3 - *Comparison of %FW of reducing sugars and %FW of sucrose over 3 years of data for L. Rosetta (I and II) and VR 808 (III and IV). Mean values with different letters were significantly different (L. Rosetta: $HSD_{0.05}$ = 0.004 for %FW of reducing sugars and $HSD_{0.05}$ = 0.008 for %FW of sucrose; VR 808: $HSD_{0.05}$ = 0.002 for %FW of reducing sugars and $HSD_{0.05}$ = 0.003 for %FW of sucrose). The median is shown as a thick darker line.*

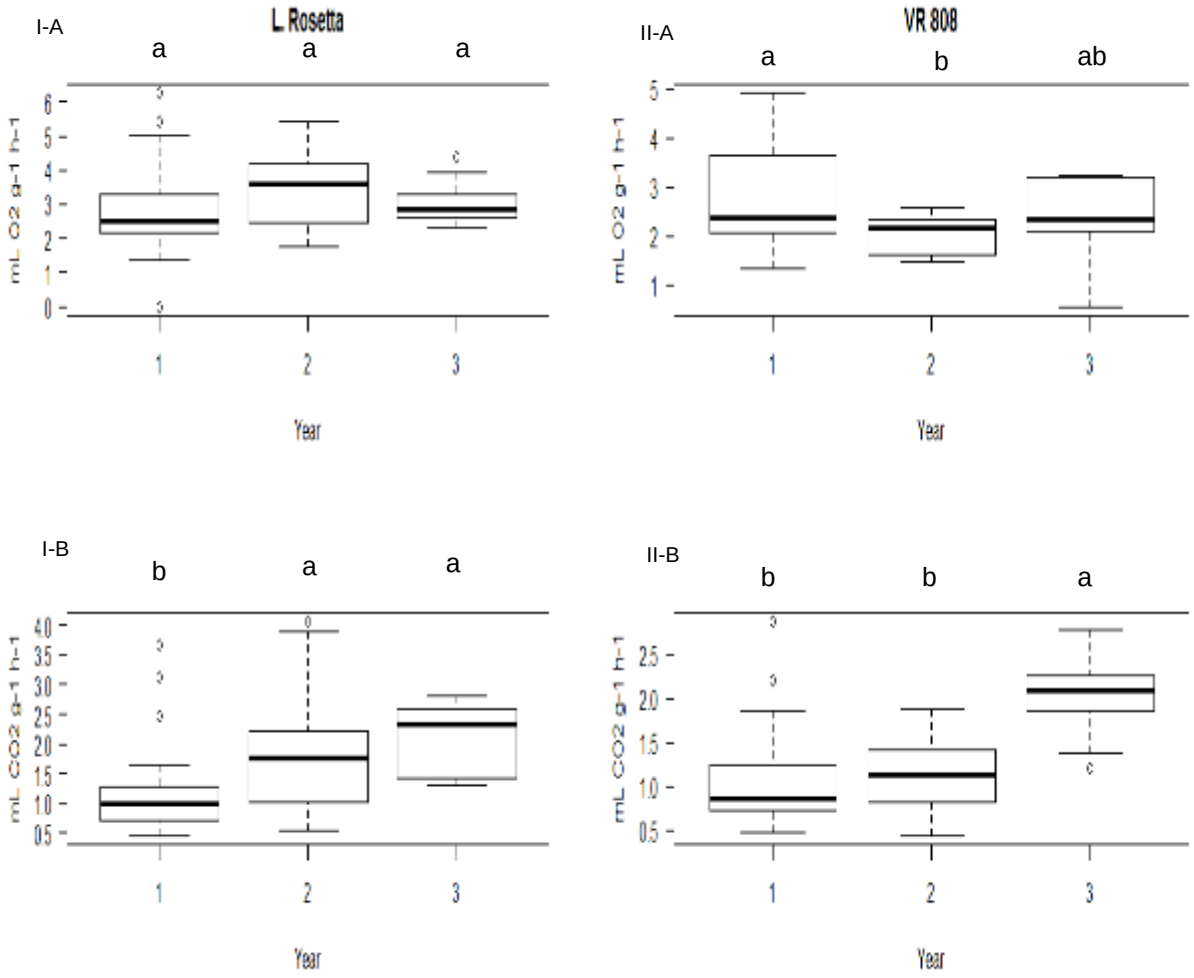


Figure 4-4 - *Comparison of O_2 consumption (I-A) and CO_2 production (I-B) for L. Rosetta, and the O_2 consumption (II-A) and CO_2 production (II-B) for VR 808, over 3 years of data. Mean values with different letters were significantly different ($HSD_{0.05}$ = 1.115 for O_2 consumption and $HSD_{0.05}$ = 0.250 for CO_2 production for L. Rosetta; $HSD_{0.05}$ = 0.820 for O_2 consumption and $HSD_{0.05}$ = 0.219 for CO_2 production for VR 808). The median is shown as a thick darker line.*

4.1.3. Changes in tuber physiology during storage

4.1.3.1. 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 (8.6 Appendix VI).

VR 808 tubers retained a low sugar profile for over 7 months of storage (May) (8.2 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 (8.8 Appendix VIII). A temporary dip in respiration rates were

observed prior to the extended rise in respiration rate which denotes the onset of sugar accumulation (Figure 4-5 and 8.9 Appendix IX) 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 (8.2 Appendix II) of storage at 10°C (March) (Figure 4-6 and 8.8 Appendix VIII). A similar, U shape curve in both O_2 consumption and CO_2 production was observed (Figure 4-6). Again, the timing of the rise and rate of 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 (8.9 Appendix IX). Sucrose and fructose accumulated at a slower rate, then respiration with an increase from base line measurements recorded after 7 months (May 2014) (Figure 4-6 and 8.8 Appendix VIII).

The relationship with changes in tuber respiration and changes in the reducing sugar and sucrose profile was dependent on variety. A significant increase in fructose and glucose (Figure 4-7) was observed from May (> 7 months in storage) (8.3 Appendix III) in P. Dell (8.8 Appendix VIII) while sucrose started to increase from January (> 3 months storage) (Figure 4-7, 8.8 Appendix VIII). 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 not such a difference between December (2 months is storage) and January CO_2 production levels as the ones observe for O_2 consumption (8.9 Appendix IX).

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 4-8 and 8.8 Appendix VIII). However, there was significant differences between months for sucrose (Figure 4-8) and fructose accumulation (8.8 Appendix VIII). In this variety, the levels of sucrose started to increase from January (> 3 months in storage) (8.3 Appendix III) and fructose levels from May (> 7 months in storage) (8.8 Appendix VIII). 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 (8.9 Appendix IX) was observed.

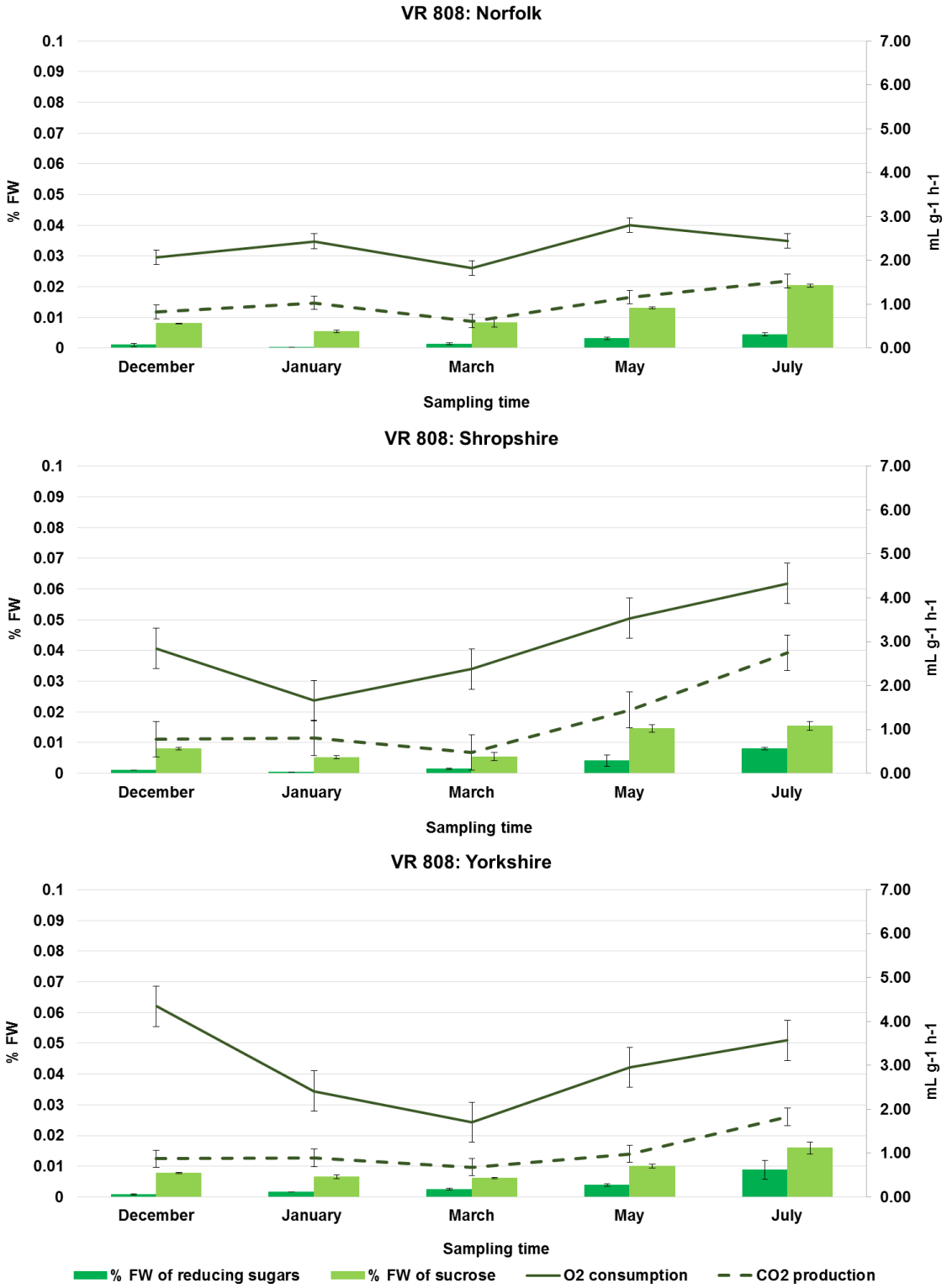


Figure 4-5 - Accumulation of reducing sugars (%FW of reducing sugars) and sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 1st year of the study (season 2013/14) from VR 808, with SE bars. For stats see 8.8 Appendix VIII and 8.9 Appendix IX.

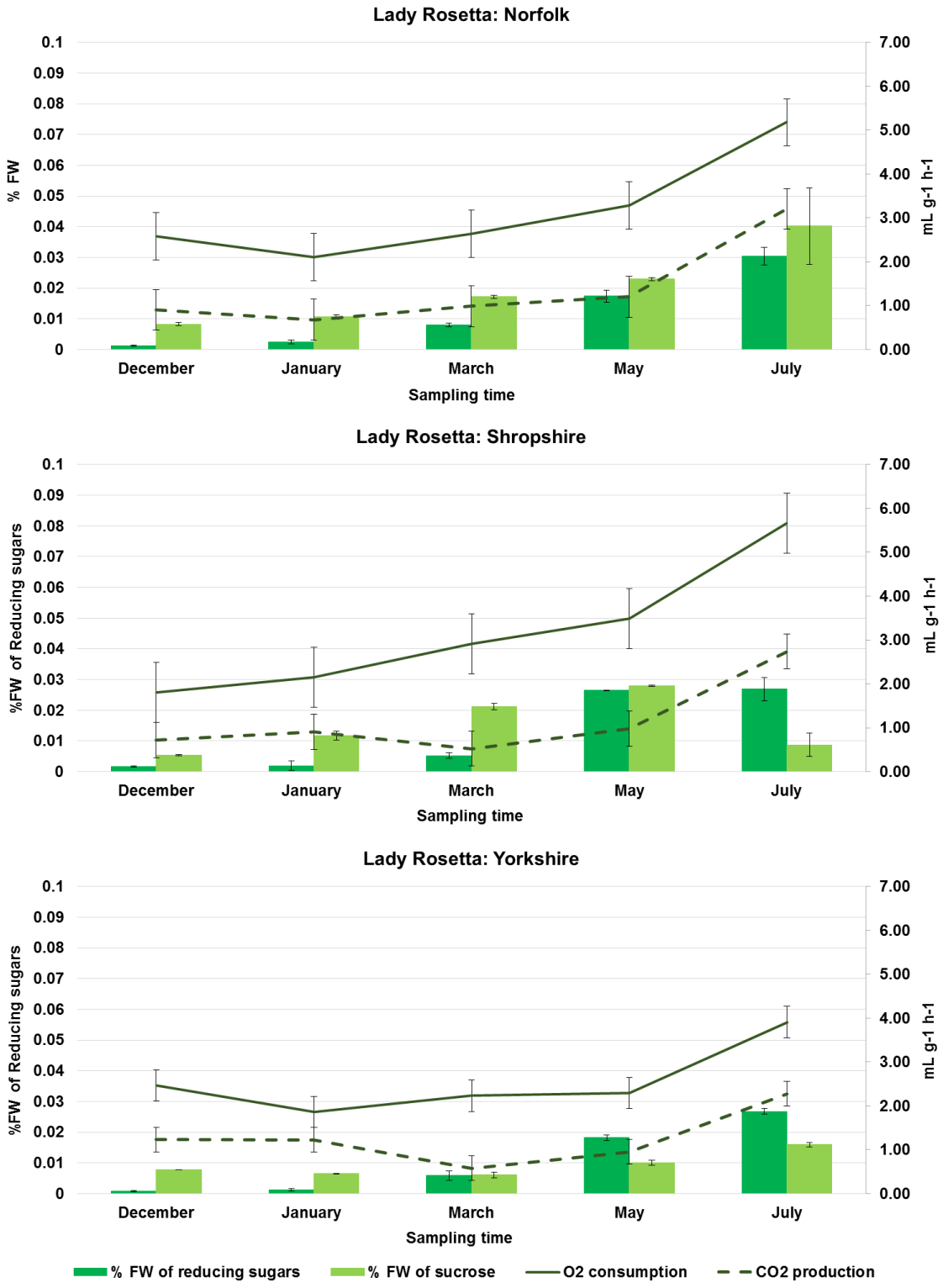


Figure 4-6 - Accumulation of reducing sugars (%FW of reducing sugars) and sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 1st year of the study (season 2013/14) from L. Rosetta, with SE bars. For stats see 8.8 Appendix VIII and 8.9 Appendix IX.

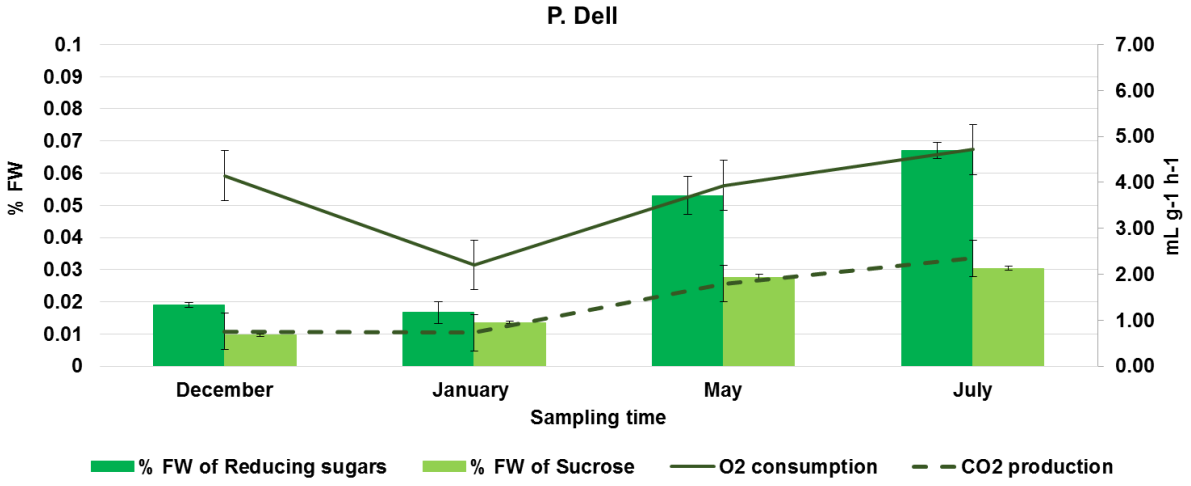


Figure 4-7 - Accumulation reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 1st year of the study (season 2013/14) for P. Dell, with SE bars. For stats see 8.8 Appendix VIII and 8.9 Appendix IX.

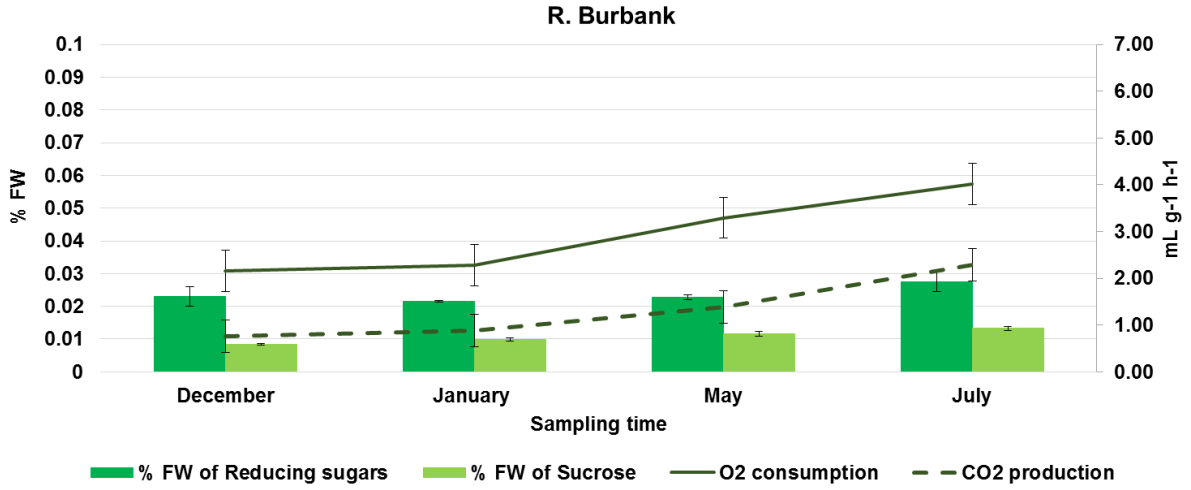


Figure 4-8 - Accumulation reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 1st year of the study (season 2013/14) for R. Burbank, with SE bars. For stats see 8.8 Appendix VIII and 8.9 Appendix IX.

Storage season November 2014 to July 2015

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 (8.4 Appendix IV) mirrored by a reduction in respiration rates. Thereafter, sugars rise and respiration increased. The magnitude of these changes was variety dependent.

The length of apical sprouts increased in line 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 (8.10 Appendix X and 8.11 Appendix XI). P. Dell was the only variety where ethylene production increased during storage time (8.11 Appendix XI). Patterns of O_2 consumption and CO_2 production were variety dependent.

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 0.02% FW (Figure 4-9).

O_2 consumption for VR 808 was lower than the other varieties and remained low during storage (Figure 3.10 and 8.10 Appendix X). 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 4-9 and 8.10 Appendix X).

In contrast, to VR 808 reducing sugar content of L. Rosetta was higher (0.009 % FW) at harvest (November) and started to increase 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 h^{-1} and reached 5.5 mL g 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 (8.10 Appendix X and 8.11 Appendix XI).

CIPC application (8.1 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 4-10 and 8.10 Appendix X) probably related with sprout growth (8.11 Appendix XI).

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) (8.4 Appendix IV) onwards (Figure 4-10, 8.10 Appendix X and 8.10 Appendix X).

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 commercial threshold of 0.02 %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 4-11). CO_2 production was higher than L. Rosetta and VR 808 at harvest (3.5 mL g h^{-1}) (8.11 Appendix XI), 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 4-11).

R. Burbank exhibited a stable reducing sugar profile during storage with reducing sugars remaining below commercial threshold of 0.02 %FW (Figure 4.12). Unlike VR 808, CO_2 production was higher at harvest 3.5mL g h^{-1} before dipping to 2,5 ml g 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 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.

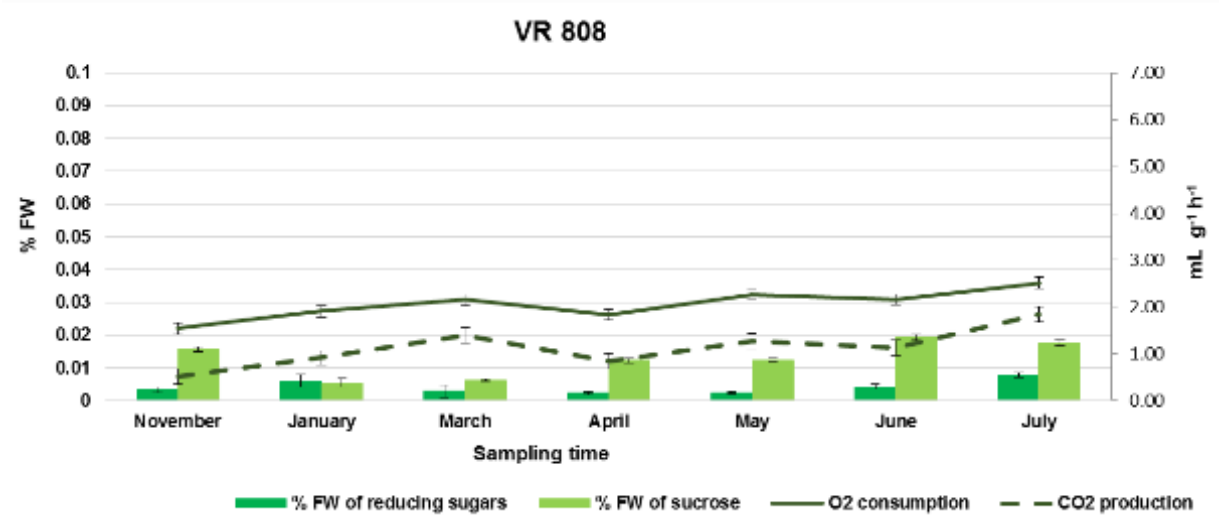


Figure 4-9 - Accumulation of reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 2nd year of the study (season 2014/15) for VR 808, with SE bars. For stats see 8.10 Appendix X and 8.11 Appendix XI.

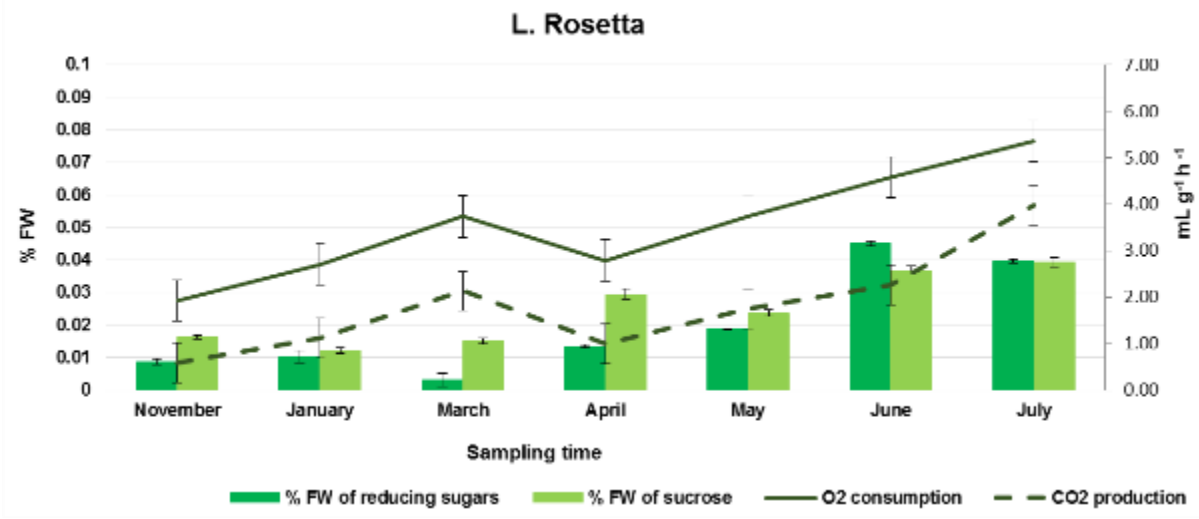


Figure 4-10 - Accumulation of reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 2nd year of the study (season 2014/15) for L. Rosetta, with SE bars. For stats see 8.10 Appendix X and 8.11 Appendix XI.

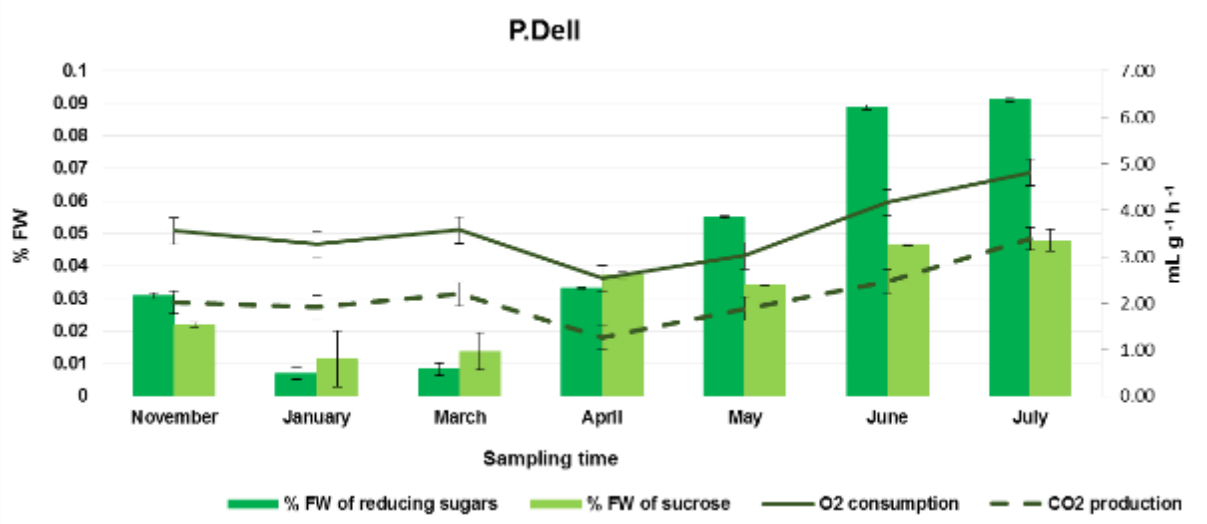


Figure 4-11 - Accumulation of reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1})) of the 2nd year of the study (season 2014/15) for P. Dell, with SE bars. For stats see 8.10 Appendix X and 8.11 Appendix XI.

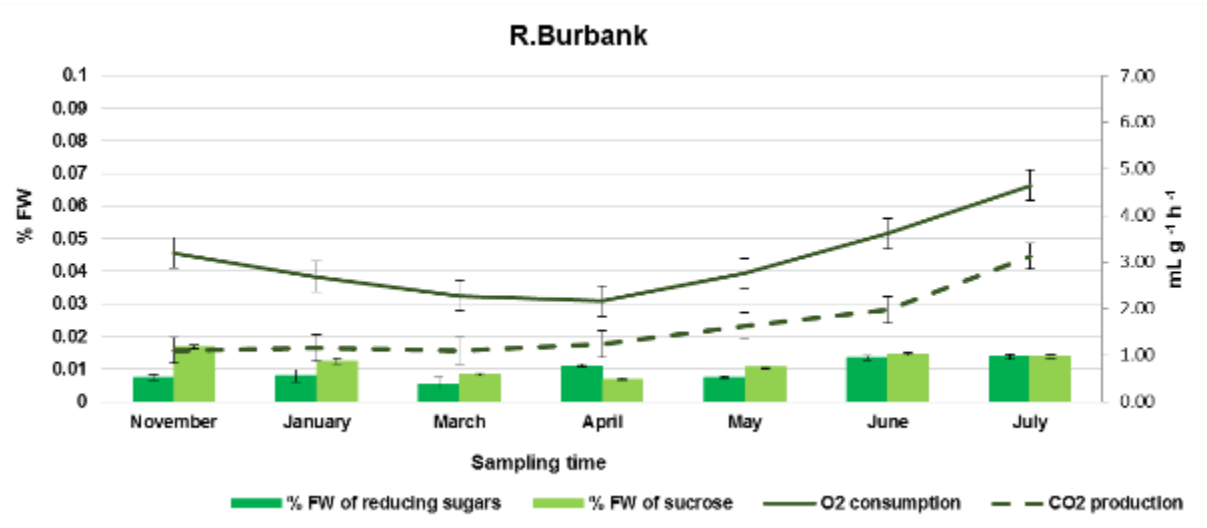


Figure 4-12 - Accumulation of reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 2nd year of the study (season 2014/15) for R. Burbank, with SE bars. For stats see 8.10 Appendix X and 8.11 Appendix XI.

4.1.3.2. 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 (8.7 Appendix VII).

Reducing sugars in VR 808 remained low (0.003 %FW) and declined to even lower concentrations after 4-6 months' storage (March-April, 8.5 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 season 2013/14, O_2 consumption over the 8 month storage season for VR 808 displayed a U shaped pattern (Figure 4-13).

The decrease in O_2 consumption mid-season correlated with a decrease in fructose and glucose (Figure 4-13, 8.12 Appendix XII and 8.13 Appendix XIII), 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 4-13, 8.12 Appendix XII and 8.13 Appendix XIII). 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 4-13, 8.12 Appendix XII and 8.13 Appendix XIII).

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 (8.12 Appendix XII). 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 4-13).

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 4-14). Interesting at the same time in March there was an increse in glucose accumulation (8.12 Appendix XII). This season was the one with the lowest %FW of reducing sugars for L. Rosetta (Figure 4-3-I), however still higher than VR 808 (0.024 %FW and 0.022 %FW, respectively, $HSD_{0.05} = 0.003$).

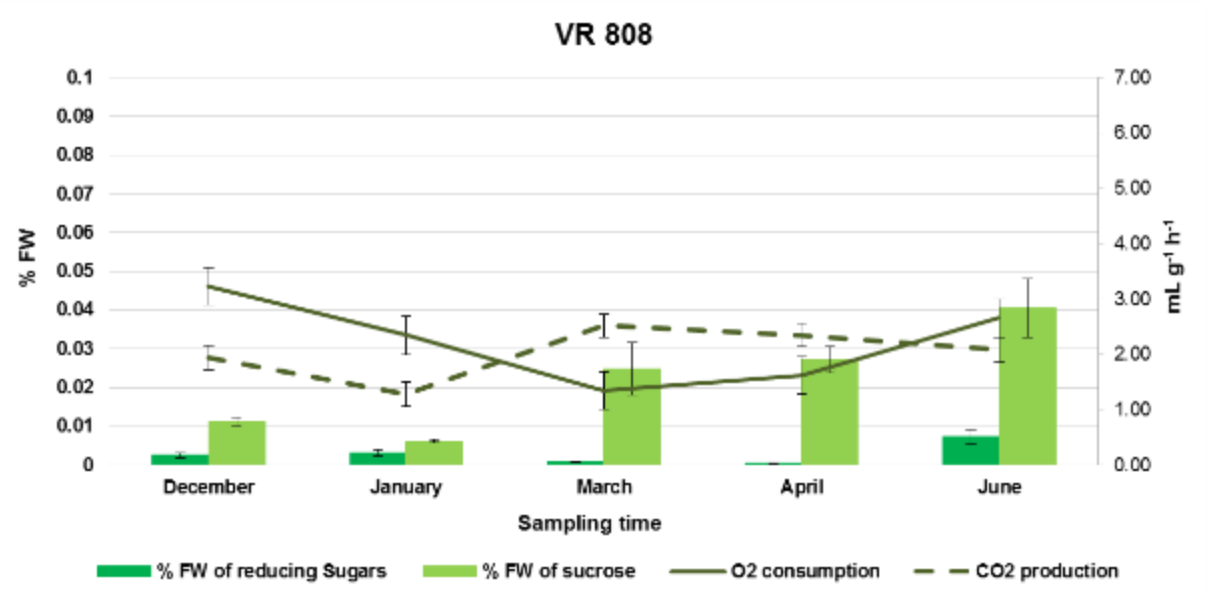


Figure 4-13 - Accumulation of reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 3rd year of the study (season 2015/16) for VR 808, with SE bars. For stats see 8.12 Appendix XII and 8.13 Appendix XIII.

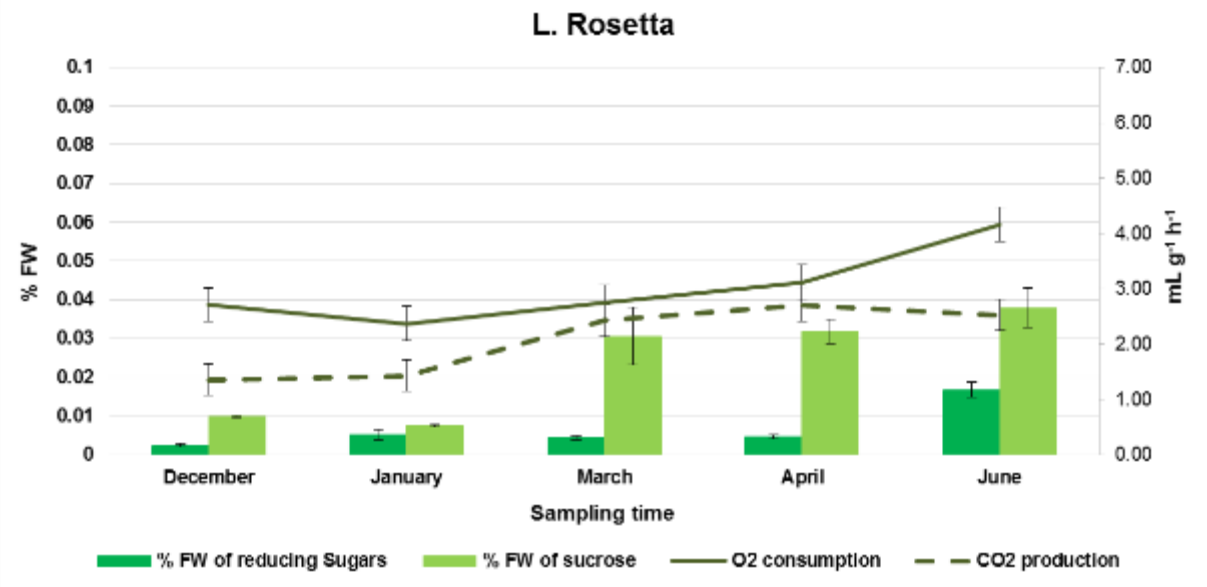


Figure 4-14 - Accumulation of reducing sugars (%FW of reducing sugars), sucrose (%FW of sucrose), consumption of O_2 (mL O_2 g^{-1} h^{-1}) and production of CO_2 (mL CO_2 g^{-1} h^{-1}) of the 3rd year of the study (season 2015/16) for L. Rosetta, with SE bars. For stats see 8.12 Appendix XII and 8.13 Appendix XIII.

