Membrane studies in Japanese plums (*Prunus salicina* Lindl.)

by

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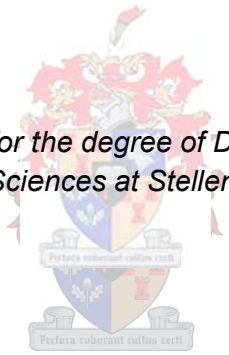

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SUMMARY

Membrane studies in Japanese plums (*Prunus salicina* Lindl.)

The export of Japanese plums from South Africa is challenging, since most cultivars are prone to develop chilling injury (CI) when stored at low temperatures. This injury manifests as gel breakdown or internal browning in the mesocarp tissue of the fruit on removal from low storage temperature conditions, i.e. in the consumer's fruit basket, who subsequently does not buy plums again.

Loss of cell membrane integrity and oxidative stress are, respectively, the primary and secondary physiological responses to CI. The main aim of this study was to investigate changes in cell membrane composition and levels of antioxidants in plums throughout fruit development and maturation, during forced air cooling (FAC) and storage under different temperature regimes.

'Sapphire' (a chilling susceptible cultivar) accumulated high levels of glutathione and polyunsaturated fatty acids (PUFAs) during fruit development. Therefore, the cultivar is protected against lipid peroxidation while developing on the tree, but the high levels of PUFAs, which are easily oxidised, may cause this cultivar to be chilling susceptible when stored at low temperatures. It is suggested that the high levels of monounsaturated fatty acids (MUFAs), which are not easily oxidised, and ascorbic acid that accumulated in 'Angeleno' (a chilling resistant cultivar) during fruit development, render this cultivar CI resistant during long-term cold-storage.

When stored at -0.5 °C, CI development increased at a higher rate, ethylene evolution rates were higher and water soluble antioxidant activity (HAA), ascorbic acid and glutathione levels, and the MUFA:PUFA ratio were lower in H2 (more mature) 'Sapphire' plums than H1 fruit (less mature). Therefore, concurrent with H2 fruit having lower levels of antioxidants to quench free radicals caused by chilling stress, their cell membranes were more vulnerable to oxidation due to their phospholipid fatty acid composition. H2 fruit also had higher levels of saturated fatty acids, and hence less fluid cell membranes than H1 fruit when stored at -0.5 °C.

An intermittent warming (IW) regime delayed symptom appearance and reduced CI severity in plums significantly compared to storage at -0.5 °C. Fruit stored under the IW regime had a more

optimal phospholipid fatty acid composition and lower membrane sterol levels under shelf-life conditions to keep the membranes fluid. It also had higher levels of HAA and lipid soluble antioxidant activity, ascorbic acid and glutathione, which rendered fruit better protected against oxidation.

Elevated storage temperatures (2.5 °C to 7.5 °C) caused higher levels of lipid peroxidation or low ascorbic acid levels and poor fruit quality compared to the IW regime in 'Sapphire' plums. 'Laetitia' plums stored at 5 °C and 7.5 °C had significantly less CI than under the IW regime, but softened quicker due to higher ethylene evolution rates.

'Sapphire' tolerated both long and short FAC durations, but a slower initial FAC rate prevented CI manifestation and caused a higher HAA after cold-storage in this fruit. 'Laetitia' cooled with a slower initial FAC rate and for a longer duration resulted in the best fruit quality and had higher HAA, total phenolic, phospholipid and saturated phospholipid fatty acid concentrations during storage.

OPSOMMING

Membraanstudies in Japanese pruime (*Prunus salicina* Lindl.)

Die uitvoer van Japanese pruime vanaf Suid-Afrika hou talle uitdagings in, want die meeste van die kultivars ontwikkel koueskade wanneer hulle by lae temperature opgeberg word. Koueskade manifesteer as gelverval of interne verbruining in die mesokarpweefsel van die vrugte wanneer die vrugte verwyder word uit die lae opbergingstemperatuuromstandighede, m.a.w. in die verbruiker se vrugtemandjie, wat nie weer pruime koop nie.

Verlies aan selmembraanintegriteit en oksidatiewe druk is, respektiewelik, die primêre and sekondêre fisiologiese reaksies op koueskade. Die hoofdoel van hierdie studie was om die veranderinge in selmembraansamestelling en antioksidantkonsentrasie in pruime te ondersoek tydens vrugontwikkeling en volwassewording, tydens geforseerde lugverkoeling (GLV) en tydens opberging onder verskillende temperatuurregimes.

'Sapphire' ('n koueskade sensitiewe kultivar) het hoër konsentrasies van glutatioon en poli-onversadigde vetsure (POV) tydens vrugontwikkeling geakkumuleer. Dié kultivar is dus voldoende beskerm teen lipiedperoksidasie tydens vrugontwikkeling aan die boom, maar die hoë konsentrasies van POVs, wat maklik oksideer, mag veroorsaak dat hierdie kultivar meer koueskadesensitief is wanneer dit by lae temperature opgeberg word. Die hoë konsentrasies van mono-onversadigde vetsure (MOV), wat nie maklik oksideer nie, en askorbiensuur wat in 'Angeleno' ('n koueskade weerstandbiedende kultivar) geakkumuleer het tydens vrugontwikkeling, verleen moontlik weerstandbiedendheid teen koueskade aan hierdie kultivar tydens langtermyn koelkopbering.

Tydens opberging by -0.5 °C het koueskade ontwikkeling vinniger toegeneem, was etileenvrystellingstempos hoër en die wateroplosbare antioksidantaktiwiteit (HAA), askorbiensuur- en glutatioonkonsentrasies en die MOV:POV verhouding laer in H2 (meer volwasse) 'Sapphire' pruime as in die H1 vrugte (minder volwasse). Dus, tesame met die laer antioksidantkonsentrasies in die H2 vrugte om die vry radikale veroorsaak deur koelopbering te verminder, was hul selmembrane ook meer vatbaar vir oksidasie a.g.v. die vetsuursamestellling van hul membraanfosfolipiede. Die H2 vrugte het ook 'n hoër konsentrasie van versadigde vetsure, en dus minder vloeibare membrane as die H1 vrugte gehad tydens opberging by -0.5 °C.

Die dubbeltemperatuurregime (DT) het simptoomontwikkeling vertraag en koueskade-intensiteit betekenisvol verminder in vergelyking met pruime wat by -0.5 °C opgeberg is. Vrugte wat met die DT regime opgeberg is, het ‘n meer optimale fosfolipiedvetsuursamestelling en laer konsentrasie van membraansterole tydens gesimuleerde raklewe gehad wat meer vloeibare membrane verseker het. Hierdie behandeling het ook hoër HAA en lipiedoplosbare antioksidantaktiwiteit (LAA), askorbiensuur- en glutatioonkonsentrasies gehad wat die vrugte beskerm het teen oksidatiewe druk.

Verhoogde opbergingstemperature het hoër vlakke van lipiedperoksidasie of lae askorbiensuurkonsentrasies asook swak vrugkwaliteit in ‘Sapphire’ pruime veroorsaak in vergelyking met die DT regime. ‘Laetitia’ pruime wat by 5 °C en 7.5 °C opgeberg is, het betekenisvol minder koueskade gehad in vergelyking met die DT regime, maar het vinniger sag geword a.g.v. hoër etileenvrystellingstempos.

‘Sapphire’ kon lang en kort GLV tye weerstaan, maar ‘n stadiger inisiële GLV spoed het die manifestasie van koueskade voorkom en het ‘n hoër HAA in die vrugte tot gevolg gehad na koelopberging. ‘Laetitia’ wat met ‘n stadiger inisiële GLV spoed en oor ‘n langer tyd verkoel is, het die beste vrugkwaliteit, en hoër HAA, totale fenool-, fosfolipied- en versadigde fosfolipiedvetsuurkonsentrasies as die ander behandelings tydens koelopberging gehad.

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NOTE

This dissertation presents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters, therefore, has been unavoidable.

GENERAL INTRODUCTION AND OBJECTIVES

In South Africa 67 000 tons of Japanese plums (*Prunus salicina* Lindl.) are produced annually for fresh consumption (HortgroServices, 2011). Of this, 76% (> 9 million 5.2 kg equivalents cartons) is exported primarily to the EU and Russia, the UK, Middle and Far East and Asia. The total 2011 season exported plum fruit value was R667.2 million CIF (Cost, Insurance and Freight).

The export of fresh plums from South Africa comes with challenges. Firstly, the markets are distant - approximately 17 days by ship. Secondly, the cold-storage of plums is complex as they are perishable products with a maximum cold-storage life of 3 to 7 weeks, due to their high respiration rate. However, this problem is overcome with strict handling protocols. These include optimum harvest windows according to mainly flesh firmness of the fruit, the placement of fruit under cooling as soon as possible after harvest and the maintenance of low pulp temperatures (-0.5°C) during the entire handling chain. The biggest challenge to overcome, however, is that most plum cultivars exported from South Africa are sensitive to storage under low temperatures, which causes chilling injury (CI). In more mature fruit, CI manifests as gel breakdown and in less mature fruit as internal browning (Taylor, 1996). Gel breakdown manifests as a gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue has a healthy appearance. Internal browning exhibits a brown discolouration of the mesocarp tissue directly beneath the skin of the fruit, but spreads throughout the entire mesocarp tissue in severe cases. For both defects the fruit has a normal external appearance. These defects usually appear when the fruit is removed from the low storage temperature conditions, i.e. in the fruit basket of the consumer, who subsequently does not buy plums again.

According to literature the first physiological response to CI is loss of cell membrane integrity, which is caused, amongst others, by changes in lipid composition (Sevillano et al., 2009; Marangoni et al., 1996). The secondary response to CI is oxidative stress, which further enhances the loss of cell membrane integrity (Sevillano et al., 2009). It is well known that, compared to chilling susceptible cultivars, chilling resistant cultivars are able to adapt or acclimatise better to chilling temperatures, due to proficient modification of their membrane composition and/or antioxidant profile under low-temperature storage conditions (Caldwell, 1990; Lurie, 2003; Upchurch, 2008). However, to our knowledge, no information exists on differences in membrane composition and antioxidant levels between plum cultivars differing in their susceptibility to CI, during plum fruit development, or how it is affected by harvest maturity and storage duration and postharvest treatments such as intermittent warming. Such information could help to establish or

improve current orchard and/or postharvest handling practices to alleviate the occurrence of CI in susceptible cultivars.

Therefore, one of the main aims of this study was to determine how the integrity of the cell membranes is influenced during fruit development, forced air cooling and storage of plum fruit under various temperature regimes. Hence, levels of lipid peroxidation, total phospholipids, total sterols and phospholipid fatty acids were determined in this study since it has been reported that they influence membrane fluidity and, therefore, also the movement of electrolytes through the cell membranes and can be used as a direct measure to establish membrane deterioration (Lester, 1996).

In order to prolong the shelf-life period and to prevent the development of internal disorders in plum fruit, it will be of great benefit to determine when oxidative stress is triggered, and when elevated stress conditions cause cellular redox homeostasis not to be maintained in the plum fruit tissue. In fruit an elaborate system of active oxygen species (AOS) scavenging enzymes (e.g. superoxide dismutase, catalase and peroxidase), lipid soluble membrane-associated antioxidants (e.g. α-tocopherol and ß-carotene) as well as water-soluble reductants (e.g. glutathione and ascorbate) regulates AOS that can cause damage to cellular components such as the cell membranes (Purvis, 2003). Although the production and elimination of AOS is well regulated at cellular level by above-mentioned antioxidants, situations arise (e.g. chilling temperatures for extended periods) in which the production of AOS exceeds the capacity of the fruit to maintain cellular redox homeostasis (Hodges, 2003). The other aim of the study was, therefore, to determine when and how the water and lipid soluble antioxidant activity and the total phenolic, ascorbic acid and glutathione concentrations are affected in the postharvest handling chain.

Consequently, to gain a better understanding of the development of CI, as well as how cell membrane composition and antioxidant levels are affected, numerous trials were conducted in this study. In Paper 1 we studied how the cell membrane composition and antioxidant levels differed during fruit development of a chilling susceptible and chilling resistant plum cultivar from 40 days after full bloom until the optimum harvest dates of fruit. This was done to establish if the cultivars already differed in their cell membrane composition and antioxidant levels at harvest, prior to storage. In Paper 2 we investigated at which point CI was induced in a chilling susceptible plum cultivar during storage at -0.5 °C and how the severity of CI was influenced by storage duration and the maturity of the fruit. In Paper 3 we studied CI incidence in chilling susceptible and less

susceptible plum cultivars stored at -0.5 °C (a temperature known to induce CI in South African plums) and the commercially used intermittent warming regime (a regime known to reduce the incidence of CI) and how the cell membrane composition and antioxidant levels in the fruit were influenced by the different treatments. In Paper 4 we studied the effect of elevated storage temperatures in comparison to the commercially used intermittent warming regime to establish if the elevated temperatures induce CI, as suggested by various research papers, or if they could alleviate CI symptoms, as is observed commercially with export fruit. In Paper 5 we investigated fast and slow initial forced air cooling rates in combination with a short and long forced air cooling duration of plum fruit to establish if these factors influenced CI manifestation. Alone or in combination these results can aid in the design of better postharvest strategies to avoid, reduce, or at least delay the incidence of CI in South African plums.

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LITERATURE REVIEW

Lipid biosynthesis and function in plant cells and their role in chilling injury

1. Introduction

Lipids are soluble in nonaqueous solvents (e.g. chloroform), are a vital ingredient of all plant cells and include products of several distinct biosynthetic pathways (Cullis and Hope, 1985; Ohlrogge and Browse, 1995; Somerville et al., 2002). Table 1 summarizes different lipid types and their functions in higher plants. The most abundant lipid types in cells are derived from the fatty acid and glycerolipid biosynthetic pathway (Ohlrogge and Browse, 1995) and these lipids will be the subject of this paper. Other lipid classes are products of the isoprenoid pathway, most of which are considered "secondary" metabolites since they are not found in all cells and are possibly not vital to cell growth. The sterols, gibberellins, abscisic acid and the phytol side chain of chlorophyll are products of the isoprenoid pathway.

There is considerable variation in the lipid content of different plant organs (Ohlrogge and Browse, 1995). For example, 70% of the fresh mass of oil seeds may consist of lipids of which the major part is triglycerides with polar lipids seldom comprising more than 5% of the total extract while leaves generally contain less than 1% (fresh weight) of total lipid of which most are polar lipids with triglycerides making out less than 5% of the total lipids (Nichols, 1964). Lipids comprise only 0.09% of the fresh mass of the pulp of pre-and post-climacteric apples (Hitchcock and Nichols, 1971). Of the total lipid extract of this tissue, 75% is composed of phospholipids and glycolipids, 5% of triglycerides, while 25% contains no fatty acyl residues. Generally lipids make up a very small proportion of the total mass of plant tissue (Table 2) and almost all of this weight is found in the membranes (Ohlrogge and Browse, 1995; Somerville et al., 2002).

Each plant cell also contains a diverse range of lipids which are located in specific structures in the cell (Somerville et al., 2002). For example, chloroplast membranes contain mainly galactolipids compared to other membranes containing mixtures of phospholipids.

2. Lipid function

Membranes are only 5 to 8 nm thick, but notwithstanding these slight dimensions, they have very important functions within the cell (Ohlrogge and Browse, 1995). Each membrane in a plant cell

has a unique lipid composition, and each class of lipid has a distinct fatty acid composition which affects plant form and function (Somerville et al., 2002).

The most important function of lipids is to form a hydrophobic barrier which separates cells from their surroundings as well as the contents of organelles from the cytosol, therefore creating compartments wherein the chemical composition can differ from the surroundings and can be optimized for a specific activity (Staehelin and Newcomb, 2002). This function depends on the polar lipids to form a bilayer that prevents uncontrolled diffusion of hydrophilic molecules between cellular organelles and in and out of cells and is essential to maintain the integrity of cells and organelles.

Short-chain fatty acids play a role in processes such as fruit ripening, flower senescence, pollen germination, and auxiliary bud growth (Gibson et al., 1994).

Lipids, mainly triacylglycerols stored in seeds, serve as a significant reserve of free energy since fatty acids are considerably more reduced molecules than carbohydrates and, therefore, have a higher potential for producing energy (Somerville et al., 2002). They are, therefore, important stores of carbon and contribute up to approximately 60% of the dry weight of seeds (Ohlrogge and Browse, 1995) and play an important role in seed germination (Gibson et al., 1994).

Lipids also serve as precursors for other important components of plant metabolism, e.g. the waxes (formed by esterification of fatty acids to fatty alcohols) that coat and protect plants from moisture loss, pathogen attack and other environmental stresses, and the cutin (a polymer of primarily 16- and 18-carbon hydroxyl fatty acids cross-linked by esterification of their carboxyl groups to hydroxyl groups on neighboring acyl chains) and suberin layers of epidermal cells (Ohlrogge and Browse, 1995; Somerville et al., 2002).

Some fatty acids have important functions in specific signal transduction pathways and defensive processes, e.g. phosphatidylinositols (Somerville et al., 2002). Extracellular signals activate a phosphatidylinositol-specific phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) (the principal active molecule) to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol, each of which acts as a second messenger. Also, the growth regulator jasmonic

acid (an oxylipin), which is synthesized from linolenic acid, is a key component of a wound-signaling pathway that allows plants to protect themselves against insect attack (Ohlrogge and Browse, 1995; Somerville et al., 2002). Jasmonate is also an important signal in pollen development (Somerville et al., 2002). Another oxylipin, traumatin, triggers cell division at the site of wounds, leading to callus development.

A variety of cellular processes may be regulated by fatty acids through their acylation of proteins (Ohlrogge and Browse, 1995; Somerville et al., 2002). Membranes also serve as scaffolding for certain proteins in which the proteins can perform a wide range of functions, e.g. as transporters of molecules, transmitters of signals across the membrane, enzymatic processors of lipids, assemblers of glycoproteins and polysaccharides, and providers of mechanical links between cytosolic and cell wall compounds (Staehelin et al., 2002).

Lipids also play a role in plant form, e.g. the *fad2* mutant of *Arabidopsis* is a miniature plant as a result of the accumulation of C18:0 in its membrane lipids (Somerville et al., 2002). The decrease in leaf size results from decreased size of mesophyll and epidermal cells. The mechanism by which the increased C18:0 levels produces the miniature plants is unknown, but when the mutant is grown at 35°C, it develops typical palisade and spongy mesophyll layers and more closely resembles the wild type.

One of the most widely researched topics in membrane biology is the relationship between lipid composition and the ability of organisms to adjust to temperature changes. It was found that the highly unsaturated fatty acids, C18:3 and C16:3, account for approximately 70% of all the thylakoid membrane fatty acids and more than 90% of the fatty acids in monogalactosyldiacylglycerol, the most abundant chloroplast lipid (Somerville et al., 2002). Loss of these two fatty acids has noticeable effects on photosynthesis at low (below 10 °C) and high (above 30 °C) temperatures. Research on *Arabidopsis* mutants indicated that plants require polyunsaturated lipids to maintain the photosynthetic machinery. The chloroplast lipids of *fad5* and *fad6* mutants of *Arabidopsis* lack desaturation and develop chlorotic leaves due to defective plastid development when exposed to chilling temperatures (Gibson et al., 1994). The presence of double bonds in the fatty acids of lipids and certain lipid headgroups protect the plant against chilling injury. For example, the *fad2* mutant of *Arabidopsis* lacks the production of polyunsaturated fatty acids and is killed by long-term exposure to chilling temperatures. In studies with five different *Arabidopsis* mutants, increased saturation resulted in plants that thrived at 22 °C, but struggled when grown at 2 to 5 °C

(Somerville et al., 2002). Where there was an increased concentration of disaturated phosphatidylglycerol in transgenic *Arabidopsis*, the damaging effects of low temperature became evident more quickly. It was found that a C16:1-*trans* at the *sn*-2 position confers chilling sensitivity on plants because the trans-double bond leaves the C16:1-*trans* fatty acid with a structure similar to C16:0 (thus termed disaturated phosphatidylglycerol). However, other research results indicated that the relationship between membrane unsaturation and plant temperature responses is subtle and complex. For example, in the *fad1* mutant of *Arabidopsis,* disaturated phosphatidylglycerol accounts for 43% of the total leaf phosphatidylglycerol (a much higher percentage than is found in many chilling-sensitive plants), however, the mutant was completely unaffected by a range of low-temperature treatments that led to the death of other chilling-sensitive plants.

3. Lipid structure

Fatty acids consist of highly reduced hydrocarbon chains (Somerville et al., 2002). Typical fatty acids in the membranes of plants contain 16 or 18 carbons (Table 3).

Unsaturated fatty acids comprise approximately 50% of the fatty acid chains in phospholipids (Kates, 1970; Kaneko et al., 1998). They promote the fluidity and permeability of the cell membrane because their acyl chains are more flexible than those of saturated fatty acids. The largest percentage of fatty acids in plants are the polyunsaturated fatty acids linoleic and α-linolenic acid (Somerville et al*.,* 2002). The main lipid type in plants, glycerolipids, consists of fatty acids esterified to derivatives of glycerol. Four types of glycerolipids are found in plants, namely triacylglycerols, glycerophospholipids (in which the head group contains phosphate), glyceroglycolipids (in which sugars form the head group), and sphingolipids (Kates, 1970; Somerville et al*.,* 2002).

Lipids are stored as triacylglycerols in which 3 fatty acids are esterified to glycerol (Somerville et al., 2002). They are primarily found in seeds and pollen where they serve as energy and carbon stores. Triacylglycerols are nonpolar (neutral) and, therefore, not soluble in the aqueous phase of cells which means that they do not contribute to the osmotic potential of the cell.

Phospholipids amount to approximately 20% of the total lipids and are synthesized by esterification of fatty acids to the 2 hydroxyl groups of *sn*-glycerol 3-phosphate to form phosphatidic acid (Kates, 1970; Somerville et al., 2002). All phospholipids are produced from phosphatidic acid by esterification of a polar "head group" to the phosphoryl group. There are 6 classes of phospholipids (phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, diphosphatidylglycerol) which are distinguished by the structure of the head group (Kates, 1970; Cullis and Hope, 1985; Taiz and Zeiger, 2010). Each class is composed of distinct molecular species defined by the fatty acids attached to the *sn*-1 and *sn*-2 positions of the glycerol backbone. In contrast to triacylglycerols, phospholipids are amphipathic, meaning that they contain both hydrophobic (noncharged, nonpolar) fatty acids and a hydrophilic (charged, polar) head group (Staehelin et al., 2002). This property allows phospholipids (and other amphipathic glycerolipids) to form a bilayer in which the polar head groups are in contact with an aqueous environment (e.g. the cytosol), and the neutral fatty acids/tails remain in contact with other hydrophobic fatty acids within the three dimensional membrane bilayer.

Glyceroglycolipids, another amphipathic glycerolipid, contain a galactosyl or sulfoquinovosyl group instead of the phosphoryl head group of the phospholipids (Cullis and Hope, 1985; Somerville et al., 2002; Taiz and Zeiger, 2010). There are 4 classes of glyceroglycolipids, namely monogalactosyldiacylglycerol, digalactocyldiacylglycerol, glucosylceramide and sulfolipid (or sulfoquinovosyldiacylglycerol). Galactolipids have high concentrations of polyunsaturated fatty acids and are mainly localized in the plastid membranes.

Sphingolipids are concentrated in the plasma membrane where they can make up as much as 26% of the mass of plasma membrane lipids (Somerville et al., 2002). Sphingolipids are not esters of glycerol, but consist of a long-chain amino alcohol that forms an amide linkage to a fatty acid. The acyl group is often longer than 18 carbons.

4. Fatty acid biosynthesis

The fatty acid biosynthetic pathway is found in every plant cell and is vital for growth (Ohlrogge and Browse, 1995). It is lethal to cells when this pathway is inhibited. In plants fatty acid biosynthesis takes place in plastids and the individual enzymes of the pathway are located in the stroma of these organelles (Robinson, 1985; Ohlrogge and Browse, 1995; Somerville et al., 2002). The plant cell must, therefore, have mechanisms to export fatty acids from the plastids and it must also be able to control the production and export of fatty acids from the plastid to other localities in the cell

(Ohlrogge and Browse, 1995). It is still unknown how the demand for extraplastidial fatty acids is communicated to the plastid.

It is hypothesized that all the enzymes involved in fatty acid biosynthesis are held together in a complex known as “fatty acid synthase” (Taiz and Zaiger, 2010). It is suggested that this complex allows the reactions in the pathway to occur more efficiently compared to when the enzymes are physically separated from each other.

The usual sequence of fatty acid synthesis is the formation of a saturated acid (since they are slightly better energy stores than unsaturated fatty acids) followed by sequential desaturation and possible chain elongation after desaturation (Hitchcock and Nichols, 1971). Generally, a natural fatty acid is composed of an even number of carbon atoms. In nature C16 and C18 fatty acids are in the majority and unsaturated fatty acids are characterized by one to three *cis* double bonds near the center of the chain (Hitchcock and Nichols 1971; Ohlrogge and Browse, 1995). The following five fatty acids make up over 90% of the acyl chains of the glycerolipids of almost all plant membranes: C18:1 (oleic acid) , C18:2 (linoleic acid), C18:3 (α-linolenic acid), C16:0 (palmitic acid), and in some species, C16:3 (hexadecatrienoic acid) (Ohlrogge and Browse, 1995).

The carbon atoms needed for fatty acid synthesis come from the pool of acetyl-coenzyme A (CoA) in the plastid (Stumpf, 1987; Ohlrogge and Browse, 1995). However, the concentration of acetyl-CoA in chloroplasts is only 30 to 50 μM – enough to supply the needs of fatty acid biosynthesis for only a few seconds. Nevertheless, the concentration of the acetyl-CoA pool remains relatively stable, even when rates of fatty acid synthesis vary greatly – in leaves it is six times higher in the light than in the dark. It is still unknown exactly how this pool of acetyl-CoA is generated. The most obvious pathway would be through the action of plastidial pyruvate dehydrogenase (PDH) acting on pyruvate, but this route has been questioned. Firstly, its activity in isolated chloroplasts of some species is insufficient to account for rates of fatty acid synthesis. Furthermore, chloroplasts have an extremely active acetyl-CoA synthetase, and free acetate has been found to be a better precursor for fatty acid synthesis in isolated chloroplasts compared to pyruvate and other substrates.

Therefore, a number of alternating pathways have been suggested for fatty acid synthesis, including the production of acetyl-CoA by a mitochondrial PDH followed by transport of free acetate or acetylcarnitine to the plastid. The free acetate that enters the plastid is then activated to acetyl-CoA by acetyl-CoA synthetase which has an activity that is 5 to 15 times higher than the *in vivo*

rate of fatty acid synthesis. Cytosolic malate and glucose-6-phosphate have also been proposed as precursors of the plastid acetyl-CoA pool in oilseeds. Since acetyl-CoA plays a central role in many metabolic pathways, it could be that more than one pathway may contribute to maintain the acetyl-CoA pool in the plastids, and which pool is used may vary with tissue, developmental stage, light/dark conditions, and species.

The fatty acid biosynthetic pathway, in which at least 30 enzymatic reactions are required, mainly involves the cyclic condensation of two-carbon units derived from acetyl-CoA into an acyl chain that is eventually 16 or 18 carbons long (Ohlrogge and Browse, 1995; Somerville et al., 2002).

The first reaction in fatty acid synthesis is catalysed by the enzyme acetyl-CoA carboxylase (ACCase) which has three functional regions, namely biotin carboxylase, biotin carboxyl carrier protein and carboxyltransferase (Fig. 1) (Stumpf, 1987; Ohlrogge and Browse, 1995; Sommerville et al., 2002). Fatty acids are assembled two carbons at a time in a two-step reaction. The first reaction is ATP-dependent and a biotin prosthetic group attached to the ε-amino group of a lysine residue is carboxylated – CO_2 from HCO_3^- is transferred to one of the nitrogens of the biotin prosthetic group. The reaction is catalysed by the biotin carboxylase portion of ACCase. In the second reaction the activated CO_2 is transferred from the carboxybiotin to acetyl-CoA to produce malonyl-CoA (the central carbon donor for fatty acid synthesis). This reaction is catalysed by the carboxyltransferase portion of ACCase. ACCase activity is highly regulated and subject to feedback and other biochemical controls which determines the rate of fatty acid synthesis. The ACCase reaction in plastids is the first committed step for fatty acid synthesis (Law and Snyder, 1972; Somerville et al., 2002). Although malonyl-CoA is mainly consumed in the plastids for fatty acid synthesis, it also serves as a substrate for flavonoid biosynthesis, fatty acid elongation at the ER, malonylation of some amino acids, and for the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Stumpf, 1987; Somerville et al., 2002). However, in the plastid, fatty acid biosynthesis is the only fate for malonyl-CoA.

In the second step (Fig. 2), the malonyl group of malonyl-CoA is transferred from CoA to an essential protein cofactor, namely acyl carrier protein (ACP), to yield malonyl-ACP (Ohlrogge and Browse, 1995; Somerville et al., 2002; Taiz and Zeiger, 2010). This reaction is catalysed by malonyl-CoA:ACP transacylase (Ohlrogge and Browse, 1995). From this point forward all the reactions of the fatty acid synthesis pathway involve ACP until the 16- or 18-carbon tails are ready for transfer to glycerolipids or export from the plastid.

Subsequently the malonyl-thioester enters into a series of condensation reactions (Fig. 2) with acyl-ACP or acetyl-CoA acceptors (Ohlrogge and Browse, 1995). The reactions entail that the malonyl-thioester is decarboxylated (the CO_2 that was added by the ACCase reaction is released) which, in turn, drives a condensation reaction in which a carbon-carbon bond forms between C-1 of an acetate "primer" (acetyl CoA) and C-2 of the malonyl group on ACP to produce acetoacetyl-ACP (Ohlrogge and Browse, 1995; Somerville et al., 2002). The release of the CO_2 helps to take the reaction forward, making it an irreversible reaction (Ohlrogge and Browse, 1995).

The condensation reaction is catalysed by 3-ketoacyl-ACP synthase (KAS) (Ohlrogge and Browse, 1995; Somerville et al., 2002). Plants contain three KAS isoenzymes (I, II and III), each distinguished by its substrate specificity. KAS I is active with C4-C14 acyl-ACPs, KAS II accepts only longer-chain (C10-C16) acyl-ACPs and KAS III has a preference for acetyl-CoA rather than acyl-ACP (Somerville et al., 2002). The characterization of KAS III raises the question of the role of acetyl-ACP in fatty acid biosynthesis, as the favored route of fatty acid biosynthesis bypasses acetyl-ACP because KAS III can use acetyl-CoA directly at a rate several times greater than that with acetyl-ACP. Nevertheless, KAS III initiates fatty acid biosynthesis by catalyzing the condensation of acetyl-CoA and malonyl-ACP to form a four-carbon product (Ohlrogge and Browse, 1995; Somerville et al., 2002). KAS I extends the acyl chain to C12-C16 and KAS II completes the synthesis to C18 (Somerville et al., 2002).

The first product of each condensation reaction is a 3-ketoacyl-ACP (Fig. 2) followed by a series of three reactions (reduction, dehydration, and reduction again) to form a saturated fatty acid (Ohlrogge and Browse, 1995; Somerville et al., 2002). In the first reduction reaction 3-ketoacyl-ACP is converted to a 3-hydroxyacyl-ACP by the enzyme 3-ketoacyl-ACP reductase and uses NADPH as the electron donor. 3-Hydroxyacyl-ACP dehydrase catalyses the removal of water from the 3-hydroxyacyl-ACP to form 2,3-*trans*-enoyl-ACP (Somerville et al., 2002). In the final reduction reaction, enoyl-ACP reductase uses NADH or NADPH to reduce the *trans-2* double bond of the 2,3-*trans*-enoyl-ACP to form the corresponding saturated acyl-ACP (Ohlrogge and Browse, 1995; Somerville et al. 2002). These four reactions lead to the lengthening of the fatty acid by adding two carbons to the acyl chain while it is still attached to ACP as a thioester.

Usually, fatty acid synthesis ends at C16:0 or C18:0, when one of several reactions stops the process (Somerville et al., 2002). The most common terminating reactions are hydrolysis of the acyl moiety from ACP by a thioesterase to release a free fatty acid, transfer of the acyl group from

ACP to a glycerolipid by an acyl transferase, or double-bond formation on the acyl moiety by an acyl-ACP desaturase. When a thioesterase releases the fatty acid from ACP, the free fatty acid is able to leave the plastid (Ohlrogge and Browse, 1995). It is suggested that the free fatty acids leave the plastid by simple diffusion across the envelope membrane. It is further suggested that an acyl-CoA synthetase on the outer membrane of the chloroplast envelope assembles an acyl-CoA thioester that is then available for acyltransferase reactions to form glycerolipids in the ER. It is not known how the acyl-CoA is transported from the outer chloroplast envelope to the ER, but acyl-CoA binding proteins may be involved.

Fatty acyl chains and their derivatives are some of the most reduced molecules in plant cells and, therefore, its biosynthesis is an energy-consuming process (Law and Snyder, 1972; Somerville et al., 2002). Each cycle of two-carbon addition involves two reduction steps, as mentioned earlier. Therefore, for a typical C18 fatty acid, 16 molecules of NAD(P)H are used.

5. Desaturation and elongation of C16:0 and C18:0 fatty acids

In membranes containing only saturated or trans-unsaturated fatty acids, the fatty acid tails will form a semi-crystalline gel, inhibiting the permeability and the mobility of the membrane components (Somerville et al., 2002). Fatty acids with *cis*-double bonds, which introduce 'kinks' or bends into a fatty acid chain, enhance membrane fluidity by lowering the temperature at which the fatty acid melts. For example, desaturation of stearic acid (C18:0) to form oleic acid (C18:1$^{\Delta 9}$), decreases the melting point of the fatty acid from 69 °C to 13.3 °C. Double bonds at other positions in the chain also exert large effects on the melting temperature of fatty acids, for example phosphatidylcholine in which the two C18 fatty acids contain double bonds at various positions along the chains: when the two acyl groups have double bonds between C-2 and C-3, the transition temperature is around 40 °C, but when the double bonds are nearer the centre of the acyl groups, the melting temperature is decreased by approximately 60 °C.

Although more than 75% of the fatty acids in most plant tissues are unsaturated, the major glycerolipids are first synthesized using only C16:0 and C18:0 fatty acids (Ohlrogge and Browse, 1995). Formation of double bonds (desaturation) is catalysed by various membrane-bound desaturases of the chloroplast and ER (Ohlrogge and Browse, 1995; Somerville et al., 2002). The first double bond in the fatty acid biosynthesis pathway is introduced by the soluble enzyme, stearoyl-ACP desaturase, a distinct family of structurally similar enzymes that catalyze the introduction of double bonds at various positions along the acyl chain. For example, unsaturated

C18 fatty acids in membrane lipids throughout the plant cell are downstream products of a stearoyl-ACP ∆9-desaturase (Somerville et al., 2002). Except for the stearoyl-ACP desaturase enzyme family which is soluble, all other fatty acid desaturases in plants are integral membrane proteins localized in the ER or the plastid (Ohlrogge and Browse, 1995; Somerville et al., 2002). During the desaturation reaction, the reduced iron center of the enzyme binds oxygen and the high valent iron-oxygen complex abstracts hydrogen from the C-H bond in the acyl chain (Ohlrogge and Browse, 1995).

There are different factors that determine the extent of desaturation of glycerolipids in membranes (Somerville et al., 2002). At this stage it seems that the regulation of the composition of many intracellular membranes in plants may require posttranscriptional mechanisms that can adjust the composition of each membrane independently. Most plants increase the extent of glycerolipid desaturation when grown at low temperature. Low temperature stimulates desaturase gene expression.

6. Membrane lipid (glycerolipid) synthesis

Each cell synthesizes its own lipids and there is no general transport of fatty acids or glycerolipids between cells (Somerville et al., 2002). However, membrane formation involves a substantial movement of fatty acids and lipids between different organelles. The most significant movement takes place between the ER and the chloroplasts, however, the mechanism(s) by which movement takes place between these organelles is not known. “Free” fatty acids are almost never found in cells; instead, their carboxyl group is esterified or otherwise modified (Ohlrogge and Browse, 1995). Almost all the fatty acids in membranes are esterified to glycerol to form the hydrophobic portion of glycerolipids.

Lipid pathways in plants are complex and still not well understood due to cellular compartmentalization of the pathways and because of extensive intermixing of the lipid pools between the different compartments (Fig. 3) (Somerville et al., 2002). There are two pathways for lipid synthesis in higher plants named the “prokaryotic pathway” and the “eukaryotic pathway” (Fig. 4). In both pathways lipid synthesis is initiated by the synthesis of phosphatidic acid (PA).

In the prokaryotic pathway C16:0 and C18:0 fatty acids are synthesized in the inner envelope of chloroplasts using 18:1-ACP, 16:0-ACP and glycerol 3-phosphate for the synthesis of glycerolipid components of the chloroplast membranes (Gibson et al., 1994; Ohlrogge and Browse, 1995; Somerville et al., 2002). The prokaryotic pathway starts with two acylation reactions that transfer fatty acids to glycerol-3 phosphate to form PA (Fig. 4) (Ohlrogge and Browse, 1995).

In the first acylation reaction of the prokaryotic pathway acyl-ACP is condensed with glycerol 3-phosphate (G3P) by the enzyme G3P acyltransferase to form lysophosphatidate (LPA) (Somerville et al., 2002). LPA rapidly partitions into the membranes where it is converted to PA by a membrane-localised LPA acyltransferase. Since the plastid acyltransferases are very specific for their acyl-ACP substrates, PA produced by the prokaryotic pathway usually has palmitic acid (C16:0) at the *sn-2* position and oleic acid (C18:1) at the *sn-1* position (Ohlrogge and Browse, 1995). There is, however, a hypothesis that G3P acyltransferases from cold-sensitive plant species have the same affinity for saturated and monounsaturated fatty acids while that from cold-tolerant species tend to have a much greater affinity for monounsaturated fatty acids than for saturated fatty acids (Gibson et al., 1994).

The initial reactions in the eukaryotic pathway are similar to the prokaryotic pathway except that acyl-CoA (instead of acyl ACP) is used, and the G3P acyltransferase is associated with the ER (and not the plastid) (Ohlrogge and Browse, 1995; Somerville et al., 2002). The first steps of this pathway involve the export of C16:0 and C18:0 fatty acids from the chloroplast to the ER (as acyl-CoAs) (Somerville et al., 2002). The PA that is subsequently produced is highly enriched with 18-carbon fatty acids at the *sn-2* position and when palmitic acid (C16:0) is present, it usually is restricted to the *sn-1* position (Ohlrogge and Browse, 1995).

During phospholipid biosynthesis in plants the attachment of the phospholipid head group can occur via two pathways, namely the diacylglycerol (DAG) and the cytidine diphosphate diacylglycerol (CDP-DAG) pathways (Fig. 5) (Somerville et al., 2002). The energy for the attachment of the polar headgroup during glycerolipid synthesis comes from nucleotide activation (Ohlrogge and Browse, 1995). A specific phosphatase located in the inner plastid envelope converts PA to DAG (Ohlrogge and Browse, 1995; Somerville et al., 2002). Alternatively, PA can react with the pyrimidine ribonucleotide, cytidine 5'-triphosphate, to produce the nucleotide-activated CDP-DAG. When DAG is the lipid substrate, it is the headgroup that is activated by the nucleotide (Ohlrogge and Browse, 1995). In the DAG pathway the *sn*-3 hydroxyl of DAG attacks

the ß-phosphate of a CDP-activated head group (Somerville et al., 2002). The major plastid membrane lipids, namely, monoglactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), which is synthesised from MGDG, and sulfoquinovosyldiacylglycerol (SL) are derived from the DAG pool in the prokaryotic pathway (Ohlrogge and Browse, 1995). In the case of the CDP-DAG pathway, a hydroxyl group located on the head group carries out a nucleophilic attack on the ß-phosphate group of CDP-DAG. The phospholipid produced via the CDP-DAG pathway in the prokaryotic pathway is phosphatidylglycerol (PG), the only phospholipid found in the thylakoids (Somerville et al., 2002). ER-derived PA include the phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) from the DAG pathway and phosphatidylserine (PS) and phosphatidylinositol (PI), which are characteristic of the various extrachloroplast membranes, from the CDP-DAG pathway (Ohlrogge and Browse, 1995; Somerville et al., 2002). The newly formed lipids subsequently move from the ER to the other organelles (Somerville et al., 2002). However, the DAG moiety of PC can also be returned to the chloroplast envelope to enter the DAG pool and contribute to the synthesis of plastid lipids (Ohlrogge and Browse, 1995). The eukaryotic pathway is the main route of glycerolipid synthesis in all non-photosynthetic tissues as well as in the photosynthetic tissues of many higher plants (Somerville et al., 2002).

Although the reactions described here seems to be a linear series of simple enzymatic steps, the actual biochemistry involved is much more complicated due to the possibilities of headgroup modification and exchange (Ohlrogge and Browse, 1995).

There is evidence that lipid exchange between the ER and the chloroplast is reversible to some extent (Ohlrogge and Browse, 1995). For example, in *Arabidopsis* mutants deficient in ER desaturases, extrachloroplastic membranes contain polyunsaturated fatty acids which originate in the chloroplasts. The fluctuation between the two pathways varies between plant species (Gibson et al., 1994; Ohlrogge and Browse, 1995). It was found that approximately 39% of fatty acids in wild type *Arabidopsis* are used for lipid synthesis within the chloroplast, 27% of the remaining fatty acids are used for synthesis of lipids for extrachloroplast membranes (e.g. the ER, Golgi, and plasma membrane) and 34% are transferred to the chloroplast for lipid synthesis (Somerville et al., 2002). However, in a mutant of *Arabidopsis* lacking plastid G3P-acyltransferase activity (needed to catalyze the first step of lipid synthesis), most of the fatty acids are exported to the cytoplasm and then ca. 61% are re-imported into the chloroplast which suggest that there is a mechanism that adjusts the flow of lipid between the membranes when necessary. In barley and pea the prokaryotic pathway makes only a small contribution to the total cellular lipid synthesis making the plastids almost entirely dependent upon the eukaryotic pathway (Gibson et al., 1994).

This indicates that even if there is a major disruption of one of the pathways of glycerolipid synthesis, mechanisms exist to ensure the synthesis and transfer of enough glycerolipids to support normal rates of membrane synthesis (Somerville et al., 2002).

In a number of plant species, including *Arabidopsis* and spinach, both pathways synthesize the same amount of MGDG, DGDG and SL (Ohlrogge and Browse, 1995). The leaf lipids of these plants typically contain substantial amounts of hexadecatrienoic acid (C16:3), which is only found in MGDG and DGDG molecules produced by the prokaryotic pathway (Ohlrogge and Browse, 1995). Therefore, these plants have been termed C16:3 plants to differentiate them from other angiosperms (C18:3 plants), whose galactolipids contain mainly linolenate (Ohlrogge and Browse, 1995). In lower plants the contribution of the eukaryotic pathway to MGDG, DGDG and SL synthesis is reduced. It is not yet known how the fluctuation between the two pathways is regulated and why there is variation between species, or in the same species under different environmental conditions (Gibson et al. 1994). It was, however, shown by Ramli et al. (2002) that enzymatic regulation of lipid biosynthesis is distributed between both parts of the pathway (fatty acid synthesis that takes place in the plastids and lipid assembly that predominantly takes place in the ER) which means that manipulation of a single step in the overall lipid biosynthetic pathway will only have a small effect on lipid yield. It is also not yet known how the hydrophobic lipid molecules from the ER are transported to other sites, especially the chloroplasts (Ohlrogge and Browse, 1995). Questions also remain on what signals are used to communicate a demand for increased glycerolipid synthesis to the lipid biosynthetic pathways and what mechanisms are used to transmit the signal from the different membranes in the cell to the chloroplast envelope and stroma and the ER during membrane expansion or repairs (Ohlrogge and Browse, 1995; Somerville et al., 2002).

Superimposed onto these two main lipid pathways are a number of additional pathways for the synthesis of other lipids, such as waxes, cutin, sphingolipids, and sterols, but little is known about the cellular localization of these pathways (Somerville et al., 2002).

Alternatively, existing lipid molecules may also be altered according to the cell's requirements by 'exchange reactions' which involve the redistribution of lipid material in the cell (e.g. under chilling conditions) or *in situ* transformation of lipid molecules already available in the membrane (Law and Snyder, 1972; Gurr and James, 1975). The *in situ* transformation of lipid molecules in the membrane involves lipid-transforming enzymes (Law and Snyder, 1972). For example, phosphatidylserine is decarboxylated to phosphatidylethanolamine, which, in turn, can be

transmethylated to phosphatidylcholine. As will be explained in the following section, these lipolytic enzymes can either catalyze the complete breakdown of the lipid, or they can catalyze the turnover and recycling of the molecule.

7. Metabolism of membrane glycerolipids

Little is known about lipid turnover and degradation (Galliard, 1980; Ohlrogge and Browse, 1995), however, it is known that it is a general metabolic reaction appearing in developmentally regulated processes (such as seed and fruit maturation, seed germination and seedling development, chloroplast development, maturation of green tissue and senescence) and as a response to biotic and abiotic stresses (which may cause membrane reorganization and degradation and the production of secondary messengers) (Hitchcock and Nichols, 1971; Chapman, 1998; Andreou and Feussner, 2009). For example, it is usually found that organisms exposed to chilling temperatures produce higher levels of unsaturated fatty acids to allow membrane phospholipids to become more fluid at the lower temperature (Stanley, 1991). This 'retailoring' of the membrane can happen within minutes in protozoans and within hours in higher plants due to fatty acid desaturation. The enzymes involved in this process are phospholipase A, fatty acyl CoA synthetase and acyltransferase. Due to this process of membrane 'retailoring' at low temperatures, postharvest practices such as mild heat treatments, temperature preconditioning such as storing produce for short durations at decreasing temperatures (gradual acclimation), and/or intermittent warming cycles can prevent sensitive produce, such as tomatoes, from developing chilling injury (Stanley, 1991; Toivonen, 2004). Although the enzymes involved, including phospholipases, galactolipases and lipoxygenases, have been studied for a number of years, it is not clear how they and other enzymes are regulated during senescence of plant organs (Ohlrogge and Browse, 1995). For example, it was found that almost half of the lipid content of a leaf is metabolized during senescence, most probably through β-oxidation (the same process that happens in germinating seeds when reserve triglycerides are broken down to support the growing seedling) of the fatty acyl chains (Beevers, 1980; Ohlrogge and Browse, 1995). Lipids are not transported out of an organ and, therefore, the entire process of lipid turnover must be controlled to facilitate the orderly dismantling of membranes and export of lipid breakdown products that can be used as precursors elsewhere in the plant.

The metabolism of membrane lipids not only results in nonspecific breakdown products, but also in the production of specific cellular signals such as phosphatidylinositol 4,5-biphosphate (Ohlrogge and Browse, 1995). This product may act as a precursor for signal molecules such as DAG and inositol 1,4,5-triphosphate (IP3). IP3 causes the release of Ca^{2+} into the cytoplasm and thereby

regulates cellular processes in numerous plant systems. Jasmonate is another plant growth regulator derived from linolenic acid (C18:3) during wounding of plant cells (Ohlrogge and Browse, 1995; Chapman, 1998). A very low concentration of jasmonic acid is required to strongly induce specific genes, including proteinase inhibitors and other plant defense genes as well as vegetative storage proteins. In plants jasmonate is produced from C18:3 (which is presumably released from membrane lipids by phospholipase A2) by a pathway that starts with the action of lipoxygenase on C18:3 (Ohlrogge and Browse, 1995; Chapman, 1998).

Abiotic (e.g. temperature) or biotic (e.g. pathogen attack) stresses may cause lipid peroxidation which includes a complex group of enzymatic and non-enzymatic reactions that occur simultaneously and competitively (Stanley, 1991). As mentioned earlier, polyunsaturated fatty acids (PUFAs) are conjugated to phospho- and galactolipids (Spiteller, 2003) and are the main targets of oxidative stress (Reis and Spickett, 2012). The products of the oxidation of PUFAs are collectively called oxylipins (Andreou and Feussner, 2009).

Any introduction of stress results in the elevation of the levels of reactive free radicals which abstract hydrogen atoms from methylene carbons in unsaturated fatty acids that lead to membrane degradation (Buege and Aust, 1978; Stanley, 1991). Fe^{2+}, Fe^{3+} and singlet oxygen are the primary initiators of lipid peroxidation (Shewfelt and Purvis, 1995). The hydrogen atoms on methylene groups immediately adjacent to double bonds in unsaturated fatty acid chains have low strength carbon-hydrogen (C-H) bonds, and those on methylenes between two double bonds have even weaker C-H bonds (Reis and Spickett, 2012). Consequently, these hydrogen atoms are effortlessly removed by reactive free radicals, which give rise to phospholipid radical species, also called conjugated dienes (due to the rearrangement of the double bonds in the fatty acid due to the oxidation reaction) (Buege and Aust, 1987; Reis and Spickett, 2012). On the other hand, saturated fatty acid chains are much more resistant to oxidative stress as their hydrogen atoms are not weakly bound since they do not contain double bonds. However, they can also eventually be auto-oxidised, although a long induction period is needed (Hitchcock and Nichols, 1971). Subsequently, the conjugated dienes that were formed quickly react with molecular oxygen to form peroxy free radicals ($LOO^{\bullet}$). The initial removal of the hydrogen followed by the addition of O_2 forms the initiation phase of lipid peroxidation (Schneider, 2009; Reis and Spickett, 2012). The addition of O_2 molecules may occur several times on a single PUFA due to the presence of other weakly bound hydrogen atoms (Reis and Spickett, 2012). In the intermediate phase or propagation phase of lipid peroxidation further phospholipid radical species are formed, including $L^{\bullet}$ (alkyl), $OL^{\bullet}$ (epoxy alkyl), $HOL^{\bullet}$ (hydroxyl-alkyl), $LO^{\bullet}$ (alkoxyl) and $OLOO^{\bullet}$ (epoxy-peroxyl) species (Schneider, 2009; Reis

and Spickett, 2012). These species propagate the damage as they are able to stabilize themselves by removing hydrogen atoms from neighboring phospholipid molecules, which cause the formation of new phospholipid radicals on adjacent phospholipids (Reis and Spickett, 2012). Therefore, a single attack by reactive free radicals can cause damage to several lipid molecules. Removal of a hydrogen atom by the peroxyl (or other) radical species converts it (the peroxyl radical) to a non-radical species, e.g. a hydroperoxide, in the termination phase of lipid peroxidation (Schneider, 2009; Reis and Spickett, 2012). The reaction of unsaturated fatty acids with atmospheric oxygen causes edible oils to develop off-flavours in air (to become rancid) while drying oils autoxidize to a hard polymer (Hitchcock and Nichols, 1971).

In the enzymatic reactions, membrane-bound phospholipases, which cleave the unsaturated fatty acids from the phospho- or galactolipids, are activated and released (Buege and Aust, 1978). Their release is important for all stages of normal plant growth and development as well as during biotic and abiotic stresses (Chapman, 1998). The phospholipases or hydrolases are grouped in families, namely phospholipase A, C or D, depending on their site of cleavage. They catalyze the production of both polar and non-polar products depending on their action on specific classes (lipids with different head groups) and molecular species (lipids with same head group, but different fatty acids).

Under stress conditions, the lipoxygenase pathway is triggered in which PUFAs freed by the phospholipase action are transformed to lipidhydroperoxides (LOOHs) by lipoxygenases (LOX) (Gurr and James, 1975; Gardner, 1995). The lipoxygenase pathway is seen as part of programmed lipid turnover and retailoring and not a major initiator of membrane damage and tissue disorders as are singlet oxygen, Fe^{2+} and Fe^{3+} (Shewfelt and Purvis, 1995). Linoleic (C18:2) and linolenic (C18:3) acid are characteristic substrates for LOX, but it is also capable of oxygenation of other PUFAs esterified to phospholipids (Hitchcock and Nichols, 1971; Gurr and James, 1975; Hildebrand, 1989; Rogiers et al., 1998). LOOHs are odourless, colourless short-lived species which break down to a number of secondary products such as malonaldehyde, volatile hydrocarbons (ethane, pentane and ethylene), jasmonic acid, etc., which represent biological signals and do not require a preceding activation of genes (Leshem, 1992; Spiteller, 2003; Wrolstad, et al., 2005). Some of the breakdown products, such as aldehydes (e.g. hexanal and nonenal) and the alcohol hexenol have characteristic flavours and odours which may be desirable or undesirable (Galliard and Chan, 1980; Hildebrand, 1989; Leshem, 1992). However, if the free PUFAs start to exceed a certain threshold, LOX “commits suicide” and the iron ions in its active center are released (Spiteller, 2003). These ions react with the surrounding LOOHs to

generate alkoxy radicals ($LO^{\bullet}$) which, in turn, induce non-enzymatic lipid-peroxidation. It is interesting to mention that a portion of the $LO^{\bullet}$ radicals produced from linoleic acid is converted to 2,4 dienals, which induce programmed cell death and the hypersensitive reaction.

For each stage of enzymatic and non-enzymatic lipid peroxidation the plant cell has a defence mechanism in the form of reducing agents such as glutathione and ascorbic acid to eliminate reactive free radicals and the toxic products of lipid peroxidation (Aust, 1986; Spiteller, 2003). Peroxidised lipids are also quickly repaired (Shewfelt and Purvis, 1995). However, under severe stress conditions the reducing agents may be overwhelmed by the amount of free radicals, and lipid peroxidation will cause uncoupling of oxidative phosphorylation in the mitochondria, alteration of the function of the ER, increased membrane permeability, alteration of the fluidity of the membrane, inactivation of membrane-bound enzymes and polymerization, cross-linking and covalent binding of proteins, which may lead to cell death (Stanley, 1991; Shewfelt and Purvis, 1995; Blokhina, et al., 1999).

As mentioned earlier, peroxidation of PUFAs may proceed through nonenzymic autoxidative pathways, or through processes that are enzymatically catalyzed (Stanley, 1991). The sequence of lipid peroxidation is as follows (Buege and Aust 1978):

$PUFA(H) + R^{\cdot} \rightarrow PUFA^{\cdot} + RH$ (1)

This reaction initiates the chain reaction with the extraction of a hydrogen atom from a PUFA by a reactive free radical.

PUFA(H) = polyunsaturated fatty acid with easily removable hydrogen atoms.

$R^{\cdot}$ = reactive free radical.

$PUFA^{\cdot}$ = conjugated diene.

RH = reduced free radical.

$PUFA^{\cdot} + O_2 \rightarrow PUFAO_2^{\cdot}$ (2)

When the conjugated dienes formed in reaction (1) are attacked by molecular oxygen, a lipid peroxy free radical is formed.

$PUFAO_2^{\cdot}$ = peroxy free radical.

$PUFAO_2^{\cdot} + XH \rightarrow PUFAO_2H + X$ (3)

The peroxy free radical formed in reaction (2) can itself abstract a hydrogen atom from a neighbouring molecule (XH), which may be another PUFA, to form a lipid hydroperoxide, or a lipid endoperoxide.

$PUFAO_2H$ = hydroperoxide.

$PUFAO_2H \rightarrow$ variety of products (4)

The hydroperoxide formed in reaction (3) can break down to a variety of products, especially when in the presence of transitional metal ions.

During senescence all the molecular components of the membrane are degraded, which includes extensive breakdown of the membrane phospholipids (Hopkins et al., 2007). This process has been found in leaves, cotyledons, flower petals and ripening fruit. The decrease in the total phospholipid levels, in fatty acid content and of selective unsaturated fatty acids, and an increase in the total sterol levels in the membrane cause membrane leakiness during the early stages of senescence and are then also used as biological markers of tissue ageing. This process seems to be mediated by enzymes. Indeed, it was found that 11 lipases/acyl hydrolases, 6 phospholipases, 2 lipoxygenases and 9 ß-oxidation enzymes are encoded during leaf senescence of *Arabidopsis* and are involved in the breakdown of membrane lipids and their conversion to energy or phloem-mobile sucrose.

The lipases de-esterify fatty acids at the *sn*-1 and *sn*-2 position of the phospholipids in the senescing membranes. The de-esterified fatty acids are not immediately removed from the membrane bilayer, because they are charged at physiological pH and remain amphipathic. Therefore, although they are liberated from the membrane lipids, they remain in the bilayer and undergo a phase separation from the liquid-crystalline phase of the bulk of the membrane to form distinct gel phase domains in the membrane, which cause the membrane to become leaky at the phase boundaries due to packing imperfections at the junctures of the two phases. The lipid phase transition of the de-esterified fatty acids is also characteristic of premature senescence caused by environmental stress such as chilling injury. Some of the liberated fatty acids are converted to steryl- and wax esters and triacylglycerol which are more easily accommodated in the bilayer (they do not carry a charge, and are therefore chemically neutral) than the de-esterified fatty acids which act as detergents and can severely disrupt the bilayer structure. It is interesting to note that the liberated fatty acids are important precursors of the acyl moieties of many phospholipids and of triglycerides (Gurr and James, 1975).

The integrity of some cellular membranes is preserved into the late stages of senescence, not only to support the production of energy to drive senescence, but also for nutrient recovery and translocation. Therefore, there must be some regulation of the de-esterification of the membrane lipids. Usually active oxygen species react with PUFAs within nanoseconds or microseconds while symptom development may take hours and even weeks, which indicates that membrane degradation is slow and incremental (Stanley, 1991; Shewfelt and Purvis, 1995). For example, in carnation petals it was found that lipid phase transitions took place in the endoplasmic reticulum

during the early part of senescence while it did not take place in the plasmalemma (Paliyath and Thompson, 1990). In leaves, chloroplasts are the first organelles to be lost due to the breakdown of the thylakoids, while the dismantling of the inner mitochondrial membranes only occurs late in leaf senescence (Kolodziejek et al., 2003). This is probably because the fatty acids of the thylakoid membranes are the most important source of carbon in leaves which must be retrieved during the early stages of senescence while most of the metabolic apparatus is still functioning to ensure its effective conversion to phloem-mobile sucrose. The thylakoid fatty acids are also converted to substrate for the mitochondria through ß-oxidation to produce ATP that drives the senescence processes.

Gel phase domains are also not uniformly distributed within the membrane bilayer, with large regions of the bilayer retaining their structural integrity by remaining in the liquid crystalline phase (Paliyath and Thompson, 1990). This would permit the functionality of ion or metabolite gradients across the cell membrane to allow for sugar and amino acid transporters to translocate these molecules from the senescing cells (Hopkins et al., 2007). Integral membrane proteins are also displaced laterally out of gel phase domains through the plane of the membrane into liquid crystalline regions and are, therefore, not immediately degraded, but remain functional. Also, lipid phase transition occurs slowly to allow gradual decompartmentalisation.

Cell membranes are completely disassembled during senescence (Hopkins, 2007). However, the insolubility of the dismantled membrane lipids and proteins in aqueous environments due to their amphipathic nature and their tendency to remain in the membrane bilayer causes a thermodynamic problem. There is, however, evidence that the dismantled lipids and proteins form macromolecules that move laterally through the plane of the membrane to form disrupting gel domains within the bilayer that are subsequently expelled from the membrane surface to form cytosolic lipid-protein particles (in the case of the plasma membrane) and plastoglobuli (in the case of thylakoids) (Robinson, 1985; Hopkins, 2007). It is, however, less clear how the lipid-protein particles and plastoglobuli are translocated to glyoxisomes for the fatty acids to be metabolized to acetyl-CoA through ß-oxidation, and sucrose through gluconeogenesis via the glyoxylate and TCA cycles.

8. Physiology and cell biology of membrane lipid composition

Polar glycerolipids are the main structural lipids in membranes (Taiz and Zeiger, 2010). Other minor structural lipids in the membrane include sphingolipids and sterols.

As mentioned earlier, glycerolipids consist of a combination of nonpolar fatty acyl chains and polar head groups which give them amphipathic properties that are essential for the formation of membrane bilayers (Cullis and Hope, 1985; Ohlrogge and Browse, 1995). It is important to note that the chemical nature of the head group and the fatty acyl chains can vary considerably (Cullis and Hope, 1985). In an aqueous medium, membrane lipids assemble with their non-polar fatty acid tails together, protected from contact with water (Staehelin et al., 2002). As a result, each membrane has one side exposed to the contents inside the compartment, and the other side in contact with the external solution. Their amphipathic property, the fact that they can assume any shape and have the ability to close on themselves give membranes the ability to form distinct compartments and to serve as permeability barriers, because most water-soluble (polar) molecules cannot readily pass through the membrane's nonpolar interior. These properties also help them to seal damaged membranes and to compartmentalize the biochemistry of the cell. It is estimated that each square cm of a leaf contains only 0.2 mg of lipid, but due to its bilayer property, this amount of lipid can account for approximately 400 cm^2 of membrane as the bilayer is only two molecules thick (Ohlrogge and Browse, 1995). Most of the other functions of membranes are performed by the membrane proteins, which define the specificity of each membrane system (Staehelin et al., 2002). The majority of membrane molecules can circulate freely within the plane of the membrane, allowing membranes to change shape and membrane molecules to rearrange rapidly. Plant cells contain 17 different membrane systems, and from the instant they are formed the cell must maintain their integrity in order to survive.

All membrane systems are passed from one generation of cells to the next according to the following rules (Staehelin et al., 2002; Taiz and Zeiger, 2010):

- Mother cells pass on a complete set of membranes to the daughter cells.
- A complete set of membranes is maintained by each potential mother cell.
- New membranes cannot be created and must be produced by growth and separation of existing membranes through cycles of membrane fusion and fission. Only the ER and chloroplasts are able to add new membrane directly through lipid and protein synthesis. The addition of new membrane material for the other organelles of the endomembrane system in the cell occurs via fusion and the subsequent fission of transport vesicles and tubules with the membranes of the named organelles. This can happen because membranes are fluid and new membrane components can be transferred to the existing membrane during fusion.

Each membrane has a unique complement of glycerolipid types and each class of lipid has a distinct fatty acid composition (Ohlrogge and Browse, 1995). There is also considerable variation

in the lipid composition of the inner and outer leaflets of membrane bilayers (Cullis and Hope, 1985). Usually the phospholipids with a net negative charge at physiological pH (phosphatidylserine and phosphatidylinositol) face the cytosolic half of the bilayer. The unique composition of each membrane and lipid class is largely conserved throughout the plant kingdom (Ohlrogge and Browse, 1995). This implies that the diversity in lipid structure is important for membrane function. As mentioned earlier, glyceroglycolipids dominate in chloroplast membranes while the other cellular membranes mainly contain glycerophospholipids (Taiz and Zeiger, 2010). However, the details of this structure/function relationship remains obscure, despite extensive effort to gain more knowledge.

Phospholipids, the most common membrane lipid, contain at least one fatty acid tail with one or more *cis* double bonds, causing 'kinks' or bends in the fatty acid, which influence the packing of the molecules in the lipid bilayer. The packing of the membrane influences the overall fluidity of the membrane (Staehelin et al., 2002). Because the individual lipid molecules are not bonded to each other covalently they are free to move and can slide past each other in the membrane bilayer (Staehelin et al., 2002). Lipid molecules can hop approximately 10^7 times a second, which means that they can travel approximately 1 μm s^{-1}. Rotation of individual molecules around their long axis is also very fast. There is also a constant flexing of the fatty acyl chains which increases toward the ends of the tails, meaning that the center of the bilayer has the greatest degree of fluidity. However, the transfer of phospholipids across the bilayer (flipping) rarely occurs, because it means that the polar head group must travel through the non-polar interior of the bilayer – an energetically unfavorable event. Some membranes do, however, contain 'flippase' enzymes which help the movements of newly synthesized lipids across the bilayer. Membrane fluidity also disperses integral and surface-associated membrane proteins laterally in the plane of the membrane, which is crucial for the functioning of the membrane as it facilitates collisions between substrate molecules and membrane-bound enzymes, of electrons between the electron transfer chain components of mitochondria (and chloroplasts) and assembly of multiprotein membrane complexes. Furthermore, many signaling pathways depend on brief interactions between specific integral membrane proteins and peripheral or lipid-anchored proteins.

Membrane sterols, on the other hand, are not only able to diffuse rapidly in the plane of the bilayer, but can also flip-flop, without enzymatic assistance, at a higher rate than phospholipids because their non-polar hydrophobic tail is much larger than their polar hydrophilic head group (a single -OH group) (Leshem, 1992; Staehelin et al., 2002).

