Summary Report

Effect of variety, harvest and storage time, and defoliation on the mechanical and biochemical properties of potato tubers in relation to bruise susceptibility

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1 SUMMARY

1.1 Aims

The main aim of this project was to investigate the relationship between bruising and the physicochemical properties of potato tubers in three varieties of potatoes known to differ in their tendency toward bruising: Lady Rosetta (LR), Maris Piper (MP) and Russet Burbank (RB). Three field trials were undertaken to investigate the effect of agricultural and storage practices on bruising incidence and tuber properties. Field trial 1 was designed to investigate the effect of harvest time and defoliation, field trial 2 was designed to investigate the effect of harvest and storage time and a third field trial was undertaken to investigate the effect of nitrogen application to soil (in variety LR only).

Additionally the research seeks to establish whether physiological and biochemical characteristics, such as weight, specific gravity, mechanical properties, phenolic acids, tyrosine and cell wall composition of skin and cortex tissue are factors that may be used as predictive indicators of bruising at harvest time and for stored potatoes. The project focused on analysing the skin and cortex, where the bruising damage occurs.

1.2 Key findings

- RB presented the highest incidence of bruising at early harvests (September), and that was associated with low mechanical strength and deformability, and higher tyrosine content. RB benefited from early harvest and short storage.
- LR presented a higher incidence of bruising when harvested in later season (October) and was significantly affected by storage. This was associated with high deformability and levels of phenolics (but not necessarily tyrosine).
- MP appeared to show moderate bruising until later harvests (October). It appeared to have tissue with strong mechanical properties that protected tubers from impact and intermediate levels of phenolics and tyrosine.
- Tubers from defoliated plants presented lower incidence of bruising if harvested after 5 weeks of defoliation.
- Short storage periods (until December/January) did not increase bruising significantly, particularly for potatoes harvested early (LR) or later (MP, RB).

- Long storage periods (March) increased incidence of bruising for all varieties, and is associated with higher specific gravity, higher tissue deformability and higher phenolic acid and tyrosine levels.
- Nitrogen application increases weight and specific gravity, and does not affect bruising if potatoes are harvested early (Sept), however, higher incidence of bruising is observed in tubers from treated soils in later harvests.
- Tyrosine levels or specific gravity were not always associated with highest bruising incidence.
- While general trends were observed, the factors determining bruising seem to be dependent upon variety and the maturity of the tubers at harvest.
- Hot dry conditions during tuber development (observed in field trial 2) were associated with early plant senescence and high tuber incidence.

2 INTRODUCTION

Bruising of potato tubers represents a major problem for the potato supply chain, being the biggest single cause of consumer complaints. It is reported that 20% of the UK annual crop is lost due to bruising (BPC, 2011). Bruising occurs as a result of mechanical impact to the cortex and medullar cells just beneath the skin, but without actually breaking it. It is generally invisible to inspection staff unless the tuber is sliced open. Bruised tubers are therefore very difficult to remove on a pack house inspection line; so whole crops are often rejected even if only a few bruised tubers are presented (Prince, 2009).

It is generally accepted that a physical impact disrupts cellular membranes sufficiently so that the enzyme polyphenol oxidase (PPO) localized within plastids (chloroplasts and amyloplasts) comes into contact with phenolic compounds present in the vacuole (Corsini *et al.*, 1992, Blessington *et a*l., 2010, Strehmel *et al.,* 2010) and starts a series of reactions that lead to quinones which are bruising pigments (Falguera *et al.,* 2010). A subcellular redistribution of PPO 12 h after impact has been found to coincide with a loss of membrane integrity and was associated with melanic deposits as the bruise developed (Partington *et al.*, 1999). The impact may be dissipated in different ways due to structural or mechanical properties of the tuber tissue (Peterson and Hall, 1975), and is dependent upon velocity and energy of impact (Skrobacki *et al*., 1989). Phenolic compounds, substrates for PPO, are present mostly between the cortex and

skin (peel) tissues of the potato (Reeve *et al.,* 1969). Phenolic content is likely to be an important factor in determining bruise development.

Potato tubers contain a large number of phenolic compounds; some of them appear in free form while others are bound to polysaccharides or other high molecular weight molecules (Cuevas *et al.*, 2010). Among the phenolic acids, chlorogenic acid contributes up to 90% of the total phenol content of potatoes tubers and most of the discussion has centred around this compound (Friedman, 1997). However, contrasting findings do not allow a clear statement to be made on the contribution of other phenolic compounds to bruise formation (Corsini *et al.,* 1992, Mondy and Munshi, 1993, Friedman, 1997).

Phenolic compounds, at low concentration, may act as antioxidants and protect foods from oxidative deterioration, providing resistance of plants to pathogens and UV damage and protecting against effects of mechanical bruising (Friedman, 1997; Shakya and Navarre, 2006). However at high concentrations, they or their oxidation products may interact with proteins, carbohydrates and minerals (Karakaya, 2004, Karakaya and El, 2006). The PPO catalysed polymerization helps to seal the injured plant surface and begin the healing process, analogous to the formation of fibrin blood clots in injured humans (Friedman, 1997).

Another phenolic compound related to bruising is free tyrosine, which has been recognized as the main substrate for PPO (Dean *et al.,* 1992 and Stevens *et al.,* 1998). Partitioning of tyrosine between tuber protein and the free amino acid pool varies with genotype (Corsini *et al.*, 1992, Mondy and Munshi, 1993). However, it is likely that tyrosine or phenolic levels are not the only factors determining bruising, and other physicochemical properties of the tubers may be important in the response of the tuber to mechanical impact.

Cell walls of potatoes have an important role in regulating the mechanical properties of potato tubers and also influence the textural quality of many processed potato products (Jarvis *et al.*, 2003). The cell wall is a rigid structure encasing plant cells which resists turgor pressure and mediates cell-cell adhesion. Specifically, unesterified galacturonic acids residues of pectin can make bridges with Ca^{+2} with formation of strong gels which increase firmness (Pardade, 2005; Ross *et al.,* 2011; Ross *et al.,* 2011b) and increase cell–cell adhesion, particularly in the pectin-rich middle lamella between cells (Fry, 1986; Jarvis, 1998).

Side chains of RG-I of pectin may also play some role in cell wall firmness. Ulvskov and co-workers (Oomen *et al.,* 2002, Skjøt *et al.*, 2002, Ulvskov *et al.,* 2005, Orfila *et al*., 2012) proposed that the components of RGI (galactan and arabinan) transmit stresses in the wall and hence play a direct role in wall rheological properties. Loss of arabinan and galactan was also associated the loss of firm texture in apples (Pena and Carpita, 2004). Alteration of arabinan content has been associated with cell adhesion defects in tomato (Orfila *et al,* 2001). The role of the cell wall in relation to bruising has not been previously investigated.

Softening is often observed after long storage of fruits and vegetables and is usually associated with a decrease in the firmness of the tissue (Pardade, 2005). The changes in texture are related to a decrease in turgor and concomitant changes to the composition and structure of cell wall components through the activity of cell wall-degrading enzymes, leading to disassembly of the cell wall and a loss of cell-cell adhesion. Softening in fruits and vegetables is accompanied by pectin solubilisation (Pardade, 2004).

Several studies have shown that genotypic differences among varieties can have a strong influence in frequency and extent of bruising (Corsini *et al*, 1992; McGarry *et al.,* 1996). Potato cultivars (Maris Piper (MP), Lady Rosetta (LR) and Russet Burbank (RB) have been shown to vary in their susceptibility to bruising in potatoes grown under controlled conditions. These varieties of potatoes are known to differ in their tendency toward bruising where MP and LR present a bruising susceptibility score of 6, and RB bruising score of 4 in ratings ranging from 0 (most susceptible) to 9 (least susceptible) (Carnegie e*t al.,* 2005, British Potato Variety Database, 2012).

Observations suggest that environmental conditions also affect bruising (McGarry *et al.,* 1996). The maturity status before the harvest is a relevant factor in bruising. Maturity is related to the time when the plant will desiccate and tuber skin is set (Pringle *et al,* 2009). It is known that the content of phenolic substrates tend to be less abundant in early (immature tubers) than in late-season (Lisinska and Leszczynski, 1989). Levels of free tyrosine in immature tubers were shown to be 0.081 and 0.038 mg/100g fresh weigh for the varieties Pontiac and Ontario harvested 9 weeks after planting and then increasing progressively, incrementing by 0.5 mg/100g fresh weigh when harvested 11 weeks after planting. Evidence shows that the concentration of tyrosine in stored tubers is highly dependent on tuber maturity at harvest (Mondy and Munshi, 1993).

In addition, maturity at harvest time is the predominant factor influencing processing quality of potatoes throughout storage (Groves *et al,* 2005). The cell walls within the tuber become weak and membranes leaky as tubers age, releasing substrates to PPO. Temperature and humidity control are important during storage to control rate of respiration and evaporation. On the other hand, bruising susceptibility tends to be higher in long, hot, and dry growing seasons apparently due to physiologically older tubers having higher levels of tyrosine (Corsini *et al.*, 1999)

One of the factors analysed in this study was defoliation. A previous Potato Council-funded project (Stalham, 2008) found that more bruising occurred in crops harvested three to five weeks after defoliation (21 and 35 days, respectively).

The use of fertilizer nitrogen can have a significant effect on a number of physiological processes in potatoes, such as crop senescence, skin set and dry matter (Pringle *et al.,* 2009). Excess nitrogen can delay maturity, reduce potato yield and delay the achievement of the dry matter content (Sun *et al.*, 2012), adversely affecting processing quality.

Early literature (De Bruyn, 1929) summarized by Mondy and Koch (1978) suggested that the incidence of bruising increased with application of nitrogen fertilizer. This effect has been recorded as an increase from 12 to 24% for an increase in nitrogen application from 30 to 100 kg/ha. (Koblet, *et al*. 1948 in McGarry, 1996). In contrast, the authors Kunkel and Dow (1961) observed that increasing nitrogen fertilizer from 100 kg/ha to 290 kg/ha was associated with a decrease in susceptibility to bruising using a falling bolt test. Rogers-Lewis (1980) observed no effect of additional nitrogen fertilizer on bruising incidence using a pendulum to damage the tubers, but recorded a decrease in incidence with additional nitrogen in one of three years experiments, when harvesting operations were used to inflict damage. Silva *et al.* (1991) also reported no effect of nitrogen on the incidence of bruising resulting from harvesting operations or from subsequent impacts in a rotating drum.

It was observed by Hole (1997) that timing of harvest can affect the incidence of bruising. With advancing crop development, tubers treated with nitrogen become less susceptible to bruising, thought the symptoms change from visible tissue fracture and brown discoloration to less obvious fracture and grey/black discoloration. This association may be due to additional nitrogen slowing the rate of crop development and maturity.

Considering that enzymatic browning is a significant problem, in the past years research has been carried out focusing on the study of bruising potato cultivars and genetic modification to develop resistant varieties, however, little attention has been paid to the defoliation, harvest time, storage time and application of nitrogen despite the fact that these are common agricultural practices that may have a relevant impact on the bruising susceptibility. There is also little understanding of the physicochemical factors that may determine bruise development. Therefore the objective of the present research was to investigate the influence of defoliation, harvest time, storage time and application of nitrogen on bruising incidence, and to investigate the relationship between observed bruising and physicochemical factors such as specific gravity, phenolic acids, tyrosine content, mechanical properties and cell wall composition of three UK potato varieties.

3 EXPERIMENTAL SECTION

3.1 Materials and Methods

3.1.1 Sampling

3.1.1.1 Field trial 1 (2010)

Potato plants from three different cultivars Maris Piper (MP), Russet Burbank (RB), and Lady Rosetta (LR) were grown at Cambridge University Farm (CUF), planted on 23 April 2010 and tubers harvested at four time points. Before the harvest, defoliation was carried out at two time points (early defoliation and late defoliation), as indicated in Table 3.1. Trials were randomized with two factors (variety, defoliation) with three replicate plots. Ten tubers per plot were collected and shipped to Leeds University on each harvest day.

Table 3.1. Field trial 1- Year 2010

Trial 1	**Harvest and Defoliation period**			
Harvest	**1st Harvest (H1)**	**2nd Harvest (H2)**	**3rd Harvest (H3)**	**4th Harvest (H4)**
Date	2nd August	16th August	9th September	20th September
Days after planted	101	115	139	150
Defoliation	D1 D2 D3	D1 D2 D3	D1 D2 D3	D1 D2 D3
Days after defoliation	0 / /	14 0 /	38 24 /	49 35 /

3.1.1.2 Field trial 2 (2011)

Potato plants from three different cultivars Maris Piper (MP), Russet Burbank (RB), and Lady Rosetta (LR) were grown at Cambridge University Farm (CUF), planted on 15 April

2011, and harvested at two time points and stored for three time points. Harvest and storage dates and periods are indicated in Table 3.2. Trials were randomized with three factors (variety, harvest and storage) with three replicate plots. Twenty tubers per plot were collected and either shipped to Leeds University or Sutton Bridge Crop Storage Research on harvest day. The tubers were stored in trays within temperatures and moisture controlled stores, at temperature below of 10º C and 95+% Relative Humidity (RH) at Sutton Bridge Crop Storage Research. Tubers were shipped from Sutton Bridge to Leeds University for analysis.

Table 3.2. Field trial 2- Year 2011/12

Trial 2	**Harvest and Storage period**					
Harvest	**1st harvest (H1)**			**2nd harvest (H2)**		
Date of harvest	19th September			12th October		
Days after planted	157			180		
Storage	Storage 1 (S1)	Storage 2 (S2)	Storage 3 (S3)	Storage 1 (S1)	Storage 2 (S2)	Storage 3 (S3)
Date of sampling	14th January	26th March	11th May	14th January	26th March	11th May
Days after harvest	H1-117	H1-189	H1-235	H2-94	H2-166	H2-212

3.1.1.3 Field trial 3 (2013)

Potato plants from cultivar Lady Rosetta (LR) were grown at Cambridge University Farm (CUF), planted on 15 April 2013, and harvested at four time points. Harvest dates are indicated in Table 2.3. Controls and application of 200kg/hectare nitrogen on the soil were studied. Trials were randomized with two factors (harvest and nitrogen application) with six replicate plots. Ten tubers per plot were collected and sent to Leeds University on each harvest day.