4.1.3.2.1. Sugar accumulation within the tuber

Significant differences between position of the samples taken within the tuber (edges 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 in the edges than in the middle during the 1st year (Table 4-1). Opposite occur during the 3rd year, with the middle part accumulating more sucrose than the edges (Table 4-1). 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 4-1).

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 4-1). In contrast sucrose accumulated more on the outer edges where the opposite eighths sections were taken in the 1st year. However, in later years of the study sucrose was higher in the edges of the tuber (Table 4-1).

The sugar content in P. Dell, was dependent on the position of the samples taken within the tuber. In particular fructose content was higher in the centre of the tuber (Table 4-1). In year 2 a higher concentration of sucrose, fructose and glucose was recorded in the middle pith of the tuber (Table 4-1).

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. In the edges 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 4-1).

Table 4-1 – %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 and 2 years of data for P. Dell and R. Burbank.

	VR 808								
	1st year			2nd year			3rd year		
	Edges	Middle	$HSD_{0.05}$	Edges	Middle	$HSD_{0.05}$	Edges	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		
	Edges	Middle	$HSD_{0.05}$	Edges	Middle	$HSD_{0.05}$	Edges	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					
	Edges	Middle	$HSD_{0.05}$	Edges	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					
	Edges	Middle	$HSD_{0.05}$	Edges	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 TukeyHSD test. Level of significance in HSD column (p<0.001 " ***" p<0.01 "**" p<0.05 "*").*

4.1.3.2.2. 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 4-15 and Figure 4-16). 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 accumulated was positively correlated with storage duration (Figure 4-15 and Figure 4-16).

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 was positively correlated with reducing sugars and with sucrose content (Figure 4-15 and Figure 4-16).

The increase in O_2 consumption rates over time was positively correlated in L. Rosetta and R. Burbank (Figure 4-15 and Figure 4-16), while O_2 consumption rates were more highly correlated d to the rate of sprout growth in P. Dell and R. Burbank rather than the duration of storage (Figure 4-16). Which suggests that rate of sprouting and effectiveness of CIPC application are just as important factors to consider than 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 4-15 and Figure 4-16), and O_2 consumption for R. Burbank (positively) and a negative relationship between O_2 consumption and VR 808 (Figure 4-15 and Figure 4-16). 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 the length of storage and respiration rates (CO_2 production and O_2 consumption), and sucrose accumulation (Figure 4-15 and Figure 4-16).

Ethylene production in tubers were 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 4-15) caused by a decrease in ethylene later in storage and moreover (Figure 4-15). R. Burbank was the only variety that had a significantly positive correlation between ethylene production and fructose and glucose accumulation (Figure 4-16). Interestingly, this variety is considered very responsive to ethylene (Haines et al., 2003).

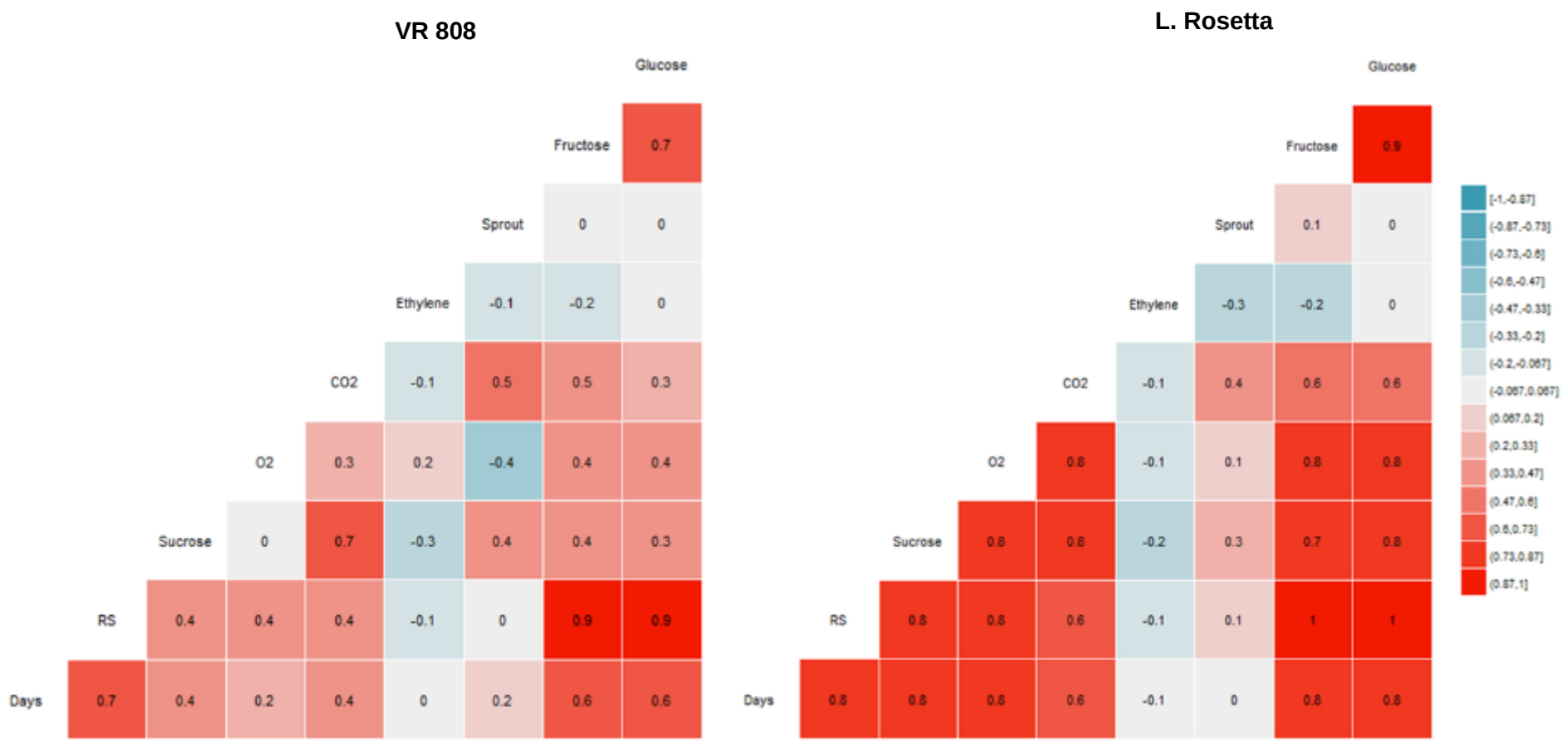


Figure 4-15 - Correlation between days in storage (Days), season (Year), O_2 consumption (O_2), CO_2 production (CO_2), Ethylene production (Ethylene), sprout growth (Sprout), %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose and %FW of glucose for the variety VR 808 and L. Rosetta (df = 25). Significant Pearson correlation coefficients from 0.32 to 1 and from -0.32 to -1 ($p < 0.05$).

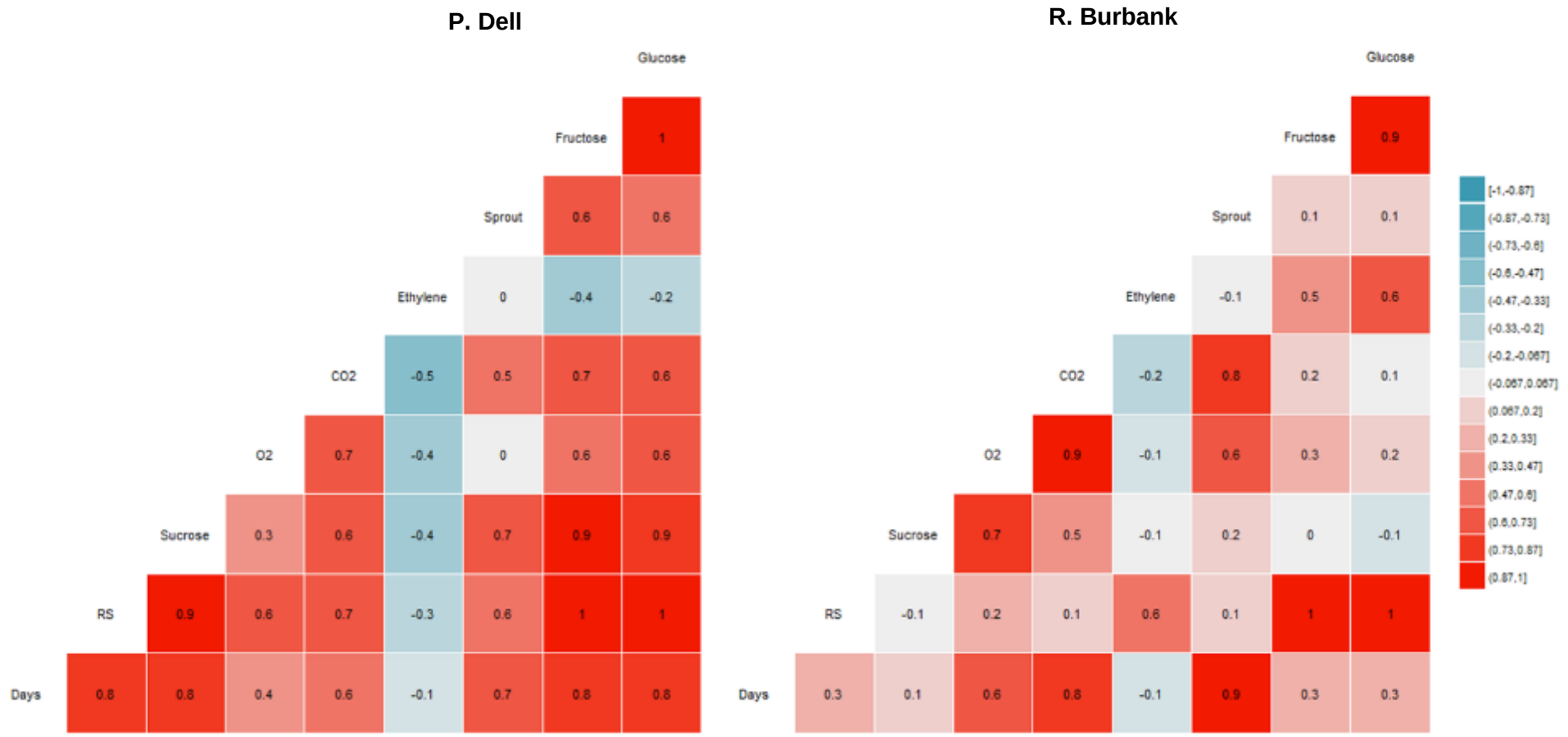


Figure 4-16 - Correlation between days in storage (Days), season (Year), O_2 consumption (O_2), CO_2 production (CO_2), Ethylene production (Ethylene), sprout growth (Sprout), %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose and %FW of glucose for the variety P. Dell and R. Burbank (df = 9). Significant Pearson correlation coefficients from 0.52 to 1 and from -0.52 to -1 ($p < 0.05$).

4.2. 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 at harvest in tubers harvested from aged Pentland Dell seed (250°C days) 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 (8.14 Appendix XIV and 8.15 Appendix XV).

Total reducing sugars and sucrose (Figure 4-19) increased with time in storage. The increase in sucrose accumulation was more linear for 250°C days than for 0°C days (Figure 4-17).

O_2 consumption follows a U shape for both treatments and CO_2 production an inverted U shape (Figure 4-18). The peak in CO_2 production in March (>3 months in storage at 10°C) corresponds to the start in the sucrose and fructose accumulation rise in both treatments (8.14 Appendix XIV and 8.15 Appendix XV). The lowest O_2 consumption value for 0°C days corresponds as well to the start in the sucrose and fructose accumulation rise (March) and in 250°C days that lowest peak is 48 days (January) before with a peak in ethylene production (8.14 Appendix XIV and 8.15 Appendix XV).

For both treatments from the chitting trial, only for glucose accumulation that the position of the samples taken within the tuber was not significant (Table 4-2). In the middle part of the tuber was the higher total %FW of reducing sugars, sucrose and fructose (Table 4-2).

Total %FW of reducing sugars and sucrose content increased over storage leading to a strong positive correlation (Figure 4-19). 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 4-19). In general, in this experiment, increase in residual sprout growth lead to a reduction of sugars and this correlation was significant in tubers from chitted seed (Figure 4-19).

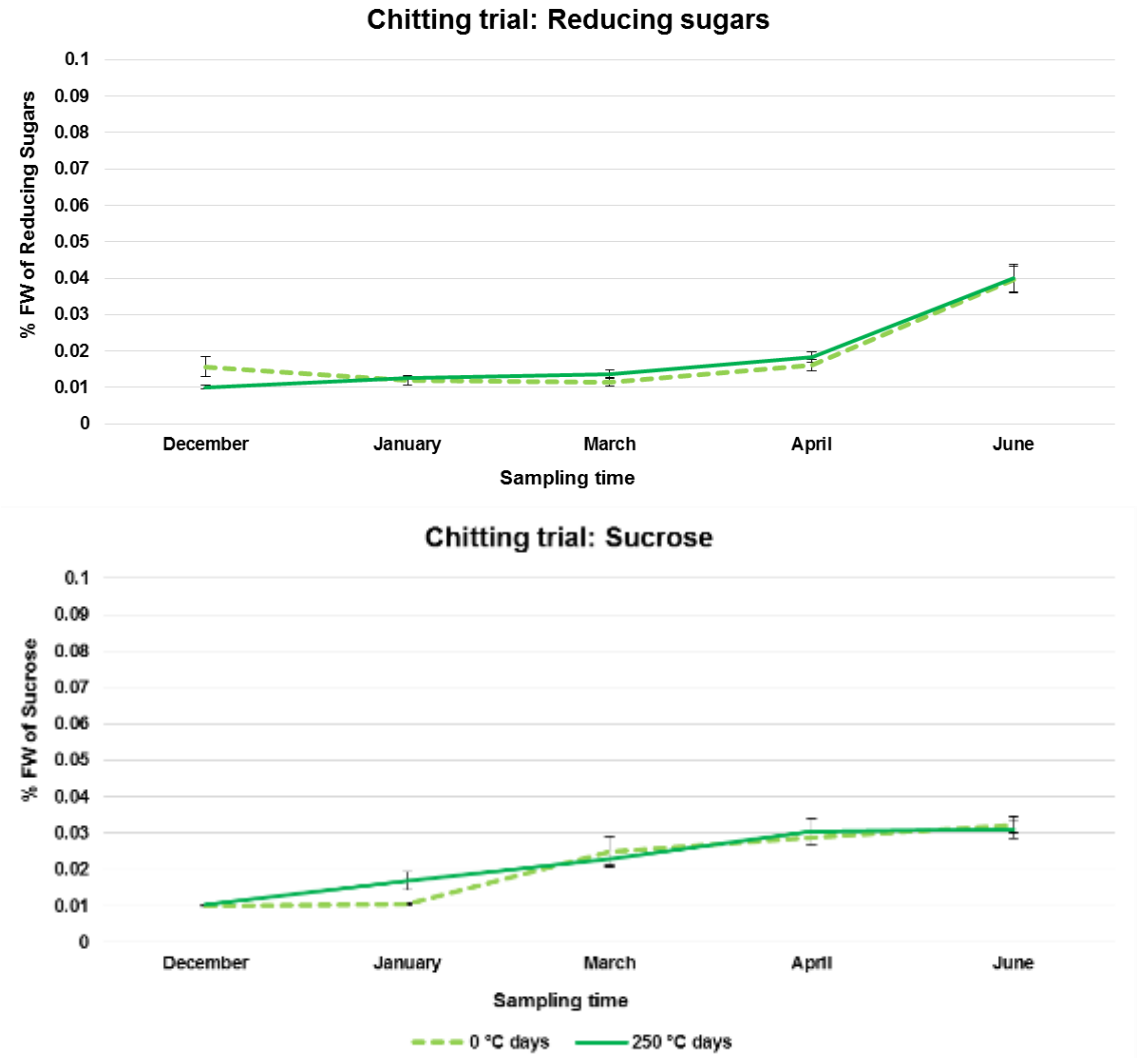


Figure 4-17 - Accumulation of reducing sugars (%FW of Reducing sugars) and sucrose (%FW of Sucrose) for P. Dell from the chitting trial, with SE bars. For stats see 8.14 Appendix XIV.

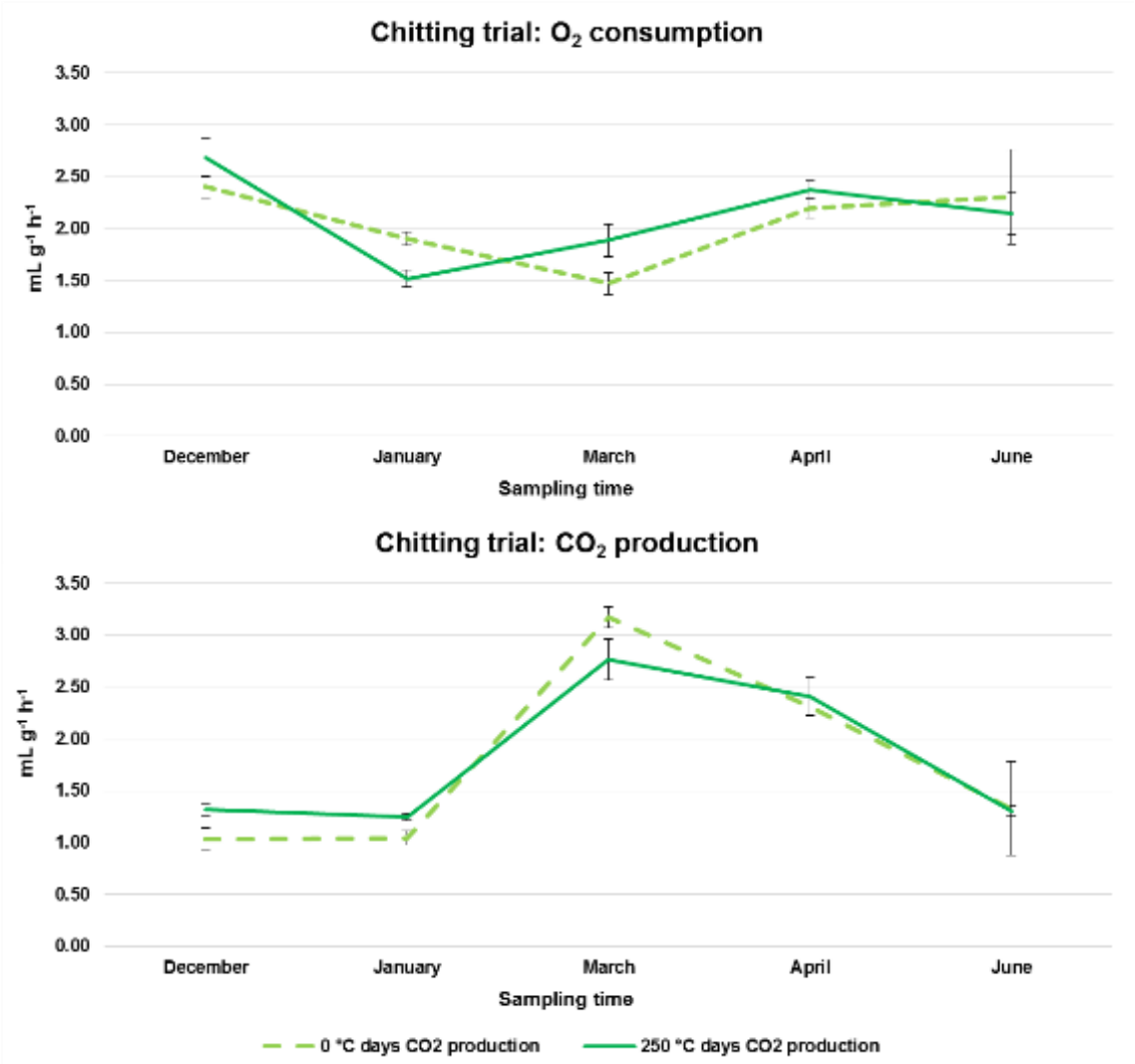


Figure 4-18 - Consumption of O_2 (mL O_2 g^{-1} h^{-1}) and CO_2 (mL CO_2 g^{-1} h^{-1}) production for P. Dell from the chitting trial, with SE bars. For stats see 8.15 Appendix XV.

Table 4-2 - %FW of reducing sugars (RS), %FW of sucrose (Suc), %FW of fructose (Fru) and %FW of glucose (Glu) for the chitting trial.

	0°C days				250°C days			
	Edges	**Middle**	**$HSD_{0.05}$**	**p-value**	**Edges**	**Middle**	**$HSD_{0.05}$**	**p-value**
RS	0.017^{b}	0.021^{a}	0.003	< 0.05	0.016^{b}	0.021^{a}	0.002	< 0.001
Suc	0.017^{b}	0.025^{a}	0.003	< 0.001	0.018^{b}	0.026^{a}	0.004	< 0.001
Fru	0.005^{b}	0.009^{a}	0.001	< 0.001	0.006^{b}	0.010^{a}	0.001	< 0.001
Glu	0.012^{a}	0.012^{a}	0.003	> 0.05	0.011^{a}	0.012^{a}	0.001	> 0.05

Mean values with different letters were significantly different according to TukeyHSD test.

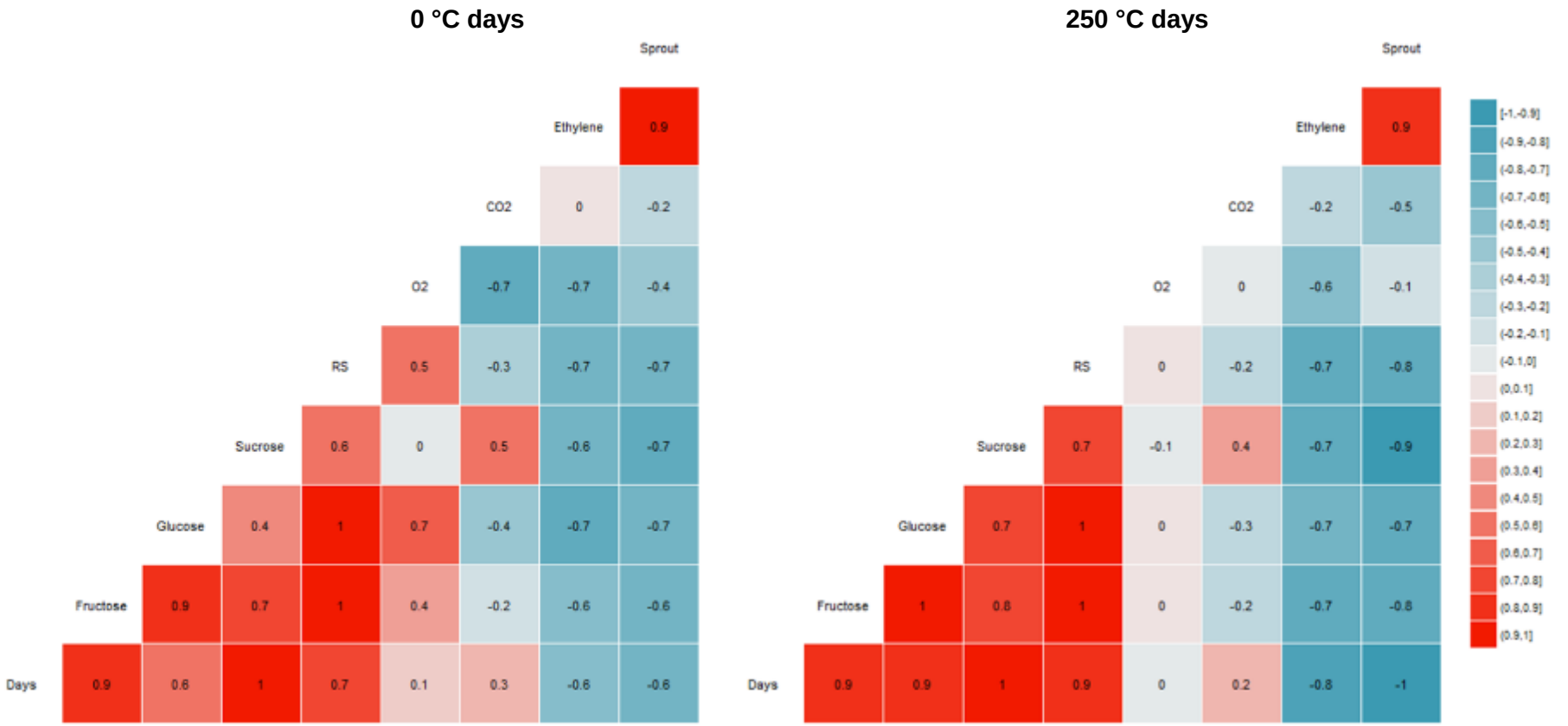


Figure 4-19 - *Correlation between days in storage (Days), %FW of fructose, %FW of glucose, %FW of sucrose (Sucrose), %FW of reducing sugars (RS), O_2 consumption (O_2), CO_2 production (CO_2), Ethylene production (Ethylene) and sprout growth (Sprout) for 0 and 250°C days from the chitting experiment (df = 3). Significant Pearson correlation coefficients from 0.81 to 1 and from -0.81 to -1 ($p < 0.05$).*

4.3. 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. DHA profiles recorded with R. Burbank the highest ranked variety, followed by P. Dell, L. Rosetta and VR 808 (Table 4-3).

In both years, L. Rosetta retained more AsA for the following season (2015/16) yielded significantly higher AsA and DHA content compared to the previous year's data and differences ($p < 0.001$) between varieties and seasons were found (Table 4-3).

In both years (2014-2016) L. Rosetta contained a significantly higher AsA content than and 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 4-3).

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 4-3). The difference could not be assigned to variance in HPLC analysis between years, as cross validation of samples by HPLC from both years was performed.

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 a peak in December and April that was not statistically significant.

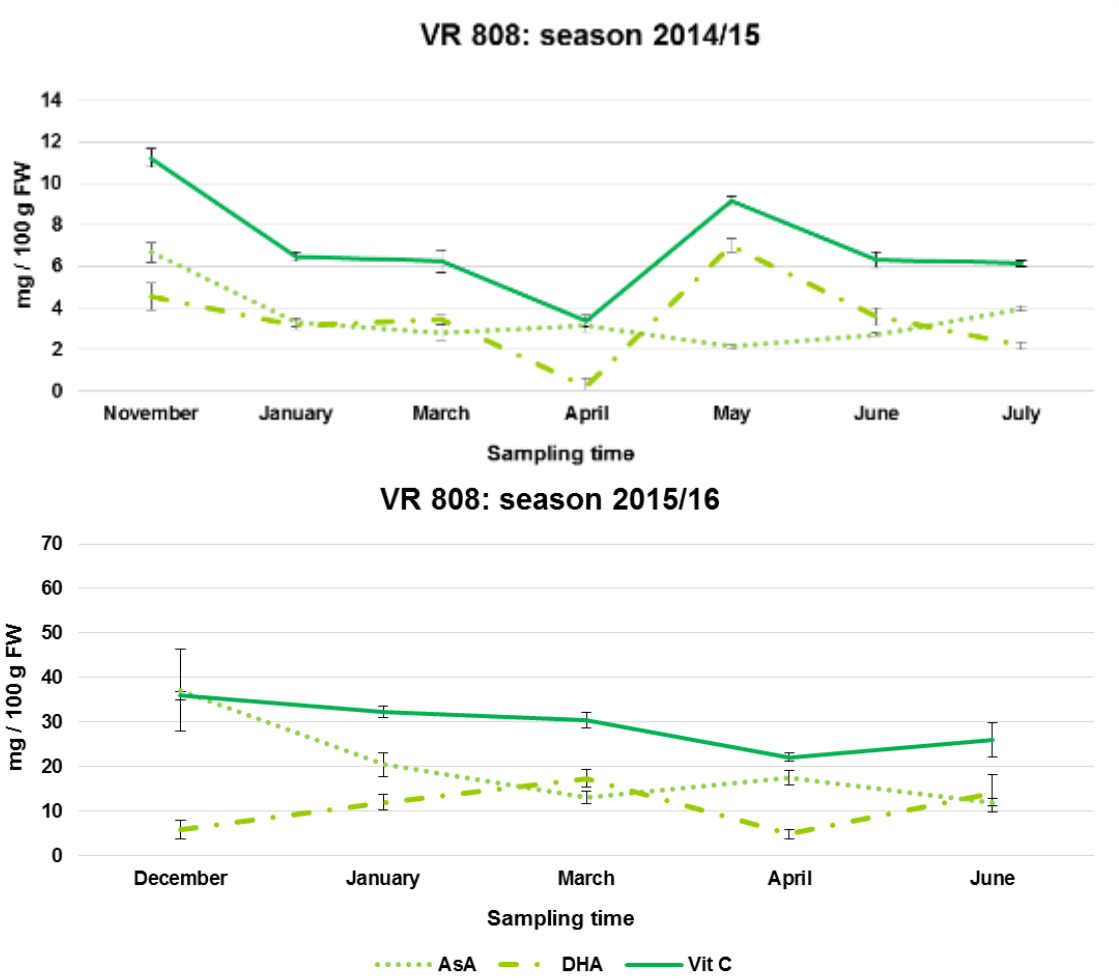
Table 4-3 - AsA accumulation (ASA, mg 100 g^{-1} FW), DHA accumulation (DHA, mg 100 g^{-1} FW) and total vitamin C accumulation (Vit C, mg 100 g^{-1} FW) for seasons 2014/15 and 2015/16.

	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 Turkeys test.

For VR 808 the highest AsA content over the two-year study was recorded at harvest and declined during storage (10°C) (8.16 Appendix XVI and 8.18 Appendix XVIII). DHA profile followed a similar pattern to AsA content in season 2014/15 with a spike 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 (8.18 Appendix XVIII). 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 3-21, 8.16 Appendix XVI and 8.18 Appendix XVIII).

Figure 4-20 - Accumulation of accumulation of ascorbic acid (mg of AsA / 100 g FW), dehydroascorbic acid (mg of DHA / 100 g FW) and total Vitamin C for seasons 2014/15 and 2015/16 for VR 808, with SE bars. For stats see 8.16 Appendix XVI and 8.18 Appendix XVIII.

The AsA content 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 content remained between 3.3 – 6.6 mg $100g^{-1}$ throughout storage (Figure 4 21 and 8.16 Appendix XVI). 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 4 21 and 8.18 Appendix XVIII). 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 slow 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).

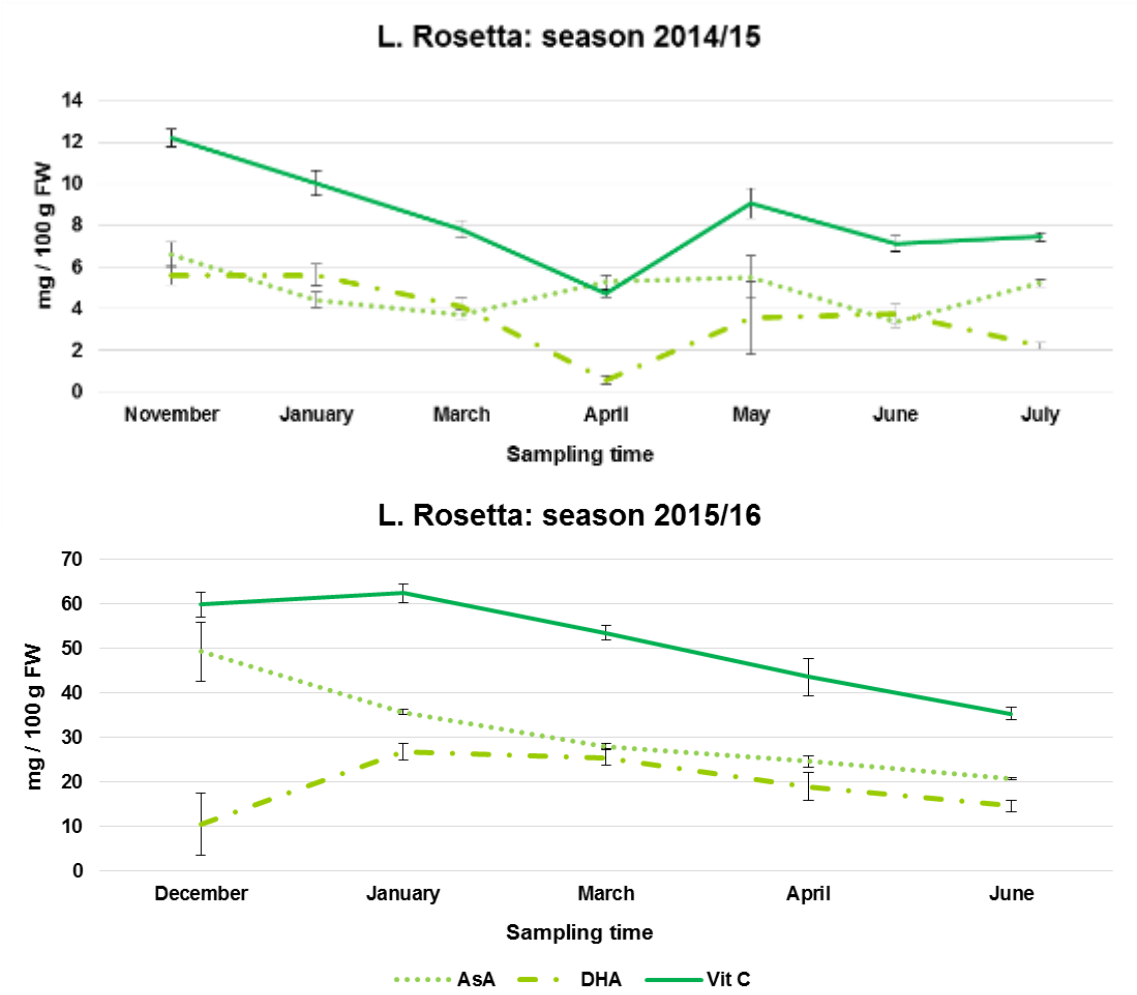


Figure 4-21 - Accumulation of accumulation of ascorbic acid (mg of AsA / 100 g FW), dehydroascorbic acid (mg of DHA / 100 g FW) and total Vitamin C for seasons 2014/15 and 2015/16 for L. Rosetta, with SE bars. For stats see 8.16 Appendix XVI and 8.18 Appendix XVIII.