Membrane bound proteins perform diverse enzymatic and structural functions (Staehelin et al., 2002) and are synthesized in much smaller amounts compared to secretory proteins in animal cells (Robinson, 1985). Proteins can be peripherally or integrally associated with membrane bilayers, they can be fatty acid linked, prenyl group-linked, or phosphatidylinositol linked to the bilayer (Staehelin et al., 2002). Peripheral proteins are water-soluble and bind either to integral proteins or lipids through salt bridges, electrostatic interactions, hydrogen bonds, or combinations of these. They also provide links between membranes and cytoskeletal systems. Peripheral proteins can be removed by washing the membrane in water, salt or acid solutions that do not disrupt the membrane bilayer. Proteins associated with the surface of membranes are synthesized on free or ER-bound ribosomes (Robinson, 1985). Extracytoplasmic membrane proteins are synthesized on ER-bound ribosomes and are transported via the Golgi apparatus from the ER to the cell surface while proteins located on the cytoplasmic side of a membrane are synthesized on free ribosomes (Robinson, 1985). Integral proteins are insoluble in water and can only be removed and solubilized with the help of detergents or organic solvents that degrade the bilayer, since these proteins are totally or partially embedded in the hydrophobic interior of the membrane (Staehelin et al., 2002). The bulk of integral membrane proteins are synthesized by ER-bound ribosomes (Robinson, 1985). Fatty acid-linked and prenyl group-linked proteins bind reversibly to the cytoplasmic surfaces of membranes to aid the regulation of membrane activities (Staehelin et al., 2002). Fatty acid-linked proteins bind either to myristic acid (C14:0) or to one or more palmitic acid (C16:0) molecules. Prenyl group-linked proteins are bound to one or more molecules of farnesyl (C15:0) or geranylgeranyl (C20:0). The phosphatidylinositol-anchored proteins are attached to the luminal/extracellular surfaces of membranes. Many arabinogalactan proteins are linked to the plasma membrane via a glycosylphosphatidylinositol anchor. Cord-like structures not only regulate, but also restrict the movement of membrane proteins (Staehelin et al., 2002). These structures may involve connections to the cytoskeleton and the cell wall, bridges between related integral proteins, or junction-type interactions between proteins in adjacent membranes. The half-life of proteins in specific membranes may vary greatly – from 3 to over 100 hours (Robinson, 1985). The half-life of ER proteins is longer than those in the plasma membrane. Protein content in the various membranes is also regulated according to the growth status of the cell, altering between synthesis and degradation.

Chilling sensitive plants (including many economically important crops such as Japanese plums) undergo physical and physiological changes induced by low temperatures (between 0 °C and 12 °C) and subsequent symptoms of stress (Somerville et al., 2002). Many studies suggest that the primary event of chilling injury is a phase transition from the liquid crystalline to the gel state in cellular membranes (Cullis and Hope, 1985; Somerville et al., 2002). Phase transition results in alterations in the metabolism of chilled cells and leads to injury and even death of the

chilling-sensitive plants. For every lipid the transition occurs at a specific temperature, namely the temperature of melting (T_m) (Staehelin et al., 2002). Since each lipid has a different T_m, low temperature can induce one type of lipid to go into the gel state and to form semicrystalline patches in the membrane, while other lipids remain in the fluid state. Gelling usually stops membrane activities and increases permeability. However, at physiological temperatures most membranes are in the liquid crystalline phase (Cullis and Hope, 1985).

To compensate for low temperatures, mechanisms to optimize membrane fluidity under such conditions include shortening of fatty acid tails, increasing the number of double bonds, and increasing the charge of the head groups (Cullis and Hope, 1985; Staehelin et al., 2002). Since desaturation of membrane lipids favours membrane fluidity and, therefore, cellular function and plant viability at low temperatures, it is important to establish the relationship between membrane composition and chilling sensitivity (Ohlrogge and Browse, 1995; Somerville et al., 2002). Although a deficiency in unsaturated lipids has severe consequences, evidence indicates that the change(s) in membrane function in *fad2* plants (mutants of *Arabidopsis* that are deficient in the activity of the ER C18:1 desaturase responsible for production of unsaturated lipids in the eukaryotic pathway of lipid synthesis) at 6 °C, begins to influence cell viability only after several days (Ohlrogge and Browse, 1995). Since symptom development is gradual in these mutants, it may indicate that the deficiency in unsaturated membrane lipids may at first have relatively limited effects in disrupting cellular function at chilling temperatures. Furthermore, the increased C16:0 content in leaves of *fad1* mutants of *Arabidopsis* (characterized by increased levels of C16:0 and C18:0) causes the synthesis of chloroplast PG that contains only C16:0, C16:1-*trans* and C18:0 which account for 42% of the PG in leaves of these mutants. Such “high-melting-point” lipids have been associated with plants that are chilling sensitive. However, it was found that *fad1* mutants do not show classic chilling sensitivity despite their increased C16:0 content and are able to grow and complete their life cycles normally at 10 °C and were also unaffected by more severe chilling treatments that caused the death of cucumber and other chilling-sensitive plants. However, when these plants were continuously grown at 2 °C they eventually showed damage. These findings suggest that high-melting-point species of PG are not, alone, responsible for chilling sensitivity of plants.

Membranes can also become too fluid and permeable at high temperatures (Staehelin et al., 2002). However, many plants can survive daily temperature fluctuations of 30 °C by altering the composition of their membranes to optimize fluidity for a given temperature. As membrane lipids

have a finite life span and have to be turned over on a regular basis, this renewal also enables plant cells to adjust the lipid composition of their membranes.

Plants can also alter their sterol composition to alter membrane responses to temperature. Sterols increase the fluidity of membranes at low temperatures by disrupting the gelling of phospholipids (since the hydrophobic, aliphatic sterol tail is inherently mobile and causes some disorder in the microdomain of the membrane where it is located), and decrease fluidity at high temperatures by interfering with the flexing motions of the fatty acid tails (because the non-polar, hydrophobic steroid cyclopentanoperhydrophenantrene skeleton comprised of the A, B, C and D rings, is rigidly planar and arranged parallel to the phospholipid fatty acid tails in the membrane) (Bloch, 1985; Leshem, 1992). Usually intact, active cells with functional membranes have a constant phospholipid:sterol ratio (Leshem, 1992). However, under stress conditions or during senescence, the most pronounced change or breakdown is usually observed in the phospholipid fraction of the membrane while the sterol fraction remains stable since it is degraded less easily. Hence, there is usually a decrease in the phospholipid:sterol ratio under stress conditions or during senescence, which could serve as an indicator of the integrity of the cell membrane.

9. Transport

As mentioned earlier, the hydrophobic nature of lipid bilayers ensures that hydrophilic compounds can be sequestered on one side of the membrane or the other (Staehelin et al., 2002). Compartmentalization not only concentrates reactants and catalysts, but also segregates incompatible processes. Division of all the specialized organelles with membranes therefore facilitates metabolic flexibility and efficiency. Controlled transport of metabolites across the different cellular membranes is required for integrated organism- and cellular metabolism, which is made possible by membrane-spanning proteins. These transport proteins can either operate as channels, carriers or pumps and are specific for the solutes they transport (Taiz and Zeiger, 2010).

Channels are responsible for the controlled, passive diffusion of water and ions across the membrane. Channel activity (the channels can be ‘open’ or ‘closed’) is regulated by membrane potential changes, phosphorylation, pH, calcium concentration, ligands, hormones, reactive oxygen species and light. Carrier proteins transport specific, uncharged ions or organic metabolites over the membrane. Conformational changes in the carrier protein are required for this type of membrane transport, which also makes it a much slower type of transport than through an open ion channel. The protein pumps (H^+-ATPase in the plasma membrane and the H^+-ATPase

(V-ATPase) and H^+-pyrophosphatase (H^+-PPase) in the tonoplast) are responsible for primary active transport (since it is coupled to a source of energy, e.g. ATP hydrolysis) of mainly H^+ (but also Ca^{2+}) outward across the membrane that causes a membrane potential and a pH gradient across the membrane. The electrochemical potential created across the membrane is then utilized by an assortment of secondary active transport proteins to move many other ions and organic substrates across the membrane against their gradient of electrochemical potential into the cell or organelle by simultaneously carrying one or two H^+ down the energy gradient. The protein pumps are regulated by the concentration of ATP, pH and temperature. The transport proteins can be regarded as conventional enzymes with the important exception that transport events are vectorial (i.e. defined by a magnitude and a direction in space) and not scalar (i.e. defined only by magnitude) as is the case with conventional enzymes (Staehelin et al., 2002). Membrane transport underlies many indispensable cellular processes such as (Staehelin et al., 2002; Taiz and Zeiger, 2010):

- Turgor generation
- Cell growth
- Cytoplasmic pH
- Stomatal opening
- Nutrient acquisition
- Waste product excretion
- Metabolite distribution
- Compartmentalization of metabolites
- Energy transduction
- Signal transduction

10. Membrane systems in the plant cell

The following membrane systems, which in some cases envelop specific organelles and distribute membranes and proteins via vesicles between the different cellular organelles, have very important functions in the cell (Taiz and Zeiger, 2010). Collectively this membrane system in eukaryotic cells is known as the endomembrane system. The intactness of these membrane systems plays a very important role in the normal functioning of the cell, and damage to them could hold dire consequences for the cell. Usually the chloroplasts are the first organelles of the endomembrane system to show signs of chilling injury (thylakoids swell and distort, starch granules disappear) while the mitochondria, nuclei and other organelles in the system are less susceptible (Kratsch and Wise, 2000). The functions of the following membrane systems are discussed briefly to give an indication of their importance in the functioning of the cell, and hence the plant.

10.1 The plasma membrane

Functions of the plasma membrane include (Staehelin et al., 2002):

- Regulation of the transport of molecules into and out of the cell
- Transmittance of signals from the environment to the cell interior
- Participation in the synthesis and assembly of cell wall molecules
- Provision of physical links between elements of the cytoskeleton and the extracellular matrix
- Production of plasmodesmata that cross cell walls and provide channels of communication between adjacent cells

The plasma membrane consists of lipids, proteins and carbohydrates in a ratio of approximately 40:40:20 (Staehelin et al., 2002). The lipid fraction is composed of phospholipids, glycolipids and sterols. The ratio between the different fractions varies among different organs in a specific plant and among the same organs in different plants. This dissimilarity in ratios gives an indication of the wide variety of lipid environments in which plasma membrane enzymes can function. One of the most important and critical alterations that occurs during cold acclimation in plant cells is changes in lipid composition of plasma membranes. Cold acclimation has been reported to cause an increase in the fraction of phospholipids and a decrease in the fraction of glucocerebrosides in cold-hardy herbaceous and woody species as well as an increase in the mole percentage of phospholipids carrying two unsaturated fatty acids. Species with cold-acclimated plasma membranes containing the most di-unsaturated phospholipids and the lowest fraction of glucocerebrosides tend to be the most cold-hardy.

Common fatty acids in plant plasma membranes are palmitic (C16:0), linoleic (C18:2), and linolenic acids (C18:3) (Staehelin et al., 2002). The most important free sterols of plant plasma membranes are campesterol, sitosterol and stigmasterol, with cholesterol being a minor component in the majority of plant species. Sterol esters, sterol glycosides and acylated sterol glycosides are more abundant in plants than in animals.

Most plasma membrane proteins involved in transmembrane activities (e.g. transport and signaling, anchoring of cytoskeletal elements to cell wall molecules) are integral proteins and often form larger complexes with peripheral proteins (Staehelin et al., 2002). The extracellular domains of many integral proteins are often glycocylated, bearing N- and O-linked oligosaccharides. The primary active transport system of plant cells is the plasma membrane H^+-ATPase which couples ATP hydrolysis to transmembrane transport of protons from the cytosol to the extracellular space.

The two effects of the proton pumping are firstly to acidify cell walls and alkalize the cytosol, which affects cell growth and expansion and other cellular activities, and secondly, to produce an electrochemical gradient across the plasma membrane. Electrochemical gradients consist of an electrical potential (inside negative in this case) and a concentration gradient (in this case a pH gradient outside acidic) which can be used to drive the transport of ions and solutes against their respective concentration gradients by secondary active transport systems. Furthermore, plant cell plasma membranes also contain proteins known as aquaporins, which act as water channels. Interaction of plasma membrane proteins with the cell wall includes (Staehelin et al., 2001):

- formation of physical links to cell wall molecules (known as Hechtian strands) which increases during cold acclimation to protect the protoplast against freeze-induced dehydration, and
- synthesis and assembly of cell wall polymers. Cellulose synthase and callose synthase complexes are both plasma membrane proteins and their products, cellulose (ß-1,4-linked glucose) and callose (ß-1,3-linked glucose), are secreted directly into the cell walls.

10.2 The endoplasmic reticulum (ER)

The ER is a three-dimensional network of continuous tubules and flattened sacs (cisternae) that not only spreads through the entire cytoplasm, but extends beyond the boundaries of individual cells via the plasmodesmata (Staehelin et al., 2002). It is associated with the nuclear envelope, and its tubules are physically connected to the plasma membrane (Staehelin et al., 2002; Taiz and Zeiger, 2010). The main functions of the ER are:

- the synthesis, processing and sorting of proteins intended for membranes, vacuoles or the secretory pathway,
- the addition of N-linked glycans to many proteins,
- together with the chloroplast, the major source of phospholipids for the endomembrane system through the eukaryotic phospholipid pathway situated in the ER,
- provision of anchoring sites for the actin filaments that drive cytoplasmic streaming, and
- regulation of calcium concentrations in the cytosol.

The ER also gives rise to the endomembrane system which transports secreted molecules to the cell surface, vacuolar proteins to the vacuoles, and distributes membrane proteins and lipids from their sites of synthesis to their sites of action (Staehelin et al., 2002). The principal membrane structures involved in this system include the nuclear envelope, membranes of the secretory pathway (ER, Golgi, *trans*-Golgi network, plasma membrane, vacuole, and different types of vesicles), and membranes involved in the endocytic pathway (plasma membrane, endocytic vesicles, multivesicular bodies, partially coated reticulum, vacuoles, and transport vesicles).

10.3 The Golgi-apparatus

The Golgi-apparatus consists of a polarized stack of cisternae and has a central position in the secretory pathway, receiving newly synthesized proteins and lipids from the ER (by receiving tubules and vesicles form the ER) and directing them to either the cell surface or the vacuoles (Staehelin et al., 2002; Taiz and Zeiger, 2010). Exocytosis is the process by which secretory vesicles from the Golgi-apparatus fuse with the plasma membrane and release their contents into the extracellular space (Staehelin et al., 2002). This process delivers the polysaccharides, glycoproteins and lipids needed for the expansion of the plasma membrane and cell wall growth. Since exocytosis delivers more membrane to the cell surface than is needed for the expansion of the plasma membrane, the process of endocytosis retrieves excess membrane for recycling through the formation of plasma membrane infoldings that give rise to endocytic vesicles. Endocytosis is also used to turn over plasma membrane and cell wall molecules and to remove activated receptors from the cell surface. The main functions of the Golgi-apparatus in plants are:

- assembly of complex polysaccharides of the cell wall matrix,
- synthesis and processing of the O-and N-linked oligosaccharide side chains of membrane, cell wall, and vacuolar glycoproteins,
- production of glycolipids for the plasma membrane and tonoplast – the glycosyltransferases and glycosidases that carry out these reactions are integral proteins of the Golgi cisternae.

10.4 The tonoplast

Vacuoles are fluid-filled organelles encompassed by the tonoplast and usually occupy more than 30% of the cell volume (Staehelin et al., 2002; Taiz and Zeiger, 2010). To maintain the turgor pressure in plant cells solutes must be actively transported into the vacuole to maintain osmolality. The driving force behind this uptake of solutes is an electro-chemical gradient across the tonoplast which is produced and maintained by two electrogenic proton pumps in the tonoplast, namely a V-type H^+-ATPase and vacuolar H^+-pyrophosphatase. The movement of water across the tonoplast is mediated by aquaporin channels (intrinsic proteins in the tonoplast). In addition to its role in cell growth (by taking up water and creating stiff, load-bearing structures that expand the primary cell wall) the vacuole has the following important functions:

- Storage organ for inorganic ions (include K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, PO_4^{3-}, NO^{3-}), sugars, pigments, amino acids, organic acids and proteins due to the occurrence of specific transporters in the tonoplast.
- Contains acid hydrolases such as proteases, nucleases, glycosidases and lipases which allow for the breakdown and recycling of almost all cellular components during senescence.

- Serves as reservoir of protons – by controlling the release of protons and other ions in the cytosol, cytosolic pH, the activity of enzymes, the assembly of cytoskeletal structures and membrane fusion events are regulated.
- Stores toxic compounds (phenolic compounds, alkaloids, cyanogenic glycosides, protease inhibitors, cell wall-degrading enzymes, latexes, etc.) to reduce feeding by herbivores and to destroy microbial pathogens.
- Sequesters toxic compounds that the plant cannot excrete.
- Contains pigments.

10.5 The nuclear membrane

The nucleus, which contains the cell's genetic information and serves as the regulatory centre, is bounded by two bilayer membranes, the nuclear envelope (Staehelin et al., 2002). The nuclear envelope separates the genetic material in the nucleus from protein synthesis in the cytoplasm and controls nucleo-cytoplasmic exchange by means of complex nuclear pores. The nuclear pores are channels consisting of > 100 different nucleoporin proteins that stretch across both membranes of the nuclear envelope (Taiz and Zeiger, 2010). These pores act as supramolecular sieves. The outer membrane of the nuclear envelope is continuous with the membranes of the ER.

10.6 Microbody (peroxisome and glyoxisome) membranes

These organelles have specialized metabolic functions in leaves and seeds and are surrounded by a single membrane (Taiz and Zeiger, 2010). Peroxisomes and glyoxisomes specialize in ß-oxidation of fatty acids and the metabolism of glyoxylate (to yield the amino acid glycine). The glyoxisomes are associated with mitochondria and oil bodies while the peroxisomes are associated with mitochondria and chloroplasts.

10.7 Oil body/ Oleosome/ Lipid body/ Spherosome membranes

Oil synthesized and designated for seed germination is stored in oil bodies which originate from the ER and are surrounded by a phospholipid monolayer (Taiz and Zeiger, 2010). Subsequent to the stored oil being consumed during seed germination, the oil bodies combine with the glyoxisomes which contain the enzymes for lipid oxidation.

10.8 Chloroplast membranes

Chloroplasts are bound by two membranes (an outer and inner membrane) and contain their own DNA and ribosomes (Taiz and Zeiger, 2010). Extensive metabolite traffic flows in both directions across the two membranes (Staehelin et al., 2002). It also contains a third membrane system, namely the thylakoids, which contain chlorophylls, carotenoids and their associated proteins that are responsible for photosynthesis (Taiz and Zeiger, 2010). The envelope and the thylakoid membranes of the chloroplast are poor in phospholipids and rich in galactolipids (Staehelin et al., 2002). The outer envelope membrane contains a nonspecific protein that freely permits movement of water and a variety of ions and metabolites while the inner membrane is freely permeable to small, uncharged molecules and to low-molecular mass, undissociated monocarboxylic acids. However, most metabolites cross the inner membrane with the help of specific transporters. The inner membrane also contains enzymes involved in the assembly of thylakoid membrane lipids. Plastids containing high levels of carotenoids are called chromoplasts while nonpigmented plastids are called leucoplasts of which the most abundant is the amyloplasts which store starch (Taiz and Zeiger, 2010). Plastids are responsible for photosynthesis, storage of an assortment of products and biosynthesis of chlorophylls, carotenoids, purines, pyrimidines and fatty acids (Staehelin et al., 2002). The downward growth of roots is caused by specialized amyloplasts in the root cap which serve as gravity sensors (Taiz and Zeiger, 2010).

10.9 Mitochondrial membranes

Mitochondria house the respiratory machinery that generates ATP, and also supply various compounds such as organic acids and amino acids that are used during biosynthesis elsewhere in the cell (Staehelin et al., 2002). Similar to chloroplasts, mitochondria are bound by two membranes (an outer and inner membrane) and house their own DNA and ribosomes (Taiz and Zeiger, 2010). The outer membrane is smooth and contains a small percentage of the total mass of the mitochondrial lipid and few enzymes while the inner membrane is more convoluted and contains 80 to 95% of the total membrane-coupled protein and more that 90% of the total mitochondrial lipid (Staehelin et al., 2002; Taiz and Zeiger, 2010). The inner membrane also contains the respiratory electron transport chain and ATP synthase that utilizes the electron transport chain to produce ATP. It also encloses the mitochondrial matrix which contains the Krebs cycle (Taiz and Zeiger, 2010). The outer membrane is highly permeable while the inner membrane has very low permeability (Staehelin et al., 2002).

11. Conclusion

Although lipids contribute only 0.09% of the total fresh weight of e.g. apple pulp, they are a vital ingredient of plant cells. Furthermore, although the cell membranes are only approximately 5 to 8 nm thick, they create unique structures in the cell that house specific chemical reactions and maintain the integrity of organelles and cells. Apart from this important function, membrane lipids also have numerous other functions such as the storage of reserve energy, signal transduction, acting as support structures for proteins in the membranes, etc. However, from a postharvest fruit storage perspective, one of the most important functions of lipids is the protection of the fruit against chilling injury. Desaturation of membrane lipids favors membrane fluidity and, therefore, cellular function and fruit viability at low temperatures. In this regard it is important to establish the relationship between membrane composition and chilling sensitivity.

Desaturase enzymes in the chloroplast and ER are stimulated to catalyse the desaturation of fatty acids when the fruit is introduced to low storage temperatures. However, it seems that G3P acyltransferases in the prokaryotic pathway (situated in the inner envelope of the chloroplast) of cold-sensitive plant species has the same affinity for saturated and monounsaturated fatty acids compared to those of cold-tolerant species which have a greater affinity for monounsaturated fatty acids. Cold-tolerant species would, therefore, have higher levels of glycerolipids containing monounsaturated fatty acids (rendering the cellular membranes more fluid under low temperature conditions) than those of cold-sensitive species.

The fact that membrane lipids are desaturated during the process of membrane retailoring in response to low storage temperatures makes the employment of postharvest handling practices, such as gradual acclimation at decreasing temperatures and/or intermittent warming cycles, viable options to keep the membranes selectively permeable and to prevent or reduce the severity of chilling injury. Antioxidants also play an important role in the control of the membrane retailoring process, since if they are overwhelmed by the number of free radicals, the process can cause severe membrane damage and death of the fruit. While it is still not clear how the complex enzymatic and non-enzymatic reactions that occur during membrane retailoring are regulated, postharvest practices such as those named above, need to be tested to ascertain that the practice works optimally for the specific product. Hence, postharvest practices to prevent chilling injury must be designed in such a way that the antioxidant system is kept intact in order for membrane retailoring, and not overall fruit deterioration, to be favored in reaction to low temperature storage.

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Table 1

Lipid types in higher plants and their functions (Somerville et al., 2002).

Function	**Lipid type**
Membrane structural components	Glycerolipids Sphingolipids Sterols
Storage compounds	Triacylglycerols Waxes
Compounds active in electron transfer reactions	Chlorophyll and other pigments Ubiquinone, plastoquinone
Photoprotection	Carotenoids (xanthophyll cycle)
Protection of membranes against damage from free radicals	Tocopherols
Waterproofing and surface protection	Long-chain and very-long chain fatty acids and their derivatives (cutin, suberin, surface waxes) Triterpenes
Protein modification	
• Addition of membrane anchors	
o Acylation	Mainly C14:0 and C16:0 fatty acids
o Prenylation	Farnesyl and geranylgeranyl pyrophosphate
o Other membrane anchor components	Phosphatidylinositol, ceramide
• Glycosylation	Dolichol
Signaling	
• Internal	Abscisic acid, gibberellins, brassinosteroids C18:3 fatty acid precursors of jasmonate Inositol phosphates Diacylglycerols
• External	Jasmonate Volatile insect attractants
Defense and antifeeding compounds	Essential oils Latex components (rubber, etc.) Resin compounds (terpenes)

Table 2

Distribution of cellular components and lipid types in leaf tissue of *Arabidopsis* (Somerville et al., 2002).

Cellular constituent	**Percentage of total dry weight**
Carbohydrates	58.7
Protein	19.4
Minerals	9.0
Other	7.0
Lipid	5.9
Lipid types	**Percentage of total lipids by weight**
Glycerolipids	56.4
Chlorophyll	25.9
Cutin monomers	6.3
Plastoquinone	4.1
Carotenoids	3.4
Tocopherols	1.2
Sterols	1.4
Sphingolipids	1.3

Table 3

Fatty acids generally found as the main constituents of membrane lipids (Somerville et al., 2002).

Common name	**Systematic name**	**Abbreviation**
Palmitic acid	*n*-Hexadecanoic acid	C16:0
Stearic acid	*n*-Octadecanoic acid	C18:0
Oleic acid	*cis*-9-Octadecenoic acid	$C18:1^{\Delta 9}$
Linoleic acid	*cis,cis*-9,12-Octadecatrienoic acid	$C18:2^{\Delta 9,12}$
α-Linolenic acid	*all-cis*-9,12,15-Octadecatrienoic acid	$C18:3^{\Delta 9,12,15}$

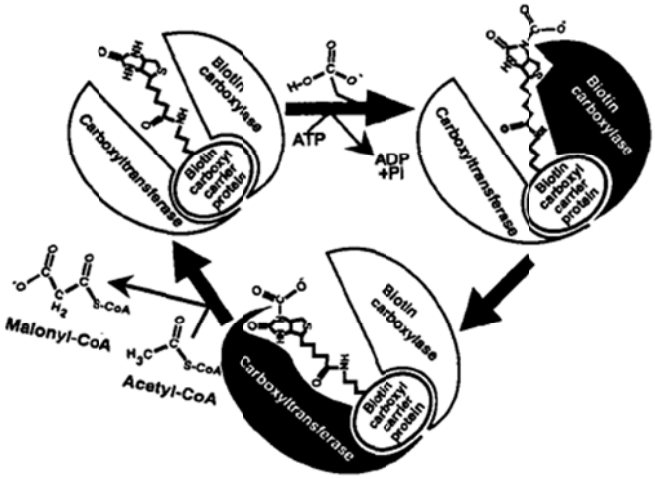

Fig. 1. The ACCase complex with its three functional groups, namely biotin carboxylase (which catalyses the carboxylation of biotin in an ATP-dependent reaction), biotin carboxyl carrier protein and carboxyltransferase (which catalyses the transfer of the activated CO_2 from biotin to acetyl-CoA) (Adapted from Ohlrogge and Browse, 1995).

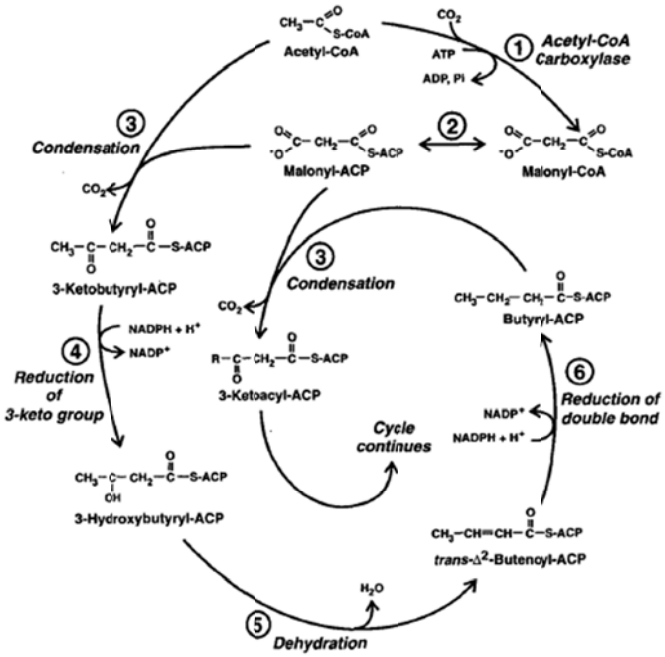

Fig. 2. The different reactions in the biosynthetic pathway of saturated fatty acids. Acetyl-CoA enters the biosynthetic pathway both as a substrate for acetyl-CoA carboxylase in reaction 1 and as a primer for the first condensation reaction in reaction 3. In reaction 2, catalysed by malonyl-CoA:ACP transacylase, malonyl is transferred from CoA to the protein cofactor ACP to form malonyl-ACP. Malonyl-ACP acts as the carbon donor for the subsequent elongation reactions. After each condensation reaction (reaction 3) the 3-ketoacyl-ACP product is reduced (reaction 4), dehydrated (reaction 5) and reduced again (reaction 6) by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase, respectively (Ohlrogge and Browse, 1995)

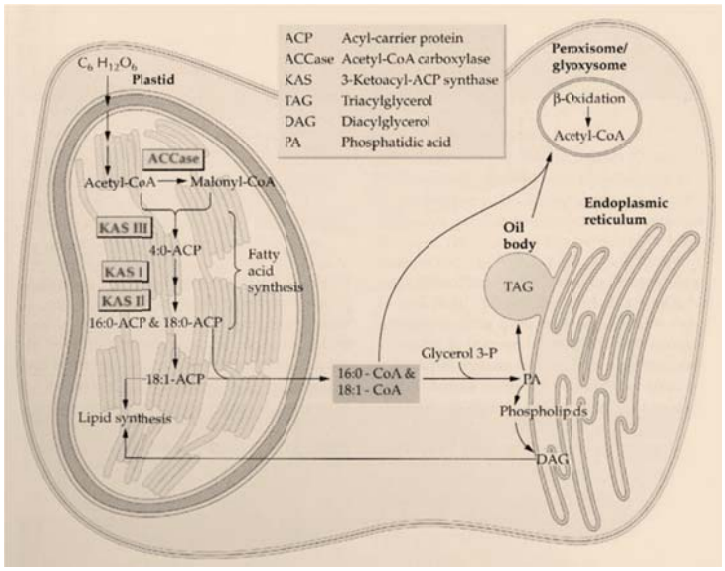

Fig. 3. Summary of cellular compartmentalization of lipid synthesis pathways and metabolism in plants (Somerville et al., 2002).

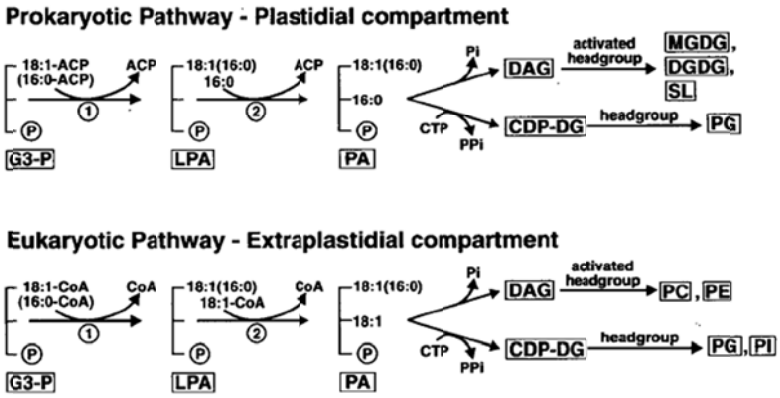

Fig. 4. Summary of the prokaryotic and eukaryotic pathways of glycerolipid synthesis (Ohlrogge and Browse, 1995). The prokaryotic pathway takes place in the plastids and uses acyl-ACPs as substrates. The eukaryotic pathway takes place outside the plastid (mainly at the ER) and uses acyl-CoAs as substrates. CDP-DG, cytidine diphosphate-diacylglycerol; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; G3-P, glycerol-3-phosphate; LPA, monoacylglycerol-3-phosphate; MGDG, monoglalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SL, sulfoguinovosyldiacylglycerol.

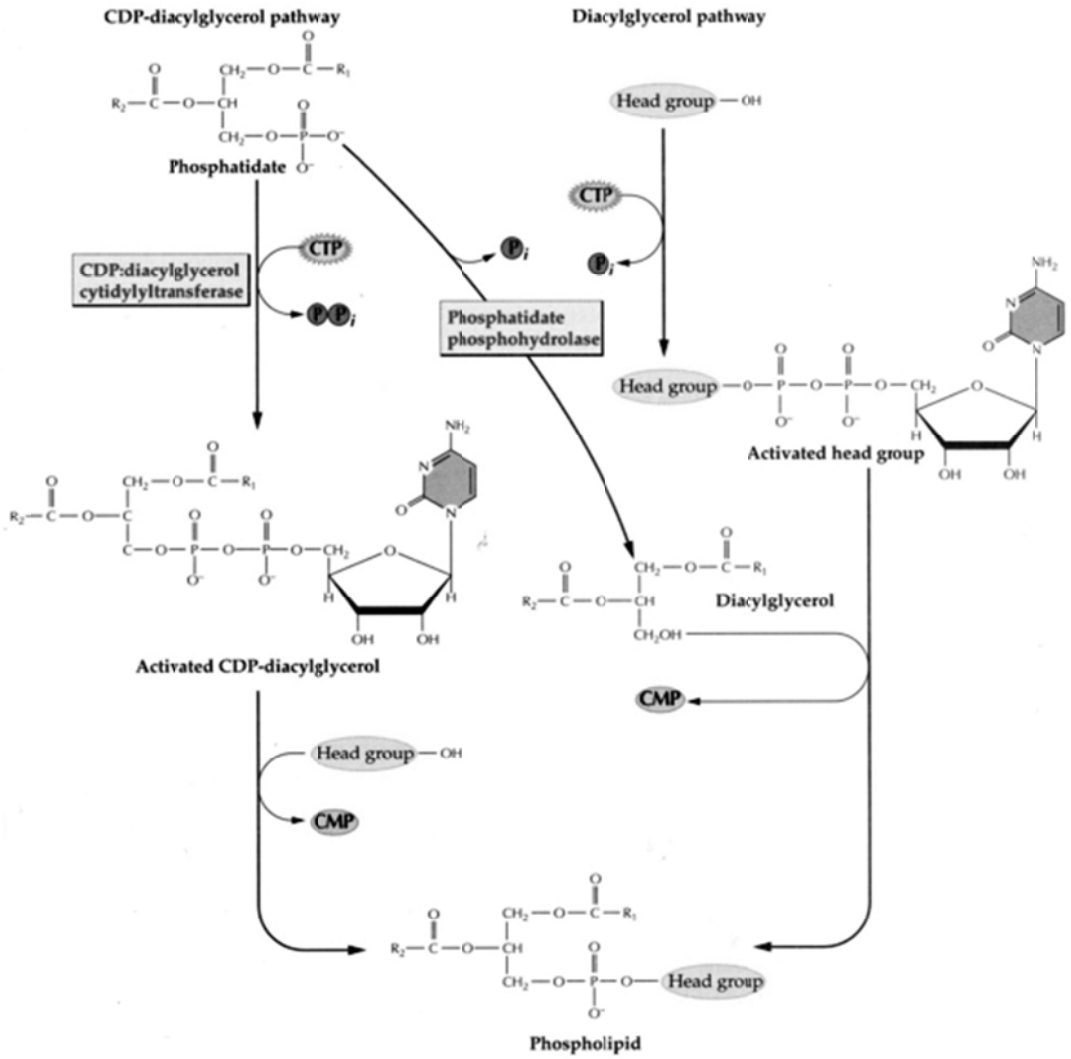

Fig. 5. Summary of the two pathways for attachment of the polar head group during glycerolipid synthesis (Somerville et al., 2002).

PAPER 1

Comparison of antioxidant levels and cell membrane composition during fruit development in two plum cultivars (*Prunus salicina* Lindl.) differing in chilling resistance

Abstract

It is known that chilling resistant cultivars are able to modify their membrane composition and/or antioxidant profile under low-temperature storage conditions unlike chilling susceptible cultivars. Knowledge of the differences in membrane composition and antioxidant levels during plum fruit development in both chilling resistant and susceptible plum cultivars could help to establish or improve current orchard and/or postharvest handling practices to alleviate the occurrence of chilling injury in susceptible cultivars. The aim of this study was to determine if apparent differences in cell membrane composition and antioxidant levels between chilling susceptible (in this study 'Sapphire') and chilling resistant (in this study 'Angeleno') plum cultivars already exist during fruit development. Regarding antioxidants, 'Sapphire' accumulated higher levels of glutathione, while 'Angeleno' accumulated higher levels of ascorbic acid. The driving factor for chilling susceptibility in 'Sapphire' plums seems to be the cultivar's ability to accumulate higher levels of linoleic acid compared to oleic acid. Linoleic acid (a polyunsaturated fatty acid) is more susceptible to lipid peroxidation than oleic acid (a monounsaturated fatty acid). 'Angeleno' accumulated higher levels of oleic acid, compared to linoleic acid, which probably renders the cultivar more tolerant to low temperature storage. The difference in the antioxidants and phospholipid fatty acids that were accumulated in the two cultivars is related to the time the fruit spend on the tree during fruit development. 'Sapphire' is an early season cultivar which, therefore, experiences lower average daily temperatures during fruit development. Glutathione and linoleic acid accumulate under lower temperatures. 'Angeleno' is a late season cultivar, experiencing higher average daily temperatures during the latter half of its fruit development period. Higher temperatures favour the accumulation of ascorbic acid and oleic acid.

Key words

Ascorbic acid, glutathione, linoleic acid, monounsaturated fatty acids, oleic acid, polyunsaturated fatty acids

1. Introduction

It is well known in the South African plum industry that 'Angeleno' is much less CI susceptible compared to cultivars such as 'Pioneer', 'Sapphire' and 'Songold'. This fact was confirmed by the research conducted by Kruger (2002). Abdi et al. (1997) speculated that cultivars with a long fruit development period are less susceptible to CI compared to fruit with a short fruit development period based on his storage results of plums with varying periods of fruit development.

The first physiological response to CI is loss of cell membrane integrity, which is caused, amongst others, by changes in lipid composition (Sevillano et al., 2009; Marangoni et al., 1996). The secondary response to CI is oxidative stress, which further enhances the loss of cell membrane integrity (Sevillano et al., 2009). It is well known that, compared to chilling susceptible cultivars, chilling resistant cultivars are able to adapt or acclimatise better to chilling temperatures, due to proficient modification of their membrane composition and/or antioxidant profile under low-temperature storage conditions (Caldwell, 1990; Lurie, 2003; Upchurch, 2008). However, to our knowledge, no information exists on the differences in membrane composition and antioxidant levels during fruit development in chilling resistant and sensitive plum cultivars. Such information could help to establish or improve current orchard and/or postharvest handling practices to alleviate the occurrence of CI in susceptible cultivars.

The aim of this study, therefore, was to determine if differences in cell membrane composition and antioxidant levels already exist between a chilling susceptible ('Sapphire') and chilling resistant ('Angeleno') plum cultivar during fruit development.

2. Materials and Methods

2.1 Sampling of fruit

'Sapphire' and 'Angeleno' plums (*Prunus salicina* Lindl.) were sampled from a commercial stone fruit farm in Wellington, Western Cape region, South Africa, over two seasons (2005 and 2007). The row orientation was north-south, and the trees were trained according to a palmette system. A complete randomised design was used. From 40 days after full bloom (dafb), fruit of similar size and colour were harvested once a week up to approximately 54 dafb ('Sapphire') or up to approximately 112 dafb ('Angeleno') and thereafter twice a week until the optimum harvest date. Fruit were sampled randomly in the orchard from both sides of the tree rows and from the outside of the canopy approximately 1.5 m above the orchard floor. Due to the small fruit size, 90 fruit

were harvested during the first 3 weeks and thereafter 30 fruit per sampling date, until the optimum harvest date of each cultivar. In both seasons, from 40 dafb until the optimum harvest dates, temperature and relative humidity were logged hourly with Temptale 4 loggers (Sensitech, Beverly, MA, USA) placed in Stevenson screens in the respective orchards.

At the laboratory, fruit were divided into three replicates containing 30 fruit each for the first three sampling weeks and thereafter 10 fruit each until the optimum harvest date. On the day of sampling, non-destructive measurements were taken on individual fruit. The fruit of each replicate were pooled, peeled and the pulp flash frozen, milled to a fine powder in liquid nitrogen, and stored at -80 °C for further analysis.

2.2 Non-destructive measurements on each sampling date

Individual fruit weight (g) was determined only in the 2007 season using a calibrated balance (Mettler PE 3600, Switzerland). The increase in fruit size over time was quantified by fruit diameter (mm) using a Mitutyo calliper (Japan). Hue angle, measured with a calibrated colorimeter (Minolta chroma meter CR-400, Japan), was used to quantify the change in ground colour during fruit development and maturation on the tree. The mean and standard deviation values were determined for each quality parameter.

2.3 Analyses conducted on the samples stored at -80 °C

2.3.1 Determination of total phenolic concentration

Total phenolic concentration was determined using the Folin-Ciocalteu assay (Kim et al., 2003; Tsao and Yang, 2003). The method involved the addition of 10 mL 80% ethanol to 1 g frozen sample (-80 °C), which was homogenized for 5 s with an Ultra Turrax. The mixture was then stirred for 1 h at 4 °C and filtered through a Whatman nr. 2 filter paper (Whatman, Maidstone, England). Next, 50 µL sample and 450 µL Folin-Ciocalteu reagent: MQ H_2O (1:9 v/v) were mixed in a cuvette and allowed to stand at room temperature for 5 min. Subsequently, 500 µL 5.6% Na_2CO_3 was added to the solution in the cuvette, mixed, and left to stand at room temperature for 90 min. Total phenolic concentration was determined spectrophotometrically (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Victoria, Australia) using a gallic acid standard curve with absorbance readings at 750 nm. Samples were analysed in triplicate. Total phenolic concentration was expressed as mg gallic acid equivalents (GAE) g^{-1} fresh weight (FW).

2.3.2 Determination of lipid peroxidation

The degree of lipid peroxidation was measured in terms of malondialdehyde (MDA, a product of lipid peroxidation) concentration by 2-thiobarbituric acid (TBA) reaction with minor modifications of the Heath and Packer (1968) method. Frozen sample (-80 °C) was weighed (1 g), 25 mL 80% ethanol was added and kept on ice while the other samples were prepared. Samples were replicated three times. Each sample was homogenized with an Ultra Turrax for 10 s and then centrifuged at 3000 x g for 10 min at 4 °C. To 1 mL of the supernatant 1 mL TBA solution (0.5% TBA (w/v), 2% trichloroacetic acid (TCA) (w/v) and 0.01% butylated hydroxytoluene (BHT) (w/v) in MQ H_2O) and 10 µL of 1% BHT (w/v) were added. The blank contained 1 mL 80% ethanol, 1 mL TBA solution and 10 µL BHT. The samples were mixed vigorously, heated at 95 °C for 25 min in a block heater and immediately cooled on ice for 10 min to stop the reaction. Finally the samples were centrifuged for 10 min at 3000 x g at 4 °C in an Eppendorf 5810R centrifuge (Hamburg, Germany) and the absorbance of the supernatant was read at 532 nm and 600 nm in glass cuvettes. MDA equivalents were calculated as follows:

MDA equivalents (nmol mL^{-1}) = $[(A_{532} – A_{600})/155\ 000] \times 10^6$

where A_{532} represents the maximum absorbance of the TBA-MDA complex, A_{600} the correction for nonspecific turbidity, and 155 000 the molar extinction coefficient for MDA. MDA equivalents were expressed as nmol g^{-1} FW.

2.3.3 Determination of ascorbic acid and glutathione in both oxidised and reduced forms

Plant tissue contains both oxidised and reduced forms of ascorbic acid and glutathione. The following abbreviations will be used throughout: L-AA = ascorbic acid (reduced form), DHA = dehydroascorbic acid (oxidised form of ascorbic acid), L-AA + DHA = total ascorbic acid, GSH = glutathione (reduced form), GSSG = glutathione disulfide (oxidised form of glutathione) and GSH + GSSG = total glutathione. The reduced forms of ascorbic acid and glutathione are the predominant forms in plant tissue (Dixon et al., 1998; Foyer, 1993) and it is only these that can be determined directly on HPLC. The oxidised forms must first be reduced to L-AA and GSH in order to be analysed indirectly by HPLC. The sample extract (Ex) and the reduced sample extract (REx), therefore, need to be analysed in separate HPLC runs. The analysis of the REx thus represents the total ascorbic acid (L-AA + DHA) and total glutathione (GSH + GSSG). DHA and GSSG levels were, therefore, determined by the following calculations:

$DHA = (L\text{-}AA + DHA)_{REx} – (L\text{-}AA)_{Ex}$ (Equation 1)

$GSSG = (GSH + GSSG)_{REx} – (GSH)_{Ex}$ (Equation 2)

Ascorbic acid and glutathione concentration were simultaneously quantified by high-performance liquid chromatography (HPLC) analysis based on the method of Davey et al. (2003) with minor modifications (M.W. Davey, personal communication).

2.3.3.1 Extraction from plum pulp

Frozen sample (-80 °C) was weighed (5 g) and mixed with 10 ml chilled (4 °C) extraction buffer and kept at 4 °C while the other samples were prepared. The extraction buffer comprised 3% metaphosphoric acid (MPA), 1 mM ethylenediaminetetracetic acid (EDTA) and 2% insoluble polyvinylpolypyrrolidone (PVPP) in MQ H_2O and was stirred well before adding it to the sample. Subsequently, 1.8 mL of the sample extract (Ex) was pipetted into a 2 mL plastic microcentrifuge tube and centrifuged at 20 000 x g for 15 min at 4 °C in an Eppendorf 5417R centrifuge (Hamburg, Germany). The supernatant (1 mL) was then pipetted into a 1.5 mL microcentrifuge tube and centrifuged again at 20 000 x g for 15 min at 4 °C. A clear 600 µL-aliquot of the supernatant was transferred to an HPLC vial for HPLC analysis.

2.3.3.2 Reduction of sub-sample of extract

Reduction of the subsample of the extract was carried out at room temperature by mixing 40 µL of Ex with 20 µL of a stock solution of 400 mM DL-dithiothreitol (DTT) in 400 mM Tris base (pH ~ 6 to 6.8). The reduction reaction was stopped after 20 min by acidification with 20 µL of 8.5% *o*-phosphoric acid, resulting in a twofold dilution of the original sample. This extract was called the reduced sample extract (REx) described earlier (see Section 2.3.3). REx was then directly analysed by HPLC. A double volume was injected to compensate for the dilution effect.

2.3.3.3 HPLC analysis

Reverse phase high-performance liquid chromatography (RP-HPLC) analysis was performed by injecting 10 µL of the sample extract (Ex, see Section 2.3.3.1) and 20 µL of the reduced sample extract (REx, see Section 2.3.3.2) immediately after preparation into an Agilent Series 1100 HPLC system (Agilent Technologies, Inc., Waldbronn, Germany). The autosampler was thermostatted at 4 °C and the column temperature was 16 °C. Detection was carried out using a photodiode array detector. The system was controlled and data were collected and integrated using the Chemstation for LC 3D systems software (Rev. B.10.03 (204), Agilent Technologies, Inc., Waldbronn, Germany). Separations were performed on a 250 x 4.6 mm CapcellPak HPLC column packed with C_{18} 5-µm stationary phase protected by a 4.6 mm x 12.5 mm guard cartridge (Zorbax

SB-C18, Agilent, USA). Solvents used for elution were: (A) 6 mM *o*-phosphoric acid, 0.1 mM EDTA (to complex metal ions which might promote oxidation of L-AA) in MQ H_2O (pH ~ 2.5) and (B) 6 mM o-phosphoric acid, 0.1 mM EDTA in MQ H_2O:acetonitrile (70:30, v/v). The flow rate was 1.0 mL min^{-1}. The mobile phase was 100% A (isocratic from 0 to 7 min), 70% A and 30% B (gradient from 7 to 10 min), 100% B (isocratic from 10 to 13 min) and 100% A (isocratic from 13 to 22 min). The L-AA and GSH peaks were eluted within the first 7 min whereafter the column was regenerated. L-AA was quantified at its UV absorption maximum of 243 nm, while GSH was quantified at 197 nm. The identities of the L-AA and GSH peaks were confirmed by coelution with authentic standards and by the characteristic absorption spectra recorded over the range of 195 nm to 300 nm. Concentrations of L-AA and GSH were calculated from standard curves prepared daily.

2.3.3.4 Standard curves

Stock solutions of 0.05 mg mL^{-1} (w/v) L-AA and GSH were prepared in a solution of 1 mM EDTA and 2.5 mM dithiothreitol DTT in 3% MPA. Aliquots (450 µL) of the stock solutions were stored at -20 °C and used to calibrate the detector response daily.

2.3.3.5 Determination of dehydroascorbic acid/oxidised ascorbic acid (DHA) and glutathione disulfide/oxidised glutathione (GSSG)

The concentration of DHA and GSSG were calculated by the 'subtractive' method (Equations 1 and 2, Section 2.3.3) after determination of the total L-AA (L-AA + DHA) and total GSH (GSH + GSSG) levels in the REx (see Sections 2.3.3.2 and 2.3.3.3) as well as L-AA and GSH in the Ex (see Sections 2.3.3.1 and 2.3.3.3), respectively. Results were expressed as µg total, reduced or oxidised ascorbic acid and µg total, reduced or oxidised glutathione per g FW.

2.3.4 Determination of total (hydrophilic and lipophilic) radical-scavenging activity

The total antioxidant activity (TAC) of plum pulp was determined using the $ABTS^{\cdot+}$ scavenging assay of Vinokur and Rodov (2006) with minor modifications.

2.3.4.1 Stepwise extraction from plum pulp

The extraction procedure allowed the isolation of hydrophilic and lipophilic antioxidant fractions by stepwise extraction of the plum pulp with acetate buffer, acetone and hexane. Firstly, 2 mL acetate buffer (pH 4.3) and 1 mL acetone were immediately added to 1 g frozen sample (-80 °C), mixed and kept at 4 °C while the other samples were prepared. The extract was homogenised, 3 mL acetone was added to the homogenate and centrifuged for 5 min at 3000 x g at 4 °C in an Eppendorf 5810R centrifuge (Hamburg, Germany). The supernatant was collected in a 10 mL graded test tube. The sample residue was re-extracted with 0.6 mL acetate buffer and 1.4 mL acetone, centrifuged for 5 min at 3000 x g at 4 °C and the supernatant added to the first supernatant fraction. Subsequently the sample residue was re-extracted with 2 mL acetone, centrifuged and the supernatant added to the first two supernatant fractions called the hydrophilic (water/acetone) extract. The dehydrated sample residue was then extracted twice with 2 mL hexane and the supernatants collected and pooled in a separate glass test tube after centrifugation and called the lipophilic (hexane) extract. Traces of lipophilic compounds were then extracted from the hydrophilic extract by mixing 2 mL hexane vigorously with the pooled acetone-water extract and the top layer was added to the lipophilic extract. The hydrophilic extract in the graded test tube was filled to the 10 mL mark with 1:3 (v/v) acetate buffer: acetone and mixed. The lipophilic extract was dried under a stream of N_2 in a warm water bath at 40 °C. Hexane (0.5 mL) was added to the dried lipophilic extract, capped and mixed.

2.3.4.2 $ABTS^{\cdot+}$ generation

Acidified ethanol (0.1% H_2SO_4 in 99% ethanol (v/v)) was incubated at 45 °C in a water bath until the solution was on temperature. Subsequently, 200 µL 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (0.15 mM in MQ H_2O) and 200 µL 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (1.77 mM in MQ H_2O) (a radical initiator) were added to the acidified ethanol and mixed. The reaction mixture was incubated at 45 °C in the water bath for 75 min. The $ABTS^{\cdot+}$ reaction mixture was prepared fresh daily.

2.3.4.3 Assay performance

Samples were analysed in triplicate. The decolouration test was performed in plastic cuvettes by adding a 10 µL aliquot of either hydrophilic or lipophilic extract to 990 µL $ABTS^{\cdot+}$ reaction mixture. The samples were incubated at room temperature for 15 min and absorbance was subsequently measured at 734 nm using acidified ethanol as a blank. Trolox (6-hydroxy-2,5,7,8-tetramethylchorman-2-carboxylic acid) was used as a standard and the radical scavenging activity

of the hydrophilic and lipophilic antioxidant fractions were expressed as mg Trolox equivalents g^{-1} FW.

2.3.5 Lipid extraction from plum pulp

The total lipid fraction was extracted using the Folsch et al. (1957) method. All the steps took place at room temperature. Only glassware and Teflon lined caps were used throughout. Firstly, 6 mL methanol with 0.01% butylated hydroxytoluene (BHT) (w/v) was immediately added to 3 g frozen sample (-80 °C) and mixed well. Next, 200 µL internal standard (2 mg mL^{-1} heptadecanoic acid in HPLC grade hexane (v/v)) and 12 mL chloroform with 0.01% BHT (w/v) were added to the extract and mixed well. The sample extract was transferred to a Buchner funnel lined with a glass microfiber filter (Whatman GF/A) and the extraction tube was rinsed with 12 mL chloroform: methanol (2:1, v/v) mixture which was added to the contents in the Buchner funnel. The mixture in the Buchner funnel was then filtered. Subsequently, 7.5 mL 0.88% KCl was added to the filtrate, shaken well and allowed to separate into two phases. The upper, aqueous layer was removed by vacuum aspiration. The remaining chloroform layer, which contained the total lipid fraction, was evaporated to complete dryness under vacuum, redissolved in 2 mL chloroform with 0.01% BHT (w/v) and stored at -20 °C for further analysis.

2.3.6 Determination of total sterols

The method of Courchaine et al. (1959) was used to quantify the total sterol concentration with minor modifications. All the steps took place at room temperature. Firstly, a ferric chloride stock solution was prepared by dissolving 2.5 g $FeCl_3 \cdot 6H_2O$ in concentrated phosphoric acid (w/v), and diluting it to 100 mL. Next, in a fume hood, 8 mL iron stock solution was diluted to 100 mL with concentrated H_2SO_4 to obtain the ferric chloride colour reagent. An aliquot (400 µL) of the total lipid fraction (see Section 2.7) was dried under a stream of N_2 in a fume hood and redissolved in 6 mL glacial acetic acid. To this mixture 4 mL ferric chloride colour reagent was added and mixed well. The mixture was allowed to stand for 10 min to allow the colour to develop. Total sterol concentration was then determined spectrophotometrically (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Victoria, Australia) at 550 nm using a stigmasterol standard curve. Plastic cuvettes were used. Samples were analysed in triplicate. Total sterol concentration was expressed as µmol stigmasterol equivalents g^{-1} FW.

2.3.7 Determination of total phospholipids

Total phospholipid concentration was quantified according to the methods of Ames (1966) and Duck-Chong (1979) with minor modifications. A 50 µL aliquot of the total lipid fraction (see Section 2.7) was added to 30 µL of a 10% Mg $(NO_3)_2$ $6H_2O$ in 95% methanol solution and evaporated to complete dryness in a heating block at 95 °C in a fume hood. The sample was then digested by heating it over a Bunsen flame in a fume hood until the brown fumes disappeared (approximately 10 s) and left to cool (5 min). Pyrophosphate which was formed during the digestion step was then hydrolysed to phosphate by adding 300 µL of 0.5 M HCl and heating the sample in a heating block at 100 °C for 15 min after which the sample was immediately cooled to room temperature by immersion in tap water. After the tube had cooled 700 µL of a 1:6 (v/v) mixture of 10% ascorbic acid and 0.42% ammonium molybdate $4H_2O$ in 1 N H_2SO_4 was added to the sample and it was incubated at 45 °C for 20 min in a heating block. The absorbance of the resulting blue phosphomolybdate complex was then measured at 820 nm (Varian, 50 Bio-UV-Visible Spectrophotometer, Mulgrave, Victoria, Australia). The total phospholipid concentration was determined using a sodium phosphate standard curve. Plastic cuvettes were used. Total phospholipid concentration was expressed as µmol g^{-1} FW.

2.3.8 Determination of phospholipid fatty acids

2.3.8.1 Separation of lipid classes

Lipid classes in the total lipid extract were separated according to the methods of Kaluzny et al. (1985), Pietsch and Lorenz (1993) and Laffargue et al. (2007). A vacuum manifold (Visiprep™24, Supelco, Bellefonte, PA, USA) was used and a vacuum of -20 kPa was applied for elution of the columns. The 6 mL 500 mg NH_2 cartridges (Chromabond, Düren, Germany) were preconditioned with hexane (2 x 2mL). A 300 µL aliquot of the total lipid fraction (See Section 2.7) was deposited onto the cartridge and allowed to sink into the column. The solvent (chloroform) was then completely sucked through the solid phase to leave the entire lipid fraction on the column. First, the neutral lipids were eluted (4 x 1 mL) with chloroform: isopropanol (2:1, v/v) and discarded. The free fatty acids were then eluted (5 x 1 mL) with diethyl ether: acetic acid (98:2, v/v) and collected as this fraction contained the internal standard (heptadecanoic acid added during the extraction of the total lipid fraction). Finally, the phospholipids were eluted (3 x 1 mL) with methanol and collected. The SPE columns were regenerated by washing first with 2 mL diethyl ether: acetic acid (98:2, v/v), then with 2 mL chloroform: isopropanol (2:1, v/v) followed by 2 mL hexane. Subsequently the free fatty acid and the phospholipid eluates were dried under a stream of N_2 in a fume hood in a water bath at 40 °C.

2.3.8.2 Methylation and gas chromatographic analysis of fatty acids

Immediately after it was dried, 0.4 mL 10% BF_3 in methanol was added to the phospholipid and free fatty acid fractions, respectively and heated at 100 °C for 5 min in a heating block to methylate the fatty acids. The tubes were immediately cooled in tap water to stop the reaction. Fatty acid methyl esters (FAMEs) of the phospholipid and free fatty acid fractions, respectively, were extracted from the methanol solution by the addition and thorough mixing of 2 mL HPLC-grade hexane and 1 mL MQ H_2O. An aliquot (1 mL) of the top hexane layer (in which the FAME's were partitioned) was transferred to a GC vial. A 2 µL aliquot was injected into an Agilent GC system (Model 7890A, Agilent Inc., Wilmington, USA) fitted with a flame ionisation detector (FID) and automatic sampler (Model 7683B, Agilent Inc., Wilmington, USA) by split injection (20:1) with a split vent flow rate of 68.3 mL min^{-1} and septum purge flow rate of 3 mL min^{-1}. Helium was used as a carrier gas at a column flow rate of 3.4 mL min^{-1} and a column inlet pressure of 270 kPa. Both the injector and detector were at 260 °C. Fatty acid separations were done on an HP-Innowax capillary column (30 m x 0.25 I.D. and 0.25 µm film thickness, coated with 100 % polyethylene glycol, J&W Scientific, Folsom and Agilent Technologies Inc., Wilmington, USA). The oven temperature was programmed from 150 °C (isothermal for 1 min) to 170 °C at 1.2 °C min^{-1}, from 170 °C to 180 °C at 5 °C min^{-1} and from 180 °C to 250 °C (isothermal for 6 min) at 20 °C min^{-1}. The system was controlled and data were collected and integrated using the GC Chemstation software (Rev. B.04.02 (96), Agilent Technologies, Inc., Waldbronn, Germany). The Supelco 37 component FAME Mix (Supelco Inc., Bellefonte, PA, USA) was used to identify the fatty acid methyl esters by comparison of their retention times. Quantification was done by setting up a standard curve for the standard mix and calculating the ratio between these and the internal standard (unmethylated heptadecanoic acid) which was contained in the free fatty acid fraction.

2.4 Statistical analysis

Discriminant analysis (DA) was performed on the four groups namely, 'Sapphire' 2005, 'Sapphire' 2007, 'Angeleno' 2005 and 'Angeleno' 2007 created for 'Sapphire' and 'Angeleno' plums over the two fruit growing seasons. This was done to establish if the variables were able to discriminate between the seasons, and to determine which parameters significantly influenced seasonal differences.

Subsequently, the cultivar data were pooled for the two seasons and a DA was performed on the two resultant groups, namely 'Sapphire' and 'Angeleno', to establish if the variables could discriminate between the two cultivars on the grounds of their membrane and antioxidant

composition (and hence their differing susceptibility to CI), irrespective of the fact that they were sampled over two seasons.

To test the robustness of each model, the data of each group were split into two unique subsets. One subset, comprising 70% of the data which was chosen randomly from the data set, was used to build the model before testing it by cross validation on the second subset comprising 30% of the data. Variables that occurred most frequently in the 20 'best' models from the best subsets procedure were then used to describe the differences between the cultivars and seasons.

The following variables were used for the best subsets selection procedure: hue angle, fruit diameter (mm), total phenolic concentration, MDA equivalents, total ascorbic acid, L-AA, DHA, total glutathione, GSH, GSSG, water soluble radical-scavenging activity (HAA), lipid soluble radical-scavenging activity (LAA), total sterols, total phospholipids, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9c), elaidic acid (C18:1n9t), linoleic acid (C18:2n6c), linolenic acid (C18:3n3), total fatty acids of phospholipids, and percentage unsaturated fatty acids of phospholipids.

Statistical analysis was performed using STATISTICA version 10 (Statsoft, Inc., 2011).

3. Results

3.1 Discriminant analyses regarding cultivar differences

According to the discriminant analyses procedure six of the 23 variables, namely total glutathione, GSSG, total ascorbic acid, oleic acid, linoleic acid and hue angle, occurred five or more times in the 20 best models selected by the best subsets selection procedure (Fig. 1.1). Of these six variables, total glutathione, GSSG, total ascorbic acid and oleic acid occurred the most frequently in the 20 'best' models. The best subset of the 20 'best' models indicated that the six variables correctly predicted 100.00% of the samples into the four groupings (Table 1.1A). Validation with an independent data set showed that the model was accurate and predicted 100.00% of the samples into the correct groupings (Table 1.1B). The observations plot of the best subset of the 20 'best' models shows that the two cultivar groupings were clearly separated from each other (Fig. 1.22). This indicates that the six variables could be used to accurately discriminate between the two cultivars. In the following presentation and discussion of the results regarding the differences between the two plum cultivars, emphasis will be placed on these six variables.

3.2 Discriminant analyses regarding seasonal differences

According to the discriminant analyses procedure nine of the 23 variables, namely total glutathione, GSSG, GSH, DHA, L-AA, total ascorbic acid, hue angle, oleic acid, and total phenolic concentration, occurred five or more times in the 20 'best' models selected by the best subsets selection procedure (Fig. 1.3). Of these six variables, total glutathione and GSSG occurred in all of the 20 'best' models. The best subset of the 20 'best' models indicated that the nine variables correctly predicted 94.85% of the samples into the four groupings (Table 1.2A). Validation with an independent data set showed that the model was accurate and predicted 96.61% of the samples in the correct groupings (Table 1.2B). The observations plot of the best subset of the 20 'best' models shows that the four cultivar/seasonal groupings were clearly separated from each other (Fig. 1.4). This indicates that the nine variables could discriminate between the two seasons. In the following presentation and discussion of the results regarding the differences between the seasons for the two plum cultivars, emphasis will be placed on these nine variables.

3.3 Variables selected to discriminate between cultivars and seasons

3.3.1 Total glutathione

Total glutathione was one of the most important variables that significantly discriminated between the two cultivars and the two seasons (Fig. 1.1 and Fig. 1.3). Concentrations were lower in 2005 compared to 2007, irrespective of cultivar (Fig. 1.5A, B, C and D, and Table 1.3). On average 'Sapphire' had higher total glutathione concentrations compared to 'Angeleno' in the respective seasons (Table 1.3). In 2005 total glutathione concentration almost halved from 40 dafb until the optimum harvest date in 'Angeleno' (Fig. 1.5C). Total glutathione concentration varied more in the 2007 season compared to the 2005 season, and in the 2007 'Angeleno' fruit towards the middle and end of the fruit growing season (Fig. 1.5D). Generally total glutathione content per fruit increased in 'Sapphire' and 'Angeleno' fruit over the sampling periods, except for 'Angeleno' in the 2005 season when total glutathione content was low throughout the sampling period (data not shown; Refer to Appendix A, Fig. 1).

3.3.2 GSH

Although GSH significantly discriminated between cultivars, it was a more important variable regarding the discrimination between seasons (Fig. 1.1 and Fig 1.3). Similar to total glutathione, GSH concentrations were lower in 2005 compared to 2007, irrespective of cultivar (Fig. 1.5A, B, C and D and Table 1.3). On average 'Sapphire' had higher GSH concentrations compared to 'Angeleno', irrespective of season, which explains why this variable also significantly discriminated

between the cultivar groupings. Except for the 'Sapphire' 2005 grouping, GSH concentration decreased over the growing season in the other groupings (Fig. 1.5A, B, C and D). Similar to total glutathione, GSH content generally increased over the sampling periods in both cultivars, except for 'Angeleno' 2005 which had low levels throughout the sampling period (data not shown; Refer to Appendix A, Fig. 1).