Table 3.3. Field trial 3 – Year 2013

Trial 3	**Harvest period**			
Event	1st harvest (H1)	2nd harvest (H2)	3rd harvest (H3)	4th harvest 4 (H4)
Days of harvest	22th July	5th August	22th August	5th September
Days after planted	98	112	129	143

3.1.1.4 Tuber preparation

Tubers were cleaned upon arrival to Leeds University to remove excess soil. Measurements of physical and mechanical properties and bruising assessment (except

optical density) were conducted with fresh tubers. Fresh tissue to be used for microscopy was processed by exposure to fixative.

Potatoes used to analyse biochemical properties and composition were prepared as follows. Potatoes were cut transversely into slices of 1 cm thickness and the stolon and bud ends were discarded. The skin was peeled and the cortex was separated from the internal section (medullary layer) using a scalpel blade. The internal part was cut in cubes (0.5 cm^3). The three different tissues were fast frozen in liquid nitrogen, kept frozen at -20ºC and freeze dried for 48 h (SB4 Freeze drier, temperature -30°C, pressure 1,5 Torr). Each sample used in further analysis (oxidative potential, tyrosine, phenolic acids and cell wall composition) was obtained by mixing equal proportions from 3 potato tubers from each plot. The freeze dried tissue was ground to a fine powder using a food processor for 5 min and stored at -20°C until use.

3.2 Bruising assessment

3.2.1 Assessment of severe bruising using the falling bolt method – field trial 1

The incidence of bruising was assessed following the falling bolt damage test (Stalham, 2008). Tubers were cooled down to 6°C in the fridge. The impact was made using a steel coach bolt of 182.6 g in weight with a regular hexagon with diameter of 13 mm, and overall bolt height of 11.5 mm. The bolt was dropped from a height of 335 mm inside an aluminium guide tube of 40 mm internal diameter onto the flat surface of the tuber leading to a force applied of 0.6 J. The guide tube was held by a pair of retort stand clamps, one acting as a guide, the other clamping the tube at the correct height above the tuber. The impacting surface was a MDF work surface.

‘Hot boxing’ was performed by placing the potatoes following impact for 48 hours in an incubator at 33ºC, >95% RH. The potatoes were brought to room temperature two hours before being examined by peeling. A single peel (depth 1.2-1.5 mm) was removed at the site of impact using an Oxo Good Grips Swivel Peeler and then a further three peeler strokes were made to detect deeper damage. Calibrations were performed on the peel thickness by measuring 50 random peel slices with a Mercer England Thickness Gage (reading 0.1mm). Bruising was classified following peeling based on a procedure developed at the Sutton Bridge Crop Storage Research (2008).

- No bruise: no visible bruise following initial exploratory peel with a domestic peeler.

- Slight bruise: no visible bruise after two additional strokes of a domestic peeler following initial exploratory peel.
- Severe bruise: bruise visible after two additional strokes of a domestic peeler following initial exploratory peel.

3.2.2 Assessment of severe bruising using the falling bolt method– field trials 2 and 3

Adaptations of the method from year 1 were made due to high incidence of bruising on field trial 2. Impact tests were carried out at 20-22ºC with the steel coach bolt used in field trial 1, impacting on the stolon end of potatoes. Different energy level (0.3 J) and incubation time and temperature (25ºC for 20 h, humidity > 95% RH) were used. After bruising development, potatoes were analysed as previously described on 3.2.1. Determination of Bruising Index (BI) was also carried out following procedure (Croy, 2007). The impact zone of each tuber was analysed and a measure of the width and the depth of pigmented tissue was taken with a visual assessment of the bruise pigment intensity compared with the surrounding tissue, using the following scale:

0 – No visible pigmented tissue at site of mechanical impact

1 – Low level of pigmented tissue (typically pink, red, red-brown, grey)

2 - Intermediate level of pigmented tissue (typically brown or brown-black)

3 - High level of pigmented tissue (typically blue-black or black)

Colour intensity was classified using Munsell Atlas Hue 9R to standardize the assessment of colours as shown in figure 3.1.

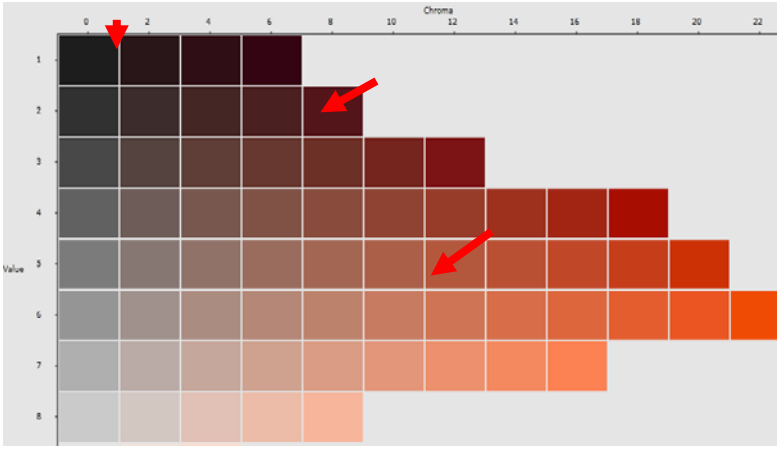
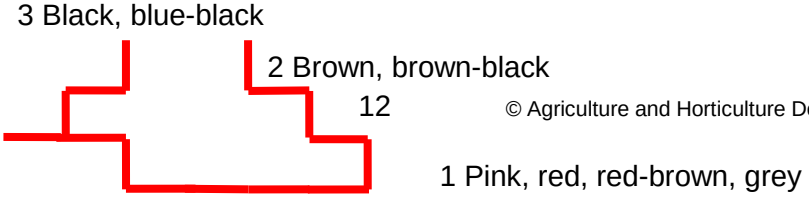


Figure 3.1 Scale used to classify colour intensity of pigmented tissue after falling bolt impact and incubation.



The mean for bruise depth, width and pigment assessment were calculated and used for comparing bruise susceptibility between different varieties and harvest and storage (trial 2) and nitrogen application (trial 3) and also on tubers from the same variety. Bruising index was calculated using equation 1:

Equation 1 $$\frac{\pi \times \frac{1}{2}(\text{bruise width})^2 \times \text{bruise depth} \times \text{bruise pigment intensity}}{235.6}$$

This assumes a cylindrical shaped bruise zone and by dividing by 235.6 this compares the bruise indices on a scale of 0 – 10 to a bruise with diameter 10mm, depth 10mm and bruise intensity 3 – the highest value observed in practice

3.2.3 Falling bolt impact captured by high speed camera

Images of falling bolt impact were recorded with a Phantom high speed camera V9 (Dantec), setting of 1000 frames-per-second (FPS) at the School of Mechanical Engineering, University of Leeds. Samples from harvest 2 (H2) stored for 212 days (S3) were used, 3 replicates for each variety. The assessment of bruising of these tubers followed the protocol described in 3.2.2.

3.3 Physical properties

3.3.1 Weight and specific gravity

Weight of individual potatoes was measured using a semi-analytical scale (field trial 1 n=9 and field trial 2 and 3 n=30, 10 and 5 samples per each plot respectively).

Specific gravity was determined on individual potatoes from field trial 2 and 3 (n=30 per variety, 10 and 5 samples per each plot respectively) using weight in air and weight in water method (Fong, 1973). Specific gravity was calculated by using equation 2.

Equation 2 $$\textbf{Specific gravity} = \frac{\text{Weight in air (g)}}{\text{Weight in water (g)}}$$

3.4 Mechanical properties

3.4.1 Energy required to break the potato skin and cortex tissue

Mechanical properties were investigated using a penetration test with a TA.XT plus Texture Analyser (Stable Micro Systems Ltd, Surrey, UK). For sample preparation, a tuber was taken from ambient temperature, cut transversely in slices of 1 cm in thickness and the slice from the middle of the tuber was separated for penetration test. The probe used to perform the test was a cylinder of 2 mm in diameter, test speed was 20 mm/sec

with tagged mode distance 5 mm, with trigger type auto (force) and trigger force 0.5 N. For each potato one test was carried out on the cortex and one test on the skin as shown in figure 3.2. To perform the test on the skin, 1 cm of the side of the potato was cut and placed vertically on the texture analyser plate. Three samples were measured for each plot on potatoes from field trial 1, ten replicates per plot on potatoes from field trial 2 and five replicates from each plot from field trial 3. Force and distance were obtained from the curve plotted from the software of the TA.XT plus Texture Analyser at the yield point (point where rupture of tissue occurs). Energy (mJ) was calculated by multiplying force (N) by distance (mm).

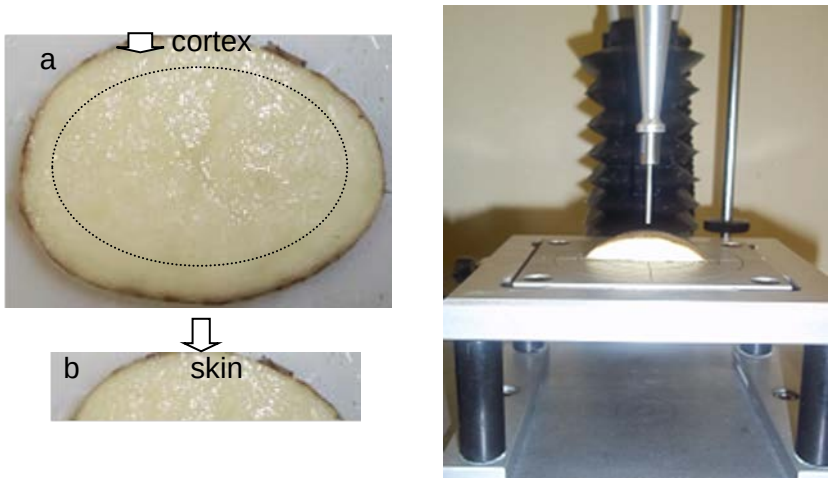


Figure 3.2 Transverse section with the points analysed with penetration test. a, cortex; b, skin cortical layer**.**

3.5 Phenolic composition

Chemicals ethylenediaminetetraacetic acid (EDTA) and formic acid were purchased from Fisher Bioreagents and methaphosporic acid from Alpha Aesar. Acetonitrile HPLC grade was purchased from VWR. Milli-Q purified water was used for dilutions and solvent preparation. Standards of phenolic acids were purchased from Sigma. The external standard method of calibration was used, with each curve prepared from 8 different concentrations of standard solutions. A representative range of phenolic acids known to be present in potato was selected for this study namely chlorogenic acids (3-, 4-, and 5-caffeoylquinic acid (CQA)), ferulic acid (FA), vanillic acid (VA), caffeic acid (CA) and *p* coumaric acid (*p*Cou). Sinapic acid was used as internal standard. Stock solutions of phenolic acids were prepared in duplicate at a concentration of 10 mg/mL in 50% ethanol and the dilutions made with Milli-Q purified water. The stock solution of dl-tyrosine was prepared in duplicate at concentration of 10mg/ml in 0.1N HCl and dilutions made with

Milli-Q purified water. Stock solutions of the standard solutions were stored in darkness at 4 ˚C.

3.5.1 Extraction of phenolic compounds

The method of extraction was adapted from Shakya and Navarre (2006). Phenolic compounds were extracted in triplicate from freeze dried cortex of three tubers with 1.5 mL of extraction buffer (50% MeOH, 2.5% metaphosphoric acid, 1 mM EDTA) and 500 mg of glass beads 1.0 mm in diameter. Tubes were shaken using a vortex for 10 min and sonicated at 10 °C for 10 min. After sonication, tubes were shaken again with the vortex for 10 min. Tubes were centrifuged at 4000 rpm at 4°C for 10 minutes and the supernatant was collected. Extractions were repeated three times and supernatants combined. The supernatants were dried under vacuum using a centrifugal evaporator at room temperature and low boiling point (BP) condition (Genevac SP Scientific, Ipswich, Suffolk, UK), resuspended in 0.5 mL of with Milli-Q purified water and filtered in 0.45um PTFE filter prior to HPLC analysis. Samples were kept chilled at all times and not exposed to bright light.

3.5.1.1 Analysis of phenolic acids using high performance liquid chromatography

Analysis of phenolic compounds was performed according to the method of Farrel *et al.,* (2011). Reversed phase HPLC Agilent 1200 Series HPLC consisting of a solvent degassing unit, binary pump, autosampler, thermostatic column oven and diode array detector was used to analyse phenolic acids. The column used was an Agilent Zorbax Eclipse plus C18, 4.6 mm x 100 mm, 1.80 micron internal diameter and 600 bar maximum pressure. Column temperature was 35°C, flow rate of 0.26 mL/min and injection volume of 5 µL. The 61-min elution program consisted of a isocratic elution from 0-17.5 min with 100% solvent A (0.1% formic acid, 5% acetonitrile and 94.9% water), followed by linear gradient from 17.5-51 min to 25% solvent B (0.1% formic acid, 5% water and 94.9% acetonitrile), linear gradient from 51-51.1 min up to 100% solvent B, isocratic elution from 51.1-56 min with 100% solvent B, linear gradient from 56-56.1 to 0% solvent B and isocratic elution from 56.1-61 min with 0% solvent B. The photo-diode array detection spectra was recorded at wavelengths of 220, 260, 280, 300, 310 and 325 nm.

3.5.1.2 Analysis of tyrosine using high performance liquid chromatography

The method for tyrosine analysis was adapted from Shakya and Navarre (2006). Reversed phase HPLC Shimadzu (Prominence) consisting of a solvent delivery unit,

column oven, autosampler, UV-Vis detector, photo-diode array detector, and on-line degassing unit was used to analyse tyrosine. The column used was Phenomenex Onyx, 4.6mm x 150 mm, 5 micron internal diameter. Column temperature was 30°C, flow rate of 1.5 mL/ min and injection volume of 10 µL. The 22-min elution program consisted of isocratic elution from 0-9 min with 100% solvent A (10 mM formic acid, pH 3.5, with ammonium hydroxide), followed by a linear gradient from 9-10.5 min 35% buffer B (100% methanol with 5 mM ammonium formate), linear gradient from 10.5 -14 min with 65 % solvent B; linear gradient from 14-16.5 min up to100% solvent B, linear gradient from 16.5 -18 min to 0 % B and isocratic gradient from 18-22 min with 0% solvent B. UV-VIS detection spectra was recorded at a wavelength of 280 nm.