AsA and DHA content of P. Dell in season 2014/15 was 5.4 mg $100g^{-1}$ at the beginning of the storage and thereafter declined during the first 4 months of storage at 10°C to 2.2 mg $100g^{-1}$ while DHA content remained constant (Figure 4-22 and 8.17 Appendix XVII). After 6 months storage (May), AsA/DHA ratio has its higher value (data not shown), at the same time the ASA had risen and DHA declined to reach a final concentration of 3.7 mg 100 g^{-1} in July (Figure 4-22 and 8.17 Appendix XVII).

A similar pattern in AsA and DHA decline ($p < 0.001$) was observed with R. Burbank with an intermittent peak in DHA content in after 6 months storage (Figure 4-22 and 8.17 Appendix XVII). Such peaks and troughs in AsA and DHA are seen in other varieties and may be associated with sprouting or late season CIPC application.

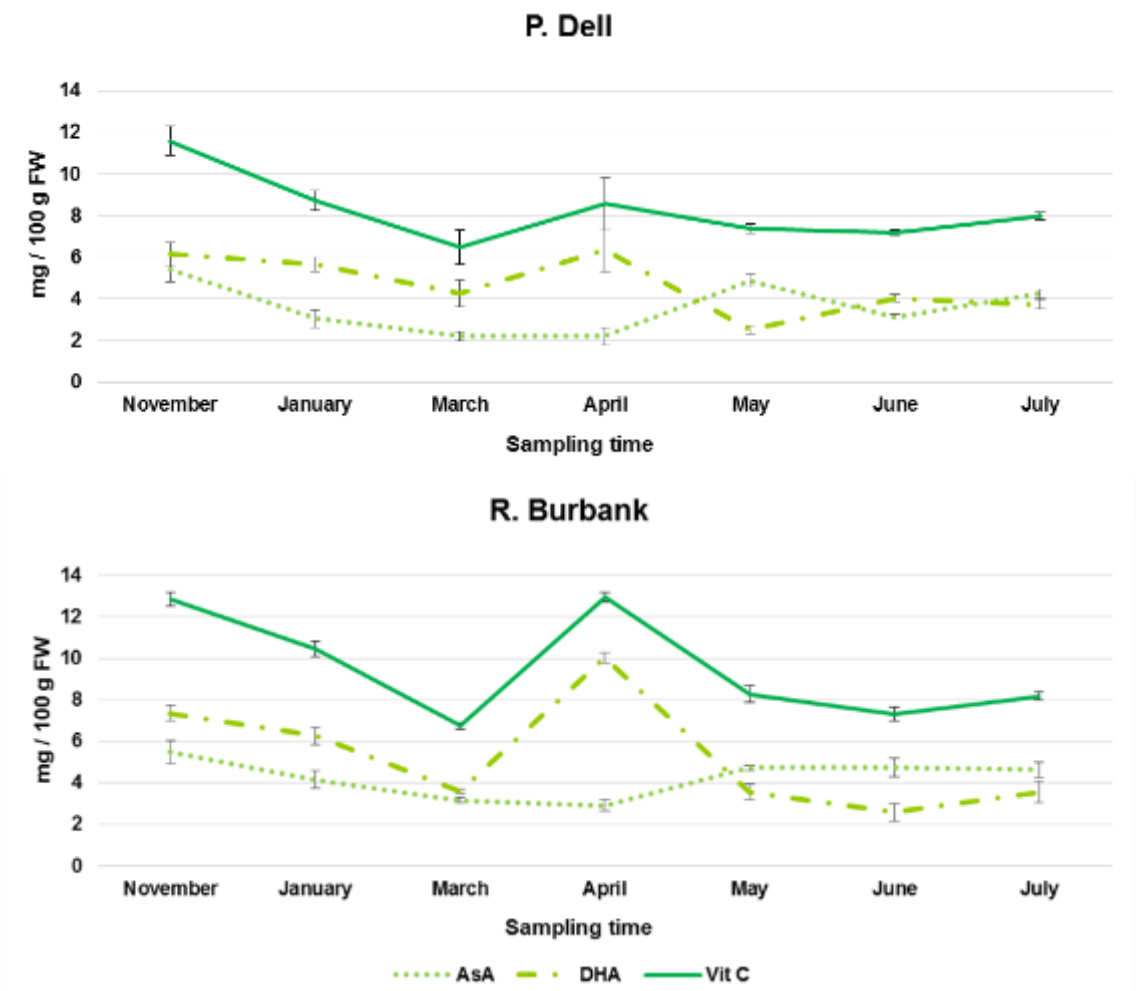


Figure 4-22 - Accumulation of accumulation of ascorbic acid (mg of AsA / 100 g FW), dehydroascorbic acid (mg of DHA / 100 g FW) and total Vitamin C for season 2014/15 for P. Dell and R. Burbank, with SE bars. For stats see 8.17 Appendix XVII.

4.3.1. The effect of physiological ageing (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 4-4).

In both cases, storage length was significant for AsA accumulation ($p < 0.001$), DHA accumulation ($p < 0.001$) and total vitamin C accumulation (8.19 Appendix XIX).

AsA content of tubers was highest in tubers sampled after a month's storage (December) for chitted (250°C) an non-chitted (0°C) tubers (Figure 4-23 and 8.19 Appendix XIX), then a sudden drop in AsA content occurred during the following month (January) (days and 250°C days) and April (250°C days). A decrease in the AsA accumulation was mirrored in an increase in DHA content (Figure

4-23). The lowest AsA/DHA ratios were observed in January and April (Figure 4-23 and 8.19 Appendix XIX).

Table 4-4 - 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 TukeyHSD test.

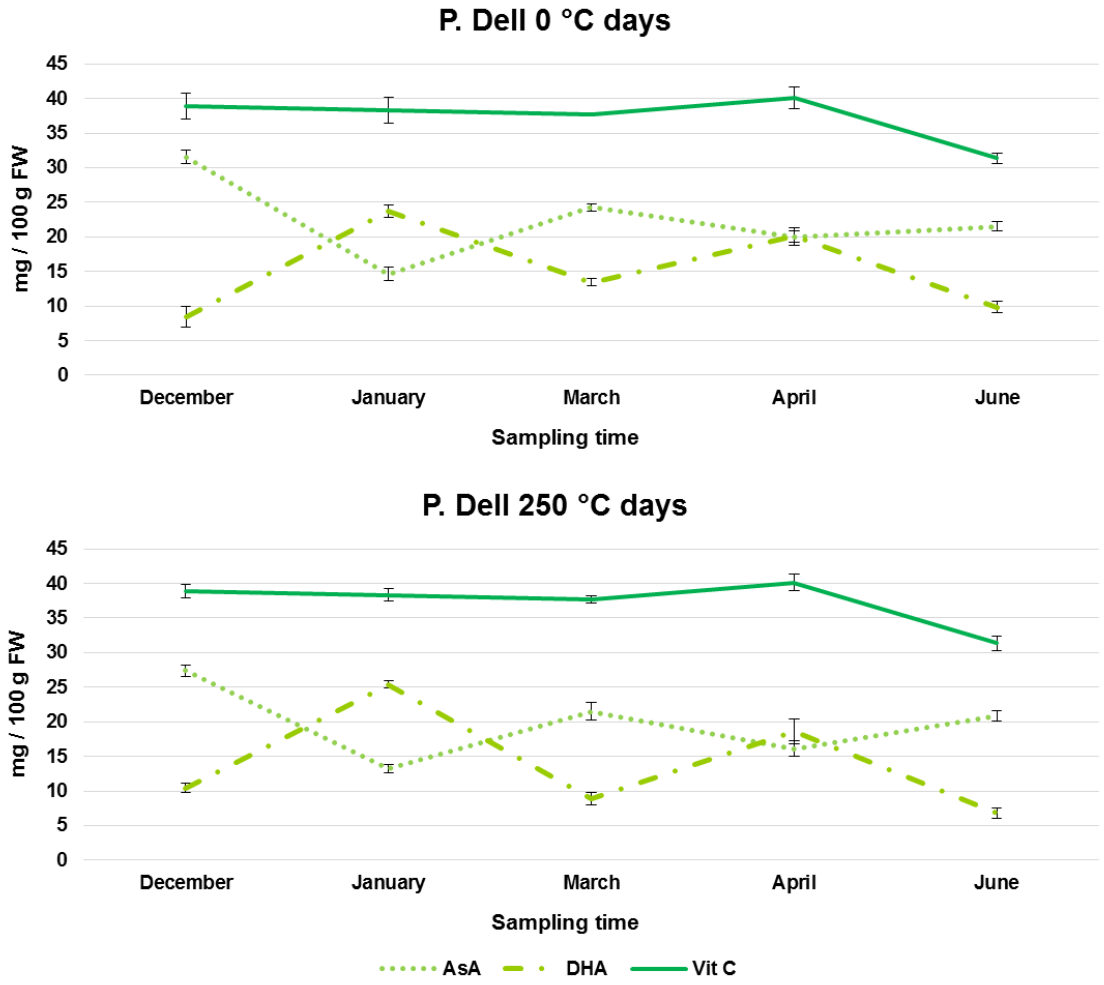


Figure 4-23 - Accumulation of accumulation of ascorbic acid (mg of AsA / 100 g FW), dehydroascorbic acid (mg of DHA / 100 g FW) and total Vitamin C for the season 2015/16 for P. Dell, 0°C days and 250°C days (chitting trial), with SE bars. For stats see 8.19 Appendix XIX.

4.4. 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) resulted in the formation of a brown polymerization product.

Hydrogen peroxide (H_2O_2) staining for season 2014/15 can be seen in Plate 4-1, Plate 4-2, Plate 4-3 and Plate 4-4, 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 superoxide content (O_2^-) (2014/15) depicted as a purple/blue precipitate was more prevalent in the middle cortex shown in Plate 4-5, Plate 4-6, Plate 4-7 and Plate 4-8 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^- content was observed during the third year of experiments.

Plate 4-1 - DAB staining (H2O2 detection) 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 4-2 - DAB staining (H2O2 detection) on VR 808, 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 4-3 - DAB staining (H2O2 detection) on P. Dell, 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).

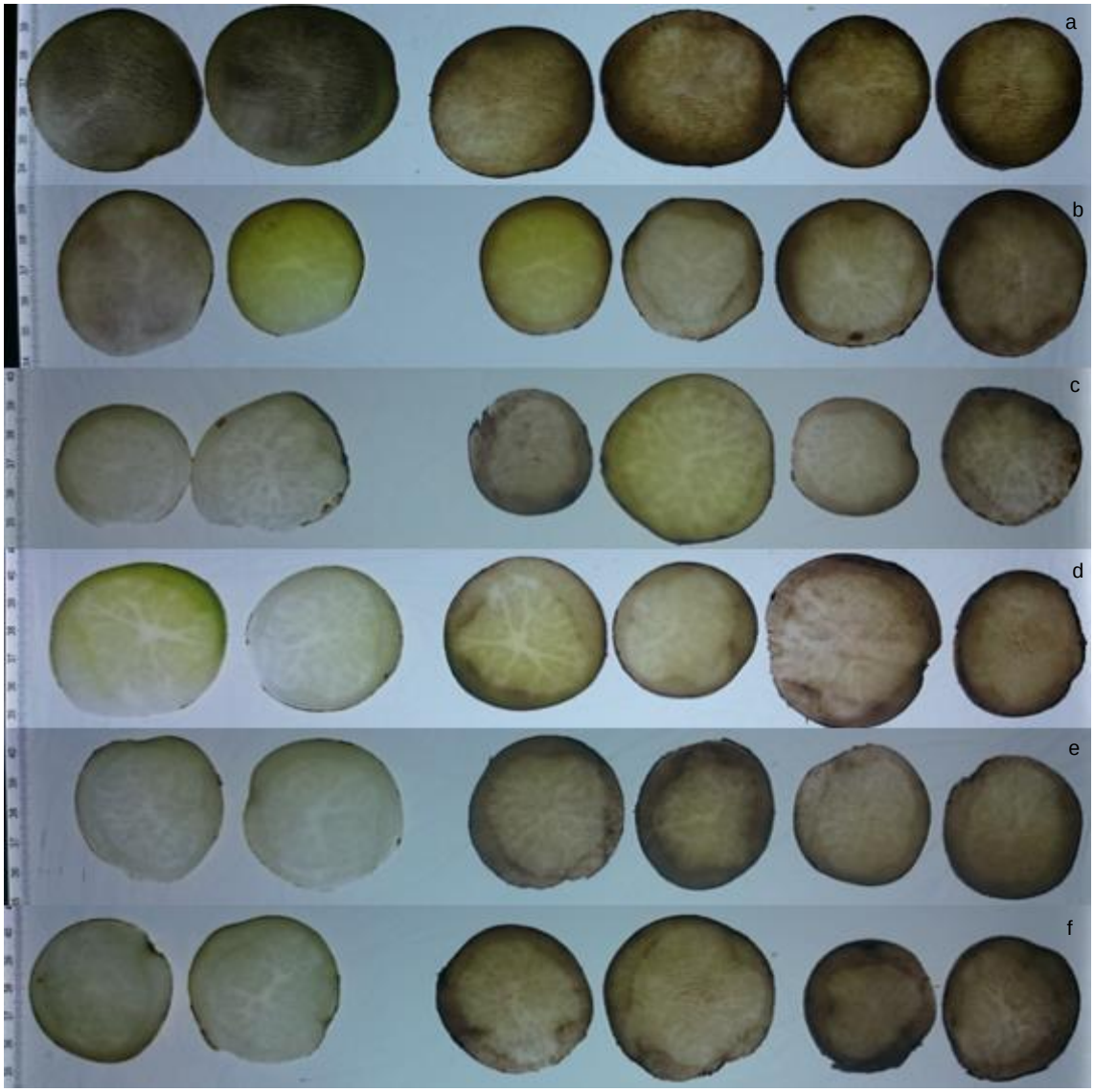


Plate 4-4 - DAB staining (H2O2 detection) on R. Burbank, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

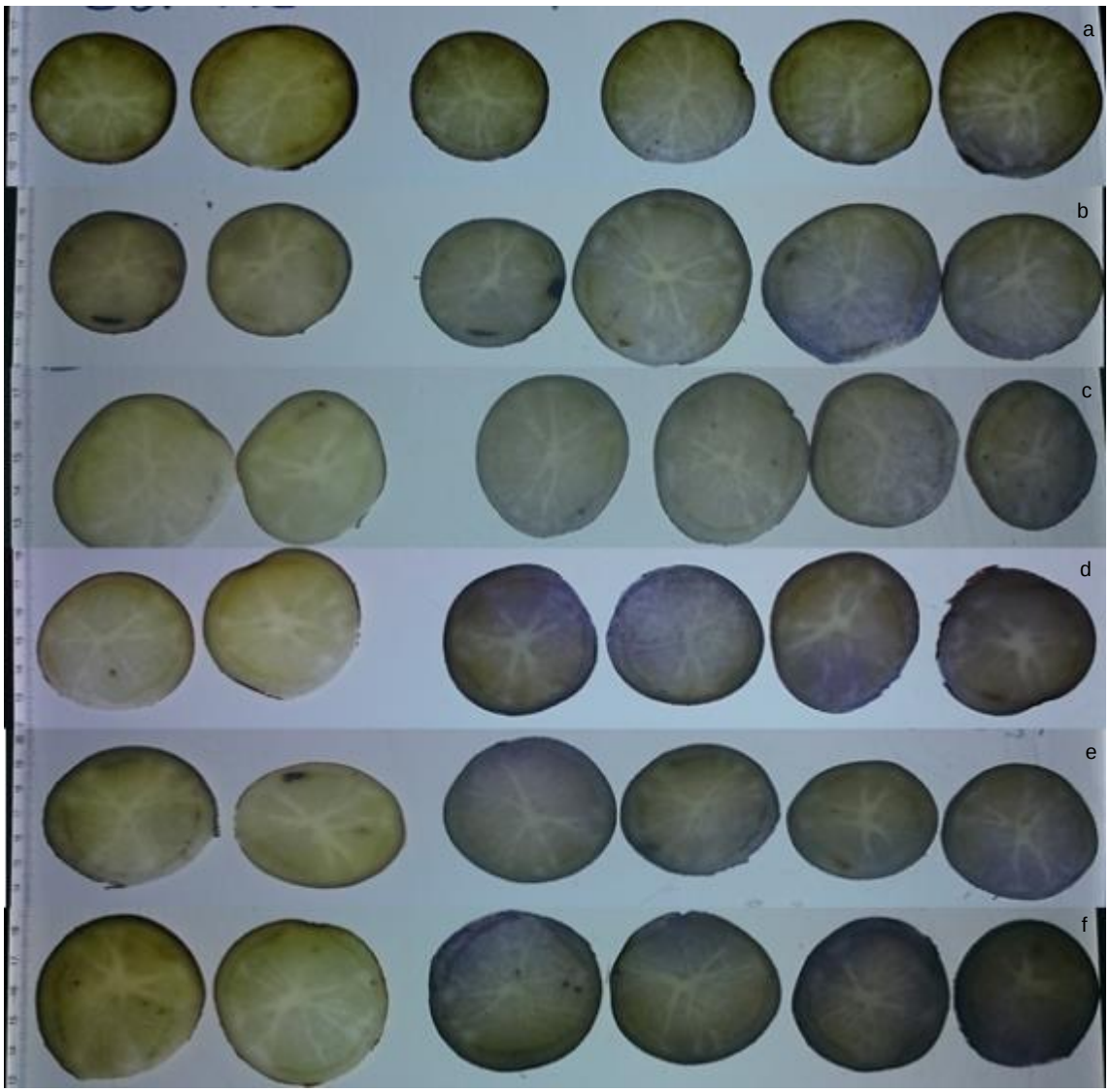


Plate 4-5 - NBT staining ($O2^-$ detection) on L. Rosetta, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

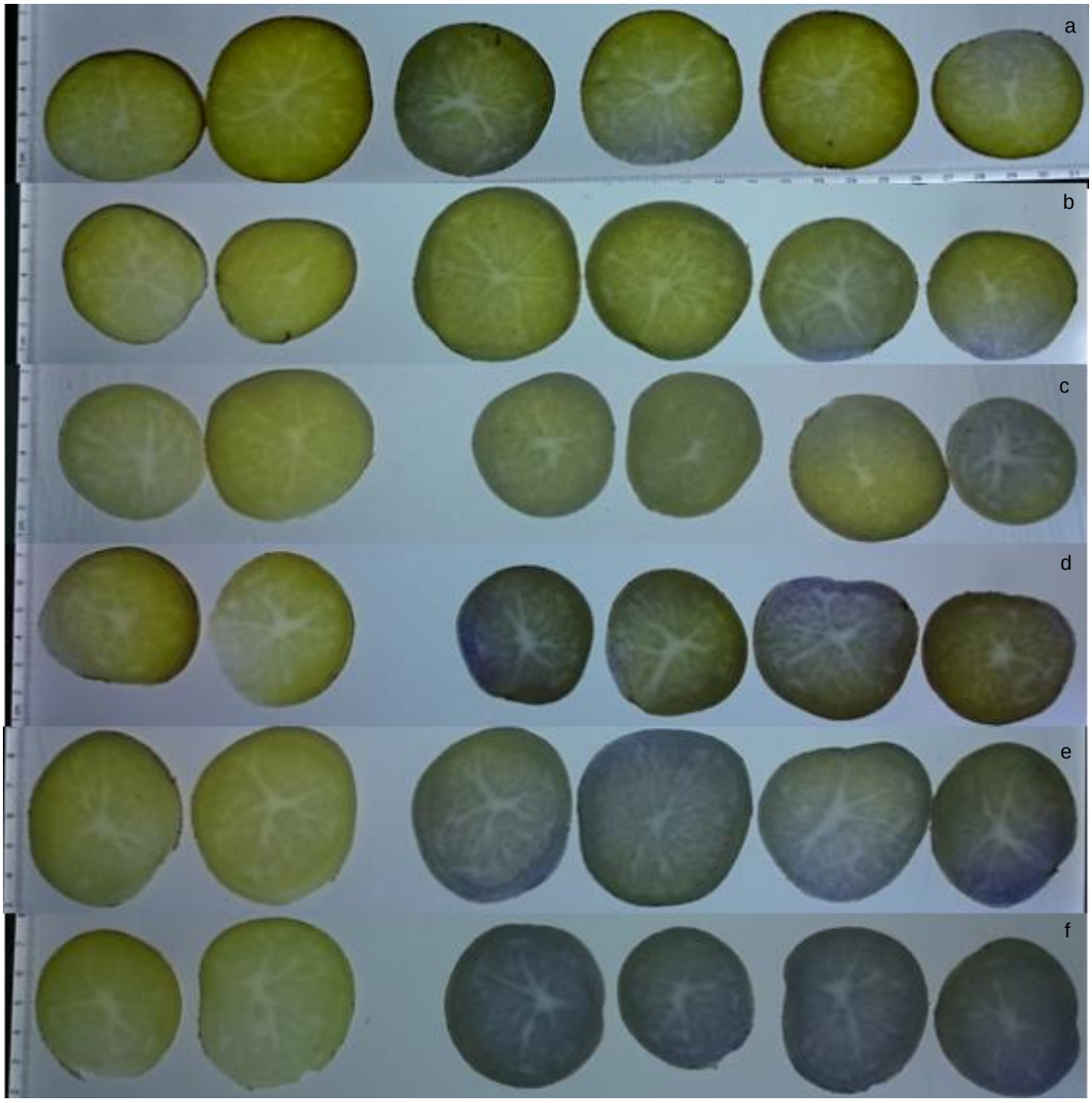


Plate 4-6 - NBT staining ($O2^{-}$ detection) on VR 808, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

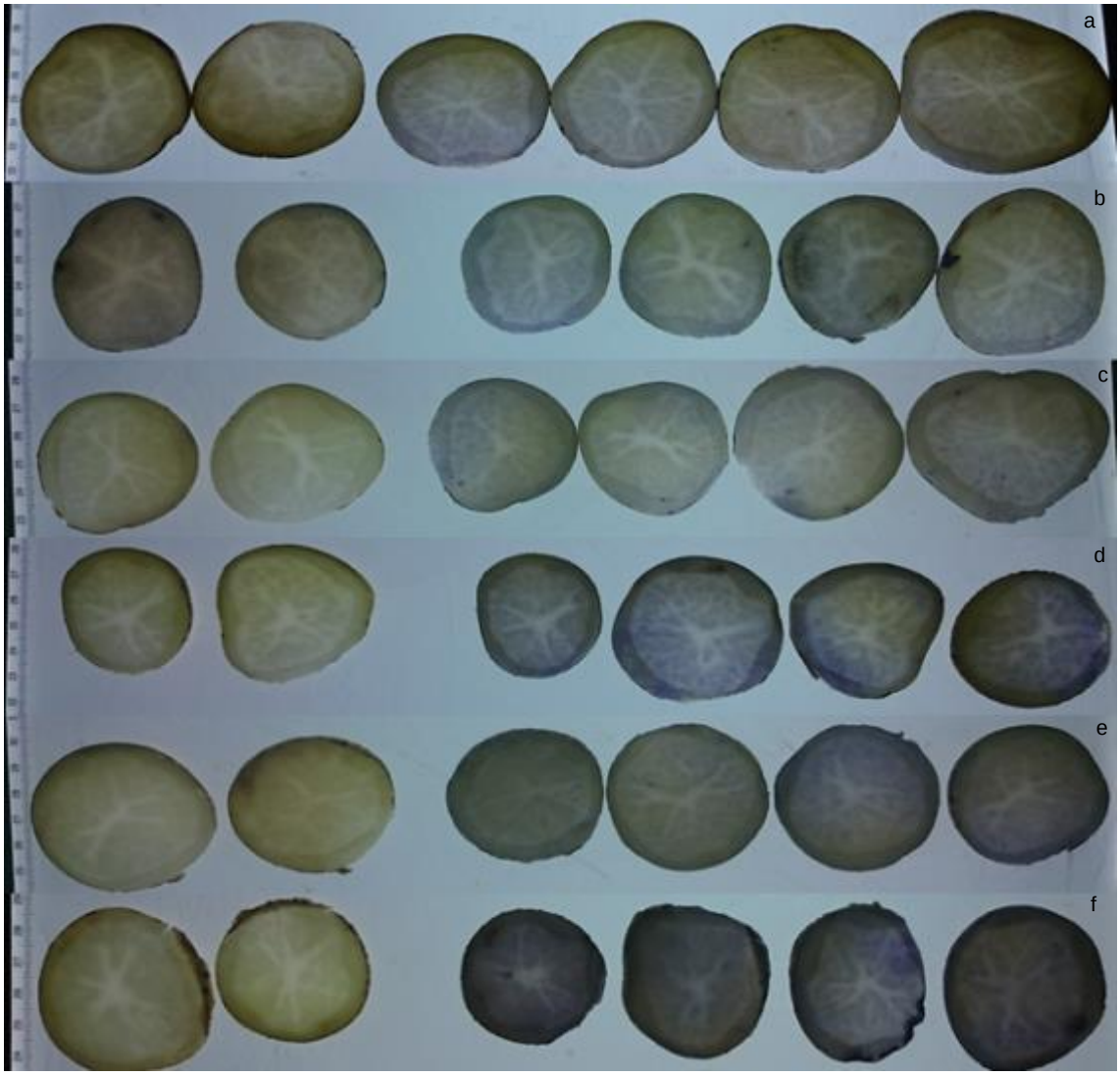


Plate 4-7 - NBT staining ($O2^-$ detection) on P. Dell, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

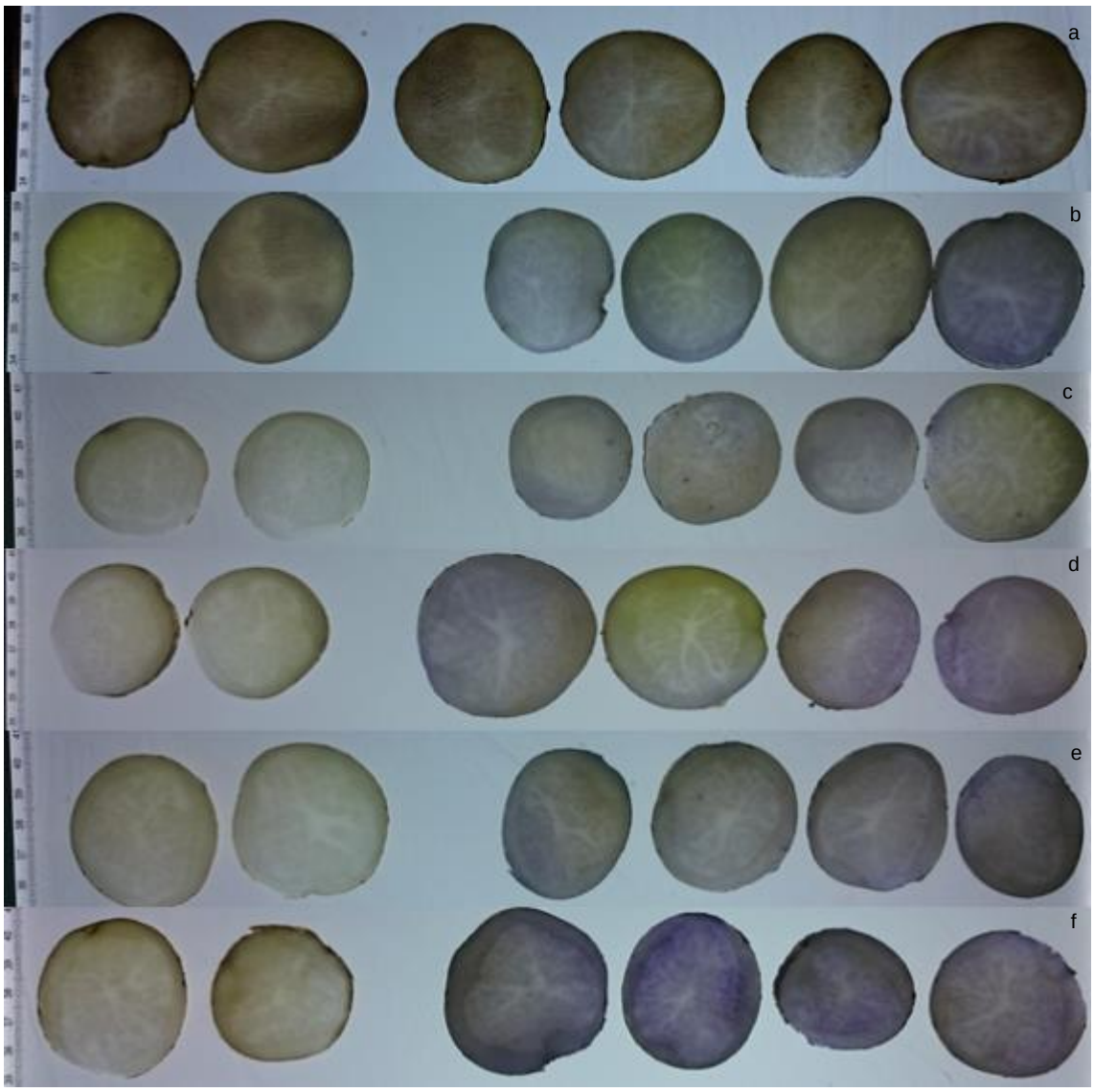


Plate 4-8 - NBT staining ($O2^-$ detection) on R. Burbank, a) February, b) March, c) April, d) May, e) June and f) July (season 2014/15). 1st two slices on the left are the control (no stain).

Development of purple and brown precipitates on 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 detection of H_2O_2) and purple (NBT detection 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 possible compare into 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).

The rate of H_2O_2 ($p < 0.001$) and O_2^- content varied ($p < 0.05$) between varieties 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 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 4-5).

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 4-5). During storage, an increase in H_2O_2 but not in O_2^- content ($p < 0.001$) was observed in both varieties.

Table 4-5 - H_2O_2 and O^{2-} content for seasons 2014/15 and 2015/16.

	Season 2014/15	
	H_2O_2	**O_2^-**
L. Rosetta	39.97[a]	38.93[a]
VR 808	27.19[a]	-0.87[b]
P. Dell	39.27[a]	48.57[a]
R. Burbank	27.01[a]	28.84[a]
$HSD_{0.05}$	*15.36*	*35.10*
	Season 2015/16	
	H_2O_2	**O_2^-**
L. Rosetta	38.28[a]	17.32[a]
VR 808	30.46[a]	9.19[b]
$HSD_{0.05}$	*14.08*	*24.17*

Mean values with different letters were significantly different according to TukeyHSD 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 4-24). When ROS activity was plotted against AsA and DHA content, DHA content raised soon after ROS (Figure 4-24).

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 4 24). H2O2 and $O2^-$ content depicted by staining patterns decreased after the early rise returning to content levels seen at or soon after harvest (Figure 4 24).

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 4 24).

In year 1 (2014/15), for L. Rosetta, content of H_2O_2 and O_2^- was very low during early storage (4 months), increasing from March (5 months) until July (7 months storage) (Figure 4-27). 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 4-27). 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 4-28).

In P. Dell and R. Burbank, O_2^- and H_2O_2 content increased with the length of storage (Figure 4-30). Changes in the content of O_2^- and H_2O_2 were similar. Interesting to note, especially in P. Dell, the increase in ROS corresponded with an increase in AsA and a decrease in DHA (Figure 4-30). In all varieties, there was a tendency within the tuber to overcome excessive ROS with increasing AsA concentration.

Multiple level correlation outputs between H_2O_2 and O_2^- with AsA/DHA and sugar profiles can be viewed in Figures 4-26, 4-27, 4-29, 4-30, 4-32 and 4-33. A significant correlation between H_2O_2 and O_2^- content depicted through staining profiles and sucrose concentration in VR 808, L. Rosetta and P. Dell in season 2014/15. It was interesting to notice that in that same season reducing sugars concentration positively correlated with H_2O_2 and O_2^- content in L. Rosetta and P. Dell (Figure 4-28 and Figure 4-31). And for R. Burbank even though H_2O_2 and O_2^- content positively correlated with reducing sugars but not sucrose (Figure 4-32). R. Burbank was the only variety where an increasing ratio of AsA/DHA was correlated with higher H_2O_2 content (Figure 4-32). P. Dell and R. Burbank, O_2^- and H_2O_2 content increased with the length of storage and glucose concentration (Figure 4-31 and Figure 4-32). However, just in P. Dell that O_2^- and H_2O_2 content positively correlated with fructose concentration (Figure 4-28).

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 4 26 and Figure 4-29), with a negative correlation between a decreasing H_2O_2 content and an increase in sucrose concentration. In VR 808 a decrease O_2^- content was correlated with a rise in sucrose concentration.