3.3.3 GSSG

Together with total glutathione, GSSG was selected as one of the most important variables to discriminate between fruit growing seasons, and between the two cultivars (Fig. 1.1 and Fig. 1.3). GSSG concentrations did not follow the same pattern for the two cultivars over the two seasons (Fig. 1.5A, B, C and D). The standard deviations calculated for this parameter show that it varied considerably throughout both seasons for both cultivars (Table 1.3). For 'Sapphire' the average GSSG concentrations in 2005 were almost three times higher compared to the 2007 season. In contrast, average GSSG concentrations were almost 80% lower in 2005 compared to 2007 for 'Angeleno'. These huge differences between the seasons explain why the variable significantly discriminated between the seasons. Similar to total glutathione, GSSG concentrations varied considerably in the 'Angeleno' plums from the middle to the end of the 2007 fruit growing season (Fig. 1.5D). On average, GSSG concentration was higher in the 'Angeleno' fruit compared to the 'Sapphire' fruit, irrespective of season, which explains why it significantly discriminated between the cultivars. GSSG content was low and did not show a marked increase or decrease over the sampling periods, except for 'Angeleno' in the 2007 season which had relatively high GSSG content which varied considerably during the last half of the fruit development period (data not shown, Refer to Appendix A, Fig. 1.1).

3.3.4 Total ascorbic acid

Although total ascorbic acid was an important variable discriminating between seasons, it was a much more important variable regarding the discrimination between cultivars (Fig. 1.1 and Fig. 1.3). Total ascorbic acid concentration was, on average, higher in 2007 compared to 2005, irrespective of cultivar (Fig. 1.6A, B, C and D, and Table 1.3). Regardless of the season, 'Sapphire' plums had lower average total ascorbic acid concentrations compared to 'Angeleno'. Total ascorbic acid concentration exhibited a decreasing trend in the 'Angeleno' fruit in both seasons, with the most pronounced decrease in the 2007 season (Fig. 1.6C and D). Total ascorbic content generally increased in both cultivars and seasons over the sampling period (data not shown; Refer to Appendix A, Fig. 2). Therefore, the decreasing trend of total ascorbic

acid concentration during 'Angeleno' fruit development is probably due to a dilution effect of the growing fruit since ascorbate content increased during fruit development in both seasons.

3.3.5 L-AA

L-AA was one of the nine important variables which significantly discriminated between the two growing seasons (Fig. 1.3). However, it was of less importance discriminating between the two cultivars (Fig. 1.1). L-AA concentration showed similar trends to the total ascorbic acid concentration (Fig. 1.6A, B, C and D). Average concentrations were much higher in the 2007 season compared to the 2005 season (Table 1.3), which explains why this was an important variable discriminating between growing seasons. 'Angeleno' fruit had higher average concentrations, albeit not much higher, compared to the 'Sapphire' fruit in the respective seasons which explains why it was not selected as an important discriminator between the two cultivar groupings. L-AA concentrations exhibited a decreasing trend in the 'Angeleno' fruit as the fruit increased in size in both seasons, with the most pronounced decrease in the 2007 season (Fig. 1.6C and D). L-AA content generally increased during the fruit development period in both cultivars (data not shown, Refer to Appendix A, Fig. 2).

3.3.6 DHA

DHA was an important variable to discriminate between seasons (Fig. 1.3), but of lesser importance regarding the discrimination between cultivars (Fig. 1.1). Average DHA concentration was higher in the 2005 season for both cultivars compared to the 2007 season and followed a decreasing trend over time in both seasons (Table 1.4 and Fig. 1.6A, B, C and D). 'Angeleno' had higher average DHA concentrations compared to 'Sapphire' in both seasons (Table 1.3). DHA content generally stayed constant in 'Sapphire' fruit while it tended to increase over the fruit development period in 'Angeleno' fruit (data not shown, Refer to Appendix A, Fig. 2).

3.3.7 Hue angle

The hue angle of the fruit skin helped to discriminate between seasons (Fig. 1.3) and, as was expected, between cultivars as the two cultivars have different skin colours (Fig. 1.1). 'Sapphire' has a green-yellow ground colour during fruit development, which turns yellow-green with a red blush towards the optimum harvest date. This change in ground colour was clearly seen in both seasons as the hue angle turned from a green-yellow 120 ° at 40 dafb to a yellow-red 53 ° in 2005 (Fig. 1.7A) and a yellow 108 ° ground colour in 2007 (Fig. 1.7B) on the optimum harvest dates

(McGuire, 1992). During fruit development 'Angeleno' has a yellow-green ground colour which turns red with the start of fruit maturation and develops to a dark red or purple ground colour. The ground colour development from a yellow-green 117 ° at 40 dafb to a dark red 353 ° on the optimum harvest date is clearly shown by the 2005 and 2007 results (Fig. 1.7C and D). Fruit harvested in 2005 tended to have a more yellow hue angle compared to a more yellow-green hue angle in 2007 (Table 1.3).

3.3.8 Oleic acid (C18:1n9c) of phospholipids

Oleic acid was an important variable to discriminate between the two cultivars (Fig. 1.1) and of lesser importance in the separation of the seasons (Fig. 1.3). Average values indicate that oleic acid concentration was higher in 'Angeleno' than in 'Sapphire' plums for the respective seasons (Table 1.3 and Fig. 1.7A, B, C and D). This difference between the two cultivars was considerable in the 2007 season (Table 1.3). Average oleic acid concentration did not differ much between the two seasons for 'Sapphire' plums. However, there was a sizeable difference between the two seasons for 'Angeleno' plums. In both seasons there was a steep increase in oleic acid concentration 2 to 3 weeks prior to the optimum harvest date in 'Angeleno' plums followed by a steep decline (Fig. 1.7C and D). In both seasons and for both cultivars there was a general increase in oleic acid content during the fruit development period with a decrease in the last 7 to 14 days prior to harvest (data not shown; Refer to Appendix A, Fig. 3).

3.3.9 Linoleic acid (C18:2n6c) of phospholipids

Linoleic acid was an important variable to discriminate between the two cultivars (Fig. 1.1), but of no significant importance to distinguish between the two seasons (Fig. 1.3). On average 'Sapphire' had a higher concentration of this unsaturated phospholipid fatty acid in both seasons compared to 'Angeleno' (Table 1.3 and Fig. 1.8A, B, C and D). Although this variable did not significantly distinguish between the two seasons, average linoleic acid concentrations were higher in the 2007 season compared to the 2005 season in the respective cultivars (Table 1.3). In both seasons and for both cultivars there was a general increase in linoleic acid content during the fruit development period (data not shown; Refer to Appendix A, Fig. 3). Generally linoleic acid content decreased the last 7 to 14 days prior to harvest, except for 'Sapphire' in the 2005 season, which showed a steady increase up to the optimum harvest date.

3.3.10 Total phenolic concentration

Total phenolic concentration was indicated as an important variable to discriminate between seasons (Fig. 1.3), but of no significant importance to distinguish between the two cultivars (Fig. 1.1). The phenolic concentration decreased over time in both cultivars, irrespective of season (Fig. 1.9A, B, C and D). The average total phenolic concentration was slightly higher in 2005 than in 2007 in both cultivars (Table 1.3). In both seasons average total phenolic concentration was higher in 'Angeleno' compared to 'Sapphire' fruit.

4. Discussion

4.1 Discrimination between cultivars

4.1.1 Antioxidant levels

It is widely accepted that CI is a consequence of oxidative stress in plant tissues (Purvis, 2004). In the respiratory electron transport chain in the mitochondria between 85 to 95% of the O_2 consumed by the plant is reduced to H_2O. An estimated 2 to 4% of the remaining O_2 is reduced to superoxide ($O_2^{\cdot -}$) due to leakage of electrons from the electron transfer system in the mitochondria. $O_2^{\cdot -}$ is rapidly dismutated to H_2O_2. Although the mitochondria are important sites for the production of active oxygen species (AOS), relatively high levels are also produced by the chloroplasts, endoplasmic reticulum (ER), peroxisomes, glyoxisomes, plasma membrane and cell walls. Although an increase in AOS has useful functions in the cell such as serving as signals to activate defence pathways, their levels must be kept under tight control. Over-accumulation of AOS can cause cell death by peroxidation of membrane lipids and proteins and the induction of lesions in DNA. One of the major AOS scavenging pathways of plants is the ascorbate-glutathione or Halliwell-Asada cycle (Hancock and Viola, 2005). This cycle is found in the chloroplasts, peroxisomes, mitochondria and cytoplasm and is very important in the detoxification of H_2O_2 (Potters et al., 2002; Lurie, 2003). It involves the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAHR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) in a succession of cyclic reactions that detoxify H_2O_2 and regenerate ascorbate and GSH through the reducing power of NAD(P)H (Cheng and Ma, 2004; Becana et al., 2010).

Interestingly, this study found that the main variables to discriminate between 'Sapphire' and 'Angeleno' were constructs of the two antioxidants involved in the ascorbate/glutathione cycle, namely total glutathione, total ascorbic acid and the oxidized form of glutathione, GSSG. It was

found that ‘Sapphire’ had, on average, higher concentrations of total glutathione than ‘Angeleno’ in both seasons. Although ‘Angeleno’ had a smaller pool of glutathione than ‘Sapphire’, it had higher concentrations of GSSG, and, consequently a much lower GSH:GSSG ratio than ‘Sapphire’. On the other hand, ‘Angeleno’ had much higher levels of total ascorbic acid than ‘Sapphire’ in both seasons.

In the case of ‘Sapphire’ a large pool of total glutathione is significant since one of the most important functions of glutathione in plants is defence against oxidative stress which is mediated by the sulfhydryl group of cysteine (Hausladen and Alscher, 1993; Noctor and Foyer, 1998; Xiang et al., 2001; Potters et al., 2002). It not only scavenges for AOS such as $^1O_2$, $OH^{\bullet}$, and H_2O_2, but also reduces DHA back to L-AA (the active form of ascorbic acid) in the ascorbate/glutathione cycle (Hausladen and Alscher, 1993; Gill and Tuteja, 2010). Hence, it not only protects the cell membranes by maintaining α-tocopherol in its reduced state, but also disulphide bonds in proteins under stress conditions (McKersie and Leshem, 1994; Potters et al., 2002). Generally it is found that glutathione levels increase in response to an increase in AOS and are higher in plants adapted to stress (e.g. in spruce during winter and in chilling-tolerant maize genotypes compared to sensitive genotypes during cool spring periods in the field, during heat stress in wheat and maize, during water deficit in sunflower seedlings and salt treatment of groundnut cell lines) (Noctor and Foyer, 1998; Potters et al., 2002; Szalai et al., 2009). A number of studies on various plant species subjected to different abiotic stresses indicated that a high GSH:GSSG ratio, maintained by increased GSH synthesis and/or GSSG reduction, is necessary for efficient protection of plants against the accumulation of free radicals (Szalai et al., 2009). Under optimal growth conditions the GSH:GSSG ratio is high which results in an overall reducing environment in the cells. According to the glutathione data accumulated in this study, it seems that ‘Sapphire’ is well protected against AOS, not only during fruit development, but also on the optimum harvest date.

Schupp and Rennenberg (1988) and Foyer (1993) found that the diurnal rhythm in glutathione concentration in spruce needles was dependent on light levels, and independent of daily fluctuations in temperature. Other studies also found that glutathione production is light dependent (Hausladen and Alscher, 1993). However, we found that ‘Angeleno’ experienced the same average number of sunshine hours daily as ‘Sapphire’ (Table 1.4) throughout the trial and could, therefore, not explain the difference in the GSH/GSSG ratio and GSSG levels between the two cultivars. Since ‘Angeleno’ is a late season cultivar spending approximately 53 days longer on the tree than ‘Sapphire’, the last two months of ‘Angeleno’ fruit development correspond with the warmest two months of the plum season in South Africa, namely January and February. Indeed, ‘Angeleno’ experienced higher average maximum and daily temperatures in both seasons

compared to 'Sapphire' plums in this study (Table 1.4). Studies on white pine and spruce needles found that glutathione concentration was low during summer, but high during the winter and early spring (Esterbauer and Grill, 1978; Schupp and Rennenberg, 1988; Anderson et al., 1992). This seasonal variation in glutathione concentration was found to be dependent on temperature since high GSH levels prevent transformation of –SH groups of inter- or intramolecular protein S-S bonds during freezing temperatures. Therefore, from these results it seems that the higher total glutathione and GSH levels in 'Sapphire' compared to 'Angeleno' are related to the cooler temperatures during fruit development of 'Sapphire' compared to the higher temperatures during 'Angeleno' fruit development – especially during the last part of the latter cultivar's fruit development period.

The lower GSH/GSSG and higher GSSG levels in 'Angeleno' do not necessarily mean that this cultivar is less adapted to stress conditions since a decreased GSH:GSSG ratio also protects the cell by activating various defence mechanisms, including several oxidants, antioxidants and stress hormones (e.g. salicylic acid and abscisic acid), through a redox signaling pathway under unfavourable stress conditions (Szalai et al., 2009; Gill and Tuteja, 2010). Noctor et al. (2002) also state that the GSH:GSSG ratio is more influential in the control of gene expression and protein function than the size of the glutathione pool. Likewise, plant cells are able to maintain high total ascorbic acid:DHA ratios concurrent with low GSH:GSSG ratios in the cytoplasm due to efficient GSH-independent pathways to regenerate ascorbic acid and/or the difference in redox potential between the GSH/GSSG and total ascorbic acid/DHA couples (Foyer and Noctor, 2011).

Although 'Angeleno' had a smaller glutathione pool, it had a substantially larger ascorbic acid pool than 'Sapphire' plums. Ascorbic acid is considered to be the main water soluble antioxidant as well as an important redox buffer and enzyme cofactor in plants (Smirnoff, 2000; Hancock and Viola, 2005; Ishikawa et al., 2006). Important external factors that affect the ascorbic acid pool size in plant tissues are light intensity and duration (Smirnoff, 2000; Hancock and Viola, 2005; Foyer and Noctor, 2011). Ascorbic acid levels increase at high light intensities because it needs to detoxify H_2O_2, regenerate oxidized α-tocopherol and be a cofactor for violaxanthin de-epoxidase (Smirnoff, 2000; Pérez et al., 2002; Hancock and Viola, 2005). Also, the synthesis of $_L$-Galactose, an important precursor of ascorbate, increases under high light intensities (Ishikawa et al., 2006). In our study it was found that 'Angeleno' experienced a similar average number of sunshine hours daily as 'Sapphire' in both seasons (Table 1.4). Differences in light intensities, is therefore ruled out as a reason for the difference in ascorbic acid levels between the two cultivars. However, temperature, water, mineral and nitrogen availability, atmospheric composition, pathogens, seed germination, tuber induction, fruit setting, fruit ripening, and senescence also effect substantial

changes in ascorbic acid levels in plant tissues (Smirnoff, 2000; Hancock and Viola, 2005; Ishikawa and Shigeoka, 2008). In greenhouse grown tomatoes it was found that fruit that were grown under higher temperature conditions had higher ascorbic acid levels (Venter, 1977). In this study 'Angeleno' fruit did experience higher average day temperatures than 'Sapphire' which could be the reason why the 'Angeleno' fruit accumulated higher ascorbic acid levels than 'Sapphire' fruit.

4.1.2 Cell membrane composition

Two other important variables to discriminate between the two cultivars were the phospholipid fatty acids oleic acid (C18:1n9c) and linoleic acid (C18:2n6c). In the respective seasons oleic acid levels (the precursor of unsaturated fatty acids) were higher in 'Angeleno' while linoleic acid levels, an essential fatty acid for humans (Stumpf, 1981; Ayaz et al., 2002), were higher in 'Sapphire' plums. Generally in all fruit species, the predominant and most abundant fatty acids during fruit development, maturation and ripening are C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid) (Ayaz et al., 2002). However, the levels and ratios of these fatty acids during the different fruit development stages are dependent on the fruit type. Similar to medlar fruit (*Mespilus germanica* L.) and olives (Ayaz et al., 2002; Beltrán et al., 2004), the content of oleic and linoleic acid increased in 'Sapphire' and 'Angeleno' plums from 40 dafb until the optimum harvest date when calculated as content per fruit. This increase is due to intense metabolic activity that needs lipids for cell expansion (Ayaz et al., 2002).

In virgin olive oils it was found that the oleic:linoleic ratios are responsible for the oxidative stability of the oil (Beltrán et al., 2004). The reason for this is that monounsaturated fatty acids (MUFAs), such as oleic acid, are more resistant to oxidation than polyunsaturated fatty acids (PUFAs), e.g. linoleic acid. Based on peroxide formation it has consequently been determined that the oxidation ratio for oleate, linoleate and linolenate is 1:12:25 (Aparicio, et al., 1999). Therefore, it has been found that olive oil is more stable when oleic acid content is high and linoleic content is low. It has also been found that pea, which is chilling resistant, contains higher levels of saturated fatty acids and has a lower fatty acid double bond index than tomato and maize, which are chilling sensitive (Bishop et al., 1979). In this study it was found that the average ratio of oleic acid:linoleic acid of phospholipids was two times higher in 'Angeleno' compared to 'Sapphire'.

Abiotic (e.g. temperature) or biotic (e.g. pathogen attack) stresses may cause lipid peroxidation which includes a complex group of enzymatic and non-enzymatic reactions that occur

simultaneously and competitively (Stanley, 1991). As mentioned earlier, PUFAs are oxygen sensitive molecules and are conjugated to phospho- and galactolipids (Spiteller, 2003). In the enzymatic reactions, membrane-bound phospholipases, which cleave the conjugates, are activated and the released, free PUFA's are transformed to lipidhydroperoxides (LOOHs) by lipoxygenases (LOX) (Buege and Aust, 1978; Spiteller, 2003; Wrolstad, et al., 2005). Linoleic acid is the best known substrate for LOX (Gurr and James, 1975). LOOHs are odourless, colourless short-lived species which break down to a number of secondary products such as alkanals, alkenals, hydroxyalkenals, ketones, alkanes, etc., which represent biological signals and do not require a preceding activation of genes (Spiteller, 2003; Wrolstad, et al., 2005). However, if the free PUFAs start to exceed a certain threshold, LOX "commit suicide" and the iron ions in its active centre are released (Spiteller, 2003). The iron ions react with the surrounding LOOHs to generate alkoxy radicals (LO**.**) which, in turn induce non-enzymatic lipid-peroxidation. It is interesting to mention that a portion of the LO**.** radicals produced from linoleic acid is converted to 2,4 dienals which induce programmed cell death and the hypersensitive reaction. In the non-enzymatic reactions AOS react with the unsaturated fatty acids (Stanley, 1991). Any introduction of stress results in the elevation of the AOS levels which abstract hydrogen atoms from methylene carbons in unsaturated fatty acids that lead to membrane degradation. The lipid peroxidation radicals may involve new PUFA molecules in the process which initiates the propagation phase of lipid peroxidation. For each stage of these enzymatic and non-enzymatic reactions the plant cell has a defence mechanism in the form of reducing agents such as glutathione and ascorbic acid to eliminate AOS and the toxic products of lipid peroxidation (Aust, 1986; Spiteller, 2003). However, under severe stress conditions the reducing agents may be overwhelmed by the amount of free radicals, and lipid peroxidation will cause uncoupling of oxidative phosphorylation in the mitochondria, alteration of the function of the ER, increase membrane permeability, alter the fluidity of the membrane, inactivate membrane-bound enzymes and cause polymerization, cross-linking and covalent binding of proteins which may lead to cell death (Stanley, 1991; Blokhina, et al., 1999). In this study it was seen that 'Sapphire' has high levels of glutathione during all the stages of fruit development – even higher than 'Angeleno' plums. It is, therefore, suggested that 'Sapphire' is properly protected against lipid peroxidation while developing on the tree, but that the relatively high levels of linoleic acid, a PUFA which is easily oxidised, may cause this cultivar to be chilling-sensitive due to the biochemical reactions described above during long-term cold-storage. The relatively high levels of oleic acid, a MUFA, which is not easily oxidised, the high oleic:linoleic acid ratio as well as the high ascorbic acid levels in 'Angeleno' compared to 'Sapphire', may very well be the factors that renders 'Angeleno' plums chilling resistant during long-term cold-storage. It will be interesting to compare oleic:linoleic acid levels in cultivars developing in the early part of the plum season to cultivars harvested in the later part of the season in South Africa as well as their susceptibility to CI.

During the season cell membranes, as the main targets of temperature adaptation, make compositional changes (Vigh and Maresca, 2002; Vigh et al., 2005). This causes the membrane to have similar fluidity at any given growth temperature (Vigh and Maresca, 2002). It enables poikilothermic organisms to optimise the barrier functions and the permeability properties of the membranes, and the activity of the membrane bound enzymes. For example, *E. coli* exhibits a lower ratio of unsaturated: saturated fatty acid under higher growth temperature. In this study, 'Sapphire' experienced lower average day temperatures than 'Angeleno' in both seasons, and it was found that lower growth temperatures increase lipid unsaturation in order to maintain membrane fluidity at low temperatures (Beltrán et al., 2004; Upchurch, 2008). The fact that 'Sapphire' fruit development takes place under lower average day temperatures could be the reason why this cultivar accumulates higher levels of linoleic acid than oleic acid compared to 'Angeleno'.

4.2 Discrimination between seasons

As was expected, there were not only differences between the two plum cultivars, but also between the two seasons within a cultivar. The DA showed that the variation in the data between the two seasons could mainly be explained by total glutathione and GSSG concentrations and to a lesser extent by the concentration of GSH, DHA, L-AA, total ascorbic acid, the hue angle and total phenolic concentration.

4.2.1 Glutathione

An increase in GSH levels was shown to be dependent on light (Hausladen and Alscher, 1993). In this study it was found that glutathione levels were lower in 2005 than in 2007, irrespective of cultivar. On average there were more sunshine hours in the 2005 season during the fruit development periods of 'Sapphire' and 'Angeleno' compared to the 2007 season (Table 1.4). This finding, therefore, does not correlate well with the finding that an increase in GSH levels is light dependent since more sunshine hours were available in the season with the lowest glutathione levels. Indeed, it is found that although higher irradiance may increase glutathione levels, ascorbate is much more responsive to high light levels than glutathione (Foyer and Noctor, 2011).

The two-step synthesis of GSH is catalysed by γ-glutamylcysteine synthetase (GCS) and glutathione synthetase with GCS being the rate-limiting enzyme (Noctor and Foyer, 1998; Myhrstad et al., 2002). In a study conducted on cell cultures Myhrstad et al. (2002) found that flavonoids, which are phenolic compounds, are important regulators of glutathione levels through

GCS gene regulation. They observed a significant increase in GSH concentration when the cell cultures were incubated with extract of yellow onion (known for its high concentrations of flavonoids) or pure flavonoids. Interestingly, in our study it was found that total phenolic concentration, an important variable to discriminate between the two seasons, and glutathione levels were higher in the 2005 than the 2007 season in both cultivars. However, whether the flavonoids included in the total phenolic concentration of the two plum cultivars really had a role in the regulation of their glutathione levels is uncertain, since 'Angeleno' fruit in both seasons had higher total phenolic concentration, but lower glutathione levels than 'Sapphire' fruit.

It was found that the availability of cysteine, one of the amino acid substrates of GCS, may be a key limitation in glutathione synthesis, particularly in the light (Noctor et al., 2002). When leaf discs of poplars were incubated with cysteine, glutathione concentration increased substantially. Indeed, it is speculated that GCS activity as well as cysteine availability are the most important factors that control plant glutathione levels. Since GCS activity and cysteine availability were not determined in this study, it is uncertain if it was responsible for the distinct difference in glutathione levels between the two seasons. As was mentioned earlier, studies on white pine and spruce needles found that glutathione concentration was low during summer, but high during the winter and early spring (Esterbauer and Grill, 1978; Schupp and Rennenberg, 1988; Anderson et al., 1992). This seasonal variation in glutathione concentration was found to be dependent on temperature since high GSH levels prevents transformation of –SH groups of inter- or intramolecular protein S-S bonds during freezing conditions. During the 2007 season, when glutathione levels were higher, lower average minimum daily temperatures were measured. Therefore, from these results it seems that the higher total glutathione and GSH levels in 2007 compared to 2005 are related to the cooler average daily temperatures measured in the 2007 season.

4.2.2 Ascorbic acid

It is well known that ascorbate levels vary between seasons in plant tissues (Foyer, 1993). Important external factors that affect AsA pool size of plant tissues are light intensity and duration (it influences substrate availability) (Gillham and Dodge, 1987; Smirnoff, 2000; Hancock and Viola, 2005; Foyer and Noctor, 2011). AsA levels increase at high light intensities because it needs to detoxify H_2O_2, regenerate oxidized α-tocopherol and be a cofactor for violaxanthin de-epoxidase (Smirnoff, 2000; Pérez et al., 2002; Hancock and Viola, 2005). It was found that ascorbic acid accumulated in tomatoes under high light exposure – tomatoes grown in greenhouses usually have lower ascorbic acid levels than field-grown tomatoes due to the lower light intensity in the greenhouses (Raffo et al., 2006). In this study it was found that both total ascorbic acid and L-AA

levels were higher in the 2007 season in both plum cultivars when fewer sunshine hours were experienced compared to the 2005 season. Unfortunately, light intensity data are not available for this study, which could possibly explain the difference in ascorbic acid levels between the two seasons. Annual fluctuations in temperature, water status, mineral and nitrogen availability, atmospheric composition, and pathogens also influence AsA levels (Gillham and Dodge, 1987; Smirnoff, 2000; Hancock and Viola, 2005). In our study it was found that DHA (the oxidised form of ascorbic acid) levels were the highest in 2005. This season had higher average minimum daily temperatures as well as longer sunshine hours compared to the 2007 season for both cultivars. The exceptionally high DHA levels measured in cherry tomatoes grown in a green house in Italy was also ascribed to the area's relatively high temperatures (Raffo et al., 2006). Therefore, the higher levels of total ascorbic acid and L-AA in the 2007 season could be ascribed to the season's lower average night temperatures and fewer sunshine hours compared to the 2005 season.

4.2.3 Hue angle

It is well known that various factors have an effect on the hue angle of fruit and may cause seasonal variation in the skin colour. Main factors that have an effect on skin colour are fruit light exposure, temperature, excessive nitrogen, and leaf number per fruit (Crisosto et al., 1999; Tromp and Wertheim, 2005). In the 2007 season fruit of both cultivars had a yellow-green hue angle compared to a more yellow hue angle in the 2005 season. Fruit of both cultivars experienced slightly higher average maximum daily temperatures and fewer sunshine hours in the 2007 season compared to the 2005 season. Carotenoids are the pigments responsible for the yellow colour in many fruits (Salisbury and Ross, 1985). They not only act as accessory pigments during light harvesting, but also protect the chlorophyll membranes from oxidative stress under high light conditions (McKersie and Leshem, 1994; Lurie, 2003). It is, therefore, suggested that the fruit in this trial accumulated more carotenoids, and hence a more yellow hue angle in the 2005 season to protect the chloroplast membranes against oxidative stress compared to the 2007 season when there were fewer sunshine hours.

4.2.4 Total phenolic concentration

Phenolic compounds are known to have antioxidant properties since they chelate metal ions, inhibit lipid oxidation and radical-forming enzymes, and quench free radicals (Robards et al., 1999; Kim et al., 2004). In this study it was shown that total phenolic concentration in the two plum cultivars, together with other variables, significantly discriminated between the two seasons. Total phenolic concentration was higher in the 2005 than the 2007 season in both cultivars. It is known that an increase in solar radiation, especially UV radiation, increases phenolic levels in

plants (Raffo et al., 2006). Light increases the activity of phenylalanine ammonia-lyase, the enzyme that converts phenylalanine into coumaric acid (the precursor of phenolic components in plants) (Toor et al., 2006). Toor et al. (2006) found that the mean total phenolic concentration of greenhouse-grown tomatoes was much higher in summer than spring and speculated that increased temperature may also enhance the accumulation of phenolics. In this study both cultivars had more sunshine hours in the 2005 season compared to the 2007 season which may explain the difference in phenolic concentration between the two seasons. Although it was not one of the variables to significantly discriminate between the two cultivars, it is interesting to note that 'Angeleno', the CI resistant cultivar, had a higher average total phenolic concentration than 'Sapphire' plums in both seasons. Kim et al. (2004) found in their study that dark blue and purple skin plum cultivars generally had higher total phenolic concentration than yellow and red skin plum cultivars. Tomás-Barberán et al. (2001) also found that 'Angeleno' as well as 'Black Beaut' plums had exceptional amounts of phenolics.

5. Conclusion

From the results it can be concluded that the much higher linoleic concentrations resulting in a much lower MUFA:PUFA ratio in 'Sapphire', compared to 'Angeleno', is probably the main reason why 'Sapphire' is prone to develop CI under long term cold-storage conditions. There are ample results from literature to demonstrate that linoleic acid, a polyunsaturated fatty acid, is more prone to lipid peroxidation under abiotic stresses than the monounsaturated fatty acids. Likewise, that membranes with a high MUFA:PUFA ratio are less prone to oxidative damage under stress conditions. The higher oleic/linoleic acid ratio, higher total and reduced ascorbic acid concentrations and total phenolic concentration in all probability render 'Angeleno' plums much more CI resistant under long term cold-storage conditions compared to 'Sapphire' plums. The results of this study confirm the observation of Abdi et al. (1997) that fruit with a long fruit development period are less susceptible to CI compared to fruit with a short fruit development period. In our study it was found that the longer fruit development period causes 'Angeleno' to experience higher average day temperatures than 'Sapphire' which, in turn, causes higher levels of MUFA instead of PUFA to accumulate in the 'Angeleno' fruit. To confirm this result, it is recommended that early, mid and late season plum cultivars be tested for their MUFA:PUFA ratios as well as their susceptibility to CI.

'Sapphire' contains higher concentrations of total and reduced glutathione, an important antioxidant in plant tissues, during fruit development than 'Angeleno' plums. The reason why 'Sapphire' accumulates glutathione to higher levels than ascorbic acid seems to be related to the fact that it is

an early season cultivar experiencing lower average daily temperatures. If measures can be found, e.g. by harvesting the fruit at an optimum maturity, treating the fruit with 1-methylcyclopropene and/or by preconditioning the fruit at specific temperatures to increase the MUFA:PUFA ratio and/or to maintain glutathione levels during postharvest handling and cold-storage in 'Sapphire' plums, the effect of CI may be reduced to a great extent in this cultivar.

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Table 1.1

Classification matrix of the best subset of the 20 best models indicating the percentage correctly predicted samples (A) on a sub-sample representing 70% of the data to build the model, and (B) on a sub-sample representing 30% of the data to validate the robustness of the model built in (A). Rows contain observed classifications and columns predicted classifications.

(A)

Class	Percent correct	Sapphire	Angeleno
Sapphire	100.00	46	00
Angeleno	100.00	0	90
Total	100.00	46	90

(B)

Class	Percent correct	Sapphire	Angeleno
Sapphire	100.00	20	00
Angeleno	100.00	0	39
Total	100.00	20	39

Table 1.2

Classification matrix of the best subset of the 20 best models indicating the percentage correctly predicted samples (A) on a sub-sample representing 70% of the data to build the model, and (B) on a sub-sample representing approximately 30% of the data to validate the robustness of the model built in (A). Rows contain observed classifications and columns predicted classifications.

(A)

Class	Percent correct	Sapphire ‘05	Sapphire ‘07	Angeleno ‘05	Angeleno ‘07
Sapphire ‘05	100.00	25	0	0	0
Sapphire ‘07	90.48	2	19	0	0
Angeleno ‘05	100.00	0	0	40	0
Angeleno ‘07	90.00	5	0	0	45
Total	94.85	32	19	40	45

(B)

Class	Percent correct	Sapphire ‘05	Sapphire ‘07	Angeleno ‘05	Angeleno ‘07
Sapphire ‘05	100.00	11	0	0	0
Sapphire ‘07	100.00	0	9	0	0
Angeleno ‘05	94.18	0	0	16	1
Angeleno ‘07	95.45	1	0	0	21
Total	96.61	12	9	16	22

Table 1.3

Average levels of variables that significantly discriminated between the 2005 and 2007 seasons and between 'Sapphire' and 'Angeleno' plums sampled from the Wellington area. Averages were calculated on the values obtained from 40 dafb until the optimum harvest date. S.D. is presented in brackets.

Variable	'Sapphire' 2005	'Sapphire' 2007	'Angeleno' 2005	'Angeleno' 2007
Total glutathione (µg g^{-1} FW)	16.32 (1.19)	24.16 (3.74)	7.20 (2.78)	15.86 (4.90)
GSH (µg g^{-1} FW)	15.58 (1.20)	24.74 (3.88)	6.35 (0.75)	11.86 (0.51)
GSSG (µg g^{-1} FW)	0.74 (0.36)	0.25 (0.26)	0.84 (0.86)	4.18 (0.55)
GSH:GSSG ratio	26.58 (11.86)	126.67 (173.08)	11.68 (7.21)	10.57 (16.65)
Total ascorbic acid (µg g^{-1} FW)	49.54 (4.60)	56.92 (10.55)	67.56 (14.24)	70.14 (31.86)
L-AA (µg g^{-1} FW)	39.93 (4.32)	51.35 (9.60)	42.36 (9.36)	60.01 (29.62)
DHA (µg g^{-1} FW)	9.60 (4.58)	5.59 (3.26)	25.08 (10.58)	10.12 (1.83)
Hue angle	111.96 (18.76)	120.03 (10.79)	88.00 (76.74)	150.98 (136.58)
Oleic acid (µg g^{-1} FW) of phospholipids	3.24 (0.82)	3.69 (0.76)	3.66 (0.39)	5.38 (0.67)
Linoleic acid (µg g^{-1} FW) of phospholipids	18.59 (5.44)	21.34 (5.18)	10.44 (3.30)	15.40 (5.68)
Total phenolics (mg gallic acid equivalents g^{-1} FW)	3.31 (1.64)	2.82 (1.58)	4.69 (1.78)	3.70 (1.85)

Table 1.4

Average maximum, minimum and daily temperatures and relative humidity measured hourly in the 'Sapphire' and 'Angeleno' orchard in the 2005 and 2007 seasons. Average sunshine hours were obtained from Landau, a weather station close to the experimental orchard. Averages were calculated on the values obtained from 40 dafb until the optimum harvest date. S.D. is presented in brackets.

Variable	'Sapphire' 2005	'Sapphire' 2007	'Angeleno' 2005	'Angeleno' 2007
Maximum temperature (°C)	29.18 (3.14)	29.73 (5.23)	30.90 (3.82)	31.59 (5.18)
Minimum temperature (°C)	13.14 (2.62)	11.82 (2.57)	14.91 (2.85)	13.36 (2.82)
Average daily temperature (°C)	20.80 (2.30)	20.77 (3.09)	22.74 (2.96)	22.48 (3.48)
Maximum relative humidity (%)	91.76 (7.85)	94.82 (4.12)	89.01 (9.22)	94.45 (4.74)
Minimum relative humidity (%)	41.93 (9.76)	35.10 (13.37)	40.31 (10.67)	32.35 (10.78)
Average daily relative humidity (%)	65.11 (6.72)	64.96 (7.29)	62.05 (9.16)	63.40 (6.33)
Average sunshine hours	7.04 (4.08)	6.06 (4.37)	6.55 (4.58)	6.04 (4.76)

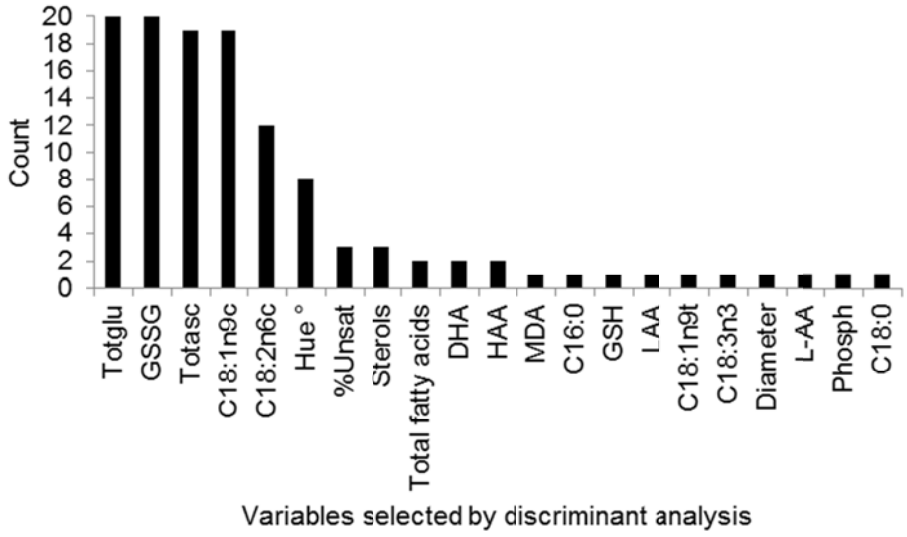


Fig. 1.1. Bar chart presenting the variables that occurred most frequently in the 20 best DA models for the discrimination between the 'Sapphire and 'Angeleno' groups. The y-axis indicates the count or number of times the individual variables occurred in the 20 best DA models. Compound abbreviations were as follows: Totglu = total glutathione, GSSG = oxidised glutathione, Totasc = total ascorbic acid, C18:1n9c = oleic acid, C18:2n6c = linoleic acid, %Unsat = percentage unsaturated phospholipid fatty acids, Sterols = total sterols, Total = total phospholipid fatty acids, DHA = oxidised ascorbic acid, HAA = water soluble radical-scavenging activity, MDA = malondialdehyde equivalents, C16:0 = palmitic acid, GSH = reduced glutathione, LAA = lipid soluble radical-scavenging activity, C18:1n9t = elaidic acid, C18:3n3 = linolenic acid, L-AA = reduced ascorbic acid, Phosp = total phospholipids, C18:0 = stearic acid.

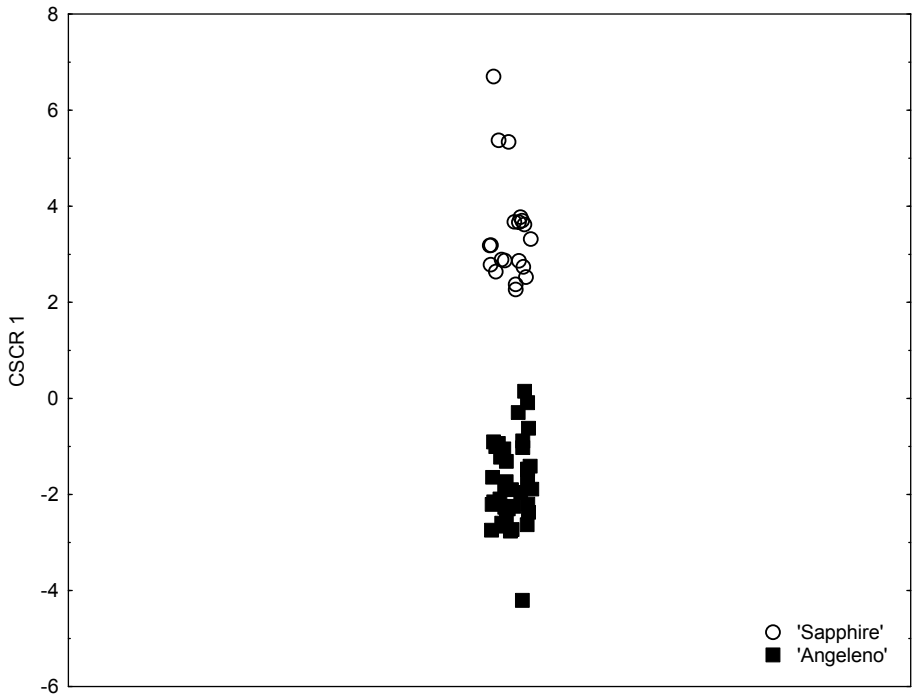


Fig. 1.2. Observations chart for the best subset of the 20 best models for the ‘Sapphire’ and ‘Angeleno’ groupings using the six variables that occurred most frequently in the best 20 DA models. Data of the two seasons were pooled for each cultivar.

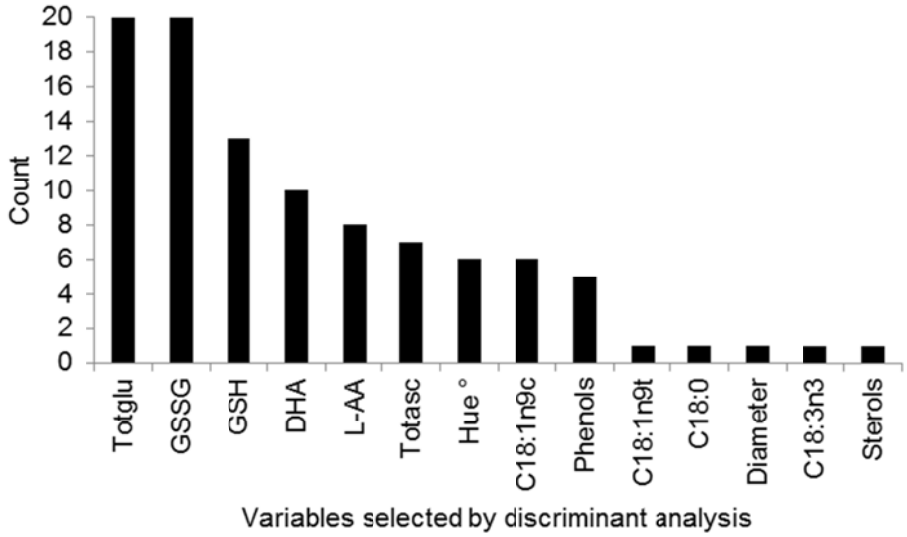


Fig. 1.3. Bar chart presenting the variables that occurred most frequently in the 20 best DA models for the discrimination between the 'Sapphire' 2005, 'Sapphire' 2007, 'Angeleno' 2005 and 'Angeleno' 2007 groupings. The y-axis indicates the count or number of times the individual variables occurred in the 20 best models. Compound abbreviations were as follows: Totglu = total glutathione, GSSG = oxidised glutathione, GSH = reduced glutathione, DHA = oxidised ascorbic acid, L-AA = reduced ascorbic acid, Totasc = total ascorbic acid, C18:1n9c = oleic acid, Phenols = total phenolic concentration, C18:1n9t = elaidic acid, C18:0 = stearic acid, Diameter = fruit diameter, C18:3n3 = linolenic acid, Sterols = total sterols,

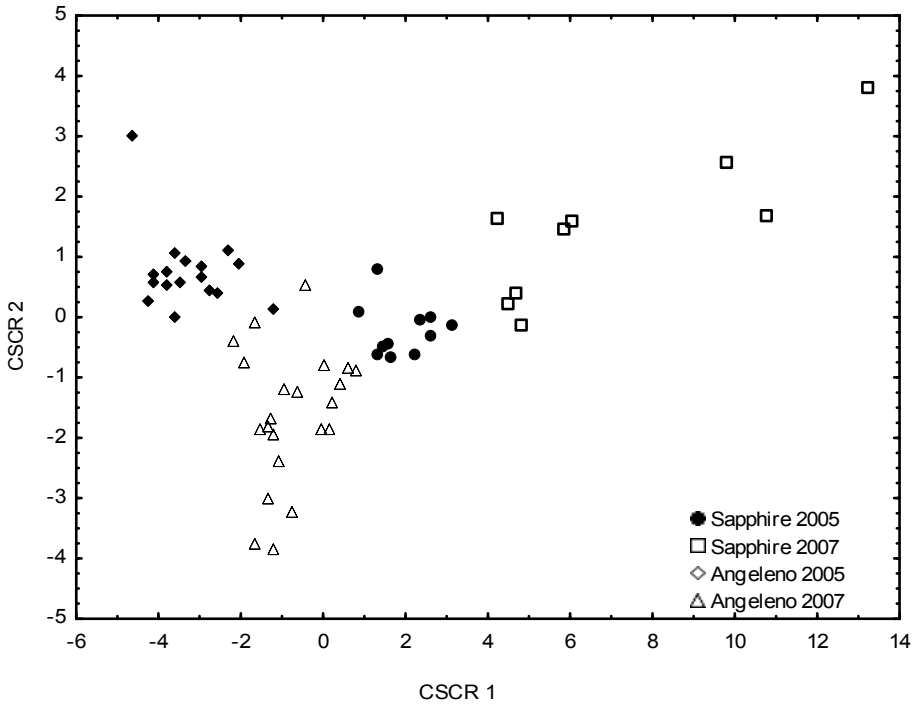


Fig. 1.4. Observations chart for the best subset of the 20 best models for the 'Sapphire' and 'Angeleno' plums sampled in the 2005 and 2007 seasons using the nine variables that occurred most frequently in the best 20 DA models.

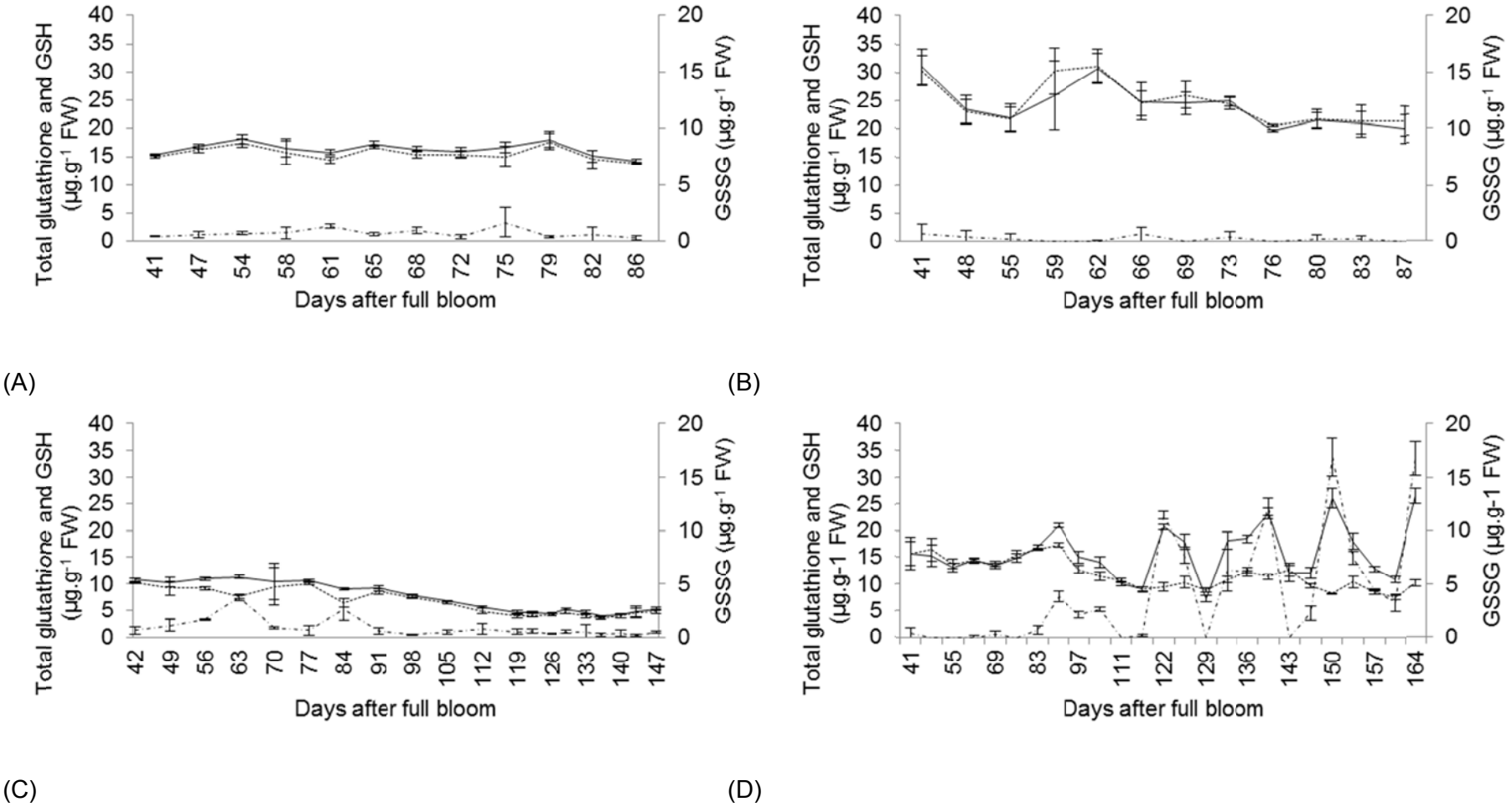


Fig. 1.5. Total glutathione (———), GSH (- - - - -) and GSSG (— · — · —) concentration determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.

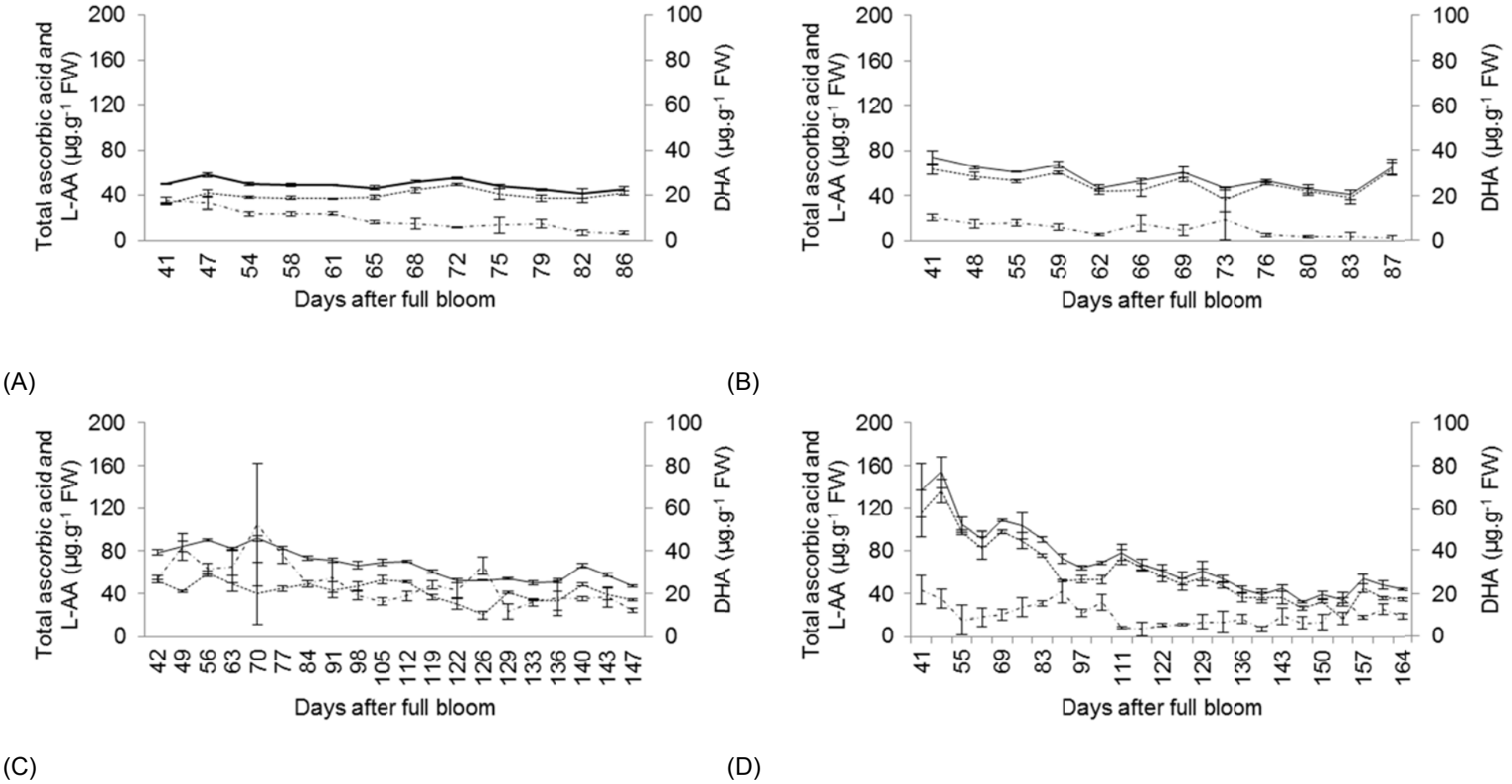
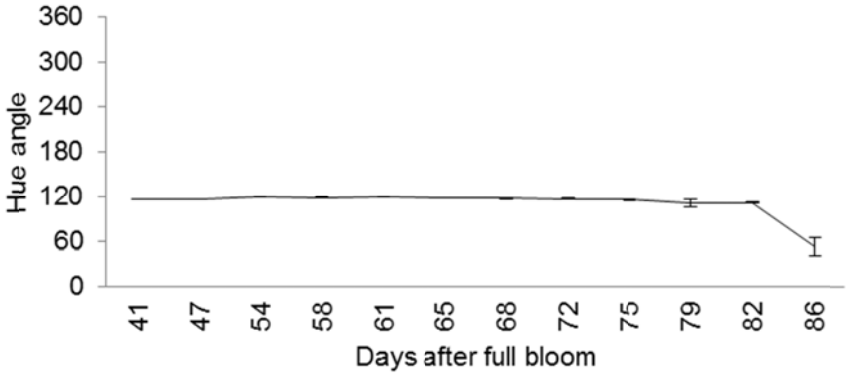
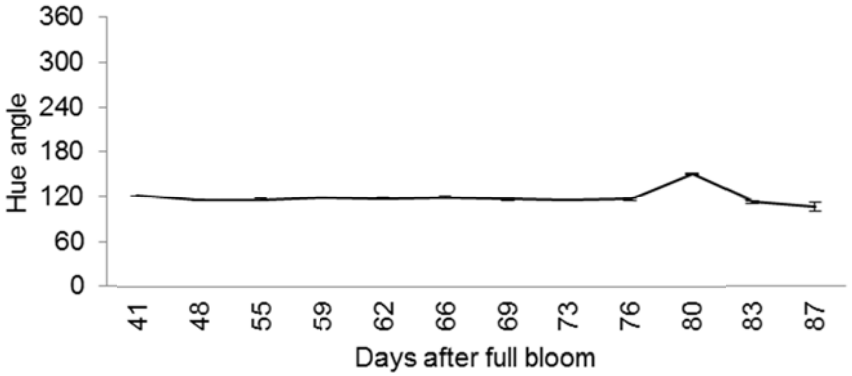


Fig. 1.6. Total ascorbic acid (———), L-AA (- - - - -) and DHA (— · — · —) concentration determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.



(A)



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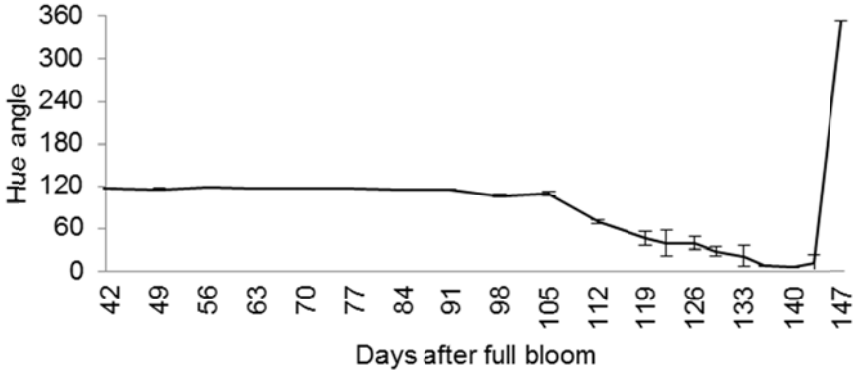

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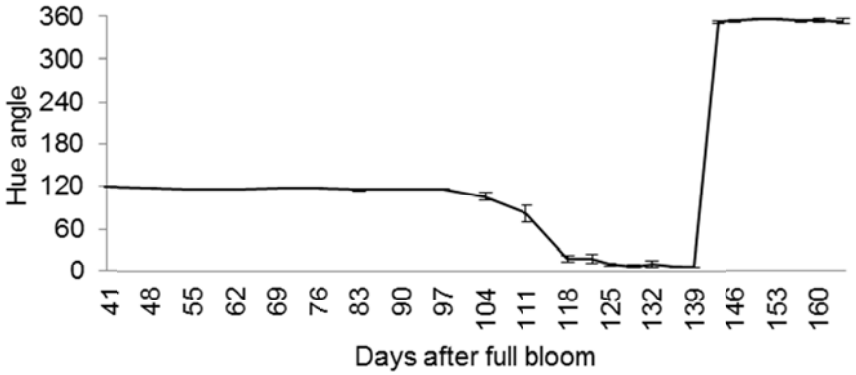


(D)

Fig. 1.7. Hue angle determined on the sampling dates for (A) ‘Sapphire’ in the 2005 season, (B) ‘Sapphire’ in the 2007 season, (C) ‘Angeleno’ in the 2005 season and (D) ‘Angeleno’ in the 2007 season. Vertical bars represent S.D.

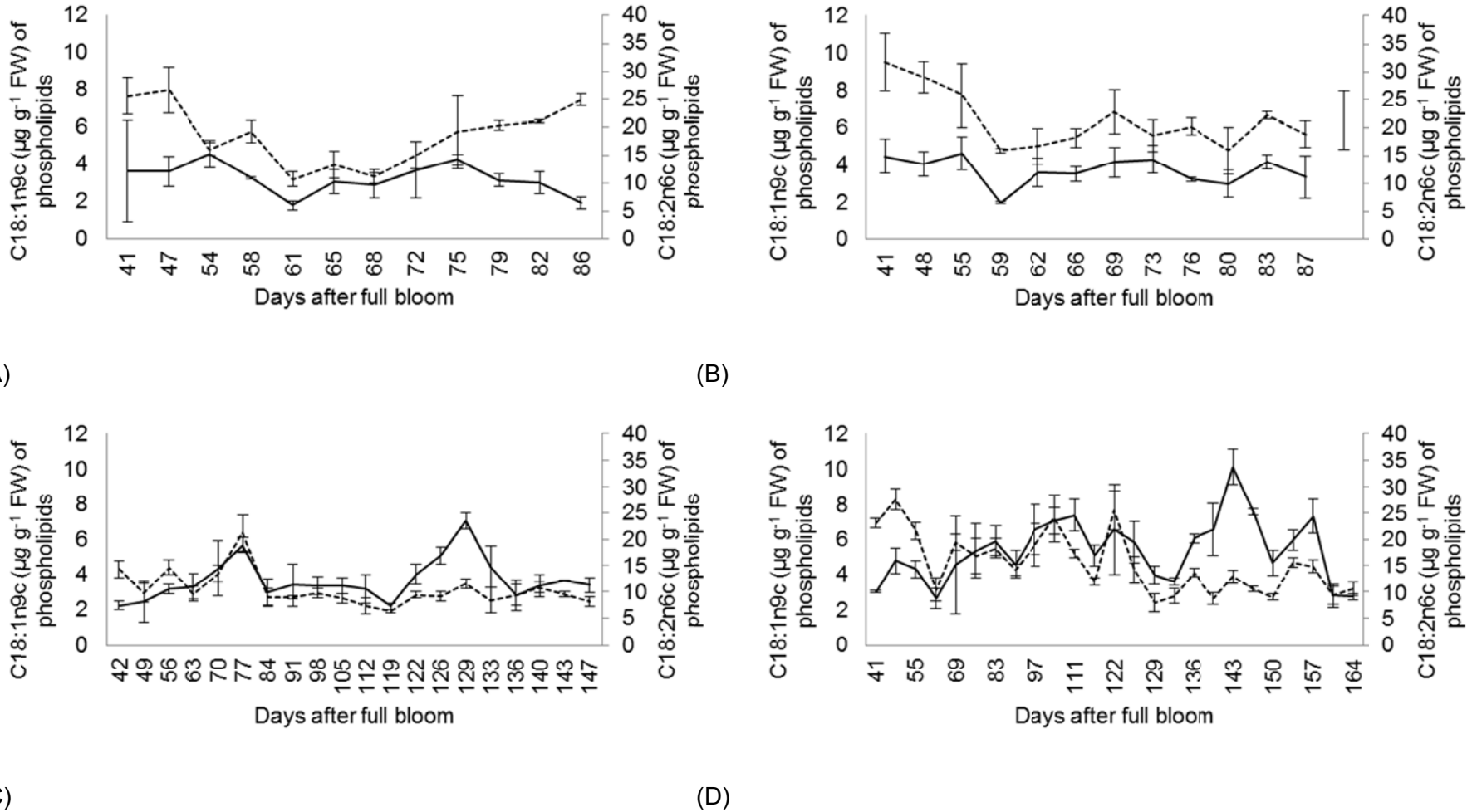
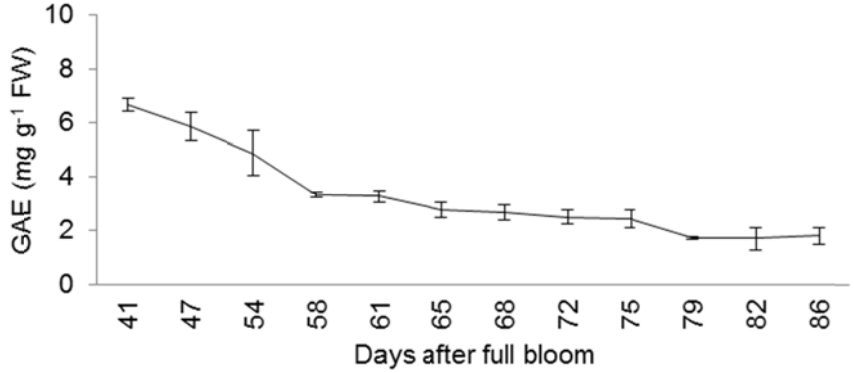
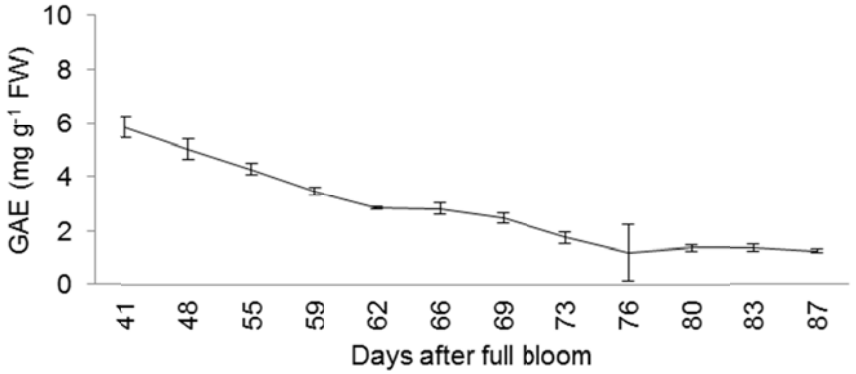


Fig. 1.8. Concentrating of phospholipid oleic acid (C18:1n9c) (———) and linoleic acid (C18:2n6c) (- - - - -) determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.



(A)



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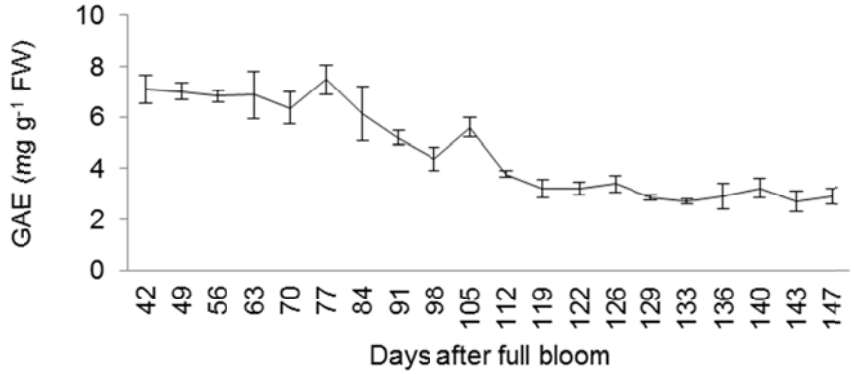
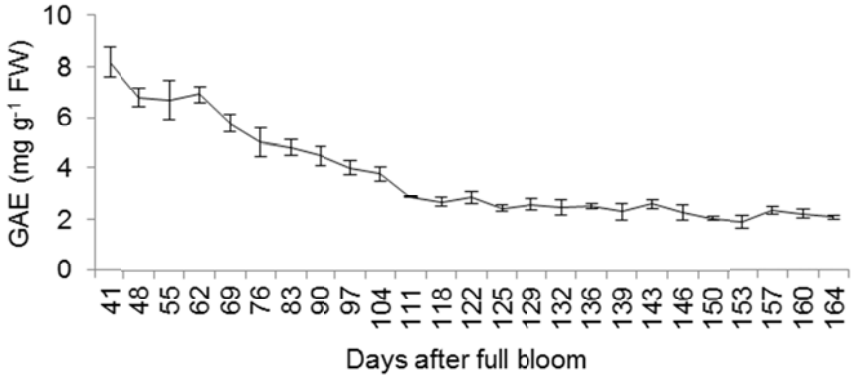


(C)



(D)

Fig. 1.9. Total phenolic content, determined as Gallic acid equivalents (GAE), determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.