3.6 Cell wall ultrastructure and composition

3.6.1 Immunofluorescence localization of cell wall polymers

Fresh tuber specimens (0.5 mm^3) were fixed in 4% formaldehyde in PEM buffer (50mM Pipes, 5 mM MgSO4 and 10mM EGTA, pH 6.9). Fixative was removed with PEM and the samples was washed with phosphate buffer saline (PBS), dehydrated in ethanol 30-70% series and embedded in Steadman wax (9:1 polyethylene glycol 400 distearate and 1-hexadecanol). Wax embedded periderm samples were sectioned using a microtome with blade at 11 degrees and 50 µm thickness. Sections were placed onto polysine-coated glass slides, followed by dewaxing with ethanol 97-50% series. Prior to the labelling procedure, sections were incubated with 150 uL of 3% (w/v) milk protein in PBS for 1 hour to reduce nonspecific binding. Monoclonal antibodies JIM5 and JIM7 were kindly provided from Professor Paul Knox (Centre for Plant Sciences, University of Leeds, UK). The sections were incubated overnight at 4°C in the primary antibodies, diluted 1:5 in PBS with milk. Control sections were incubated in PBS alone. Samples were washed twice with 0.1% v/v tween 20 in PBS for 10 min. All sections were incubated at room temperature for 1 hour in secondary antibody anti-rat FITC (Sigma), diluted 1:100 in PBS. Samples were washed 10 min with 0.1% v/v tween 20 in PBS plus 10 min in PBS. Samples were then stained with 0.1% Toluidine Blue for 10 min, washed for 10 min in PBS, mounted with anti-fading glycerol phosphate buffered solution (Citifluor AF1, Agar Scientific, UK) and covered with a glass cover slip. Observations were made with a BH2 Olympus microscope equipped with blue epifluorescence and Confocal Zeiss Axioplan Imaging LSM 510 Meta.

3.6.2 Optical localization of biological wall membranes

Fresh samples were hand cut and embedded samples (as described on 3.6.1) were used. Samples were cut using a microtome to thickness varying from 12-35 um. Fresh and dewaxed samples were stained with 0.1% Toluidine Blue for 10 min, washed for 10 min in PBS, mounted on glass slides and covered with a glass cover slip. Observations were made using light with optical BH2 Olympus microscope.

3.6.3 Analysis of Cell Wall Material (CWM)

3.6.3.1 Extraction

Isolation of the cell walls material was carried out according adaptations from methods to analyse the cell wall (Jardine *et al.,* 2002; Obro *et al.,* 2004; Ross *et al.,* 2011) and commercial enzymatic protocols (Megazyme methods) to analyse total starch (amyloglucosidase/ α-amylase method) and total dietary. The enzymes used to hydrolyse starch and protein are summarised in table 3.4. Lyophilized cortex (1 g) was homogenised using a homogenizer (Ultra Turrax , IKA, Staufen, Germany) at 13.500 rpm with 5 mL of mixed-cation buffer (MCB) (10mM NaOAC, 3mM KCl, 2mM $MgCl_2$ and 1 mM $CaCl_2$, pH 6.5) containing Triton X-100 (2mg/ml). The adequate disruption was achieved with up to 5 minutes of homogenisation and checked under the light microscopy using toluidine blue for staining the cells membranes. All procedures were carried out at 4˚C. The detergent suspension was removed by washing through a 45 µm metal sieve with 10 mL of chilled MCB without Triton X100. The residue was washed with 10 mL of 50% chilled acetone. To deproteinate samples, two procedures were tested. On the first, the washed residue was stirred with 80% (v/w) saturated phenol for 30 minutes following by filtration and washes with MCB. After this step, gelatinisation was carried out in 10 mL MCB at 80°C for 45 minutes following by incubation with α-amylase as described below. On the second method tested the washed residue was gelatinised with 10 mL MCB at 80°C for 45 minutes and incubate at 40°C for 45 min with 400 ul of pancreatin solution (10mg/mL) (P7545 Sigma) before starch digestion with 11.700 U heat stable α-amylase (A3306 Sigma)**.** The temperature was cooled down to 20°C following incubation for 2h.

Table 3.1 The enzymes involved in hydrolysis of starch and protein

Source	Substrate	Specific Activity Unit/ Portion	Optimum pH	Stable pH	Optimum temperature	Stability temperature
α – Amylase – One unit hydrolyse 1 mg of maltose						
Bacillus licheniformis	p-nitrophenyle maltoheptaoside	39,000 U/mL	6.9	5.1-8.2	20°C	< 75°C
Amyloglucosidase – one unit liberate 1.0 mg of glucose						
Aspergillus niger	starch	2,725 U/mL	5.0		25°C	
Pullulanase – one unit liberate 1 umole of maltotriose						
Klebiela pneumonia	pullulan	32,877 U/mL	5.0		25°C	
Pancreatin 8 × USP specifications - amylase, trypsin, lipase, ribonuclease and protease.						
Porcine pancreas	proteins, starch and fats	>250/mg solid	7.5		40°C	

After the α-amylase incubation step, the suspension was adjusted to pH 5.0 with 1M acetic acid and a combination of pullulanase (12 U) (P5420- Sigma) and amyloglucosidase (12 U) (A9913 Fluka) were added to enzymatically degrade branched starch. After incubation for 14 hour at 25˚C, the presence of starch was monitored by removing small aliquots of the insoluble material and staining with 0.2% iodine to visualise the starch using light microscope. The cell suspension was washed using a metal sieve 45 µm with 2L of water and 10mL of 50% acetone.

After washing, the purified cell wall material (CWM) was dried overnight in an oven at 35°C. CWM hydrolysis was performed in duplicate in two steps. 2 mg of CWM were first hydrolysed with 1mL of 0.1M trifluoroacetic acid (TFA) for 1h at 100˚C. Samples were centrifuged at 4000 rpm at 4°C for 10 minutes and the supernatant was collected. The CWM solid residue from step one was then hydrolysed with 2 M TFA for 1h at 100°C. Tubes were centrifuged at 4000 rpm at 4°C for 10 minutes and the supernatant was collected. 500 µl from each supernatant (0.1 and 2M TFA) were combined and the TFA was removed using a centrifugal evaporator (Genevak, Surrey, UK). Dried samples were resuspended with 1 mL of milli-Q purified water and filtered using a 0.45 um nylon filter prior to Dionex analysis. Samples were kept chilled at all times and not exposed to bright light.

3.6.3.2 Analysis of monosaccharide composition using high performance anion exchange chromatography amperometric detection (HPAEC - PAD) - Dionex

The method used was adapted from Obro *et al.,* 2004. The monosaccharide composition was determined with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The column used was PA20 (Dionex, Thermo Scientific). Column temperature was 30°C, flow rate of 0.30 mL/min and injection volume of 10 uL. The 65-min elution program consisted of linear gradient with 10 uM NaOH from 0 – 1.5 min, followed by isocratic gradient with 5 uM NaOH from 1.5 – 30 min, linear gradient up to 1 M NaOH from 30-40 min, column washing with 1M from 40-45 min, linear gradient to 10mM from 45-55 min following equilibration of the column with 10 mM NaOH from 55 to 65 min. Monosaccharides were detected using a pulsed amperometric detector with gold working electrode and silver reference electrode. Monosaccharide standards were L-Fucose, L-Rhamnose, L-Arabinose, D-Galactose, D-Glucose, D-Xylose, D-Manose, D-Galacturonic acid and D-Glucuronic acid. Fructose was used as an internal standard. A standard mixture run was performed before sample analysis to determine response factor.

3.7 Statistical analysis

Mixed effects analysis of variance ANOVA was explored being randomised factors harvest time (field trial 1, 2 and 3) and storage time (only field trial 2) and considered as fixed factor the variety (field trial 1 and 2), defoliation (field trial 1) and supplement of nitrogen (field trial 3). Multiple comparisons have been performed with Student-Newman-Keuls (SNK) and confirmed with REGWQ - Ryan/Einot and Gabriel/Welsch test procedure. Individual variety results were explored using a factorial 2-way ANOVA to analyse the effect of factors harvest and defoliation (field trial 1), storage (field trial 2) and supplement of nitrogen (field trial 3). The relationships between results were summarised using Principal Component Analysis (PCA). Statistical analysis is performed using R for Windows (R-project). The Student's t test (Excel, Microsoft 2010) was performed to compare two samples on method development. Error bars shown on the graphs are standard errors of the mean.

4 RESULTS

4.1 Bruising assessment

4.1.1 Assessment of severe bruising using the falling bolt method – field trial 1

For potatoes of field trial 1 harvested early in the season (H1 and H2) no bruising was observed amongst any varieties or defoliation regime studied as shown in Figure 4.1. Potatoes were not subjected to 'hot boxing' at these times. At the 3rd harvest the highest incidence of severe bruising was found for RB and at the 4th harvest the highest was found for LR. The general trend observed in relation to defoliation was higher incidence of bruising in defoliated samples at H3 (D2 after 24 days of defoliation and D1 after 38 days of defoliation), only except RB D2. This was expected as previous studies from Stalham (2008) showed higher incidence of bruising when potatoes where harvested 3 to 5 weeks after defoliation. However at the 4th harvest, tubers from defoliated plants presented lower incidence of bruising than undefoliated (35 and 49 days after defoliation), except MP D2. The meteorological data collected during this field trial indicates that this growing season was characterised by bright warm days early during tuber initiation, with rainfall and cloud cover later in the season. These conditions are thought to be associated with low bruising incidence.

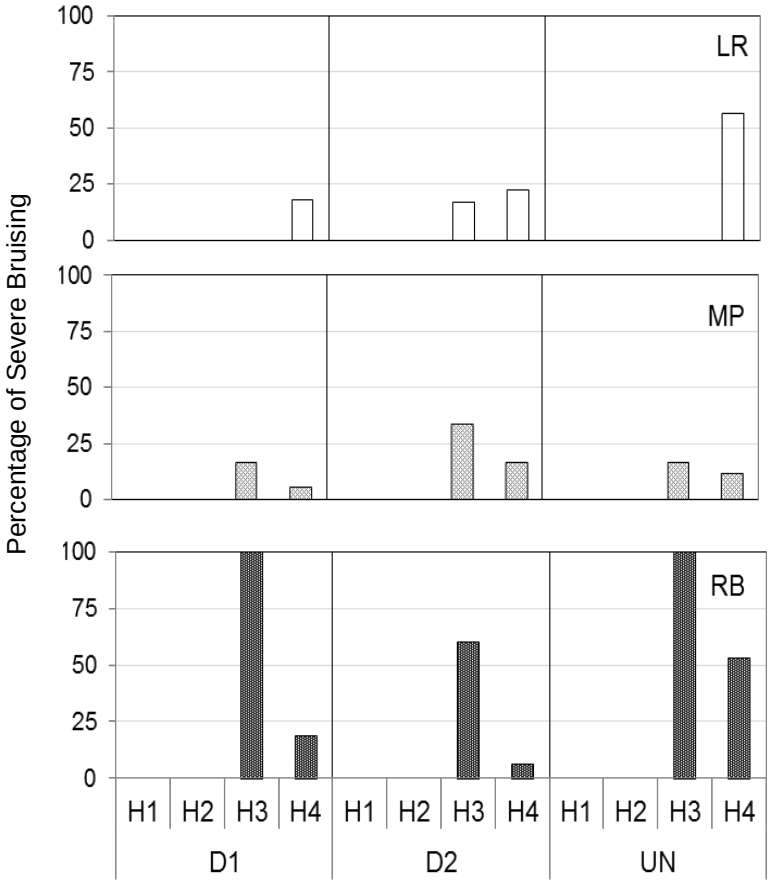


Figure 4.1 Effect of variety, harvest time and defoliation regime on percentage of severe bruising following damage using a falling bolt method in potatoes from crops harvested in early August (H1), late August (H2), early September (H3) and late September (H4), defoliated early August (D1), late August (D2) and undefoliated (UN), field trial 1. No bruising was found at H1 and H2, measured without hot boxing. Values show incidence (H1-H3 n=9 and H4 n=18).

4.1.2 Assessment of bruising index and severe bruising using the falling bolt method– field trial 2 and 3

Field trial 2. At the first harvest time, highest incidence of severe bruising has been found in RB (93%), followed by MP (61%) and LR (52%). At the second harvest, similar results were found for the three varieties. The highest incidence was found in RB (88%), which nevertheless showed a decrease in incidence of severe bruising by 5%, followed by MP (85%) showing an increase of 24% and LR (82%) with increase of 29% as shown in figure 4.2. This may mean that RB reaches its 'bruising maximum' earlier than the other two varieties.

The incidence of bruising in stored samples was influenced by the harvest time. More bruising was observed in the variety LR when harvested late. The opposite was found for the varieties MP and RB, presenting in general lower incidence of bruising when tubers were harvested late. Also, in these two varieties, potatoes showed a lower

incidence of bruising after a short period of storage compared to fresh potatoes, for both early and late harvested tubers.

A strong positive correlation between the incidence of severe bruising and Bruising Index was found for RB (R^2=0.82) and for MP (R^2=0.68). There was a moderate correlation for LR (R^2=0.37). The meteorological data collected during this field trial indicates that this growing season was characterised by bright warm days early during tuber initiation, with very little rainfall later in the season. These conditions are thought to be associated with high bruising incidence.

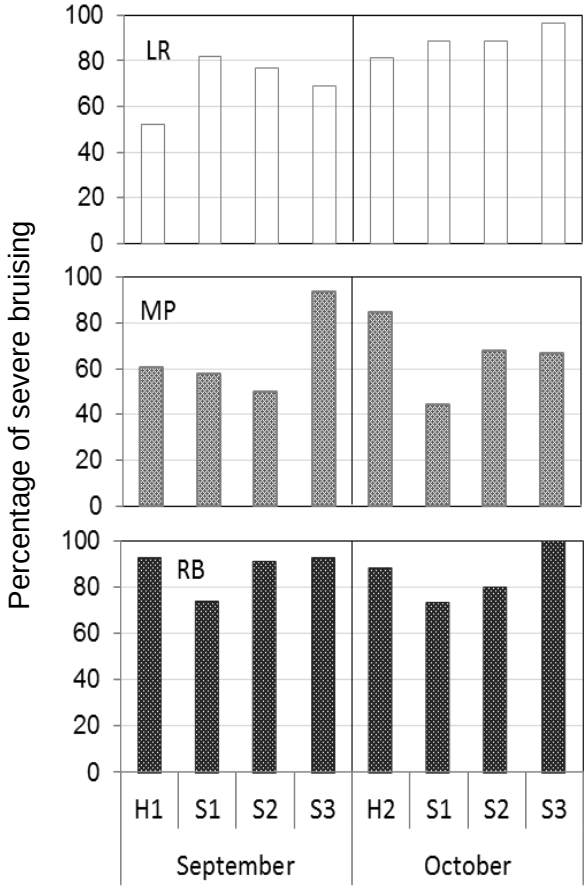


Figure 4.1 Effect of variety, harvest and storage time on the incidence of severe bruising following damage with the falling bolt in potatoes from crops harvested in September (H1) and October (H2) and stored until January (S1), March (S2) and May (S3), field trial 2. Values show incidence (n>21).