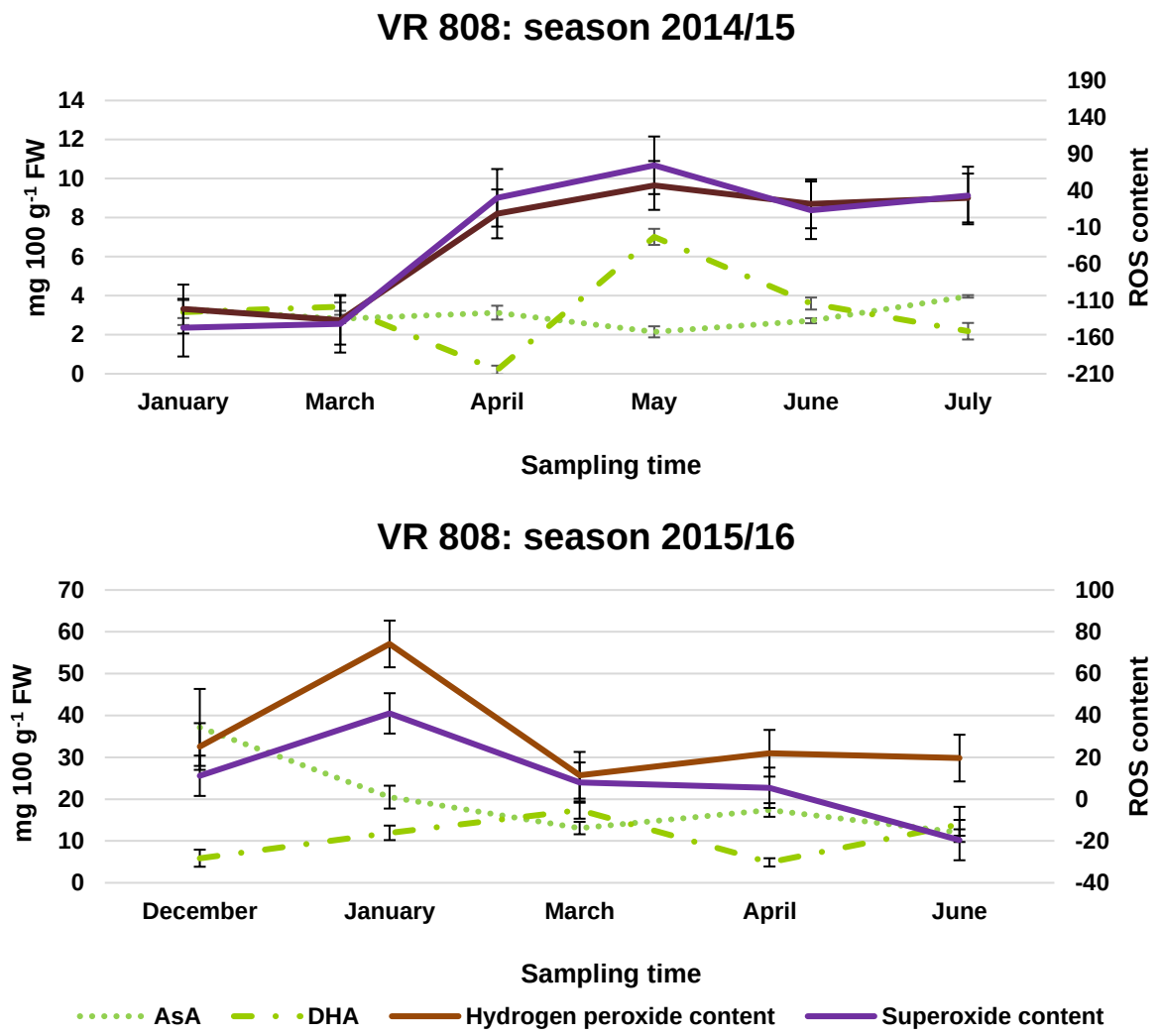


Figure 4-24 – Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) content in relation to AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) concentration for VR 808 tubers from seasons 2014/15 and 2015/16.

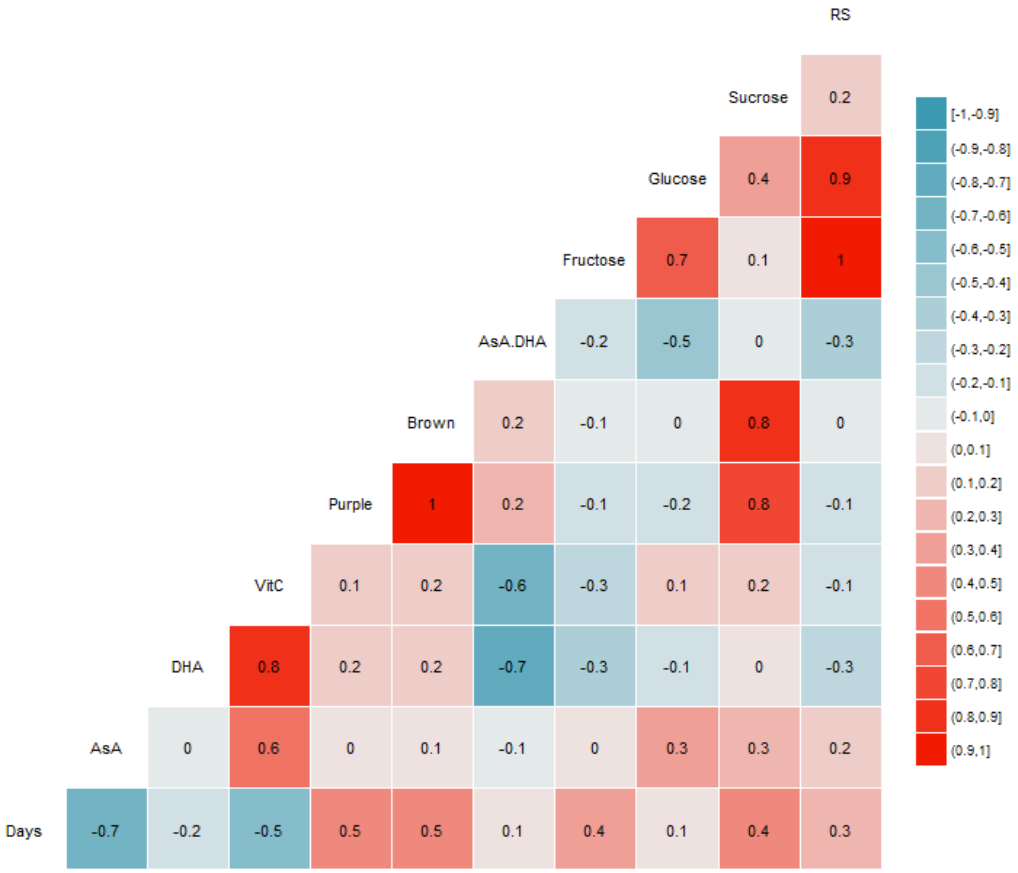


Figure 4-25 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for VR 808 (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

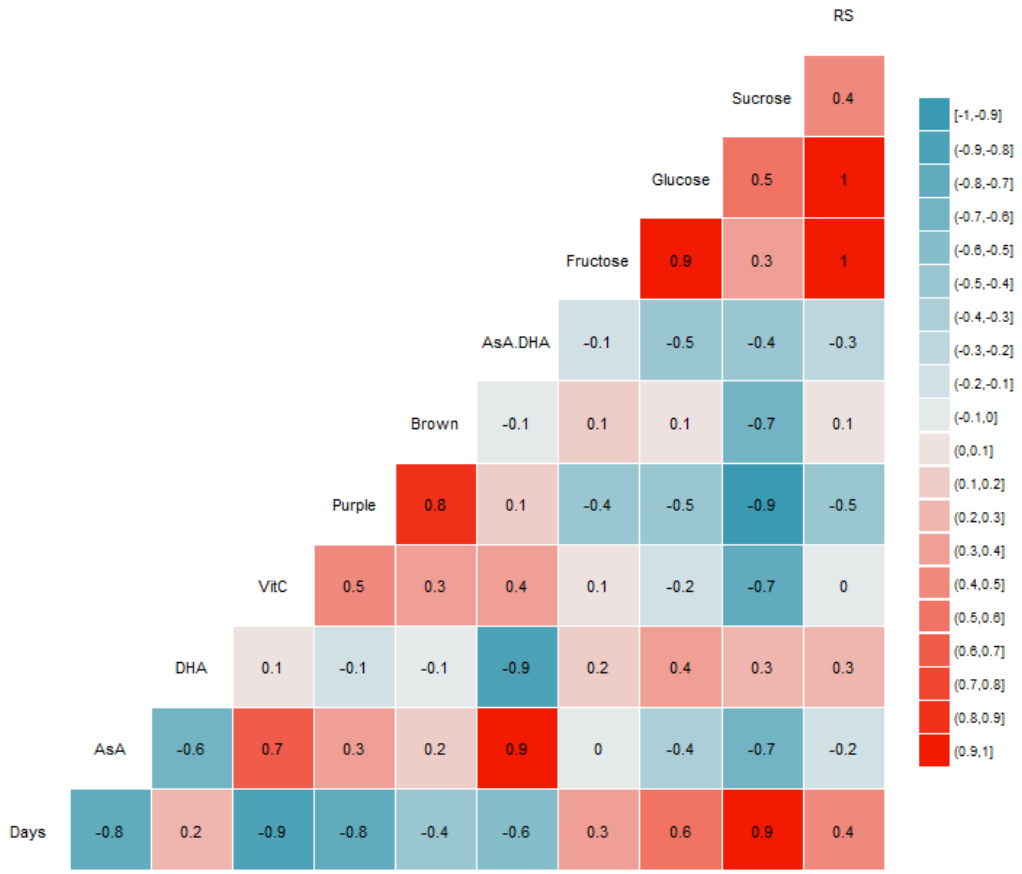


Figure 4-26 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for VR 808 (2015/16) (df = 4). Significant Pearson correlation coefficients from 0.73 to 1 and from -0.73 to -1 ($p < 0.05$).

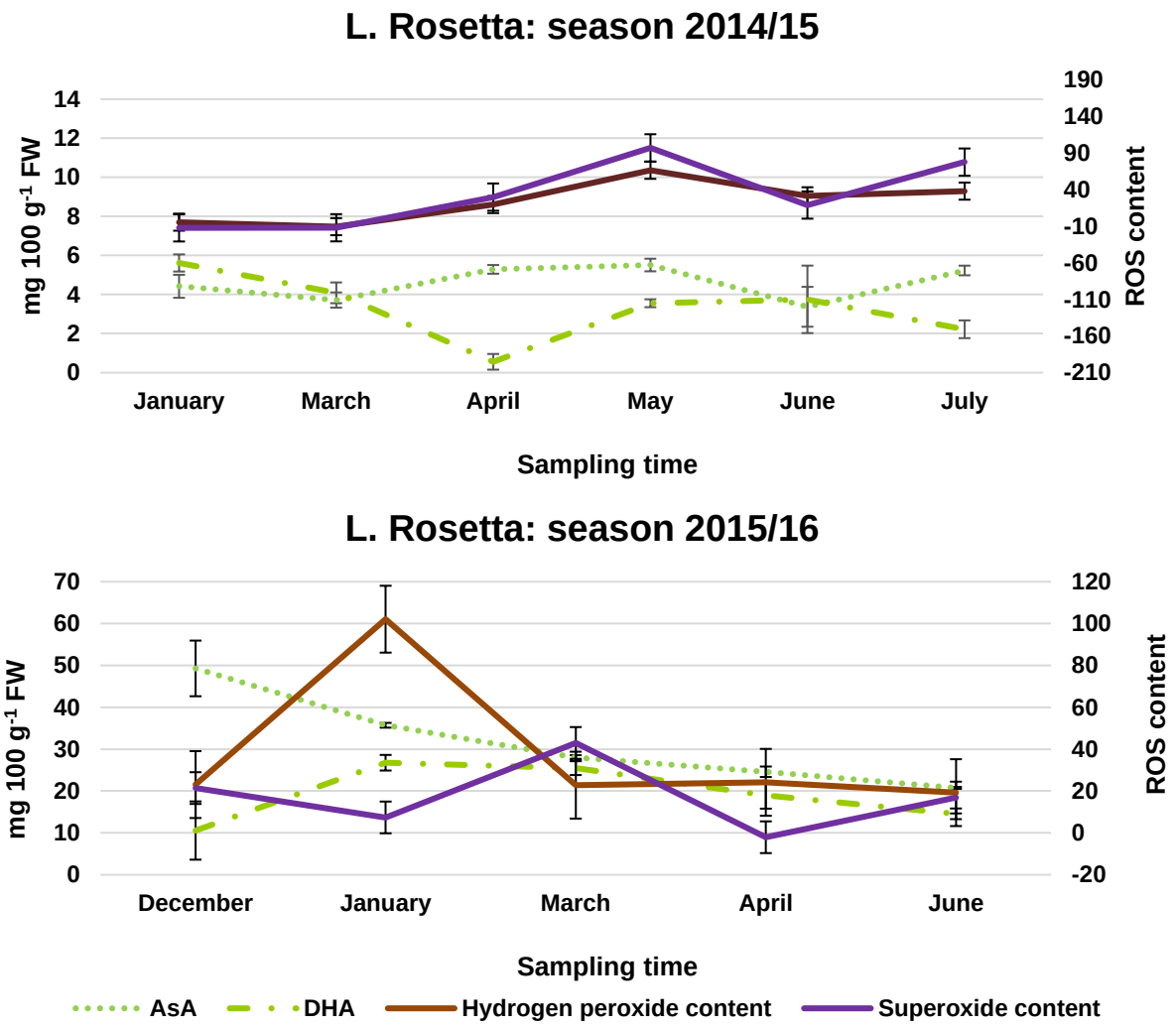


Figure 4-27 – Superoxide (O_2^-) and hydrogen content (H_2O_2) content in relation to AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) concentration for L. Rosetta tubers from seasons 2014/15 and 2015/16.

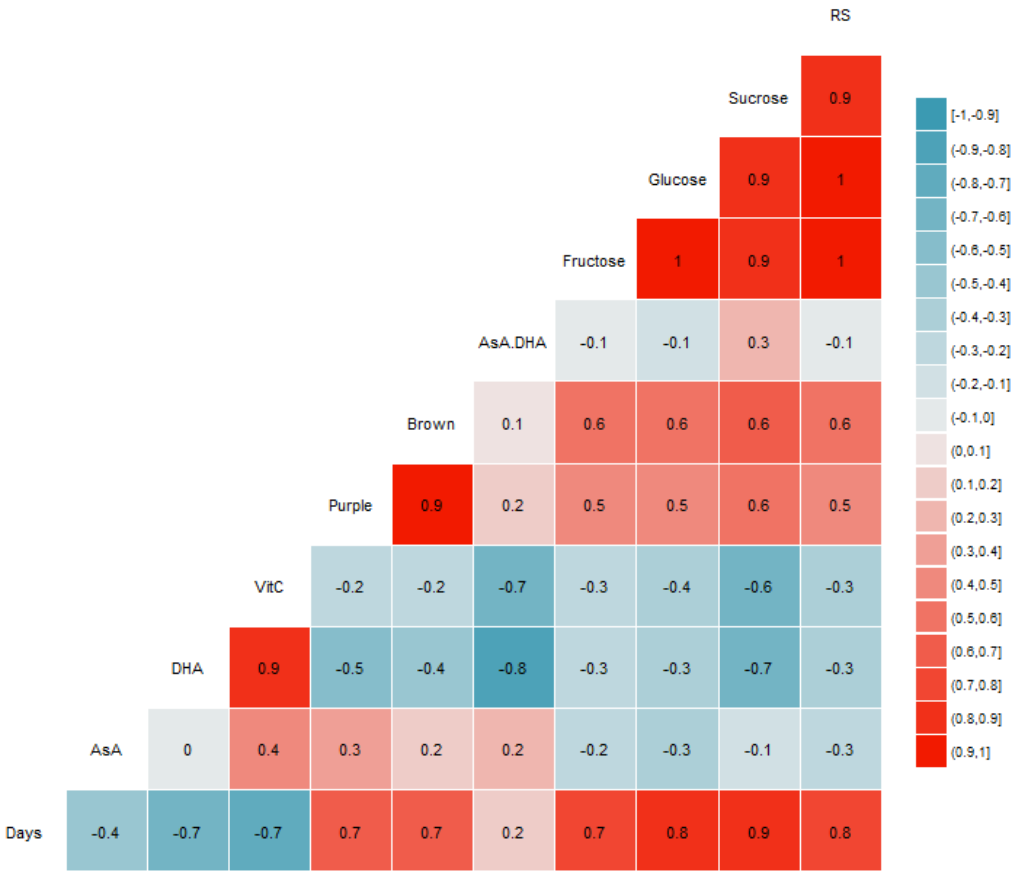


Figure 4-28 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for L. Rosetta (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

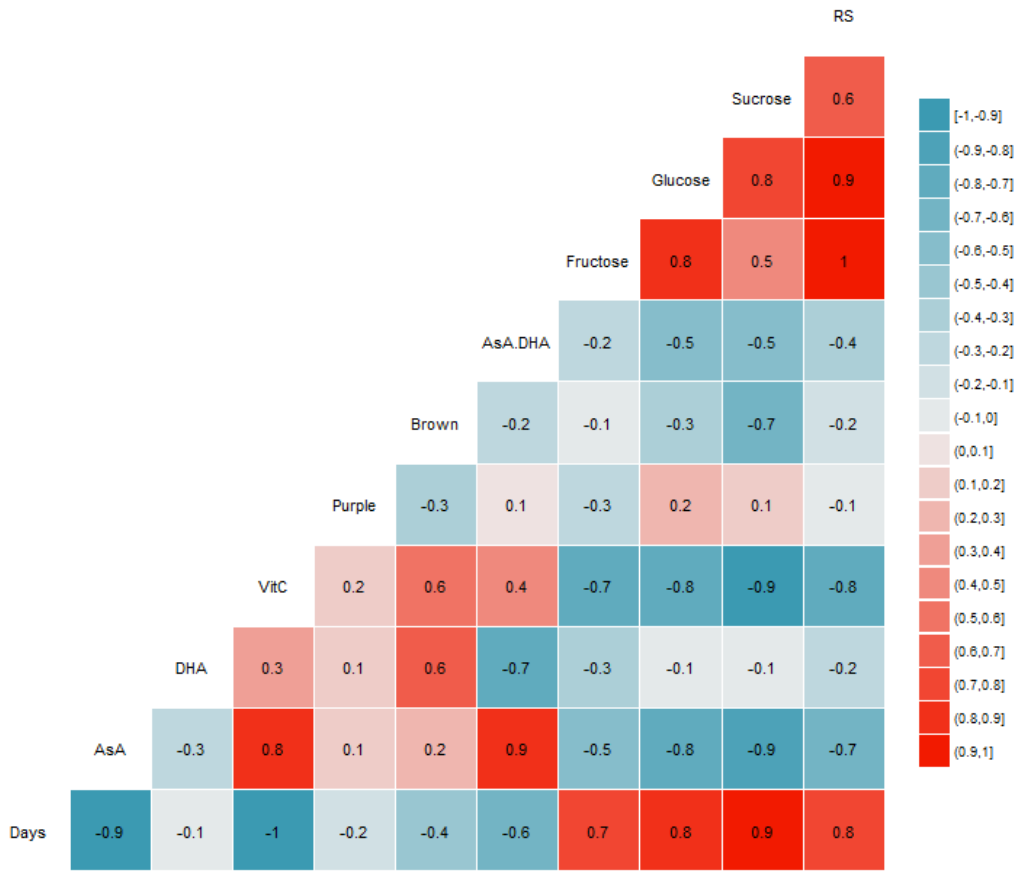


Figure 4-29 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for L. Rosetta (2015/16) (df = 4). Significant Pearson correlation coefficients from 0.73 to 1 and from -0.73 to -1 ($p < 0.05$).

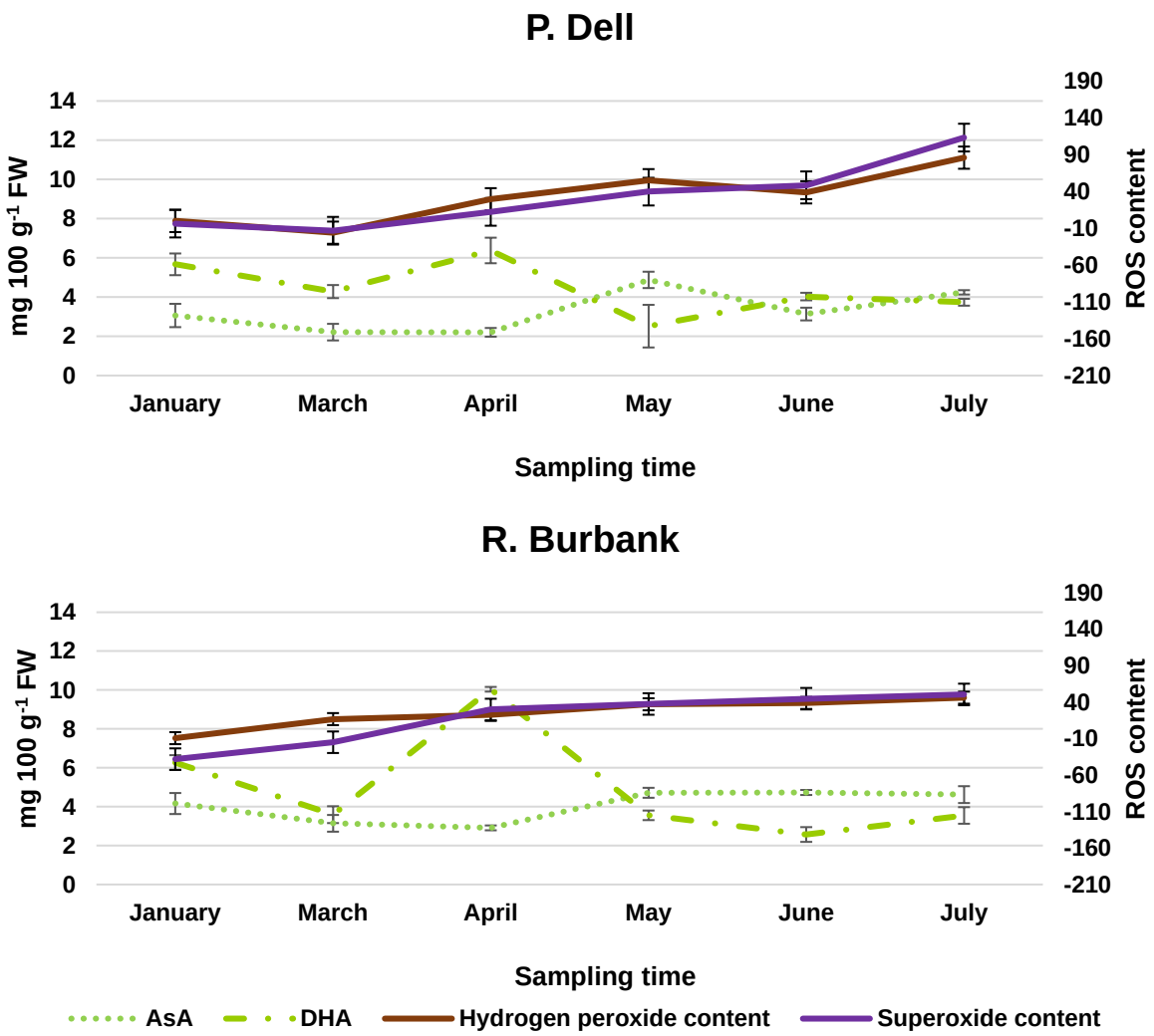


Figure 4-30 – Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) content in relation to AsA (mg 100 g^{-1} FW) and DHA (mg 100 g^{-1} FW) concentration for P. Dell and R. Burbank tubers from season 2014/15.

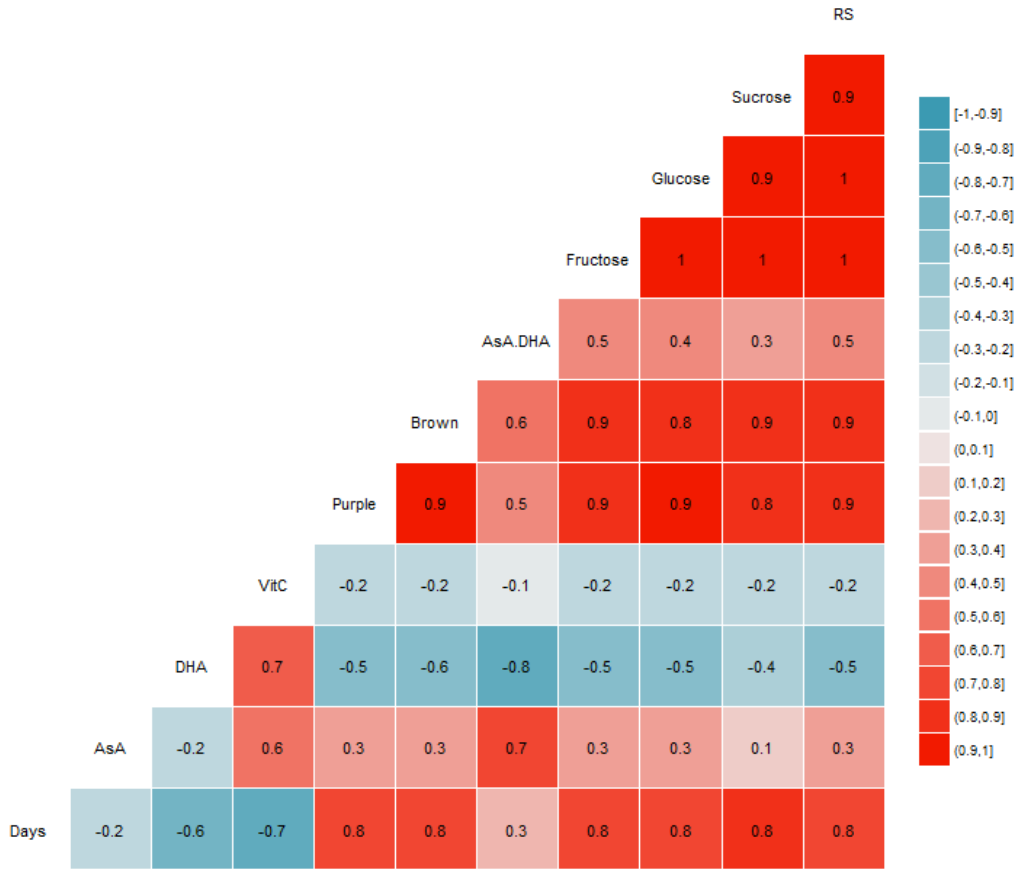


Figure 4-31 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for P. Dell (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

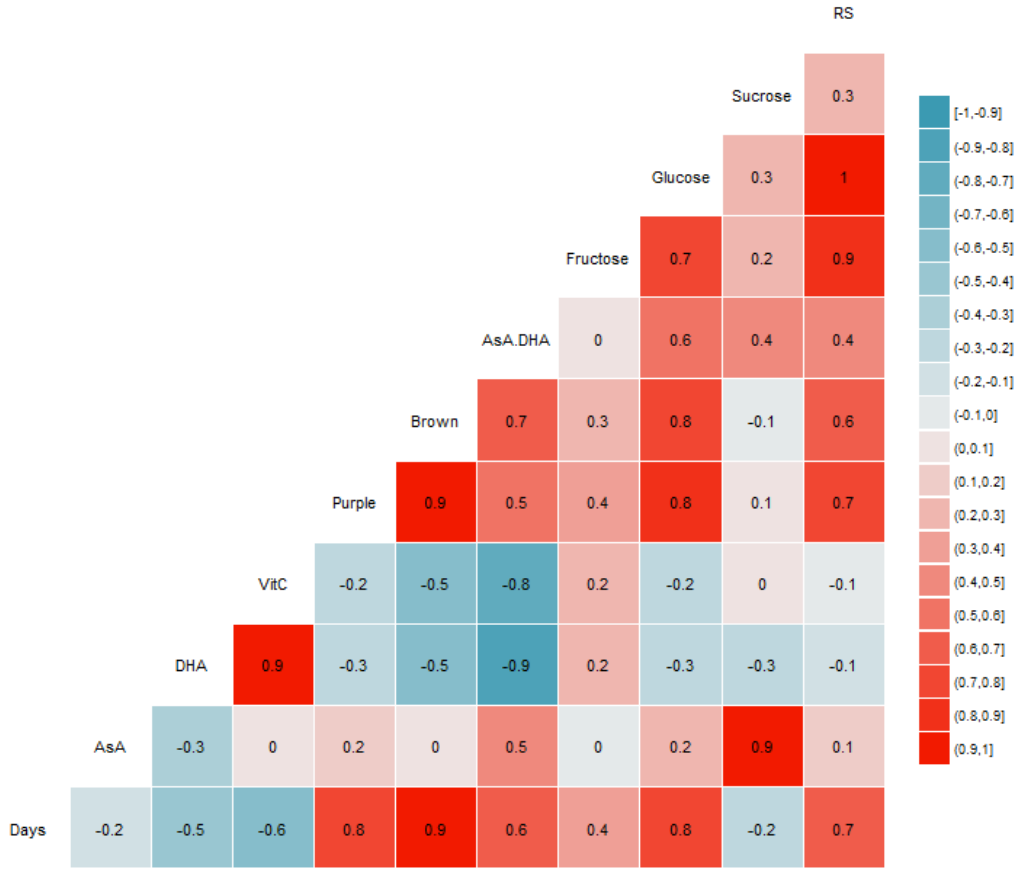


Figure 4-32 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %FW of reducing sugars (RS) for R. Burbank (2014/15) (df = 5). Significant Pearson correlation coefficients from 0.67 to 1 and from -0.67 to -1 ($p < 0.05$).

4.5. The effect of physiological aging (Chitting) of seed potatoes on ROS accumulation

Physiologically aging of seed tubers (chitted) prior to planting influenced O_2^- activity ($p < 0.01$), but not H_2O_2 activity in tubers harvested from chitted plants. An overall increase in H_2O_2 activity ($p < 0.001$) was observed across treatments after 2 months (January) of storage and then declined afterwards (Figure 4-33-c).

Multiple correlation analysis of ROS, antioxidant content and sugars performed against tubers from plants grown from chitted and non-chitted seed found a decrease in H_2O_2 activity over time in non-physiologically aged seed tubers was at the same time an increase in DHA accumulation, and all the sugars (Figure 4-34).

For physiologically aged seed tubers H_2O_2 activity decreased over the storage period in storage while fructose and glucose increased. O_2^- activity was negatively correlated with DHA accumulation and positively correlated with sucrose accumulation (Figure 4-35).

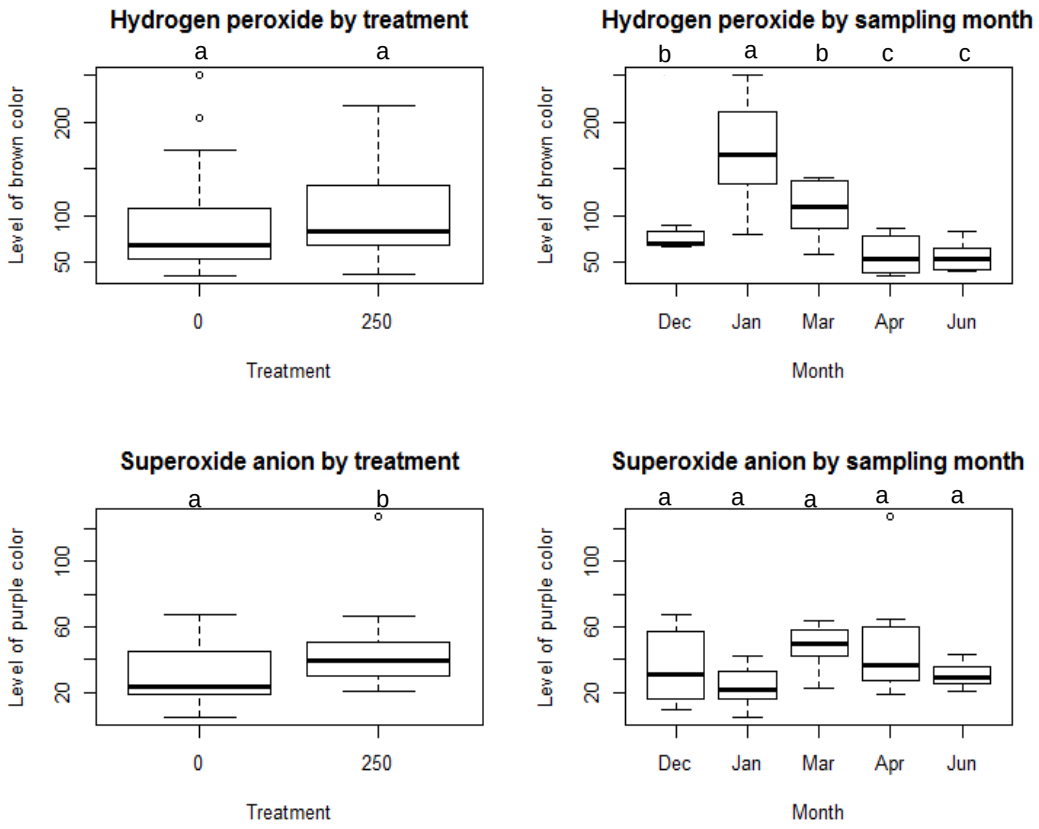


Figure 4-33 – Hydrogen peroxide (H2O2) activity by treatment (0°C days and 250°C days) (a) and by sampling month (b), and superoxide anion ($O2^-$) levels by treatment (0°C days and 250°C days) (c) and by sampling month (d), for P. Dell from the chitting trial. Mean values with different letters were significantly different (a) HSD0.05 = 17.42, b) HSD0.05 = 13.06, c) HSD0.05 = 39.13 and d) HSD0.05 = 29.33). The median is shown as a thicker dark line.

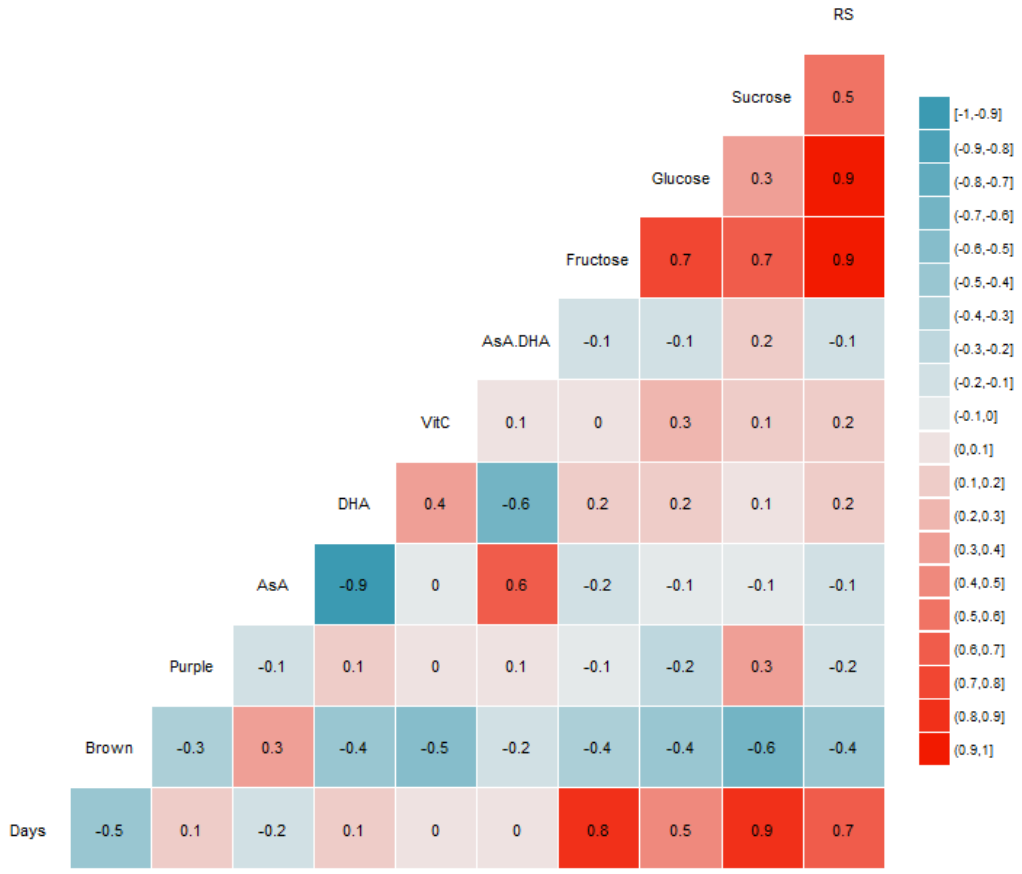


Figure 4-34 – Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %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 ($p < 0.05$).