PAPER 2

Effect of harvest maturity on chilling injury incidence, membrane composition and antioxidant levels in a chilling susceptible plum cultivar (*Prunus salicina* Lindl.)

Abstract

A number of plum (Prunus salicina Lindl.) cultivars exported from South Africa are chilling sensitive. Therefore, the aim of this study was to investigate the changes in cell membrane composition and antioxidant levels in the fruit of a chilling susceptible plum cultivar during storage. Fruit were harvested at two maturities, cold-stored at -0.5 °C (to induce chilling injury), and examined at weekly intervals for six consecutive weeks after storage and after a simulated shelf-life period at 10 °C. It was found that chilling injury incidence was higher in more mature fruit, that it increased with an increase in storage duration and was higher after a simulated shelf-life period at 10 °C than after the preceding storage duration at -0.5 °C. More mature fruit also had higher levels of shrivel and ethylene evolution rates than less mature fruit. Due to higher levels of saturated fatty acids, the membranes of mature fruit were probably less fluid, and due to its lower monounsaturated:polyunsaturated phospholipid fatty acid ratio, its membranes were more prone to oxidative stress than those of less mature fruit. Concurrent with its membranes being more vulnerable to oxidative stress, the more mature fruit had lower levels of antioxidants to quench the free radicals caused by the chilling stress. However, although the less mature fruit had higher levels of antioxidants and linolenic acid, as well as higher unsaturated:saturated and monounsaturated:polyunsaturated phospholipid fatty acid ratios than the more mature fruit, it did not prevent them from developing CI, albeit at much lower levels.

Key words

Ascorbic acid, ethylene, glutathione, linoleic acid, monounsaturated fatty acids, oleic acid, polyunsaturated fatty acids, shrivel

1. Introduction

Chilling injury (CI) adversely affects the quality of plums exported from South Africa. The cold-storage of these plums is complex as it is a perishable product with a maximum cold-storage life of 3 to 7 weeks, due to its high respiration rate. To counteract the high respiration rate, and concomitant rapid ripening, the fruit is stored at -0.5 °C. However, most plum cultivars exported from South Africa are sensitive to storage under low temperatures, as they develop CI. CI manifests as gel breakdown (GB) or internal browning (IB) in the mesocarp of plums (Taylor, 1996). Affected fruit has a normal external appearance. These defects usually appear when the fruit is removed from the low storage temperature conditions, i.e. in the fruit basket of the consumer, who subsequently does not buy plums again.

CI symptoms are the result of cellular disorganisation due to the loss of normal cell function (Bramlage, 1982). The thermodynamics of every molecule in each cell of the plant are changed by chilling temperatures (Kratsch and Wise, 2000). Hence, due to decreased substrate diffusion rates, enzymatic reactions are slowed, membrane properties are altered and transport across membranes is interrupted (Franks, 1981; Kratsch and Wise, 2000).

Alterations in the physical and chemical characteristics of cell membranes, which, in turn, lead to alterations in cellular metabolism and accelerated senescence, are found central in the process of fruit deterioration (Borochov et al., 1994; Marangoni et al., 1996; Thompson et al., 1997). In a cell wall study, Taylor et al. (1993, 1995) found that GB in ‘Songold’ plums is caused by increased cell membrane permeability, which causes cell fluids to leak into the cell wall area, together with the availability of water-soluble pectins that bind the cell fluids. Characterisation of the total free sterols, total phospholipids and/or phospholipid fatty acid saturation index gave a good indication of membrane integrity, and, hence, senescence and/or deterioration in apple fruit (Lurie and Ben-Arie, 1983), peaches (Izzo et al., 1995), muskmelon fruit (Lester and Stein, 1993), and carnation flowers (Fobel et al., 1987). It was found that these factors influence membrane fluidity, and, therefore, also the movement of electrolytes through the cell membranes.

According to Lester (2003) lipid peroxidation is often the first measurable process that indicates a loss in membrane integrity, which ultimately results in cellular death. Active oxygen species (AOS) scavenging enzymes (e.g. superoxide dismutase, catalase and peroxidase), lipid-soluble membrane-associated antioxidants (e.g. alpha-tocopherol and beta-carotene) as well as water-soluble reductants (e.g. glutathione and ascorbate) regulate the AOS in the fruit tissue. Although the production and elimination of AOS are well regulated, situations occur (e.g. chilling

temperatures for extended periods) in which the production of AOS exceeds the capacity of the fruit to maintain cellular redox homeostasis (Hodges, 2003; Masia, 2003).

In this study, fruit of a CI susceptible plum cultivar, harvested at two maturities, was cold-stored at -0.5 °C (to induce chilling injury) and examined at weekly intervals for six consecutive weeks. This was done to gain a better understanding of the development of chilling injury in plum fruit, as well as the role of cell membranes and antioxidants in these processes. Knowledge of these processes will help in the design of better postharvest strategies to avoid, reduce, or at least delay the incidence of CI in South African plums.

2. Materials and Methods

2.1 Sampling of fruit

'Sapphire' plums (*Prunus salicina* Lindl.) were sampled from a commercial stone fruit farm in Wellington, Western Cape region, South Africa, over two seasons (2005 and 2007). The row orientation was north-south, and the trees were trained according to a palmette system. The fruit was harvested at two maturities, based on flesh firmness. The two harvest maturities were selected to represent fruit harvested at the upper and lower end of the harvesting window for export, respectively (DAFF, 2012). The same randomised complete block design with six blocks per harvest maturity was used in both seasons. The fruit were packed into count 30 pulp trays. Two layers were packed per carton (MO5I) and were covered with a perforated, high density polyethylene (HDPE) shrivel sheet. This was done to prevent moisture loss from the fruit as 'Sapphire' plums are prone to shrivel during storage. Directly after harvest the packed cartons were transported to the laboratory using covered, uncooled transport.

At the laboratory the fruit were cold-stored at -0.5 °C for 6 weeks. On each harvest date, and at weekly intervals afterwards, a subsample of fruit per harvest maturity was placed at 10 °C for 7 days to simulate shelf-life conditions. The plastic shrivel sheet was removed before the fruit were placed at 10 °C to prevent condensation of water and fruit decay. Fruit evaluation was done on each harvest date, and at weekly intervals afterwards on the fruit stored at -0.5 °C and the fruit stored under the simulated shelf-life conditions at 10 °C. Destructive and non-destructive measurements were made on each evaluation date as described below. Furthermore, five fruit per replicate per evaluation date were pooled, peeled and the pulp flash frozen, milled to a fine powder in liquid nitrogen, and stored at -80 °C for further analysis.

2.2 Measurements made on each evaluation date

Rate of ethylene (C_2H_4) evolution (µL kg^{-1} h^{-1}) was measured on two fruit per block. The fruit were sealed in airtight 0.5 L glass jars for 1 h. A headspace gas sample was taken from each jar with a 10 mL airtight syringe, and injected into a Varian GC system (Model 3300, Varian Instrument Group, Palo Alto, California, USA) fitted with a flame ionisation detector (FID) and 2 m PoropakQ column. Nitrogen was used as a carrier gas. The oven temperature was programmed from 60 °C (isothermal for 2 min) to 70 °C at 1 °C min^{-1}. The injector temperature was at 65 °C and the detector temperature at 250 °C. A 1 ppm standard gas was used to identify and quantify C_2H_4. For the rest of the variables, measurements took place on 10 fruit per block unless stated differently. Hue angle was measured on one cheek per fruit with a calibrated colorimeter (Minolta chroma meter CR-400, Japan). Shrivel and decay (expressed as %) were determined subjectively. Shrivel was counted when shrivelled skin extended over the shoulder of the fruit. Flesh firmness (N) was measured with a Southtrade fruit pressure tester (Model FT327, Alphonsine, Italy) fitted with an 11.1 mm tip, on one peeled cheek per fruit. Total soluble solids (TSS) and titratable malic (TA) acid were determined only on the respective harvest dates, and on a pooled, juiced sample of the 10 fruit per block. For TSS determination, a temperature-controlled, digital refractometer (Palette PR-32 ATAGO, Bellevue, USA) was used. TSS was expressed as %Brix. TA was expressed as % and determined by titrating a 10 g aliquot of the juice sample with 0.1 N NaOH to a pH end-point of 8.2 using an automated titrator (Metrohm 719 S Titrino, Herisau, Switzerland). Internal defects (%) were determined by cutting the fruit around the equatorial axis, and separating the two halves of the fruit. A gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue had a healthy appearance, was classified as GB. A brown discolouration of the mesocarp tissue, associated with a loss in juiciness, was classified as IB. To obtain the total CI, the sum of the percent GB and IB per block were calculated. For the biochemical analyses conducted on the samples stored at -80 °C, see Paper 1, Section 2.3.

2.3 Statistical analysis

A two-way analysis of variance was conducted on the harvest maturity parameters determined on each harvest date using the SAS program (SAS Institute Inc., 2002 – 2003). Correlation analysis was done between percent CI incidence and the pooled values of each parameter determined on the respective fruit evaluation dates. Although the Spearman r-values of the variables that were correlated with %CI were low (they explained 10% or less of the variation in the data), parameters that correlated significantly ($P \leq 0.05$) with %CI were used as covariates in the ANOVA for the CI data. A mixed model repeated measures analysis of variance was performed on all the data using STATISTICA version 10 (Statsoft, Inc., 2011). The effect of the three factors, namely harvest maturity, storage duration and temperature (simulated storage at -0.5 °C and the subsequent

simulated shelf-life at 10 °C), were determined on the different variables. The data of the two seasons were pooled. ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

The following variables were used in the analysis: shrivel (%), decay (%), hue angle, flesh firmness (N), GB (%), IB (%), CI (%) (which was the sum of GB and IB), rate of C_2H_4 evolution (µL kg^{-1} h^{-1}), water soluble radical-scavenging activity (HAA) (mg g^{-1} FW), lipid soluble radical-scavenging activity (LAA) (mg g^{-1} FW), lipid peroxidation (nmol MDA equivalents mL^{-1} g^{-1} FW), total phenolic concentration (mg GAE g^{-1} FW), total ascorbic acid (µg g^{-1} FW), L-AA (µg g^{-1} FW), DHA (µg g^{-1} FW), total glutathione (µg g^{-1} FW), GSH (µg g^{-1} FW), GSSG (µg g^{-1} FW), GSH:GSSG ratio, total sterols (µmol g^{-1} FW), total phospholipids (µmol g^{-1} FW), palmitic acid (C16:0) (µg g^{-1} FW), stearic acid (C18:0) (µg g^{-1} FW), oleic acid (C18:1n9c) (µg g^{-1} FW), elaidic acid (C18:1n9t) (µg g^{-1} FW), linoleic acid (C18:2n6c) (µg g^{-1} FW), linolenic acid (C18:3n3) (µg g^{-1} FW), total fatty acids of phospholipids (µg g^{-1} FW), unsaturated fatty acids of phospholipids (%), saturated fatty acids of phospholipids (%), saturated: unsaturated fatty acid ratio, and monounsaturated:polyunsaturated fatty acid ratio.

3. Results

3.1 Harvest maturity

In both seasons there was a significant difference between the two harvest maturities regarding hue angle, flesh firmness and TA (Table 2.1).

3.2 Chilling injury

3.2.1 Internal browning

There was a significant interaction between harvest maturity, storage duration and storage temperature for IB (Fig. 2.1A and B). No IB manifested during the first 2 weeks of storage at -0.5 °C or during the subsequent simulated shelf-life period at 10 °C. After 3 weeks of storage, IB started to manifest in the H2 fruit stored at both -0.5 °C and the subsequent shelf-life period at 10 °C. IB started to manifest in H1 fruit 1 week later (after 4 weeks of storage) at -0.5 °C and 10 °C. When IB manifested, levels were always higher in H2 fruit, and when stored at 10 °C. At both storage temperatures and in both fruit maturities, IB levels increased with an increase in storage duration.

3.2.2 Gel breakdown

There were statistically significant interactions between storage duration and storage temperature (Fig. 2.2A) as well as storage duration and harvest maturity (Fig. 2.2B) for GB incidence. The first GB manifested after harvest during the simulated shelf-life period (Fig. 2.2A). GB levels remained low and did not differ significantly between the fruit stored at -0.5 °C and the subsequent simulated shelf-life period at 10 °C for the first 4 weeks of storage. Thereafter GB levels increased significantly in the fruit stored at 10 °C as storage duration progressed, while levels remained low in the fruit stored at -0.5 °C. Regarding the effect of storage duration and harvest maturity, GB levels remained low and did not differ significantly between harvest maturities for the first 4 weeks of storage (Fig. 2.2B). Thereafter, GB levels increased at a significantly faster rate in H2 than in H1 fruit.

3.2.3 Total chilling injury

There were statistically significant interactions between storage temperature and storage duration (Fig. 2.3A) as well as harvest maturity and storage duration (Fig. 2.3B) for total CI (sum of GB and IB). For both storage temperatures there was an increase in CI levels with an increase in storage duration (Fig. 2.3A). When CI manifested, levels were always higher in the fruit stored under the shelf-life conditions compared to the preceding storage duration at -0.5 °C. CI first started to manifest in the fruit stored under simulated shelf-life conditions after 0 days of storage. During the first 14 days of storage, very low CI incidence was evident in both harvest maturities (Fig. 2.3B). However, after 21 days of storage CI levels started to increase with an increase in storage duration in fruit of both harvest maturities. CI increased at a faster rate in H2 than H1 fruit.

3.3 Fruit quality variables that significantly ($P < 0.05$) correlated with CI

Table 2.2 presents the results of the correlation analysis of the measured variables and CI. Only the variables that significantly correlated with CI will be discussed in this paper.

3.3.1 Shrivel

Despite packing the fruit with perforated HDPE sheets, shrivel manifested. There was a statistically significant interaction ($P = 0.0001$) between harvest maturity and storage duration for shrivel (Fig. 2.4). In both fruit maturities shrivel incidence increased with an increase in storage duration – following the same trend as CI (Fig. 2.3B). It manifested earlier during storage, and increased at a significantly faster rate in H2 compared to H1 fruit (Fig. 2.4). Shrivel was positively

correlated with CI (Table 2.2). Covariate analysis indicated that shrivel affected CI significantly (P = 0.0000), however, other factors also played a role in the manifestation of CI as the second order interactions were still significant (Fig. 2.3A and B).

3.3.2 Hue angle

There were statistically significant interactions between harvest maturity and storage duration (P = 0.0000) as well as harvest maturity and storage temperature (P = 0.0225) for the hue angle (Fig. 2.5A and B). The hue angle did not change significantly in H2 fruit during the storage duration, while H1 fruit skin colour significantly changed from yellow to yellow-red (McGuire, 1992) (Fig. 2.5A). H2 fruit had more advanced skin colour than H1 fruit at both storage temperatures. Both fruit maturities had more advanced skin colour after storage at 10 °C compared to -0.5 °C (Fig. 2.5B).

3.3.3 Flesh firmness

There was a statistically significant interaction (P = 0.0000) between harvest maturity, storage duration and storage temperature for flesh firmness (Fig. 2.6A and B). Flesh firmness of H1 fruit decreased by almost 1 kg during the first week of storage at -0.5 °C, and remained relatively unchanged for the next 3 weeks of storage (Fig. 2.6A). Thereafter it decreased again by approximately 1 kg after 5 weeks of storage which coincided with the first substantial incidence of GB in the H1 fruit (Fig. 2.2B). Under the simulated shelf-life conditions the flesh firmness of H1 fruit remained high for the first week after harvest (i.e. no storage at -0.5 °C), but then decreased by > 2 kg after only 1 week's storage at -0.5 °C (Fig. 2.6B). After the initial fast rate of decrease, H1 flesh firmness decreased only marginally at 10 °C until the end of the storage duration. Except for the first week after harvest, H1 flesh firmness was significantly lower under the shelf-life conditions than under the storage conditions at -0.5 °C. H2 flesh firmness remained relatively constant during storage at -0.5 °C and the subsequent shelf-life periods at 10 °C (Fig 2.6A and B). However, H2 flesh firmness was significantly lower at 10 °C than at -0.5 °C. H2 fruit had significantly lower flesh firmness at -0.5 °C and 10 °C than H1 fruit.

3.4 Biochemical parameters significantly (P < 0.05) correlated with CI

3.4.1 Ethylene evolution

There was a significant interaction (P = 0.0012) between harvest maturity, storage duration and storage temperature for C_2H_4 evolution (Fig. 2.7A and B). C_2H_4 evolution rate was generally lower

at -0.5 °C than at 10 °C. Coinciding with the first manifestation of IB (Fig. 2.1A), H2 fruit had a significant increase in C_2H_4 evolution rate after 3 weeks of storage at -0.5 °C. After the increase, C_2H_4 evolution rates in the H2 fruit decreased and remained low (Fig. 2.7A). H1 fruit stored at -0.5 °C did not show an increase in C_2H_4 levels when IB first manifested after 4 weeks of storage. However, after 3 weeks of storage, C_2H_4 evolution rates at 10 °C became significantly higher than at -0.5 °C, which was also when the first CI was observed in the H1 fruit (Fig. 2.3B). C_2H_4 evolution rates were always higher in the H2 than the H1 fruit at 10 °C – in most cases this difference was statistically significant (Fig. 2.7B). In both fruit maturities, C_2H_4 evolution rates increased with an increase in storage duration during the simulated shelf-life storage, but decreased in the last shelf-life period after 42 days of storage. Covariate analysis indicated that C_2H_4 affected CI significantly ($P = 0.0226$), however, other factors also played a role in the manifestation of CI as two of the second order interactions were also statistically significant (Fig. 2.3A and B).

3.4.2 Antioxidants

3.4.2.1 Water soluble antioxidant activity

There was a significant interaction ($P = 0.0004$) between harvest maturity, storage duration and storage temperature for HAA (Fig. 2.8A and B). During storage at -0.5 °C, HAA levels were higher in H1 than H2 fruit. HAA levels decreased in H1 fruit during the first 3 weeks of storage at -0.5 °C, increased significantly after 4 weeks of storage and then decreased again until the end of the 6-week storage duration (Fig. 2.8A). The increase in HAA after 4 weeks of storage coincided with the first incidence of IB (Fig 2.1A), but lagged a week behind the first manifestation of CI in H1 fruit (Fig 2.3A and B). HAA levels generally declined in H2 fruit, but increased during the last week of storage at -0.5 °C (Fig. 2.8A). During storage in the subsequent shelf-life periods, HAA levels decreased after the first week of storage in H1 fruit, reaching its lowest level (Fig. 2.8B). Thereafter it increased in the following week of storage, and remained at the elevated level for the remainder of the storage duration, following the same pattern as the manifestation of CI at 10 °C (Fig. 2.3A). HAA levels in H2 fruit increased during the shelf-life periods following the first 2 weeks of storage (Fig. 2.8B). For the rest of the storage duration, however, HAA levels steadily decreased in H2 fruit. This decrease in HAA levels was inversely proportional to the increase in IB levels in the same fruit during the same time period in storage at 10 °C (Fig. 2.1B).

3.4.2.2 Total and reduced ascorbic acid

There was a statistically significant interaction between harvest maturity, storage duration and storage temperature for total (Fig. 2.9A and B) and reduced ascorbic acid (Fig. 2.10A and B).

H1 fruit had significantly higher levels of total and reduced ascorbic acid on the harvest dates than H2 fruit (Fig. 2.9A and Fig. 2.10A). At both storage temperatures, total ascorbic acid levels were higher in H1 than in H2 fruit for the entire storage duration (Fig 2.9 A and B). Although this was also generally true for the L-AA levels, H1 levels were lower, albeit not significantly, than H2 levels after 35 days at -0.5 °C (Fig. 2.10A). At both storage temperatures and in both fruit maturities, there was a decrease in total and reduced ascorbic acid with an increase in storage duration. For the first 14 days of storage at -0.5 °C, the rate of decrease was fast followed by a moderate decrease for the rest of the storage duration for both fruit maturities (Fig. 2.9A and Fig. 2.10A). One week after the decrease in total and reduced ascorbic acid slowed down (at day 21), the first significant CI was observed in the fruit stored under the shelf-life conditions (Fig. 2.3A). Total and reduced ascorbic acid levels were higher or similar to those in the subsequent shelf-life evaluations in both fruit maturities for the analysis after 0 and 7 days (Fig. 2.9A and B; Fig. 2.10A and B). However, from 14 days of storage until the end of the storage duration, total and reduced ascorbic acid levels were higher in the fruit stored at 10 °C compared to -0.5 °C.

3.4.2.3 DHA

There was a significant interaction (P = 0.0451) between storage temperature and duration for oxidised ascorbic acid (Fig. 2.11). DHA levels varied more, albeit within a very small margin, at 10 °C than at -0.5 °C. Generally, DHA levels were higher at 10 °C than at -0.5 °C. Mostly there was a slight increase in DHA levels with an increase in storage duration at -0.5 °C.

3.4.2.4 Total glutathione

There was a statistically significant interaction (P = 0.0004) between harvest maturity, storage temperature and storage duration for total glutathione (Fig. 2.12A and B). The two harvest maturities did not differ significantly regarding total glutathione levels on the harvest dates (Fig. 2.12A). Total glutathione levels significantly decreased during the first 7 days of storage where after it increased with an increase in storage duration at -0.5 °C in H1 fruit. The increase in total glutathione levels in H1 fruit after 2 weeks of storage coincided with the first manifestation of CI in this fruit (Fig. 2.3B), but preceded the first significant incidence of CI in the shelf-life-stored fruit by 1 week (Fig. 2.3A). Conversely, total glutathione levels remained fairly unchanged in H2 fruit for the entire storage duration at -0.5 °C. Hence, total glutathione levels were generally higher in H1 than H2 fruit, and the difference became statistically significant after 28 days of storage at -0.5 °C. Total glutathione levels generally increased at 10 °C in fruit of both harvest maturities with a progression in storage duration (Fig. 2.12B).

3.4.2.5 GSH

There was a statistically significant interaction (P = 0.0138) between harvest maturity, storage temperature and storage duration for GSH (Fig. 2.13A and B). Levels of GSH did not differ significantly between the fruit of the two harvest maturities at harvest (Fig. 2.13A). Directly after harvest, GSH levels decreased significantly in H1 fruit, where after it increased with an increase in storage duration at -0.5 °C (Fig. 2.13A). Similar to total glutathione levels, the start of the increase in GSH levels coincided with the first manifestation of CI in H1 fruit (Fig. 2.3B), and preceded the first statistically significant manifestation of CI in shelf-life stored fruit by 1 week (Fig. 2.3A). In H2 fruit, GSH levels remained constant for the first 2 weeks whereafter it increased significantly after 3 weeks, but decreased again and remained relatively constant for the rest of the storage duration at -0.5 °C. The increase in GSH levels after 3 weeks of storage coincided with the first significant levels of CI that manifested during the simulated shelf-life after 3 weeks of storage. At 10 °C, GSH levels increased with an increase in storage duration, and the levels did not differ much between the two harvest maturities (Fig. 2.13B).

3.4.2.6 GSSG

There was a statistically significant interaction (P = 0.0004) between harvest maturity, storage temperature and storage duration for GSSG (Fig. 2.14A and B). GSSG levels were similar on the harvest date and after 7 days of storage at -0.5 °C for the two fruit maturities (Fig. 2.14A). In H1 fruit GSSG levels peaked significantly on two occasions during storage at -0.5 °C. The first peak occurred after 14 days of storage at -0.5 °C (Fig. 2.14A) – which coincided with the first incidence of CI in this fruit (Fig. 2.3B). The second peak occurred after 35 days of storage at -0.5 °C (Fig. 2.14A) when CI levels were already unacceptably high in H1 fruit stored at -0.5 °C (Fig.2.3B). Generally, GSSG levels had an increasing trend in H1 fruit. In contrast, GSSG levels in H2 remained low and increased only slightly towards the end of storage at -0.5 °C (Fig. 2.14A). Hence, GSSG levels were generally higher in H1 than H2 fruit during storage at -0.5 °C. Following harvest, during the simulated shelf-life period at 10 °C, GSSG levels in H1 fruit remained the same as at harvest, but increased significantly in H2 fruit (Fig. 2.14A and B). The increase in GSSG levels after harvest in H2 fruit at 10 °C coincided with the first incidence of CI observed in this fruit (Fig. 2.3A and B). Subsequently, there was a peak in GSSG levels in H1 fruit in the simulated shelf-life period after 7 days of storage, which preceded the first incidence of CI in this fruit by 1 week (Fig. 2.3B). After this peak, GSSG levels decreased and remained low during the simulated shelf-life periods at 10 °C, but increased significantly during the last shelf-life period for H1 fruit (Fig. 2.14B). After the initial peak in GSSG levels in H2 fruit stored at 10 °C, levels decreased significantly, but increased again during the simulated shelf-life periods after 3 and 4 weeks of storage.

3.4.3 Cell membrane components significantly (P < 0.05) correlated with CI

3.4.3.1 Total phospholipids

There was a statistically significant interaction (P = 0.0000) between storage temperature and storage duration for total phospholipids (Fig. 2.15). Except for 28 and 42 days of storage, total phospholipid levels were always significantly higher in the simulated shelf-life period than in the preceding storage durations at -0.5 °C. Excluding a significant spike after 4 weeks of storage, total phospholipid levels gradually increased with an increase in storage duration at -0.5 °C. The spike in total phospholipid levels coincided with the first GB incidence at -0.5 °C (Fig. 2.2A). At 10 °C, total phospholipid levels increased slightly after harvest, remained relatively constant for the following 4 weeks of storage and then decreased significantly after 6 weeks of storage.

3.4.3.2 Total sterols

There was a significant interaction (P = 0.0457) between harvest maturity, storage temperature and storage duration for total sterol levels (Fig. 2.16A and B). Generally total sterol levels were higher in H2 than in H1 fruit during storage at -0.5 °C and 10 °C. Total sterol levels were only higher in H1 than H2 fruit after the simulated shelf-life period at 10 °C following storage at -0.5 °C for 7 days, albeit this difference was not significant (Fig. 2.16B). At -0.5 °C there were two peaks in total sterol levels in H1 fruit, namely after 7 and 28 days of storage (Fig. 2.16A). The peak at 7 days was also visible in the following shelf-life period at 10 °C (Fig. 2.16B), and preceded the first incidence of CI in H1 fruit with 1 week (Fig. 2.3B). The peak at 28 days of storage at -0.5 °C coincided with the first incidence of IB in the H1 fruit (Fig 2.1A). In the simulated shelf-life at 10 °C following 35 days of storage at -0.5 °C, there was a significant decrease in total sterol levels in H1 fruit, which increased again in the following shelf-life period (Fig. 2.16B). Total sterol levels in H2 fruit varied very little over the storage duration at -0.5 °C as well as during the following simulated shelf-life durations at 10 °C (Fig. 2.16A and B). There was a significant decrease in total sterols in H2 fruit after 7 weeks of storage at -0.5 °C (Fig. 2.16A), but they increased again in the following shelf-life period at 10 °C (Fig. 2.16B).

3.4.3.3 Phospholipid fatty acids significantly (< 0.05) correlated with CI

3.4.3.3.1 Oleic acid (C18:1n9c)

There was a statistically significant interaction (P = 0.0015) between harvest maturity, storage temperature and storage duration for oleic acid levels (Fig. 2.17A and B). On the harvest dates oleic acid levels were significantly higher in H1 than H2 fruit (Fig. 2.17A). Levels decreased

significantly in H1 fruit during the first week of storage at -0.5 °C, increased again during the following week of storage and remained at the increased levels for 2 weeks. Thereafter oleic acid levels decreased significantly after 4 weeks of storage which coincided with the first incidence of IB in H1 fruit stored at -0.5 °C (Fig. 2.1B). Oleic acid levels increased significantly in H2 fruit during the first week of storage at -0.5 °C, whereafter they decreased with an increase in storage duration (Fig. 2.17A). The start of decrease after 2 weeks of storage at -0.5 °C coincided with a sharp increase in CI in H2 fruit (Fig. 2.3B). After shelf-life oleic acid levels were generally lower than in the preceding -storage durations at -0.5 °C for both fruit maturities (Fig. 2.17A and B). While oleic acid levels generally declined with an increase in storage duration in H1 fruit stored at 10 °C, levels in H2 fruit remained relatively constant with a significant increase at 35 days of storage, whereafter they decreased again (Fig. 2.17B).

3.4.3.3.2 Elaidic acid (C18:1n9t)

There was a statistically significant interaction (P = 0.0410) between harvest maturity and storage duration for elaidic acid (Fig. 2.18). At harvest elaidic acid levels were significantly higher in H1 than H2 fruit. Elaidic acid levels remained fairly stable in the H2 fruit for the duration of cold-storage. However, they decreased significantly after 4 weeks of storage in H1 fruit and remained at the reduced level for the remainder of the storage duration. The decrease in elaidic acid after 4 weeks of storage in H1 fruit coincided with the first incidence of IB in this fruit (Fig. 2.1A and B).

3.4.3.3.3 Linolenic acid (C18:3n3)

There was a statistically significant interaction (P = 0.0007) between harvest maturity, storage duration and storage temperature for linolenic acid (Fig. 2.19A and B). Levels were significantly higher in H1 than in H2 fruit on the harvest date (Fig. 2.19A), and generally higher at 10 °C than at -0.5 °C for both fruit maturities (Fig. 2.19A and B). Linolenic acid levels remained relatively stable during the first 3 weeks of storage at -0.5 °C, whereafter they increased with an increase in storage duration in H1 fruit (Fig. 2.19A). This increase started at the same time and followed the same trend as IB incidence in H1 fruit stored at -0.5 °C (Fig. 2.1A). During the first week of storage at -0.5 °C, linolenic acid levels increased significantly in H2 fruit and followed the same trend as the H1 fruit for the duration of the storage period (Fig. 2.19A). However, the increase in linolenic acid levels in H2 fruit occurred at a significantly slower rate and lagged 1 week behind the H1 fruit and 2 weeks behind the first incidence of IB in the H2 fruit (Fig. 2.1A). Except for a significant decrease in the last week of storage, there was a general increase in linolenic acid levels in H1 fruit at 10 °C with an increase in storage duration (Fig. 2.19B). In H2 fruit there was an increase after the first week of storage, similar to what was observed at -0.5 °C (Fig. 2.19A and B). However, there was

a significant depression in linolenic acid levels in H2 fruit after 3 weeks of storage, which coincided with the first incidence of IB in this fruit (Fig. 2.19B).

3.4.3.3.4 Percentage saturated and unsaturated phospholipid fatty acids, the unsaturated:saturated phospholipid fatty acid ratio, and the mono-unsaturated:polyunsaturated phospholipid fatty acid ratio

There was a statistically significant (P = 0.0000) interaction between harvest maturity and storage temperature for the percentage saturated (data not shown; Refer to Appendix B, Fig. 1) and unsaturated phospholipid fatty acids (data not shown; Refer to Appendix A, Fig. 2). For both storage temperatures, levels of saturated and unsaturated phospholipid fatty acids, respectively, did not differ significantly for H1. However, H2 fruit had significantly higher levels of saturated and significantly lower levels of unsaturated fatty acids than H1 fruit at -0.5 °C. Under the simulated shelf-life storage at 10 °C, H2 fruit had similar levels of saturated and unsaturated phospholipid fatty acids compared to the H1 fruit. Consequently, the unsaturated:saturated fatty acid ratio of the H2 fruit was significantly lower compared to the H1 fruit while stored at -0.5 °C, but similar when stored at 10 °C (Fig. 2.20). H2 fruit also had a significantly lower monounsaturated:poly-unsaturated fatty acid (MUFA:PUFA) ratio than H1 fruit while stored at -0.5 °C, however, the ratio was similar at 10 °C for the two fruit maturities (Fig. 2.21A). Generally the MUFA:PUFA ratio was higher at -0.5 °C than at 10 °C (Fig. 2.21B). The unsaturated:saturated fatty acid ratio had a significant (P = 0.0267) effect on CI as a covariate (Fig. 2.3A and B). However, other factors than the unsaturated:saturated fatty acid ratio also contributed to the manifestation of CI as two of the second order interactions also significantly influenced CI.

4. Discussion

4.1 Chilling injury manifestation

In chilling sensitive tissues there is usually an interaction between time and temperature, with greater severity of injury as temperature is lowered, or as exposure to chilling temperatures is extended (Saltveit and Morris, 1990; Sevillano et al., 2009). Symptom development also becomes more apparent and accelerates after transfer of the fruit to non-chilling/ ripening temperatures (Lyons, 1973; Morris, 1982; Raison and Orr, 1990; Sevillano, et al., 2009). Moreover, the type and severity of CI that manifests are influenced by the developmental stage of the fruit (Bramlage, 1982). Generally it is found that fruit are most sensitive to CI during the climacteric rise and at the climacteric peak, while pre- and post-climacteric fruit are less susceptible, with post-climacteric fruit being the least susceptible (Wade, 1979; Wang, 1982). In avocados it was found

that the threshold temperature for CI changes during ripening of fruit, being the highest in the climacteric peak and the lowest in the post-climacteric stage (Wade, 1979). In two studies on plums, higher levels of CI were found in the more mature fruit (Taylor, 1996; Abdi et al., 1997). It is proposed that changes in the membrane lipid composition in the different fruit maturation and ripening stages account for the changes in the chilling threshold temperature, and that chilling temperatures may have a severely disruptive effect on the intense metabolic activity during fruit ripening (Wade, 1979). In agreement with the findings of previous studies, this study found that CI incidence escalated with an increase in storage duration, was generally higher at 10 °C following prior storage at -0.5 °C than at -0.5 °C alone, and increased at a higher rate in the more mature fruit.

4.2 Shrivel manifestation

The deficit between the saturated vapour pressure inside the fruit and the actual vapour pressure of the surrounding atmosphere, determines the rate of evaporation from the product (Paull, 1999). The approximate RH in the cold-rooms used for the trial was 80%, while an RH of 95% is desirable for stone fruit (Mitchell, 1986). Therefore, the shrivel incidence in fruit of both maturities was probably due to the sub-optimal RH in the cold-room. It is suspected that the H2 fruit developed shrivel symptoms earlier and at a faster rate than H1 fruit, because it had a higher respiration rate than the less mature H1 fruit. Unfortunately fruit respiration could not be determined in this study. Therefore, it can only be speculated that the respiratory heat could have caused the H2 fruit to remain at a slightly higher temperature than the surrounding cold room air. As the HDPE shrivel sheet reduces air movement over the fruit, and also moisture loss compared to no barrier (Moelich and Taylor, 2012), it is assumed that respiration heat could not be adequately removed from the fruit, causing a small, positive temperature gradient favouring water loss (Kays and Paull, 2004a). Moisture stress resulting from moisture loss induces higher C_2H_4 and abscisic acid production (Tandler et al., 1989; Morgan and Drew, 1997; Aked, 2002; Burg, 2004). Both hormones accelerate senescence and, hence, membrane leakiness. When shrivel was used as a covariate in the analysis of CI (Fig. 2.3A and B), it had a significant ($P = 0.0000$) effect on CI. However, two of the second order interactions were still significant ($P = 0.0000$), indicating that not only shrivel, but other factors also played a role in the development of CI. Since shrivel levels in the H2 fruit reached unacceptable levels (> 10%), and the fruit was more prone to develop CI, a postharvest strategy will have to be found to improve air movement over the fruit, or to reduce the respiration rate of the fruit, so that the heat of respiration can be removed without causing excessive moisture loss during long-term storage.

4.3 Hue angle

The decrease in hue angle value and flesh firmness over the storage duration and during the simulated shelf-life periods were expected, and correlate with the findings of another plum study (Abdi et al., 1997). The change in skin colour is probably due to the degradation of chlorophyll with the concurrent unmasking or production of carotenoids and anthocyanins, and is a normal phenomenon found in many fruit types during storage (Pentzer and Allen, 1944; Monreal et al., 1999; Kays and Paull, 2004; Varasteh et al., 2012). It is known that increased C_2H_4 production causes changes in chlorophyll, carotenoid and flavonoid concentrations as well as fruit texture (Czarny et al., 2006). The more advanced skin colour in fruit of both maturities at 10 °C than at -0.5 °C is probably due to the higher rates of C_2H_4 evolution at the higher temperature observed in this study. While the breakdown of pectic substances is hampered at -0.5 °C, cellulose degradation is not inhibited and may be the reason for the decrease in the flesh firmness of plums under cold-storage (Taylor, 1996). Since changes in the fruit texture and skin colour during storage are direct effects of C_2H_4 production, it is probably not directly involved in the initiation of CI – which is confirmed by the two variables not having a significant impact on CI when used as covariates in the analysis of the CI data.

4.4 C_2H_4 evolution

It has been observed that C_2H_4 production is stimulated by chilling temperatures (Wang, 1982; Sevillano et al., 2009). C_2H_4 has been shown to increase the respiration rate, change the activity of certain enzymes, increase membrane permeability, and alter cellular compartmentalization, all which accelerate product senescence and deterioration (Kays and Paull, 2004b). Usually 1-aminocyclopropane-1-carboxylic acid (ACC), ACC synthase activity and C_2H_4 production remain low at the chilling temperature, but increase rapidly when the product is transferred to a higher temperature (Wang, 1982; Sevillano et al., 2009). This is because the mRNA encoding for ACC-synthase is stimulated by low temperatures, and the translation of these messages occurs immediately after transfer of the product to higher temperatures (Sevillano et al., 2009), which explains the higher C_2H_4 evolution rate at 10 °C than at -0.5 °C in this study.

Usually CI symptoms are also more apparent and their development accelerates after transfer of the fruit to non-chilling or ripening temperatures (Sevillano et al., 2009). Candan et al. (2008) found a relation between CI symptoms in ‘Larry Ann’ plums and a concomitant increase in membrane permeability and C_2H_4 production after transfer of the fruit to a higher temperature. This study found that H2 fruit produced higher levels of C_2H_4 and had a higher incidence of CI at 10 °C than the H1 fruit. C_2H_4 evolution and CI were also significantly correlated in this study, and

as a covariate, C_2H_4 had a significant influence on CI. However, as mentioned previously, moisture loss and chilling temperatures stimulate the production of C_2H_4. The moisture stress experienced by the H2 fruit (demonstrated by the fruits' high shrivel levels) could, therefore, have enhanced the effect of C_2H_4 on the development of CI in the H2 fruit. In this study, the effect of C_2H_4 production on CI manifestation could, therefore, have been a secondary effect of moisture stress. Tandler et al. (1989), in their study on the effect of chilling and water stress on potted plants of clerodendrum, also concluded that the enhanced production of C_2H_4 they observed was a secondary effect and not the cause of CI.

In this trial it was observed that C_2H_4 production decreased in both fruit maturities after the final week of storage in the simulated shelf-life period. Other researchers also found that chilling induced C_2H_4 production declines after the initial stimulation, and it is speculated that the step converting ACC to C_2H_4 is easily damaged by chilling temperatures (Wang, 1982; Chen and Patterson, 1985). It was found that ACC synthase, the enzyme which is stimulated by chilling temperatures and which is responsible for the production of ACC from S-adenosyl methionine (SAM), is a soluble enzyme and not membrane-bound (Wang, 1982). However, Wang (1982) speculated that ACC oxidase, the enzyme responsible for the conversion of ACC to C_2H_4, is membrane bound. Hence, if the membrane is damaged by the chilling temperature, ACC oxidase would be inhibited, and, consequently, C_2H_4 production would decrease. However, it was subsequently found that ACC oxidase is not associated with cell membranes, but that L-AA and Fe(II) are required as cofactors for ACC oxidase (John, 1997). In this study the levels of L-AA were relatively low after 6 weeks of storage, but it is unclear whether levels were too low for the proper functioning of ACC oxidase. Rather, it is suggested that the decrease in C_2H_4 production after 6 weeks of storage in fruit of both harvest maturities, is probably due to a time-dependent collapse of the cellular membranes. Such a collapse could, e.g., cause the C_2H_4 receptors, which are membrane-bound (Czarny et al., 2006), not to be optimally functional.

4.5 Antioxidant levels

4.5.1 Water soluble antioxidant activity

As mentioned earlier, storing fruit at chilling temperatures causes oxidative stress (Lester, 2003). It is suggested that the low temperatures reduce the product's demand for ATP (Purvis, 2004; Wismer, 2004). This results in a decrease in oxygen consumption and a build-up of a high concentration of oxygen in the mitochondria while some of the components of the electron transport chain remain reduced (Purvis, 2004). This situation favours electron leakage to molecular oxygen which, in turn, results in an increased production of AOS. If allowed to

accumulate, the AOS levels can exceed the capacity of the antioxidant systems of the plant product, and can cause damage to cellular components, such as the cell membranes (Anderson et al., 1995). Low temperature storage reduces the activity of antioxidant enzymes which further weakens the product's ability to overcome the increased levels of AOS (Purvis, 2004). In our study we found that H1 fruit, which had much lower CI incidence than H2 fruit, generally also had higher HAA levels compared to H2 fruit. This is in accordance with the findings of a study on internal browning in apples (Toivonen, 2004). However, in a study on tomatoes it was found that HAA did not differ between harvest maturities (Illić et al., 2009).

Some studies found that HAA increases at higher storage temperatures (Kalt et al., 1999), while others found that it was higher at chilling temperatures (Kondo et al., 2005). In two apple cultivars differing in their susceptibility to internal browning disorders, it was found that HAA decreased significantly in the susceptible cultivar, while it was stable in the resistant cultivar (Toivonen, 2004). Although 'Sapphire' is a chilling sensitive cultivar, HAA levels did not differ much between the two storage temperatures for the two individual harvest maturities in this study. In their study on eight plum cultivars, Díaz-Mula et al. (2009) also did not find significant losses in HAA over storage duration. Although the levels were similar to the levels at -0.5 °C, we found that HAA first increased and then decreased at 10 °C in H2 fruit, and that the decline was inversely proportional to the increase in IB levels in these fruit for the same storage duration. Similarly, in a study on mango ginger rhizomes, it was found that HAA levels increased for the first 30 days under chilling temperatures, whereafter it decreased over time, and that the peak coincided with the onset of CI (Policegoudra and Aradhya, 2007). Antioxidant capacity usually increases under low stress or during acclimation before a higher degree of stress is introduced (Hodges, 2001). However, a similar trend was not observed for the H1 fruit in this study at either temperature, although CI also manifested in these fruit.

In numerous studies on various fresh produce, including plums, it was found that HAA is strongly correlated with the total phenolic content of the product (Kalt et al., 1999; Gil et al., 2002; Kondo et al., 2005; Díaz-Mula et al., 2009). Due to the higher concentration of phenolic compounds compared to other antioxidants, it has been suggested that they are of greater value in protecting fresh produce against oxidative stress (Hodges, 2001). However, phenolic compounds are primarily found in the vacuole, while most of the AOS are produced outside the vacuole. AOS have very short life spans, and therefore, would tend to react with the cellular contents in their near vicinity rather than travel over the tonoplast where they could be quenched by the phenolic compounds. This may also be the reason why only slight decreases in HAA levels over storage duration were observed in this study. It is suggested that the high concentration of the phenolics

relative to the other antioxidants, masked the effect of the other antioxidants such as ascorbic acid and glutathione, which were more readily available to quench the AOS. Therefore, in this study it seems that harvest maturity definitely influenced HAA levels, with fruit harvested at the upper end of the harvest window having a better capacity to scavenge for AOS, and being less susceptible to CI than fruit harvested at the middle or lower end of the harvest window. However, except for HAA levels in the H2 fruit at 10 °C which started to decrease when IB incidence was first noted, HAA levels over storage duration generally did not give a clear indication of the onset of chilling damage in H1 fruit. These discrepancies between the two harvest maturities towards their differing trends in HAA levels compared to CI incidence, are strengthened by the observation that HAA as a covariate did not have a significant influence on CI.

4.5.2 Ascorbic acid

Ascorbic acid levels are usually maintained at a relatively constant level, and are often increased under stress conditions in the chloroplasts of leaves (Foyer, 1993). A decrease in ascorbic acid levels is usually an indicator that the product is experiencing severe stress. In this study, total and reduced ascorbic acid levels decreased at a fast rate during the first 14 days of storage, whereafter the decreasing trend slowed down for the rest of the storage duration for both fruit maturities. This decline was not due to an increase in oxidation of ascorbic acid, but rather to a general decline in ascorbic acid levels. It was found that ascorbic acid generally decreases in fruits and vegetables during storage since it is extremely labile (Lurie 2003; Toivonen, 2003), that the decrease may be more pronounced in chilling sensitive cultivars or products (Walker and McKersie, 1993; Anderson et al., 1995; Wang, 1996), and that it depends on the storage temperature and storage condition (e.g. regular atmosphere vs. controlled atmosphere storage) (Veltman et al., 2000). Similar to our study Kondo et al. (2005) found that ascorbic acid levels were lower at the lower storage temperature compared to the higher storage temperature in mangoes and bananas. Wang (1996) and Franck et al. (2003) found that the highest rate of ascorbic acid losses occurred early after harvest during storage followed by a slower rate of loss for the rest of the storage duration, which is similar to our findings.

Toivonen (2003) suggested that this postharvest decline in ascorbic acid is due to its double role as a scavenger of AOS and regenerator of other antioxidant systems such as flavonols and tocopherols. The reason for DHA not increasing, or for L-AA declining, may be because DHA can be easily degraded to tartrate and oxalate if it is not swiftly reduced back to L-AA through the ascorbate glutathione cycle (Walker and McKersie, 1993; Anderson et al., 1995; Potters et al., 2002; Hancock and Viola, 2005; Ishikawa, 2006). The decline in total and reduced ascorbic acid in

our study could, therefore, also have been due to high levels of oxidation (although masked by the quick degradation of DHA to tartrate and oxalate) and/or decreased ascorbic acid synthesis.

Our study also found that the less mature fruit generally had higher ascorbic acid levels than the more mature fruit, which agrees with the findings of Veltman et al. (2000) on pears. It has been found that the activity of galactono-1,4-lactone dehydrogenase (GalLDH), the mitochondrial enzyme responsible for the final biosynthetic step of ascorbic acid, declines with tissue aging and senescence of leaves (Conklin, 2001). This may be the reason for the H1 fruit having higher ascorbic acid levels than the H2 fruit, and even for the decline in ascorbic acid in both maturities with an increase in storage duration. GalLDH is localised on the inner membrane of the mitochondria (Conklin, 2001). Although the mitochondrial membranes are not as sensitive to chilling temperatures as the chloroplast membranes (Kratsch and Wise, 2000), it is suggested that, if the inner mitochondrial membrane is influenced by the low storage temperatures, it could have influenced the activity of the enzyme, and hence the synthesis of ascorbic acid.

Veltman et al. (2000) found that tissue browning in pears was initiated when the ascorbic acid level decreased to between 2 to 6 mg $100g^{-1}$ FW. However, Franck et al. (2003) refrained from supporting the ascorbic acid threshold hypothesis, and was not able to link low ascorbic acid levels directly with core breakdown incidence in 'Conference' pears. According to De Gara (2004) there is ample evidence that ascorbic acid plays an important role in redox sensing and signalling pathways in the cell, and, that through that role, its levels eventually alters plant cell metabolism. Indeed, in this study the levels of total and reduced ascorbic acid started to change soon after cold-storage commenced, probably because it sensed the higher levels of AOS that was caused by the low storage temperatures, and therefore, could have served as a signal for other chilling responses.

4.5.3 Glutathione

Total glutathione levels in H1 fruit tended to increase, while it did not change much in the H2 fruit when stored at -0.5 °C. However, at 10 °C total glutathione levels increased in both harvest maturities with an increase in storage duration. GSH levels increased with an increase in storage duration in both fruit maturities, and storage temperatures. This increase in GSH was not due to a significant decrease in GSSG levels. GSSG levels did not follow a similar trend at either temperature for the two fruit maturities, although CI increased in both maturities with an increase in storage duration.

Several other studies found that glutathione levels increase under chilling conditions (Esterbauer and Grill, 1978; Anderson et al., 1992; Hausladen and Alscher, 1993; Gómez et al., 2009). It is suggested that the reason for this increase in glutathione levels is because the activity of glutathione reductase (GR), the enzyme responsible for the reduction of GSSG to GSH and the maintenance of a high GSH:GSSG ratio, is upregulated under chilling conditions (Wang, 1995; Gómez et al., 2009). Increased activity of GR and the simultaneous increase of GSH levels under low-temperature conditions were reported in a number of studies (Esterbauer and Grill, 1978; Wang, 1995). Esterbauer and Grill (1978) suggest that the reason for this phenomenon is the protection of the -SH groups in enzymes and structural proteins by GSH to prevent the formation of S-S bonds under freezing conditions. Since GSSG forms mixed disulphides with proteins – which inactivates many biosynthetic enzymes - GR is needed for the quick reduction of GSSG (Noctor et al., 2002). It was also found that the activity of one of the two enzymes that catalyses the synthesis of glutathione, namely γ-glutamylcysteine synthetase (γECS), increases under chilling conditions (Szalai et al., 2009). Hodges (2003) remarks that an increase in glutathione levels in chilled plant tissue is important, as it scavenge for H_2O_2 and other toxic oxygen compounds together with ascorbic acid. A large GSH pool would, therefore, allow for sufficient reduction of ascorbic acid, so that an equally large pool of GSH would not be needed. Since glutathione levels tended to increase in H1 fruit, but not in H2 fruit, at -0.5 °C, H1 fruit was better protected against oxidative stress than the H2 fruit, and therefore, were less susceptible to CI. However, Hodges (2003) also remarks that increased levels of GSH alone would not ensure enough reducing power in all cases to prevent injury under elevated stress conditions. This is confirmed by this study, since although GSH levels increased over storage duration at -0.5 °C in H1 fruit, CI still manifested in these fruit. GSH levels did increase significantly over storage duration in H2 fruit at 10 °C, but it was probably too late to prevent or reduce the CI that was induced at -0.5 °C.

4.6 Cell membrane composition

Fruit tissue makes some adaptations regarding the difference in temperature conditions in the orchard (ranging between approximately 10 °C to 35 °C in this study), and a storage temperature of -0.5 °C (Lurie et al., 1987). Indeed, temperature has a direct effect on the 'fluidity' of cellular membranes – e.g. increased temperature causes an increase in the rate of molecular motion and a decrease in order in the membrane (Cossins, 1981). It also causes reversible gel to liquid-crystalline membrane phase transitions.

4.6.1 Phospholipid fatty acids

Since the cell membranes are composed of a mixture of phospholipid head groups, hydrocarbon chains, sterols and membrane-bound proteins, phase transition usually occurs over a wide range of temperatures. Hence, separate areas of gel and liquid-crystalline phases usually coexist in the same membrane. In the gel state (occurs at low temperatures) the fatty acid chains exist in a straight conformation, and are aligned at approximately right angles to the plane of the membrane, which limits the movement of the chains (Cossins, 1981; Murata and Yamaya, 1984). In this phase, the membrane becomes leaky to small electrolytes which negatively influence the selective permeability of the membrane to the detriment of physiological activities of the cell (Murata and Yamaya, 1984). However, in the liquid-crystalline state (occurs at higher temperature), the fatty acid chains are more flexible and rotate about their carbon-carbon bonds, which create free space that allows flexing and motion of adjacent chains (Cossins, 1981).

Furthermore, membranes containing only saturated fatty acids (e.g. C16:0 and C18:0), undergo the gel to liquid-crystalline phase transition above 40 °C (Murata and Nishida, 1990). However, membranes containing phospholipids with one mono-unsaturated and one saturated fatty acid have phase transition near 0 °C, while two mono-unsaturated fatty acids decrease the phase transition temperature to -20 °C. Double bonds, which are usually of the *cis*-configuration in plants, cause 'kinks' or bends in the fatty acid which influence the packing of the fatty acids in the membrane bilayer, and hence, help the membrane to stay fluid at low temperatures (Staehelin and Newcomb, 2000). Consequently, it has been found that the degree of phospholipid fatty acid saturation and oxidation of unsaturated fatty acids are strongly linked to CI incidence in chilling prone produce (Parkin and Kuo, 1989; Whitaker, 1995). Likewise, chilling tolerant plants accumulate polyunsaturated fatty acids (PUFA) - specifically C18:3n3 - in response to low temperatures through the upregulation of fatty acid desaturases (Mazliak, 1979; Los and Murata, 2004; Upchurch, 2008). It is hypothesized that low storage temperatures favours the synthesis of linolenic acid (C18:3) at the cost of oleic (C18:1) and linoleic (C18:2) acid (Mazliak, 1979; Cyril et al., 2002). During the unsaturation of fatty acids, stearic acid (C18:0) is desaturated to oleic acid (C18:1), which, in turn, is desaturated to linoleic acid (C18:2) which is desaturated to linolenic acid (C18:3). According to Mazliak (1979), low temperatures favour the accumulation of linolenic acid, while higher temperatures favour the accumulation of oleic and linoleic acid.

In this study there was an increase in linolenic acid levels after 28 days for H1 and 35 days for H2 of storage at -0.5 °C. These increases followed a decrease in the monounsaturated fatty acids (MUFA), oleic and elaidic acid, after 21 days for H1 and 14 days for H2 fruit of storage at -0.5 °C.

The increase in C18:3n3 at -0.5 °C followed approximately 1 to 2 weeks after the manifestation of CI at 21 days of storage. According to Nishida and Murata (1996) it is critical for membrane lipids to become unsaturated at low temperatures in order for their proper functioning, and hence, the survival of the organism. In this study H2 fruit had a significantly lower unsaturated:saturated fatty acid ratio compared to the H1 fruit while stored at -0.5 °C. This could have had a detrimental effect on the fluidity of the membranes of the H2 fruit, causing it to become more permeable, and more prone to develop CI.

Moreover, H2 fruit also had a significantly lower MUFA:PUFA ratio than the H1 fruit at -0.5 °C. This result agrees with the finding of Beltrán et al. (2004) that more mature olives have a lower MUFA:PUFA ratio than less mature fruit. In virgin olive oils the oleic/linoleic ratios are responsible for the oxidative stability of the oil. The reason for this is that monounsaturated fatty acids (MUFAs), such as oleic acid, are more resistant to oxidation than polyunsaturated fatty acids (PUFAs), e.g. linolenic acid. Based on peroxide formation it has consequently been determined that the oxidation ratio for oleate, linoleate and linolenate is 1:12:25 (Aparicio, et al., 1999). Therefore, it has been found that olive oil is more stable when oleic acid content is high and linoleic content is low. In this study, H1 fruit had a significantly higher MUFA:PUFA ratio at -0.5 °C as well as significantly lower CI incidence than the H2 fruit. Based on the olive oil research, it is possible that the higher MUFA:PUFA ratio caused the H1 membranes to be more resistant to oxidative stress than the H2 fruit. Therefore, the relatively high levels of saturated fatty acids, which caused the membranes to be less fluid, as well as a lower MUFA:PUFA ratio, which caused the membranes to be more prone to oxidative stress at -0.5 °C, probably caused the H2 fruit to develop higher levels of CI than the H1 fruit. However, it should also be noted that the H1 fruit also did not have optimal levels of linolenic acid and/or MUFA:PUFA ratio, since it also developed CI, albeit at lower levels. It will be interesting to determine the linolenic acid and MUFA:PUFA ratio in fruit of different maturities stored with the commercially used dual-temperature regime which reduces CI in plum fruit to a great extent.

4.6.2 Total phospholipid and total sterol concentrations

Cold acclimation of plant tissues usually also involves an increase in the membrane phospholipid concentration (Staehelin and Newcomb, 2000). It is also known that free sterols increase membrane fluidity below the phase transition temperature (because its aliphatic tail is mobile and causes a certain degree of disorder in the hydrophobic part of the membrane), and decreases the fluidity above the phase transition temperature (because the steroid skeleton is rigidly planar) (Bloch, 1985; Leshem, 1992; Marangoni et al., 1996). Therefore, sterols stabilise membrane

fluidity over a wide range of temperatures (Marangoni et al., 1996). The effect of sterols on membrane fluidity is usually due to changes in the sterol:phospholipid ratio, and therefore indirect (Bloch, 1985). In this study the total phospholipid concentration started to increase after 3 weeks of storage at -0.5 °C in both fruit maturities, which indicates that the membranes attempted to acclimate to the low storage temperature. Simultaneously, the total sterol concentration remained relatively constant in both harvest maturities at -0.5 °C, which caused the sterol:phospholipid ratio to decrease with an increase in storage duration (data not shown; Refer to Appendix B, Fig. 3). Since less sterols were available in relation to the phospholipids, the membranes would have been less fluid, causing more gel phases. However, at the same time the sterol:phospholipid ratio started to decrease, linolenic acid levels were increased, albeit to a greater extent in H1 than H2 fruit. It is, therefore, clear that complex changes took place in the membranes to adapt the membrane fluidity to the lower storage temperatures. Whitaker (1988) found that sterol content increased as tomato fruit ripened. This could be the reason why the H2 fruit in this study, which was harvested more mature, had higher levels of total sterols than the H1 fruit. However, the H2 fruit also had lower levels of C18:3n3 and a significantly lower unsaturated:saturated fatty acid ratio in their membranes than the H1 fruit, which could have caused the H2 cell membranes to be less fluid, and therefore, more prone to leak at -0.5 °C.

5. Conclusion

From the results obtained in this study, it can be concluded that CI incidence was higher in the more mature than the less mature fruit. CI also increased with an increase in storage duration and was higher after a simulated shelf-life period of 7 days at 10 °C. Therefore, to reduce CI incidence, ‘Sapphire’ plums must be harvested less mature (> 60.0 N) and not be stored for extended durations (> 21 days) at chilling temperatures.

More mature fruit also had higher levels of shrivel than less mature fruit, probably due to inadequate removal of respiratory heat from the fruit. Since moisture stress induces higher levels of C_2H_4 to be produced, which accelerates senescence and membrane deterioration, a postharvest strategy will have to be found to reduce moisture loss from ‘Sapphire’ plums. Such a strategy may include shrivel sheets or bags made from other plastic materials and/or with larger perforations, or by reducing the respiration rate by e.g. treating the fruit with 1-methylcyclopropene (1-MCP).

Ethylene evolution rates, together with CI incidence, were higher in the more mature fruit at 10 °C than in the less mature fruit. It is known that low temperatures and moisture stress stimulate C_2H_4

production. C_2H_4 causes the cell membranes to be more permeable, and its higher levels within the more mature fruit could have enhanced the incidence of CI. It is, therefore, recommended that postharvest treatments to reduce moisture loss and/or reducing C_2H_4 evolution (e.g. by treating the fruit with 1-MCP) in the more mature fruit must be investigated to determine if it reduces CI in 'Sapphire' plums.

HAA levels were lower in the more mature fruit, and, therefore, had less capacity to scavenge for AOS caused by the chilling temperatures. The more mature fruit also had lower ascorbic acid levels than the H1 fruit, again indicating the poorer AOS scavenging capacity of the H2 fruit. The fast decrease in ascorbic acid levels during the first 14 days of storage in fruit of both maturities also indicated that the fruit was experiencing severe stress. Of all the parameters measured, it was the first to indicate that the fruit was experiencing stress. It, therefore, could have acted as a signal of chilling stress. Glutathione levels also increased in H1 fruit while stored at -0.5 °C, while levels remained relatively constant in the H2 fruit. Again, the data indicated that the H1 fruit was better equipped to scavenge for AOS than the H2 fruit. Since the less mature fruit had higher levels of antioxidants than the H2 fruit, it could better prevent damage by AOS, and, consequently, had a lower incidence of CI.

The more mature fruit had less fluid membranes due to higher levels of saturated fatty acids. This situation is strongly linked to CI incidence. Furthermore, the H2 fruit had a lower MUFA:PUFA ratio than the H1 fruit. This indicates that the membranes of the H2 fruit were more prone to oxidative stress. Concurrent with membranes being more vulnerable to oxidative stress, the H2 fruit had lower levels of antioxidants to quench the free radicals caused by the chilling stress. However, the higher levels of antioxidants and linolenic acid, as well as the higher unsaturated:saturated and MUFA:PUFA ratios in H1 fruit, did not prevent the fruit from developing CI, albeit at much lower levels than the H2 fruit. It would, therefore, be interesting to repeat this trial using the commercially used dual-temperature regime to determine why it renders the fruit less susceptible to CI. However, other treatments such as conditioning the fruit at room temperature or low, but not chilling temperatures, or a high temperature treatment before storage should also be tested. This will give an indication if the fruit are able to adapt their fatty acid ratios and antioxidant levels to survive storage at chilling temperatures.

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Table 2.1

Harvest maturity of ‘Sapphire’ plums on the harvest dates in the 2005 and 2007 seasons.

Harvest maturity	Hue angle	Flesh firmness (N*)	TSS (% Brix)	TA (%)	C_2H_4 (µL kg^{-1} h^{-1})
2005					
H1	113.67a	86.04a	12.63a	2.04a	0.04
H2	53.99b	58.21b	11.03b	1.76b	0.02
P-value	***<0.0001***	***<0.0001***	***0.0022***	***0.0036***	*0.6201*
LSD (P≤0.05)	*11.16*	*4.90*	*0.71*	*0.14*	*0.12*
2007					
H1	63.16a	69.38a	11.23	1.61a	0.00a
H2	27.48b	52.92b	11.85	1.40b	0.24b
P-value	***0.0065***	***0.0063***	*0.0703*	***0.0003***	***0.0129***
LSD (P≤0.05)	*20.48*	*9.02*	*0.69*	*0.06*	*0.16*

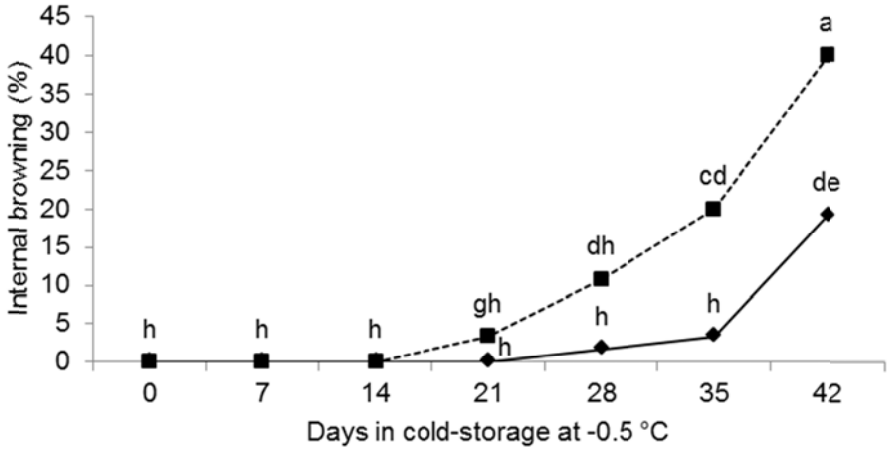
* To convert Newton to kilogram, divide by 9.80.

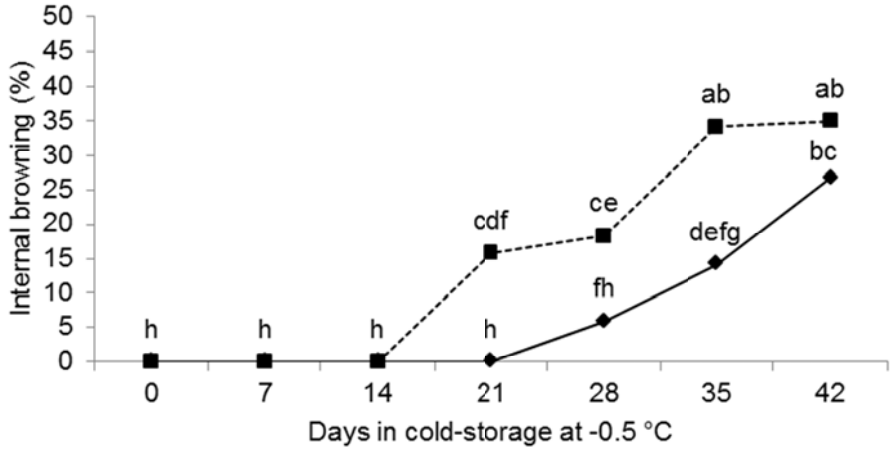
Table 2.2

Correlation of chilling injury with variables determined on each evaluation date during storage and after shelf-life. The Spearman r-values and corresponding *p*-values indicate the correlation and significance, respectively, between CI incidence and the different variables measured.