Field trial 3. Application of nitrogen reduced bruising incidence of the variety LR when tubers were harvested earlier than 112 days after planting (H2), as shown in Figure 4.3. After this period (H3 and H4), tubers grown in fertilized soil showed higher bruising incidence by 5 and 10 % than the control (no treatment) tubers. Results of the bruising index were slightly different than assessment of severe bruising. Tubers from treated soil presented lower BI when harvested up to late August (H3) and higher incidence when

harvested in September (H4). However, very strong positive correlation between results from incidence of severe bruising and Bruising Index was found for control tubers (R^2=0.92) and strong for treated tubers (R^2=0.64). The meteorological data collected during this field trial indicates that this growing season was characterised by bright warm days early during tuber initiation, with significant rainfall and cloud cover later in the season. These conditions are thought to be associated with low bruising incidence.

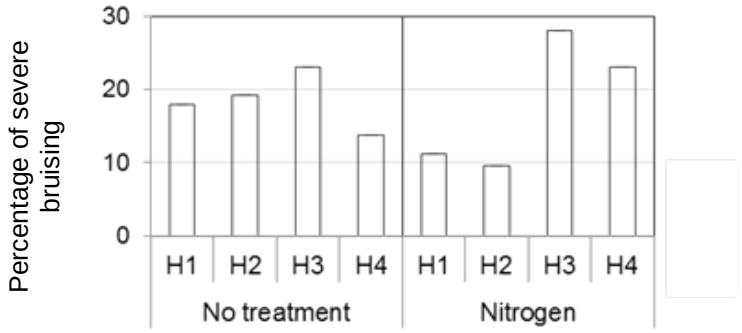


Figure 4.3 Effect of harvest and nitrogen treatment (200 kg/ha) on the incidence of severe bruising(%) following damage with the falling bolt the variety LR harvested in July (H1), August (H2 and H3) and September (H4), field trial 3. Values show incidence (n>21).

4.1.3 Falling bolt impact captured by high speed camera

It was found that the bolt impacts the tubers twice when it is dropped on the stolon end of a potato as indicated in Figure 4.4, transmitting to the tuber more energy than predicted (0.3-0.6J) by potential energy (E=m.g.h). The coefficient of restitution was calculated from the height of the bolt reached after the first impact for each variety and very strong positive correlation was found with bruising index (R^2 =0.97).

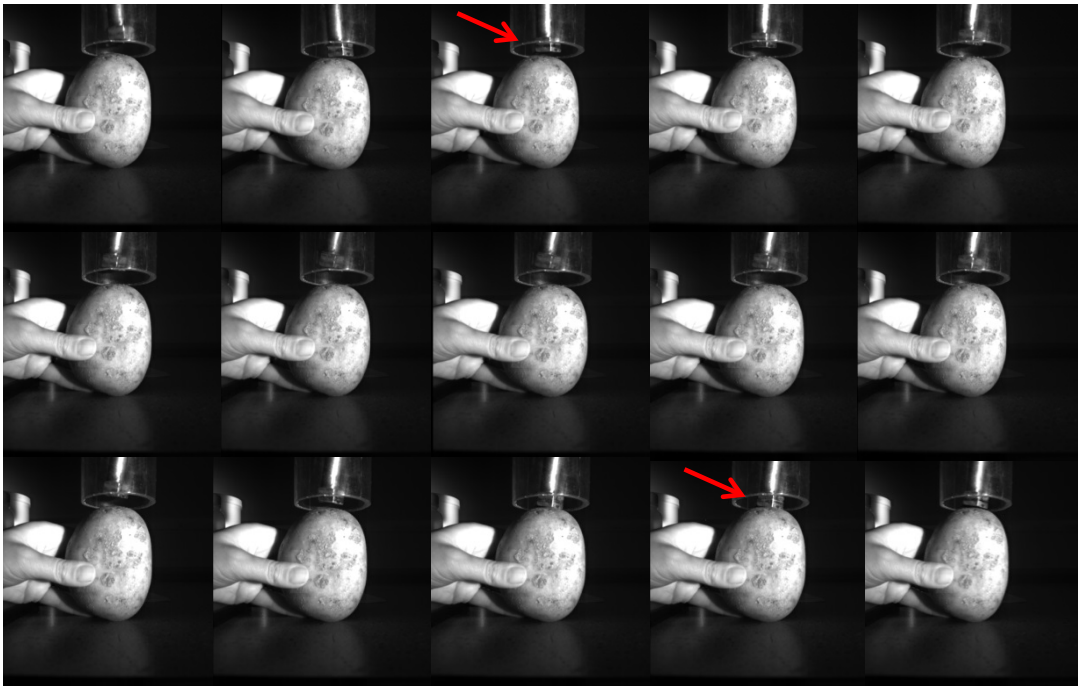


Figure 4.4. Impact on the stolon end of the potato using falling bolt. Images from high-speed camera every 0.02 sec. Arrows indicate contact between bolt and tuber.

4.2 Physical properties

4.2.1 Weight and specific gravity

4.2.1.1 Weight

Field trial 1. Results showed that the weight of tubers from all varieties studied were significantly affected by early defoliation comparing to undefoliated samples ($p<0.05$). The differences in weight of samples defoliated early (D1) and undefoliated at the 4th harvest were 33, 31 and 14 % for MP, LR and RB, respectively. Smaller differences were found when plants were defoliated later (D2), being 26, 14 and 2% for MP, LR and RB respectively. MP showed the largest differences among the varieties studied.

Field trial 3. Significant differences were found in the yield between treated and untreated samples at the 3th and 4th harvest ($p<0.01$) where tubers treated with nitrogen presented ~ 35% more weight than untreated.

There was no significant correlation between weight and bruising incidence in any field trial.

4.2.1.2 Specific gravity (SG)

Field trial 1. No SG data was collected.

Field trial 2. The results from H1 and H2 showed higher specific gravity in LR followed MP and RB (Figure 4.5). No significant difference ($p<0.05$) was found when comparing H1 to H2 for each variety studied. Although increments in SG at S1 were observed for all varieties compared to the respective harvest time (except MP H1S1), no significant difference was found at this time point ($p>0.05$). After a long storage period (S3), a decrease in SG was observed in potatoes harvest late. The decrease in SG at H2S3 was related to higher bruising in LR and RB compared to H1S3 but not for MP. Weak and negligible correlations were found for all the varieties between SG and the incidence of severe bruising (R^2 <0.25) and BI (R^2 <0.19) respectively.

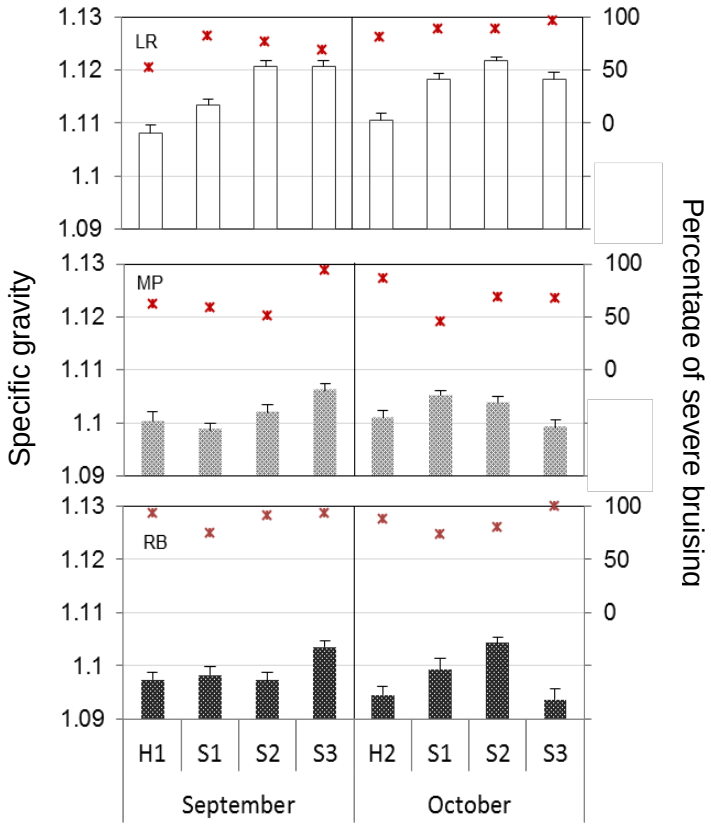


Figure 4.5 Effect of variety, harvest and storage time on the specific gravity of samples (bars) and percentage of severe bruising (scatter) from crops harvested in September and October, stored until January (S1), March (S2) and May (S3), field trial 2. Value bars show means (n=30), error bars are SE and scatter shows incidence of severe bruising (n>21).

Field trial 3. Significant differences were found in the SG between tubers treated and untreated with nitrogen only at the 3th harvest ($p<0.001$) where tubers treated with nitrogen presented higher SG than untreated. Along harvest times, significant increases in SG ($p<0.05$) were found in treated tubers between H1-H2, H2-H3 and a significant decreases between H3 and H4 ($p<0.05$) for both samples (treated/untreated), Figure 4.6. The decrease in dry matter may be due to an increase in turgor at the last harvest due high rainfall by end of August. A strong correlation was found for both treatments between SG and the incidence of severe bruising (R^2 =0.7) and a weak correlation with BI (R^2 =<0.49).

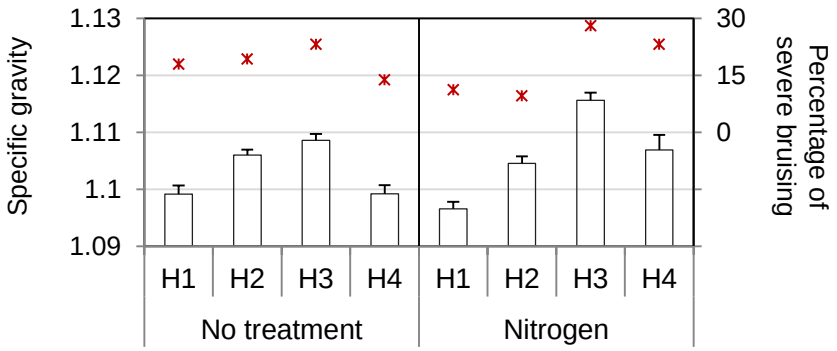


Figure 4.6 Effect of harvest and nitrogen treatment (200 kg/ha) on the specific gravity of the variety LR (bars) and percentage of severe bruising (scatter) harvested in July (H1), August (H2 and H3) and September (H4), field trial 3. Values show incidence (n>21).

4.3 Mechanical properties

4.3.1 Energy required to break the potato skin and cortex tissue

Field trial 1. Amongst the varieties the skin of the RB, variety that bruised the most, required on average lower energy to break, followed by LR and MP for all harvest times as shown in Figure 4.7 (top). The energy required to break the skin decreased along harvests for the varieties MP and RB. LR presented a trend in all treatments investigated, decreasing the energy to break the skin until H3, followed by slight increase at H4. This trend was also found on RB D1 samples but these results were not always associated with higher bruising incidence. Deformability (distance to break tissue) presented a slight increase in defoliated samples (D1) for RB and MP and LR all tubers. A moderate negative correlation was found for MP (R^2=-0.52) when analysing correlations between the energy to break the skin tissue and the incidence of bruising but not found for LR (R^2=0.06) and RB (R^2=0.28).

Measurement of mechanical properties of the cortex of potatoes from the first year's trial showed a decrease in the energy required to break the tissue along harvests (Figure 4.7 bottom). Significantly different levels of energy were required to break the cortex between the varieties studied ($p<0.05$), being lowest for RB, followed by MP and LR. A weak negative correlation between the energy required to break the cortex tissue and severe bruising incidence was found only for the variety MP (R^2=-0 38), not found for LR (R^2=0.19) and RB (R^2=0.13).

Comparing all varieties, there was no significant effect of defoliation on the energy required to break the skin or cortex along the harvest times.

Field trial 2. RB required on average a lower energy to break the skin and cortex tissues, followed by MP and LR as shown in picture 4.8. Significant increase in energy required to break the skin tissue was observed in stored samples ($p<0.01$) for all varieties, with exception of MP H1S2 compared to MP H1 as shown in Figure 4.8 (top). Comparing storage period, the only significant difference was found between S1 and S2 for MP harvested in September ($p<0.001$). Weak and moderate correlations were found when contrasting incidence of severe bruising and energy to break skin tissue for MP (R^2= -0.11) and LR (R^2=0.36) and no correlation was found for RB (R^2= -0.09). Results from energy to break the cortex tissue and incidence of severe bruising showed weak correlation for LR (R^2=0.20) and no correlation for RB (R^2 = -0.03) and MP (R^2= -0.13).

From the results of distance to rupture the skin tissue, LR presented higher degree of deformability, followed by MP and RB (data not shown). Increase in distance to rupture the skin was observed for LR along harvest whereas no changes were observed for the varieties MP and RB. Substantial increase was observed comparing fresh to stored tubers but no significant ($p>0.05$) differences were observed comparing storage periods.

Field trial 3. In general, the energy required to break the skin decreased with harvest in treated (nitrogen application) and untreated (control) tubers as shown in Figure 4.9. Significant differences were found between treatments ($p<0.05$) at H2 and H3, with treated samples requiring more energy to break the skin. Increased energy appears to be associated with higher incidence of severe bruising. Negligible and very strong negative correlations were found when contrasting incidence of severe bruising and energy to break skin tissue for control (R^2= -0.01) and treated (R^2=-0.77).

On average a slight decrease in the energy required to break the cortex was observed in tubers grown in non-treated soil but was not significantly different among harvests for both samples ($p>0.05$). The energy required to break the cortex tissue was not significantly different between the treatments studied ($p<0.05$). According to the results from previous trials, it was expected that the force to break the cortex tissue would diminish along harvests, particularly at H4, however this was not observed and may be due to those tubers being after a wetter month (August 2013) than other trials. Results from energy to break the cortex tissue and incidence of severe bruising showed no correlation for control (R^2=0.01) and very strong positive correlation for nitrogen treated tubers (R^2 = 0.88).