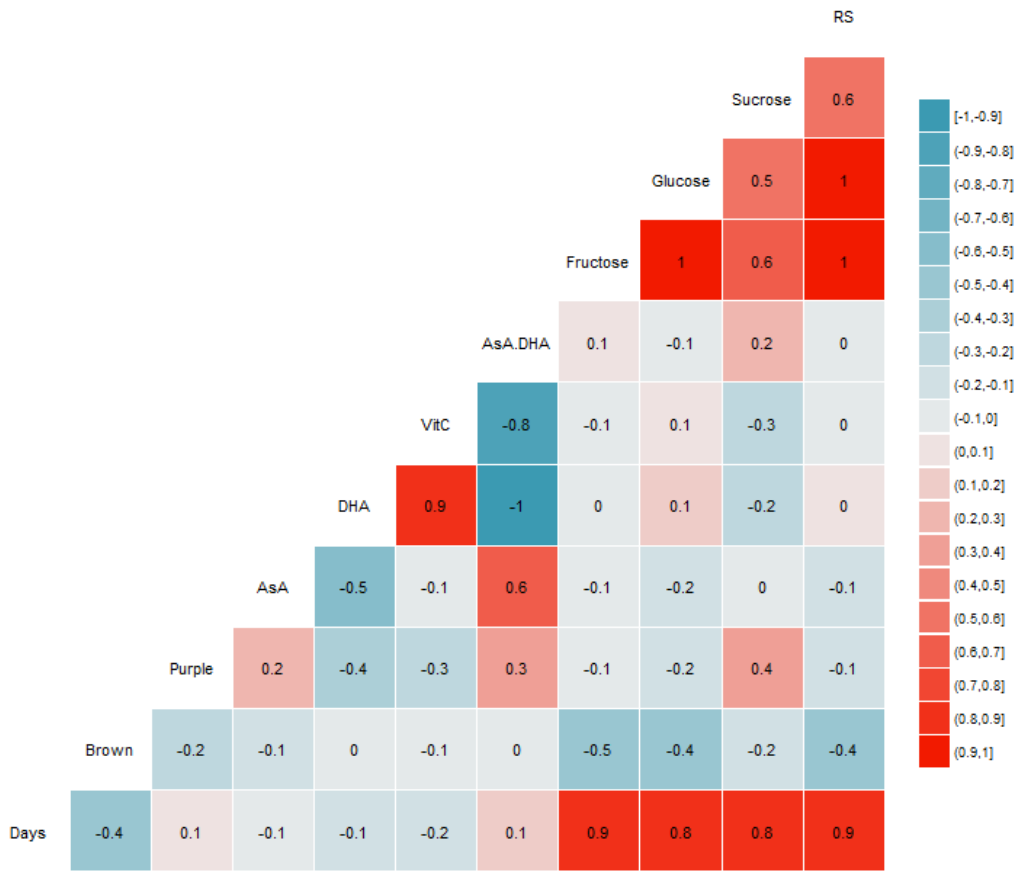


Figure 4-35 - Correlation between days in storage (Days), AsA concentration (AsA), DHA concentration (DHA), total vitamin C concentration (VitC), O_2^- content (Purple), H_2O_2 content (Brown), AsA/DHA ratio (AsA.DHA), %FW of fructose (Fructose), %FW of glucose (Glucose), %FW of sucrose (Sucrose) and %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 ($p < 0.05$).

4.6. Assessment of tuber texture during storage

Wedge 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 4-36 and Figure 4-37). P. Dell (Figure 3-39) required the most energy to induce cell fracture due to increased deformation of tissue (Plate 4-9), with tubers sampled at the end of storage compared to samples taken at harvest.

Location of cultivation had no impact on firmness for VR 808 (Figure 4-39) 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 4-39). With increasing storage duration, there was an increased in resistance to fracture (Figure 4-36).

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 4-37). Resistance to cell fracture increased over a 7-8 months period during storage at 10°C and then decreased by the final sampling at 9-10 months (July).

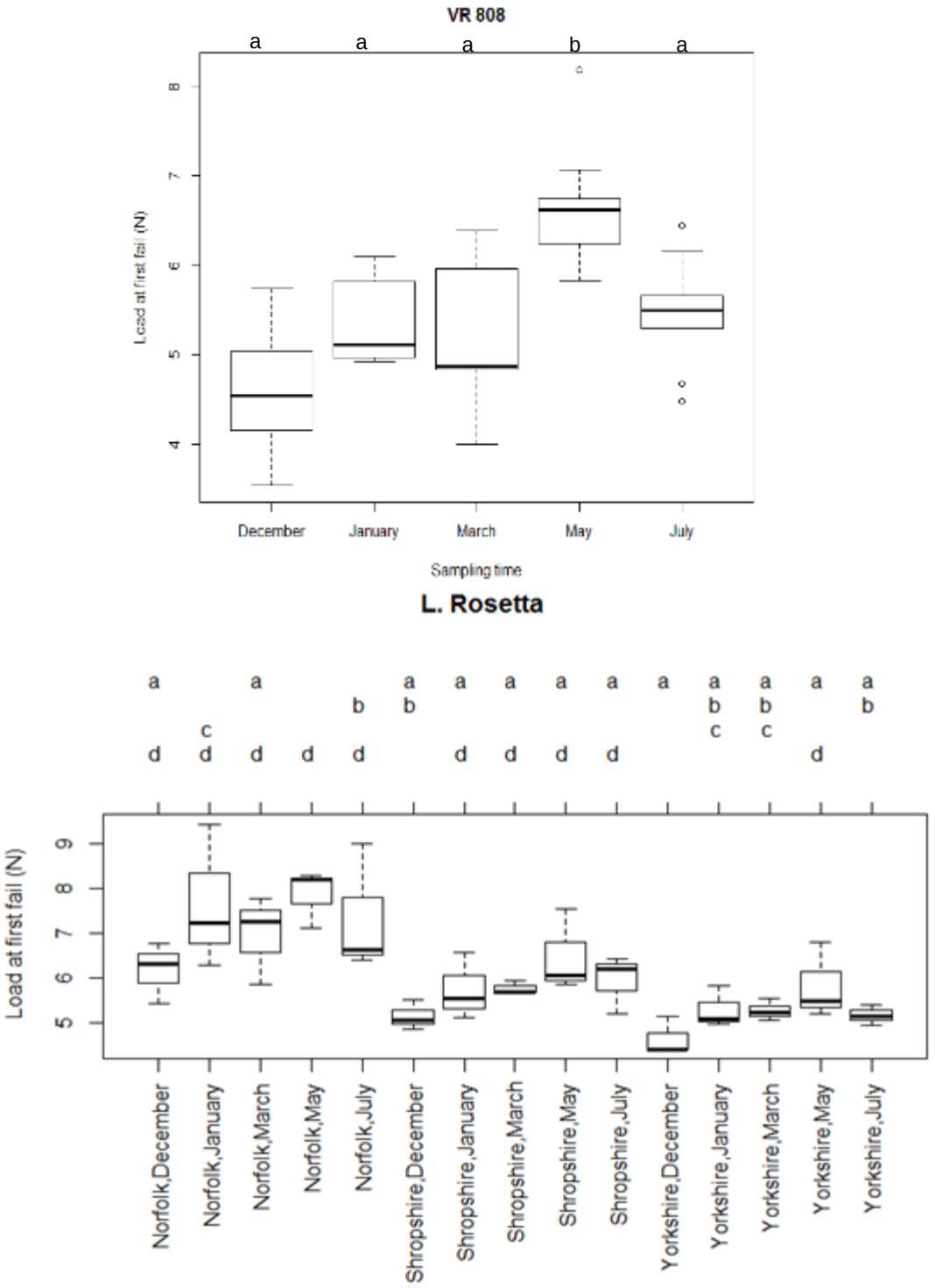


Figure 4-36 - Comparison of the variation in firmness (Load at first fail (N)) per sampling occasion for season 2013/14 for VR 808 and L. Rosetta. Mean values with different letters are significantly different (HSD0.05 VR 808 = 0.99 and HSD0.05 L. Rosetta= 1.1). The median is shown as a thicker dark line.

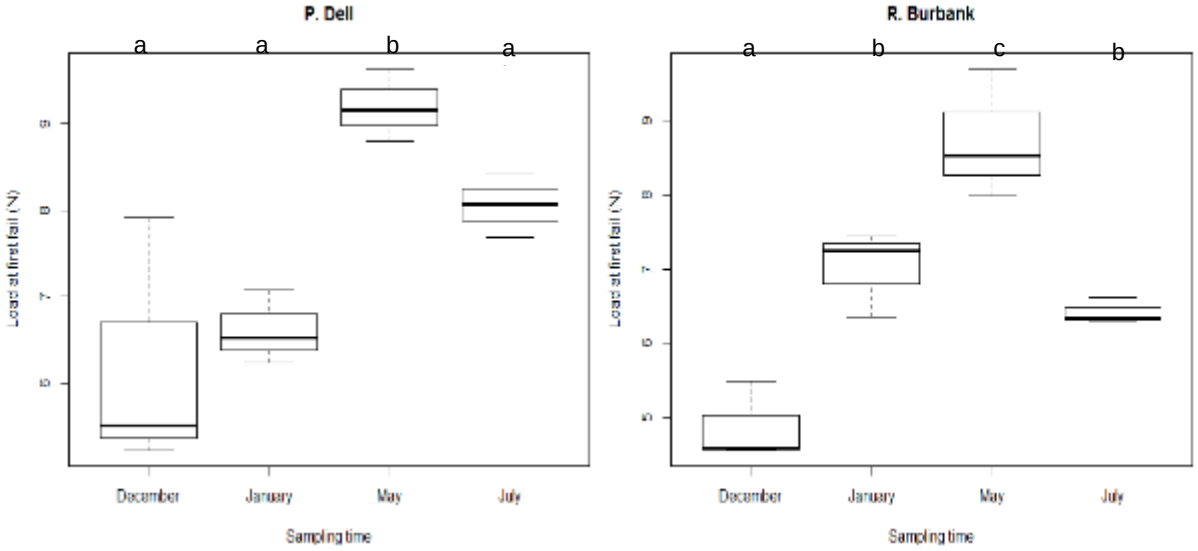


Figure 4-37 - Comparison of the variation in firmness (Load at first fail (N)) per sampling occasion for season 2013/14 for (HSD0.05 P. Dell = 2.13 and HSD0.05 R. Burbank = 1.55). The median is shown as a thicker dark line.

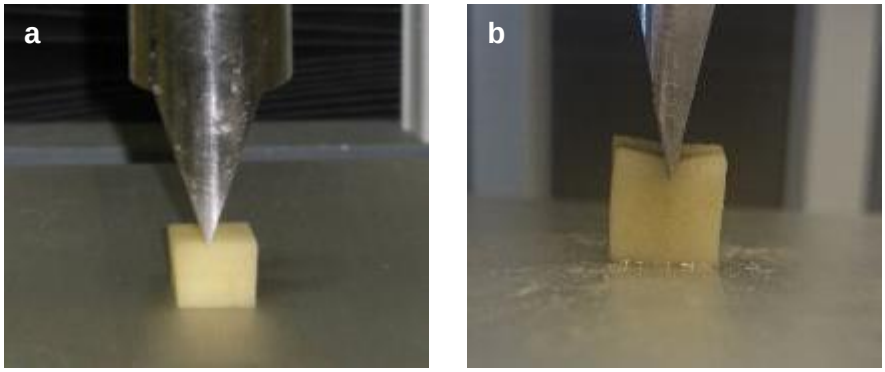


Plate 4-9 – VR 808 sample in Lloyd LRX-plus texture analyser from December 2013 (a) and May 2014 (b)

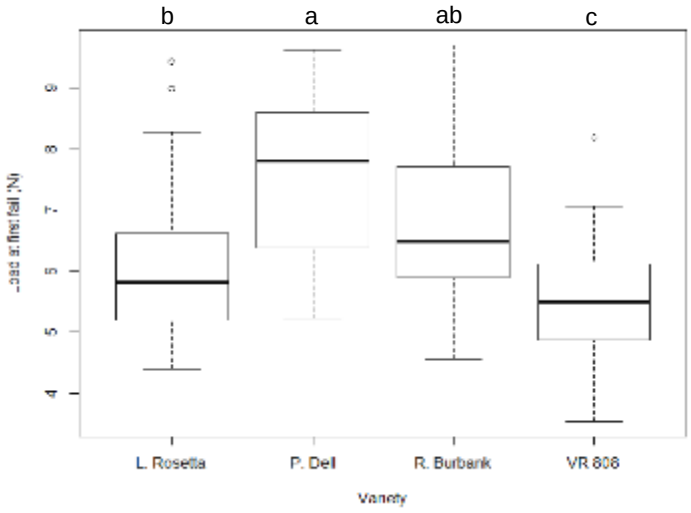


Figure 4-38 - Comparison of the variation in firmness (Load at first fail (N)) for the 4 varieties used during season 2013/14. Mean values with different letters are significantly different (HSD0.05 = 0.76). The median is shown as a thicker dark line.

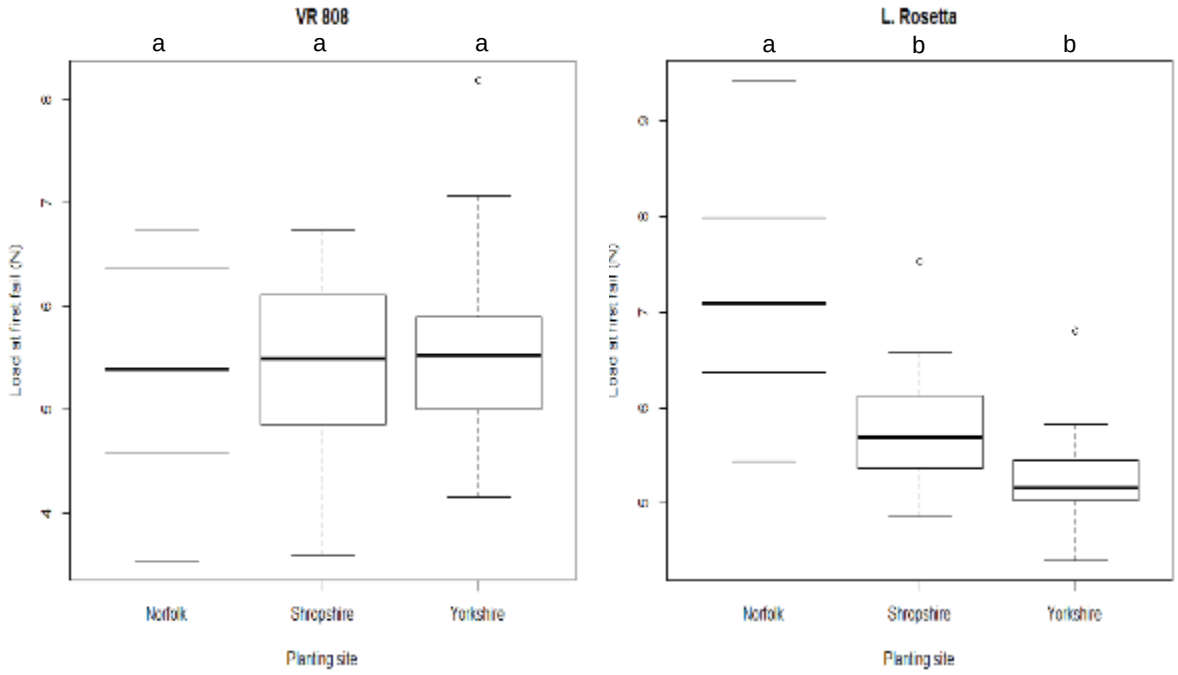


Figure 4-39 - Comparison of the variation in firmness (Load at first fail (N)) for the 3 planting sites of season 2013/14 for VR 808 and L. Rosetta. Mean values with different letters are significantly different (HSD0.05 VR 808 = 0.65 and HSD0.05 L. Rosetta = 0.72). The median is shown as a thicker dark line.

4.7. Assessment of amyloplast and cellular changings during storage using scanning electron microscope (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 were first observed at the end of storage (9 months at 10°C).

In 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 to freeze dry powder (Plate 4-10). 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 instable under the beam.

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. However, fractures started to appear in January (4 months) (Plate 4-11 and 8.20 Appendix XX) in samples of L. Rosetta (8.21 Appendix XXI) and VR 808 (Plate 4-11), but such symptoms were delayed until March (5 months) for P. Dell (8.22 Appendix XXII) and R. Burbank (8.23), 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 4-11).

The first signs in changes in amyloplast membrane integrity were the appearance of surface fractures. The timiming of onset of fractures was dependent 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 from middle of the tuber (inner core – medulla or pith) was first observed later after 170 days of storage (March, 8.20 Appendix XX). In contrast, for 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 4-12), 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 4-13 and Plate 4-14). 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.

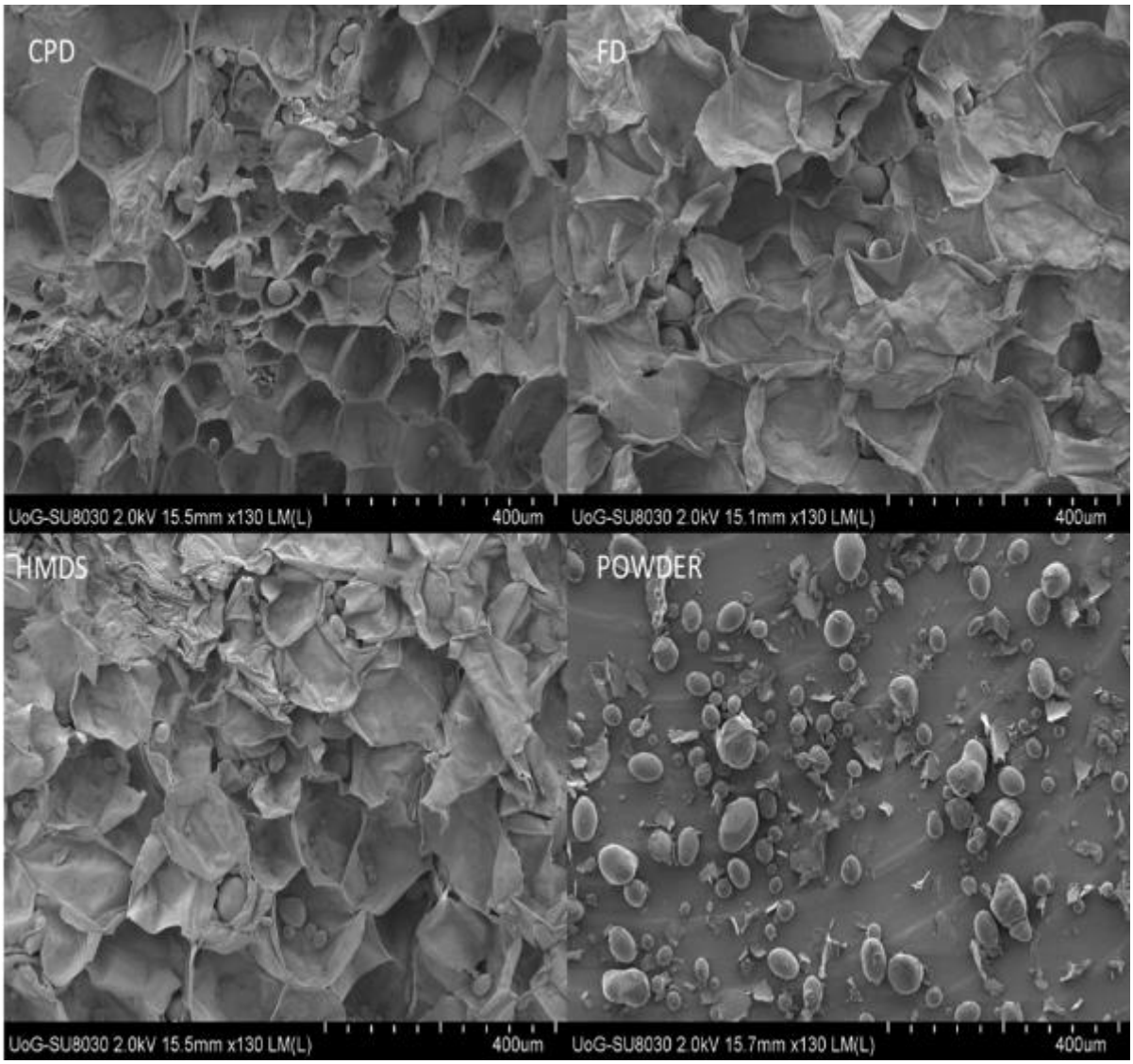


Plate 4-10 - *SEM images obtained from the different methods of fixation/dehydration of samples (CPD, Critical Point Dry; FD, Dehydration by freeze drying; HMDS, Hexamethyldisilazane, and Powder, Powder freeze dry tissue)*

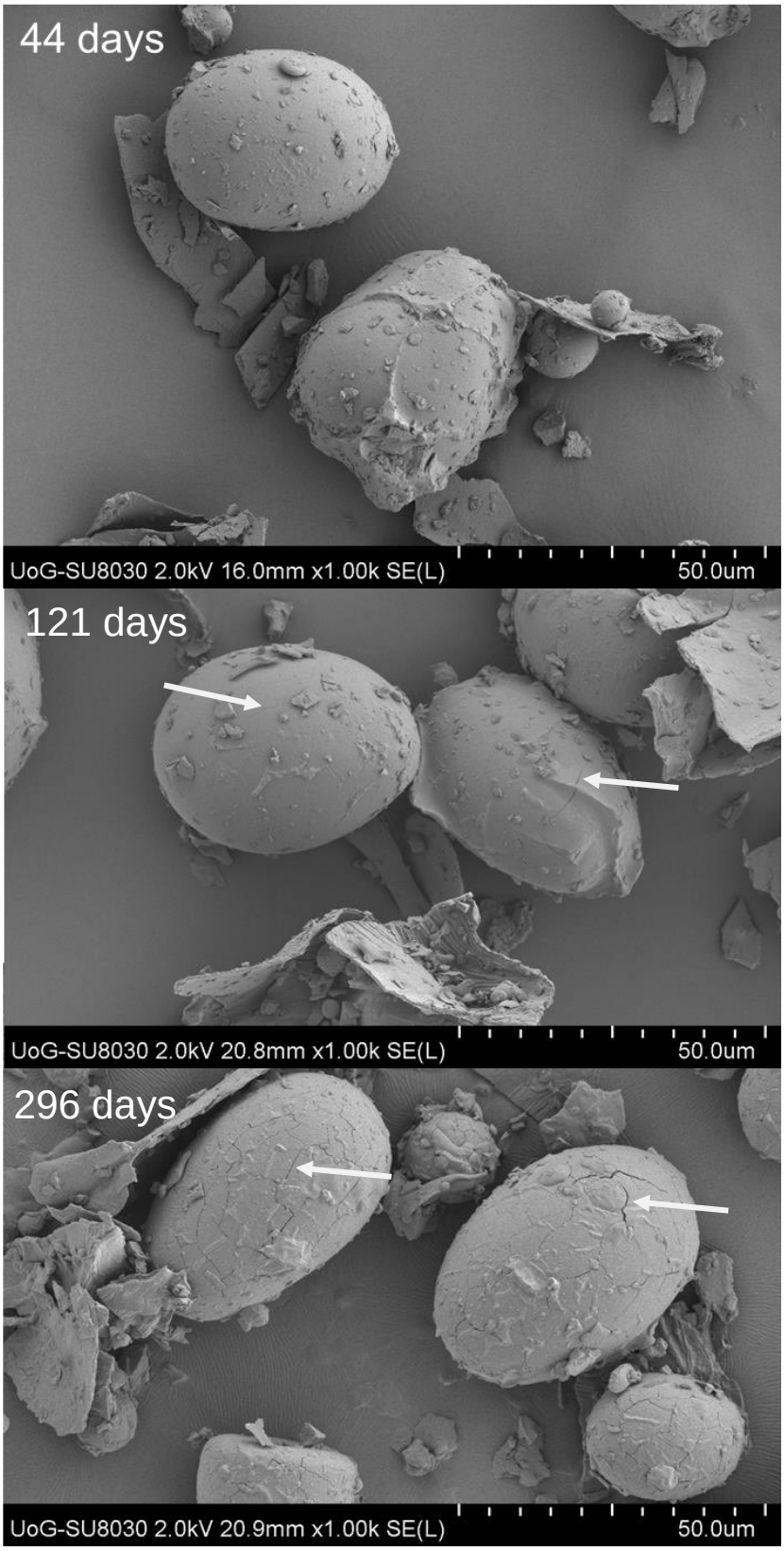


Plate 4-11 - SEM images from edge section of VR 808 with 44 days (November 2014), 121 days (January 2015, 1st visible fractures) and 296 days (July 2015) of storage. White arrows point to fractures.

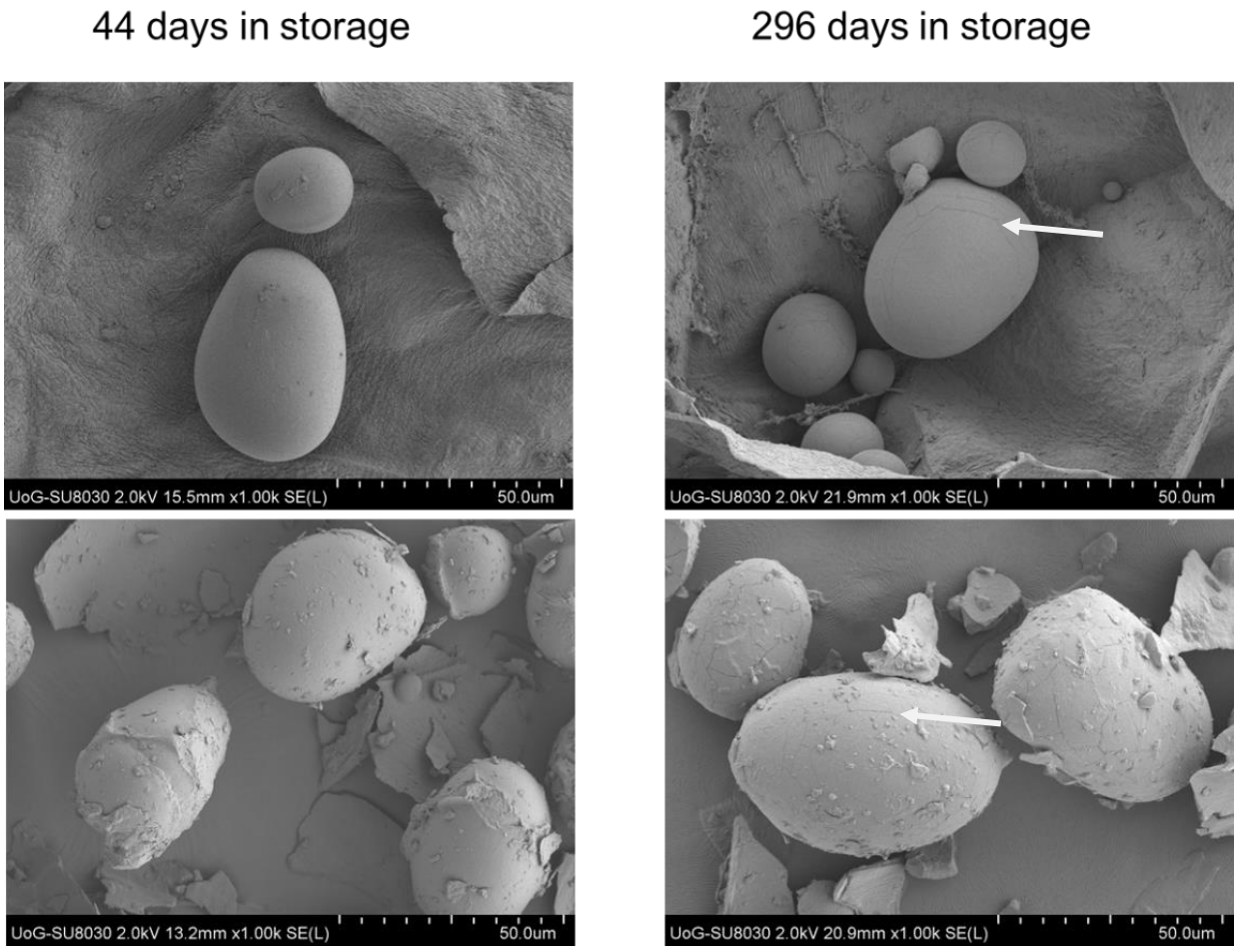


Plate 4-12 - Samples from VR 808 (storage season 2014/15), top images obtained with CPD and down images obtained with powder freeze dry tissue. White arrows point to fractures.

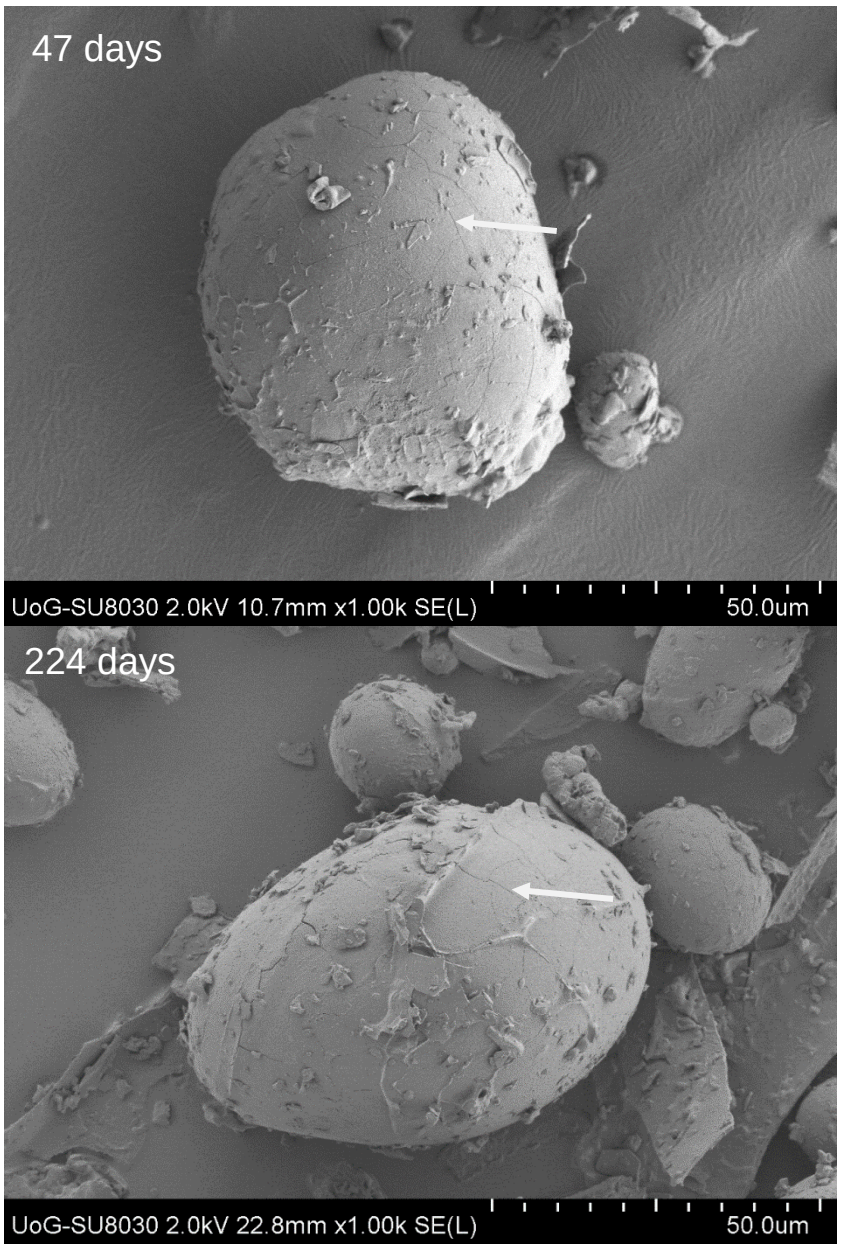


Plate 4-13 - SEM image from edge section of VR 808 with 47 days (November 2015, with visible fractures) and 224 days (June 2016, with deeper fractures) of storage. White arrows point to fractures.