Variable*	Correlation value r (Spearman)	*p*-value
Shrivel (%)	0.33	**<0.01**
Hue angle	-0.16	**<0.01**
Flesh firmness (kg)	-0.37	**<0.01**
C_2H_4 evolution (μL kg^{-1} h^{-1})	0.29	**<0.01**
HAA (mg Trolox equivalents g^{-1} FW)	-0.21	**<0.01**
Total ascorbic acid (μg g^{-1} FW)	-0.23	**<0.01**
L-AA (μg g^{-1} FW)	-0.31	**<0.01**
DHA (μg g^{-1} FW)	0.26	**<0.01**
Total glutathione (μg g^{-1} FW)	0.18	**<0.01**
GSH (μg g^{-1} FW)	0.23	**<0.01**
Total sterols (μmol g^{-1} FW)	0.16	**<0.01**
C18:1n9c (μg g^{-1} FW)	-0.23	**<0.01**
C18:1n9t (μg g^{-1} FW)	-0.17	**<0.01**
C18:3n3 (μg g^{-1} FW)	0.18	**<0.01**
Saturated fatty acids (%)	-0.14	**0.01**
Unsaturated fatty acids (%)	0.14	**0.01**
Unsaturated:Saturated fatty acids	0.14	**0.01**
Monounsaturated:polyunsaturated fatty acids	-0.25	**0.00**
GSSG (μg g^{-1} FW)	0.12	**0.03**
Total phospholipids (μmol g^{-1} FW)	0.12	**0.03**
C18:0 (μg g^{-1} FW)	0.10	0.07
Lipid peroxidation (nmol MDA equivalents ml^{-1} g^{-1} FW)	0.06	0.27
C18:2n6c (μg g^{-1} FW)	0.03	0.54
C16:0 (μg g^{-1} FW)	-0.03	0.60
Total fatty acids (μg g^{-1} FW)	0.03	0.63
Total phenolic content (mg GAE g^{-1} FW)	-0.02	0.72
Decay (%)	0.01	0.83
GSH:GSSG	0.01	0.86
Total sterols:Total phospholipids	0.01	0.86
LAA (mg Trolox equivalents g^{-1} FW)	-0.00	0.93

* Variable abbreviations are as follows: HAA = water soluble radical-scavenging activity, L-AA = reduced ascorbic acid, DHA = oxidised ascorbic acid, GSH = reduced glutathione, C18:1n9c = oleic acid, C18:1n9t = elaidic acid, C18:3n3 = linolenic acid, GSSG = oxidised glutathione, C18:2n6c = linoleic acid, C16:0 = palmitic acid, LAA = lipid soluble radical scavenging activity.



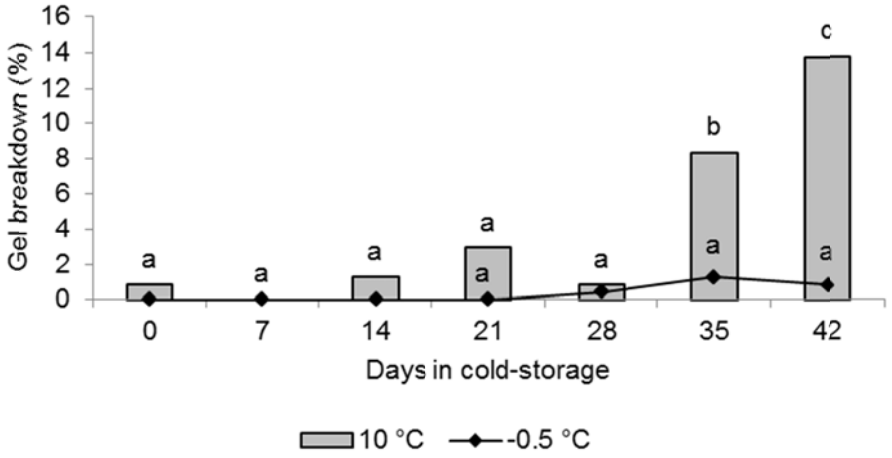


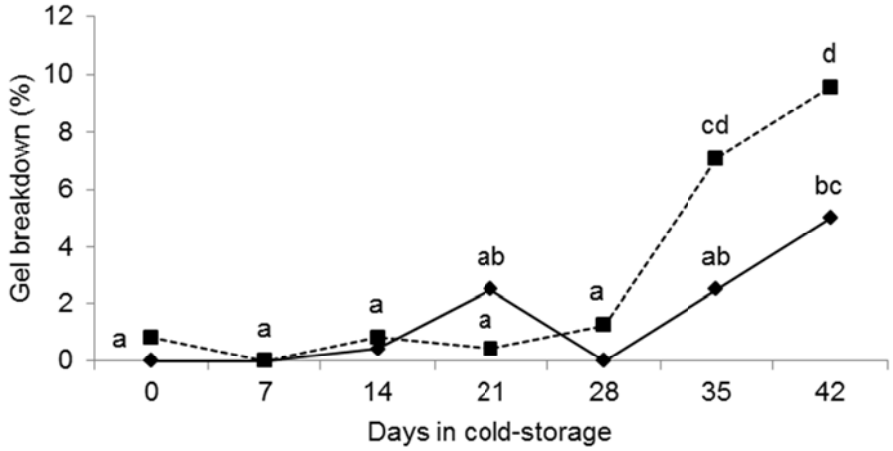
(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	12.16	0.0058
Storage temperature	12.80	0.0050
Storage duration	18.40	0.0000
Harvest maturity x Storage temperature	0.21	0.6541
Harvest maturity x Storage duration	2.04	0.0729
Storage temperature x Storage duration	4.18	0.0014
Harvest maturity x Storage temperature x Storage duration	2.53	**0.0298**

Fig. 2.1. Manifestation of internal browning in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the incidence of IB during simulated shelf-life at 10 °C is presented at the same time-point as the IB incidence levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.

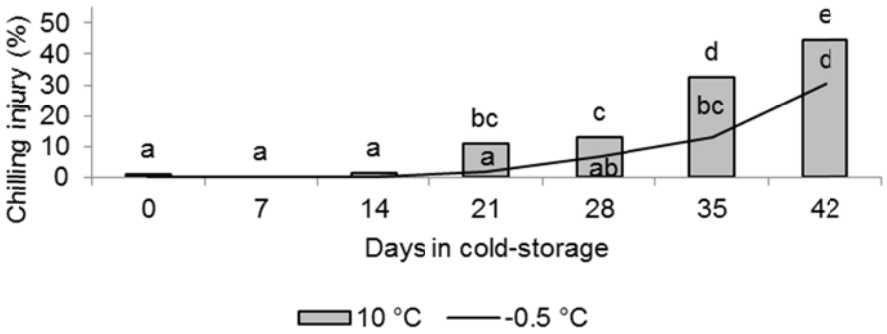




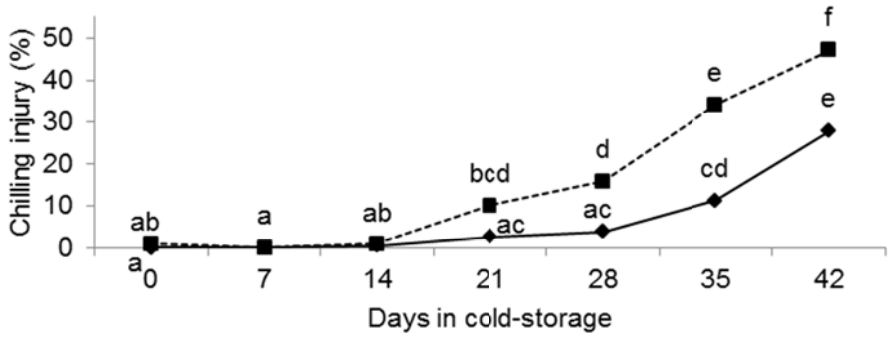
(A) **(B)**

Effect	F	*p*
Harvest maturity	4.05	0.0718
Storage temperature	31.95	0.0002
Storage duration	13.25	0.0000
Harvest maturity x Storage temperature	1.03	0.3320
Harvest maturity x Storage duration	2.56	**0.0283**
Storage temperature x Storage duration	10.01	**0.0000**
Harvest maturity x Storage temperature x Storage duration	1.12	0.3595

Fig. 2.2. (A) Manifestation of gel breakdown (GB) in 'Sapphire' plums as influenced by storage duration and storage temperature. For clarity, the manifestation of GB during simulated shelf-life at 10 °C is presented at the same time-point as the GB levels that manifested in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later. (B) Manifestation of GB in 'Sapphire' plums as influenced by harvest maturity and storage duration. H1 (———) and H2 (- - - - -).



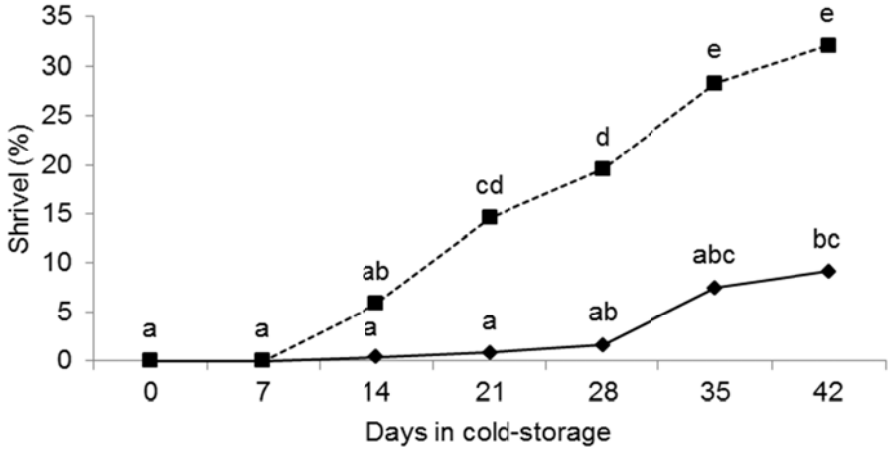
(A)



(B)

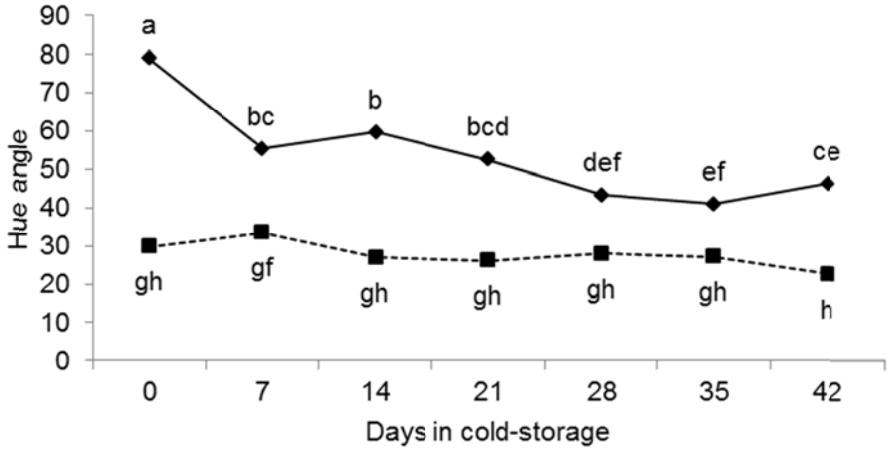
Main effect P ≤ 0.05	Covariate	Harvest maturity	Storage temp.	Storage duration	Harvest maturity x Storage temp.	Harvest maturity x Storage duration	Storage temp. x Storage duration	Harvest maturity x Storage temp. x Storage duration
Without covariate	-	0.0017	0.0000	0.0000	0.2433	**0.0033**	**0.0000**	0.3672
With single analysed covariates								
Shrivel (%)[x]	**0.0000**	0.0000	0.0000	0.0000	**0.0497**	**0.0000**	**0.0000**	0.0594
C_2H_4 (µL kg^{-1} h^{-1}) [x]	**0.0226**	0.0045	0.0027	0.0000	0.9856	**0.0050**	**0.0000**	0.4072
Unsaturated:Saturated fatty acid ratio [x]	**0.0267**	0.0012	0.0000	0.0000	0.3563	**0.0013**	**0.0000**	0.2655

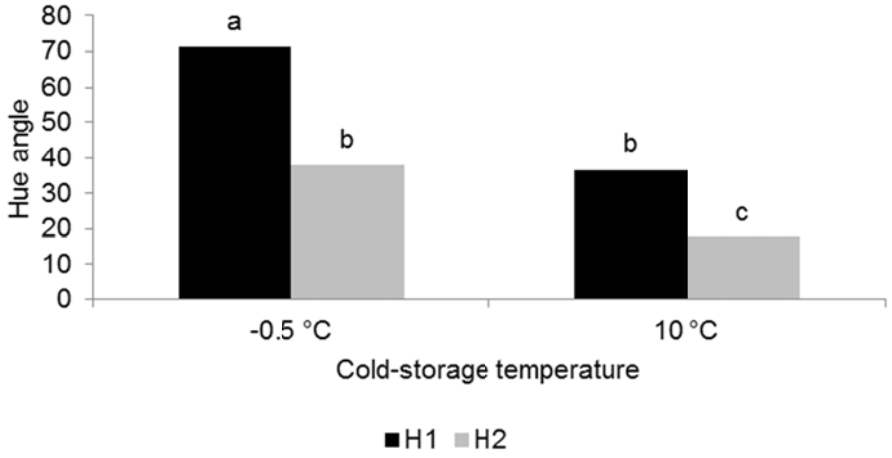
Fig. 2.3. (A) Manifestation of chilling injury (CI) in 'Sapphire' plums as influenced by storage duration and storage temperature. In this graph the manifestation of CI during simulated shelf-life at 10 °C is presented at the same time-point as the CI levels that manifested in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later. (B) Manifestation of CI in 'Sapphire' plums as influenced by storage duration and harvest maturity. H1 (———) and H2 (-- - - -). [X] With variable as a single covariate.



Effect	F	*p*
Harvest maturity	26.96	0.0004
Storage temperature	3.21	0.1033
Storage duration	16.16	0.0000
Harvest maturity x Storage temperature	3.57	0.0877
Harvest maturity x Storage duration	5.51	**0.0001**
Storage temperature x Storage duration	1.80	0.1140
Harvest maturity x Storage temperature x Storage duration	1.98	0.0822

Fig. 2.4. Manifestation of shrivel in 'Sapphire' plums as influenced by storage duration and harvest maturity. H1 (———) and H2 (-- - - -).

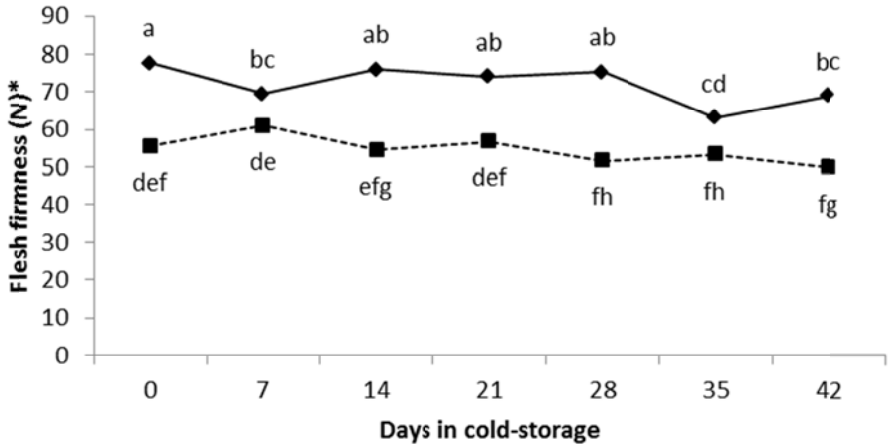


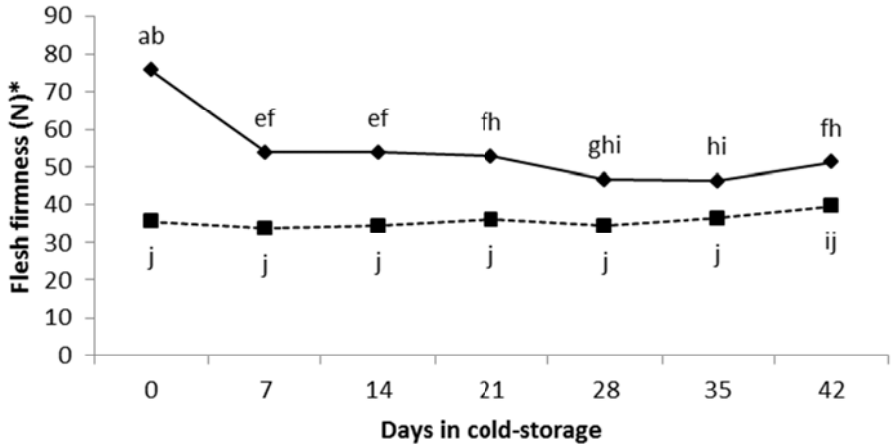


(A) **(B)**

Effect	F	*p*
Harvest maturity	88.72	0.0000
Storage temperature	102.83	0.0000
Storage duration	10.28	0.0000
Harvest maturity x Storage temperature	7.25	**0.0225**
Harvest maturity x Storage duration	6.98	**0.0000**
Storage temperature x Storage duration	1.63	0.1523
Harvest maturity x Storage temperature x Storage duration	1.76	0.1226

Fig. 2.5. Change in hue angle of 'Sapphire' plums as influenced by (A) storage duration and harvest maturity, and (B) storage temperature and harvest maturity. H1 (———) and H2 (-- - - -).



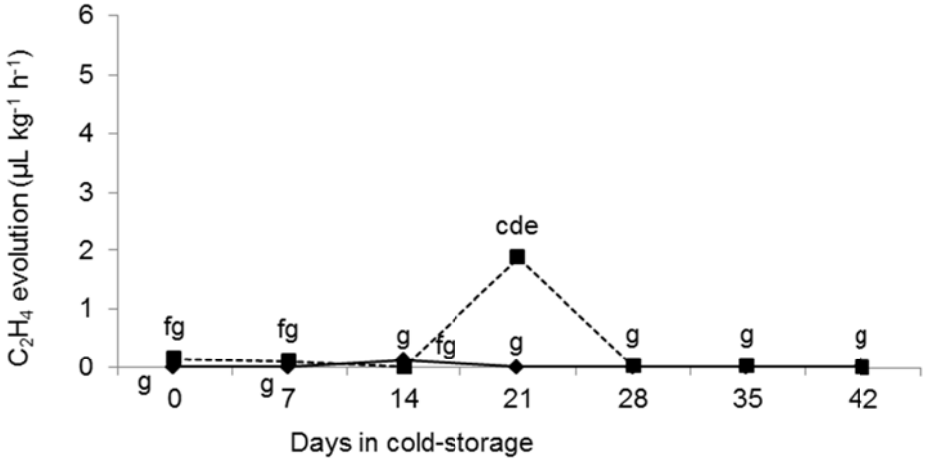


(A) [1] **(B)**[1]

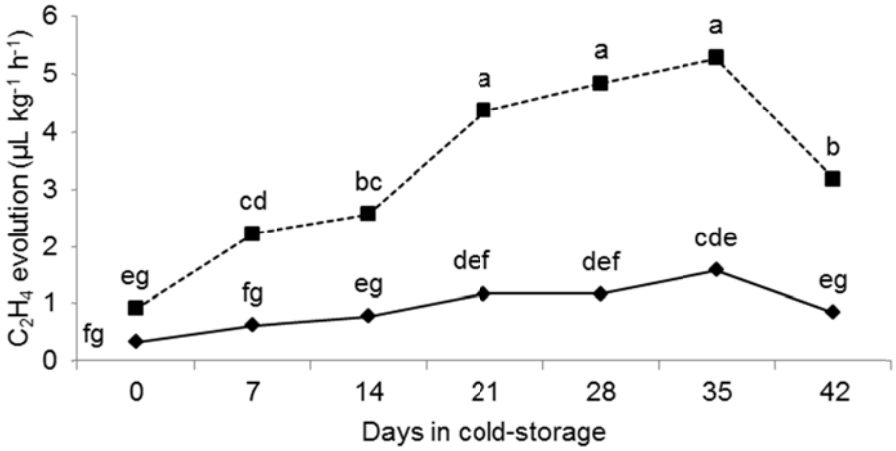
Effect	F	*p*
Harvest maturity	109.86	0.0000
Storage temperature	151.80	0.0000
Storage duration	5.76	0.0000
Harvest maturity x Storage temperature	0.15	0.7032
Harvest maturity x Storage duration	5.00	0.0003
Storage temperature x Storage duration	5.82	0.0000
Harvest maturity x Storage temperature x Storage duration	7.61	**0.0000**

Fig. 2.6. Change in flesh firmness of 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (——) and H2 (- - - - -).
* To convert Newton to kilogram, divide by 9.80.

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the flesh firmness during simulated shelf-life at 10 °C is presented at the same time-point as the flesh firmness that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



(A) [1]

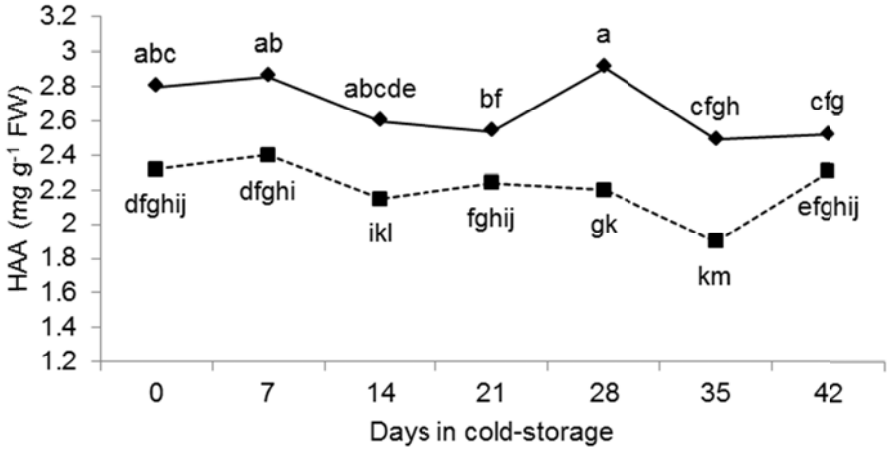


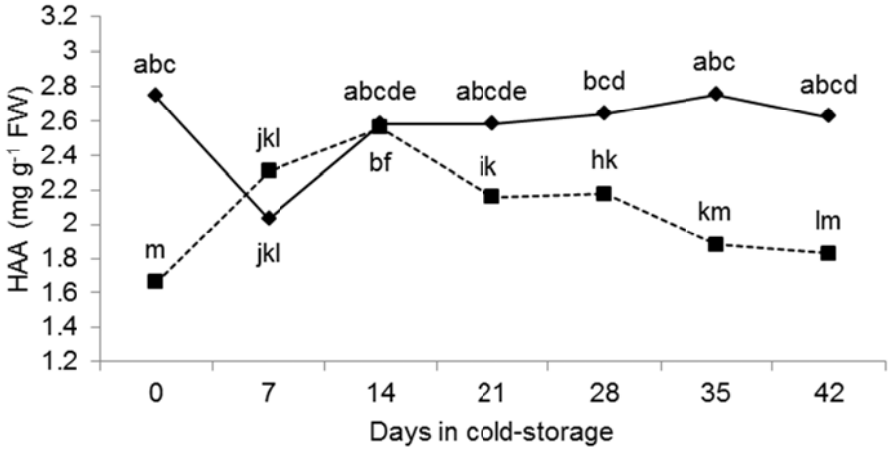
(B)[1]

Effect	F	*p*
Harvest maturity	29.29	0.0002
Storage temperature	9.41	0.0000
Storage duration	9.41	0.0000
Harvest maturity x Storage temperature	20.46	0.0011
Harvest maturity x Storage duration	4.31	0.0011
Storage temperature x Storage duration	10.98	0.0000
Harvest maturity x Storage temperature x Storage duration	4.25	**0.0012**

Fig. 2.7. C_2H_4 evolution rates in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity C_2H_4 evolution rates during simulated shelf-life at 10 °C is presented at the same time-point as the C_2H_4 evolution rates that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



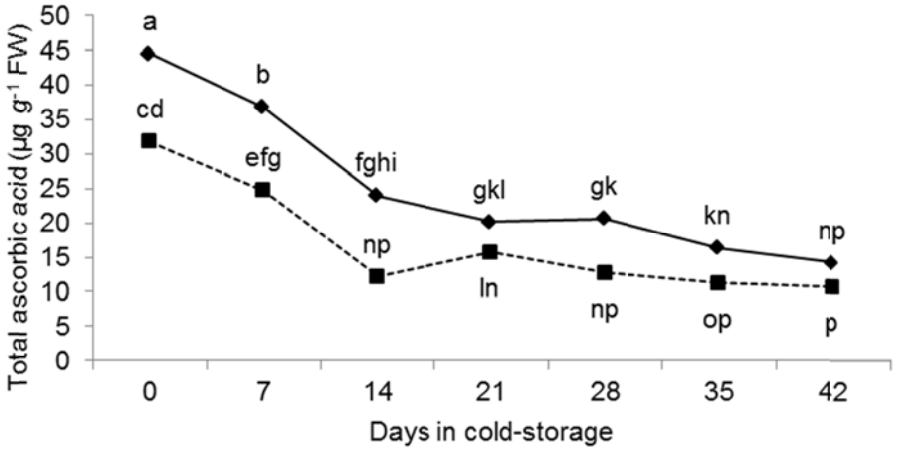


(A) [1] **(B)**[1]

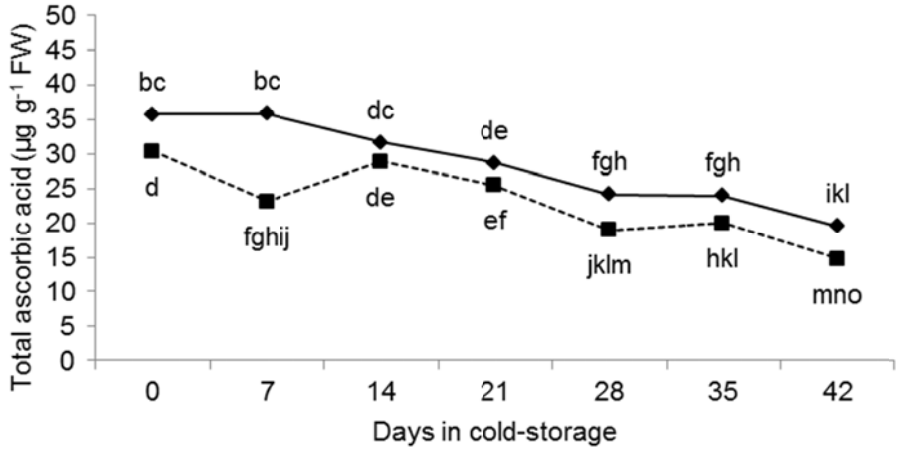
Effect	F	*p*
Harvest maturity	29.29	0.0002
Storage temperature	9.41	0.0000
Storage duration	9.41	0.0000
Harvest maturity x Storage temperature	20.46	0.0011
Harvest maturity x Storage duration	4.31	0.0011
Storage temperature x Storage duration	10.98	0.0000
Harvest maturity x Storage temperature x Storage duration	4.25	**0.0012**

Fig. 2.8. Water soluble antioxidant activity (HAA) in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the HAA during simulated shelf-life at 10 °C is presented at the same time-point as the HAA that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



(A) [1]

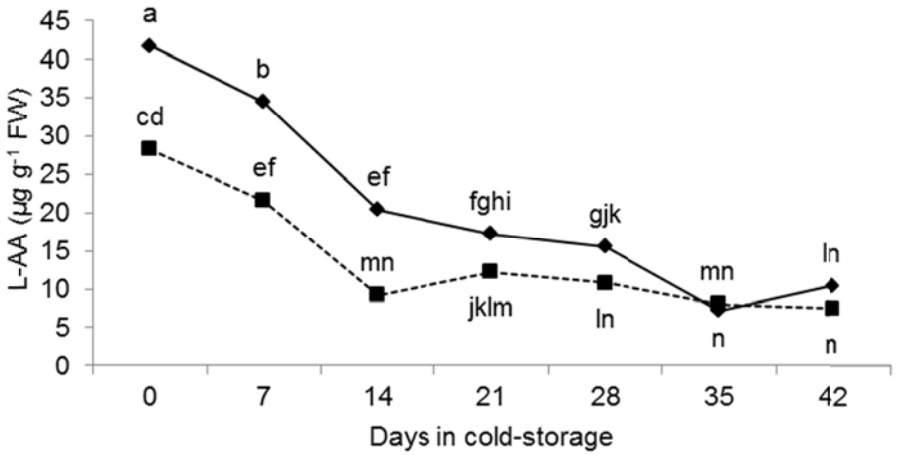


(B)[1]

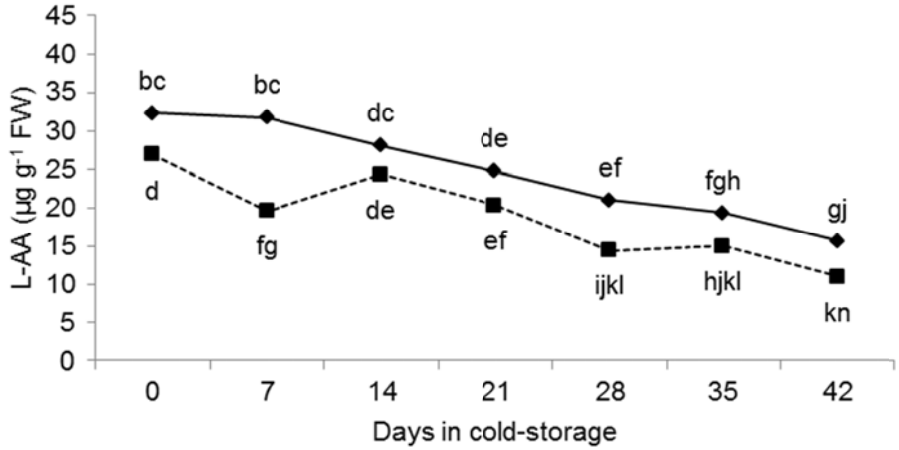
Effect	F	*p*
Harvest maturity	62.49	0.0000
Storage temperature	51.46	0.0000
Storage duration	64.14	0.0000
Harvest maturity x Storage temperature	4.65	0.0563
Harvest maturity x Storage duration	2.91	0.0145
Storage temperature x Storage duration	21.98	0.0000
Harvest maturity x Storage temperature x Storage duration	2.35	**0.0416**

Fig. 2.9. Total ascorbic acid levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the total ascorbic acid levels during simulated shelf-life at 10 °C is presented at the same time-point as the total ascorbic acid levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



(A) [1]

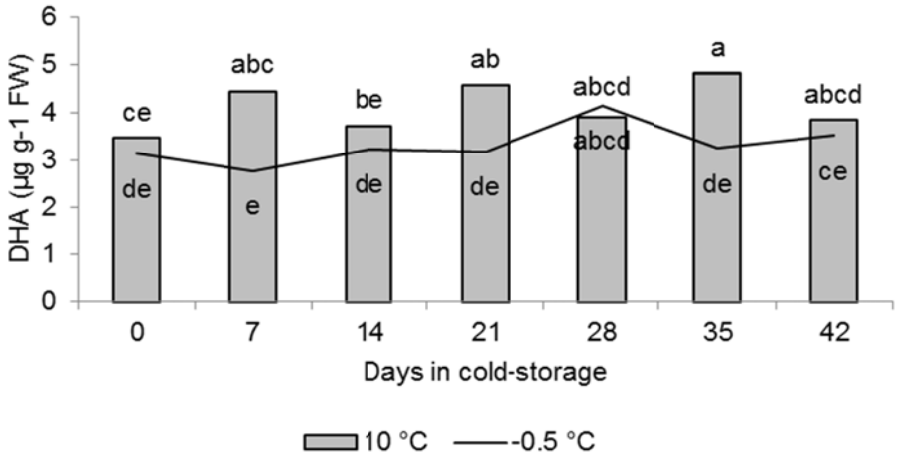


(B)[1]

Effect	F	*p*
Harvest maturity	64.69	0.0000
Storage temperature	49.43	0.0000
Storage duration	66.95	0.0000
Harvest maturity x Storage temperature	0.89	0.3672
Harvest maturity x Storage duration	3.73	0.0031
Storage temperature x Storage duration	24.26	0.0000
Harvest maturity x Storage temperature x Storage duration	3.89	**0.0023**

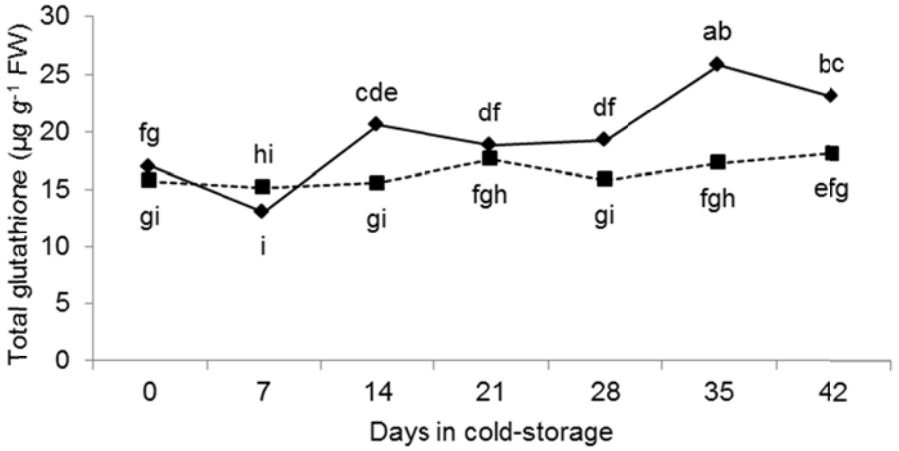
Fig. 2.10. L-AA levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

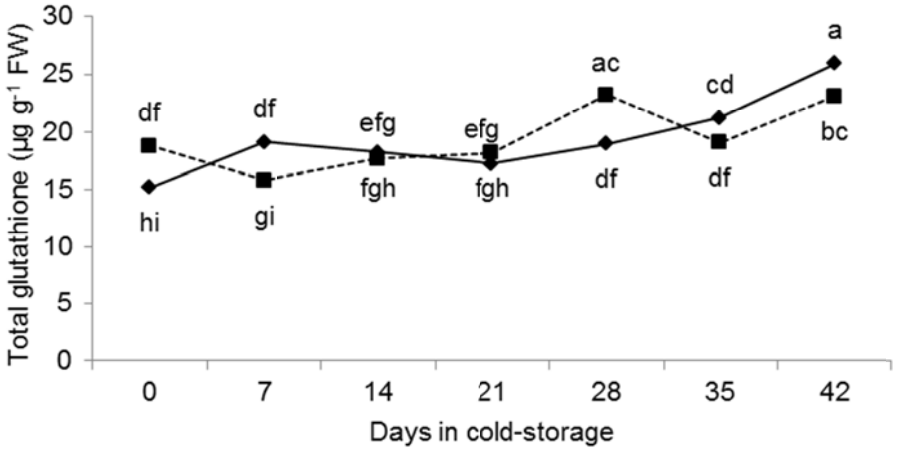
[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the L-AA levels during simulated shelf-life at 10 °C is presented at the same time-point as the L-AA levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



Effect	F	*p*
Harvest maturity	203.69	0.0000
Storage temperature	14.64	0.0033
Storage duration	1.04	0.4068
Harvest maturity x Storage temperature	3.80	0.0798
Harvest maturity x Storage duration	0.79	0.5801
Storage temperature x Storage duration	2.30	**0.0451**
Harvest maturity x Storage temperature x Storage duration	1.53	0.1813

Fig. 2.11. DHA levels in 'Sapphire' plums as influenced by storage duration and storage temperature. In this graph the DHA levels during simulated shelf-life at 10 °C are presented at the same time-point as the DHA levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



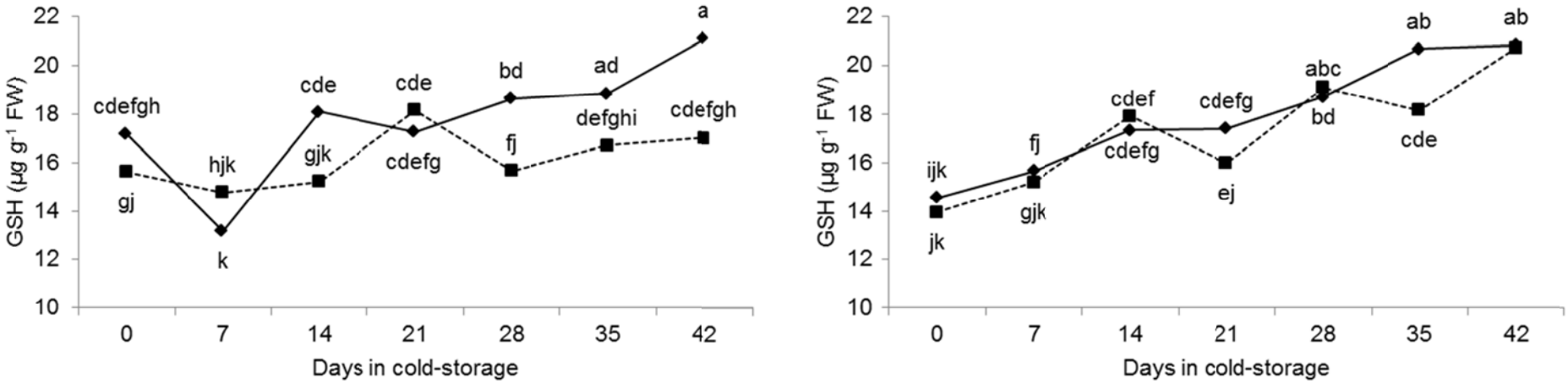


(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	14.20	0.0036
Storage temperature	11.43	0.0069
Storage duration	22.97	0.0000
Harvest maturity x Storage temperature	16.31	0.0023
Harvest maturity x Storage duration	6.11	0.0000
Storage temperature x Storage duration	5.00	0.0003
Harvest maturity x Storage temperature x Storage duration	4.84	**0.0004**

Fig. 2.12. Total glutathione levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the total glutathione levels during simulated shelf-life at 10 °C is presented at the same time-point as the total glutathione levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.

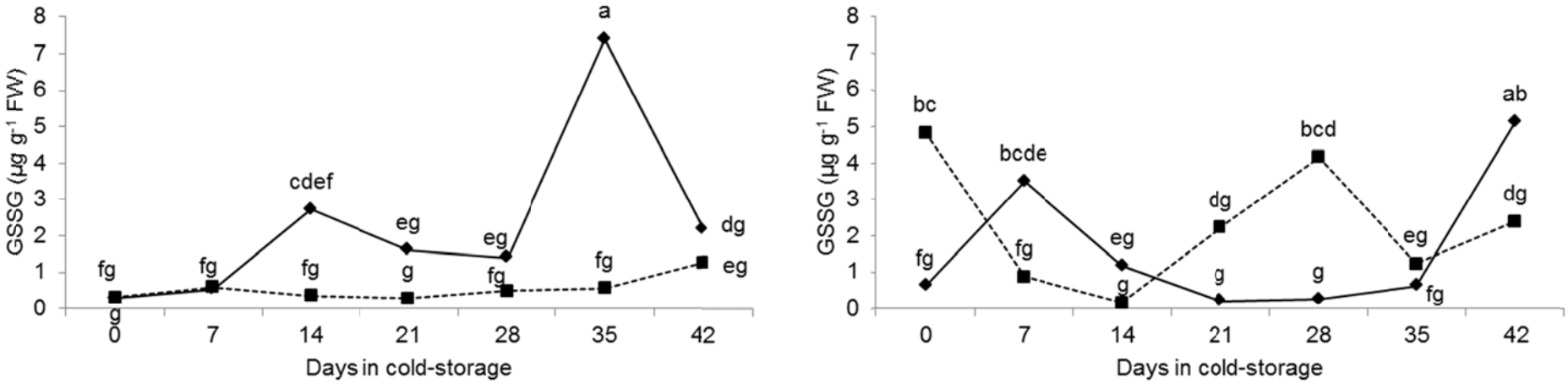


(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	4.63	0.0568
Storage temperature	4.33	0.0639
Storage duration	19.34	0.0000
Harvest maturity x Storage temperature	2.96	0.1159
Harvest maturity x Storage duration	1.50	0.1932
Storage temperature x Storage duration	4.03	0.0018
Harvest maturity x Storage temperature x Storage duration	2.94	**0.0138**

Fig. 2.13. GSH levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the GSH levels during simulated shelf-life at 10 °C is presented at the same time-point as the GSH levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.

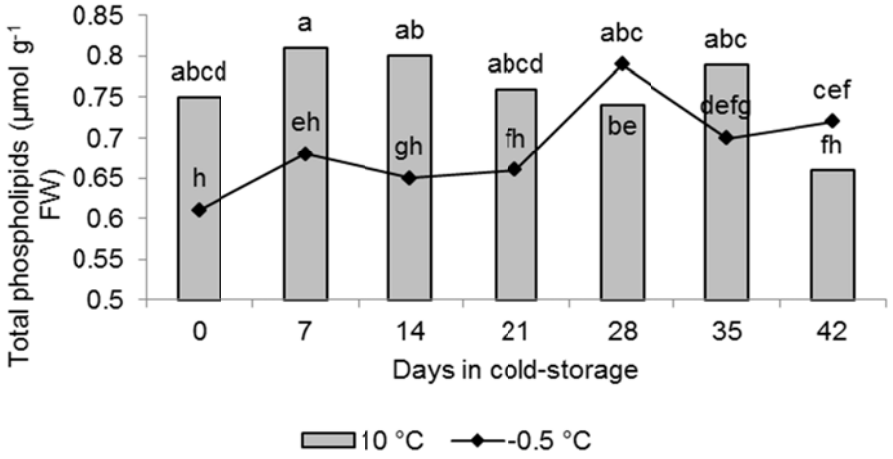


(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	2.59	0.1381
Storage temperature	2.38	0.1536
Storage duration	2.47	0.0334
Harvest maturity x Storage temperature	11.69	0.0065
Harvest maturity x Storage duration	5.26	0.0002
Storage temperature x Storage duration	5.38	0.0001
Harvest maturity x Storage temperature x Storage duration	4.83	**0.0004**

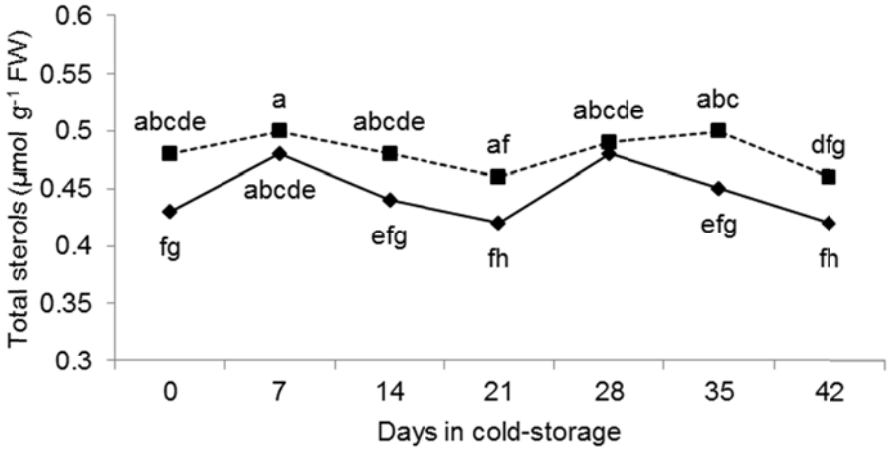
Fig. 2.14. GSSG levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

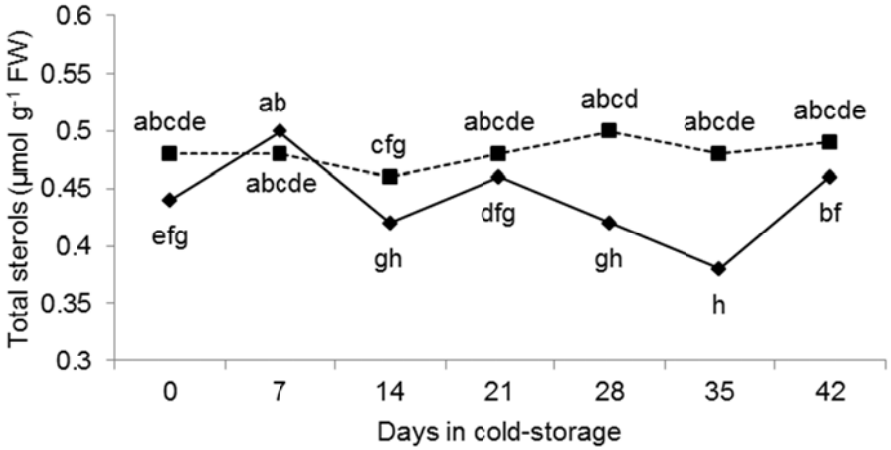
[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the GSSG levels during simulated shelf-life at 10 °C is presented at the same time-point as the GSSG levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



Effect	F	*p*
Harvest maturity	0.40	0.5388
Storage temperature	21.04	0.0009
Storage duration	3.31	0.0069
Harvest maturity x Storage temperature	0.29	0.6012
Harvest maturity x Storage duration	1.46	0.2045
Storage temperature x Storage duration	7.54	**0.0000**
Harvest maturity x Storage temperature x Storage duration	0.85	0.5322

Fig. 2.15. Total phospholipid levels in 'Sapphire' plums as influenced by storage duration and storage temperature. In this graph the total phospholipid levels during simulated shelf-life at 10 °C are presented at the same time-point as the total phospholipid levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



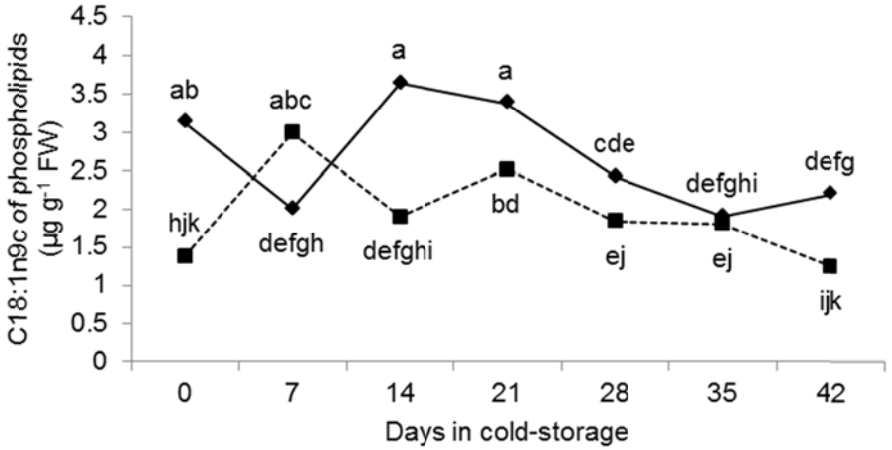


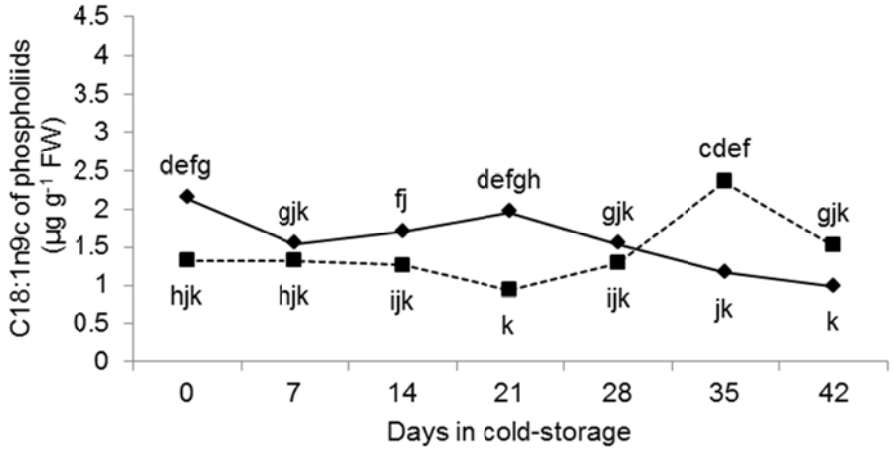
(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	45.11	0.0000
Storage temperature	0.81	0.3865
Storage duration	2.99	0.0125
Harvest maturity x Storage temperature	0.05	0.8155
Harvest maturity x Storage duration	2.21	0.5354
Storage temperature x Storage duration	3.51	0.0048
Harvest maturity x Storage temperature x Storage duration	2.30	**0.0457**

Fig. 2.16. Total sterol levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the total sterol levels during simulated shelf-life at 10 °C is presented at the same time-point as the total sterol levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



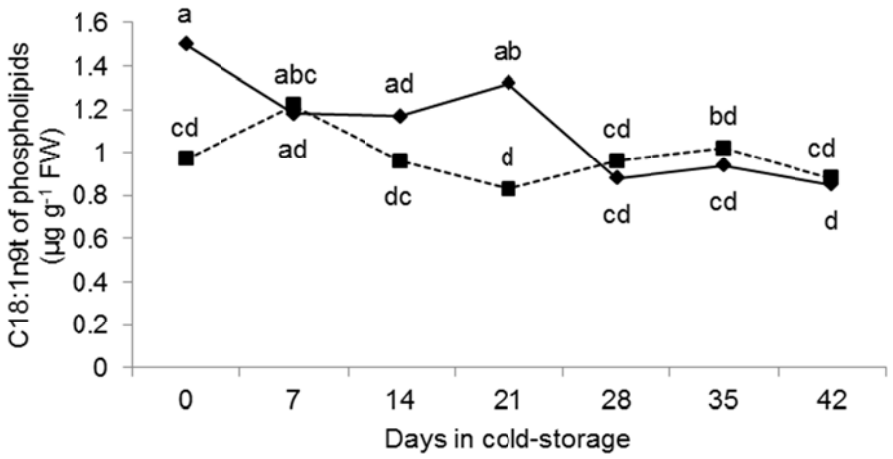


(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	22.09	0.0008
Storage temperature	79.34	0.0000
Storage duration	4.12	0.0015
Harvest maturity x Storage temperature	9.64	0.0111
Harvest maturity x Storage duration	8.95	0.0000
Storage temperature x Storage duration	4.29	0.0011
Harvest maturity x Storage temperature x Storage duration	4.13	**0.0015**

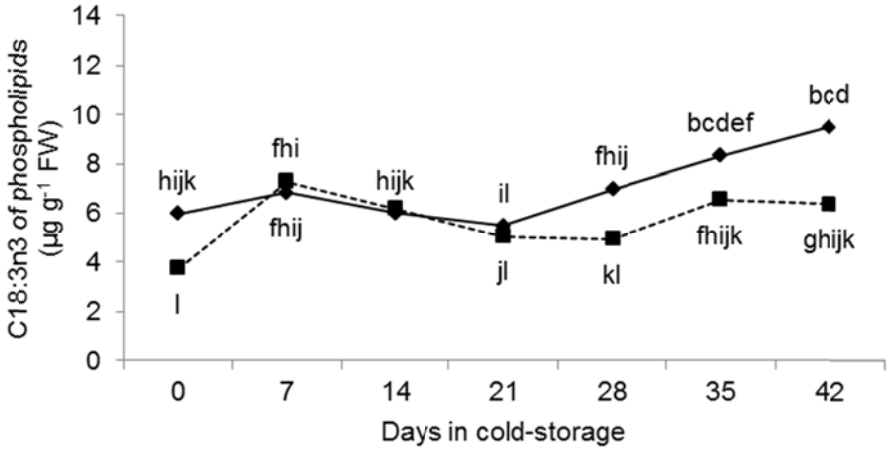
Fig. 2.17. Phospholipid oleic acid levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

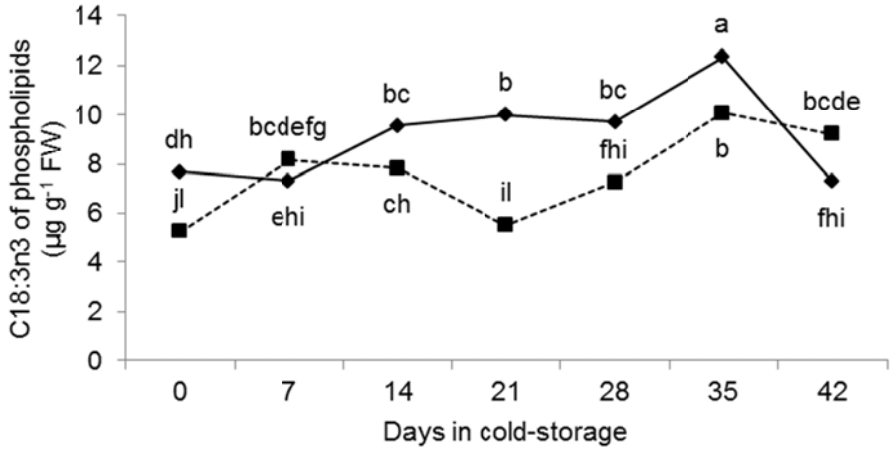
[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the oleic acid levels during simulated shelf-life at 10 °C is presented at the same time-point as the oleic acid levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



Effect	F	*p*
Harvest maturity	4.45	0.0609
Storage temperature	17.55	0.0018
Storage duration	2.38	0.0395
Harvest maturity x Storage temperature	0.33	0.5765
Harvest maturity x Storage duration	2.36	**0.0410**
Storage temperature x Storage duration	1.31	0.2626
Harvest maturity x Storage temperature x Storage duration	1.85	0.1031

Fig. 2.18. Elaidic acid levels in 'Sapphire' plums as influenced by harvest maturity and storage duration. H1 (———) and H2 (-- - - -).



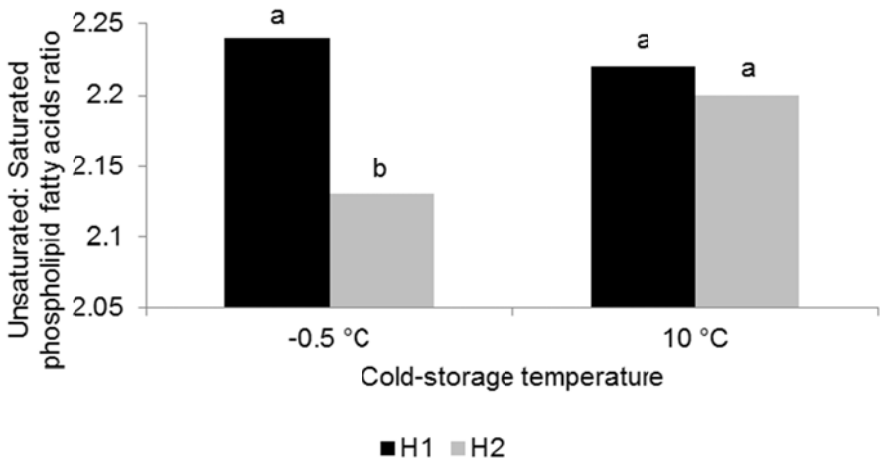


(A) [1] **(B)**[1]

Effect	F	*p*
Harvest maturity	15.92	0.0025
Storage temperature	64.96	0.0000
Storage duration	12.28	0.0000
Harvest maturity x Storage temperature	0.18	0.6739
Harvest maturity x Storage duration	3.17	0.0089
Storage temperature x Storage duration	3.34	0.0065
Harvest maturity x Storage temperature x Storage duration	4.52	**0.0007**

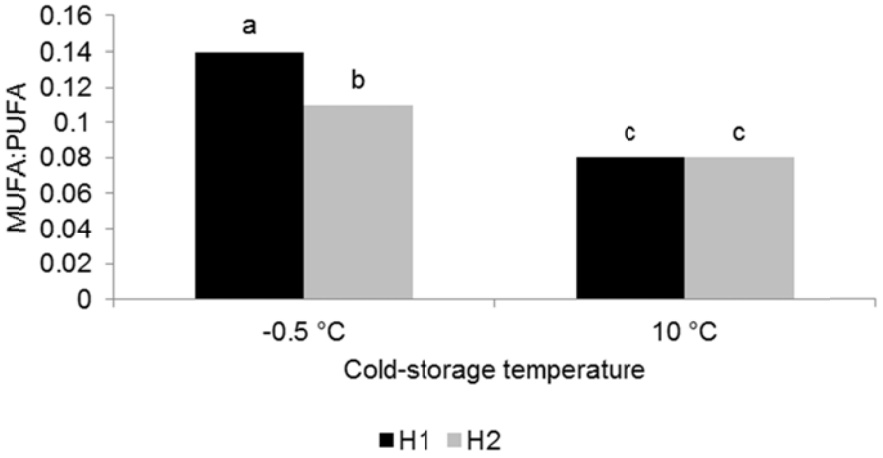
Fig. 2.19. Phospholipid linolenic acid levels in 'Sapphire' plums as influenced by harvest maturity, storage duration and storage temperature. (A) Fruit stored at -0.5 °C and (B) fruit stored at -0.5 °C plus a subsequent simulated shelf-life of 7 days at 10 °C. H1 (———) and H2 (- - - - -).

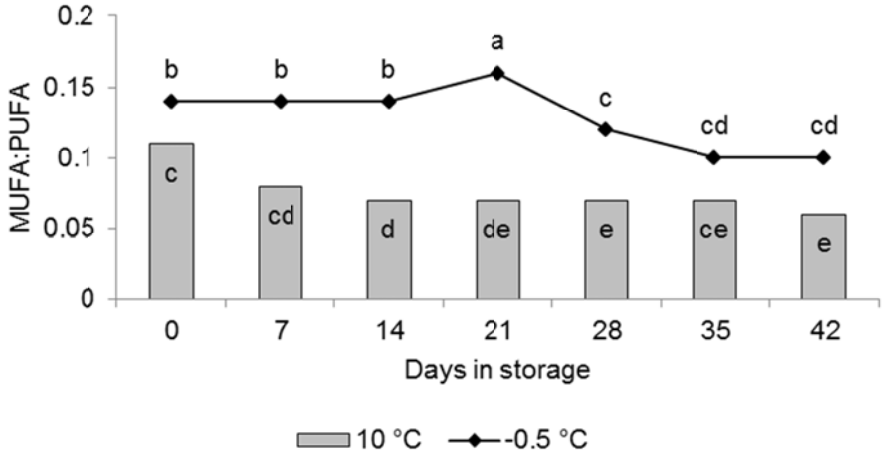
[1] In order to present the third order interaction more clearly, the effect of the storage temperature was divided between Panel A (effect of storage duration at -0.5 °C x harvest maturity) and Panel B (effect of storage duration at -0.5 °C plus a subsequent shelf-life of 7 days at 10 °C x harvest maturity). Therefore, the two graphs should be seen as a unit and not as two separate sets of data. For clarity the linolenic acid levels during simulated shelf-life at 10 °C is presented at the same time-point as the linolenic acid levels that were measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.



Effect	F	*p*
Harvest maturity	1.75	0.2145
Storage temperature	3.78	0.0029
Storage duration	6.52	0.0286
Harvest maturity x Storage temperature	7.72	**0.0000**
Harvest maturity x Storage duration	4.52	0.0592
Storage temperature x Storage duration	0.68	0.6619
Harvest maturity x Storage temperature x Storage duration	1.03	0.4115

Fig. 2.20. Unsaturated:saturated phospholipid fatty acid ratio in ‘Sapphire’ plums as influenced by harvest maturity and storage temperature.





(A) **(B)**

Effect	F	*p*
Harvest maturity	1.10	0.3176
Storage temperature	70.34	0.0000
Storage duration	8.94	0.0002
Harvest maturity x Storage temperature	7.70	**0.0195**
Harvest maturity x Storage duration	2.88	0.0519
Storage temperature x Storage duration	7.22	**0.0008**
Harvest maturity x Storage temperature x Storage duration	1.49	0.2362

Fig. 2.21. Monounsaturated phospholipid fatty acid:polyunsaturated phospholipid fatty acid (MUFA:PUFA) ratio in 'Sapphire' plums as influenced by (A) harvest maturity and storage temperature, and (B) storage temperature and storage duration. In graph B the MUFA:PUFA ratio during simulated shelf-life at 10 °C are presented at the same time-point as the MUFA:PUFA ratio that was measured in the preceding storage duration at -0.5 °C, although the shelf-life evaluation was conducted 7 days later.

PAPER 3

Comparison of cell membrane composition, antioxidant status and the manifestation of chilling injury in two plum cultivars (*Prunus salicina* Lindl.) stored under a single-temperature regime at -0.5 °C and an intermittent warming regime

Abstract

For the past 60 years an intermittent warming regime has been used to export most Japanese plum cultivars from South Africa. This is done to reduce chilling injury (CI) and to increase the storage potential of these chilling susceptible fruit. It is not known by which mechanism the intermittent warming regime reduces chilling injury. Therefore, the aim of the study was to compare the effect of the intermittent warming regime and a single-temperature regime at -0.5 °C on the cell membrane composition, antioxidant status, and chilling injury incidence of two plum cultivars differing in susceptibility to chilling injury ('Sapphire' – more susceptible; 'Laetitia' - less susceptible). Fruit was sampled from a packhouse, cold-stored with a commercially used intermittent and single-temperature regime, and examined after 28 days (simulated arrival of fruit at overseas destination), after the maximum recommended commercial cold-storage duration of each cultivar, and at the end of a simulated shelf-life of 7 days at 10 °C. It was found that the intermittent warming regime not only delayed the onset of CI symptom appearance, but also reduced chilling severity significantly compared to single-temperature storage at -0.5 °C. It is suggested that this was mainly accomplished by the maintenance of the antioxidant levels in fruit by the intermittent warming regime. Nevertheless, the intermittent warming treatment could not prevent chilling injury completely as the defect still developed during the shelf-life period in both cultivars, albeit at much reduced levels.

Key words

Ascorbic acid, glutathione, phospholipids, sterols, saturated fatty acids, unsaturated fatty acids

1. Introduction

Chilling injury (CI) adversely affects the quality of plums exported from South Africa. The cold-storage of these plums is complex as they are perishable products with a maximum cold-storage life of 3 to 7 weeks, due to their high respiration rate. To counteract the high respiration rate, the fruit is stored at -0.5 °C. However, most plum cultivars exported from South Africa are sensitive to storage under low temperatures, as they develop CI. CI manifests as gel breakdown (GB) or internal browning (IB) in the mesocarp of South African plums (Taylor, 1996). Affected fruit has a normal external appearance. These defects usually appear when the fruit is removed from the low storage temperature conditions, i.e. in the fruit basket of the consumer, who subsequently does not buy plums again. Consequently, an intermittent warming (IW) regime replaced single-temperature (ST) storage at -0.5 °C for most plum cultivars exported from South Africa since the early 1950s (Taylor, 1996). The so called 'dual-temperature' regime, in conjunction with harvesting the fruit less mature, not only reduces the levels of internal defects, but also increases the storage potential of plums exported from South Africa. IW during storage also proved to successfully reduce or delay the onset of CI in various other fresh produce such as oranges (Schirra and Cohen, 1999), apples (Rudell et al., 2011), tomatoes (Artés et al., 1998), peaches and nectarines (Sevillano et al., 2009).

IW comprises the interruption of cold-storage with short periods of warming (Lurie, 2002; Sevillano et al., 2009; Wang, 2010). The length of the warming period depends on the cultivar, the maturity of the fruit and the growth conditions (Sevillano et al., 2009). It was determined that longer periods of warming are needed if temperatures ≤ 12 °C are used, compared to 20 °C (Lurie, 2002). For South African export plums 7.5 °C is used as the warming temperature (Taylor, 1996), and the length of the IW period varies between 5 to 10 days (PPECB, 2012). The introduction of the warming period should occur before the damage caused by CI is irreversible (Sevillano et al., 2009; Wang, 2010). For South African plums it is recommended that the IW should be introduced after a maximum of 10 to 14 days of storage at -0.5 °C (Hortgro Services, 2012).

It is not known by which mechanism IW reduces CI (Sevillano et al., 2009), but a number of hypotheses exist. It is suggested that while IW maintains the quality of the fruit, it increases the levels of mRNA encoding the two enzymes of the ethylene biosynthetic pathway, namely ACC oxidase and ACC synthase, which permits the fruit to have a normal ethylene production, and hence ripening, after storage (Lurie, 2002; Sevillano et al., 2009). Another hypothesis is that the warming period increases metabolic activities which allow tissues to break down surplus intermediates and replenish shortages which would aid in the repair of damaged membranes,

organelles or metabolic pathways (Wang, 2010). The fluctuation in temperature would also increase the synthesis of polyunsaturated fatty acids (Wang, 2010), which helps the membranes to stay fluid at low temperatures (Staehelin and Newcomb, 2000).

The aim of this study was to compare the effect of the IW regime and a ST regime at -0.5 °C on the cell membrane composition, antioxidant status, and CI incidence of two plum cultivars differing in their susceptibility to chilling injury. This knowledge will help to gain a better understanding of the processes involved in the development of CI, as well as in the design of better postharvest strategies to avoid, reduce, or at least delay the incidence of CI in South African plums.