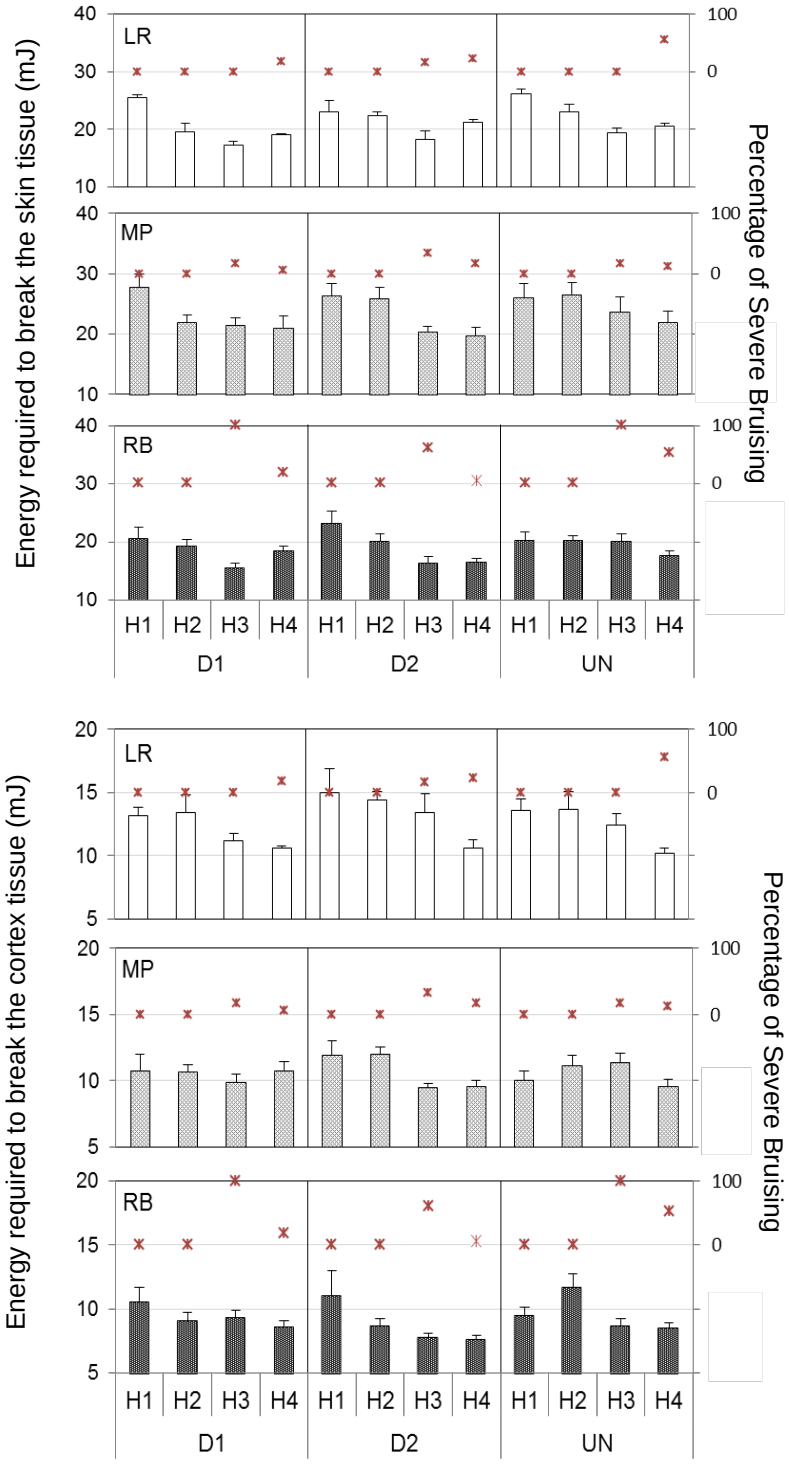


Figure 4.2 Effect of variety, harvest time and defoliation regime on energy to break the cortex tissue in mJ (bars) and percentage of severe bruising (scatter) from crops harvested in early August (H1), late August (H2), early September (H3) and late September (H4), defoliated early August (D1), late August (D2) and undefoliated (UN) , field trial 1. Values bars show mean (n=9), errors bars are SE and scatter shows incidence (H1-H3 n=9, H4 n=18).

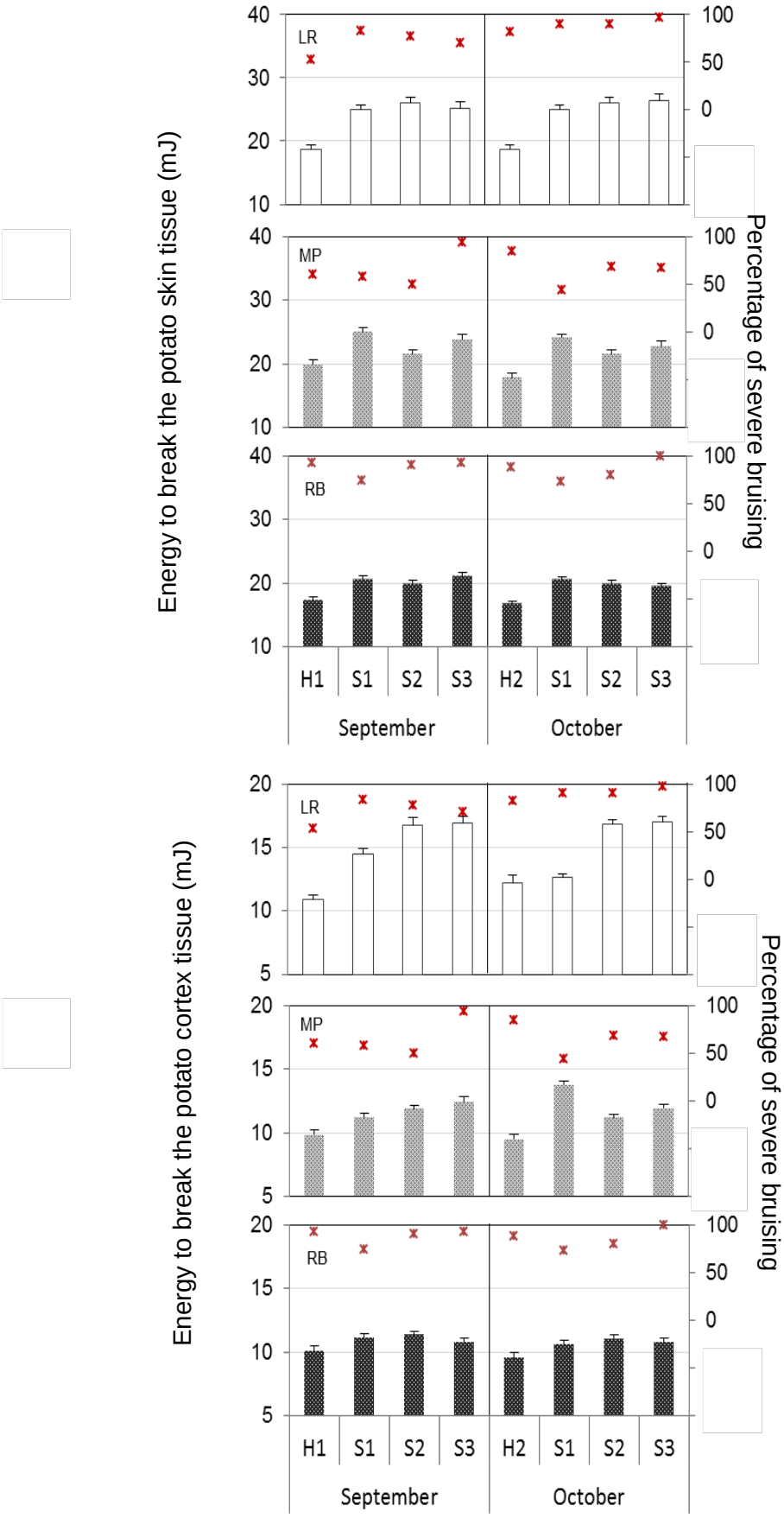


Figure 4.8 Effect of variety, harvest and storage time on the energy to break the potato cortex tissue (mJ) (bars) and percentage of severe bruising (scatter) from crops harvested in September and October, stored until January (S1), March (S2) and May (S3), field trial 2. Value bars show means (n=30), error bars are SE and scatter shows incidence of severe bruising (n>21).

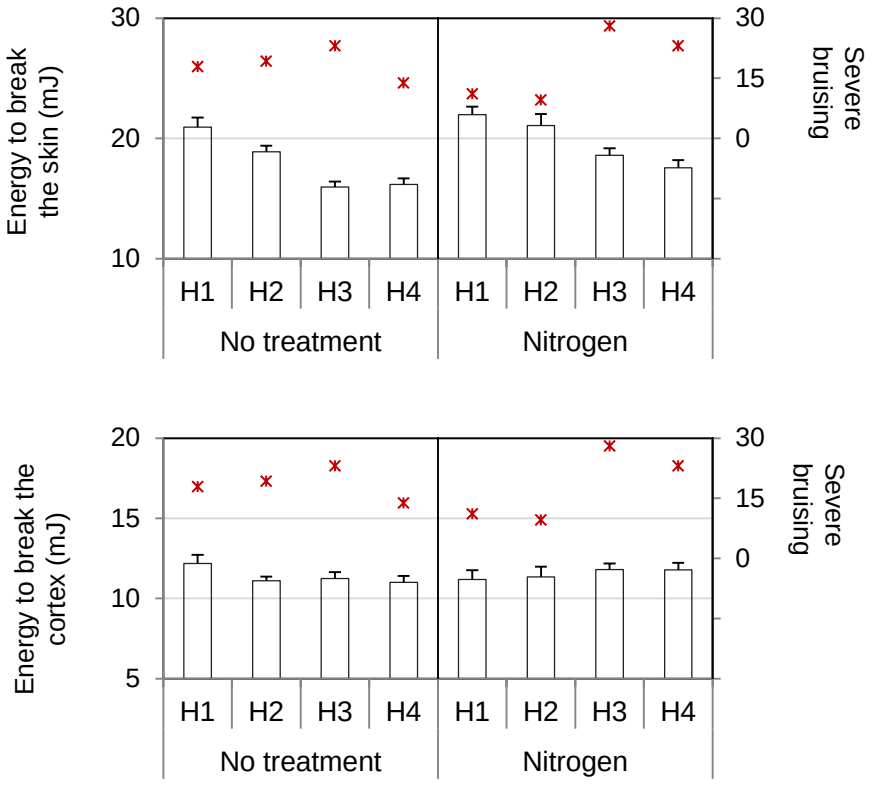


Figure 4.3 Effect of harvest and nitrogen treatment (200 kg/ha) on the energy required to break the skin tissue of the variety LR (bars) and percentage of severe bruising (scatter) harvested in July (H1), August (H2 and H3) and September (H4), field trial 3. Values show incidence (n>21).

4.3.2 Phenolic composition

4.3.2.1 Analysis of phenolic acids using high performance liquid chromatography

The most abundant compound found for all varieties was 5-O-caffeoylquinic acid (5-CQA), referred to commonly as a chlorogenic acid (CGA) (Clifford, 2000), as has previously been reported to be present in potato (Shakya and Navarre, 2006). Of the varieties examined, the highest amount of 5-CQA was found in LR, followed by RB and MP.

Field trial 1. Chlorogenic acid (5-CQA) concentration increased up to the 3rd harvest time and diminished from the 3rd to the 4th harvest for all varieties and defoliations. Defoliation significantly affected the content of 5-CQA for the varieties MP and RB compared to undefoliated samples ($p<0.01$) at H3 (higher amounts found in undefoliated) and H4 (higher amounts in undefoliated RB and defoliated MP). LR was not significant different ($p>0.05$) when defoliated late (D2) at the 3rd harvest and when defoliated early (D1) and late (D2) at the 4th harvest compared to undefoliated samples.

Defoliation showed significant differences ($p<0.05$) in the composition of the isomers of CQA compared to undefoliated samples at the 3rd and the 4th harvest times. In general defoliated samples presented an increase in the content of 4-CQA and decreased the content of 3-CQA. A strong correlation between the incidence of severe bruising and concentration of chlorogenic acids in the cortex was found for the varieties RB ($R^2=0.64$) and MP ($R^2=0.52$), but not for LR ($R^2=0.09$).

Field trial 2. The varieties presented different metabolic profile of 5-CQA, as shown in Figure 4.11. The content of 5-CQA of MP was not changed significantly when comparing harvests, storage periods to harvests and periods between storage, with the only exception for the short storage H1S1 compared to H1 and H1S2 ($p<0.001$).

LR presented a significant decrease ($p<0.001$) in the content of 5-CQA when harvested late (October). Storage periods presented significant lower content 5-CQA ($p<0.01$) compared to the respective harvest (except H2S3 to H2) and significant differences between storage ($p<0.01$). However, comparing storage periods of tubers harvested in September and October, significant different contents of 5-CQA were found only in tubers stored until March ($p<0.001$).

RB showed a significant increase ($P>0.001$) during harvest. Significant increase ($p<0.001$) in the content of 5-CQA was found in tubers harvested early upon storage and significant decrease ($p<0.001$) in tubers harvested late, with exception of RB H2S2. Significant different contents of 5-CQA were found in tubers stored until January and March ($p<0.001$) when compared storage periods of both harvest.

Both 3-CQA and 4-CQA presented higher levels at stored tubers compared to fresh tubers irresrespective of harvest time. The results from three caffeoylquinic acids quantitated (CQA's) showed a strong negative correlation with incidence of severe bruising for LR ($R^2=0.75$), moderate for RB ($R^2= 0.69$) and no correlation for MP ($R^2= 0.03$).