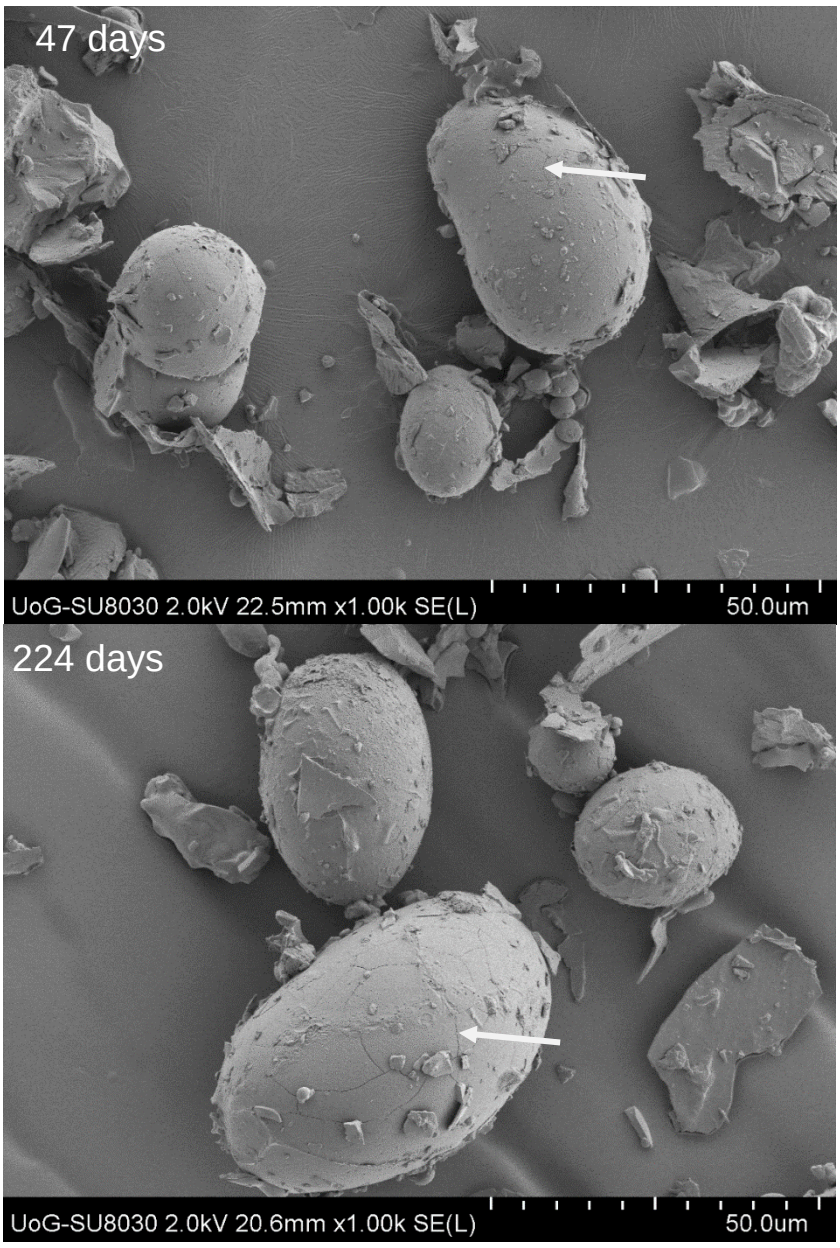


Plate 4-14 - SEM image from edge section of L. Rosetta with 47 days (November 2015, with visible fractures) and 224 days (June 2016, with deeper fractures) of storage. White arrows point to fractures.

4.8. 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 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) when averaged across the two samples taken from the edges 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 4-6).

Other mineral elements such as Mg and K can compete and displace calcium from binding sites and influence the overall calcium activity even when 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 characteristic could not be fully explained by the availability of calcium antagonists. While, P. Dell, tubers accumulated greater amounts of K (1226 mg/100g) the lowest content was found in L. Rosetta (978.6 mg/100g). However, L. Rosetta (54 mg/100g) and P. Dell (53 mg/100) were higher in Mg (Table 4-6).

In other stored crops, such as apple the ration 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 mg/100g) (Table 4-6).

For all the varieties Ca_{total} and Ca_{bound} content in the edges of the tuber were significantly different from the inner core of the tuber (Table 4-7) suggesting that availability of calcium may change across the tuber. Comparison of nutrients sampled at the opposite edges (eighths) 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 4-7). No clear relationship between mineral distribution and the propensity to sweeten could be concluded from the data with the exception that K 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 4-8 show no increase in calcium binding, and in 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 4-8).

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 4-40 and Figure 4-41). Interesting to notice that just in R. Burbank was observed a negative correlation between %FW of sucrose and %FW of reducing sugars (Figure 4-41).

Table 4-6 – 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/100g FW	Ca_{bound} mg/100g FW	% Ca_{bound}	K mg/100g FW	Mg mg/100g FW	K+Mg / Ca	Cu mg/100g FW	Fe mg/100g FW	P mg/100g FW	Zn mg/100g 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 TukeyHSD test.

Table 4-7 – Catotal, Cabound, %Cabound, 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/100g FW	Ca_{bound} mg/100g FW	% Ca_{bound}	K mg/100g FW	Mg mg/100g FW	K+Mg / Ca	Cu mg/100 g FW	Fe mg/100 g FW	P mg/100 g FW	Zn mg/100 g FW
VR 808	Edges	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	Edges	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	Edges	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	Edges	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 TukeyHSD test.

Table 4-8 – 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/100g FW	Ca_{bound} mg/100g FW	% Ca_{bound}	K mg/100g FW	Mg mg/100g FW	K+Mg / Ca	Cu mg/100 g FW	Fe mg/100 g FW	P mg/100 g FW	Zn mg/100 g 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 TukeyHSD test.

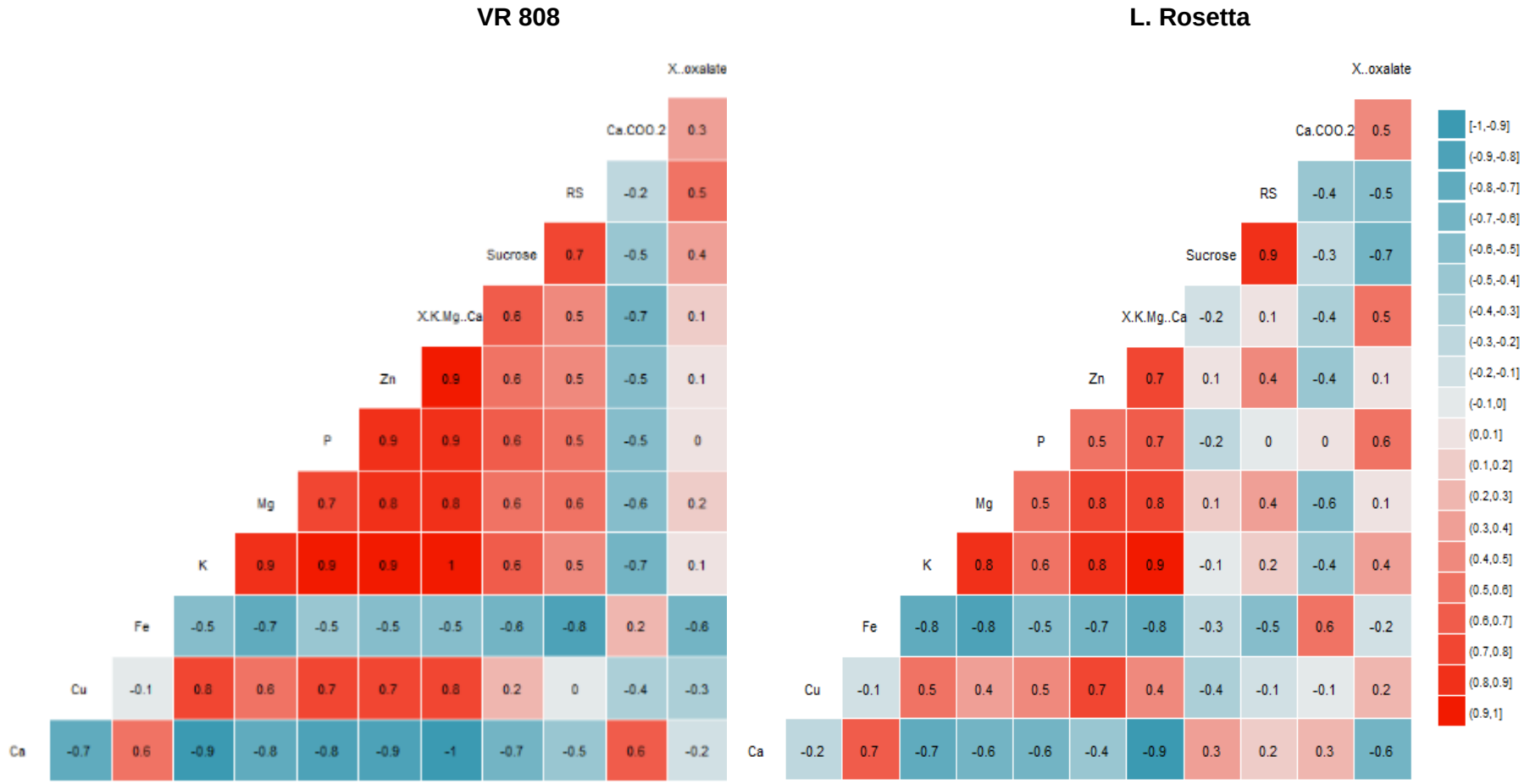


Figure 4-40 – Correlation between Catotal (Ca), Cu, Fe, K, Mg, P, Zn, (K+Mg)/Ca (X.K.Mg..Ca), Ca bound (Ca.COO.2), %Cabound (X.oxalate), sucrose and reducing sugars (RS) during the storage season 2014/15 for the varieties VR 808 and L. Rosetta. Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$, $df = 18$).

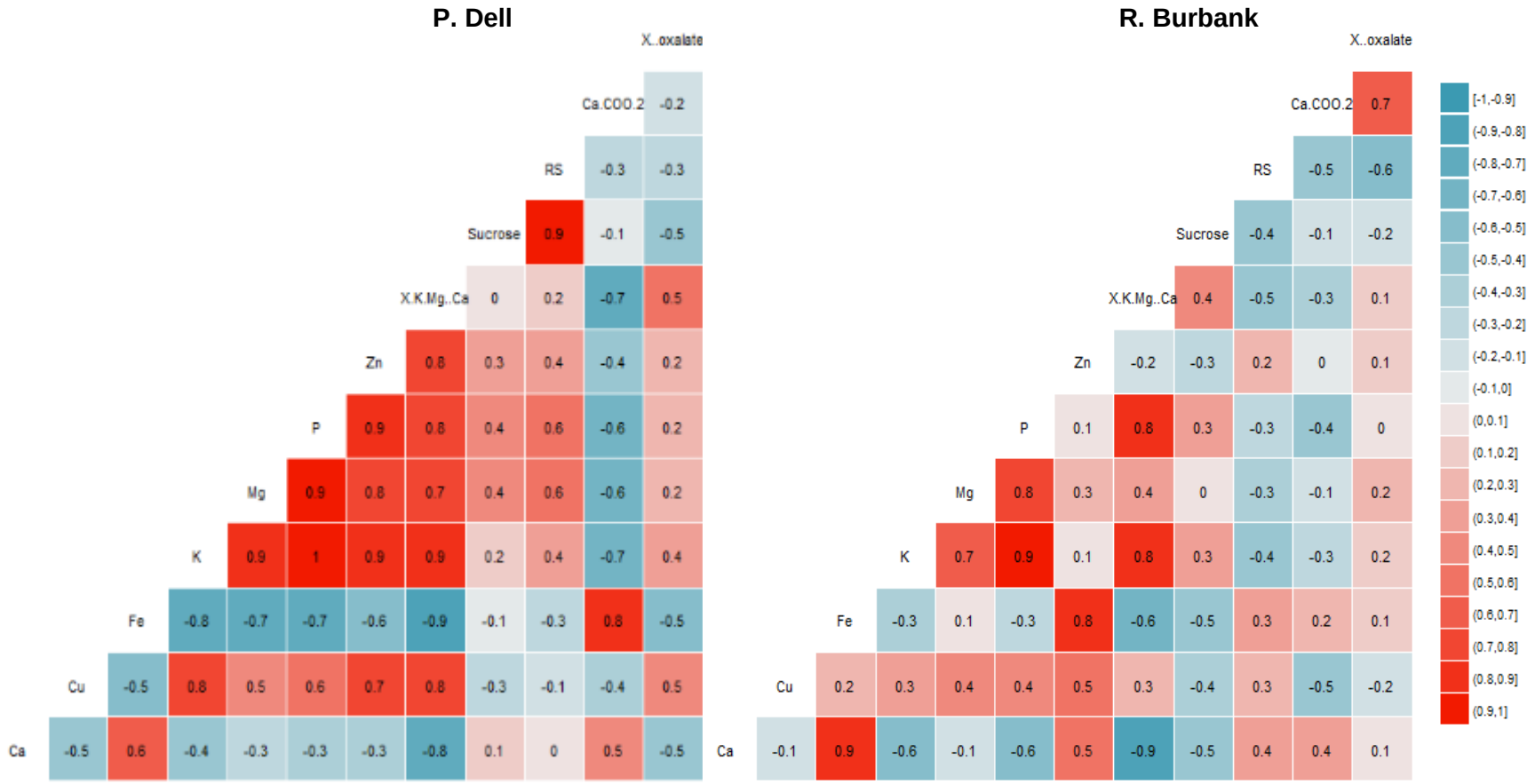


Figure 4-41 - Correlation between Catotal (Ca), Cu, Fe, K, Mg, P, Zn, (K+Mg)/Ca (X.K.Mg..Ca), Cabound (Ca.COO.2), %Cabound (X..oxalate), sucrose and reducing sugars (RS) during the storage season 2014/15 for the varieties P. Dell and R. Burbank. Significant Pearson correlation coefficients from 0.38 to 1 and from -0.38 to -1 ($p < 0.05$, $df = 18$)

4.9. Investigation of the effect of calcium in the storage potential of potato tubers

4.9.1. 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 4-9).

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 4-10).

Fructose increased after 3 months storage at 10°C (Table 4-9). Fructose and sucrose accumulation was higher in the middle cortex of the tuber than the ends (Table 4-10).

Table 4-9 - %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 fertilisation trial

	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 TukeyHSD test.

Table 4-10 - %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 fertilisation trial

	RS	Sucrose	Fructose	Glucose
Edges	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 TukeyHSD test.

4.9.2. Ascorbic acid accumulation

Tubers that received calcium fertilisation (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 4-11).

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 4-11).

Table 4-11 - 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 trial.

	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	20.22^{a}	8.07^{b}	25.90^{a}
HSD$_{0.05}$	*1.29*	*1.20*	*0.94*
p-value	*<0.01*	*<0.01*	*>0.05*

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

4.9.3. **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 4-12). Control tubers had significantly higher accumulation of Mg and B than the ones fertilized with Tropicote (Table 4-12).

Table 4-12 – Mineral concentrations in P. Dell potato tubers from the calcium fertilisation trial, sampled in December 2015 (beginning of storage season).

	Ca mg/100g FW	N mg/100 g FW	N/Ca	K mg/100g FW	Mg mg/100 g FW	K+Mg / Ca	P mg/100g FW	B mg/100 g FW	Zn mg/100 g FW
Tropicote	58.71^{a}	1447^{a}	24.83^{a}	1644^{a}	85.81^{b}	29.72^{a}	157^{a}	0.60^{b}	1.63^{a}
Control	66.09^{a}	1462^{a}	22.23^{a}	1815^{a}	93.21^{a}	28.98^{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 TukeyHSD test.

4.9.4. **ROS determination**

Calcium treatment significantly reduced ($p < 0.05$) H_2O_2 content visualized through DAB staining in P. Dell tubers during storage (Table 4-13). 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 4-43). 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 4-43). 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 4-13 - 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.

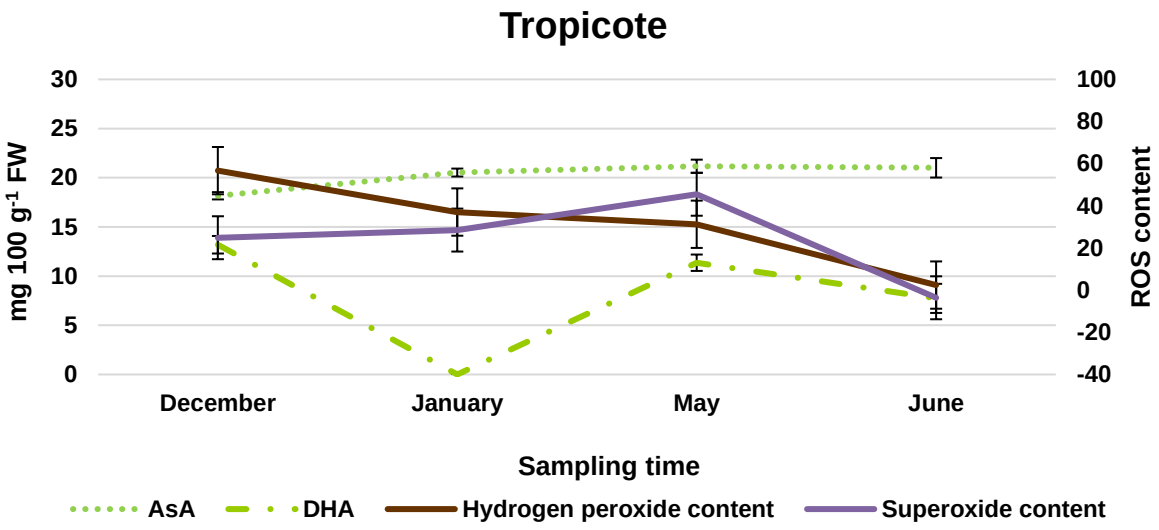


Figure 4-42 - Hydrogen peroxide content (H_2O_2), superoxide content (O_2^-), AsA and DHA concentration during storage at 10°C for P. Dell tubers treated with calcium fertilization (Tropicote), with SE bars.

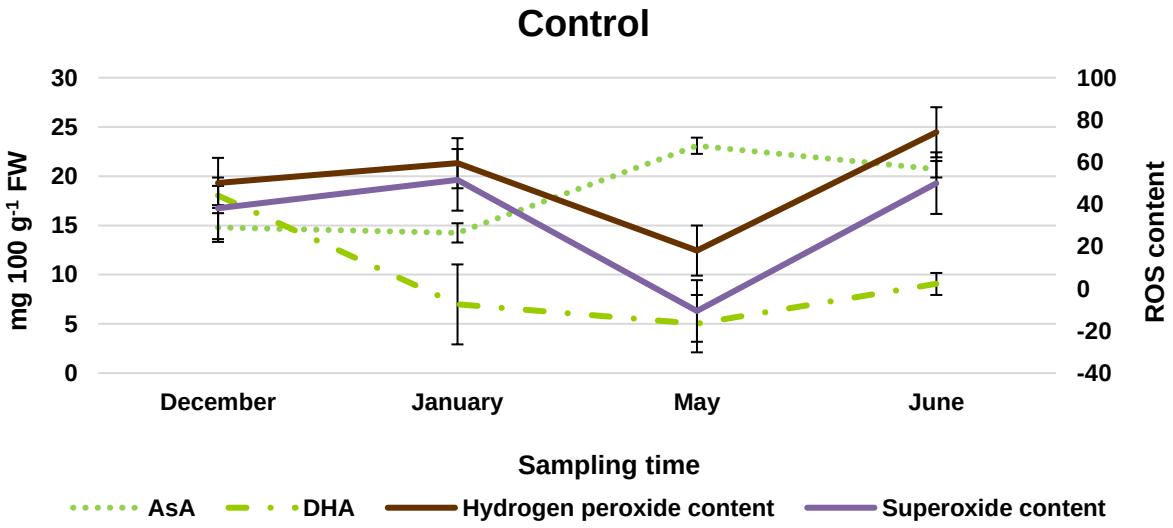


Figure 4-43 - Hydrogen peroxide content (H_2O_2), superoxide content (O_2^-), AsA and DHA concentration during storage at 10°C for P. Dell tubers treated without calcium fertilization (Control), with SE bars.

4.9.5. **Assessment of amyloplast and cellular changes due to calcium fertilisation 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 4-15). However, at this point calcium treated tubers showed no signs of cracking on the amyloplast (Plate 4-16); 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.

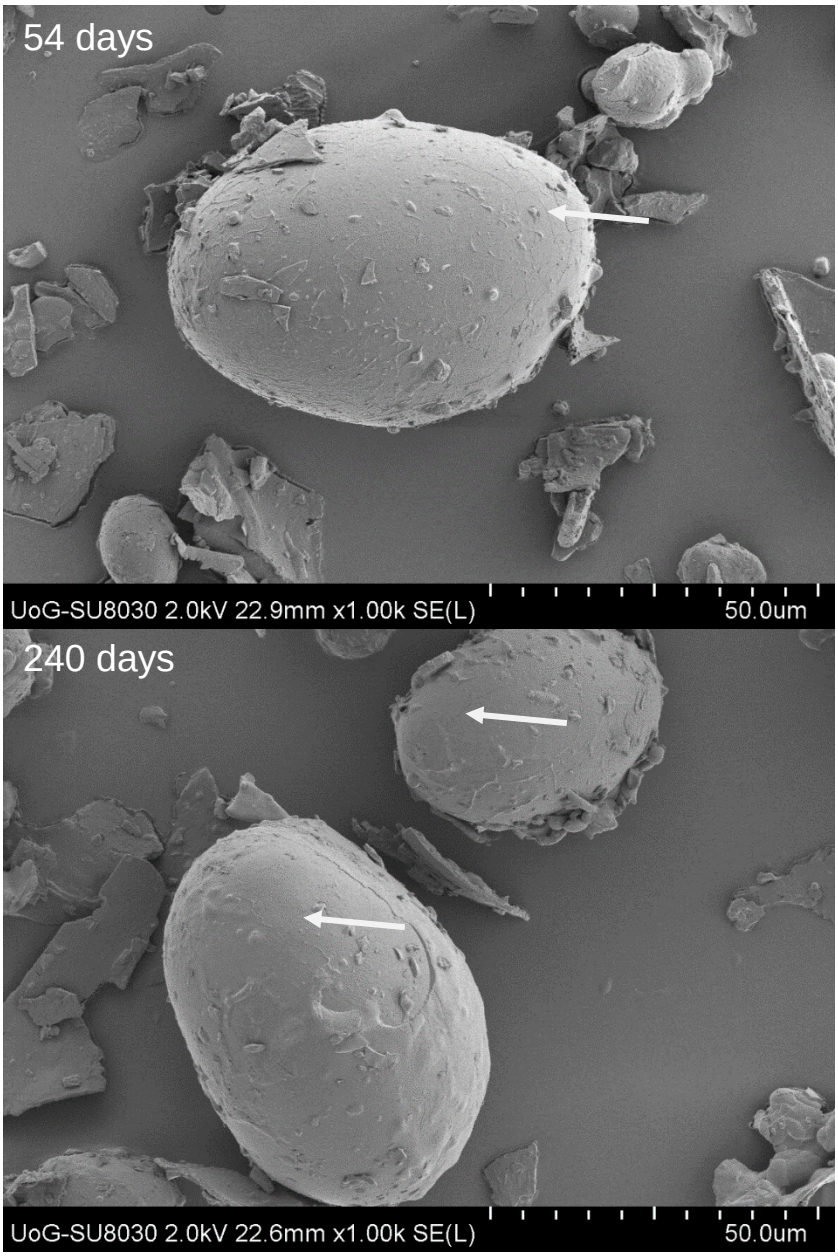


Plate 4-15 - SEM image from the tuber ends section of P. Dell (control) with 54 days (December 2015) and 240 days (June 2016) of storage with fractures on amyloplast surface. White arrows point to fractures.

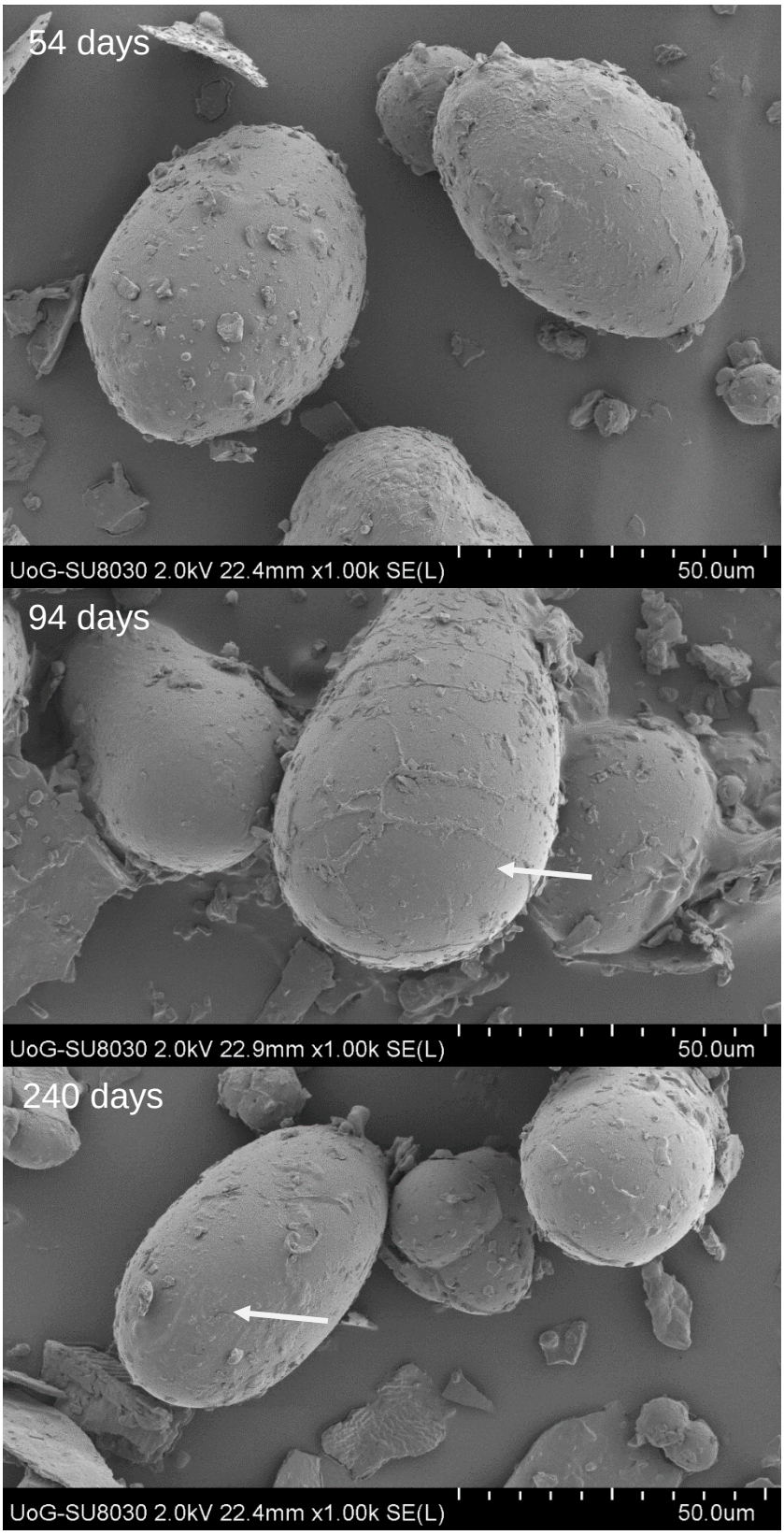


Plate 4-16 - SEM image from the tuber ends section of P. Dell with calcium fertilization (Tropicote) with 54 days (December 2015, no visible fractures), 94 days (January 2016, with 1st visible fractures) and 240 days (June 2016) of storage with few visible fractures on the surface of the amyloplast. White arrows point to fractures.

4.10. Investigation of the effect of calcium in dormancy and sprouting

4.10.1. 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 dissicated and deteriorate much faster than buds incubated with Ca^{2+}.

Buds incubated in Ca^{2+} initiated rapid shoot growth 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 dependent on the position taken from the tuber. Apical buds had a significantly ($p < 0.001$) higher growth rates than lateral buds selected from either mid- or stolen end of the tuber. Apical buds growth increased more rapidly than lateral buds with an increase in sprout vigour by the 8th day of incubation (1.05 mm), lateral buds from mid-whorl 11th day (1.44 mm), and stolen buds by the 13th day (1.63 mm).

Growth rates of lateral buds from the stolen end were stimulated by the addition of 40 mM of Ca^{2+} (Table 4-16), however, in general, increasing calcium above 30 mM of Ca^{2+} suppressed growth 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 growth (Figure 4-45 and Figure 4-46). The application of the calcium chelator EGTA and the $LaCl_3$ channel blocker delayed dormancy break and reduced sprout growth 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 4-16). A similar effect is observed in $LaCl_3$ mid-whorl and stolen buds (Table 4-16). Interestingly buds excised from the mid-whorl did not respond to EGTA treatments (Table 4-16). When $LaCL_3$ was applied its effects were observed across buds excised from all locations, which is in contrast to of Ca^{2+} and EGTA application.

In general, 30mM of Ca^{2+} was the treatment with the best performance for bud growth opposed 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 $LaCL_3$.

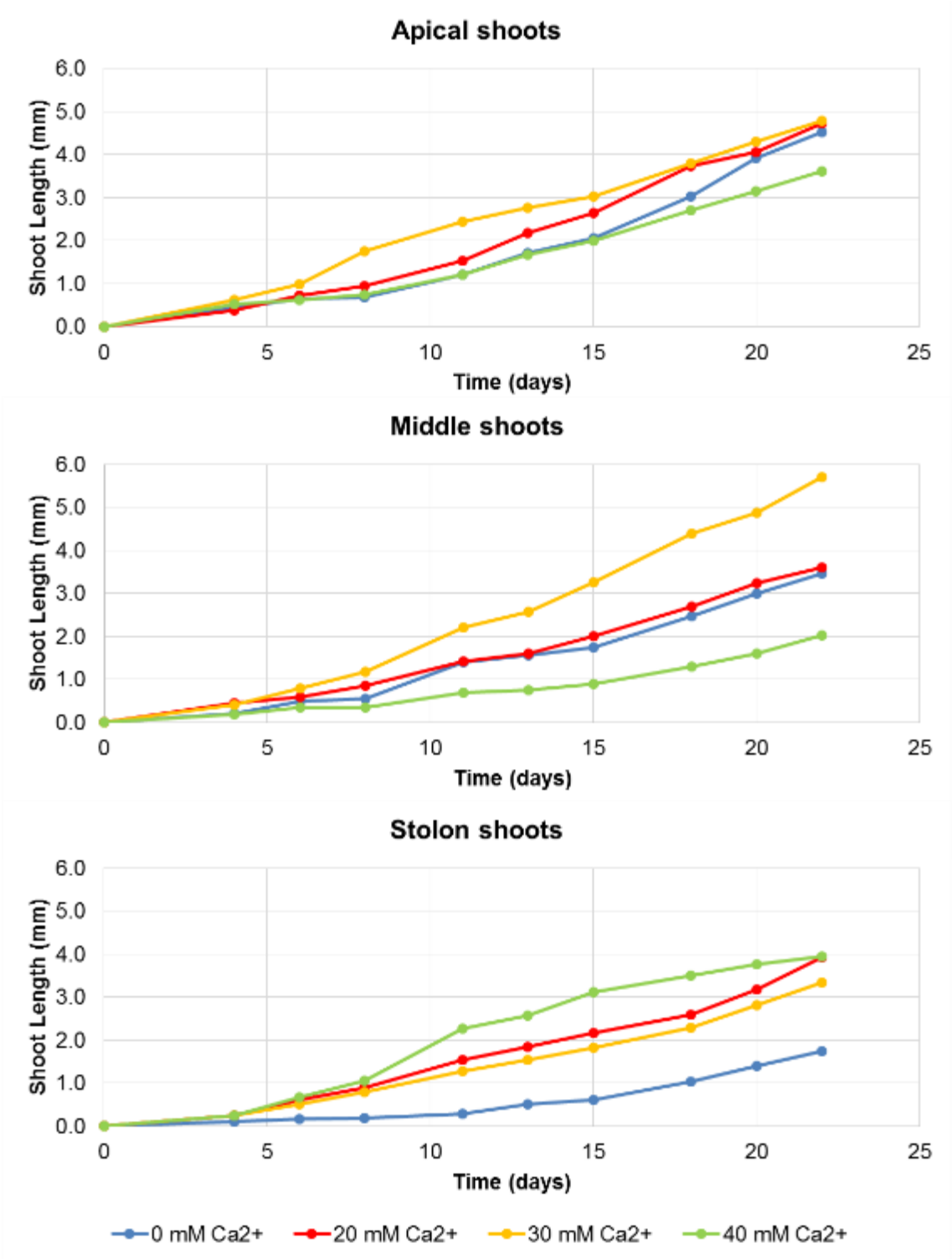


Figure 4-44 - Shoot length (mm) after the application of different concentrations of calcium chloride (Ca2+) to apical, middle and stolon excised buds from Lady Balfour tubers

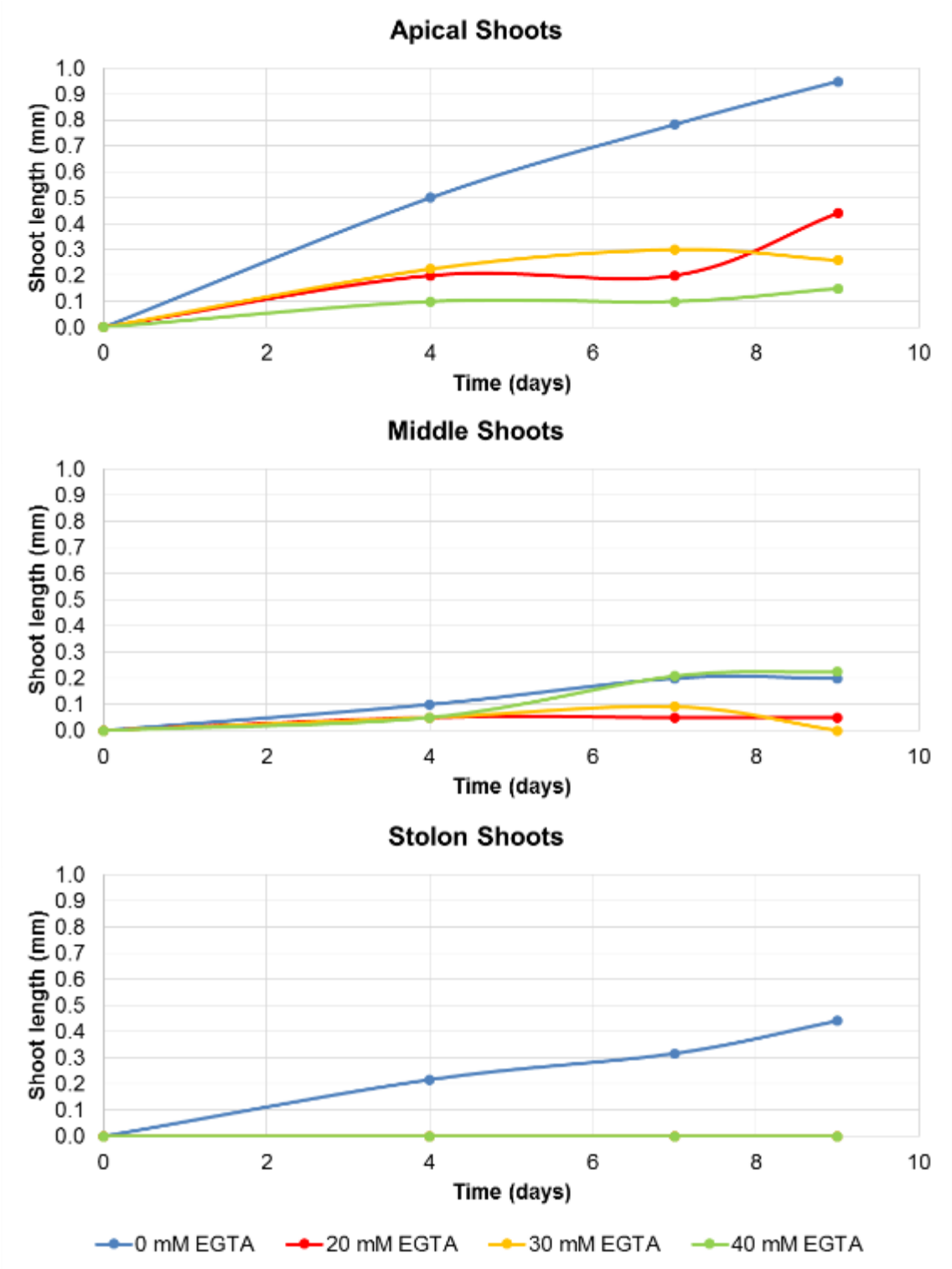


Figure 4-45 - Shoot length (mm) after the application of different concentrations of Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) to apical, middle and stolon excised buds from Lady Balfour tubers

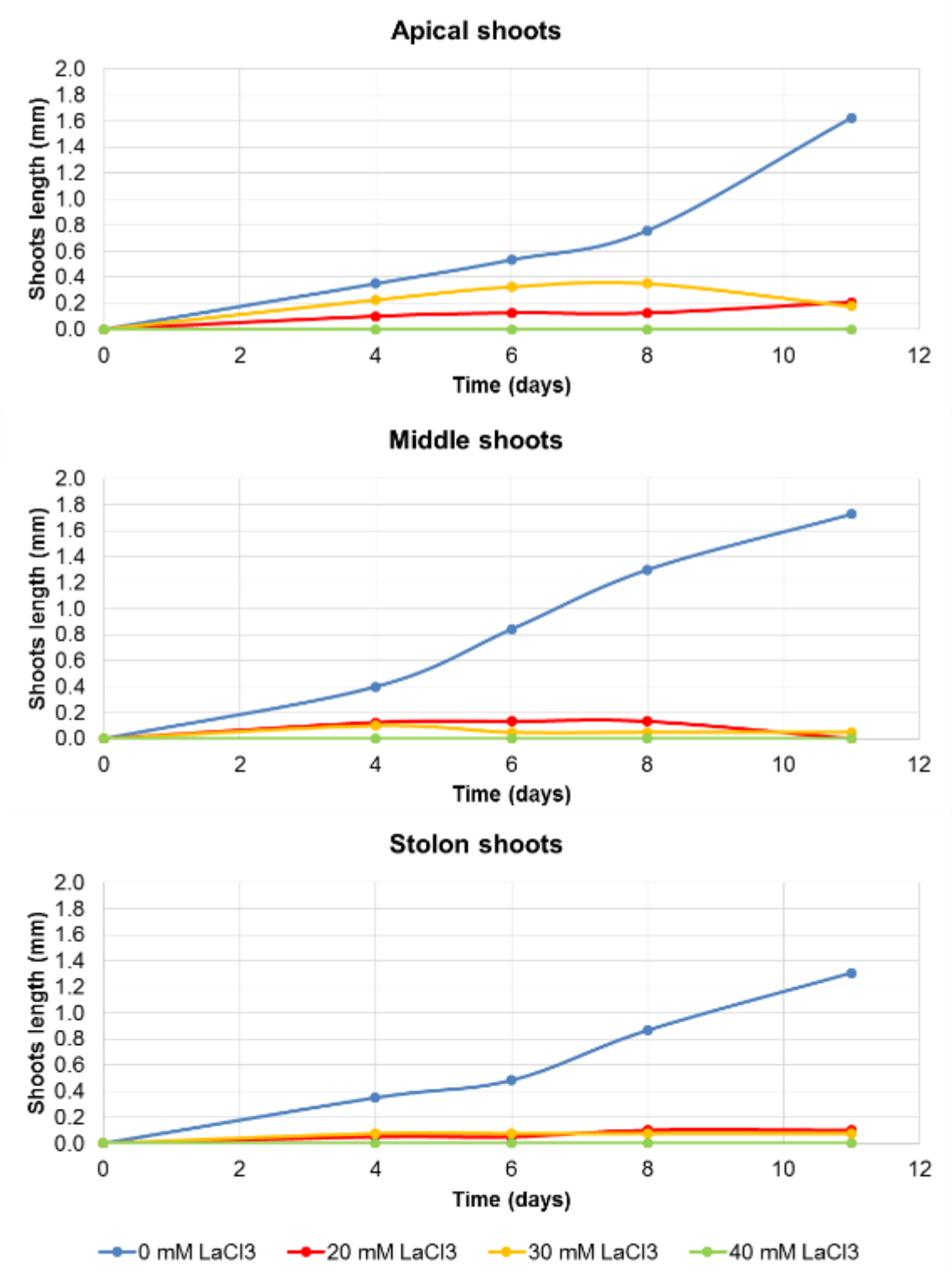


Figure 4-46 - Shoot length (mm) after the application of different concentrations of Lanthanum (III) chloride heptahydrate (LaCl3) to apical, middle and stolon excised buds from Lady Balfour tubers

Table 4-16 – Shoot length (mm) from buds excised from different regions of potato tubers and treated with Ca^{2+}, EGTA and $LaCl_3$.