2. Materials and Methods

2.1 Sampling of fruit and measurements made on each evaluation date

'Sapphire' (susceptible to CI) and 'Laetitia' (less susceptible to CI) plums (*Prunus salicina* Lindl.), packed according to export standards (DAFF, 2012), were sampled from a commercial packhouse in Simondium, Western Cape, South Africa in the 2009 season. The fruit was sampled on the day of harvest. Only size A (50 to 55 mm diameter) fruit were used. Fruit of both cultivars were packed with perforated, high density polyethylene (HDPE) shrivel sheets. This was done to prevent moisture loss from the fruit as 'Sapphire' and 'Laetitia' plums are prone to shrivel during storage. The fruit did not receive any pre-cooling prior to it being sourced for the trial. The packed cartons were transported to the laboratory using covered, uncooled transport. A complete, randomised design with six replicates per storage treatment was used.

At the laboratory the fruit were cold-stored under two cold-storage regimes, namely the IW regime and the ST regime at -0.5 °C as depicted in Fig. 3.1. To simulate commercial conditions, 'Sapphire' plums were cold-stored for a maximum period of 35 days, and 'Laetitia' plums for 49 days before commencement of the simulated shelf-life period. The plastic shrivel sheet was removed before the fruit were placed at 10 °C to prevent condensation of water and fruit decay. Fruit were evaluated on the sampling date, after 28 days of cold-storage (which simulates the arrival of the fruit at the overseas destination), after cold-storage (day 35 for 'Sapphire' and day 49 for 'Laetitia') and after the simulated shelf-life of 7 days at 10 °C. On each evaluation date 35 fruit per replicate per treatment were evaluated.

Shrivel and decay (expressed as %) were determined subjectively on 30 fruit per replicate. Shrivel was counted when shrivelled skin extended over the shoulder of the fruit. Hue angle was measured on one cheek of five fruit per replicate with a calibrated colorimeter (Minolta chroma meter CR-400, Japan). Flesh firmness (N) was measured with a Southtrade fruit pressure tester (Model FT327, Alphonsine, Italy) fitted with an 11.1 mm tip, on one peeled cheek of 10 fruit per replicate. Internal defects (%) were determined by cutting 20 fruit per replicate around the equatorial axis, and separating the two halves of the fruit. A gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue had a healthy appearance, was classified as GB (Taylor, 1996). A brown discolouration of the mesocarp tissue, associated with a loss in juiciness, was classified as IB. Fruit were classified as overripe (OR) when abnormally soft to the touch with excessive amounts of free juice, when the mesocarp tissue in the sub-epidermal region developed a translucent breakdown while the inner tissue exhibited a normal appearance and/or when cut around the equatorial axis, and the two halves of the fruit were twisted in opposite directions, the skin and sub-epidermal layers of the mesocarp separated from the inner mesocarp which remained attached to the stone. To obtain the total CI, the sum of the percent GB and IB per replicate were calculated, while the total internal defects were calculated by adding the total CI and the percent OR.

Five fruit per replicate were pooled, peeled and the pulp flash frozen, milled to a fine powder in liquid nitrogen, and stored at -80 °C for further analysis. For the biochemical analyses conducted on the samples stored at -80 °C, see Paper 1, Section 2.3. Please note that lipid peroxidation levels and total phenolic concentration were not determined for this paper.

2.2 Statistical analysis

Factorial analysis of variance (Factorial ANOVA) was used to calculate statistical differences between all data that resulted from fruit analysis after the different storage periods. All Factorial ANOVAs were performed using SAS version 9.1 (SAS Institute, Inc. 2000). ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

3. Results

3.1 Effect of storage regime and duration on the fruit quality of 'Sapphire' and 'Laetitia' plums

3.1.1 Hue angle

For all storage durations 'Sapphire' plums stored under the IW regime were redder (McGuire, 1992), albeit not always statistically significant, compared to the fruit stored under the ST regime (Table 3.1). Although it was not statistically significant, 'Laetitia' plums stored under the IW regime were also redder compared to the fruit stored under the ST regime (Table 3.2).

3.1.2 Flesh firmness

The flesh firmness of the 'Sapphire' plums decreased by approximately 25 N (Table 3.1), while that of 'Laetitia' decreased by approximately 8 N (Table 3.2) during the first 28 days of storage after the sampling date. 'Sapphire' plums stored under the ST regime had a significantly higher flesh firmness compared to the IW regime on each evaluation date during storage (Tables 3.1). Although it did not differ significantly, 'Laetitia' plums stored under the ST regime also had a higher average flesh firmness than the fruit stored under the IW regime (Table 3.2). Flesh firmness decreased significantly with an increase in storage duration for both cultivars.

3.1.3 Shrivel

Shrivel levels were negligible for the 'Sapphire' fruit (Table 3.1). For 'Laetitia' fruit there was a significant interaction between storage regime and duration for shrivel (Table 3.2). For both regimes shrivel levels were similar on day 28, but increased significantly until day 49. On day 49, which simulates the maximum storage duration for 'Laetitia' plums, shrivel levels were significantly higher in the fruit stored under the ST regime than under the IW regime. However, the shrivel levels decreased during the simulated shelf-life period between days 49 and 56, and in the case of the ST regime, this difference was significant.

3.1.4 Decay

Although decay levels were negligible for both cultivars, levels were higher, albeit not statistically significant, for the fruit stored under the ST regime (Tables 3.1 and 3.2). Decay only manifested at the end of the simulated shelf-life periods.

3.1.5 Internal browning

IB was the only CI and internal defect that manifested in both cultivars. For 'Sapphire' and 'Laetitia' there was a statistically significant interaction between storage regime and storage duration for IB (Tables 3.1 and 3.2). When IB manifested, levels were always higher in the fruit stored under the ST regime, albeit not always statistically significant. For fruit stored under the IW regime, IB only manifested after the simulated shelf-life period for both cultivars. For 'Sapphire' plums IB was already visible after 28 days of storage under the ST regime, and increased with an increase in storage duration (Table 3.1). For 'Laetitia' plums the first IB manifested after 49 days of storage under the ST regime (Table 3.2).

3.2 Effect of storage regime and duration on the cell membrane composition of 'Sapphire' and 'Laetitia' plums

3.2.1 Total phospholipids

For 'Sapphire' plums there was a slight increase in the total phospholipid concentration during the first 28 days of storage, irrespective of the storage regime used (Table 3.3). This increase was more pronounced in the fruit stored under the ST regime. Although this was also true for the 'Laetitia' plums stored under the ST regime, fruit stored under the IW regime had a slightly lower total phospholipid concentration after 28 days of storage compared to the sampling date (Table 3.4). The total phospholipid concentration was significantly higher in the 'Sapphire' fruit stored under the ST regime than under the IW regime during storage (Table 3.3). For 'Laetitia' plums there was a statistically significant interaction between storage regime and duration for the total phospholipid concentration in the fruit (Table 3.4). While the total phospholipid concentration remained unchanged in the fruit stored under the ST regime, it increased significantly from day 28 to 49, but decreased again during the shelf-life period in the 'Laetitia' plums stored under the IW regime.

3.2.2 Total sterols

The total sterol concentration increased significantly in the 'Sapphire' plums during the first 28 days of storage in both regimes (Table 3.3). After 28 days of storage, the total sterol concentration was significantly higher in the 'Sapphire' plums stored under the IW regime compared to the ST regime. However, the total sterol concentration decreased with an increase in storage duration in the 'Sapphire' fruit stored under the IW regime, while the levels remained unchanged in the fruit stored under the ST regime. For the 'Laetitia' fruit the total sterol concentration remained almost unchanged during the first 28 days of storage under both regimes (Table 3.4). Although the total

sterol concentration did not change after 28 and 49 days of storage, the concentration was significantly higher in fruit stored under the ST regime and significantly lower in the fruit stored under the IW regime after the simulated shelf-life period between days 49 and 56.

3.2.3 Total sterols:total phospholipid ratio

Since the fruit stored under the IW regime had significantly lower levels of phospholipids than the fruit stored under the ST regime during storage, the former treatment had a significantly higher total sterol:total phospholipid ratio compared to the latter treatment (Table 3.3). 'Sapphire' fruit stored for 35 days, which simulates the maximum storage period for this cultivar, had the lowest total sterol:total phospholipid ratio, although it did not differ significantly from the harvest date. There was a statistically significant interaction between storage regime and duration for the total sterol:total phospholipid ratio of 'Laetitia' plums (Table 3.4). In the fruit stored under the IW regime, the ratio decreased significantly from 28 to 49 days of storage, but increased, albeit not significantly, during the shelf-life period. For the ST regime, the ratio remained unchanged from 28 to 49 days of storage, but increased significantly during the shelf-life period.

3.2.4 Saturated phospholipid fatty acids

'Sapphire' plums stored under the IW regime had a significantly lower percentage saturated fatty acids compared to the fruit stored under the ST regime (Table 3.3). Levels of saturated fatty acids remained unchanged during storage in the 'Sapphire' plums. For 'Laetitia' plums there was a slight increase in the percentage saturated fatty acids in the fruit stored under the IW regime, while there was a slight decrease for the fruit stored under the ST regime for the first 28 days of storage, although the difference was not significant (Table 3.4). The percentage saturated fatty acids was generally higher in the 'Laetitia' plums stored under the IW regime compared to the ST regime, however, the difference was not always significant. Levels also increased significantly between day 28 and 49 of storage, but decreased again, albeit not significantly, during the simulated shelf-life period between days 49 and 56 for both storage regimes (Tables 3.3 and 3.4).

3.2.5 Unsaturated phospholipid fatty acids

'Sapphire' plums stored under the IW regime had a significantly higher percentage unsaturated fatty acids than the fruit stored under the ST regime (Table 3.3). The unsaturated fatty acid levels remained unchanged during storage in the 'Sapphire' plums. For 'Laetitia' plums stored under the IW regime there was a slight, but non-significant, decrease in the percentage unsaturated fatty

acids during the first 28 days of storage, while levels increased slightly, albeit not significantly, in the fruit stored under the ST regime (Table 3.4). For the evaluations during storage the percentage unsaturated fatty acids was higher, albeit not always significantly, in the fruit stored under the ST regime compared to the IW regime.

3.2.6 Unsaturated:saturated phospholipid fatty acid ratio

The unsaturated:saturated fatty acid ratio increased slightly, albeit not significantly, in the 'Sapphire' plums stored under the IW regime, while it decreased slightly, albeit not significantly, in the fruit stored under the ST regime during the first 28 days of storage (Table 3.3). The ratio remained unchanged between days 28 and 42 of storage in both regimes. The regimes also did not differ significantly regarding the unsaturated:saturated fatty acid ratio on the different fruit evaluation dates during storage. For the 'Laetitia' plums the unsaturated:saturated fatty acid ratio was lower, albeit not always significantly, in the fruit stored under the IW regime compared to the ST regime during storage (Table 3.4).

3.3 Effect of storage regime and duration on the water- and lipid soluble antioxidant activity and the ascorbic acid and glutathione concentration of 'Sapphire' and 'Laetitia' plums

3.3.1 Water soluble antioxidant activity (HAA)

For 'Sapphire' plums stored under both regimes there was a significant reduction in the HAA during the first 28 days of storage (Table 3.5). After 28 days of storage the HAA were comparable and increased significantly with an increase in storage duration in both regimes. After 35 and 42 days of storage the HAA was significantly higher in the 'Sapphire' plums stored under the IW regime compared to the ST regime. For 'Laetitia' plums the HAA increased slightly, but not significantly, in the fruit stored under the IW regime, but decreased significantly in the fruit stored under the ST regime during the first 28 days of storage (Table 3.6). While the HAA levels did not differ significantly between the different fruit evaluation dates for 'Laetitia' stored under the IW regime, they increased significantly from day 28 until day 49 in the fruit stored under the ST regime, whereafter they decreased significantly during the simulated shelf-life period between days 49 and 56. After 28 and 56 days of storage the HAA was significantly higher in 'Laetitia' plums stored under the IW regime compared to the ST regime.

3.3.2 Lipid soluble antioxidant activity (LAA)

Similar to the HAA, the LAA decreased significantly during the first 28 days of storage in 'Sapphire' plums stored under both regimes (Table 3.5). After 28 days of storage the LAA increased in 'Sapphire' plums stored under both regimes, but at a much faster rate in the fruit stored under the IW regime. The LAA was significantly higher on days 35 and 42 for fruit stored under the IW regime compared to the ST regime. For 'Laetitia' plums the LAA remained unchanged in the fruit stored under the IW regime from the sampling date until the end of the storage duration (Table 3.6). However, the LAA decreased significantly during the first 28 days of storage to levels that were too low to detect for the 'Laetitia' fruit stored under the ST regime. Subsequently the levels increased significantly from day 28 until day 49, but decreased significantly again to undetectably low levels during the simulated shelf-life period between days 49 and 56 in the ST regime fruit.

3.3.3 Ascorbic acid

There was a significant decrease in the total and reduced ascorbic acid levels in the 'Sapphire' plums during the first 28 days of storage (Table 3.5). Total, reduced and oxidised ascorbic acid levels were significantly higher in 'Sapphire' fruit stored under the IW regime compared to the ST regime. Although the fruit stored under the ST regime had significantly lower levels of total and reduced ascorbic acid compared to the IW regime, levels of oxidised ascorbic acid were similar for the two regimes during storage.

There was a significant decrease in the total and reduced ascorbic acid levels in the 'Laetitia' plums during the first 28 days of storage (Table 3.6). The total ascorbic acid levels were significantly higher in the fruit stored under the IW regime compared to the ST regime. Reduced and oxidised ascorbic acid levels were similar for fruit stored under both regimes. Levels of reduced ascorbic acid decreased significantly between days 28 and 49 and remained at the lower level for the rest of the storage duration. Levels of oxidised ascorbic acid did not change significantly after 28 days of storage in the 'Laetitia' fruit.

3.3.4 Glutathione

In 'Sapphire' plums stored under both regimes, there was a slight (significant in the case of the ST regime) decrease in total glutathione levels during the first 28 days of storage (Table 3.5). Total and reduced glutathione levels increased during the simulated shelf-life period for both regimes,

albeit not significantly in the case of the IW treatment. There was not a significant difference between storage regimes or duration for oxidised glutathione levels in 'Sapphire' plums.

In 'Laetitia' plums stored under both regimes, there was an increase in the total glutathione levels during the first 28 days of storage (Table 3.6). Total and reduced glutathione levels were significantly higher in the 'Laetitia' plums stored under the IW regime. Levels of oxidised glutathione were similar for 'Laetitia' fruit stored under both regimes and for the different storage durations.

4. Discussion

4.1 Hue angle and flesh firmness

It was expected that the fruit stored under the IW regime would be redder and have a lower flesh firmness compared to the fruit stored under the ST regime due to the warming period. It is known that increased C_2H_4 production causes changes in chlorophyll, carotenoid and flavonoid concentrations as well as fruit texture (Czarny et al., 2006). Although C_2H_4 production was not determined in this study, it is suggested that the more advanced skin colour and the lower flesh firmness in fruit stored under the IW regime are probably due to higher rates of C_2H_4 evolution during the warming period.

4.2 Shrivel manifestation

The deficit between the saturated vapour pressure inside the fruit and the actual vapour pressure of the surrounding atmosphere determines the rate of evaporation from the product (Paull, 1999). The approximate RH in the cold-rooms used for the trial was 80%, while an RH of 95% is desirable for stone fruit (Mitchell, 1986). The fact that shrivel developed progressively during storage in the 'Laetitia' plums indicates that an optimal RH in the cold-store is imperative if plums are to be stored for extended durations, even if packaging is used to retard moisture loss from the product. The reason for the 'Sapphire' plums having lower levels of shrivel compared to the 'Laetitia' plums, is ascribed to 'Laetitia' having a thin and sensitive skin which makes the cultivar more prone to injuries, and hence to decay, and moisture loss (Bester, 1991). It is suggested that the decrease in shrivel levels during the shelf-life period for 'Laetitia' plums is probably due to cell wall disassembly in conjunction with a loss in the turgidity of the mesocarp cells during fruit ripening at the higher storage temperature (10 °C), as was also observed during the ripening of melting peaches and 'Ailsa Craig' tomatoes (Saladié et al., 2007; Ghiani et al., 2011). The fruit softening that resulted

from the loss of the cell wall integrity and turgor of the mesocarp cells probably caused the skin surface to relax, making shrivel less evident.

4.3 Chilling injury manifestation

In CI there is usually an interaction between time and temperature, with greater severity of injury as temperature is lowered, or as exposure to chilling temperatures is extended (Saltveit and Morris, 1990; Sevillano et al., 2009). This is in agreement with the findings of this study that the fruit stored under the ST regime (where the fruit were constantly stored at -0.5 °C) had higher levels of CI and developed CI earlier during storage compared to the fruit stored under the IW regime (where storage at -0.5 °C was interrupted with a warming period at 7.5 °C). However, fruit of both cultivars stored under the IW regime were not totally resistant to CI as the defect manifested after the shelf-life period, albeit at significantly lower levels than in the fruit stored under the ST regime. Generally it is observed that CI symptoms are more apparent and development accelerates after transfer of the fruit to non-chilling or ripening temperatures (Sevillano et al., 2009).

4.4 Cell membrane composition

The most important function of lipids is to form a hydrophobic barrier which separates cells from their surroundings as well as the contents of organelles from the cytoplasm (Somerville et al., 2002). This function depends on the polar lipids to form a bilayer that prevents uncontrolled diffusion of hydrophilic molecules between cellular organelles and in and out of cells (Staehelin and Newcomb, 2000; Maalekuu et al., 2006). Therefore, a decrease in the total phospholipid levels could have dire consequences regarding the barrier properties of the membrane and the organisation of the biochemistry within the cell or organelle. Cold acclimation of all cold-hardy herbaceous and woody species usually involves an increase in the membrane phospholipid concentration (Yoshida, S., 1984; Staehelin and Newcomb, 2000). This was also observed during the initial storage period of apples (Lurie et al., 1987) and tomatoes (Whitaker, 1992) at chilling temperatures. It was also found that sterols stabilise membrane fluidity over a wide range of temperatures (Marangoni et al., 1996). Free sterols increase membrane fluidity below the phase transition temperature (because its aliphatic tail is mobile and causes a certain degree of disorder in the hydrophobic part of the membrane), and decreases the fluidity above the phase transition temperature (because the steroid skeleton is rigidly planar) (Bloch, 1985; Leshem, 1992; Marangoni et al., 1996).

In this study it was found that the total phospholipid concentration increased significantly in the 'Sapphire' plums stored under both storage regimes during the first 28 days of storage. The 'Sapphire' plums stored under the ST regime had a significantly higher total phospholipid concentration compared to the fruit stored under the IW regime – probably in order for the membranes to acclimatise to the low storage temperature under the ST regime. This caused the fruit stored under the IW regime to have a significantly higher total sterol:total phospholipid ratio than the fruit stored under the ST regime. Interestingly, the 'Sapphire' plums stored under the ST regime had significantly higher levels of saturated fatty acids and significantly lower levels of unsaturated fatty acids compared to the IW regime, which indicates that the membranes did not adjust their phospholipid fatty acid unsaturation to remain fluid under the low storage temperature conditions, although these fruit accumulated more phospholipids during this period. It has been found that the degree of phospholipid fatty acid saturation is strongly linked to CI incidence in chilling prone produce (Parkin and Kuo, 1989; Whitaker, 1995). Therefore, a higher concentration of saturated fatty acids in their membranes could possibly explain why the 'Sapphire' plums stored under the ST regime manifested CI after only 28 days of storage although they increased their total phospholipid concentration. Conversely, it is suggested that the higher concentration of unsaturated phospholipid fatty acids in the membranes of the 'Sapphire' plums stored under the IW regime protected the fruit against the development of CI during the first 28 days of storage. This result agrees with the findings of Wang (2010) that IW treatments increase fatty acid unsaturation.

While there were not big differences over the storage time for the total phospholipid or total sterol concentrations between the two regimes, interestingly 'Laetitia' plums stored under the IW regime had higher levels of saturated and lower levels of unsaturated phospholipid fatty acids compared to fruit stored under the ST regime. It is suggested that the higher levels of saturated fatty acids in the fruit stored under the IW regime were to ensure that the membranes had optimal permeability properties under the warmer storage conditions of the IW regime. This adjustment probably protected the 'Laetitia' fruit stored under the IW regime from the development of high levels of CI. Conversely, the higher levels of unsaturated fatty acids in the 'Laetitia' fruit stored under the ST regime compared to the IW regime were to ensure that the membranes remained fluid under the ST storage at -0.5 °C. Unfortunately, it seems these adjustments were not enough, and the 'Laetitia' fruit stored under the ST regime still developed higher levels of CI than the fruit stored under the IW regime.

The increase in the total sterol concentration in both cultivars during the shelf-life period (significant in the case of 'Laetitia' plums) stored under the ST regime while the levels decreased (albeit not statistically significant) in the fruit stored under the IW regime, is noteworthy. This was also a

phenomenon observed during ripening of apples (Lurie and Ben-Arie, 1983; Lurie et al., 1987), after rewarming of mature-green bell pepper fruit stored at chilling temperatures (Whitaker, 1995) and during aging of overwintering and summer leaves of wild strawberry during spring and summer when temperatures were higher (O'Neill et al., 1981). An increase in the total sterol concentration during ripening or senescence is associated with increased membrane viscosity and a loss in permeability (O'Neill, 1981; Lurie et al., 1997). It is suggested that increased membrane permeability due to increased total sterol concentrations caused the excessively high levels of CI observed after the shelf-life period in the fruit stored under the ST regime.

4.5 Antioxidant levels

4.5.1 Water soluble antioxidant activity

Storing fruit at chilling temperatures causes oxidative stress (Lester, 2003). In this study it was found that there was a significant reduction in HAA during the first 28 days of storage in 'Sapphire' fruit stored under both regimes as well as in the 'Laetitia' fruit stored under the ST regime, probably indicating chilling stress in the fruit. Generally HAA were higher in fruit of both cultivars stored under the IW regime, albeit not always statistically significant. HAA generally increases under stress conditions (Hodges, 2001), as was found in this study. Therefore, the higher HAA in the fruit stored under the IW regime compared to the ST regime probably protected the fruit against CI. It is suggested that the production of active oxygen species (AOS) in the fruit stored under the ST regime exceeded the scavenging potential of the HAA in these fruit, ultimately leading to the manifestation of CI in the fruit. It is interesting that HAA levels did not change in the 'Laetitia' fruit stored under the IW regime for the entire storage duration. In two apple cultivars differing in their susceptibility to internal browning disorders, it was found that HAA decreased significantly in the susceptible cultivar, while it was stable in the resistant cultivar (Toivonen, 2004). This may explain why the HAA in the 'Sapphire' fruit stored under the IW regime decreased during the first 28 days of storage – 'Sapphire' is a chilling sensitive cultivar – while no decrease was observed in the more chilling resistant 'Laetitia' fruit stored under the IW regime. This result also indicates that, while 'Laetitia' is more chilling resistant, the IW regime enhanced this trait of the cultivar further by preventing a decrease in the HAA of the fruit, maintaining the AOS scavenging potential of the fruit, compared to the 'Laetitia' plums stored under the ST regime. Similar to the HAA, the LAA of the fruit stored under the IW regime was generally higher than in the fruit stored under the ST regime, extending the protection of the fruit stored under the IW regime against chilling stress.

4.5.2 Ascorbic acid

Ascorbic acid levels are usually maintained at a relatively constant level, and are often increased under stress conditions in the chloroplasts of leaves (Foyer, 1993). Typically a decrease in ascorbic acid levels indicates severe stress in the product. It was found that the decrease may be more pronounced in chilling sensitive cultivars or products (Walker and McKersie, 1993; Anderson et al., 1995; Wang, 1996), and depends upon the storage temperature and storage condition (e.g. regular atmosphere vs. controlled atmosphere storage) (Veltman et al., 2000). In this study, total ascorbic acid levels decreased considerably during the first 28 days of storage in both regimes, for both cultivars. Wang (1996) and Franck et al. (2003) found that the highest rate of ascorbic acid losses occurred early after harvest during cold-storage followed by a slower rate of loss for the rest of the storage period, which is similar to our findings. Toivonen (2003) suggests that this postharvest decline in ascorbic acid is due to its double role as a scavenger of AOS and regenerator of other antioxidant systems such as flavonols and tocopherols. Nevertheless, fruit of both cultivars stored under the IW regime had significantly higher levels of total ascorbic acid. This result again indicates that, as for the HAA and LAA, the IW regime assisted the fruit to a great extent in the maintenance of their AOS scavenging potential compared to the fruit stored under the ST regime.

4.5.3 Glutathione

While total glutathione levels decreased slightly in ‘Sapphire’ fruit, it increased in ‘Laetitia’ fruit during the first 28 days of storage. However, ‘Sapphire’ fruit stored under the IW regime increased their total glutathione levels after the initial decrease, while the levels in the fruit stored under the ST regime remained at the lower levels until the end of cold-storage. Total glutathione levels were also significantly higher in the ‘Laetitia’ plums stored under the IW regime compared to the ST regime. Several other studies found that glutathione levels increase under chilling conditions (Esterbauer and Grill, 1978; Anderson et al., 1992; Hausladen and Alscher, 1993; Gómez et al., 2009). It is suggested that the reason for this increase in glutathione levels is because the activity of glutathione reductase (GR), the enzyme responsible for the reduction of GSSG to GSH and the maintenance of a high GSH:GSSG ratio, is upregulated under chilling conditions (Wang, 1995; Gómez et al., 2009). It was also found that the activity of one of the two enzymes that catalyses the synthesis of glutathione, namely γ-glutamylcysteine synthetase (γECS), increases under chilling conditions (Szalai et al., 2009). Hodges (2003) remarked that an increase in glutathione levels in chilled plant tissue is important, as it scavenges for H_2O_2 and other toxic oxygen compounds together with ascorbic acid. Since glutathione levels tended to be higher in the fruit stored under the IW regime, it is suggested that these fruit were better protected against oxidative stress than the fruit stored under the ST regime, and therefore, were less susceptible to CI.

5. Conclusion

From the results obtained in this study, it can be concluded that CI incidence was significantly reduced by more than 20% by the IW regime for both cultivars tested. Overall, it seems that the maintenance of the fruits' AOS scavenging potential through the maintenance of their antioxidant levels is the most important contribution of the IW regime in the protection of the fruit against CI compared to the ST regime. However, the IW regime also prevented CI manifestation during the early part of storage in 'Sapphire' plums by affecting the fruit to have a higher concentration of unsaturated than saturated phospholipid fatty acids in their membranes compared to the ST regime.

Fruit stored under the IW regime were redder and softer compared to fruit stored under the ST regime. Since the IW regime causes more enhanced fruit softening, this regime must be used with caution on more mature fruit to prevent excessive softening of the fruit. In the case of more mature fruit, a shorter IW period may be considered.

From the results it is also clear that the IW regime did not prevent the development of CI completely, as low levels manifested after the shelf-life period in both cultivars. It is, therefore, recommended that other postharvest treatments, e.g. low temperature preconditioning, heat treatments and/or application of natural compounds be tested to determine if they can reduce CI more effectively in Japanese plums intended for long-term storage at low temperatures.

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Table 3.1

Effect of storage regime and cold-storage duration on the quality of 'Sapphire' plums.

Examination parameter	Storage duration[1]	Storage regime[2] (A)		Storage duration[3] (B)				*Prob. > F*[4]		
		IW	ST	0 days	28 days	35 days	42 days	*A*	*B*	*A x B*
Hue angle	0	62.72a	62.72a					*<0.0001*	*<0.0001*	***0.0025***
	28	26.78bcd	36.46b							
	35	20.88cd	54.55a							
	42	15.39d	28.88bc							
Flesh firmness (N)[5]	0	73.45a	73.45a					*<0.0001*	*<0.0001*	***0.0034***
	28	45.28c	50.63b							
	35	29.58d	42.34c							
	42	17.23e	26.77d							
Shrivel (%)	0+28+35+42	0.74	0.58	0.00	0.00	2.66	0.00	*0.8553*	*0.0854*	*0.9916*
Decay (%)	0+28+35+42	0.14	0.30	0.00a	0.00a	0.00a	0.90b	*0.5257*	***0.0255***	*0.7468*
Internal browning (%)	0	0.00a	0.00a					*0.0099*	*0.0002*	***0.0079***
	28	0.00a	2.78a							
	35	0.00a	4.16a							
	42	6.35a	41.86b							

[1] Data pooled across storage regimes and storage durations for non-significant interactions, with 0, 28, 35 and 42 indicating number of days in storage.
[2] Values in same row followed by different superscripts indicate significant differences ($P < 0.05$) according to the LSD test. IW and ST indicating intermittent warming regime and single-temperature regime at -0.5 °C, respectively.
[3] Values in same row followed by different letters indicate significant differences where ($P < 0.05$). Simulated arrival of the fruit at the overseas destination is represented by 28 days of storage, the maximum storage time of 'Sapphire' plums is represented by 35 days of storage and 42 days of storage represents the end of the simulated shelf-life period at 10 °C between 35 and 42 days of storage.
[4] Two-way ANOVA table with complete randomised design for Factor A (storage regime) and Factor B (storage duration).
[5] To convert Newton to kilogram, divide by 9.80.

Table 3.2

Effect of storage regime and cold-storage duration on the quality of 'Laetitia' plums[1].

Examination parameter	Storage duration	Storage regime (A)		Storage duration[3] (B)				*Prob. > F*		
		IW	ST	0 days	28 days	49 days	56 days	*A*	*B*	*A x B*
Hue angle	0+28+49+56	26.72	30.72	20.28b	18.06bc	64.79a	11.74c	*0.0957*	***<0.0001***	*0.1716*
Flesh firmness (N)[2]	0+28+49+56	44.44	45.81	57.29a	49.24b	41.04c	32.94d	*0.4283*	***<0.0001***	*0.6344*
Shrivel (%)	0	0.00e	0.00e					*0.3970*	*<0.0001*	***0.0065***
	28	8.79cd	2.02de							
	49	22.72b	37.89a							
	56	16.16bc	15.15bc							
Decay (%)	0+28+49+56	0.00	0.25	0.00	0.00	0.00	0.50	*0.3233*	*0.4028*	*0.4028*
Internal browning (%)	0	0.00c	0.00c					*<0.0001*	*<0.0001*	***<0.0001***
	28	0.00c	0.00c							
	49	0.00c	7.50b							
	56	6.66b	30.00a							

[1] For definitions of headings see Table 3.1.
[2] To convert Newton to kilogram, divide by 9.80.
[3] Simulated arrival of the fruit at the overseas destination is represented by 28 days of storage, the maximum storage time of 'Laetitia' plums is represented by 49 days of storage and 56 days of storage represents the end of the simulated shelf-life period at 10 °C between 49 and 56 days of storage.

Table 3.3

Effect of storage regime and cold-storage duration on the membrane composition of 'Sapphire' plums[1].

Examination parameter	Storage duration	Storage regime (A)		Storage duration (B)				*Prob. > F*		
		IW	ST	0 days	28 days	35 days	42 days	*A*	*B*	*A x B*
Total phospholipids (µmol g^{-1} FW)	0	1.03e	1.04de					*<0.0001*	*<0.0001*	***0.0286***
	28	1.15cd	1.28b							
	35	1.24bc	1.43a							
	42	1.02e	1.26bc							
Total sterols (µmol g^{-1} FW)	0	0.36d	0.37d					*0.8152*	*<0.0001*	***0.0044***
	28	0.50a	0.44bc							
	35	0.43bc	0.43bc							
	42	0.40cd	0.45b							
Total sterols:total phospholipids	0+28+35+42	0.38a	0.34b	0.35bc	0.39a	0.32b	0.38a	***0.0030***	***0.0066***	*0.0699*
Saturated fatty acids (%)	0+28+35+42	34.89a	36.78b	37.30	36.25	34.72	35.15	***0.0175***	*0.0627*	*0.0545*
Unsaturated fatty acids (%)	0+28+35+42	65.10a	63.21b	62.70	63.74	65.27	64.84	***0.0175***	*0.0627*	*0.0545*
Unsaturated:saturated fatty acids	0	1.89a	1.55c					*0.0089*	*0.0582*	***0.0363***
	28	1.86ab	1.67bc							
	35	1.90a	1.86ab							
	42	1.80ab	1.86ab							

[1] For definitions of headings see Table 3.1.

Table 3.4

Effect of storage regime and cold-storage duration on the membrane composition of 'Laetitia' plums[1].

Examination parameter	Storage duration	Storage regime (A)		Storage duration[2] (B)				*Prob. > F*		
		IW	ST	0 days	28 days	49 days	56 days	*A*	*B*	*A x B*
Total phospholipids (µmol g^{-1} FW)	0	0.88ab	0.76d					*0.5660*	*0.0041*	***0.0065***
	28	0.77cd	0.86abc							
	49	0.94a	0.88ab							
	56	0.78cd	0.82bcd							
Total sterols (µmol g^{-1} FW)	0	0.36c	0.37bc					*0.0001*	*<0.0001*	***<0.0001***
	28	0.38bc	0.38bc							
	49	0.40b	0.40b							
	56	0.36c	0.52a							
Total sterols:total phospholipids	0	0.41d	0.48bc					*0.0008*	*<0.0001*	***<0.0001***
	28	0.50b	0.44bcd							
	49	0.42cd	0.45bcd							
	56	0.46bcd	0.64a							
Saturated fatty acids (%)	0	33.09cd	34.66bc					*0.0004*	*0.0512*	***0.0005***
	28	33.84bc	32.84cd							
	49	37.60a	33.14cd							
	56	35.84ab	31.44d							
Unsaturated fatty acids (%)	0	66.90ab	65.34bc					*0.0004*	*0.0512*	***0.0005***
	28	66.16bc	67.16ab							
	49	62.40d	66.85ab							
	56	64.15cd	68.56a							
Unsaturated:saturated fatty acids	0	2.02ab	1.89bc					*0.0003*	*0.0670*	***0.0003***
	28	1.96bc	2.04ab							
	49	1.68d	2.02ab							
	56	1.80cd	2.18a							

[1] For definitions of headings see Table 3.1.
[2] Simulated arrival of the fruit at the overseas destination is represented by 28 days of storage, the maximum storage time of 'Laetitia' plums is represented by 49 days of storage and 56 days of storage represents the end of the simulated shelf-life period at 10 °C between 49 and 56 days of storage.

Table 3.5

Effect of storage regime and cold-storage duration on the water- and lipid soluble antioxidant capacity, and the ascorbic acid and glutathione levels of 'Sapphire' plums[1].

Examination parameter	Storage duration	Storage regime (A)		Storage duration (B)				*Prob. > F*		
		IW	ST	0 days	28 days	35 days	42 days	*A*	*B*	*A x B*
HAA (mg g^{-1} FW)[2]	0	3.85a	2.69b					*<0.0001*	*<0.0001*	***0.0079***
	28	1.84cd	1.73d							
	35	3.03b	2.21c							
	42	3.66a	2.88b							
LAA (mg g^{-1} FW)[3]	0	0.080ab	0.012cd					*<0.0001*	*<0.0001*	***0.0003***
	28	0.004cd	0.000d							
	35	0.066b	0.015cd							
	42	0.090a	0.024c							
Total ascorbic acid (µg g^{-1} FW)	0+28+35+42	30.92a	25.33b	60.16a	15.86c	14.07c	22.41b	***0.0122***	***<0.0001***	*0.8467*
Reduced ascorbic acid (µg g^{-1} FW)	0+28+35+42	27.26a	22.64b	56.18a	13.48bc	11.53c	18.60b	***0.0242***	***<0.0001***	*0.6119*
Oxidised ascorbic acid (µg g^{-1} FW)	0+28+35+42	3.66a	2.68b	3.98a	2.38b	2.54b	3.80a	***0.0211***	***0.0116***	*0.2388*
Total glutathione (mg g^{-1} FW)	0	15.46bcd	19.16a					*0.6493*	*<0.0001*	***0.0495***
	28	13.10de	12.26e							
	35	15.06cde	12.70de							
	42	17.54abc	18.46ab							
Reduced glutathione (mg g^{-1} FW)	0	15.09bc	19.15a					*0.5184*	*<0.0001*	***0.0265***
	28	12.56cd	11.96d							
	35	14.91bcd	12.42cd							
	42	17.41ab	18.39a							
Oxidised glutathione (mg g^{-1} FW)	0+28+35+42	0.32	0.28	0.22	0.40	0.24	0.32	*0.7644*	*0.7141*	*0.6257*

[1] For definitions of headings see Table 3.1.
[2] HAA indicates water soluble antioxidant capacity.
[3] LAA indicates lipid soluble antioxidant capacity.

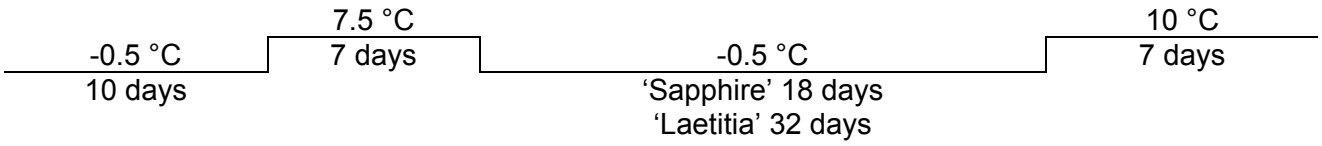
Table 3.6

Effect of storage regime and cold-storage duration on the water- and lipid soluble antioxidant capacity, and the ascorbic acid and glutathione levels of 'Laetitia' plums[1].

Examination parameter	Storage duration	Storage regime (A)		Storage duration[3] (B)				*Prob. > F*		
		IW	ST	0 days	28 days	49 days	56 days	*A*	*B*	*A x B*
HAA (mg g^{-1} FW)[2]	0	3.57abc	2.92bc					*<0.0001*	*0.3028*	***0.0053***
	28	3.64ab	1.70d							
	49	3.32abc	2.85c							
	56	4.12a	1.46d							
LAA (mg g^{-1} FW)[3]	0	0.083a	0.055a					*<0.0001*	*0.0300*	***0.0359***
	28	0.058a	0.002b							
	49	0.056a	0.054a							
	56	0.078a	0.001b							
Total ascorbic acid (µg g^{-1} FW)	0+28+49+56	50.50a	42.32b	70.12a	45.80b	34.18c	33.05c	***0.0299***	***<0.0001***	*0.3916*
Reduced ascorbic acid (µg g^{-1} FW)	0+28+49+56	20.84	19.96	40.87a	18.01b	10.90c	9.54c	*0.6301*	***<0.0001***	*0.7137*
Oxidised ascorbic acid (µg g^{-1} FW)	0+28+49+56	29.66	22.36	29.24	27.79	23.91	23.50	*0.0701*	***0.5812***	*0.4741*
Total glutathione (mg g^{-1} FW)	0+28+49+56	38.44a	29.24b	21.20c	32.84b	38.78ab	44.92a	***0.0005***	***<0.0001***	*0.0580*
Reduced glutathione (mg g^{-1} FW)	0+28+49+56	38.47a	28.90b	21.14c	32.71b	39.29ab	44.21a	***0.0003***	***<0.0001***	*0.0532*
Oxidised glutathione (mg g^{-1} FW)	0+28+49+56	0.36	0.34	0.16	0.18	0.00	0.97	*0.9507*	*0.2382*	*0.7469*

[1] For definitions of headings see Table 3.1.
[2] HAA indicates water soluble antioxidant capacity.
[3] LAA indicates lipid soluble antioxidant capacity.
[4] Simulated arrival of the fruit at the overseas destination is represented by 28 days of storage, the maximum storage time of 'Laetitia' plums is represented by 49 days of storage and 56 days of storage represents the end of the simulated shelf-life period at 10 °C between 49 and 56 days of storage.

(A)



(B)

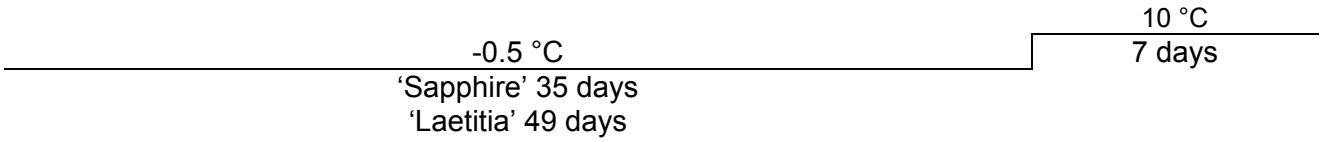


Fig. 3.1. Cold-storage regimes tested. (A) Intermittent warming regime at -0.5 °C and 7.5 °C followed by a simulated shelf-life period at 10 °C for 7 days . (B) Single-temperature (ST) storage at -0.5 °C followed by a simulated shelf-life period at 10 °C for 7 days. To simulate commercial conditions, ‘Sapphire’ plums were cold-stored for a maximum period of 35 days, and ‘Laetitia’ plums for a maximum period of 49 days before commencement of the simulated shelf-life period.

PAPER 4

Comparison of cell membrane composition, antioxidant status and the manifestation of chilling injury in two plum cultivars (*Prunus salicina* Lindl.) stored under an intermittent warming regime and elevated storage temperatures

Abstract

Japanese plums (Prunus salicina Lindl.) are exported with an intermittent warming (IW) regime from South Africa to prevent the development of chilling injury (CI) in the fruit. However, this practice is challenged by the increased use of integral reefer containers. While the containers were designed to maintain fruit temperature, they have an inadequate capacity to rapidly recool cargo, which creates temperature management problems when an IW regime is employed. While there is evidence that elevated storage temperatures are detrimental to plum fruit quality, there are indications that higher cold-storage temperatures could possibly be beneficial to plum fruit quality. Hence, the aim of this study was to investigate the effect of the commercially used IW regime and elevated storage temperatures of 2.5 °C, 5 °C and 7.5 °C on CI incidence, cell membrane composition and antioxidant status of two plum cultivars differing in their susceptibility to CI. For 'Sapphire' plums (more susceptible to CI) the IW regime performed better overall due to a higher lipid soluble antioxidant activity compared to the other treatments, and specifically lower lipid peroxidation levels, and hence slower deterioration, compared to the 5 °C and 7.5 °C treatments and higher ascorbic acid levels, and hence lower CI, than the 2.5 °C treatment. Regarding 'Laetitia' plums (less susceptible to CI), fruit stored at the elevated temperatures had comparable levels of internal defects to the IW regime. However, fruit stored at the elevated temperatures tended to develop slightly higher levels of shrivel and decay and the fruit stored under the 5 °C and 7.5 °C treatments softened quicker than the fruit stored under the IW regime.

Key words

Ascorbic acid, ethylene evolution, fruit softening, glutathione, lipid soluble antioxidant activity

1. Introduction

To overcome chilling injury (CI) that manifests when fruit are stored for extended periods at -0.5 °C, Japanese plums (*Prunus salicina* Lindl.) exported from South Africa are stored using an intermittent warming (IW) regime (Taylor, 1996). However, the use of IW is challenged by the use of integral reefer containers, which were introduced with increasing popularity in the South African export fruit industry since circa 1997. Integral containers were designed to maintain fruit temperature of properly precooled products, but have inadequate capacity to rapidly recool warm cargo (Kapp, 2008a). Although the refrigeration system has sufficient capacity to cool cargo, the air-flow system within the integral reefer containers does not allow efficient cooling of the produce (Irving, 1988). Furthermore, the vent of the integral container must be open to prevent the build-up of ethylene and CO_2 (PPECB, 2012a). This practise causes the right hand side of the container to be warmer compared to the left hand side, due to differential frosting on the coils (Kapp, 2008b).

Commercially it is observed that, due to the lack of proper cooling of the produce after the IW period, plums exported from South Africa sometimes arrive overseas with pulp temperatures within the so called 'killing zone' (2 to 7 °C), but without internal disorders. However, Mitchell (1986) found that most stone fruit cultivars experience severe "internal breakdown" when stored at temperatures between 2 °C and 7 °C. Crisosto et al. (1999) found that while three of the six plum cultivars they tested developed CI when stored at 0 °C, all six cultivars developed CI when they were stored at 5 °C. Zhang et al. (2009 and 2010) found that nectarines stored at 0 °C had less CI, had a higher fatty acid double bond index, accumulated higher levels of unsaturated fatty acids, omega-3 fatty acid desaturase mRNA, and specific proteins related to membrane stability and the breakdown of phenolic compounds compared to fruit stored at 5 °C. Apricots also develop higher levels of CI at 5 °C than at 0 °C and loss of taste when stored at temperatures between 4.4 °C to 7.2 °C (Monzini and Gorini, 1991). In agreement with the above findings, higher levels of woolliness and internal breakdown also develop in South African nectarines when stored at temperatures between 2 and 6 °C (Eksteen, 1982).

However, this is contrary to what is observed commercially in export plums. In support of the commercial results, a preliminary trial on elevated storage temperatures indicated that 'Pioneer' plums stored at 2 °C had higher flesh firmness and less internal defects compared to fruit stored with the IW regime (Kapp, 2008c). The Perishable Products Export Control Board (PPECB) of South Africa also recently added experimental temperature regimes within the 'killing zone', namely at 2 °C, 3 °C and 4 °C to their list of recommended carrying temperature regimes for sea export of plums (PPECB, 2012b). Hence, although there is evidence that elevated storage

temperatures are detrimental to plum fruit quality, there are also indications that higher cold-storage temperatures could possibly be beneficial to plum fruit quality.

The aim of this study was to investigate the effect of the commercially used IW regime and elevated single storage temperatures of 2.5 °C, 5 °C and 7.5 °C on CI incidence, the cell membrane composition and antioxidant status of two plum cultivars differing in their susceptibility to CI. This knowledge will help to gain a better understanding of the processes involved in the development of CI, as well as in the design of better postharvest strategies to avoid, reduce, or at least delay the incidence of CI in South African plums.

2. Materials and Methods

2.1 Sampling of fruit and measurements made on each evaluation date

'Sapphire' (susceptible to CI) and 'Laetitia' (less susceptible to CI) plums (*Prunus salicina* Lindl.), packed according to export standards (DAFF, 2012), were sampled from a commercial packhouse in Franschhoek, Western Cape, South Africa in the 2007 and 2008 seasons. The fruit was sampled on the day of harvest. Only size A (50 to 55 mm diameter) fruit were used. Fruit of both cultivars were packed with perforated, high density polyethylene (HDPE) shrivel sheets. This was done to prevent moisture loss from the fruit as 'Sapphire' and 'Laetitia' plums are prone to shrivel during cold-storage. The fruit did not receive any pre-cooling prior to it being sourced for the trial. The packed cartons were transported to the laboratory using covered, uncooled transport. A complete randomised design with six replicates per storage treatment was used.

At the laboratory fruit were cold-stored under a commercially used IW regime (control), and three single-temperature (ST) regimes, namely at 2.5 °C, 5 °C and 7.5 °C as depicted in Fig. 4.1. To simulate commercial conditions, 'Sapphire' plums were cold-stored for a maximum period of 35 days, and 'Laetitia' plums for 49 days before commencement of the simulated shelf-life period. The plastic shrivel sheet was removed before the fruit were placed at 10 °C (the shelf-life temperature) to prevent condensation of water and fruit decay. Fruit were evaluated on the sampling date, after 10 days of storage (which coincides with the commencement of the IW period commercially), 21 days of storage, 28 days of cold-storage (which simulates the arrival of the fruit at the overseas destination), 35 days of storage (which simulates the end of cold-storage for 'Sapphire' plums), 42 days of cold-storage (which simulates the end of the shelf-life period for 'Sapphire' plums), after 49 days (which simulates the end of storage for 'Laetitia' plums) and after

56 days (which simulates the end of the shelf-life period for 'Laetitia' plums). On each evaluation date 37 fruit per replicate per treatment were evaluated.

Shrivel and decay (expressed as %) were determined subjectively on 30 fruit per replicate. Shrivel was counted when shrivelled skin extended over the shoulder of the fruit. Flesh firmness (N) was measured with a Southtrade fruit pressure tester (Model FT327, Alphonsine, Italy) fitted with an 11.1 mm tip, on one peeled cheek of 10 fruit per replicate. Internal defects (%) were determined by cutting 20 fruit per replicate around the equatorial axis, and separating the two halves of the fruit. A gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue had a healthy appearance, was classified as GB (Taylor, 1996). A brown discolouration of the mesocarp tissue, associated with a loss in juiciness, was classified as IB. Fruit were classified as overripe (OR) when abnormally soft to the touch with excessive amounts of free juice, when the mesocarp tissue in the sub-epidermal region developed a translucent breakdown while the inner tissue exhibited a normal appearance and/or when cut around the equatorial axis, and the two halves of the fruit were twisted in opposite directions, the skin and sub-epidermal layers of the mesocarp separated from the inner mesocarp which remained attached to the stone. To obtain the total CI, the sum of the percent GB and IB per block were calculated, while the total internal defects were calculated by adding the total CI and the percent OR.

Rate of ethylene (C_2H_4) evolution (µL kg^{-1} h^{-1}) was measured on two fruit per replicate. The fruit were sealed in airtight 0.5 L glass jars for 1 h. A headspace gas sample was taken from each jar with a 10 mL airtight syringe, and injected into a Varian GC system (Model 3300, Varian Instrument Group, Palo Alto, California, USA) fitted with a flame ionisation detector (FID) and 2 m PoropakQ column. Nitrogen was used as a carrier gas. The oven temperature was programmed from 60 °C (isothermal for 2 min) to 70 °C at 1 °C min^{-1}. The injector temperature was at 65 °C and the detector temperature at 250 °C. A 1 ppm standard gas was used to identify and quantify C_2H_4.

Five fruit per replicate were pooled, peeled and the pulp flash frozen, milled to a fine powder in liquid nitrogen, and stored at -80 °C for further analysis. For the biochemical analyses conducted on the samples stored at -80 °C, see Paper 1, Section 2.3.

2.2 Statistical analysis

Factorial analysis of variance (Factorial ANOVA) was used to calculate statistical differences between all data that resulted from fruit analysis after the different storage periods. Season was used as a co-variate. Factorial ANOVAs was performed using SAS version 9.1 (SAS Institute, Inc. 2000). ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

3. Results

3.1 Effect of storage regime on the fruit quality of 'Sapphire' and 'Laetitia' plums

3.1.1 Flesh firmness

For both cultivars and under all four regimes, flesh firmness decreased with an increase in storage duration (Fig. 4.2A and B). This decrease was faster in the fruit stored at 5 °C and 7.5 °C. Hence, fruit stored under the latter two regimes had significantly the lowest flesh firmness after the simulated shelf-life period.

3.1.2 Shrivel

For 'Sapphire' plums shrivel levels did not differ between the storage regimes (Fig 4.3A). However, for 'Laetitia' plums, shrivel levels were significantly higher in the fruit stored at 5 °C (Fig. 4.3B). Although it did not differ significantly from the 2.5 °C and 7.5 °C treatments, fruit stored under the IW regime had the lowest shrivel levels. Generally shrivel levels were higher in the 'Laetitia' than in the 'Sapphire' fruit (Fig. 4.3A and B). In both cultivars shrivel levels increased with an increase in storage duration (Fig. 4.4A and B). While 'Sapphire fruit only manifested significantly higher shrivel levels after 35 days of storage, 'Laetitia' fruit had significantly higher levels after only 21 days of storage. Commercially export fruit are usually rejected when shrivel levels exceed 10%. From the results of both cultivars it is clear that, despite the protective packaging used, none of the treatments adequately controlled the manifestation of shrivel.

3.1.3 Decay

Decay levels did not differ significantly between regimes or storage durations for either cultivar (data not shown; Refer to Appendix D, Fig. 1). For 'Sapphire' plums decay only manifested in the fruit stored at 5 °C and 7.5 °C, while the highest, albeit not significant, decay levels in the 'Laetitia'

plums were noted in the fruit stored under the elevated storage temperatures. Decay levels were generally higher in the 'Laetitia' than the 'Sapphire' plums when it manifested.

3.1.4 CI

CI manifested progressively with an increase in storage duration in 'Sapphire' plums for all the regimes tested (Fig. 4.5). Although the levels did not differ significantly during storage between day 0 and 35, fruit stored under the IW regime and at 7.5 °C had significantly the lowest CI levels after the shelf-life period between days 35 and 42. 'Sapphire' plums stored at 2.5 °C had the highest levels of CI after storage, albeit not significantly higher than in the fruit stored at 5 °C. The main type of CI that manifested in all regimes tested for the 'Sapphire' plums was GB, while IB made a very small contribution (data not shown; Refer to Appendix D, Fig. 2). Surprisingly, 'Laetitia' plums stored under the IW regime had the highest levels of CI (Fig. 4.6A). Although the CI levels that developed under the IW regime were comparable to the levels that manifested at 2.5 °C, it was significantly higher than the levels that manifested at 5 °C and 7.5 °C. CI levels were relatively low in the 'Laetitia' fruit until 42 days of storage, whereafter it increased significantly (Fig. 4.6B). Similar to the 'Sapphire' plums, significantly the highest CI levels manifested after the shelf-life period in the 'Laetitia' plums. GB was the only type of CI that manifested in 'Laetitia' plums stored under the IW regime and at 7.5 °C, while IB and GB manifested in the fruit stored at 2.5 °C and 5 °C (data not shown, Refer to Appendix D, Fig. 3).

3.1.5 Total internal disorders

'Sapphire' plums stored at 7.5 °C had significantly the highest levels of internal disorders, followed by fruit stored at 5 °C (Fig. 4.7). This was mainly due to excessively high levels of OR that manifested in these fruit (data not shown, Refer to Appendix D, Fig. 4). 'Sapphire' plums stored under the IW regime had significantly the lowest levels of internal disorders after the shelf-life period. Hence, due to the risk of unacceptably low flesh firmness and the development of decay (when stored at 5 °C and 7.5 °C) and internal disorders (CI when stored at 2.5 °C and 5 °C and OR when stored at 5 °C and 7.5 °C), the elevated temperature storage regimes cannot be recommended as replacements for the IW regime for Sapphire' plums.

Levels of total internal disorders did not differ much from the CI levels that manifested in the 'Laetitia' plums (data not shown, Refer to Appendix D, Fig. 5). The reason for this is that OR did not contribute much to the total internal defects that manifested. While no OR manifested in the 'Laetitia' plums stored under the IW regime, it did develop in the fruit stored under the elevated

storage temperatures (data not shown, Refer to Appendix D, Fig. 6). Although the fruit stored under the IW regime had the highest levels of internal disorders, it did not differ significantly from the fruit stored under the elevated storage regimes. Therefore, in the case of 'Laetitia' plums, the elevated storage temperature regimes did not perform better than the IW regime regarding the development of internal disorders, decay or shrivel. However, flesh firmness did decrease faster in the fruit stored at 7.5 °C and 5 °C (Fig. 4.2B). This factor would preclude the use of both the 5 °C and 7.5 °C storage regimes from commercial use, as fruit firmness remains one of the primary quality variables considered by the fruit trade.

3.2 Effect of storage regime on the biochemical parameters measured in 'Sapphire' and 'Laetitia' plums

3.2.1 Ethylene evolution

C_2H_4 evolution rates remained relatively low and did not differ significantly for 'Sapphire' plums stored under the IW regime, 2.5 °C and 5 °C between days 0 and 35 (Fig. 4.8A). However, rates increased in fruit stored at 7.5 °C after 10 days of storage and were higher than in the other treatments until after 35 days of storage, albeit not always statistically significant. The increase in C_2H_4 evolution rates in the fruit stored at 7.5 °C coincided with the increase in OR in the same fruit. The rate of C_2H_4 evolution increased in all the treatments during the shelf-life period. After the shelf-life period, C_2H_4 evolution rates were the highest in fruit stored under the IW regime, but only differed significantly from the fruit stored at 2.5 °C, which had the lowest evolution rates. Similar to the 'Sapphire' fruit, C_2H_4 evolution rates were relatively low and did not differ significantly between days 0 and 49 for the 'Laetitia' fruit stored under the IW regime, 2.5 °C and 5 °C (Fig. 4.8B). However, the rate increased significantly in fruit stored at 7.5 °C after 35 days of storage, and remained significantly higher than in the other treatments until the end of storage. This increase in the C_2H_4 evolution rates could not be linked to the manifestation of internal disorders in this treatment during this storage period, as was the case for 'Sapphire' plums. Coinciding with the increased manifestation of internal disorders, the C_2H_4 evolution rates increased in all the treatments (albeit not significantly in the case of the 'Laetitia' plums stored under the IW regime) during the shelf-life period.

3.2.2 Antioxidants

3.2.2.1 Water- and lipid soluble antioxidant activity

For both cultivars there was a significant interaction between storage regime and duration for the HAA (data not shown; Refer to Appendix D, Fig. 7). The HAA remained stable for the entire

storage duration in all the regimes and did not differ much between the treatments. This was generally also true for the LAA of the 'Laetitia' plums stored under the different regimes (data not shown, Refer to Appendix D, Fig. 8). However, the LAA in the 'Sapphire' plums stored under the IW regime started to increase after 10 days of storage and was significantly higher than in the other treatments after 21 days of storage (Fig. 4.9). Subsequently, the LAA in this treatment decreased again to levels comparable to the other treatments for the rest of the storage duration. The LAA spike in the IW treatment coincided with the first incidence of IB in the 'Sapphire' fruit (data not shown, Refer to Appendix D, Fig. 9). Although IB also manifested in the other treatments (data not shown; Refer to Appendix D, Fig. 2B), the IW regime was the only treatment to show an increase in LAA.

3.2.2.2 Ascorbic acid

The total ascorbic acid levels in 'Sapphire' plums decreased during the first 21 days of storage in all the treatments (Fig. 4.10A). Subsequently, the levels increased in the fruit stored at 7.5 °C to be significantly higher than in the other treatments after 28 and 35 days of storage. During the same storage duration, total ascorbic acid levels decreased and levelled out in the other storage treatments, with the lowest levels, albeit not always significant, recorded in the fruit stored at 2.5 °C. After the shelf-life period the levels were comparable in the fruit stored under the IW regime, 2.5 °C and 5 °C. However, the levels in the fruit store at 7.5 °C were significantly higher than in the IW and 2.5 °C treatments. The levels of the reduced ascorbic acid in the 'Sapphire' plums remained stable for 28 days in the fruit stored under the IW regime and for 21 days at 2.5 °C after which it decreased and remained at the lower levels for the rest of the storage duration (Fig. 4.10B). The levels in the fruit stored at 5 °C remained relatively stable for the entire storage duration, while the concentration increased during the first 10 days of storage in the 7.5 °C treatment and then levelled out at the higher levels for the remainder of the storage duration. The treatments did not differ significantly regarding the levels of the oxidised ascorbic acid (data not shown; Refer to Appendix D, Fig. 10).

Total ascorbic acid levels declined during the first 21 days of storage in 'Laetitia' plums stored under all, except the 7.5 °C treatment (Fig. 4.11A). In the latter treatment the total ascorbic acid concentration remained at the harvest date levels until 42 days of storage, after which it decreased slightly and remained at the reduced level for the remainder of the storage duration. The 7.5 °C treatment generally had the highest ascorbic acid levels from 7 days of storage until the end of shelf-life, although it did not always differ significantly from the other treatments. After 28 days of storage the 'Laetitia' plums stored under the IW and 2.5 °C treatments had the lowest ascorbic

acid levels, although it did not always differ significantly from the 5 °C treatment. The total ascorbic acid levels decreased slower in the fruit stored at 5 °C after 21 days of storage, causing it to have a higher concentration of ascorbic acid between days 28 and 56 than the IW and 2.5 °C treatments, although the difference was not always statistically significant. The levels of reduced ascorbic acid followed the same trend compared to the total ascorbic acid levels in all the treatments for 'Laetitia' plums (Fig. 4.11 B). It is interesting to note that the total and reduced ascorbic acid levels were almost two times higher in the 'Laetitia' than in the 'Sapphire' plums (Fig. 4.10 and 4.11). Oxidised ascorbic acid did not follow a clear trend in any of the treatments for the 'Laetitia' plums and remained within a narrow band for all the treatments for the entire storage duration (data not shown; Refer to Appendix D, Fig. 11).

3.2.2.3 Glutathione

For 'Sapphire' the concentration of the total and reduced glutathione increased with an increase in storage duration in all the treatments (Fig. 4.12A and B). Although the total and reduced glutathione concentration also increased in the 'Laetitia' plums, the increase in the fruit stored under the IW and 2.5 °C treatments was less than in the 5 °C and 7.5 °C treatments (Fig. 4.13A and B). Subsequently, levels of total and reduced glutathione were lower in the IW and 2.5 °C treatments between days 28 and 49, although the difference was not always statistically significant. However, levels were comparable in all the 'Laetitia' treatments after the shelf-life period on day 56. Oxidised glutathione increased after 10 days of storage in all the 'Sapphire' treatments (Fig. 4.14A). Fruit stored under the IW regime had the lowest levels on this date, but it did not differ significantly from the other treatments. Subsequently, the levels decreased in all the treatments, except in the 7.5 °C treatment, in which the levels continued to increase. This increase coincided with the manifestation of high levels of OR in this treatment, while no or very low OR levels were recorded in the other treatments (data not shown; Refer to Appendix D, Fig. 4). However, the concentration of oxidised glutathione decreased again in the 7.5 °C treatment after 28 days of storage, and increased again, as did the other treatments, after 35 days of storage. The levels decreased once again in all the treatments during the shelf-life period, except in the 5 °C which had a significant increase in the levels of oxidised glutathione. In 'Laetitia' plums, levels of oxidised glutathione did not differ significantly from the harvest levels throughout storage, except for the 5 °C treatment, which had significantly the highest levels after 49 days of storage (Fig. 4.14B).

3.2.3 Cell membrane components

3.2.3.1 Total phospholipids

For both cultivars the total phospholipid concentration did not change much in any of the treatments from the harvest date until the end of storage (data not shown, Refer to Appendix D, Fig. 12).

3.2.3.2 Total sterols

For 'Sapphire' plums the total sterol levels did not differ significantly from the harvest date until the end of storage in the fruit stored under the IW regime and at 5 °C (data not shown, Refer to Appendix D, Fig. 13a). However, there was a significant spike in the total sterol concentration in the fruit stored at 2.5 °C and 7.5 °C after 35 days of storage. Notwithstanding these spikes, the total sterol levels were the same in all the storage treatments after the simulated shelf-life on day 42. For 'Laetitia' plums the total sterol levels did not change much in any of the treatments from the harvest date until the end of storage (data not shown, Refer to Appendix D, Fig. 13B).

3.2.3.3 Total sterol:total phospholipid ratio

Due to the relatively stable phospholipid and sterol concentrations during storage for both cultivars and in under all storage regimes, the ratio between the sterols and phospholipids did not differ significantly between the treatments (data not shown, Refer to Appendix D, Fig. 14).

3.2.3.4 Phospholipid fatty acids

3.2.3.4.1 Unsaturated:saturated phospholipid fatty acid ratio

The ratio between saturated and unsaturated phospholipid fatty acids differed very little during the entire storage duration for both cultivars and for the regimes tested, and stayed within a very narrow range (data not shown, Refer to Appendix D, Fig. 15).

3.2.3.4.2 Monounsaturated:polyunsaturated phospholipid fatty acid ratio (MUFA:PUFA)

There was a tendency for the MUFA:PUFA ratio to decrease slightly over the storage duration in all the 'Sapphire' treatments (Fig. 4.15A). After 28 days of storage the ratio increased in all the treatments, except in the 5 °C treatment where the ratio continued to decrease. While the ratio

decreased in the IW, 2.5 °C and 7.5 °C treatments between 28 and 35 days of storage, it increased in the 5 °C treatment. During the shelf-life period between days 35 and 42, the ratio decreased in all the treatments, except for the 2.5 °C treatment, which had significantly the highest levels after the shelf-life period. Contrary to this, the MUFA:PUFA ratio remained almost unchanged in the 'Laetitia' treatments for the entire storage duration (Fig. 4.15B).

3.2.3.4.3 Lipid peroxidation

Lipid peroxidation levels remained relatively stable during storage in the 'Sapphire' plums stored under the IW regime and 2.5 °C (Fig. 4.16A). However, lipid peroxidation levels were significantly the highest after 10 days of storage in the 5 °C treatment. The levels also increased in the 5 °C and 7.5 °C treatments from 35 days of storage until the end of the shelf-life on day 42. After the shelf-life period these two treatments had significantly the highest lipid peroxidation levels. In the 'Laetitia' plums the lipid peroxidation levels remained relatively unchanged in the fruit stored under the IW regime, 2.5 °C and 5 °C (Fig. 4.16B). However, the fruit stored at 7.5 °C had a significant spike in lipid peroxidation after 10 days of storage and also significantly higher levels after the shelf-life period compared to the other treatments.

4. Discussion

4.1 Shrivel and decay manifestation

The slightly higher shrivel and decay levels observed in the 'Laetitia' compared to the 'Sapphire' plums are probably due to 'Laetitia' having a thin and sensitive skin which makes the cultivar more prone to injuries, and hence to decay, and moisture loss (Bester, 1991).