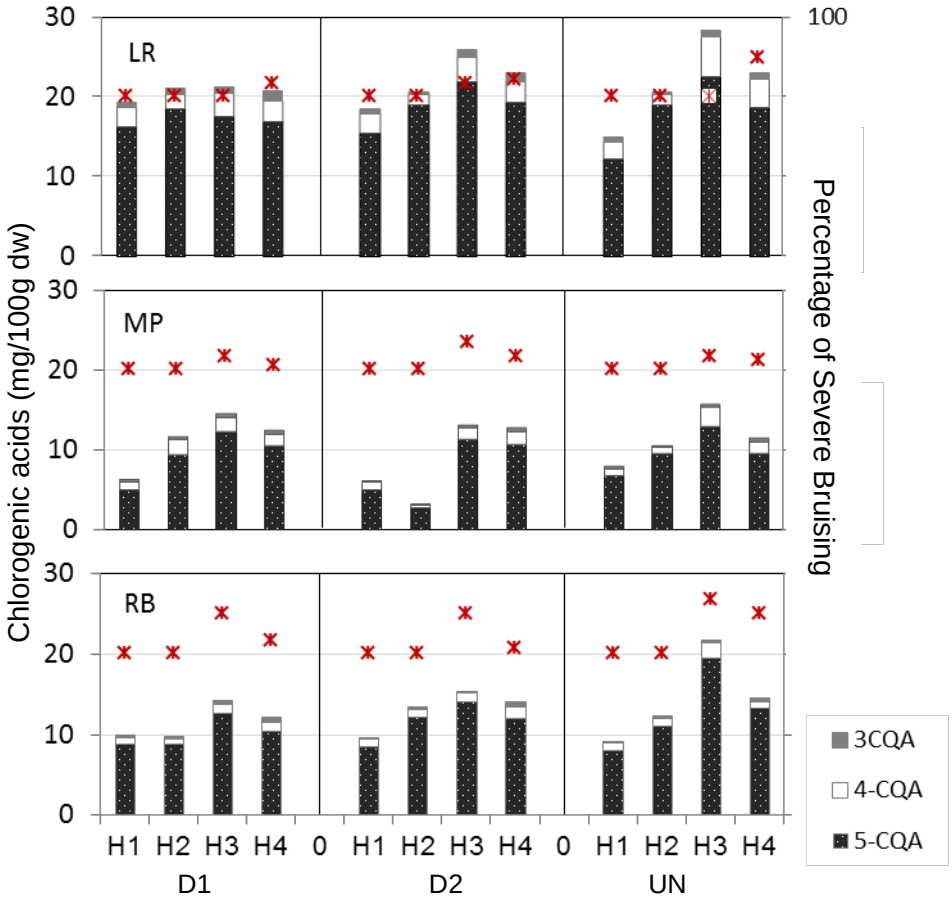


Figure 4.10 Effect of variety, harvest time and defoliation regime on chlorogenic acids (3-, 4- and 5-CQA) of lyophilized cortex (mg/100 g dw) and percentage of severe bruising from crops harvested in early August (H1), late August (H2), early September (H3) and late September (H4), defoliated early August (D1), late August (D2), and undefoliated (UN), field trial 1. Values bars show mean (n=3), errors bars are SE and scatter shows incidence (H1-H3 n=9, H4 n=18).

Field trial 3. Chlorogenic acid (5-CQA) increased in content up to the 3rd harvest time and diminished from the 3rd to the 4th harvest for both samples. These results were similar with results from the field trial 1. Significant increase was found from H2 to H3 and decrease from H3 to H4 in tubers untreated ($p<0.05$), but no significantly different were the treated samples ($p>0.05$) in 5-CQA content, shown in Figure 4.12

Tubers treated with nitrogen showed slight higher content of 5-CQA but significantly higher levels were only found at the 4th harvest ($p<0.001$). Comparing samples, 4-CQA and 3-CQA content were significantly different at the 2nd and 3rd harvest ($p<0.05$), increasing content from H1 to H3 and decreasing at H4 in treated tubers and decreasing content from H1 to H2 following increase in treated tubers. Strong

correlations were found when contrasting incidence of severe bruising and chlorogenic acids for control (R^2=0.78) and treated tubers (R^2=0.60).

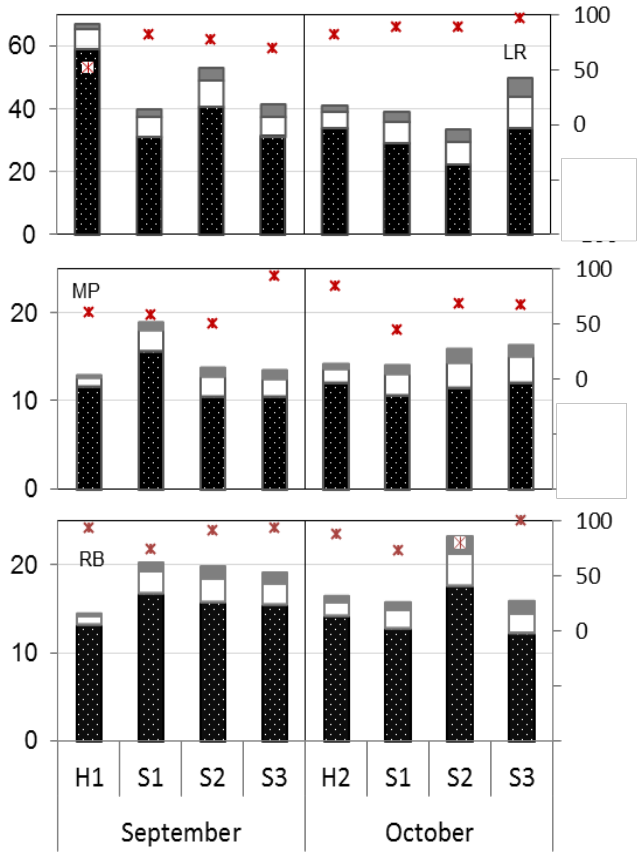


Figure 4.11 Effect of variety, harvest and storage time on chlorogenic acids (3-, 4- and 5- CQA) of lyophilized cortex (mg/100 g dw) (bars) and percentage of severe bruising (scatter) from crops harvested in September and October, stored until January (S1), March (S2) and May (S3), field trial 2. Value bars show means (n=30), error bars are SE and scatter shows incidence of severe bruising (n>21).

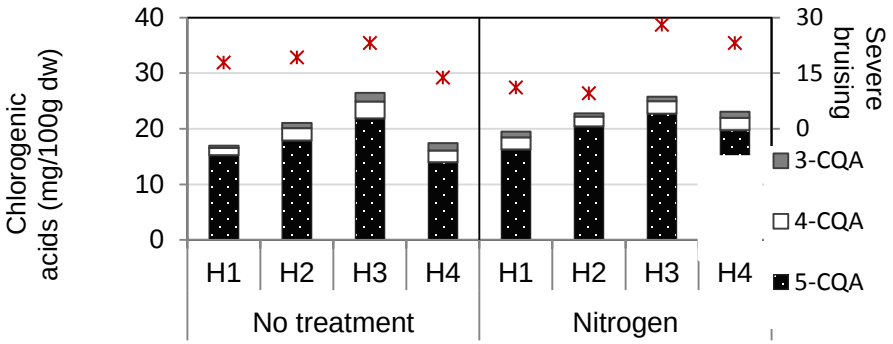


Figure 4.4 Effect of harvest and nitrogen treatment (200 kg/ha) on the chlorogenic acids (3-, 4- and 5- CQA) of lyophilized cortex (mg/100 g dw) (bars) and percentage of severe bruising (scatter) of the variety LR (bars) and percentage of severe bruising (scatter) harvested in July (H1), August (H2 and H3) and September (H4), field trial 3. Values show incidence (n>21).

4.3.2.2 Analysis of tyrosine using high performance liquid chromatography

Field trial 1. Significant variations in free tyrosine content were observed amongst all varieties ($p<0.001$), as shown in Figure 4.13. The higher amount of free tyrosine was found in RB, varying from 19.8-59.6 mg/100 g dw, followed by MP with 7.3-55.4 mg/100 g dw and LR from 4.1-30.1 mg/100g dw. These results were slight lower than cited by other the literature, with variation from 9 to 319 mg/ 100g of dw (Lisinska and Leszczynski, 1989), however, tyrosine content in early-season cultivars tended to be less than that in late-mature potatoes, as previously reported (Lisinska and Leszczynski, 1989).

Although significant differences in the content among varieties were found, all of them showed the same trend with small variation when comparing H1 and H2 and increase in the content from H2 to H4. Statistical analysis of variance showed significant differences, either in samples defoliated in early August (D1) and late August (D2) when compared to undefoliated samples ($p<0.01$) for all varieties studied. A higher content of tyrosine was found in potatoes defoliated early and later (D1 and D2 respectively) than undefoliated samples. A weak correlation between incidence of severe bruising and tyrosine content was found for LR ($R^2=0.41$), but not for other varieties ($R^2=0.10$ for MP and RB).

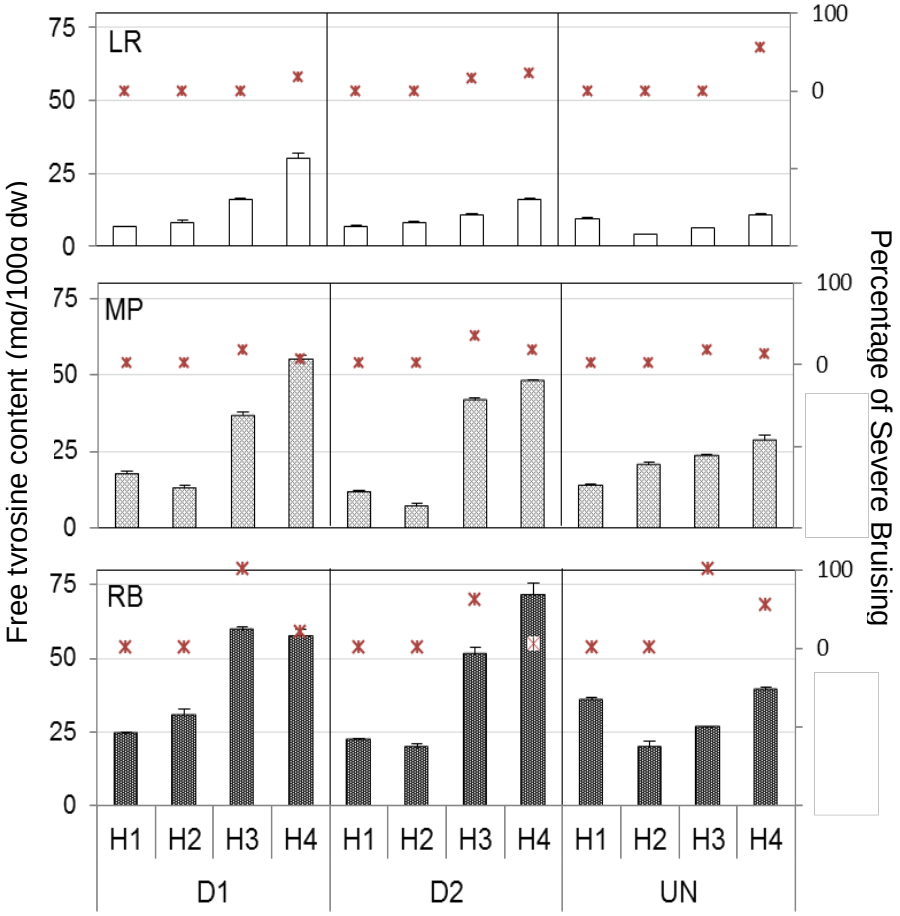


Figure 4.13 Effect of variety, harvest time and defoliation regime on free tyrosine levels of lyophilized cortex in mg/100 g dw (bars) and percentage of severe bruising (scatter) from crops harvested in early August (H1), late August (H2), early September (H3) and late September (H4), defoliated early August (D1), late August (D2) and undefoliated (UN), field trial 1. Values bars show mean (n=3), errors bars are SE and scatter shows incidence (H1-H3 n=9, H4 n=18).

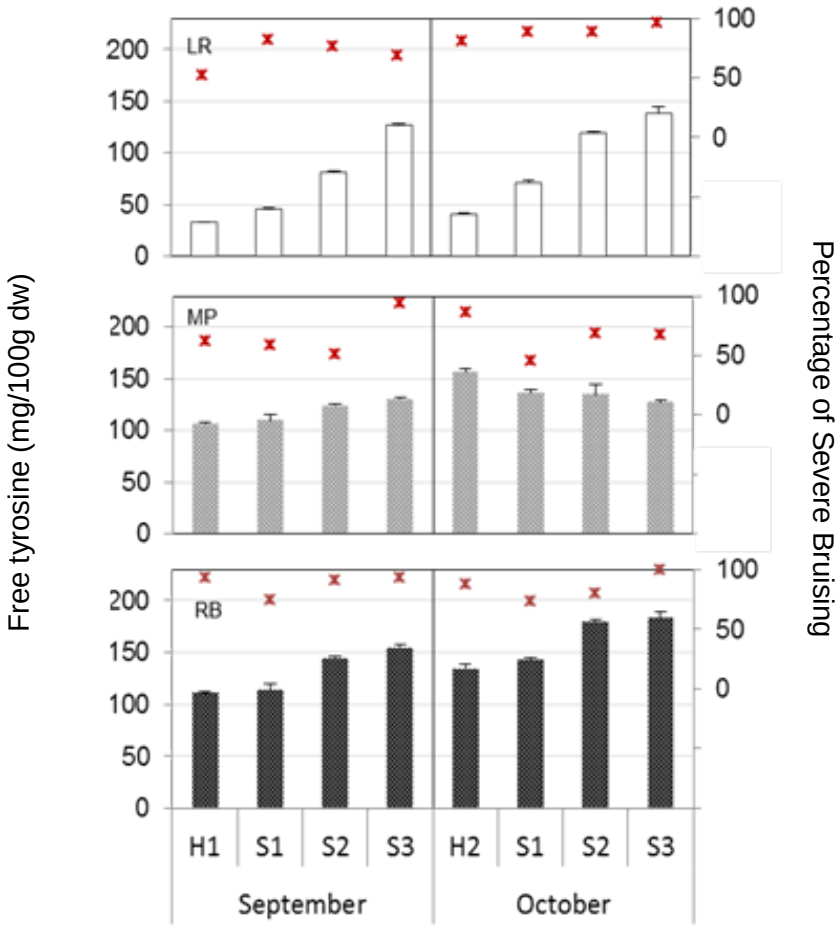


Figure 4.14 Effect of variety, harvest and storage time on free tyrosine content of lyophilized cortex (mg/100 g dw) (bars) and percentage of severe bruising (scatter) from crops harvested in September and October, stored until January (S1), March (S2) and May (S3), field trial 2. Value bars show means (n=30), error bars are SE and scatter shows incidence of severe bruising (n>21).

Field trial 2. Significant variations of free tyrosine content among all varieties was observed ($p<0.001$). The concentration of free tyrosine in the tuber cortices were on average higher for RB, the most susceptible cultivar to bruise, followed by MP and LR as shown in Figure 4.14. Higher levels of free tyrosine were found in field trial 2 compared to field trial 1.

A significant increase ($p<0.001$) during growth was found for all varieties. LR and RB showed increase of 21% on the tyrosine content at H2 compared to H1 whereas MP showed increase of 46%.