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

4.10.2. Assessment of the influence of calcium and calcium inhibitors in dormancy and sprout growth

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

A comparison of buds excised from the apical and stolen 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 4-47 and Figure 4-48).

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 4-47 and Figure 4-48).

Treatment and variety had significant effect ($p < 0.05$) on shoot growth (mm/day) as well the interactions 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 4-49 and Figure 4-50).

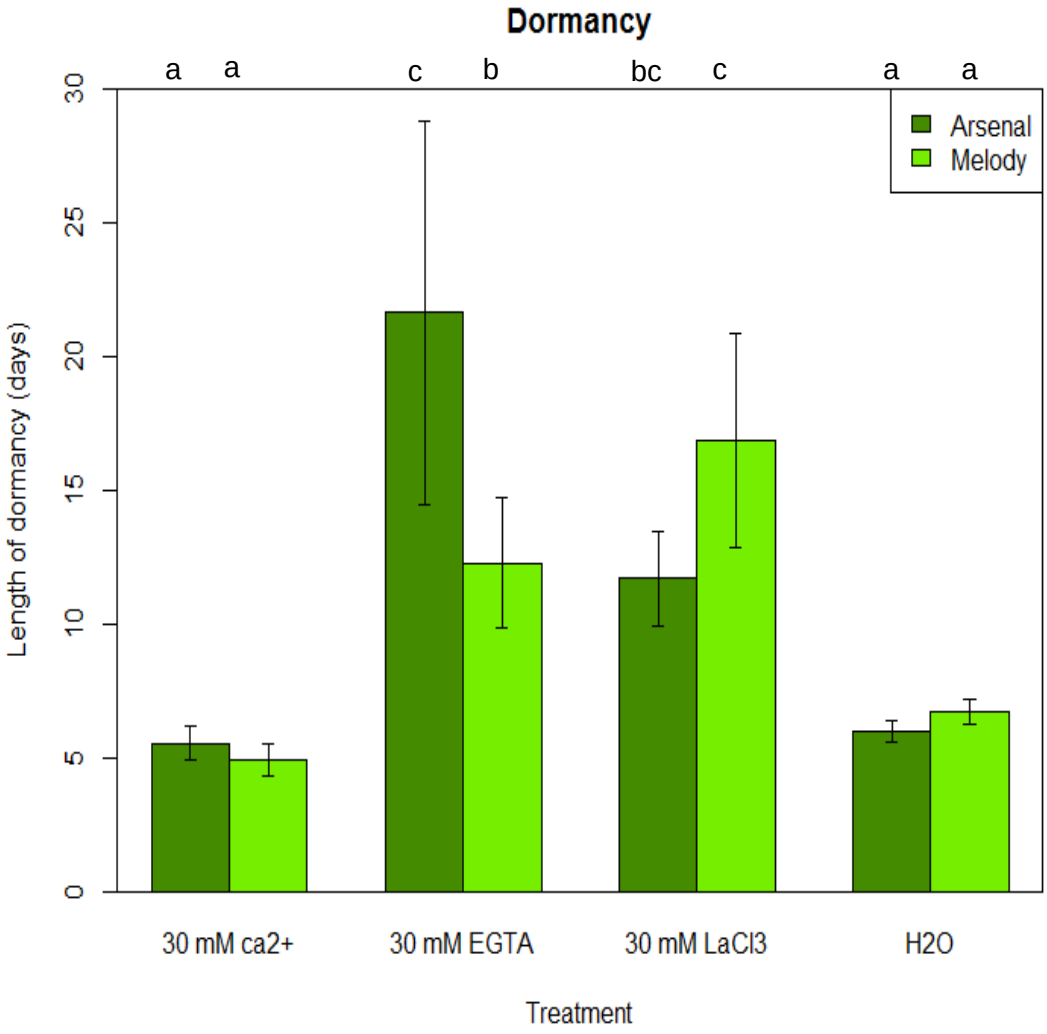


Figure 4-47 – Dormancy prediction based on Weibull distribution for the different varieties with different treatments. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package "multcomp"), with SE bars.

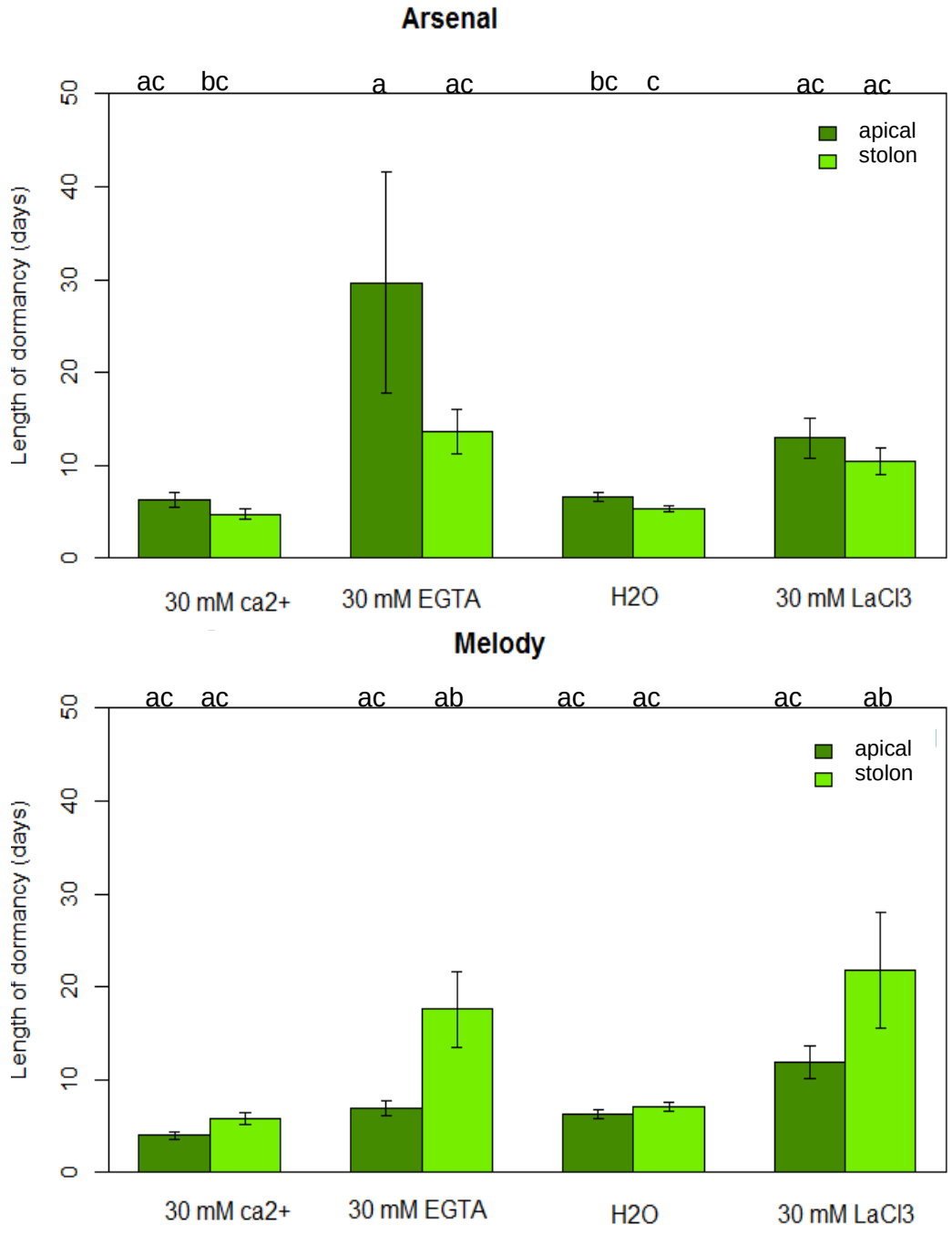


Figure 4-48 - *Dormancy prediction on Weibull distribution for the different varieties with different treatments by tuber site. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package "multcomp"), with SE bars.*

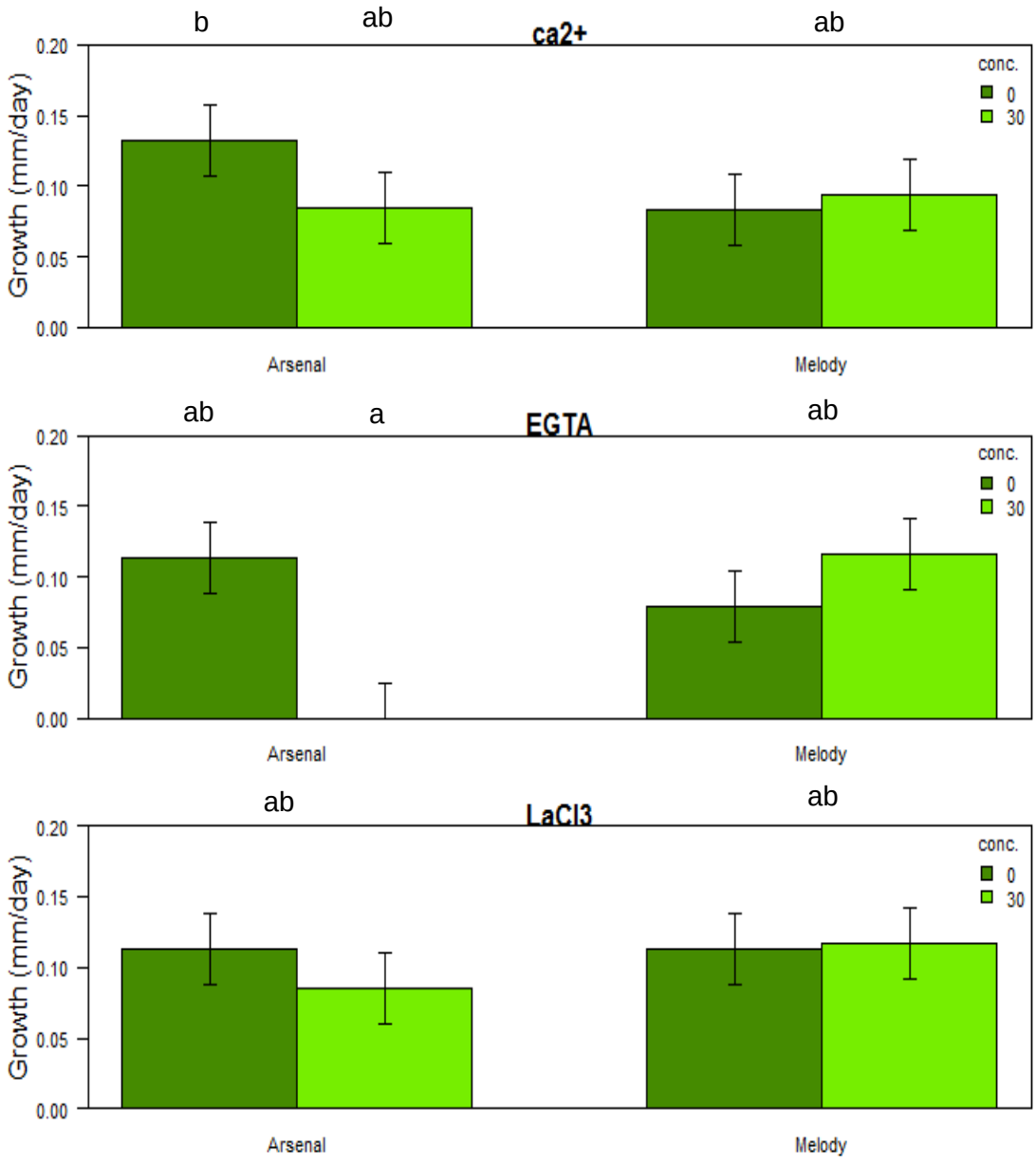


Figure 4-49 – Shoot growth (mm/day) for each variety and treatment for apical site. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package "multcomp"), with SE bars.

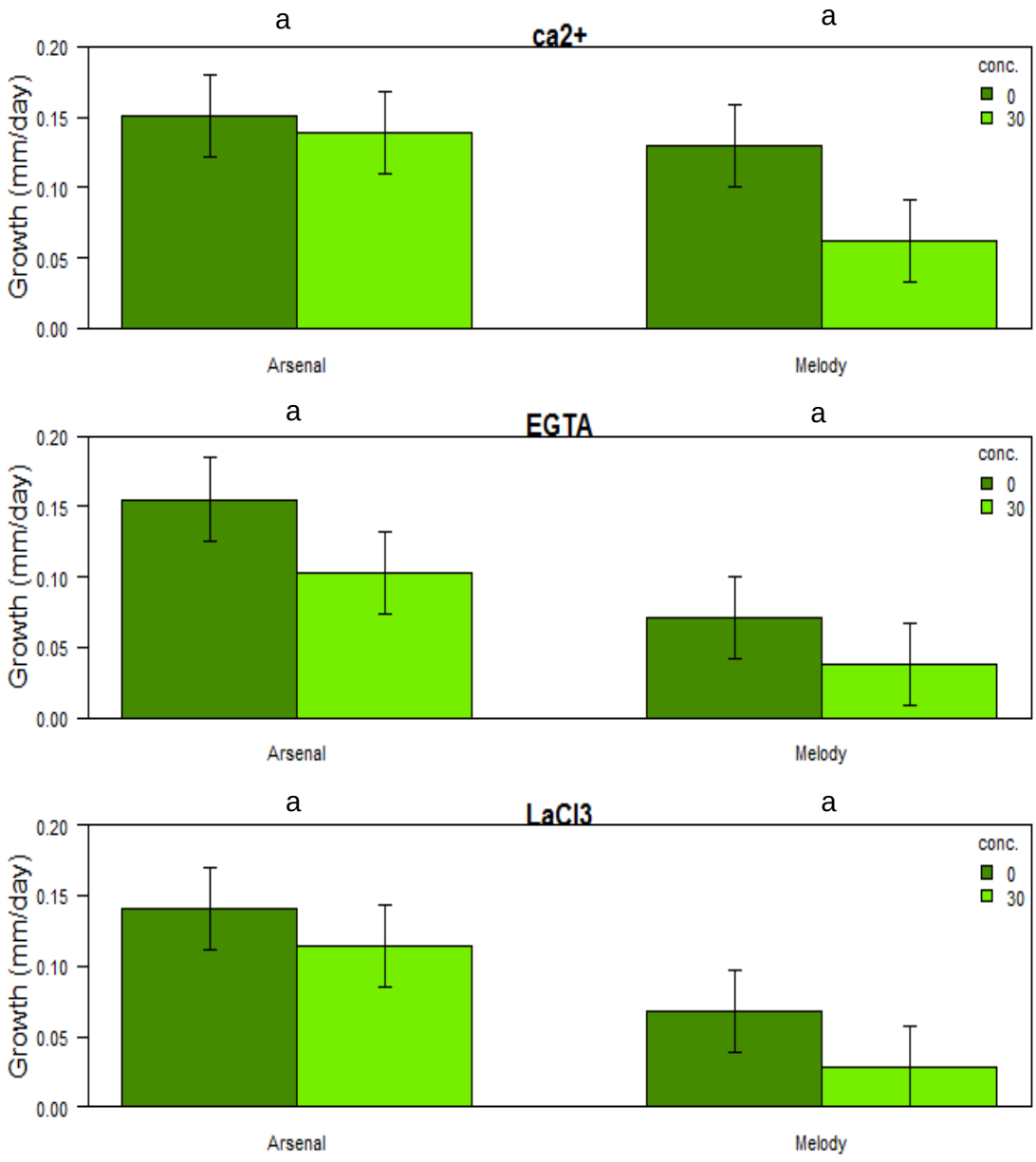


Figure 4-50 – Shoot growth (mm/day) for each variety and treatment for stolen site. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package "multcomp"), with SE bars.

4.10.3. **Assessment of the influence of calmodulin blockers in dormancy and sprout growth**

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

For 20°C, 500 µM of calmidazolium chloride was the treatment with the higher sprout growth and 30 mM $LaCl_3$ resulted in the lowest sprout growths (Table 4-17). Buds treated with $LaCl_3$ stayed dormant for longer (Table 4-18).

Incubating buds in 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 4-17). Buds treated with $LaCl_3$ stayed dormant for longer (Table 4-18). It is interesting to note that 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 growth, while sprout growth in calmidazolium chloride, was lower than untreated control buds incubated at 10°C (Table 4-19). For this incubation temperature, buds treated with $LaCl_3$ were the ones that stayed longer dormant and control buds the ones with shorter dormancy (Table 4-20). Dormancy duration was not significant different between buds treated with Ca^{2+} and the ones treated with W7. Nevertheless, calmidazolium chloride had significant dormancy duration from the Ca^{2+} buds (Table 4-20).

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/per day) compared to 10°C (0.03 mm/per day).

Buds kept at 10°C were expected to stayed dormant for longer (Figure 4-51). On average, dormancy break was just expected to happen by the 10^{th} day for buds kept at 10°C and by the 5^{th} day for the ones kept at 20°C ($HSD_{0.05} = 0.5$).

Table 4-17 – 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 TukeyHSD test ($HSD_{0.05} = 0.4$).

Table 4-18 – 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 TukeyHSD test ($HSD_{0.05} = 0.9$).

Table 4-19 – Sprout growth (mm) for buds incubated at 10°C with calmidazolium chloride (CC), W7, EGTA, $LaCl_3$, Ca^{2+} and control (dH_2O).

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

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

Table 4-20 – 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).

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

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

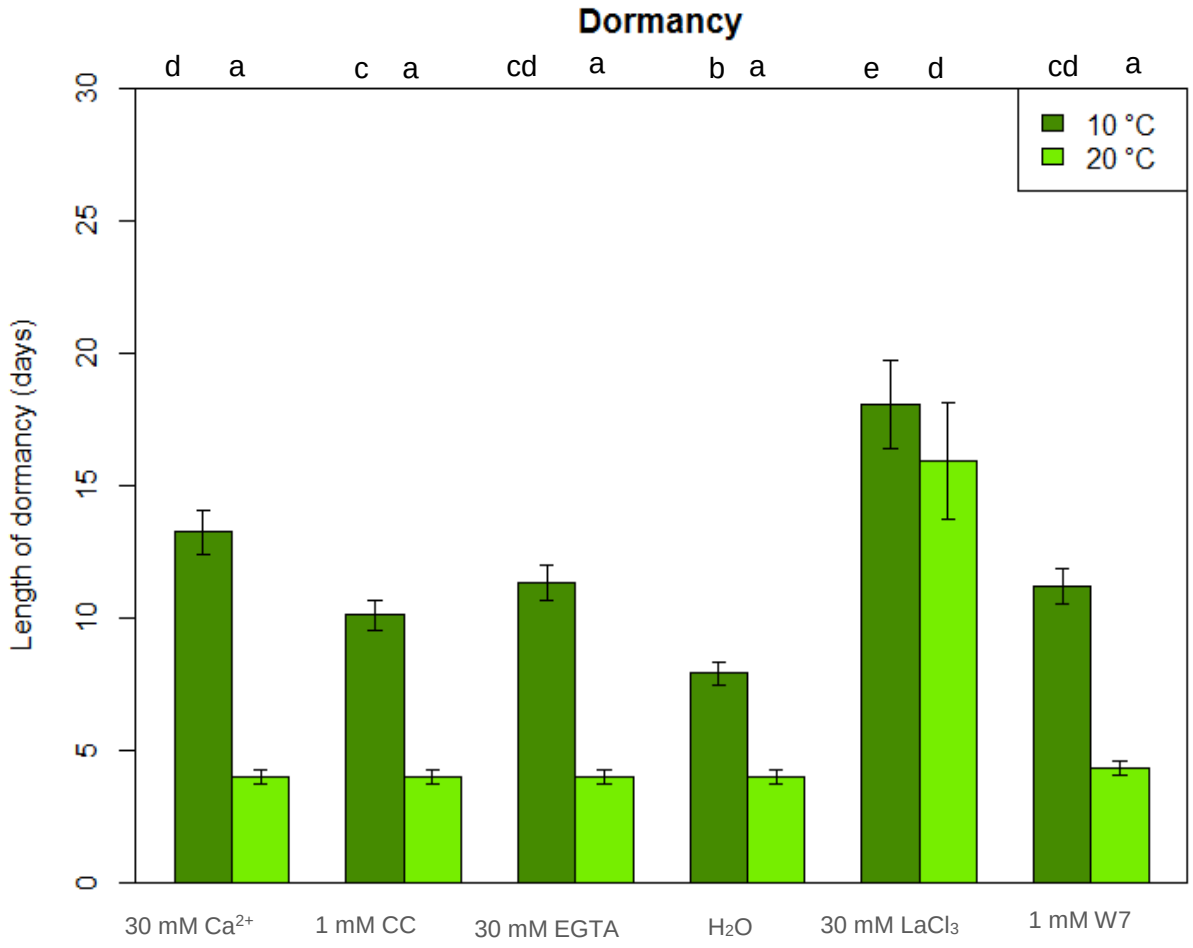


Figure 4-51 - Dormancy prediction by Weibull distribution for Lady Balfour for 10°C and 20°C with different treatments. Means with the same letter are not significantly different according to Tukey test (Tukey test calculated using package "multcomp"). Error bars indicate SE.

4.11. Variety comparison in terms of gene expression using real time Reverse Transcription Polymerase Chain Reaction (RT qPCR)

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. 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) (8.24 Appendix XXIV and 8.25 Appendix XXV).

There was a significant change in StGWD1 and StGWD3 expression in L. Rosetta tubers during storage (10°C) ($p < 0.001$), but this was not observed in the sweetening resistant VR 808 ($p > 0.05$). When comparing to the housekeeping gene (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 4-52). 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 4-52).

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 4-52).

When comparing to EF1-α, in L. Rosetta StGWD1 and StGWD3 expression was down regulated in the beginning of the storage at 10°C, and up regulation by the end of the storage season (7.5 months) for StGWD1. However, in this variety StGWD3 exhibits the tendency to up regulation 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 4-53).

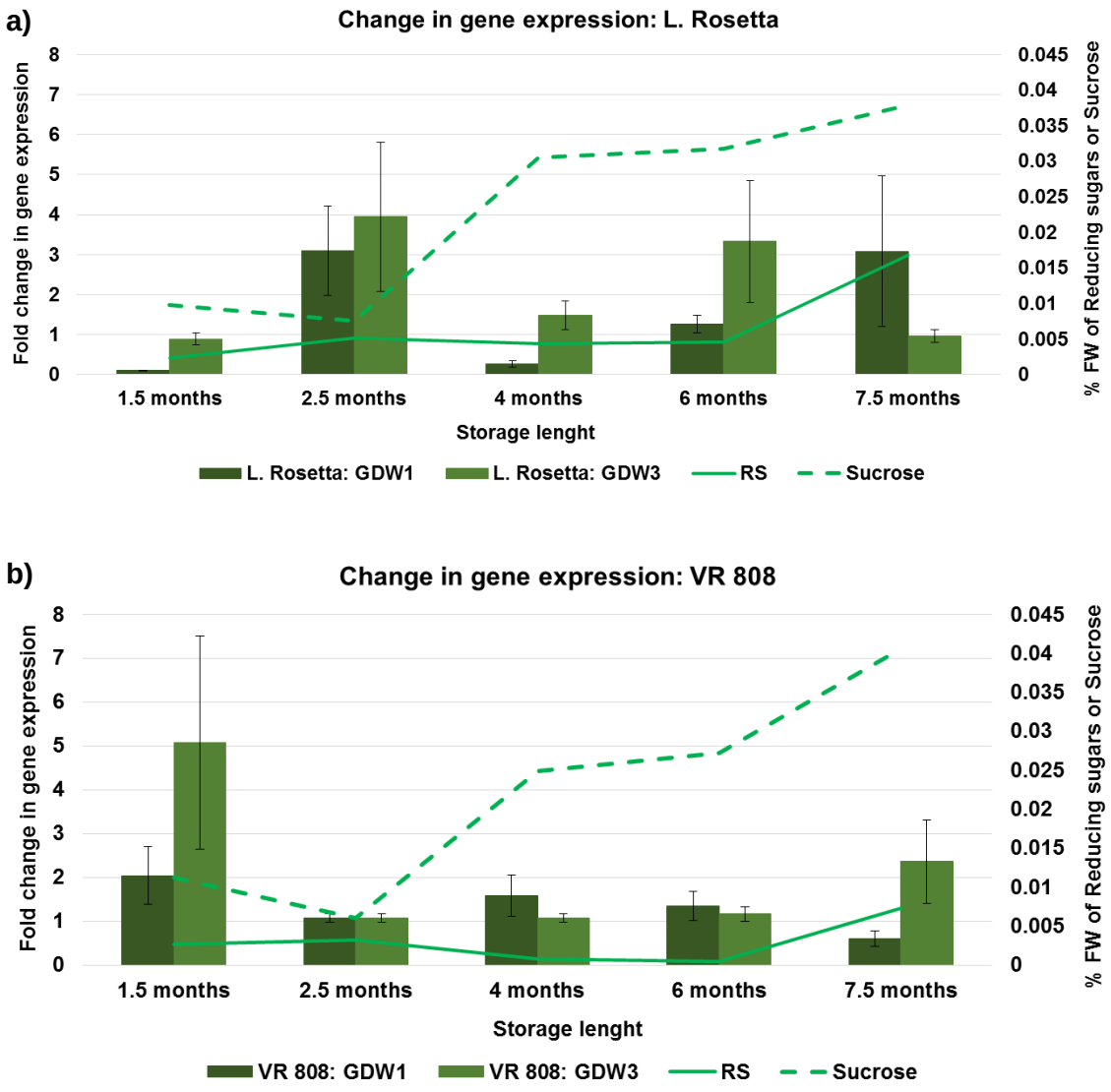


Figure 4-52 - Change in StGWD1 and StGDW3 gene expression in comparison to %FW of reducing sugars and %FW of sucrose during storage at 10°C in L. Rosetta (a) and VR 808 (b) during season 2015/16 with SE bars.

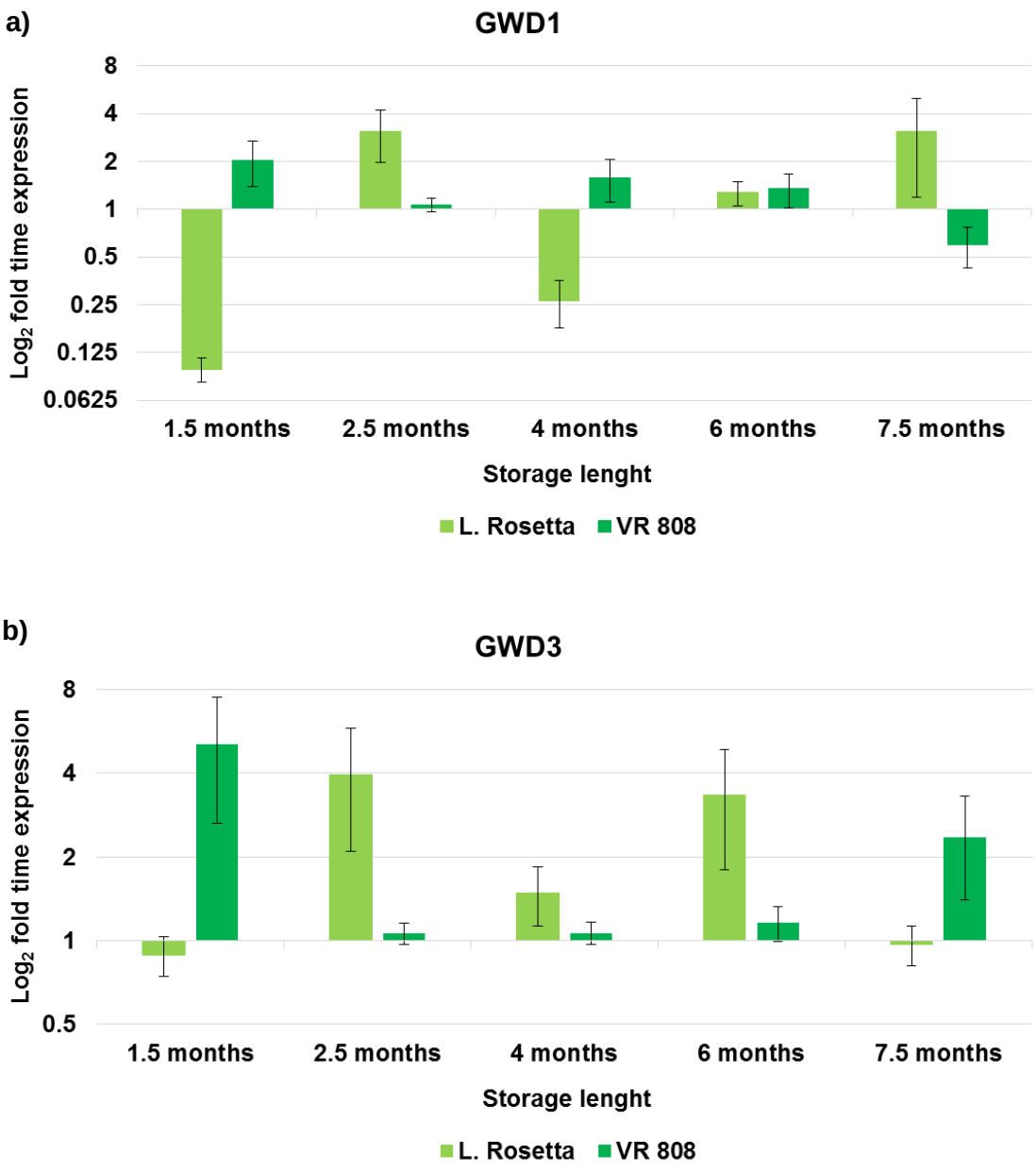


Figure 4-53 – *Log_2 fold time expression (2- ΔΔCT) of StGWD1 (a) and StGWD3 (b) for L. Rosetta and VR 808, with SE bars. Up regulation when $2^{-\Delta\Delta CT} > 2$ and down regulation when $2^{-\Delta\Delta CT} < 1$.*

4.12. Reactive Oxygen Species (ROS) generation and tuber physiological changes

4.12.1. 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^- activity as determined by purple colouration of staining. However, a significant ($p < 0.001$) change in H_2O_2 activity as observed by brown colouration was observed.