4.2 C_2H_4 evolution

It was expected that the fruit stored under the IW regime and at 2.5 °C would soften at a slower rate than the fruit stored at 5 °C and 7.5 °C, since most plant species are only sensitive to C_2H_4 at temperatures between 4°C and 38 °C (Mitchell, 1986). It is known that increased C_2H_4 production causes changes in fruit texture (Czarny et al., 2006). In this study the highest C_2H_4 evolution rates were recorded in the fruit stored at 7.5 °C, which also softened the quickest during storage. Correspondingly, the 'Sapphire' plums stored at 7.5 °C and 5 °C had the highest levels of OR, which is also ascribed to the higher C_2H_4 evolution rates at these storage temperatures.

It has also been observed that C_2H_4 production is stimulated by low or chilling temperatures (Wang, 1982; Sevillano et al., 2009). Usually 1-aminocyclopropane-1-carboxylic acid (ACC), ACC synthase activity and C_2H_4 production remain low at the chilling temperature, but increase rapidly when the product is transferred to a higher temperature. This is because the mRNA encoding for ACC-synthase is stimulated by low temperatures, and the translation of these messengers occurs immediately after transfer of the product to higher temperatures (Sevillano et al., 2009), which explains the higher C_2H_4 evolution rates measured in this study after shelf-life in all the treatments compared to the rates during the cold storage period.

Usually CI symptoms are also more apparent and their development accelerates after transfer of the fruit to non-chilling or ripening temperatures (Sevillano et al., 2009). Candan et al. (2008) found a relation between CI symptoms in 'Larry Ann' plums and a concomitant increase in membrane permeability and C_2H_4 production after transfer of the fruit to a higher temperature. This study also found that CI levels, as well as the C_2H_4 evolution rates were the highest in all the treatments after the shelf-life period. Therefore, it seems that if C_2H_4 evolution or the effect of C_2H_4 on the fruit could be controlled not only fruit softening, but also the manifestation of CI, could be controlled to a great extent in both plum cultivars.

4.3 Antioxidant levels

4.3.1 Water and lipid soluble antioxidant activity

Storing fruit at chilling temperatures causes oxidative stress (Lester, 2003). If allowed to accumulate, the levels of the active oxygen species (AOS), which are formed during oxidative stress, can exceed the capacity of the antioxidant systems of the plant product, and can cause damage to cellular components, such as the cell membranes (Anderson et al., 1995). Besides this, low temperature storage also lowers the activity of antioxidant enzymes, which further weakens the product's ability to overcome the increased levels of AOS (Purvis, 2004). In this study it was found that the HAA in both cultivars remained unchanged in all the storage regimes tested. This result agrees with the findings of Illić et al. (2009) on tomatoes stored at 5 °C and 12 °C.

The LAA also remained unchanged in all the 'Laetitia' and 'Sapphire' treatments, except for the 'Sapphire' fruit stored under the IW regime. The IW treatment caused a significant increase in the LAA of the 'Sapphire' fruit when IB started to manifest in this cultivar. In their study on eight Japanese plum cultivars, Díaz-Mula et al. (2009) only found an increase in the LAA of the flesh of

'Angeleno' plums stored at 2 °C for 35 days, while the levels remained unchanged in the other seven cultivars. They also found a strong correlation between LAA and the carotenoid concentration in the peel and pulp of the plum fruit. Carotenoids, as well as α-tocopherol, are important membrane-associated lipid soluble antioxidants which protect especially unsaturated fatty acids against oxidation by singlet oxygen (Larson, 1988; Hodges, 2001; Asensi-Fabado and Munné-Bosch, 2010). Consequently it has been found that membrane damage by temperature stress can be avoided by the maintenance of a high α-tocopherol concentration (Lurie, 2003). It is, therefore, suggested that the lower incidence of internal disorders in the 'Sapphire' fruit stored under the IW treatment may be due to a higher LAA under this regime compared to the other regimes tested.

4.3.2 Ascorbic acid

Ascorbic acid is considered to be the main water soluble antioxidant as well as an important redox buffer and enzyme cofactor in plants (Smirnoff, 2000; Hancock and Viola, 2005; Ishikawa et al., 2006). Its concentration is usually maintained at a relatively constant level, and is often increased under stress conditions in the chloroplasts of leaves (Foyer, 1993). A decrease in ascorbic acid levels is usually an indication that the product is experiencing severe stress. In this study, particularly the total ascorbic acid levels decreased at a fast rate during the first 10 and 21 days of storage, respectively for 'Sapphire' and 'Laetitia'. Wang (1996) and Franck et al. (2003) found that the highest rate of ascorbic acid losses occurred early after harvest during cold-storage followed by a slower rate of loss for the rest of the storage period, which is similar to our findings. Toivonen (2003) suggest that this postharvest decline in ascorbic acid is due to its double role as a scavenger of AOS and regenerator of other antioxidant systems such as flavonols and tocopherols.

It was found that ascorbic acid levels were lower at the lower storage temperatures compared to the higher storage temperatures in mangoes, bananas and strawberries harvested at the white tip stage (Kondo et al., 2005; Shin et al., 2008). Similar to their results, this study also found that the ascorbic acid levels in the 'Laetitia' plums stored at 7.5 °C did not decrease during the initial stages of storage and remained at approximately the same levels as on the harvest date. Likewise, the 'Sapphire' fruit stored at 7.5 °C had significantly higher ascorbic acid levels after 28 and 35 days of storage than the other treatments. The 7.5 °C treatment also had the lowest incidence of CI in both cultivars while the treatments that had the lowest ascorbic acid levels during storage had the highest incidence of CI, namely the IW and 2.5 °C treatments in the case of the 'Laetitia' plums and the 2.5 °C treatment in the case of the 'Sapphire' plums.

In greenhouse grown tomatoes it was found that fruit that were grown under higher temperature conditions had higher ascorbic acid levels (Venter, 1977). This was also observed in our study, where 'Laetitia', a late season cultivar, harvested during a warmer part of the season (in January) had higher levels of ascorbic acid than 'Sapphire', which is an early season cultivar harvested during a cooler part of the season (early December). However, the cultivars developed comparable levels of CI in the different treatments although 'Laetitia' plums had almost double the amount of ascorbic acid than the 'Sapphire' plums throughout storage.

4.3.3 Glutathione

Several studies found that glutathione levels increase under chilling conditions (Esterbauer and Grill, 1978; Anderson et al., 1992; Hausladen and Alscher, 1993; Gómez et al., 2009). It is suggested that the reason for this increase in glutathione levels is because the activity of glutathione reductase (GR), the enzyme responsible for the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH) and the maintenance of a high GSH:GSSG ratio, is upregulated under chilling conditions (Wang, 1995; Gómez et al., 2009). Increased activity of GR and the simultaneous increase of GSH levels under low-temperature conditions were detected in a number of studies (Esterbauer and Grill, 1978; Wang, 1995). Esterbauer and Grill (1978) suggest that the reason for this phenomenon is the protection of the -SH groups in enzymes and structural proteins by GSH to prevent the formation of S-S bonds under freezing conditions. It was also found that the activity of one of the two enzymes that catalyses the synthesis of glutathione, namely γ-glutamylcysteine synthetase (γECS), increases under chilling conditions (Szalai et al., 2009). An increase in glutathione levels in chilled plant tissue is important, as it scavenges for H_2O_2 and other toxic oxygen compounds together with ascorbic acid (Hodges, 2003). In this study glutathione levels increased in all the treatments during storage for both cultivars, which indicates that fruit exposed to all the treatments attempted to adapt to the low storage temperatures. The highest levels of glutathione were recorded in the 'Laetitia' plums stored at 5 °C and 7.5 °C. These two treatments also had the lowest CI incidence compared to the 'Laetitia' plums stored under the IW regime and 2.5 °C. However, Hodges (2003) states that increased levels of GSH alone would not ensure enough reducing power in all cases to prevent injury under elevated stress conditions. This is confirmed by this study, since although GSH levels increased over storage time in all the treatments, and to a greater extent in the 'Laetitia' plums stored at 7.5 °C and 5 °C, CI still manifested in these fruit. At best, the glutathione can thus be regarded as an ameliorating factor and not one preventing CI development.

4.4 Cell membrane composition

Temperature has a direct effect on the 'fluidity' of cellular membranes – e.g. increased temperature causes an increase in the rate of molecular motion and a decrease in order in the membrane (Cossins, 1981). It also causes reversible gel to liquid-crystalline membrane phase transitions. In the gel state (occurs at low temperatures) the fatty acid chains exist in a straight conformation, and are aligned at approximately right angles to the plane of the membrane, which limit the movement of the chains (Cossins, 1981; Murata and Yamaya, 1984). In this phase, the membrane becomes leaky to small electrolytes which negatively influence the selective permeability of the membrane to the detriment of physiological activities of the cell (Murata and Yamaya, 1984). However, in the liquid-crystalline state (occurs at higher temperature), the fatty acid chains are more flexible and rotate about their carbon-carbon bonds, which create free space that allows flexing and motion of adjacent chains (Cossins, 1981). Since the cell membranes are composed of a mixture of phospholipid head groups, hydrocarbon chains, sterols and membrane-bound proteins, phase transition usually occurs over a wide range of temperatures.

4.4.1 Total phospholipid and total sterol concentrations

Cold acclimation of plant tissues usually involves an increase in the membrane phospholipid concentration (Staehelin and Newcomb, 2000). A decrease in the total phospholipid levels would have dire consequences regarding the barrier properties of the membrane and the organisation of the biochemistry within the cell or organelle. In this study it was found that the phospholipid concentration remained relatively constant from the harvest date until the end of storage in both cultivars and all the treatments.

It is also known that free sterols increase membrane fluidity below the phase transition temperature (because its aliphatic tail is mobile and causes a certain degree of disorder in the hydrophobic part of the membrane), and decreases the fluidity above the phase transition temperature (because the steroid skeleton is rigidly planar) (Bloch, 1985; Leshem, 1992a; Marangoni et al., 1996). The effect of sterols on membrane fluidity is usually due to changes in the sterol:phospholipid ratio, and therefore indirect (Bloch, 1985). Since the sterol levels also remained relatively unchanged in both cultivars and for all the storage treatments in this study, there was also no change in the total sterol:total phospholipid ratio.

4.4.2 Phospholipid fatty acids

Membranes containing only saturated fatty acids (e.g. C16:0 and C18:0), undergo the gel to liquid-crystalline phase transition above 40 °C (Murata and Nishida, 1990). However, membranes containing phospholipids with one mono-unsaturated and one saturated fatty acid have phase transition near 0 °C, while two mono-unsaturated fatty acids decrease the phase transition temperature to -20 °C. Double bonds cause 'kinks' or bends in the fatty acid which influence the packing of the fatty acids in the membrane bilayer, and help the membrane to stay fluid at low temperatures (Staehelin and Newcomb, 2000). Consequently, it has been found that the degree of phospholipid fatty acid saturation and oxidation of unsaturated fatty acids are strongly linked to CI incidence in chilling prone produce (Parkin and Kuo, 1989; Whitaker, 1995). Likewise, chilling tolerant plants accumulate polyunsaturated fatty acids (PUFA) - specifically C18:3n3 - in response to low temperatures through the upregulation of fatty acid desaturases (Mazliak, 1979; Los and Murata, 2004; Upchurch, 2008). According to Nishida and Murata (1996) it is critical for membrane lipids to become unsaturated at low temperatures in order for its proper functioning, and hence, the survival of the organism. What was surprising in this study was that all the treatments for both cultivars had a relatively stable unsaturated:saturated phospholipid ratio throughout storage, indicating that all the tested regimes had a comparable effect on the unsaturated and saturated fatty acid levels in the cell membranes. From this it can be deduced that all the treatments would also have comparable membrane fluidity.

In virgin olive oils the oleic/linoleic ratios are responsible for the oxidative stability of the oil. The reason for this is that monounsaturated fatty acids (MUFAs), such as oleic acid, are more resistant to oxidation than polyunsaturated fatty acids (PUFAs), e.g. linolenic acid. Based on peroxide formation it has been determined that the oxidation ratio for oleate, linoleate and linolenate is 1:12:25 (Aparicio, et al., 1999). Therefore, it has been found that olive oil is more stable when oleic acid content is high and linoleic content is low. In this study, the MUFA:PUFA ratio did not change during storage and did not differ between the different 'Laetitia' treatments. However, the MUFA:PUFA ratio tended to first decrease, then increased again in all the Sapphire' treatments after approximately 28 days of storage, but then decreased in all the treatments during the last part of storage, except in the 2.5 °C treatment. Hence, the 'Sapphire' membranes first adapted to cold-storage in all the treatments, by accumulating polyunsaturated fatty acids in response to low temperatures, then adapted their MUFA:PUFA ratio to have a higher MUFA:PUFA ratio, probably to prevent oxidation of the polyunsaturated fatty acids, but then increased their polyunsaturated fatty acids again at the end of storage. However, these adaptations did not seem to influence the manifestation of CI in the elevated storage temperature regimes, since CI started to manifest after only 10 days of storage in these treatments. However, although the IW treatment made the same

adaptions to its MUFA:PUFA ratio as the other treatments, CI only started to manifest in these fruit after 28 days of storage. It, therefore, seems that the MUFA:PUFA ratio did not play an important role in the prevention of CI under the elevated temperatures or IW treatment in this study.

4.4.3 Lipid peroxidation

Lipid peroxidation is caused by either AOS, which leads to the formation of lipid-free radicals, or by lipoxygenase (LOX) - usually in concert with hydrolytic enzymes since it usually only oxidises free fatty acids - that forms lipid hydroperoxides (LOOH) (Shewfelt and Purvis, 1995). PUFAs are most susceptible to peroxidation, and in the case of lipoxygenase action, specifically linoleic and (9, 12, 15)-linolenic acid (Vick and Zimmerman, 1987). Due to the specific degradation of the PUFAs, the membranes may start to leak or may become more rigid (Leshem, 1992). In this study it was found that 'Sapphire' fruit stored at 5 °C and 7.5 °C, and 'Laetitia' fruit stored at 7.5 °C had the highest lipid peroxidation levels. These treatments had a lower linoleic acid concentration than the other treatments (significant in the case of 'Laetitia'), but a higher linolenic acid concentration (albeit not always significant) than the IW and 2.5 °C treatments (data not shown; Refer to Appendix D, Fig. 16 and Fig. 17). These results are, therefore, not always consistent with the finding that PUFA's are the main substrates of lipid peroxidation and are destroyed in the process. However, since damage to a single membrane system (e.g. plasma membrane, or inner mitochondrial membrane) may be sufficient to induce injury, the measurement of total PUFA's, as was done in this study, may not be an indication of the significant losses of PUFA's in the affected membrane (Shewfelt and Purvis, 1995). In this study the affected treatments were more associated with senescence than with CI, as the fruit stored under these regimes had the lowest flesh firmness (for both cultivars) and the highest decay and internal disorder levels, specifically OR, in the 'Sapphire' plums. Similarly, Zhuang et al. (1997) found that broccoli florets stored at 13 °C and 23 °C had higher levels of lipid peroxidation and accelerated deterioration compared to broccoli florets stored at 2 °C. Sung and Chiu (1995) also recorded higher levels of lipid peroxidation and more rapid deterioration in soybean seeds stored at 25 °C compared to 5 °C. It was also found in this study that especially the 7.5 °C treatment had the highest ethylene evolution rates. LOX is specifically associated with senescing plant tissues (Galliard and Chan, 1980; Hildebrand, 1989). The breakdown products, as mentioned earlier, are hydroperoxides, which are further catabolised to produce aldehydes, such as MDA (measured in this study to determine lipid peroxidation levels), hexanal and nonenal (which, among others, give rise to the typical aromas of fruit) and C_2H_4 (Leshem, 1992b). LOX, therefore, is also involved in the production of stress C_2H_4, as was observed in the 7.5 °C treatment of this study. Consequently, it seems that LOX, rather than AOS, was responsible for the higher levels of lipid peroxidation recorded in the 7.5 °C treatment in this study.

5. Conclusion

Single-temperature storage regimes within the 'killing zone' were detrimental to 'Sapphire' fruit quality, however, not always due to the manifestation of CI. When 'Sapphire' plums were stored at 7.5 °C and 5 °C it caused the fruit to soften quickly and to manifest higher levels of decay and overripeness compared to the IW regime. This was probably due to higher levels of lipid peroxidation, presumably by LOX rather than AOS, in these treatments. Storage of 'Sapphire' plums at 2.5 °C and 5 °C caused the manifestation of higher levels of CI than when the fruit was stored under the IW regime, which agrees with the results of Crisosto et al. (1999) on plums. It was shown that the fruit stored at 2.5 °C had the lowest ascorbic acid levels, while the fruit stored at 5 °C had high lipid peroxidation levels. The low levels of internal disorders that develop in the fruit stored under the IW regime is probably due to a better antioxidant status, specifically lipid soluble antioxidants, in these fruit, compared to the other regimes. Since the IW regime performed better overall, elevated storage temperatures within the 'killing zone' cannot be recommended for the postharvest storage of 'Sapphire' plums. Another solution must, therefore, be found to prevent the CI that still manifests in this cultivar when the fruit is stored under the IW regime.

'Laetitia' plums stored at 5 °C and 7.5 °C had significantly less CI than the fruit stored under the IW regime. Overall, the 'Laetitia' plums stored under the elevated storage temperatures had comparable levels of internal defects to the fruit stored under the IW regime. However, similar to the 'Sapphire' plums, fruit stored at 5 °C and 7.5 °C softened quicker – probably due to the higher C_2H_4 evolution rates in these fruit caused by presumably LOX activity. Although it did not differ significantly from the IW treatment, 'Laetitia' fruit stored under the elevated storage temperature regimes also tended to have higher shrivel and decay levels. It, therefore, seems that the elevated temperature storage regimes could hold promise for the storage of 'Laetitia' plums. However, some adaptions will probably have to be made to the regimes for them to have optimal benefits to the postharvest fruit quality of 'Laetitia' plums, since it caused more enhanced fruit softening and did not prevent the development of CI completely. In this regard it would be interesting to include 1-MCP (1-methylcyclopropene) treatment to counteract the negative effects of C_2H_4 resulting from the higher storage temperatures.

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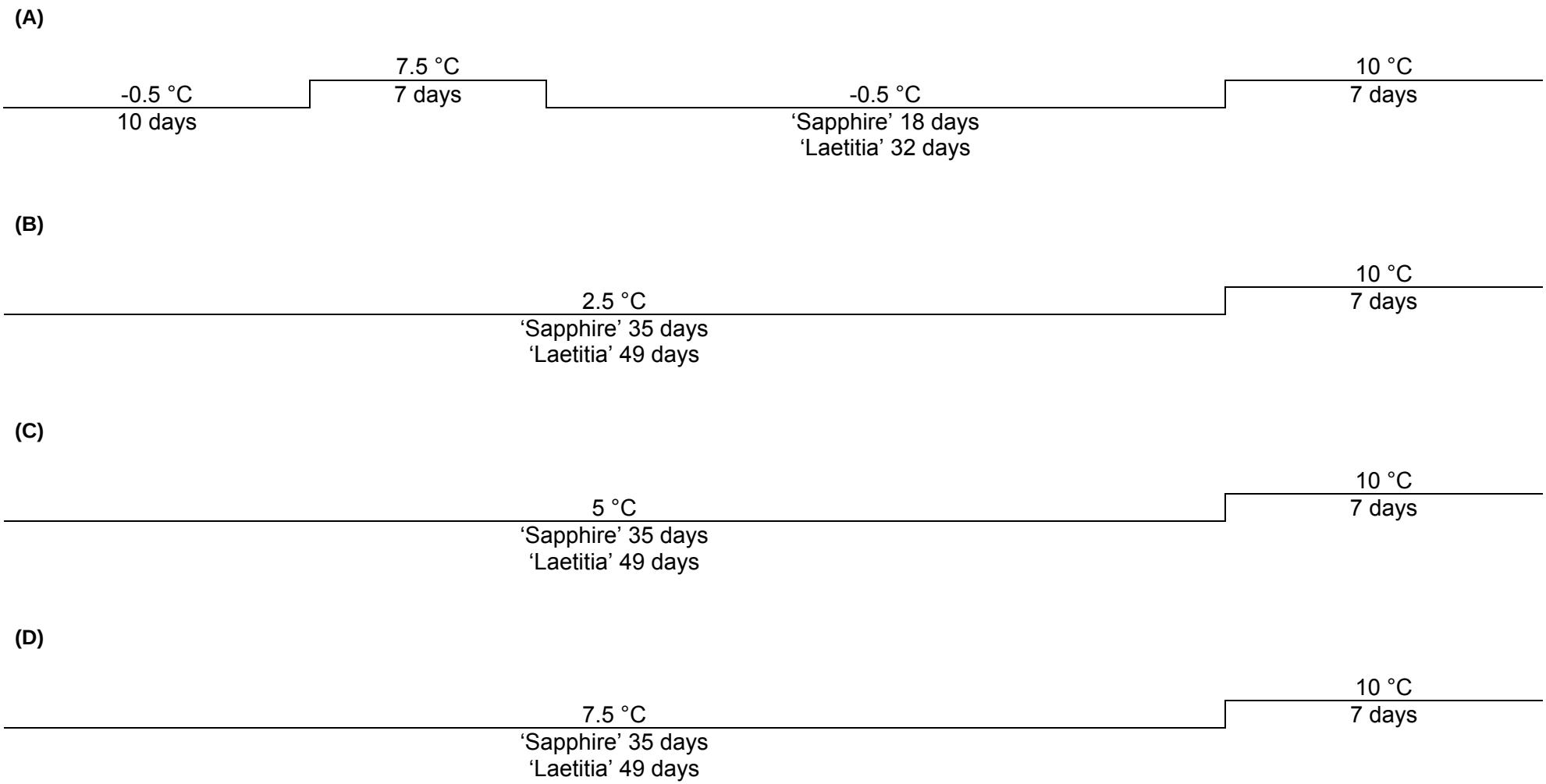
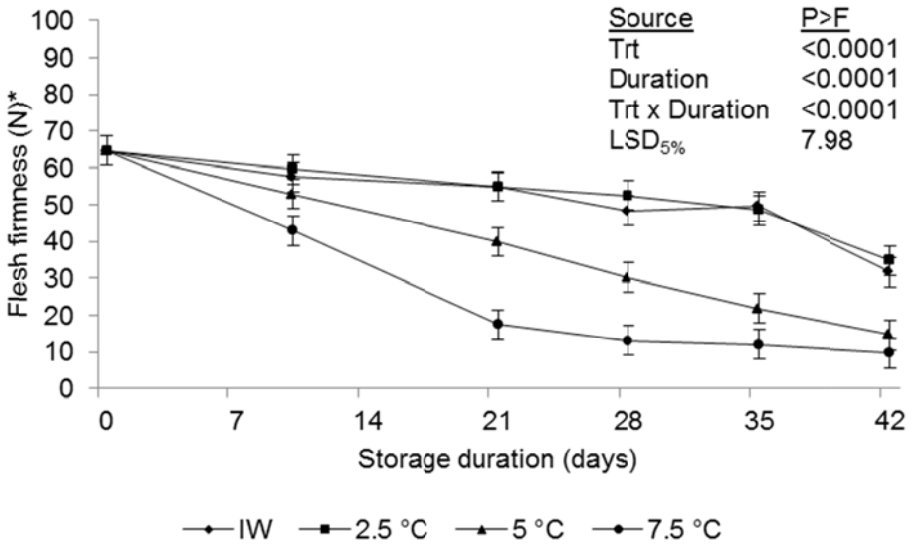


Fig. 4.1. Cold-storage regimes tested. (A) Intermittent warming regime (IW) at -0.5 °C and 7.5 °C followed by a simulated shelf-life period at 10 °C for 7 days. (B), (C) and (D) Single-temperature (ST) storage at, respectively, 2.5 °C, 5 °C and 7.5 °C followed by a simulated shelf-life period at 10 °C for 7 days. To simulate commercial conditions, 'Sapphire' plums were cold-stored for a maximum period of 35 days, and 'Laetitia' plums for a maximum period of 49 days before commencement of the simulated shelf-life period.



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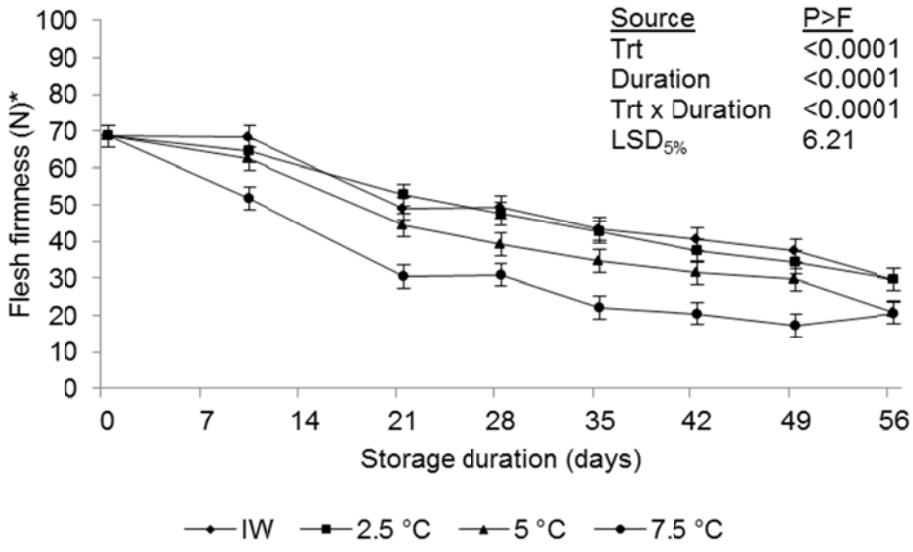
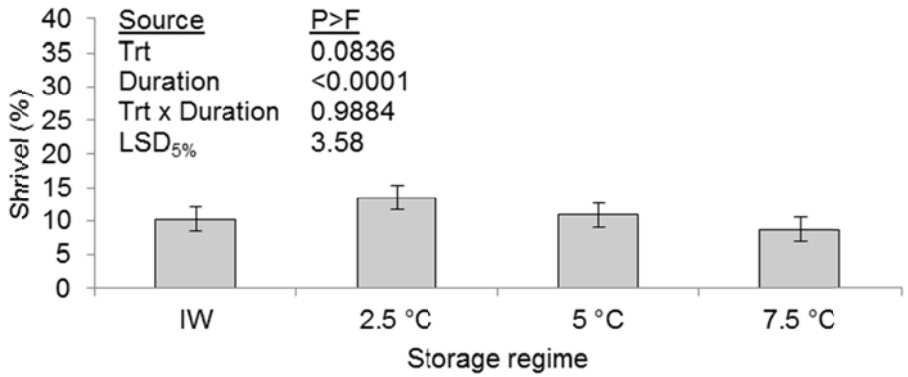
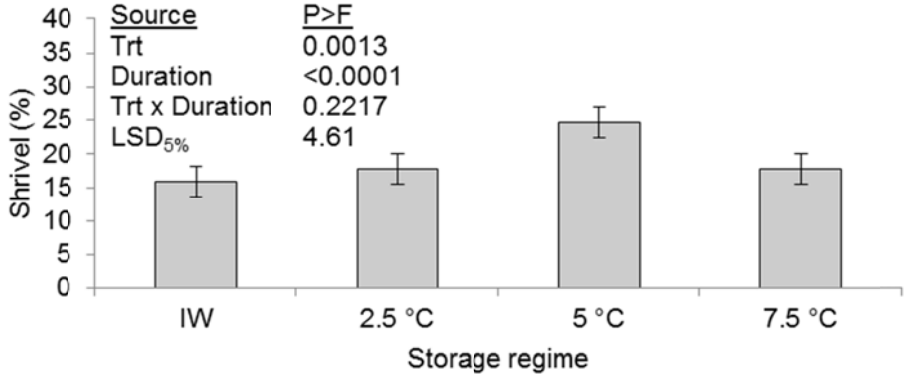


(B)

Fig. 4.2. Change in flesh firmness of (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by storage regime and storage duration. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

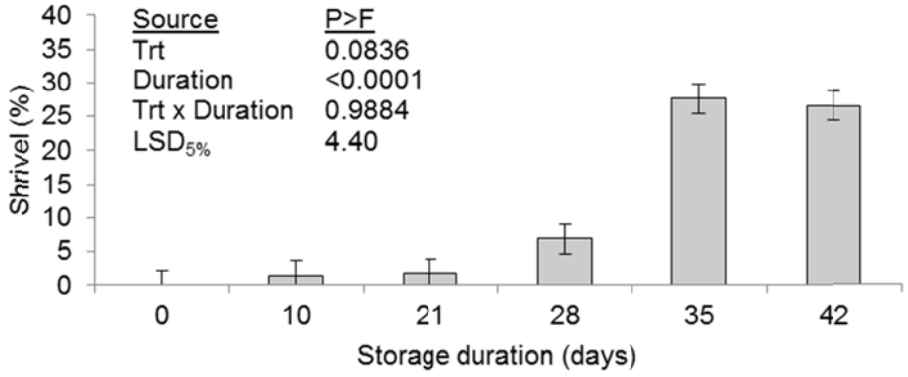


(A)

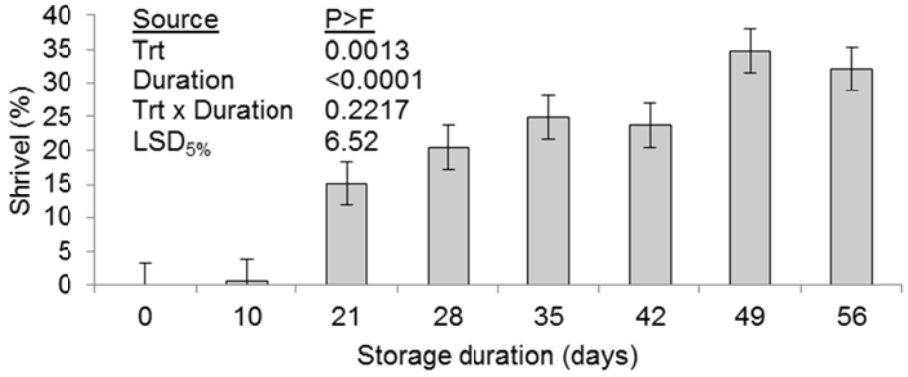


(B)

Fig. 4.3. Shrivel levels in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.



(A)



(B)

Fig. 4.4. Shrivel levels in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage duration. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

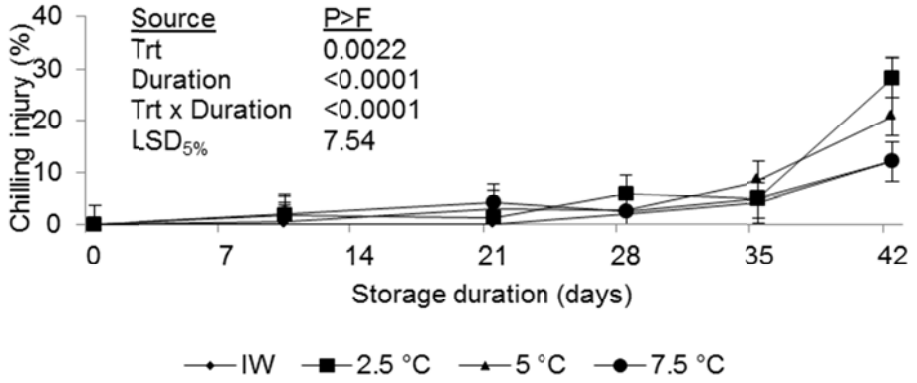
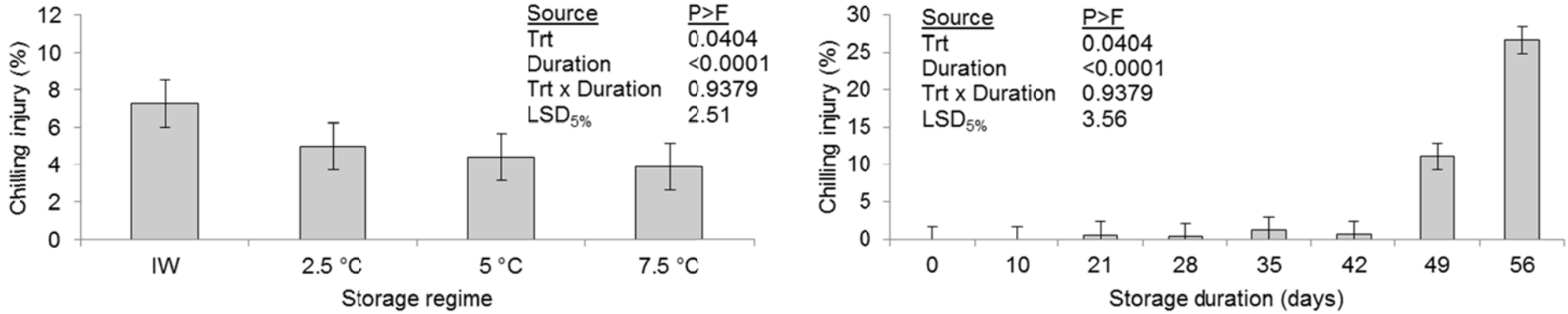


Fig. 4.5. Manifestation of chilling injury (sum of gel breakdown and internal browning) in 'Sapphire' plums as influenced by storage regime and storage duration. The fruit were stored for 35 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.



(A) **(B)**

Fig. 4.6. Chilling injury (sum of gel breakdown and internal browning) in 'Laetitia' plums as affected by (A) storage regime and (B) storage duration. 'Laetitia' plums were stored for 49 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

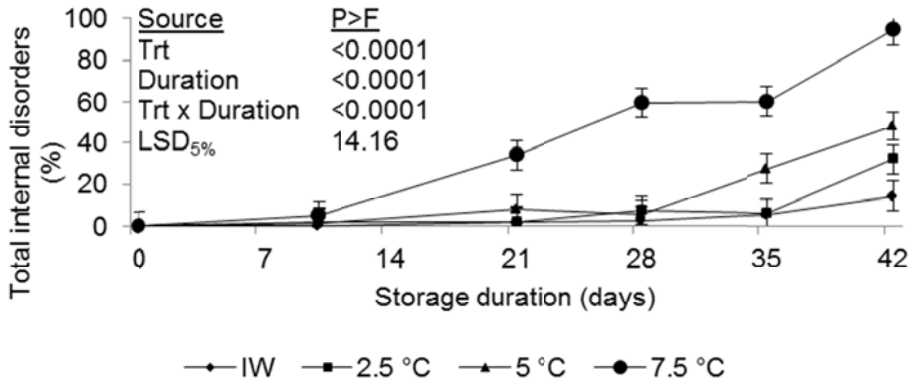
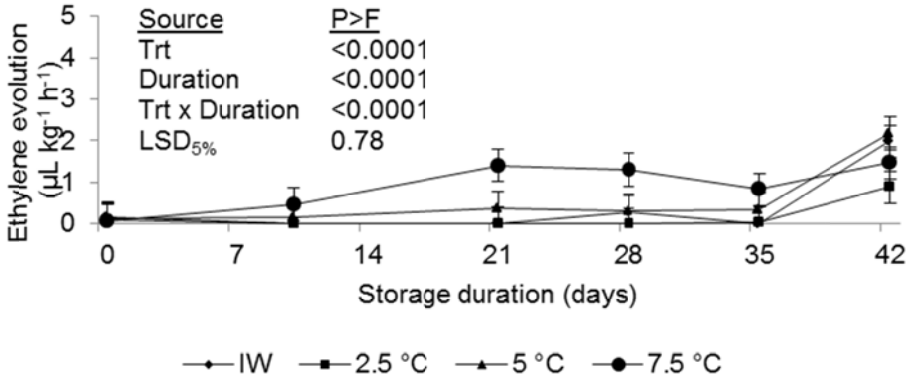


Fig. 4.7. Total internal disorders (sum of gel breakdown, internal browning and overripeness) in 'Sapphire' plums as influenced by storage regime and storage duration. The fruit were stored under the various temperatures for 35 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.



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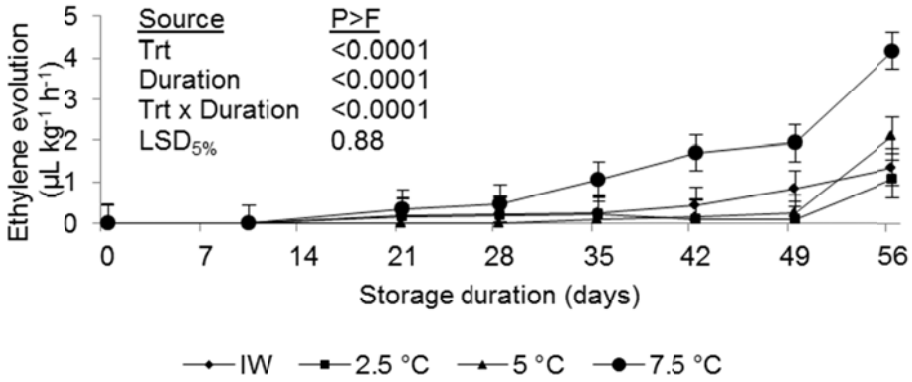


(B)

Fig. 4.8. Ethylene evolution in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

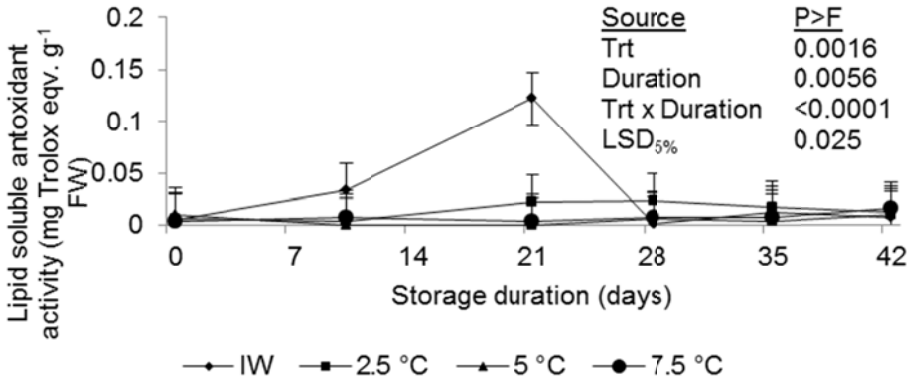
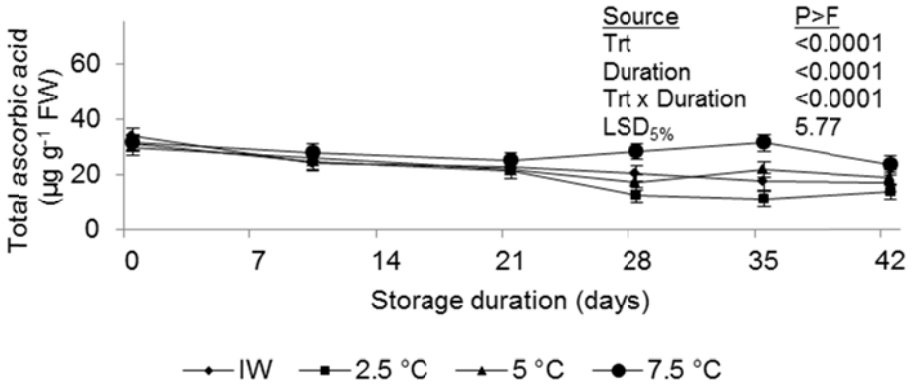
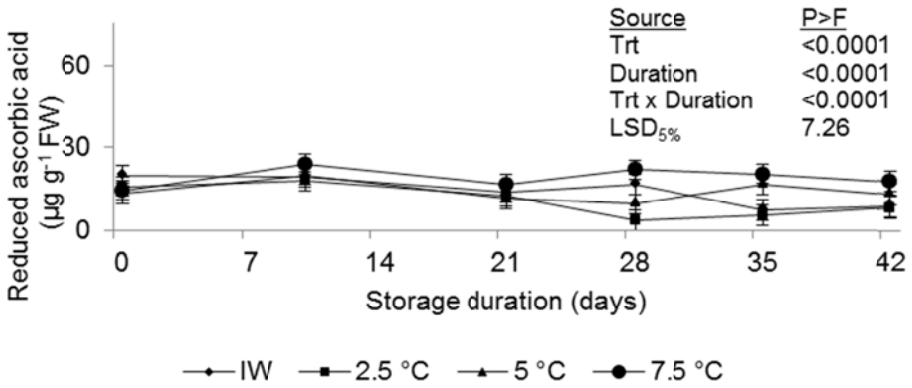


Fig. 4.9. Lipid soluble antioxidant activity in 'Sapphire' plums as affected by storage regime and storage duration. 'Sapphire' plums were stored for 35 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

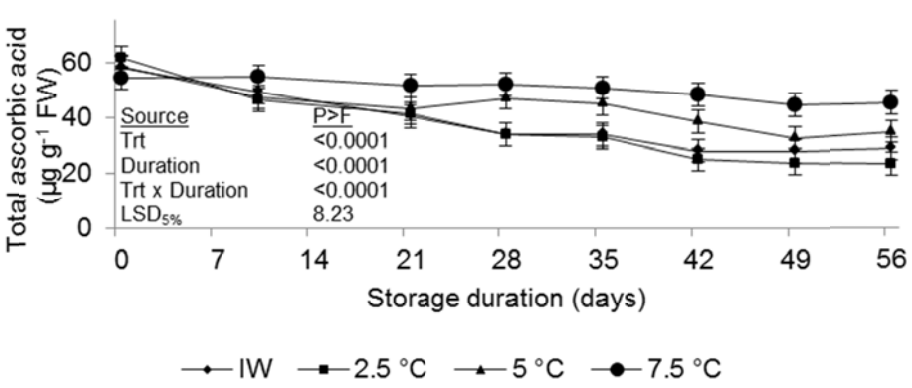


(A)

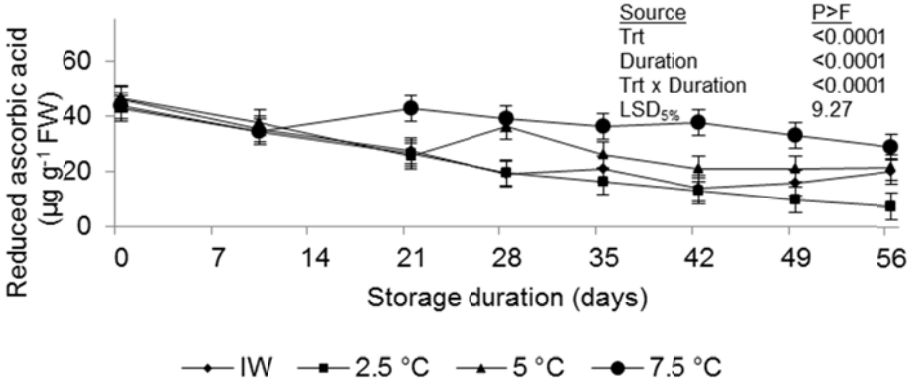


(B)

Fig. 4.10. Total ascorbic acid levels (A) and reduced ascorbic acid levels (B) in 'Sapphire' plums as affected by storage regime and storage duration. 'Sapphire' plums were stored for 35 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

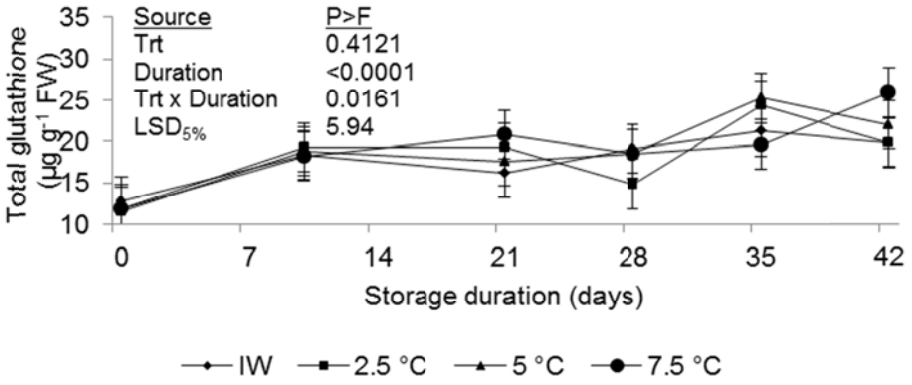


(A)

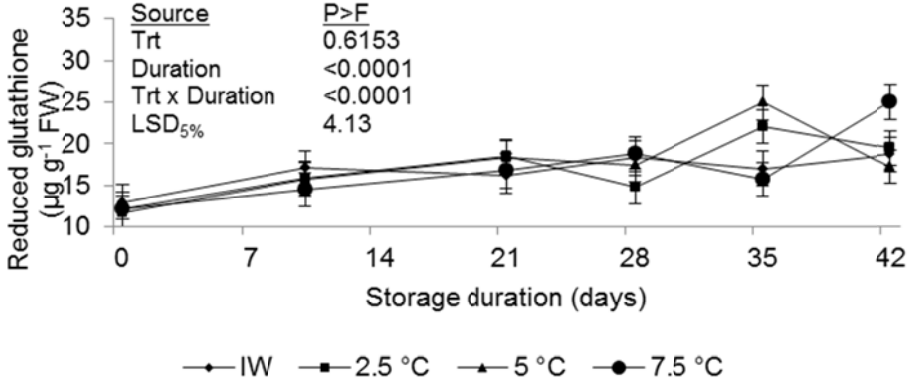


(B)

Fig. 4.11. Total ascorbic acid levels (A) and reduced ascorbic acid levels (B) in 'Laetitia' plums as affected by storage regime and storage duration. 'Laetitia' plums were stored for 49 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

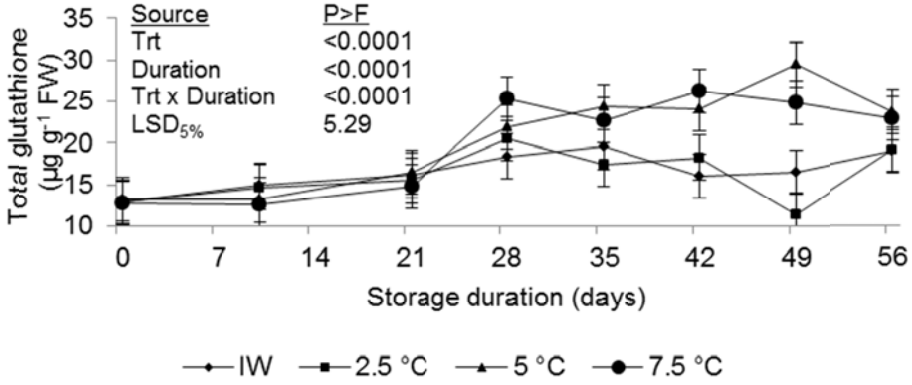


(A)

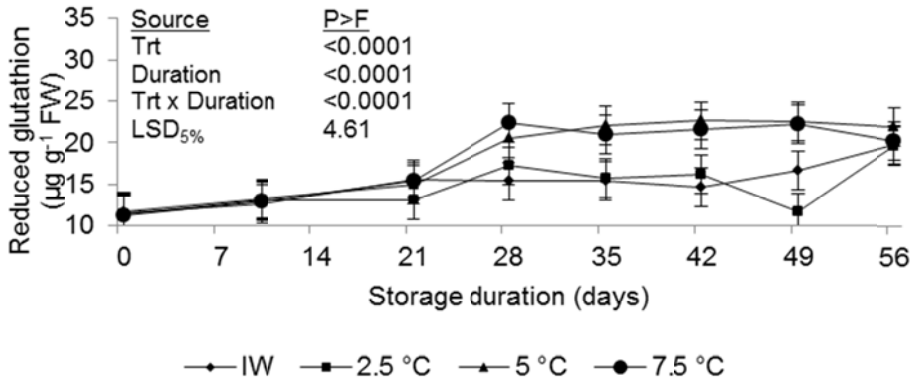


(B)

Fig. 4.12. Total glutathione (A) and reduced glutathione levels (B) in 'Sapphire' plums as affected by storage regime and storage duration. 'Sapphire' plums were stored for 35 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

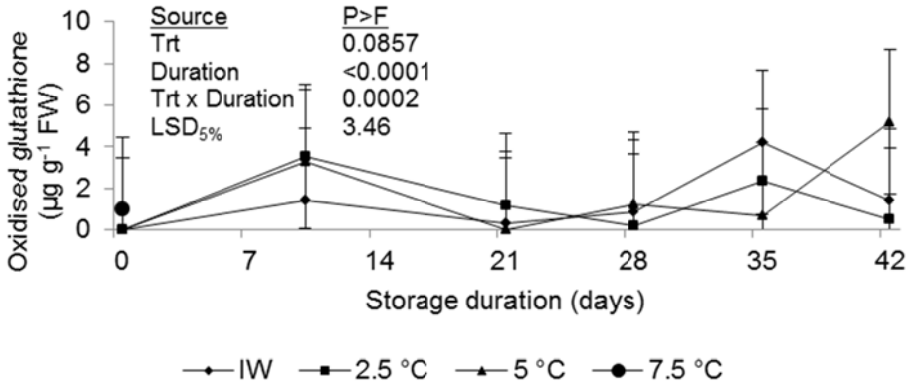


(A)

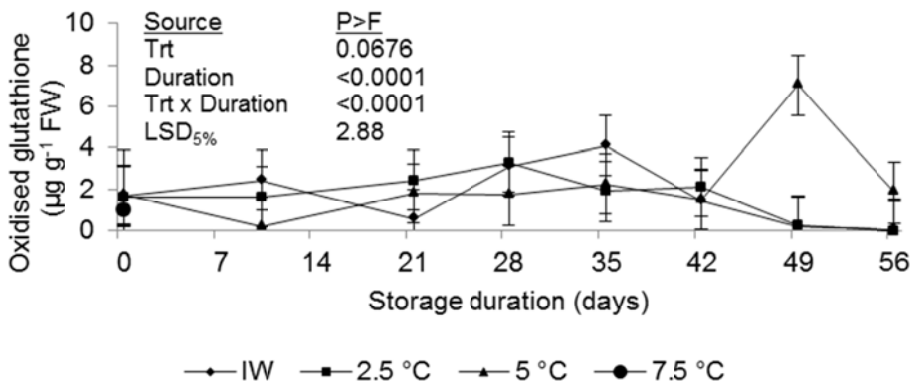


(B)

Fig. 4.13. Total glutathione (A) and reduced glutathione levels (B) in 'Laetitia' plums as affected by storage regime and storage duration. 'Laetitia' plums were stored for 49 days plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

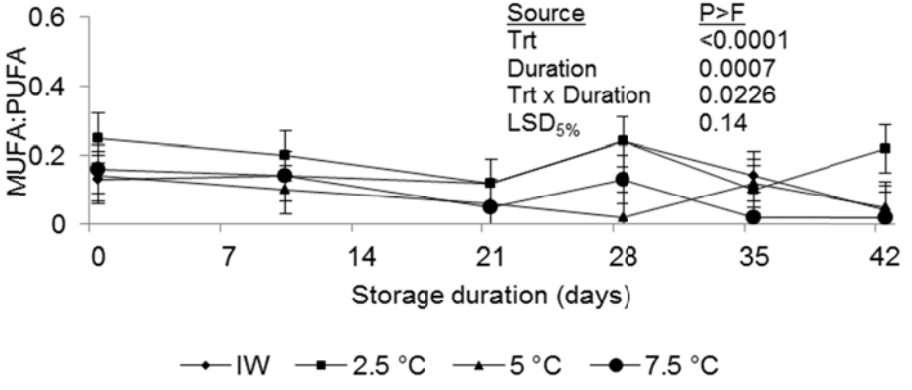


(A)

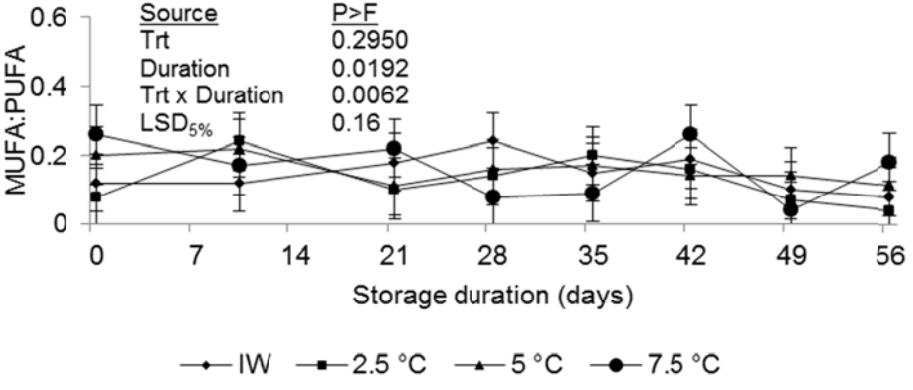


(B)

Fig. 4.14. Oxidised glutathione in 'Sapphire' (A) and 'Laetitia' (B) plums as affected by storage regime and storage duration. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

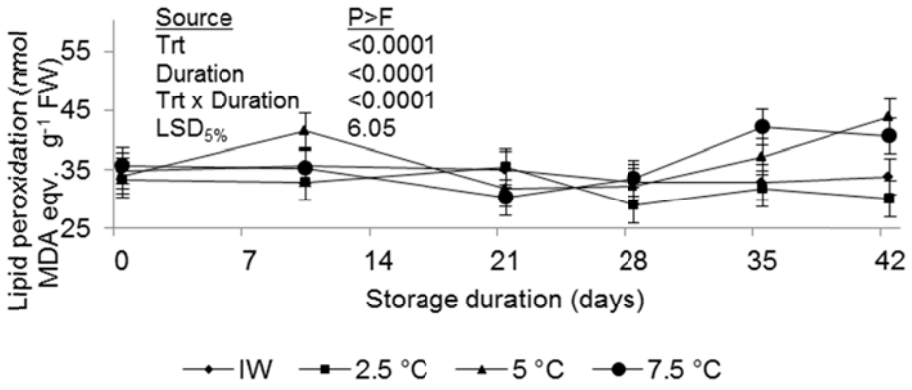


(A)

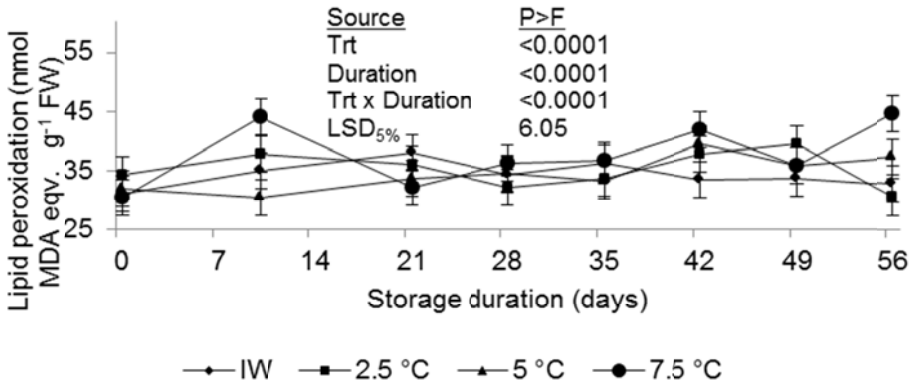


(B)

Fig. 4.15. The monounsaturated:polyunsaturated phospholipid fatty acid ratio (MUFA:PUFA) in 'Sapphire' (A) and 'Laetitia' (B) plums as affected by storage regime and storage duration. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.



(A)



(B)

Fig. 4.16. Lipid peroxidation levels in 'Sapphire' (A) and 'Laetitia' (B) plums as affected by storage regime and storage duration. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively plus a subsequent simulated shelf-life of 7 days at 10 °C. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

PAPER 5

Total forced air cooling time and initial rate affects cell membrane composition, antioxidant status and the manifestation of chilling injury in Japanese plums (*Prunus salicina* Lindl.)

Abstract

To prevent quality deterioration of fresh produce, as incidence of internal defects and decay levels after harvest and cold-storage, it is recommended that field heat is removed as soon as possible after harvest by means of rapid pre-cooling. However, two studies conducted on Japanese plums in South Africa indicated that rapid pre-cooling exacerbated the development of internal disorders and shrivel in some cultivars. Hence, the aim of this study was to determine the effect of the initial cooling rate and total forced air cooling (FAC) time on the cell membrane composition, antioxidant status, and chilling injury incidence of two plum cultivars differing in their sensitivity towards rapid FAC. For 'Sapphire' plums the FAC durations tested in this study (12 h and 24 h) did not affect fruit quality adversely, but a slower initial FAC rate (fruit cooled to a pulp temperature of 5 °C within 6 h after the commencement of FAC) prevented CI manifestation in this cultivar compared to a faster initial FAC rate (fruit cooled to a pulp temperature of 5 °C within 3 h after the commencement of FAC). For 'Laetitia' plums a faster initial FAC rate exacerbated shrivel development in this cultivar. Furthermore, it was found that the best internal and external 'Laetitia' fruit quality was obtained when fruit were cooled with a slower initial FAC rate (to a pulp temperature of 5 °C within 6 h after commencement of FAC) and for a longer duration (24 h opposed to 12 h). The beneficial effects of the FAC treatments for both cultivars may be attributed to the maintenance of specifically the water soluble antioxidant activity in the fruit.

Key words

Phospholipids, sterols, total phenolic concentration, unsaturated:saturated phospholipid fatty acid ratio, water soluble antioxidant activity

1. Introduction

Plums are highly perishable products (Crisosto and Mitchell, 2002) and can lose a day's storage life for every hour subjected to 20 °C after harvest (Taylor, 1996; Brosnan and Sun, 2001). To prevent deterioration of the product and an increase in internal breakdown and decay levels after harvest and cold-storage, it is recommended that field heat is removed as soon as possible after harvest by means of rapid pre-cooling to a pulp temperature near 0 °C (Mitchell, 1986; Monzini and Gorini, 1991; Tonini and Caccioni, 1991; Taylor, 1996; Combrink and Visagie, 1997; Brosnan and Sun, 2001). Therefore, for plums exported from South Africa it is strongly advised that the fruit is packed and that forced air cooling (FAC) commences on the same day as harvest (Hortgro Services, 2012a). Furthermore, that the fruit must be cooled to a pulp temperature below 10 °C within 10 to 12 h after commencement of FAC and to -0.5 °C within 24 h (Hortgro Services, 2012b).

Interestingly, in a study to determine the effect of the rate and duration of FAC on stone fruit quality, it was observed that the best results were obtained when 'Imperial' apricots were cooled within 6 h, that 'Songold' plums were sensitive to rapid cooling and needed to be forced air cooled for 12 h or longer and that 'Pioneer' plums were not affected by FAC rate or duration (Jooste and Khumalo, 2005). A subsequent study confirmed that 'Pioneer' and 'Sundew' plums were not sensitive to rapid cooling and may be cooled within 12 h (Khumalo et al., 2006). They also found that 'Sapphire' plums should be cooled within 24 h, while 'Laetitia' and 'Songold' plums, which are sensitive to rapid cooling, should be cooled to a pulp temperature of -0.5 °C in a time period between 24 and 48 h to prevent the development of higher levels of internal disorders. Therefore, while it is beneficial to commence FAC as soon as possible after harvest to prevent fruit deterioration, rapid pre-cooling does not always result in the maintenance of good fruit quality as is suggested by numerous researchers (Mitchell, 1986; Monzini and Gorini, 1991; Tonini and Caccioni, 1991; Taylor, 1996; Combrink and Visagie, 1997; Brosnan and Sun, 2001). This is confirmed by a study on the effect of the rate of chilling at 0 °C on the survival of microorganisms (Cao-Hoang et al., 2008). They found that a slow temperature decrease (2 °C min^{-1}) resulted in cell stabilisation and rapid cooling (2000 °C min^{-1}) in the loss of cell viability due to irreversible membrane rigidification. It, therefore, seems that the reason for the injury caused by fast cooling could be due to cell membrane alterations.

The aim of this study was to determine the effect of the initial cooling rate and total FAC time on the cell membrane composition, antioxidant status, and CI incidence of two plum cultivars differing in their sensitivity to rapid FAC. This knowledge will help to gain a better understanding of the

processes involved in the development of CI, as well as in the design of better postharvest strategies to avoid, reduce, or at least delay the incidence of CI in South African plums.

2. Materials and Methods

2.1 Sampling of fruit and measurements made on each evaluation date

'Sapphire' (medium susceptibility to injury when rapidly FAC) and 'Laetitia' (susceptible to injury when rapidly FAC) plums (*Prunus salicina* Lindl.), packed according to export standards (DAFF, 2012), were sampled from a commercial packhouse in Franschhoek, Western Cape, South Africa in the 2007 and 2008 seasons. The fruit was sampled on the day of harvest. Only size A (50 to 55 mm diameter) fruit were used. Fruit of both cultivars were packed with perforated, high density polyethylene (HDPE) shrivel sheets. This was done to prevent moisture loss from the fruit as 'Sapphire' and 'Laetitia' plums are prone to shrivel during cold-storage. The fruit did not receive any pre-cooling prior to it being sourced for the trial. The packed cartons were transported to the laboratory using covered, uncooled transport. A complete randomised design with six replicates per FAC treatment was used.

At the laboratory the fruit of each cultivar was subjected to four treatments of different FAC rates and total times at a delivery air temperature of -1 °C to a pulp temperature of 0 °C as depicted in Fig. 5.1. The FAC treatments were as follows:

- FAC1 = cooled to a pulp temperature of 5 °C within 3 h and then to a pulp temperature of 0 °C within a total time of 12 h,
- FAC2 = cooled to a pulp temperature of 5 °C within 6 h and then to a pulp temperature of 0 °C within a total time of 12 h,
- FAC3 = cooled to a pulp temperature of 5 °C within 3 h and then to a pulp temperature of 0 °C within a total time of 24 h,
- FAC4 = cooled to a pulp temperature of 5 °C within 6 h and then to a pulp temperature of 0 °C within a total time of 24 h.

After FAC fruit was immediately placed under static cold-storage. A commercially used intermittent warming (IW) regime, as depicted in Fig. 5.2, was used for cold-storage of the plums. To simulate commercial conditions, 'Sapphire' plums were cold-stored for a maximum period of 35 days, and 'Laetitia' plums for 49 days before commencement of the simulated shelf-life period. The plastic shrivel sheet was removed before the fruit were placed at 10 °C to prevent condensation of water and fruit decay. Fruit were evaluated on the sampling date, directly after FAC, after 10 days of

storage (which coincides with the commencement of the IW period commercially), 21 days of storage, 28 days of storage (which simulates the arrival of the fruit at the overseas destination), 35 days of storage (which simulates the end of cold-storage for 'Sapphire' plums), 42 days of storage (which simulates the end of the shelf-life period for 'Sapphire' plums), after 49 days (which simulates the end of the cold-storage for 'Laetitia' plums) and after 56 days (which simulates the end of the shelf-life period for 'Laetitia' plums). On each evaluation date 37 fruit per replicate per treatment were evaluated.

Shrivel and decay (expressed as %) were determined subjectively on 30 fruit per replicate. Shrivel was counted when shrivelled skin extended over the shoulder of the fruit. Flesh firmness (N) was measured with a Southtrade fruit pressure tester (Model FT327, Alphonsine, Italy) fitted with an 11.1 mm tip, on one peeled cheek of 10 fruit per replicate. Internal defects (%) were determined by cutting 20 fruit per replicate around the equatorial axis, and separating the two halves of the fruit. A gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue had a healthy appearance, was classified as GB (Taylor, 1996). A brown discolouration of the mesocarp tissue, associated with a loss in juiciness, was classified as IB. Fruit were classified as overripe (OR) when abnormally soft to the touch with excessive amounts of free juice, when the mesocarp tissue in the sub-epidermal region developed a translucent breakdown while the inner tissue exhibited a normal appearance and/or when cut around the equatorial axis, and the two halves of the fruit were twisted in opposite directions, the skin and sub-epidermal layers of the mesocarp separated from the inner mesocarp which remained attached to the stone. To obtain the total CI, the sum of the percent GB and IB per block were calculated, while the total internal defects were calculated by adding the total CI and the percent OR.

Rate of ethylene (C_2H_4) evolution (μL kg^{-1} h^{-1}) was measured on two fruit per replicate. The fruit were sealed in airtight 0.5 L glass jars for 1 h. A headspace gas sample was taken from each jar with a 10 mL airtight syringe, and injected into a Varian GC system (Model 3300, Varian Instrument Group, Palo Alto, California, USA) fitted with a flame ionisation detector (FID) and 2 m PoropakQ column. Nitrogen was used as a carrier gas. The oven temperature was programmed from 60 °C (isothermal for 2 min) to 70 °C at 1 °C min^{-1}. The injector temperature was at 65 °C and the detector temperature at 250 °C. A 1 ppm standard gas was used to identify and quantify C_2H_4.