A linear increase along storage periods was found for all varieties with the only exception for MP H2 which presented linear decrease along the storage period. In tubers harvest early, marked significant increase ($p<0.001$) was found between S1 and S2 for all varieties. In potatoes harvested late (H2), slight higher content of tyrosine was observed in stored and significant increase was found only for LR for all storage periods.

Weak correlation coefficients (R^2) between the incidence of severe bruising and free tyrosine was found for LR and MP (R^2=0.2) and no correlation found for RB (R^2=0.1).

Field trial 3. Significant variations in free tyrosine content between samples treated with nitrogen and untreated were found at the 2nd, 3rd and 4th harvest (p<0.001), being higher for untreated samples as shown on figure 4.15. Tubers from untreated soils had a spike in tyrosine content at H3 which was not observed in treated samples. Moderate and strong positive correlations were found when contrasting incidence of severe bruising and free tyrosine levels for control (R^2=0.38) and treated tubers (R^2=0.65) respectively.

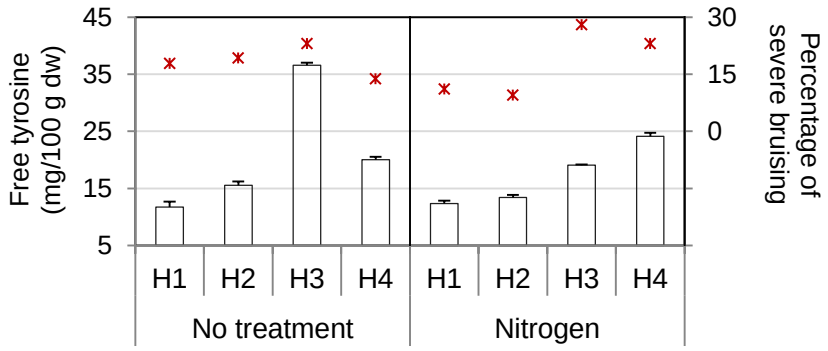


Figure 4.55 Effect of variety, harvest and storage time on free tyrosine of lyophilized cortex (mg/100 g dw) (bars) and percentage of severe bruising (scatter) from crops harvested in September and October, stored until January (S1), March (S2) and May (S3), field trial 2. Value bars show means (n=3), error bars are SE and scatter shows incidence of severe bruising (n=>21).

4.4 Cell wall ultrastructure and composition

4.4.1.1 Optical localization of cell membranes

General anatomical features of potato tissue were investigated by light microscopy in order to identify the differences between varieties and characteristics of different tissues (e.g. skin and cortex). Wax embedded sections were stained with toluidine blue to show cell membranes as presented in Figure 4.16.

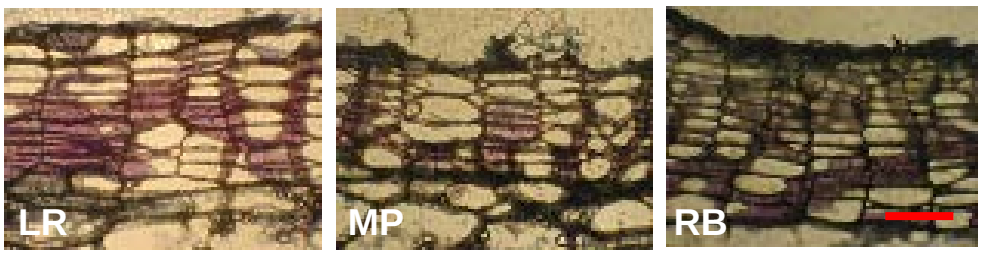
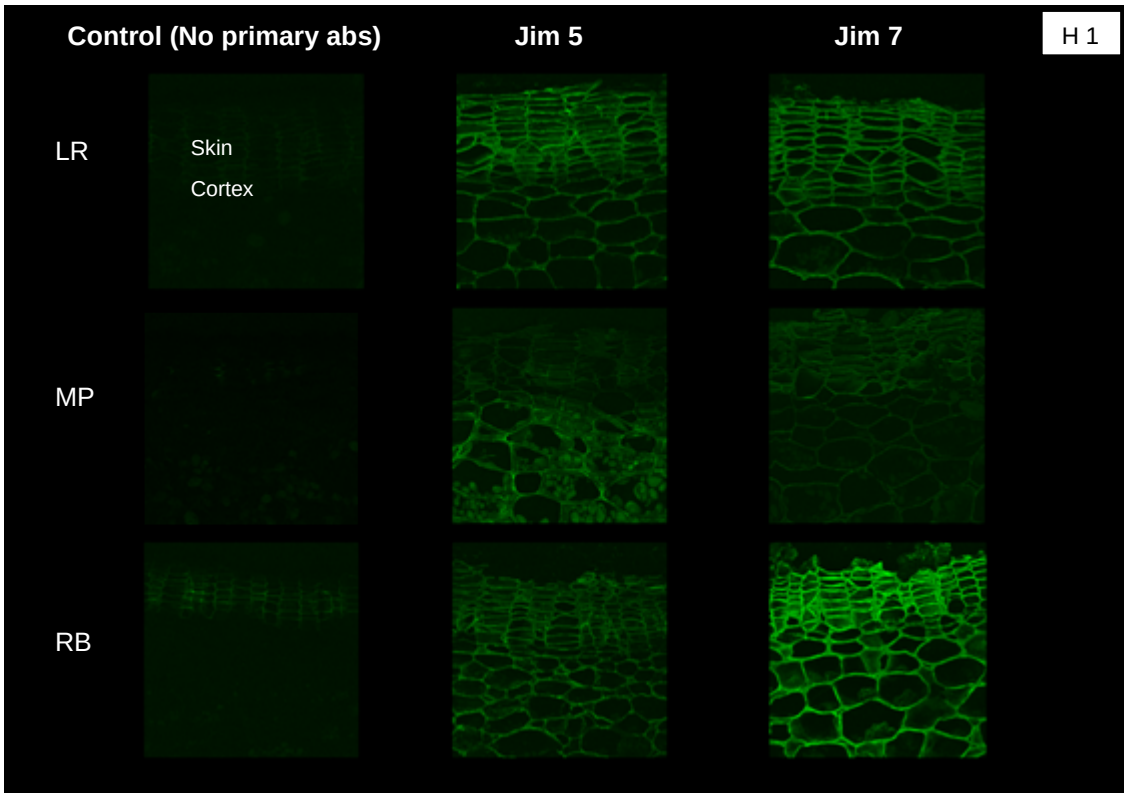


Figure 4.16 Toluidine blue staining of membranes in wax-embedded sections of skin. Magnitude 20X. Scale bar =50 uM

On the skin, RB presented suberized cells stacked with adjacent cells compressed together whereas LR and MP presented a “ragged” appearance. The “ragged” organisation may act to prevent transmission and dissipation of forces throughout the potato, leading to more energy to be absorbed at the point of impact rather than transmitting and distributing it to the rest of the tuber. Comparing the structural characteristics with the results from the energy to break the skin tissue, LR and MP required respectively 25.5 and 33.3% more energy than RB to break the skin respectively (H4, field trial 1).

4.4.2 Immunofluorescence localization of cell wall polymers – trial 2

Cortical tissues were investigated by immunofluorescence microscopy using specific antibodies JIM5 and JIM7 that recognise methyl-unesterified and methyl-esterified homogalacturonan domain of pectic polysaccharides, respectively. Immunofluorescence micrographs from LR, MP and RB from crops under two harvest time conditions (September and October) are shown in figure 4.17



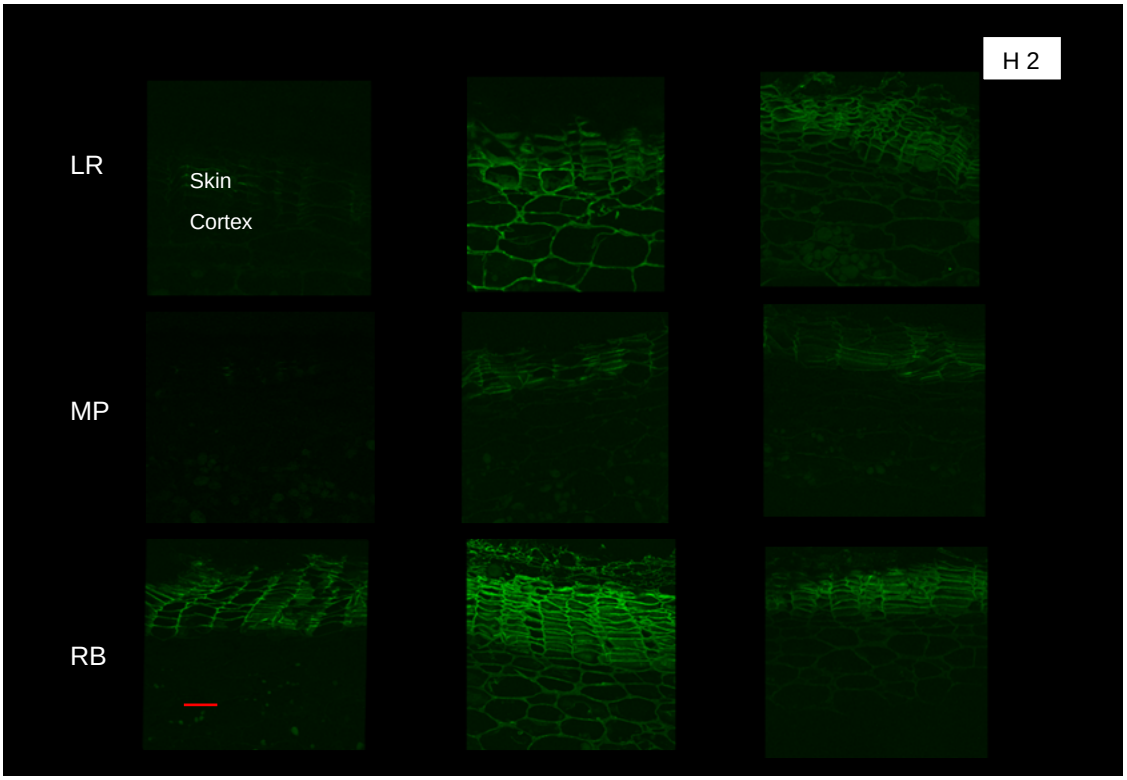


Figure 4.17 Fluorescent microscopy of potatoes cortex sections labeled with JIM5 or JIM7. Control indicates no primary antibody. Magnification 20X, scale bar=50um

Tubers from LR showed loss of methyl (JIM7) in cortex tissue when harvested in October (H2) and an increase in binding of partially methylesteriefied homogalacturan (JIM5). This can be associated with lower levels of pectin methyl esterification in cortex cell walls. This may allow pectin to ionically interact with calcium in later harvest (H2). Similar association was found for RB, where there was highly methylated pectin in H1 (JIM7) and micrographs fade in colour in H2, showing losses in methylation.

MP presented loss of partially methylesteriefied (JIM7) comparing potatoes harvested H1 to H2 but it was not associated with increase of unmethylated (JIM5).

The qualitative nature of the labelling does not allow for numerical correlations to be made with bruising incidence or mechanical properties. For this reason, cell wall composition was analysed with chromatography as described below.

4.4.3 Analysis of monosaccharide composition using high performance anion exchange chromatography amperometric detection (HPAEC - PAD) – Dionex

The molar ratio of the neutral sugars (Ara+Gal) to Uronic acids (GalA+GluA) can give information about the branching of the pectic polysaccharides, assuming that all neutral sugars are present as side chains (van Marle *et al.*, 1997). A higher molar ratio of

Ara+Gal to Uronic acids implicates the presence of more and/or longer neutral sugar side chains. The side chain extend cannot discriminate between either a small number of relatively long polymerization degree (DP>) side chains or a large number of relatively small (DP<) side chains. The molar ratio of uronic acids to rhamnose can be used as an indication for the number of side chains and represent the measure of linearity of the cell wall pectin (van Dijk *et al.,* 2002).

Field trial 1. Among all varieties, the molar ratio of Ara+Gal to UA ranged from 1.8 to 3.2 (Table 4.1). The results found were comparable with the potato varieties Nicola and Irene with molar ratios of 2.3 and 2.6, respectively when CWM were hydrolysed with 2M TFA (van Marle *et al.,* 1997). Comparing varieties, MP and RB presented more or longer side chains than LR samples (higher molar ration (Ara+Gal/UA)), LR presented higher linearity of pectin than MP and RB defoliated and undefoliated. No pattern was found when analysing defoliation. There was no correlation between incidence of severe bruising and linearity of pectin (R^2=0.00) or branching (R^2=0.01) of all varieties and defoliations together.

Table 4.1 Neutral sugars (Rham+Ara+Gal), branching (molar ratio of Ara + Gal to UA) and number of side chains (UA/Rhamnose) in CWM of the cultivars LR, MP and RB harvested in later September 2010 (H4), defoliated early August (D1), late August (D2) and undefoliated (UN) after sequential with 0.1M and 2M TFA.

	Varieties and defoliation								
Trial 1	**LR D1**	**LR D2**	**LR UN**	**MP D1**	**MP D2**	**MP UN**	**RB D1**	**RB D2**	**RB UN**
Neutral sugars (Rham+Ara+Gal)	59.5	54.6	54.7	65.4	68.0	67.5	64.2	60.3	66.6
Molar ratio (Ara+Gal/UA) Linearity	2.2:1	1.8:1	2.0:1	2.8:1	3.0:1	3.1:1	2.6:1	2.0:1	3.2:1
UA/Rhamnose Branching	31.3:1	49.1:1	62.1:1	21.6:1	27.7:1	23.3:1	21.9:1	33.7:1	19.8:1

Field trial 2. On the basis of the data presented in table 4.2, changes in cell wall composition along harvest and upon storage were observed. The LR pectin becomes less linear (the ratio of UA/Rha decreases) and more branched (the ratio of Ara+Gal /UA increases) along harvest and upon storage. MP and RB pectin becomes less linear along harvest and along storage (the ratio of UA/Rha decreases) and less branched along harvest and upon storage (the ratio of Ara+Gal /UA decreases). Very strong positive correlations was found when contrasting incidence of severe bruising and linearity of pectin for LR (R^2=0.80) and strong negative for the varieties MP (R^2=0.-52) and RB

(R^2=0.-59). When comparing incidence of severe bruising and branching of pectin, Very strong negative correlations were found for LR (R^2=-0.72) and strong negative correlation for MP (R^2=-0.36) but no correlation was found for RB (R^2=-0.09).