For the H_2O_2 activity, 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 4-21). 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 4-21).

Due to these results, it was decided that the next part of this experiment would focus on 0 and 5 µM paraquat.

Table 4-21 – 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.

4.13. Optimizing "Alethea" treatments for reducing induced oxidative stress and impact in the reducing sugars, AsA and DHA accumulation

4.13.1. Sugar accumulation

For this experiment fructose levels 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 does not affect the sugar accumulation, its relation with % of Alethea used had significant effect on %FW of reducing sugars ($p < 0.05$) (Table 4-22).

Treatments with Alethea were responsible for the lowest sucrose accumulation and appear to reduce the accumulation of reducing sugars when tubers were treated with paraquat (Table 4-22).

Table 4-22 – %FW of sucrose (Suc) and %FW of reducing sugars (RS) per paraquat and Alethea treatment, in VR 808.

	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 TukeyHSD test.

4.13.2. **Ascorbic acid 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 does not affect the AsA accumulation, its relation with % of Alethea used had significant effect on AsA accumulation ($p < 0.05$). Treating with 0% of Alethea was significant different from using 5 or 10 % of Alethea (Table 4-23).

Tuber cores treated with 0 µM of paraquat and 0% of Alethea had the higher AsA accumulation 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 4-23).

Similar to AsA accumulation, once again % 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 ($p > 0.05$). 0 % of Alethea was the treatment with the higher DHA accumulation.

Interestingly both concentration of paraquat and % of Alethea ($p < 0.01$) as well as their interaction ($p < 0.05$) were significant for total vitamin C accumulation. Lower concentration (0 µM) of paraquat was related to a greater accumulation of vitamin C than the higher concentration. The treatment of 0 µM of paraquat and 0% of Alethea was the one with the higher vitamin C accumulation, not significant different from the treatment of 0 µM of paraquat and 5% of Alethea.

Table 4-23 – 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 in VR 808.

	AsA	DHA	AsA/DHA	H_2O_2	O_2^-
5 µM paraquat + 0 %Alethea	0.16^{b}	0.06^{a}	1.02^{b}	43.3^{bc}	-148.2^{b}
5 µM paraquat + 5 %Alethea	0^{c}	0.05^{a}	0^{c}	237.8^{a}	-145.9^{b}
5 µM paraquat + 10 %Alethea	0^{c}	0.03^{a}	0^{c}	12.9^{c}	-126.9^{ab}
0 µM paraquat + 0 %Alethea	0.19^{a}	0.04^{a}	1.07^{a}	34.2^{c}	-34.2^{ab}
0 µM paraquat + 5 %Alethea	0^{c}	0.12^{a}	0^{c}	226.1^{ab}	67.0^{a}
0 µM paraquat + 10 %Alethea	0^{c}	0.06^{a}	0^{c}	27.4^{c}	-113.0^{ab}
$HSD_{0.05}$	*0.03*	*0.11*	*0.002*	*1.75.36*	*237.56*
p-value	*<0.001*	*0.08*	*<0.001*	*< 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.

4.13.3. **ROS determination**

Paraquat concentration and % of Alethea had significant effect on H_2O_2 activity (brown colour) and on O_2^- activity (purple colour) (Table 4-23). There was no significant difference between 0, 5 and 10 % of Alethea for O_2^- activity, however 5 % of Alethea had higher H_2O_2 activity than 0 and 10% of Alethea (Table 4-23).

In treatments where paraquat was combined with Alethea application no statistical differences were observed in O_2^- levels 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 activity was reduced compared to the rest of the treatments (Table 4-23).

Paraquat treatments were negatively correlated with O_2^- activity and Alethea was positively correlated with H_2O_2 activity and negatively correlated with AsA, DHA and sucrose accumulation (Figure 4-55).

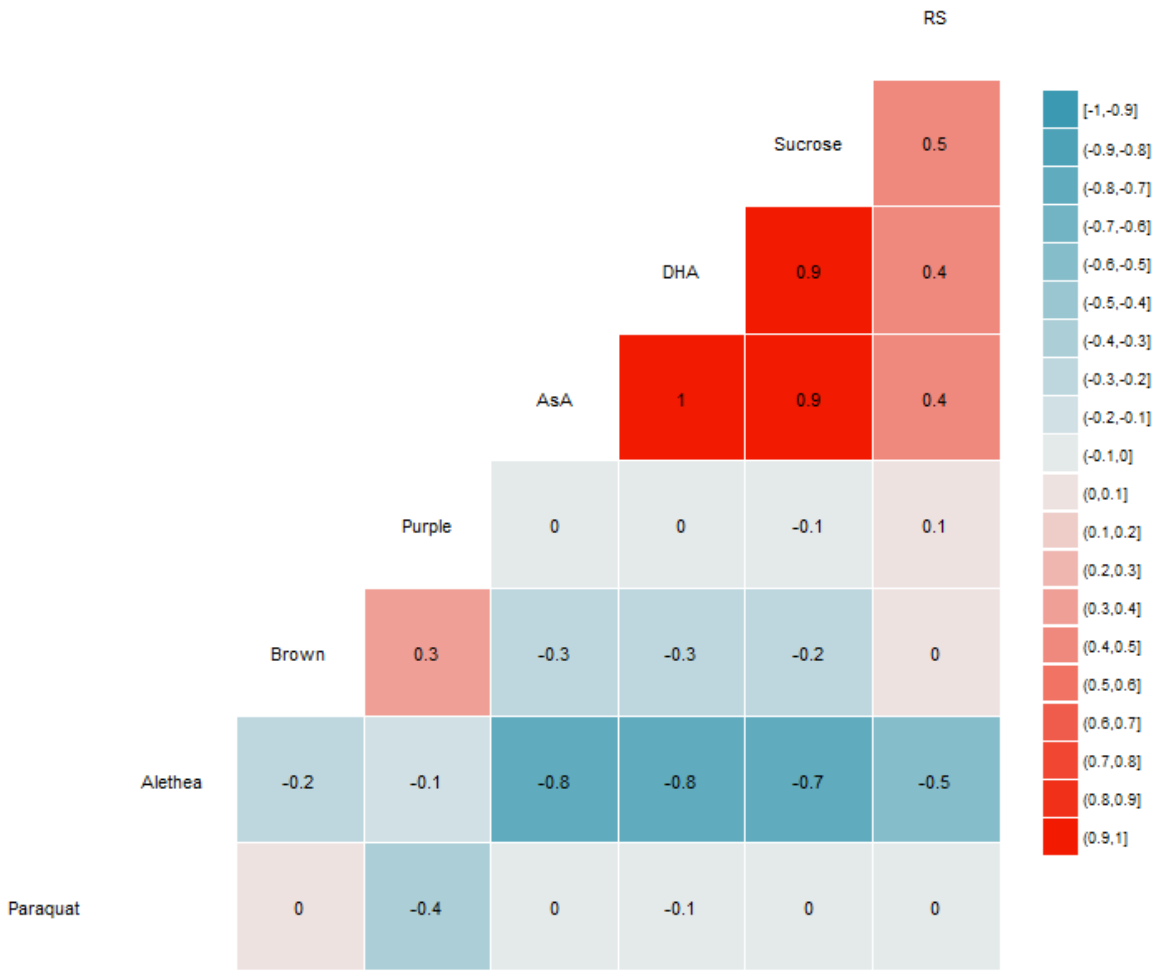


Figure 4-55 - Correlation between paraquat, Alethea, H_2O_2 content (Brown), O_2^- content (Purple), ascorbic acid content (AsA), dehydroascorbic acid content (DHA), %FW of sucrose (Sucrose), %FW of reducing sugars (RS) for VR 808 tubers harvested on October 2016, treated with 0 µM and 5 µM of paraquat, 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 ($p < 0.05$, $df = 18$).

5. DISCUSSION

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

5.1.1. 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 (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 varieties.

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, 1987; 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).

5.1.2. **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.

5.1.3. **Amyloplast changes, texture and sweetening**

5.1.3.1. 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.

5.1.3.2. 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.

5.1.4. **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 3-4). The difference could not be assigned to variance in HPLC analysis 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 whenH_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.

5.1.5. 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.

5.1.6. 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 affect 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).

5.2. Investigation of the effect of calcium in the storage potential of potato tubers

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 oxidative/reductive status of AsA/DHA (Li *et al.*, 2010). APX catalyses 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 through 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 resistance to high or low temperatures in grape leaves is accompanied 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 maintaining 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.

5.3. Investigation of the effect of calcium in dormancy and sprouting

5.3.1. Assessment of Ca^{2+}, $LaCl_3$ and EGTA concentrations

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.

5.3.2. 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).

5.3.3. Assessment of the influence of calmodulin 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+} allow 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 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 have 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.

5.4. Variety comparison in terms of gene expression using real time Reverse Transcription Polymerase Chain Reaction (RT qPCR)

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 4-13 and Plate 4-14).

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.

5.5. Reactive Oxygen Species (ROS) generation and tuber physiological changes

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 have 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 prevents the $HO^\bullet$ formation (Mittler et al., 2004), that 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.

6. CONCLUSIONS

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.

6.1. 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 location was variety dependent; hence the effect of location was not always significant nor consistent for each variety.

6.2. 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 are a loss in antioxidant capacity leading to an increase of ROS.

During the storage, variations 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 surpassed AsA under conditions of oxidative stress (higher content of H_2O_2 and O_2^-), while at the end of the storage higher AsA concentration was recorded; the proposed mechanism was 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.

6.3. 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 during storage led to increased sponginess of tuber cortex tissue.

6.4. 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 amyloplast membrane losing initial integrity via appearance of fractures from 5-8 months in storage, suggesting a relation 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 that reason as a means of assessing tuber quality, this method should be used with caution.

6.5. 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 the potato tubers. It appears that StGWD1 expression decreases with a reduction in sucrose accumulation and StGWD3 expression increases with reducing sugar accumulation.

6.6. 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 over time may influence results, moreover, redistribution of the minerals between middle and outer regions of the tuber have been reported to influence mineral analysis results 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 levels and reduction of AsA to DHA, and possibly limiting the activity of ROS activity. Ca appears to protect the amyloplast membranes, by delaying its 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 bioavailability of Ca in the tuber bud, influences 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 is reduced and thus extending dormancy.

6.7. Effects of chitting

Tubers from physiologically aged potato seed tubers (*chitted* seed) did not give rise to any important change in sugars accumulation, respiration rate, sprout growth or even ethylene production compared to non chitted seed.

However, in tubers from chitted seed a correlation between % FW of reducing sugars and storage length was observed. Chitted seed showed an adverse effect in the storage potential of the stored crop, once tubers from chitted seed accumulated less AsA and had higher O_2^-.

6.8. Developing a model to examine the role of stress in senescent sweetening.

Comparison of fluctuations and the abundance of ROS content captured by staining can help to tie in possible mechanisms for decrease in AsA concentration during 10°C storage. Further targeted analysis of ROS activity using fluorescent bioassay does provided a more quantitative method for overall changes in ROS activity over time.

Generation of ROS in tuber tissue using paraquat and to reverse its effects using a plant health activator commercially known as “Alethea” provided new insights into how ROS activity influenced starch break down. Pre-treatments of “Alethea” were able to significant reduce the glucose and sucrose accumulation in tuber cores treated with paraquat, probably because sugars were utilised for ROS scavenging.

6.9. Implications of this work

The main contributions of this work are a better understanding of the biochemical changes in potatoes during storage that can influence potato 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 (is) underlying senescent sweetening will enable strategies to mitigate the problem and provide markers for breeding programmes.

6.10. Future work

In the future, investigating different ROS detection methodologies to a better quantification, in different sections of tuber which have been identified through staining. Such a method based on the enzymes activity such as described by Melony *et al.* (2003), or based on fluorescence used by Hide *et al.* (2002) or quantifying O_2^- and H_2O_2 production rates reported by Liu *et al.* (2017). Increasing sample numbers of Ca fertilized tubers and use of tubers treated with Ca Nano-particles will provide a greater in depth study of the role of calcium uptake in maintaining tuber quality. Fortunately, some of the findings from this PhD can be explored during 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, I will have the opportunity to follow-up the Ca effect on dormancy break/sprout growth in further experiments.

7. REFERENCES

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8. APPENDICES

8.1. APPENDIX I Storage date and CIPC application dates for all the varieties

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

8.2. 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

8.3. 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

8.4. 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

8.5. 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

8.6. 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
Suk	73.484	4.62e-15 ***	24.4	4.63e-09 ***	114.504	5.04e-11 ***	14.425	8.2e-05 ***
Frock	48.062	1.26e-12 ***	260.723	< 2e-16 ***	77.979	9.14e-10 ***	3.292	0.04776 *
Glue	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 ***
Suk	49.404	< 2e-16 ***	135.150	< 2e-16 ***	97.156	< 2e-16 ***	71.137	< 2e-16 ***
Frock	2.449	0.036258 *	54.414	< 2e-16 ***	53.92	< 2e-16 ***	2.540	0.0310 *
Glue	3.485	0.00552 **	99.633	<2e-16 ***	163.96	< 2e-16 ***	42.692	< 2e-16 ***

VR 808 and L. Rosetta: 4 DP (2013/15) and 6 DP (2014/15), P. Dell and R. Burbank: 3 DP (2013/14) 6 DP (2014/15)

8.7. 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
O_2	1.453	0.341	21.91	0.00228 **
CO_2	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 ***
Suk	18.969	8.14e-09 ***	17.391	2.48e-10 ***
Frock	7.449	0.000139 ***	131.568	< 2e-16 ***
Glue	6.033	0.000677 ***	47.714	< 2e-16 ***

VR 808 and L. Rosetta: 7 DP

8.8. APPENDIX VIII %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)

Variety	Month	Means			
		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 TukeyHSD test.

8.9. APPENDIX IX O_2 consumption (O_2), CO_2 production (CO_2), ethylene production (Ethylene) and sprout growth (sprout) 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 TukeyHSD test.

8.10. APPENDIX X %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 TukeyHSD test.

8.11. APPENDIX XI O_2 consumption (O_2), CO_2 production (CO_2), ethylene production (Ethylene) and sprout growth (sprout) 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 TukeyHSD test.

8.12. APPENDIX XII %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 TukeyHSD test.

8.13. APPENDIX XIII O2 consumption (O2), CO2 production (CO2), ethylene production (Ethylene) and sprout growth (sprout) 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 TukeyHSD test.

8.14. APPENDIX XIV %FW of reducing sugars (RS), %FW of sucrose (Sucrose), %FW of fructose (Fructose) and %FW of glucose (Glucose) by sampling month for the chitting trial

	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 TukeyHSD test.

8.15. APPENDIX XV O_2 consumption (O_2), CO_2 production (CO_2), ethylene production (Ethylene) and sprout growth (Sprout) by sampling month for the chitting trial

	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 TukeyHSD test.

8.16. APPENDIX XVI AsA accumulation (ASA), DHA accumulation (DHA) and total vitamin C accumulation (Vat C) for L. Rosetta and VR 808 for season 2014/15.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vat 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 are significantly different according to the TukeyHSD test.

8.17. APPENDIX XVII AsA accumulation (ASA), DHA accumulation (DHA) and total vitamin C accumulation (Vat C) for P. Dell and R. Burbank for season 2014/15.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vat 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 are significantly different according to the TukeyHSD test.

8.18. APPENDIX XVIII AsA accumulation (ASA), DHA accumulation (DHA) and total vitamin C accumulation (Vat C) for L. Rosetta and VR 808 for season 2015/16.

		Means		
Variety	**Month**	**AsA**	**DHA**	**Vat 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*

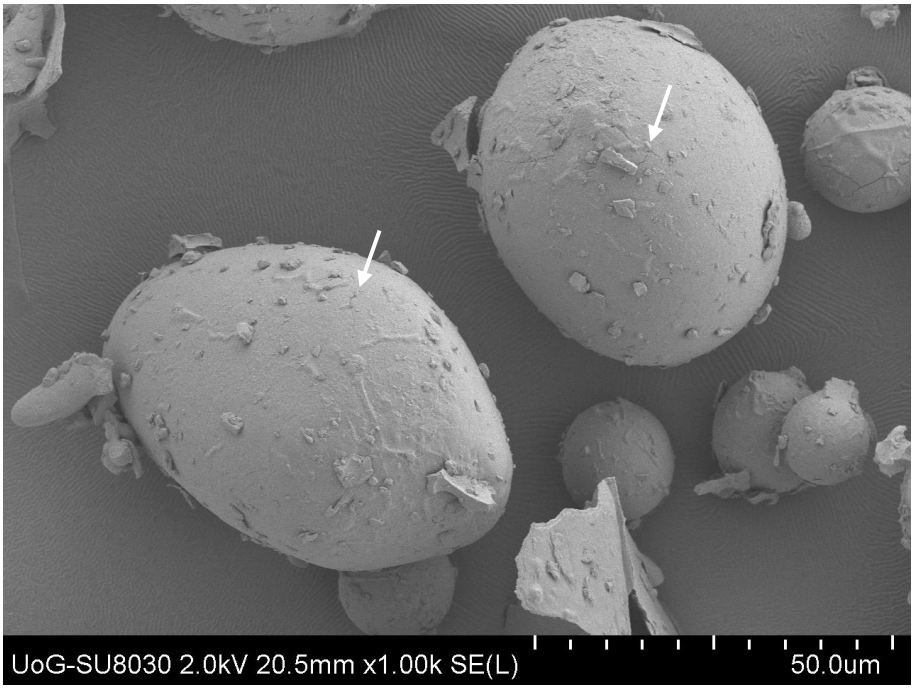
Mean values with different letters are significantly different according to the TukeyHSD test.

8.19. APPENDIX XIX AsA accumulation (ASA), DHA accumulation (DHA) and total vitamin C accumulation (Vat C) for the chitting trial

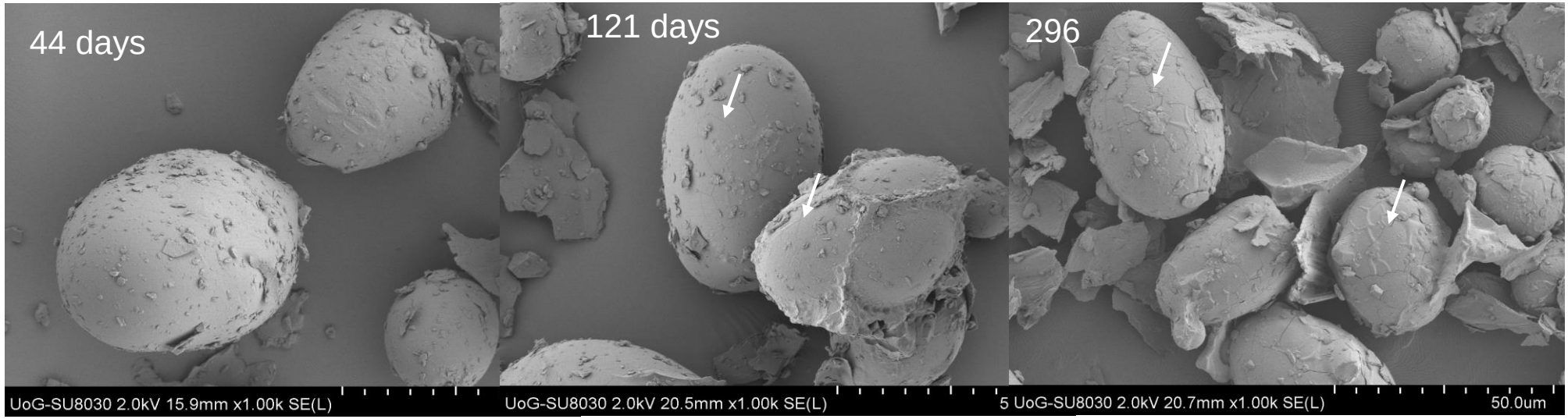
		Means		
Variety	**Month**	**AsA**	**DHA**	**Vat C**
0°C	**December**	31.5[a]	8.4[c]	38.9[a]
	January	14.6[d]	23.7[a]	38.3[a]
	March	24.3[b]	13.5[bc]	37.7[ab]
	April	20.0[c]	20.1[ab]	40.1[a]
	June	21.5[bc]	9.9[c]	31.4[b]
	p-value	*< 0.001*	*< 0.001*	*< 0.01*
	HSD$_{0.05}$	*4.1*	*6.7*	*6.5*
250°C	**December**	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 are significantly different according to the TukeyHSD test. In the AsA/DHA values are the average for the ratios for each replicate, not the ratio of the averages.

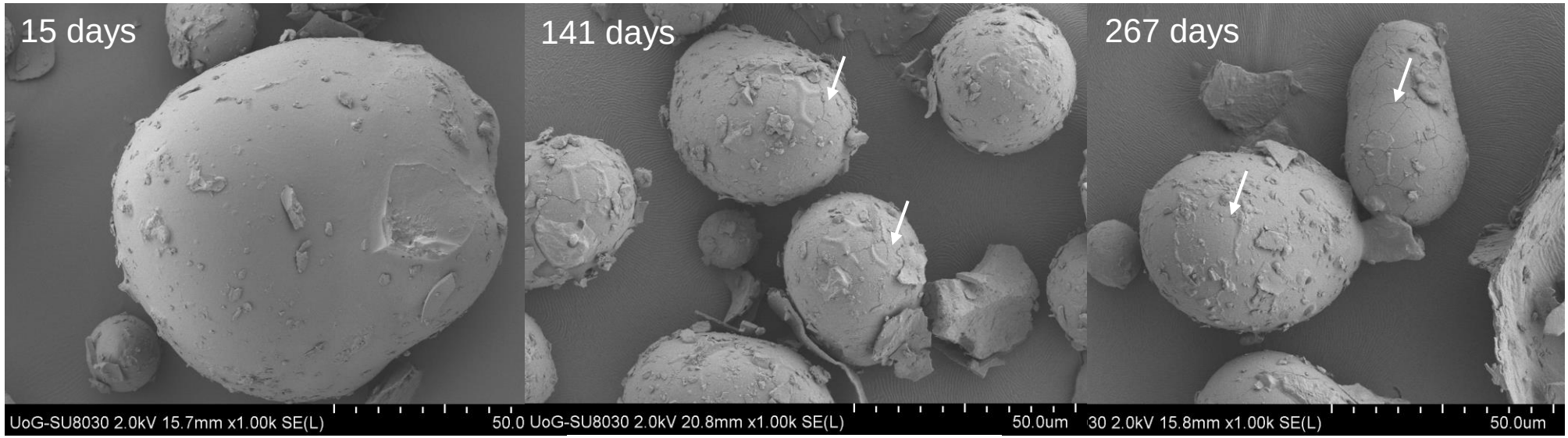
8.20. APPENDIX XX SEM image from middle section of VR 808 with 170 days of storage with 1st visible fractures on the surface of the amyloplast (March 2015). White arrows point to fractures.



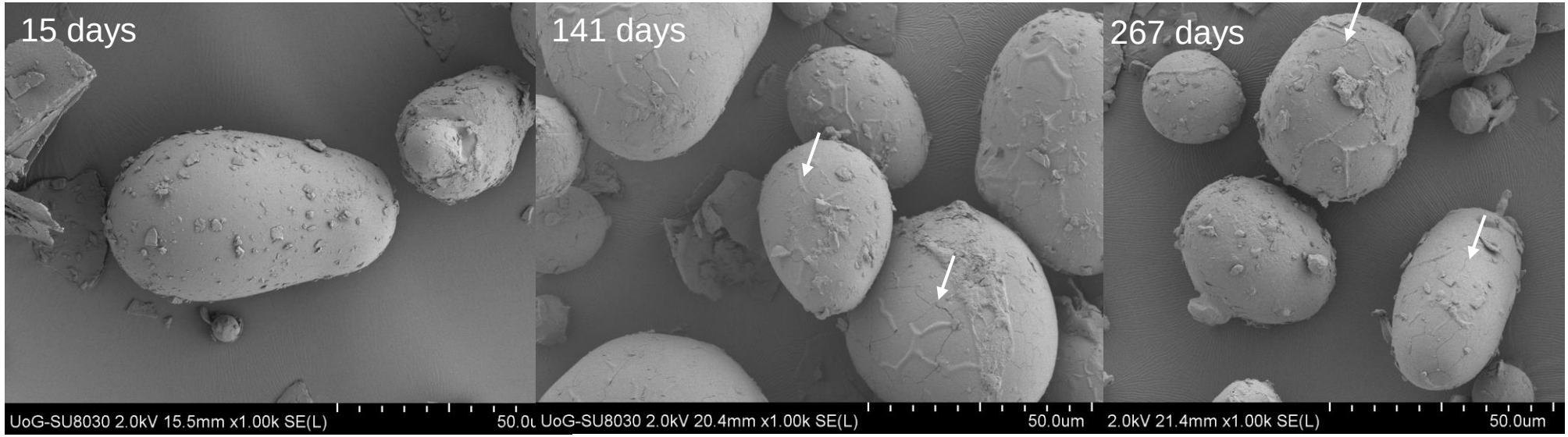
8.21. APPENDIX XXI SEM image from edge section of L. Rosetta with 44 days (November 2014), 121 days (January 2015) and 296 days (July 2015) of storage. White arrows point to fractures.



8.22. APPENDIX XXII SEM image from edge 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.

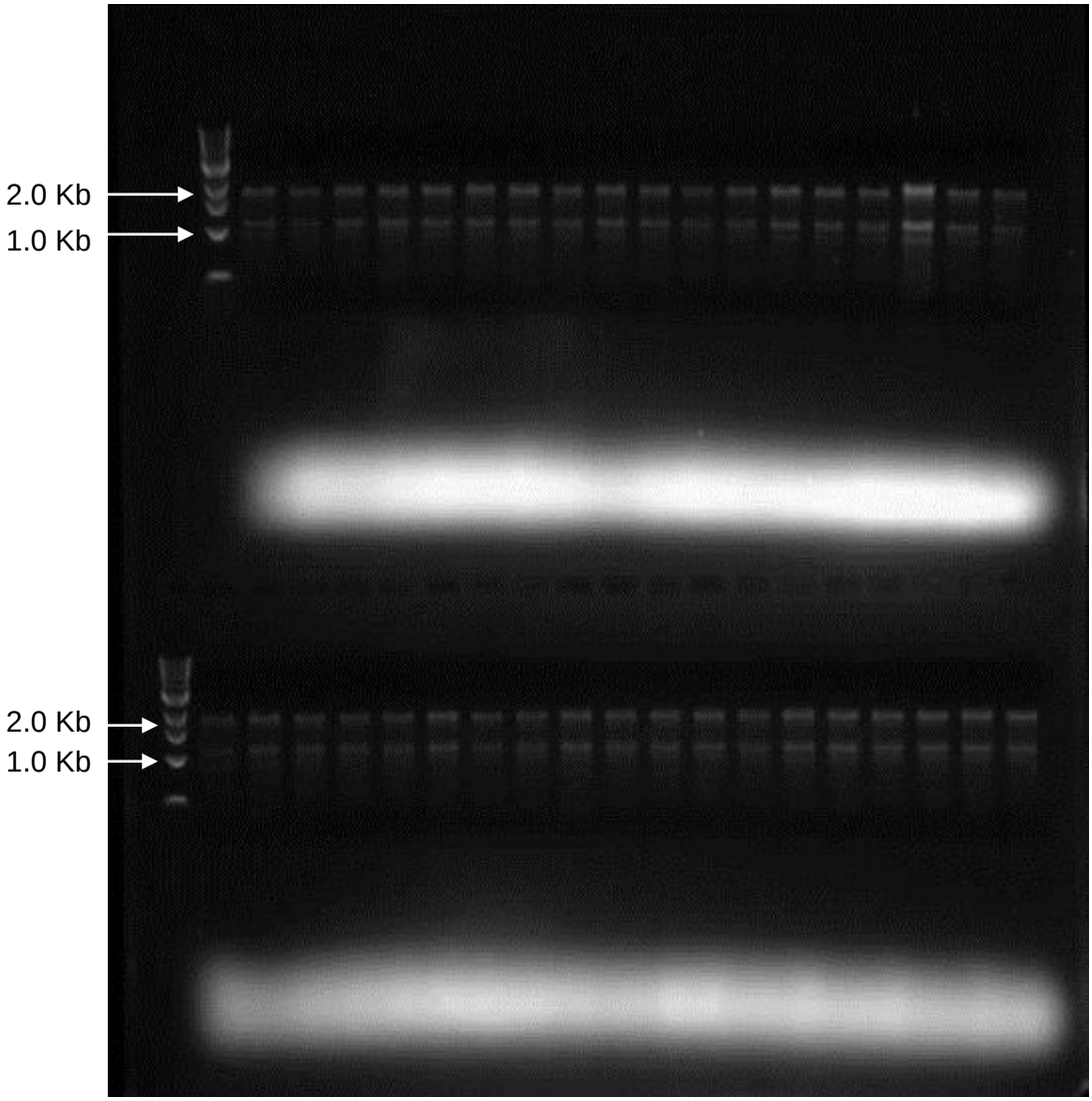


8.23. APPENDIX XXIII SEM image from edge section of R. Burbank with 15 days (November 2014), 141 days (March 2015) and 267 days (July 2015) of storage. White arrows point to fractures.



8.24. APPENDIX XXIV RNA integrity in 1% agarose gel electrophoresis:

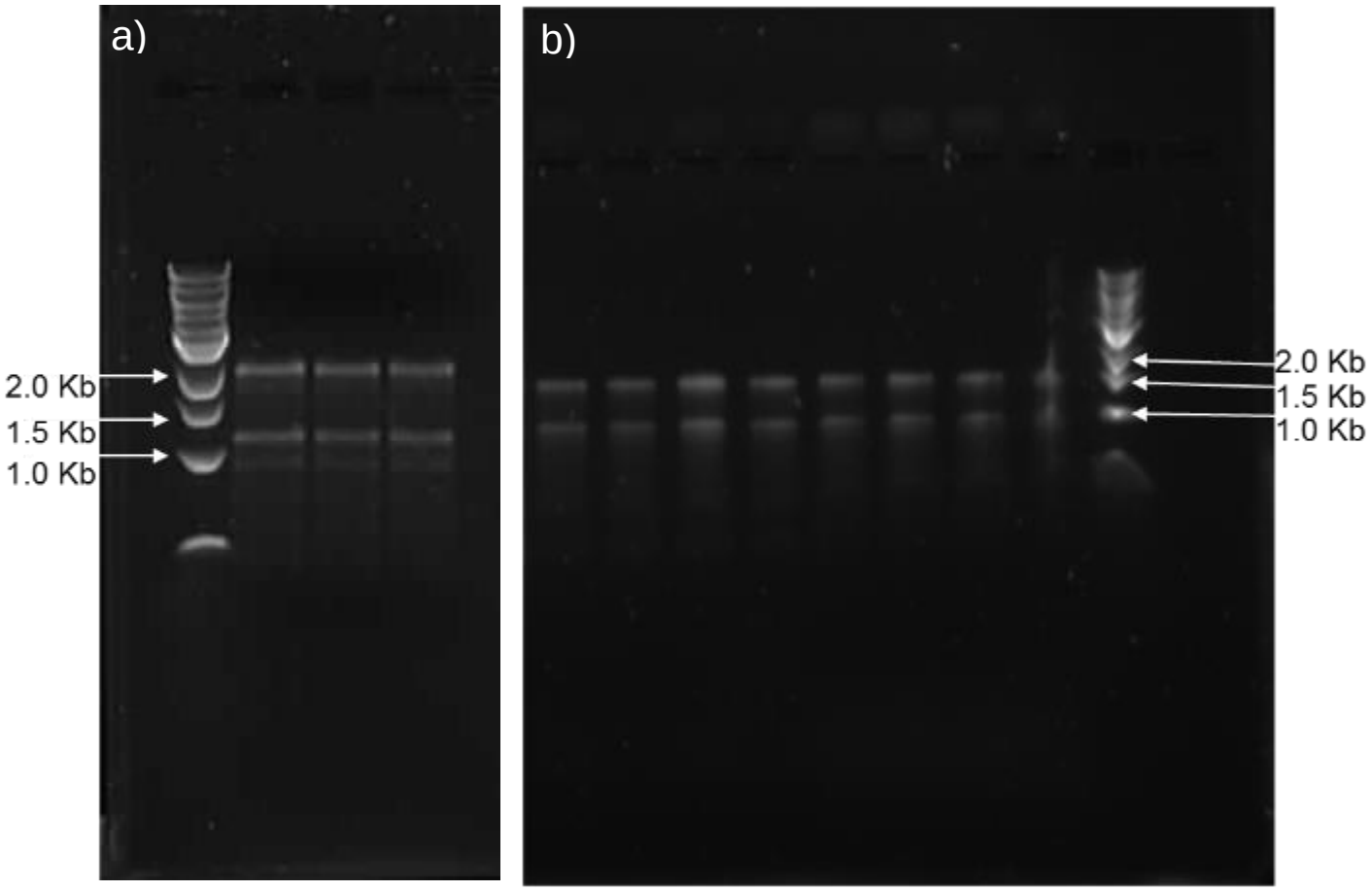
1st row: 1st well 1Kb ladder (NEB), 2nd to 6th well VR 808 from December 2015, 7th to 11th well VR 808 from June 2016, 12th to 16th well L. Rosetta from December 2015, 17th to 18th L. Rosetta from June 2016; 2nd row: 1st well 1Kb ladder (NEB), 2nd to 3rd well L. Rosetta from June 2016, 4th to 8th well 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



8.25. APPENDIX XXV 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).



8.26. APPENDIX XXVI Alethea

Alethea (Plant Impact PLC, Preston, UK) was composed by a combination of potassium dihydrojasmonate, sodium benzoate and L-arginine (family of international patents arising from PCT/GB2005/001562). In addition several plant nutrients were add to the AletheaTM composition as in the table below (values prior dilution) (Wargent et al., 2013):

Nutrient	% contribution to Alethea compound
Zinc	2.0
Iron	2.0
Manganese	1.0
Copper	0.5
Magnesium oxide	0.5
Boron	0.025

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