Field trial 3. It was observed similar molar ratio of Ara+Gal to Uronic acids in untreated and treated samples (1.1-1.2), which implicates in no difference about the presence of more and/or longer neutral sugar side chains.

The molar ratio of uronic acids to rhamnose (the measure of linearity of the cell wall pectin) was lower in tubers supplied with nitrogen, being less linear than untreated. Comparing results from H3 and H4, tubers from untreated soils showed a decrease in pectin linearity and tubers treated increased in linearity. These differences are possibly due the delay in maturity of tubes supplied with nitrogen. Moderate positive correlations was found when contrasting incidence of severe bruising and linearity of pectin (R^2=0.32) but not for branching (R^2=0.04).

Table 2.2 Neutral sugars (Rham+Ara+Gal), branching (molar ratio of Ara + Gal to UA) and number of side chains (UA/Rhamnose) in CWM of the cultivars LR, MP and RB harvested in September (H1) and October (H2) 2011 and stored until May 2012 (S3) after sequential with 0.1M and 2M TFA.

Trial 2	Varieties and time			
	LR H1	**LR H1S3**	**LR H2**	**LR H2S3**
Neutral sugars (Ara+Gal+Rha)	57.6	54.2	59.0	52.6
Molar ratio (Ara+Gal/UA)	2.0:1	2.6:1	2.5:1	4.0:1
UA/Rhamnose	82.0:1	44.4:1	58.4:1	30.8:1
	MP H1	**MP H1S3**	**MP H2**	**MP H2S3**
Neutral sugars (Ara+Gal+Rha)	51.0	51.9	54.6	52.4
Molar ratio (Ara+Gal/UA)	2.2:1	1.4:1	1.6:1	1.5:1
UA/Rhamnose	112.2:1	53.4:1	50.1:1	59.2:1
	RB H1	**RB H1S3**	**RB H2**	**RBH2S3**
Neutral sugars (Ara+Gal+Rha)	63.8	58.0	60.1	50.9
Molar ratio (Ara+Gal/UA)	2.9:1	1.9:1	2.6:1	1.4:1
UA/Rhamnose	66.7:1	95.7:1	44.4:1	66.0:1

Table 2.3 Neutral sugars (Rham+Ara+Gal), branching (molar ratio of Ara + Gal to UA) and number of side chains (UA/Rhamnose) in CWM of the cultivar LR untreated and treated with nitrogen (200 kg/ha), harvested in July (H1), August (H2 and H3) and September (H4), field trial 3 after sequential with 0.1M and 2M TFA.

	HARVEST 3		**HARVEST 4**	
Trial 3	**No treatment**	**200 kg/ha**	**No treatment**	**200 kg/ha**
Neutral sugars (Ara+Gal+Rha)	43.8	49.8	45.0	44.7
Molar ratio (Ara+Gal/UA)	1.2	1.1	1.1	1.2
UA/Rhamnose	68.8	9.3	50.9	23.7

It was expected to observed an increase in the strength of the tissue presenting less linear pectin, which may ionically interact with calcium but these observations were not enough to generate changes in mechanical properties according to the analyse studied.

4.5 PCA

Further correlation analyses were conducted to investigate the relationships. The results for the three varieties studied under four harvests, two regimes of defoliation (field trial 1), three storage periods (field trial 2) and LR grown in soil treated or untreated with nitrogen (field trial 3) are summarized in the PCA bi-plot (Figure 4.17). The labels are indicated in Table 2.4. These analyses generated a substantial number of correlations and the model of PCA explained about 60% of the data variance. However, only the first component allowed discrimination of varieties. Investigation into the relative contribution (loadings) of individual variables in the PC1 dimension highlighted components with a significant effect on bruising and tyrosine, not correlated to mechanical properties of skin (WS) or cortex (WC).

Severity of bruising and caffeic acid dimensions showed orthogonally, suggesting strongly negative correlation on this study, providing evidence of a useful link between bruising and this phenolic acid. Other phenolic acids were correlated with severe bruising incidence.

Both first and second component allowed discrimination of varieties where LR was more associated with chlorogenic acids, MP with CA and tyrosine and RB more associated with tyrosine.

Table 2.4 Labels of samples in the PCA graphs from LR, MP and RB crops harvested in early August (H1), late August (H2), early September (H3) and late September (H4), defoliated early August (D1), late August (D2) and undefoliated (UN), field trial 1.

Lady Rosetta						Maris Piper				Russet Burbank			
No.	Trial1	N.	Trial2	No.	Trial3	No.	Trial 1	No.	Trial 2	No.	Trial1	No.	Trial 2
			LR H1		LR NO		MPD1H		MP H1				RB H1
1	LRD1H1	13	Y2	21	H1	29	1	41	Y2	49	RBD1H1	61	Y2
					LR NO		MPD1H						
2	LRD1H2	14	LR H1S1	22	H2	30	2	42	MP H1S1	50	RBD1H2	62	RB H1S1
					LR NO		MPD1H						
3	LRD1H3	15	LR H1S2	23	H3	31	3	43	MP H1S2	51	RBD1H3	63	RB H1S2
					LR NO		MPD1H						
4	LRD1H4	16	LR H1S3	24	H4	32	4	44	MP H1S3	52	RBD1H4	64	RB H1S3
			LR H2				MPD2H		MP H2				
5	LRD2H1	17	Y2	25	LR N200 H1	33	1	45	Y2	53	RBD2H1	65	RB H2Y2
							MPD2H						
6	LRD2H2	18	LR H2S1	26	LR N200 H2	34	2	46	MP H2S1	54	RBD2H2	66	RB H2S1
							MPD2H						
7	LRD2H3	19	LR H2S2	27	LR N200 H3	35	3	47	MP H2S2	55	RBD2H3	67	RB H2S2
							MPH2H						
8	LRD2H4	20	LR H2S3	28	LR N200 H4	36	4	48	MP H2S3	56	RBD2H4	68	RB H2S3
	LRUNH						MPUNH				RBUNH		
9	1					37	1			57	1		
	LRUNH						MPUNH				RBUNH		
10	2					38	2			58	2		
	LRUNH						MPUNH				RBUNH		
11	3					39	3			59	3		
	LRUNH						MPUNH				RBUNH		
12	4					40	4			60	4		

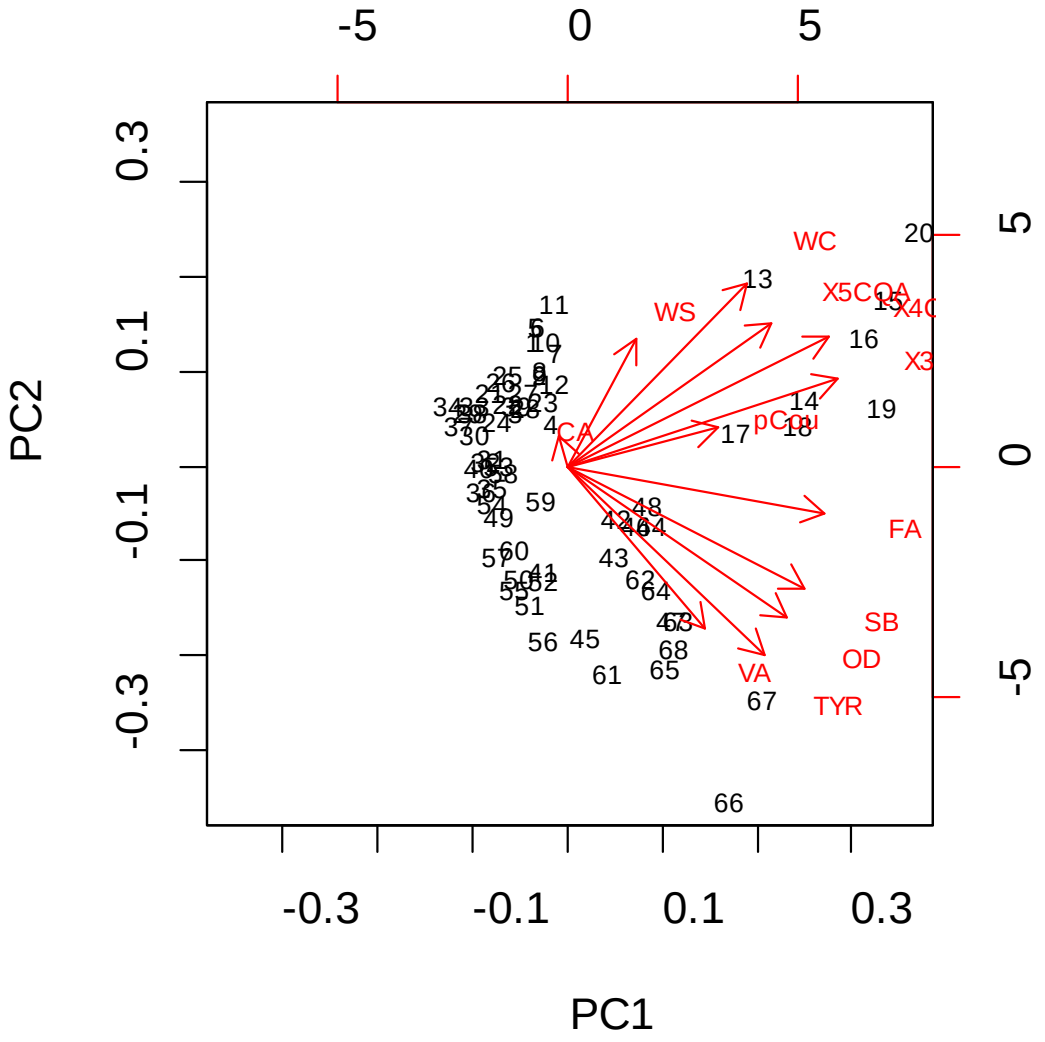


Figure 4.6 PCA bi-plot of data from the potatoes from field trials 1, 2 and 3. PC1 explains 42% of variance and PC2 18 %.

5 DISCUSSION AND CONCLUSIONS

The main aim of this study was to investigate the effect of defoliation, harvest, storage and the effect of nitrogen application on bruising incidence in three varieties of potatoes; and to investigate the physical and biochemical factors that may be associated with bruising.

Varietal differences- During field trials 1 and 2, three varieties were investigated simultaneously, and it was apparent that the varieties responded differently to the test conditions of the field trial. RB presented the highest incidence of severe bruising in field trials 1 and 2 during early to late harvests. This suggests that RB probably reached

maturation stage earlier than the other two varieties. Interestingly, in very late harvests (e.g. H4 in October), RB appeared to bruise less, with LR being the most susceptible cultivar. This suggests that there is a peak time for bruising, which was reached by RB around September, and by LR around October. LR presented the highest incidence of bruising during storage, particularly for potatoes harvested later, and this was associated with increased deformability of tubers. MP presented lower incidence of severe bruising compared to RB and LR in field trials 1 and 2. This suggests that MP may mature at a later stage than the other two varieties. All varieties bruised significantly when harvested late and when stored for a long time.

It is interesting that RB required the lowest amount of energy to break the skin and cortex tissues, and also presented the highest susceptibility to bruising. The cellular arrangement of the cells of RB may be a contributing factor. However, these mechanical properties showed no correlation to bruising incidence, indicating that other factors are more important in this variety. In this variety, tyrosine levels were the highest and the levels correlated with bruising, indicating a biochemical readiness to bruise. LR, however, required higher energy to break tissue and low levels of tyrosine but presented major changes in deformability at late harvest and along storage, which may lead to ideal conditions to initiate bruising. MP presents intermediate mechanical properties and phenolic substrate levels. Overall, these observations suggest that at early stages of tuber maturation, mechanical properties maybe important at protecting tubers, but this is overridden at later stages by high phenolic/tyrosine contents in mature tubers which promote tuber bruising regardless of mechanical properties.

Defoliation- A higher incidence of bruising in defoliated samples harvested 24 and 38 days after defoliation. This supports observations from earlier studies by Stalham (2008) which showed higher incidence of bruising when potatoes where harvested 3 to 5 weeks after defoliation. However, tubers from defoliated plants presented lower incidence of bruising than undefoliated at H4 harvest (49 days after defoliation). It was suggested that defoliation promotes skin setting which may be protective. However, no significant effects of defoliation on mechanical properties or tissue ultrastructure were observed in this study. The reduced bruising at later harvest times may be associated with a halting of tuber maturation due to forced defoliation. Defoliation did decrease tuber weight significantly.

Storage- Short storage (until January) was not associated with increased bruising incidence in any of the varieties. Longer storage (until March) did significantly increase

bruising incidence in all varieties, particularly LR harvested late. Storage increased the deformability of the cortex in all varieties, most particularly LR which bruised the most during storage. Both phenolic acids and tyrosine levels increased during storage, although the highest levels were not found in LR, but in RB. This indicates that tyrosine is not always the determining factor for bruising.

Nitrogen- Nitrogen application to the soil led to increased levels of bruising, but only in later harvests. The treatment affected mechanical properties of the tubers, most particularly skin, and tubers from treated soils showed a strong correlation between mechanical properties and bruising. Nitrogen application to soil did not affect tyrosine levels considerably.

Tuber maturity- The 2012 season (trial 2) was characterized by appreciably low rainfall (<0.8 mm) between tubers during tuber growth (before end of May) and temperature of the air and soil was slightly higher than average years. The plants experienced early senescence. All varieties were mature at harvest time based on the field indicator of decline of canopy (data not shown).

Mechanical properties- Tuber mechanical properties were mostly affected by storage whereby the tubers became more deformable and more prone to bruising. The association between mechanical properties and bruising was most apparent in MP during harvest and LR during storage. Defoliation did not affect mechanical properties, while nitrogen did and was associated with more bruising.

Cell wall properties- No relationship between mechanical properties and pectin linearity or branching was found with defoliation, harvest or storage. There was a decrease in methylation of pectin along harvest time, which is expected during cell wall maturation. However, the changes were not significant enough to explain differences in bruising incidence between varieties

Tyrosine and phenolic acids- The variations in the incidence of severe bruising did not always reflect the levels of tyrosine or phenolic acids. So, composition of these phenolics substrates at harvest could not be used as a predictor for stored samples. RB was the only variety where tyrosine levels were associated with bruising.

Specific gravity- Increase in specific gravity along harvests and storage was observed. However, there was not relationship between SG and bruising incidence during trials 1 and 2. However, it was observed that SG increased upon application of nitrogen to soil, and that was associated with higher bruising at later harvests.

In conclusion, each variety presented different mechanical and biochemical profiles associated with bruising. Further research to identify factors associated with senescence and tuber maturation is recommended.

6 Acknowledgements

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