Five fruit per replicate were pooled, peeled and the pulp flash frozen, milled to a fine powder in liquid nitrogen, and stored at -80 °C for further analysis. For the biochemical analyses conducted on the samples stored at -80 °C, see Paper 1, Section 2.3.

2.2 Statistical analysis

Factorial analysis of variance (Factorial ANOVA) was used to calculate statistical differences between all data that resulted from fruit analysis after the different storage periods. Season was used as a co-variate. Factorial ANOVAs was performed using SAS version 9.1 (SAS Institute, Inc. 2000). ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

3. Results

3.1 Effect of FAC treatment on the fruit quality of 'Sapphire' and 'Laetitia' plums

3.1.1 Flesh firmness

The FAC treatments did not have a significant effect on the flesh firmness of 'Sapphire' or 'Laetitia' plums (data not shown; Refer to Appendix E, Fig. 1).

3.1.2 Shrivel

For 'Sapphire' plums the FAC treatments did not have a significant effect on the shrivel levels of the fruit (Fig. 5.3A). The shrivel levels in the 'Sapphire' fruit were within the export norm (commercially export fruit are usually rejected when shrivel levels exceed 10%). For 'Laetitia' plums the shrivel levels were also within the norm for export fruit. However, after 49 days of storage the shrivel levels were the highest in the FAC3 treatment (Fig. 5.3B). Although the shrivel levels in the FAC3 treatment did not differ significantly from the FAC1 treatment, it was significantly higher than in the FAC2 and FAC4 treatments (slower initial FAC rate) after 49 days of storage. The result indicates that a faster initial cooling rate (within 3 h to a pulp temperature of 5 °C) exacerbates shrivel development in 'Laetitia' plums during storage, while a slower initial cooling rate (within 6 h to a pulp temperature of 5 °C) limits shrivel manifestation. However, after the shelf-life period shrivel levels decreased in the FAC1 and FAC3 treatments and did not differ significantly from the FAC2 and FAC4 treatments.

3.1.3 Decay

For 'Sapphire' plums the FAC treatments did not have a significant effect on decay development (Fig. 5.4A). For 'Laetitia' plums the FAC2 treatment (slower initial FAC rate and shorter FAC duration) had significantly higher decay levels compared to the other treatments (Fig. 5.4B).

3.1.4 CI

For ‘Sapphire’ plums the FAC1 treatment had the highest levels of CI (Fig. 5.5A). Although the CI levels in the FAC1 treatment did not differ significantly from the FAC3 treatment, it was significantly higher than in the FAC2 and FAC4 treatments. This result indicates that the faster initial rate of FAC (within 3 h to a pulp temperature of 5 °C) exacerbated the development of CI in ‘Sapphire’ plums while a slower initial FAC rate (within 6 h to a pulp temperature of 5 °C) reduced CI manifestation. It is also interesting to note that the initial rate of FAC, and not the total FAC duration, influenced the manifestation of CI in ‘Sapphire’ plums.

For ‘Laetitia’ plums the FAC1 and FAC2 treatments had significantly higher CI levels after 49 days of storage compared to the FAC3 and FAC4 treatments (Fig. 5.5B). This result indicates that a shorter FAC duration (12 h) caused severe CI manifestation in ‘Laetitia’ plums, while a longer FAC duration (24 h) prevented CI development to a large degree during cold storage. During the shelf-life period between days 49 and 56, CI levels increased significantly in the FAC1 and FAC3 treatments, increased slightly, but not significantly, in the FAC4 treatment and levelled out in the FAC2 treatment. This resulted in the FAC1 treatment to have the highest CI levels (differed significantly from FAC2 and FAC4, but not from FAC3), followed by FAC3 (did not differ significantly from FAC2 and FAC4). Contrary to the evaluation results on day 49, the after shelf-life results indicate that the FAC treatments with the fastest initial cooling rates (FAC1 and FAC3), and not the shortest cooling duration, had the highest levels of CI after shelf-life. However, FAC4, which had a slower initial cooling rate (6 h to a pulp temperature of 5 °C) and a longer FAC duration (24 h), had the lowest CI levels after both cold-storage and shelf-life.

The main type of CI that manifested in the ‘Sapphire’ and ‘Laetitia’ plums was IB, while GB made a small contribution (data not shown; Refer to Appendix E, Fig. 2 for ‘Sapphire’ and Fig. 3 for ‘Laetitia’).

3.1.5 Total internal disorders

Since OR levels were very low and did not differ between the FAC treatments (data not shown; Refer to Appendix E, Fig. 4), neither the levels nor trends of the total internal disorders that manifested differed from what was observed for CI (data not shown; Refer to Appendix E, Fig. 5).

3.2 Effect of FAC treatments on the biochemical parameters measured in 'Sapphire' and 'Laetitia' plums

3.2.1 Ethylene evolution

C_2H_4 evolution rates in the 'Sapphire' plums were very low in all the FAC treatments during the first 35 days of storage (Fig. 5.6A). However, the C_2H_4 evolution rates increased significantly in all the treatments after the shelf-life period, with the FAC3 treatment having significantly the highest rate. For 'Laetitia' plums there was not a significant difference between the C_2H_4 evolution rates of the different FAC treatments (Fig. 5.6B).

3.2.2 Antioxidants

3.2.2.1 Water- and lipid soluble antioxidant activity

For 'Sapphire' plums there was generally not a difference in the HAA of the different FAC treatments during storage, except for a significant spike in the HAA of the FAC2 and FAC4 treatments (slower initial FAC rate) after 35 days of storage (Fig. 5.7A). While this spike did not cause the HAA levels in the FAC2 treatment to be significantly higher than in the FAC1 and FAC3 treatments (faster initial FAC rate), it caused the FAC4 treatment to have significantly the highest levels of HAA. After the shelf-life period (day 42) the FAC1 and FAC2 treatments (shorter total FAC duration) had a lower HAA than the FAC3 and FAC4 treatments (longer total FAC duration), but the difference was not significant. For 'Laetitia' plums there was a significant increase in the HAA of the FAC2 and FAC4 treatments (slower initial FAC rate) at the end of the FAC treatment (1 day after the commencement of FAC), while the HAA in the FAC1 treatment remained almost unchanged and it decreased significantly in the FAC3 treatment (Fig. 5.7B). From day 1 until day 42 of storage the HAA was the highest, although it did not differ significantly from all the other treatments, in the FAC2 treatment. After 49 days of storage and after the shelf-life period the HAA was higher in the FAC2 and FAC4 treatments (slower initial FAC rate), but it did not differ significantly from the other two treatments.

For 'Sapphire' plums the FAC2 and FAC3 treatments caused a significant decrease in the LAA levels during the FAC treatment (as was measured 1 day after the commencement of FAC) (Fig. 5.8A). During storage the LAA in all the treatments were generally comparable, except for a significant spike in the LAA of the FAC4 treatment after 35 days of storage, which was also significantly higher than in the other treatments. Therefore, the FAC4 treatment had significant spikes in HAA and LAA that coincided after 35 days of storage, and which was not observed in any of the other treatments (Fig. 5.7A and 5.8A). Except for the FAC3 treatment, a significant increase

in the LAA of the other FAC treatments was observed in the 'Laetitia' plums at the end of the FAC treatment (day 1 of storage) (Fig. 5.8B). Apart from for the FAC1 treatment, which had significant spikes in LAA after 28 and 49 days of storage, and only differed significantly from the FAC2 and FAC3 treatments, the FAC treatments did not differ significantly in their LAA levels during the entire storage duration of the 'Laetitia' plums.

3.2.2.2 Ascorbic acid

For both cultivars and all FAC treatments total and reduced ascorbic acid levels decreased significantly during storage (Fig. 5.9 for 'Sapphire' and Fig. 5.10 for 'Laetitia' plums). For 'Sapphire' plums there was an increase in both the total and reduced ascorbic acid levels after the shelf-life period, although the increase was not significant in most treatments (Fig. 5.9A and B). A similar trend was not observed in the 'Laetitia' plums; on the contrary levels of total and reduced ascorbic acid continued to decrease in this cultivar during the shelf-life period (Fig. 5.10A and B). For the 'Laetitia' plums the FAC3 treatment had a significant spike in total ascorbic acid levels, but not in the L-AA levels, after 42 days of storage (Fig. 5.10A and B). For both cultivars levels of total and reduced ascorbic acid were comparable in all the treatments after the shelf-life period (Fig. 5.9A and B and Fig. 5.10A and B).

For 'Sapphire' plums levels of DHA increased, although not significantly, during storage in all the FAC treatments (Fig. 5.11A). For the 'Laetitia' plums there was a general decrease in the levels of DHA in all the treatments from day 1 until day 21 to day 28 (depending on the treatment) of storage (Fig. 5.11B). However, the decrease was not significant in any of the treatments. After the initial decrease in the 'Laetitia' plums, levels of DHA either increased again or levelled out in the different FAC treatments. Generally there was not a significant difference in the DHA levels between the different FAC treatments during storage in the 'Laetitia' plums.

3.2.2.3 Glutathione

Except for the FAC2 treatment, which caused a non-significant increase, the other FAC treatments generally caused a non-significant decrease in total and reduced glutathione levels in the 'Sapphire' plums during FAC (Fig. 5.12A and B). For all the FAC treatments the levels of total and reduced glutathione remained relatively stable during the first 28 days of storage, but then increased significantly in all the treatments until the end of storage. At the end of the shelf-life period all the FAC treatments had similar levels of total and reduced glutathione. Regarding GSSG in the 'Sapphire' plums, the FAC4 treatment had a significant spike after 10 days of storage,

but then decreased again and remained low for the rest of the storage duration (Fig. 5.13). After 35 days of storage the FAC1 and FAC2 treatments (shorter total FAC duration) had significantly higher levels of GSSG compared to the FAC3 and FAC4 treatments. After the shelf-life period the FAC2 treatment had significantly the highest levels of GSSG compared to the other treatments.

The levels of total and reduced glutathione remained unchanged in the 'Laetitia' plums after the FAC treatments were completed on day 1 of storage (Fig. 5.14A and B). After 21 days of storage all the treatments had an increase in total glutathione levels (significant in the case of FAC1 and FAC4) (Fig. 5.14A). However, for the GSH this increase was non-significant in all the FAC treatments (Fig. 5.14B). Subsequently, the total and reduced glutathione levels remained relatively stable until day 28 of storage (Fig. 5.14A and B). After 35 days of storage there was a significant increase in the total glutathione levels of the FAC2 and FAC3 treatments, while levels remained almost unchanged in the FAC1 and FAC4 treatments (Fig. 5.14A). Although the GSH levels also increased in the FAC2 and FAC3 treatments after 35 days of storage, it was only significant in the case of the FAC3 treatment (Fig. 5.14B). After 42 days of storage there was a significant decrease in the total glutathione levels of the FAC1 treatment followed by a significant decrease in the total glutathione levels of the FAC2, FAC3 and FAC4 treatments after 49 days of storage (Fig. 5.14A). Although similar decreases were observed in the levels of GSH, it was only significant in the case of the FAC3 and FAC4 treatments (longer FAC duration) (Fig. 5.14B). After the shelf-life period the FAC4 treatment had significantly the highest levels of total glutathione, and although this treatment also had the highest levels of GSH after shelf-life, it only differed significantly from the FAC2 and FAC3 treatments (Fig. 5.14A and B).

In the 'Laetitia' plums the FAC1 and FAC2 treatments (shorter FAC duration) caused a sharp, but non-significant, decrease in the levels of GSSG compared to almost no change in the FAC3 and FAC4 treatments (longer FAC duration) (Fig. 5.15). After 21 days of storage the FAC2 treatment had the lowest levels of GSSG, which was significantly lower than in the FAC1 and FAC4 treatments. This was followed by a significant spike in the levels of GSSG in the FAC1, FAC2 and FAC4 treatments after 35 days of storage. However, after 49 days of storage the levels of GSSG decreased to comparable levels in all the treatments. After the shelf-life period the levels of GSSG were very low in all the treatments, except in the FAC 4 treatment which had a significant increase during the shelf-life period to have significantly the highest levels.

3.2.2.4 Total phenolics

For ‘Sapphire’ plums the FAC3 treatment had the highest concentration of total phenolics, although it did not differ significantly from the FAC1 treatment (Fig. 5.16A). Therefore, although the difference was not necessarily significant, the FAC1 and FAC3 treatments (faster initial FAC rate) had a higher total phenolic concentration than the FAC2 and FAC4 treatments (slower initial FAC rate). For the ‘Laetitia’ plums the total phenolic concentration generally decreased in all the treatments during storage with no significant differences between the treatments on the different evaluation dates during cold-storage (Fig. 5.16B). At the end of the storage period the total phenolic concentration was significantly lower in all the treatments than on the sampling date, except for the FAC4 treatment, which had a similar concentration to the sampling date.

3.2.3 Cell membrane components

3.2.3.1 Total phospholipids

After 10 and 21 days of storage of the ‘Sapphire’ plums the FAC3 treatment had the highest levels of total phospholipids, but it did not differ significantly from all the treatments on both dates (Fig. 5.17A). Subsequently, the total phospholipid concentration gradually decreased in the FAC3 treatment until the end of storage. From day 21 to day 28 of storage, there was a significant increase in the total phospholipid concentration in the FAC2 treatment so that it had the highest concentration of phospholipids, although not significantly different from all the other treatments, until day 35 of storage after which it decreased during the shelf-life period. After the shelf-life period the FAC3 and FAC4 treatments (longer FAC duration) had a higher total phospholipid concentration than the FAC1 and FAC2 treatments (shorter FAC duration), although the difference was not always significant.

For the ‘Laetitia’ plums the total phospholipid concentration increased significantly in all the treatments, except FAC3, during cold-storage (Fig. 5.17B). At the end of cold-storage the FAC4 treatment had the highest total phospholipid concentration, but it did not differ significantly from the FAC1 treatment. After the shelf-life period the FAC3 treatment had the highest levels of total phospholipids, but it only differed significantly form the FAC4 treatment, which had the lowest levels.

3.2.3.2 Total sterols

For ‘Sapphire’ plums the total sterol concentration did not change significantly in any of the treatments during storage, except in the FAC2 treatment which had a significant spike after 21 days of storage (Fig. 5.18A).

For the ‘Laetitia’ plums there was a significant increase in the total sterol concentration of the FAC2 and FAC4 treatments (slower initial FAC rate) after 10 days of storage, which also differed significantly form the FAC1 and FAC3 treatments (Fig. 5.18B). Subsequently, the levels decreased in the FAC4 treatment, but remained relatively high, but not significantly different from all the treatments, in the FAC2 treatment until day 49 of storage. On day 49 of storage the FAC2 and FAC3 treatments had a significantly higher total sterol concentration compared to the FAC1 and FAC4 treatments. However, after the shelf-life period the FAC4 treatment had significantly the highest total sterol concentration.

3.2.3.3 Total sterol:total phospholipid ratio

Due to the relatively stable phospholipid and sterol concentrations during storage for both cultivars and in all the FAC treatments, the ratio between the sterols and phospholipids did not differ significantly between the treatments (data not shown, Refer to Appendix E, Fig. 6).

3.2.3.4 Phospholipid fatty acids

3.2.3.4.1 Unsaturated:saturated phospholipid fatty acid ratio

For ’Sapphire’ plums the FAC1, FAC2 and FAC4 treatments caused a decrease (significant in the case of FAC1) in the unsaturated:saturated fatty acid ratio during forced air cooling (Fig. 5.19A). After 10 days of storage, however, all the treatments adjusted their ratios to have similar levels compared to the sampling date. For the rest of the storage duration the FAC1 and FAC2 treatments (shorter FAC duration) had a higher, although not always significant, unsaturated:saturated fatty acid ratio than the FAC3 and FAC4 treatments (longer FAC duration).

For ‘Laetitia’ plums the different FAC treatments had comparable ratios of unsaturated:saturated fatty acids for the largest part of the storage duration (Fig. 5.19B). Interestingly, all the treatments had a decrease in the ratio after 35 days of storage, which was significant in the case of the FAC3 and FAC4 treatments (longer FAC duration). The ratio remained at the decreased levels for the

remainder of the storage period in the FAC2 and FAC4 treatments (slower initial FAC rate) while there was considerable variation in the ratio of the FAC1 and FAC3 treatments (faster initial FAC rate) for the rest of the storage duration. At the end of the shelf-life period the FAC1 treatment had the highest ratio, but it did not differ significantly from the FAC4 treatment.

3.2.3.4.2 Monounsaturated:polyunsaturated phospholipid fatty acid ratio (MUFA:PUFA)

The FAC4 treatment caused a sharp, but non-significant, increase in the MUFA:PUFA ratio, while the other treatments caused much smaller, non-significant, increases in the ratio during FAC of the 'Sapphire' plums (Fig. 5.20A). After 10 days of storage the MUFA:PUFA ratio was higher, although not significantly, in the FAC1 and FAC2 treatments (shorter FAC duration) compared to the FAC3 and FAC4 treatments (longer FAC duration). Subsequently, there was a sharp decrease in the MUFA:PUFA ratios of the FAC1, FAC2 and FAC4 treatments (significant in the case of FAC1 and FAC2 which represented shorter FAC durations), while the ratio increased significantly in the FAC3 treatment to be significantly higher than in the other treatments on day 21, and only non-significantly higher on day 28 of storage. However, after 35 and 42 days of storage all the treatments had comparable MUFA:PUFA ratios.

For 'Laetitia' plums the MUFA:PUFA ratio did not differ significantly between the FAC treatments (Fig 5.20B).

3.2.3.4.3 Lipid peroxidation

For 'Sapphire' plums there was considerable variation in the lipid peroxidation levels of the FAC1 and FAC2 treatments (shorter FAC duration) during storage (Fig. 5.21A). Nevertheless, the FAC2 treatment had the lowest lipid peroxidation levels after 28 and 35 days of storage, however, it did not differ significantly from the FAC3 and FAC4 treatments (longer FAC duration) on day 28. In the FAC3 and FAC4 treatments (longer FAC duration) the lipid peroxidation levels decreased during FAC, levelled out after 10 days of storage and then gradually increased towards the end of the storage period. At the end of the shelf-life period the lipid peroxidation levels were comparable in all the treatments.

For 'Laetitia' plums the lipid peroxidation levels were generally in a narrow band in all the treatments throughout storage and did not differ much from each other (Fig. 5.21B). After the

shelf-life period the levels were the highest in the FAC1 treatment, but it did not differ significantly from the FAC4 treatment.

4. Discussion

4.1 Shrivel and decay manifestation

The results of this study indicated that neither the initial FAC rate nor total duration had an effect on the manifestation of shrivel or decay in 'Sapphire' plums. It also indicated that 'Sapphire' plums could tolerate short (within 12 h) and long (within 24 h) FAC durations, but that a slower initial FAC rate prevented CI development compared to a fast initial rate. Therefore, it is suggested that 'Sapphire' plums can be forced air cooled within 12 to 24 h, but with a slower initial rate (to a pulp temperature of 5 °C within 6h after commencement of FAC) to prevent CI development in the fruit. This recommendation, therefore, refines the recommendation of Khumalo et al. (2006) which recommended that 'Sapphire' should be forced air cooled within 24 h, but did not recommend an initial FAC rate. For 'Laetitia' plums a faster initial FAC rate exacerbated shrivel development, while a combination of a slow initial FAC rate and short FAC duration caused significant decay development. Mitchell and Gentry (1963) found that not only air velocity, but also relative humidity (RH) played an important role in shrivel manifestation in nectarines after FAC. They found that at an RH of 95%, air velocity did not affect shrivel development, but that an RH of 75% caused severe weight loss and shrivel manifestation in nectarines. Since 'Laetitia' plums have a thin and sensitive skin which makes the cultivar more prone to injuries, and hence to decay, and moisture loss (Bester, 1991), it would be beneficial to ensure an RH of 95% in the FAC facility when this cultivar is cooled. The best internal and external fruit quality for 'Laetitia' plums was obtained when a slow initial FAC rate and a longer FAC duration were employed, which agrees with the recommendations of Khumalo et al. (2006). Overall, these findings and recommendations support the recommendation of Hortgro Services (2012b) that plums must be FAC to a pulp temperature below 10 °C within 10 to 12 h after commencement of FAC and to -0.5 °C within 24 h.

4.2 C_2H_4 evolution

In this study it was observed that C_2H_4 evolution rates were very low during cold-storage in the 'Sapphire' plums, but increased significantly after the shelf-life period in all the treatments. Usually 1-aminocyclopropane-1-carboxylic acid (ACC), ACC synthase activity and C_2H_4 production remain low at the chilling temperature, but increase rapidly when the product is transferred to a higher temperature (Wang, 1982; Sevillano et al., 2009). This is because the mRNA encoding for ACC-synthase is stimulated by low temperatures, and the translation of these messengers occurs

immediately after transfer of the product to higher temperatures (Sevillano et al., 2009), which explains the higher C_2H_4 evolution rates measured in this study after shelf-life. It is also interesting to note that FAC3, a treatment with a fast initial FAC rate which generally caused higher levels of CI in the 'Sapphire' fruit than the treatments with a slower initial FAC rate, had the highest rate of C_2H_4 evolution after the shelf-life period. In their study on the role of C_2H_4 in the manifestation of CI in plums, Candan et al. (2008) found that there was a clear relationship between cold-induced C_2H_4 and CI manifestation in specifically climacteric plum cultivars. They found that there was a concomitant increase in membrane permeability when C_2H_4 evolution rates increased after cold-storage and suggested that CI manifestation in plums is related to an increase in ripening in these fruit. To prove this they reduced CI manifestation in the climacteric plum cultivar by treating it with 1-methylcyclopropene (1-MCP). However, it is not clear why the FAC3 treatment specifically had the highest C_2H_4 evolution rate after shelf-life in this study, as the FAC1 treatment had higher levels of CI than the FAC3 treatment, but a lower rate of C_2H_4 evolution after shelf-life.

4.3 Antioxidant levels

4.3.1 Water and lipid soluble antioxidant activity

It was interesting to note that the FAC treatments which reduced the manifestation of CI in 'Sapphire' and 'Laetitia' plums, namely FAC2 and FAC4, had the highest, albeit not always significant, HAA after the FAC treatment on day 1 of storage (only for 'Laetitia' plums) and at the end of cold-storage (day 35 for 'Sapphire' and day 49 for 'Laetitia'), while the other treatments had a decrease followed by a levelling out of their HAA. For the 'Sapphire' plums the FAC4 treatment also had the highest LAA concentration after 35 days of storage. Coincidentally the first CI was also noted on day 35 of storage in the 'Sapphire' plums (data not shown; Refer to Appendix E, Fig. 7) and day 49 for 'Laetitia' plums. Antioxidant capacity usually increases under low stress or during acclimation before a higher degree of stress is introduced (Hodges, 2001). However, a similar increase in HAA was not observed in the FAC1 and FAC3 treatments, which manifested higher levels of CI than the FAC2 and FAC4 treatments. Low temperature storage reduces the activity of antioxidant enzymes which weakens the product's ability to overcome the increased levels of AOS (Purvis, 2004). It therefore seems that the slower initial FAC rate in the FAC2 and FAC4 treatments benefitted the HAA of the fruit compared to the faster initial FAC rate of the FAC1 and FAC3 treatments. However, it is not known why the FAC2 treatment, which generally had the highest levels of HAA in the 'Laetitia' plums during cold-storage, manifested high levels of CI at the end of cold-storage as well as higher levels, although not significant, of CI after the shelf-life period than the FAC4 treatment. Also, the FAC1 treatment had significant spikes in its LAA after 28 and 49 days of storage of the 'Laetitia' plums, but the highest levels of CI after the shelf-life period. The

result indicates that HAA and LAA alone were not able to protect the fruit sufficiently against CI and that other factors also played a role.

4.3.2 Total phenolic concentration

In numerous studies on various fresh produce, including plums, it was found that HAA is strongly correlated with the total phenolic content of the product (Kalt et al., 1999; Gil et al., 2002; Kondo et al., 2005; Díaz-Mula et al., 2009). However, in this study it was found that the FAC2 and FAC4 treatments (slower initial FAC rate), which had the highest HAA in the 'Sapphire' plums after cold-storage and after shelf-life and lower levels of CI, had lower average total phenolic concentrations than the FAC1 and FAC3 treatments, which caused more CI in the cultivar. Hodges (2001) suggests that due to the higher concentration of phenolic compounds compared to other antioxidants, they are of greater value in protecting fresh produce against oxidative stress, which seems not to be the case in this study. The observation in this study that more CI was observed in the treatments with the higher levels of total phenolics, may be due to phenolic compounds being primarily found in the vacuole, while most of the active oxygen species (AOS) are produced outside the vacuole (Hodges, 2001). AOS have very short life spans, and therefore, would tend to react with the cellular contents in their near vicinity rather than travel over the tonoplast where they could be quenched by the phenolic compounds. In the 'Laetitia' plums the total phenolic concentration decreased significantly in all the FAC treatments during cold-storage, but increased only in the FAC4 treatment, which is the preferred treatment for this cultivar, at the end of the shelf-life period to levels similar to the sampling date. Therefore, except for the FAC4 treatment at the end of the shelf-life period, storage duration had a bigger effect on the total phenolic concentration than the FAC treatment in the 'Laetitia' plums.

4.3.3 Ascorbic acid and glutathione

In both cultivars the storage duration and temperature had a much bigger influence on the ascorbic acid concentration than the FAC treatments. However, regarding glutathione in the 'Sapphire' plums, the FAC treatments with the shorter durations (FAC1 and FAC2) caused significantly higher oxidation of glutathione after 35 days of storage, and the FAC2 treatment had the highest levels of GSSG after the shelf-life period. Nevertheless, this did not cause significantly lower levels of GSH in the FAC1 and FAC2 treatments of the 'Sapphire' plums. Similar to this study's findings in the 'Sapphire' plums, several other studies found that glutathione levels increase under chilling conditions (Esterbauer and Grill, 1978; Anderson et al., 1992; Hausladen and Alscher, 1993; Gómez et al., 2009), purportedly because the activity of glutathione reductase (GR), the enzyme responsible for the reduction of oxidised to reduced glutathione and the maintenance of a high

GSH:GSSG ratio, is upregulated under chilling conditions (Wang, 1995; Gómez et al., 2009). Increased activity of GR and the simultaneous increase of GSH levels under low-temperature conditions were reported in a number of studies (Esterbauer and Grill, 1978; Wang, 1995). Esterbauer and Grill (1978) suggest that the reason for this phenomenon is the protection of the -SH groups in enzymes and structural proteins by GSH to prevent the formation of S-S bonds under freezing conditions. Since GSSG forms mixed disulphides with proteins – which inactivate many biosynthetic enzymes - GR is needed for the quick reduction of GSSG (Noctor et al., 2002). It was also found that the activity of one of the two enzymes that catalyses the synthesis of glutathione, namely γ-glutamylcysteine synthetase (γECS), increases under chilling conditions (Szalai et al., 2009). Hodges (2003) remarks that an increase in glutathione levels in chilled plant tissue is important, as it scavenge for H_2O_2 and other toxic oxygen compounds together with ascorbic acid. It therefore seems that the FAC1 and FAC2 treatments (shorter FAC durations) caused more stress in the 'Sapphire' plums, hence the higher production of GSSG, but that the fruit were able to adapt to the stress, since both treatments also produced higher levels of GSH. Since the FAC2 treatment had low levels of CI opposed to high levels of CI in the FAC1 treatment, it can be deduced that the higher levels of GSSG could not be the reason for the higher CI levels in the latter treatment.

The decline in the GSH levels in the FAC3 and FAC4 treatments (longer FAC duration) after 49 days of storage of the 'Laetitia' plums bears some significance. GSH aids in the maintenance of the reduced state of the cells in order to neutralise the effect of oxidative stress; hence a decline in the GSH levels indicates severe stress in these treatments (Gill and Tuteja, 2010). However, these two treatments also had significantly the lowest levels of CI after 49 days of storage compared to the FAC1 and FAC2 treatments. This is probably due to the fact that the FAC3 and FAC4 treatments had higher levels of GSH after 42 days of storage (just prior to the significant decline) and similar levels of GSH after the decline on day 49 of storage compared to the FAC1 and FAC2 treatments. Furthermore, the FAC4 treatment, which is the preferred treatment for the 'Laetitia' plums, had the highest levels of GSH and GSSG after the shelf-life period. Surprisingly, the higher GSH level after the shelf-life period in the FAC4 treatment did not differ significantly from the GSH level in the FAC1 treatment, which had the highest CI levels after the shelf-life period. It, therefore, seems that glutathione alone was not the main driver that aided the FAC4 treatment to yield the best 'Laetitia' fruit quality after storage.

4.4 Cell membrane composition

4.4.1 Total phospholipid and total sterol concentration

Cold acclimation of all cold-hardy herbaceous and woody species usually involves an increase in the membrane phospholipid concentration (Yoshida, S., 1984; Staehelin and Newcomb, 2000). This was also observed during the initial storage period of apples (Lurie et al., 1987) and tomatoes (Whitaker, 1992) at chilling temperatures. It was also found that sterols stabilise membrane fluidity over a wide range of temperatures (Marangoni et al., 1996). Free sterols increase membrane fluidity below the phase transition temperature (because its aliphatic tail is mobile and causes a certain degree of disorder in the hydrophobic part of the membrane), and decreases the fluidity above the phase transition temperature (because the steroid skeleton is rigidly planar) (Bloch, 1985; Leshem, 1992; Marangoni et al., 1996). In this study the FAC2 treatment, one of the preferred FAC treatments of 'Sapphire' plums, had a higher total phospholipid concentration than the other treatments from day 28 until the end of cold-storage as well as a significant spike in its total sterol levels on day 21 of storage. However, the FAC4 treatment, which also had low CI levels in the 'Sapphire' plums, did not have exceptionally high total phospholipid levels at the end of the cold-storage period or exceptional sterol levels during cold-storage. In 'Laetitia' plums the FAC4 treatment, the preferred treatment of the 'Laetitia' plums, had a higher total phospholipid concentration on day 49 of storage and total sterol concentration after shelf-life than the other treatments. However, the FAC3 treatment, which also had low CI levels in the 'Laetitia' plums at the end of the cold-storage period, had the lowest levels of total phospholipids and significantly higher total sterol levels than the FAC4 treatment on day 49 of storage. Interestingly, the FAC2 treatment, which had significantly higher CI after cold-storage, but comparably low CI levels to the FAC4 treatment in the 'Laetitia' plums after the shelf-life period, had relatively high sterol levels during cold-storage, but significantly lower levels than the FAC4 treatment after the shelf-life period. Therefore, due to conflicting results, the lower CI levels in specific FAC treatments cannot be definitely ascribed to sterol or phospholipid concentrations.

4.4.2 Phospholipid fatty acids

It has been found that the degree of phospholipid fatty acid saturation is strongly linked to CI incidence in chilling prone produce since it causes rigidification of the membrane under low temperature conditions (Parkin and Kuo, 1989; Whitaker, 1995). Therefore, it is interesting to note that the FAC treatments with the shorter durations had a higher unsaturated:saturated fatty acid ratio than the treatments with a longer duration in the 'Sapphire' plums. This result suggest that a shorter FAC duration caused higher levels of unsaturated phospholipid fatty acids, while a longer FAC duration caused higher levels of saturated fatty acids in this cultivar. However, for 'Sapphire' plums the initial FAC rate, and not the FAC duration influenced the fruit quality after storage.

Therefore, in the case of the 'Sapphire' plums, although the FAC treatments with a longer duration caused more unsaturated phospholipid fatty acids to be built into the cell membranes, and hence more fluid cell membranes under the low temperature conditions, it did not necessarily influenced the manifestation of CI in this cultivar. In the 'Laetitia' plums the FAC treatments with a slower initial FAC rate (preferred for 'Laetitia' plums since they manifested the lowest CI levels after cold-storage) had a lower unsaturated:saturated fatty acid ratio from day 35 until the end of the storage period. Therefore, for the 'Laetitia' plums, the FAC treatments that had the highest levels of saturated fatty acids also had significantly the lowest CI levels after cold-storage. This result is contrary to the result of Cao-Hoang et al. (2008) who found that a slow temperature decrease (2 °C min^{-1}) resulted in cell stabilisation and rapid cooling (2000 °C min^{-1}) in the loss of cell viability due to irreversible membrane rigidification. In Paper 3 it was also observed that the 'Laetitia' plums stored under the IW regime had higher levels of saturated fatty acids – probably to ensure that the membranes remain selectively permeable under the warmer storage conditions of the IW regime. The result of the current study, therefore, indicates that the FAC treatments with a slower initial cooling rate enhanced this trait of the 'Laetitia' plums.

While there were some trends observed in the MUFA:PUFA ratio, none explained why the FAC2 and FAC4 treatments performed better regarding 'Sapphire' fruit quality compared to the FAC1 and FAC3 treatments. For the 'Laetitia' plums the FAC treatments did not have a significant impact on the MUFA:PUFA ratio.

4.4.3 Lipid peroxidation

Regarding lipid peroxidation levels, the FAC2 treatment (a preferred treatment for 'Sapphire' plums) had the lowest levels after 28 and 35 days of storage. However, the other preferred treatment for 'Sapphire' plums, FAC4, had significantly higher levels of lipid peroxidation on day 35 of storage (when CI was first observed) than the FAC2 treatment. Therefore, the lipid peroxidation levels also do not consistently provide an explanation for the better 'Sapphire' fruit quality when a slower initial FAC rate was employed. The trends observed in the 'Laetitia' plums also do not explain why the FAC4 treatment had the best internal and external fruit quality compared to the other FAC treatments.

5. Conclusion

For 'Sapphire' plums the FAC durations tested in this study did not affect fruit quality adversely. However, the results showed that, irrespective of the FAC duration tested, a slower initial FAC rate

prevented CI manifestation in this cultivar. Fruit treated with a slower initial FAC rate had the highest HAA after the cold-storage period, which probably protected the fruit against oxidative stress. None of the other biochemical parameters that were determined on the 'Sapphire' plums gave a clear indication as to why the slower initial FAC rate was more beneficial to the internal fruit quality compared to the faster initial FAC rate. Therefore, in 'Sapphire' it seems that the slower initial FAC rate was beneficial to the fruit quality because it caused a higher HAA in the fruit, and not because it stabilised the cell membranes, as was found by the study of Cao-Hong et al. (2008) on microorganisms.

For 'Laetitia' plums it was clear that a faster initial FAC rate exacerbated shrivel development in this cultivar, probably due to the cultivar's thin skin which makes it more prone to injuries and moisture loss. This could probably be overcome by ensuring an RH of 95% within the FAC facility. Furthermore, it was found that the best internal and external 'Laetitia' fruit quality was obtained when the fruit were FAC with a slower initial FAC rate (to a pulp temperature of 5 °C within 6 h after commencement of FAC) and for a longer duration (24 h opposed to 12 h). It was found that this FAC treatment (FAC4) had the highest HAA after FAC and after cold-storage, an increased total phenolic concentration after shelf-life, the highest total phospholipid concentration after cold-storage and a lower unsaturated:saturated phospholipid fatty acid ratio at the end of the storage period. Therefore, it is suggested that the higher HAA after cooling and after cold-storage as well as the higher total phenolic concentration after the shelf-life period protected the fruit of this treatment against oxidative stress, while the high phospholipid and saturated phospholipid fatty acid concentrations at the end of the cold-storage period caused the membranes to stay optimally permeable and protected them against CI at the low storage temperatures.

An interesting result of this study is that rapid pre-cooling did not result in the best fruit quality of the two plum cultivars tested as is suggested by a number of other studies (Mitchell, 1986; Monzini and Gorini, 1991; Tonini and Caccioni, 1991; Taylor, 1996; Combrink and Visagie, 1997; Brosnan and Sun, 2001). Contrarily, the results of this study support the current protocol recommended by Hortgro Science (2012b) to force air cool plums to a pulp temperature below 10 °C within 10 to 12 h after commencement of FAC and to -0.5 °C within 24 h.

6. Acknowledgements

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and fruit available for the trial. We also thank the laboratory personnel at the Department of Horticultural Science, Stellenbosch University, for assistance with the sample analysis as well as Prof Karen Theron for assistance with the statistical analysis of the data.

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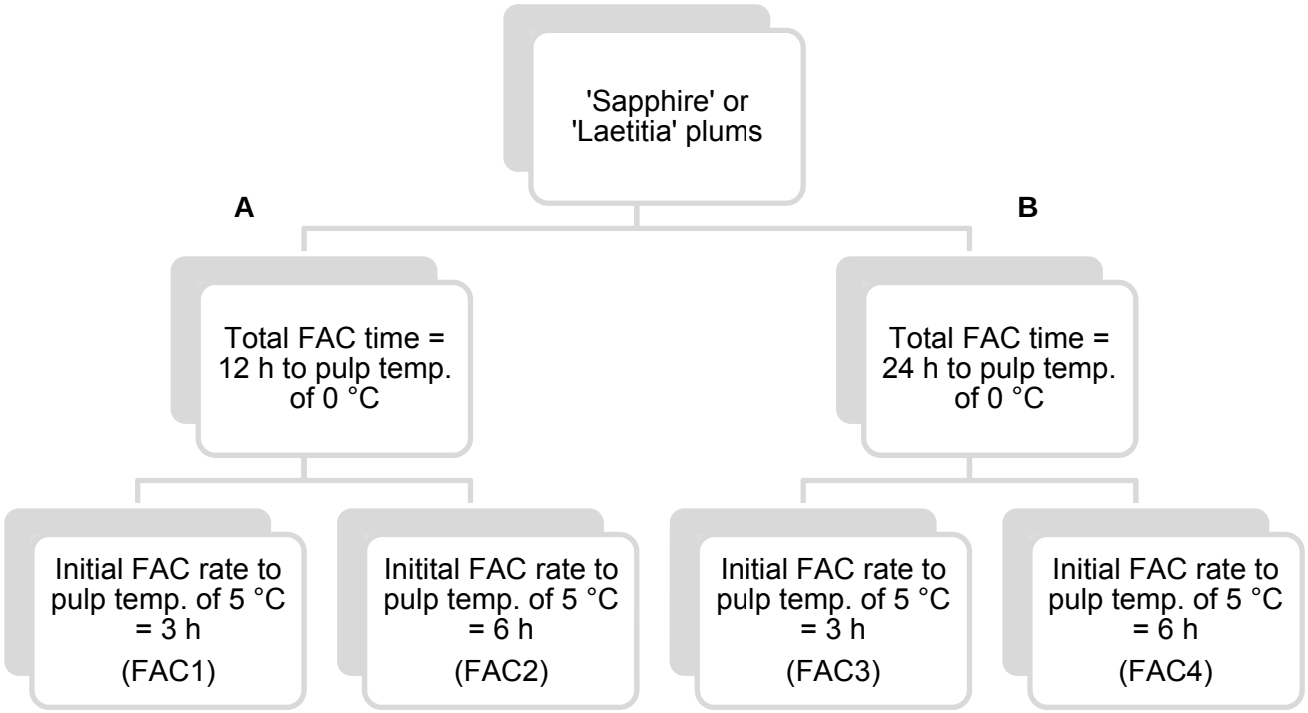


Fig. 5.1. Total forced air cooling (FAC) times and initial FAC rates to a pulp temperature of 5 °C examined in this trial. (A) Total FAC time of 12 h split into two different initial rates, namely cooled to a pulp temperature of 5 °C within 3 h or within 6 h after which the fruit were cooled to a pulp temperature of 0 °C. (B) Total FAC time of 24 h split into two different initial rates, namely cooled to a pulp temperature of 5 °C within 3 h or within 6 h after which the fruit were cooled to a pulp temperature of 0 °C.

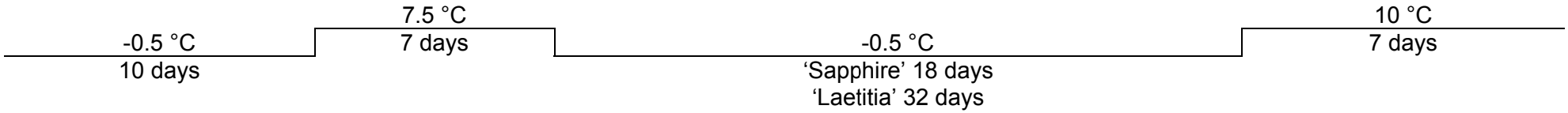
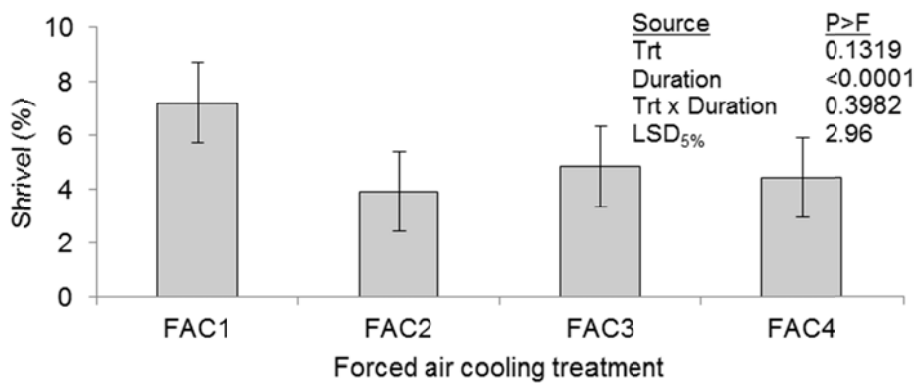
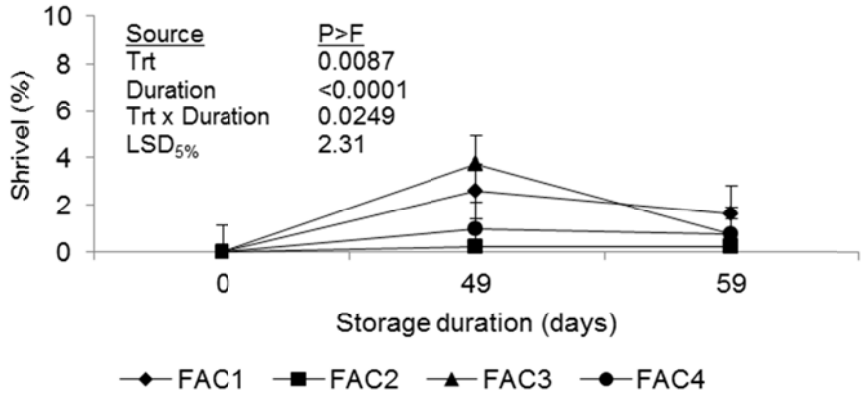


Fig. 5.2. After FAC, the fruit was stored with a commercially used intermittent warming regime at -0.5 °C and 7.5 °C followed by a simulated shelf-life period at 10 °C for 7 days. To simulate commercial conditions, 'Sapphire' plums were cold-stored for a maximum period of 35 days, and 'Laetitia' plums for a maximum period of 49 days before commencement of the simulated shelf-life period.

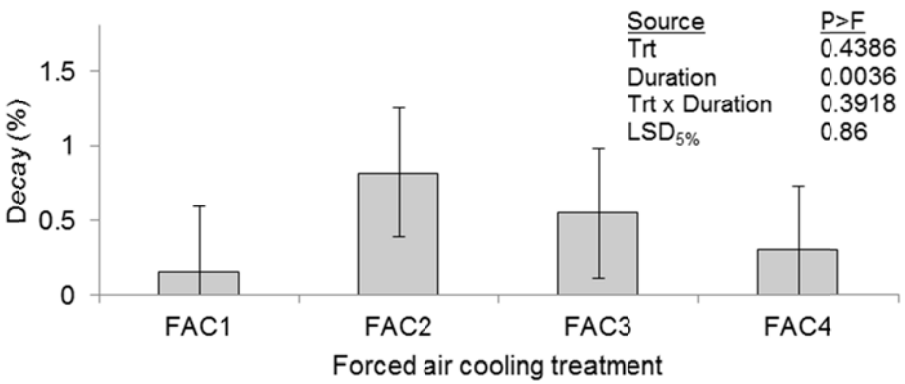


(B)

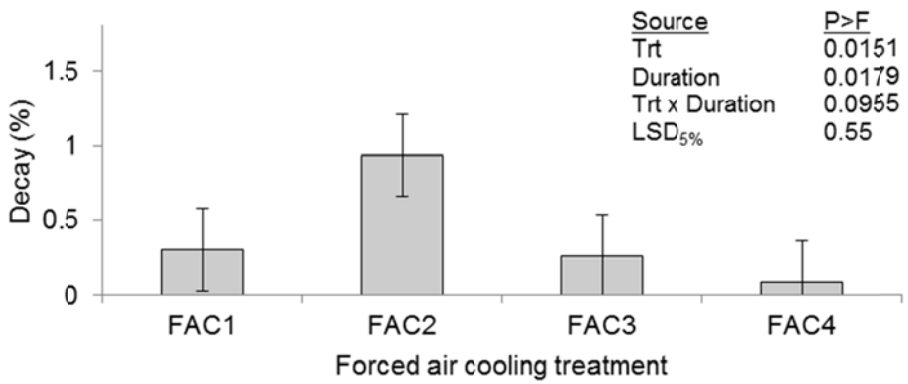


(B)

Fig. 5.3. Shrivel levels in (A) 'Sapphire' plums as affected by forced air cooling (FAC) treatment and (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. FAC1 = initial FAC to pulp temperature of 5 °C within 3 h; total FAC time to pulp temperature of 0 °C within 12 h; FAC2 = initial FAC to pulp temperature of 5 °C within 6 h; total FAC time to pulp temperature of 0 °C within 12 h; FAC3 = initial FAC to pulp temperature of 5 °C within 3 h; total FAC time to pulp temperature of 0 °C within 24 h; FAC4 = initial FAC to pulp temperature of 5 °C within 6 h; total FAC time to pulp temperature of 0 °C within 24 h. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

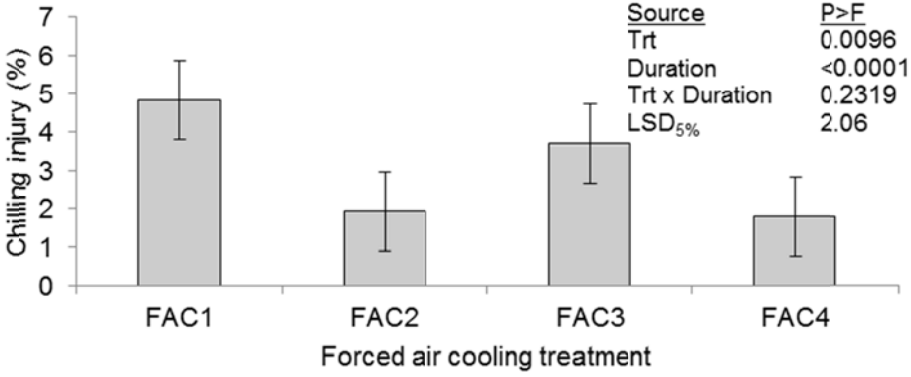


(A)

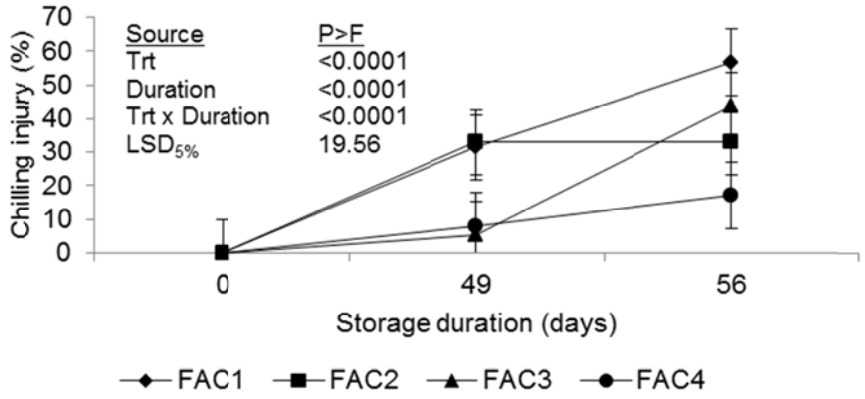


(B)

Fig. 5.4. Decay levels in (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by FAC treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

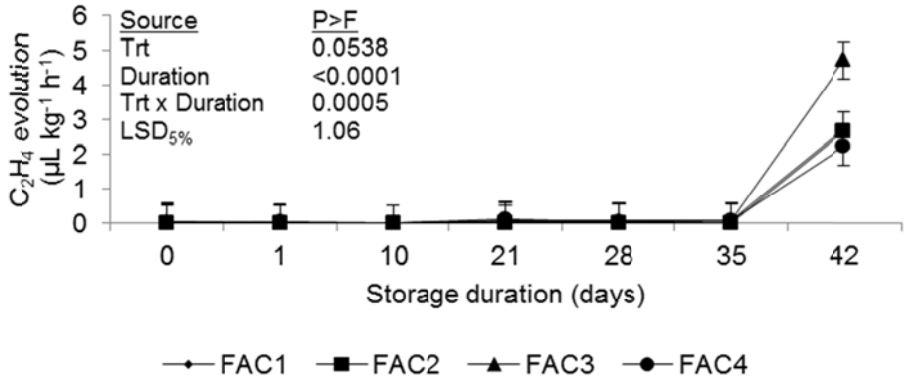


(A)

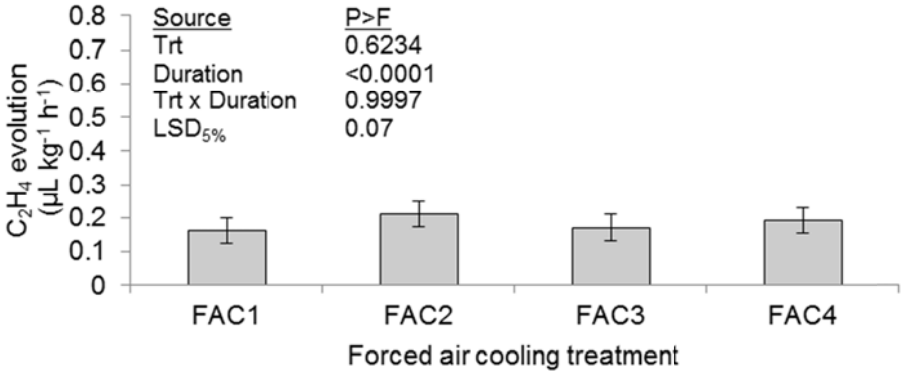


(B)

Fig. 5.5. Chilling injury manifestation in (A) 'Sapphire' plums as influenced by forced air cooling (FAC) treatment and (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

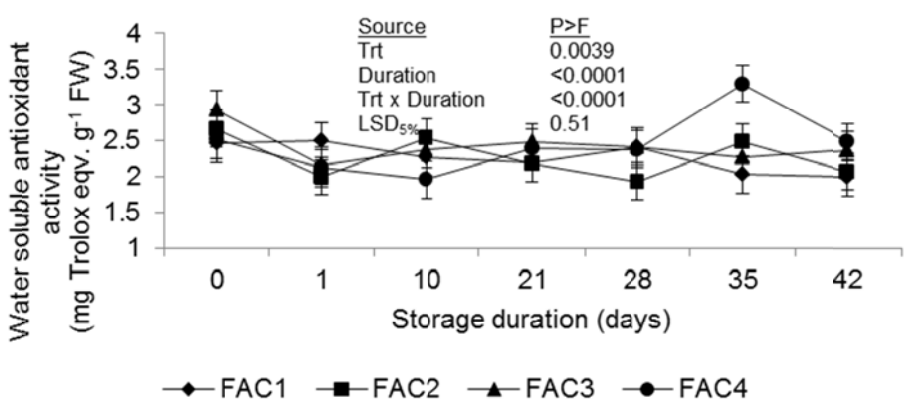


(A)

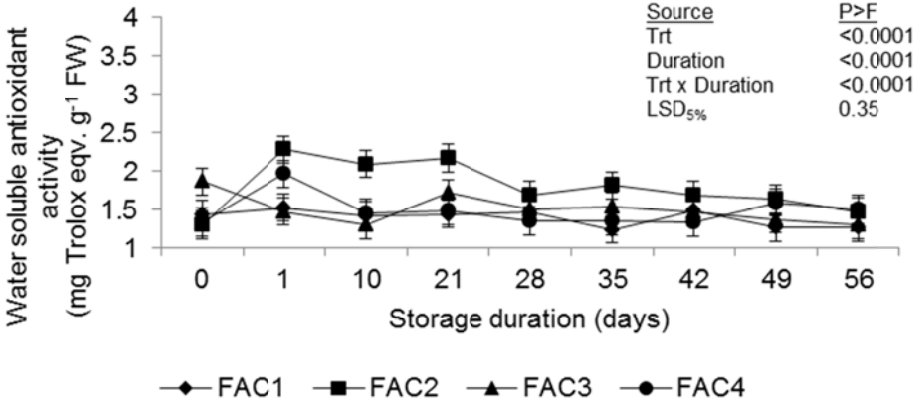


(B)

Fig. 5.6. C_2H_4 evolution rates in (A) 'Sapphire' plums as influenced by forced air cooling (FAC) treatment and storage duration, and (B) 'Laetitia' plums as influenced by FAC treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

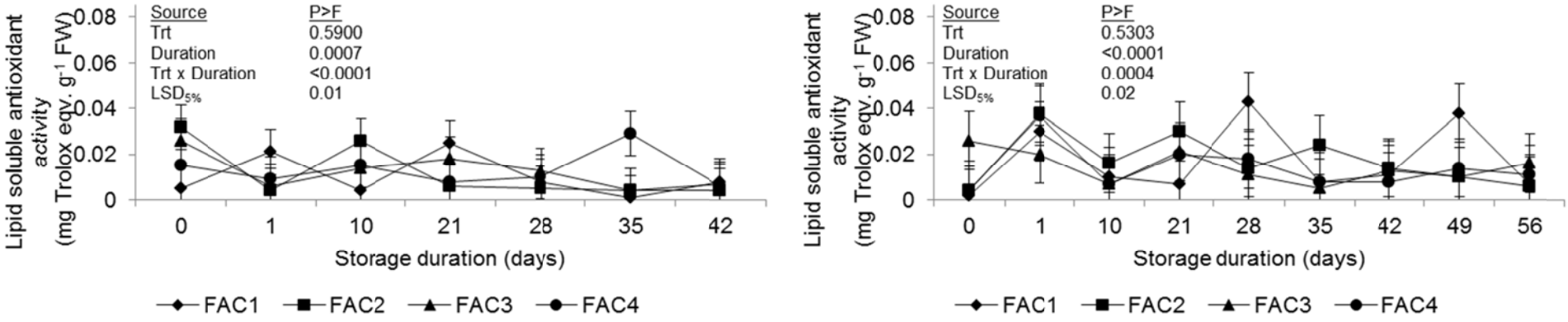


(A)



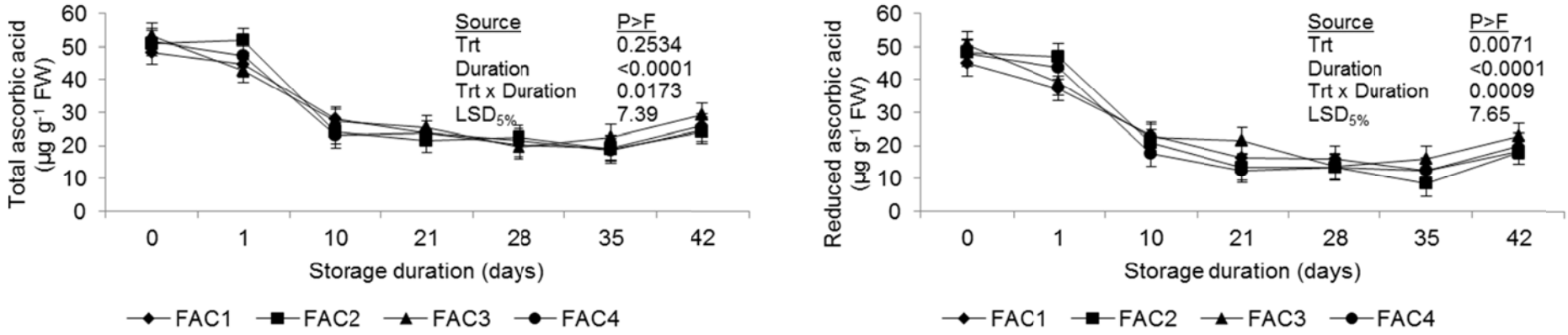
(B)

Fig. 5.7. Water soluble antioxidant activity in (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.



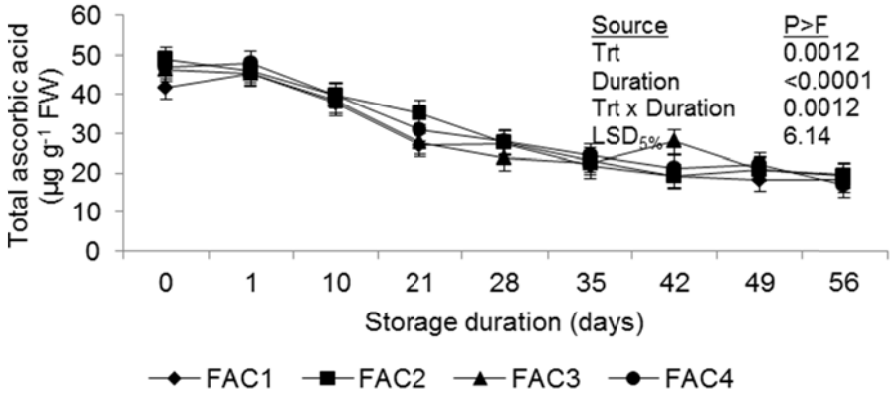
(A) **(B)**

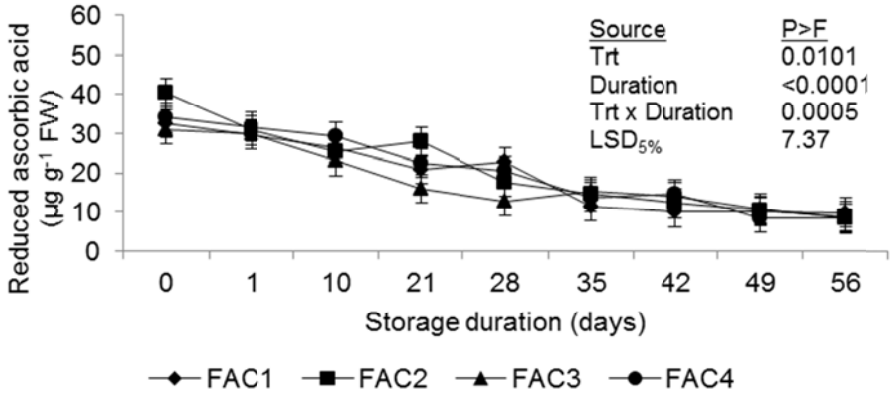
Fig. 5.8. Lipid soluble antioxidant activity in (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.



(A) **(B)**

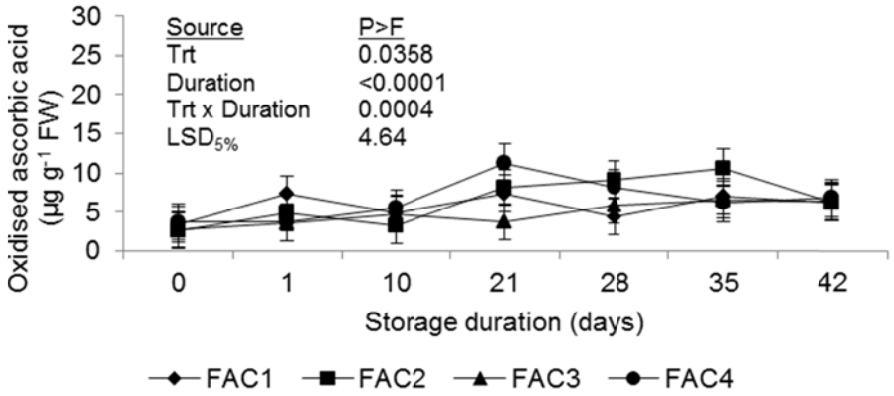
Fig. 5.9. (A) Total and (B) reduced ascorbic acid levels in 'Sapphire' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' plums were stored for 35 plus a subsequent simulated shelf-life of 7 days at 10 °C.

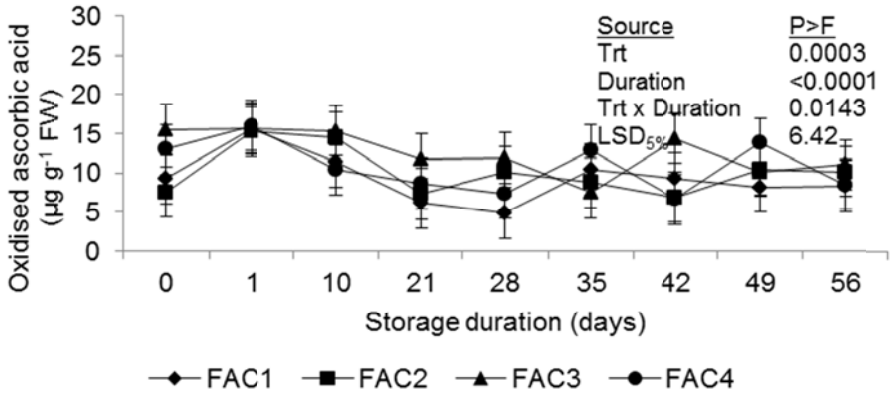




(A) **(B)**

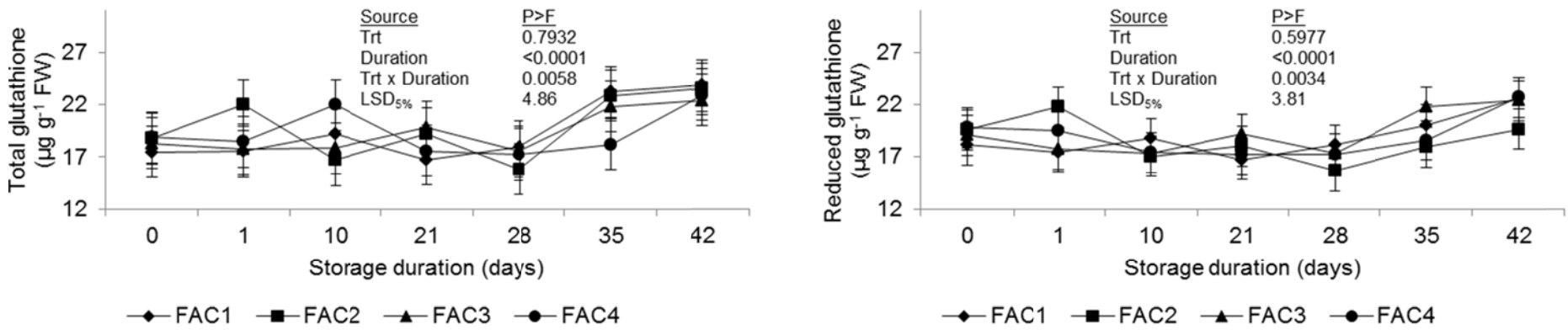
Fig. 5.10. (A) Total and (B) reduced ascorbic acid levels in 'Laetitia' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Laetitia' plums were stored for 49 plus a subsequent simulated shelf-life of 7 days at 10 °C.





(A) **(B)**

Fig. 5.11. Oxidised ascorbic acid in (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.



(A) **(B)**

Fig. 5.12. (A) Total and (B) reduced glutathione levels in 'Sapphire' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' plums were stored for 35 plus a subsequent simulated shelf-life of 7 days at 10 °C.

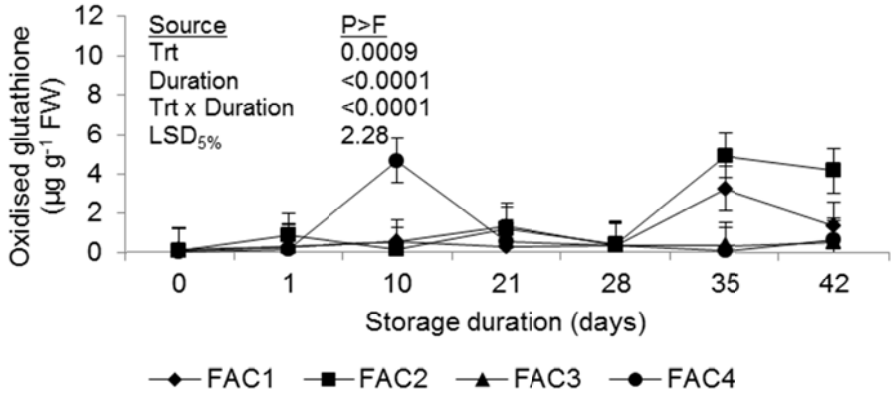
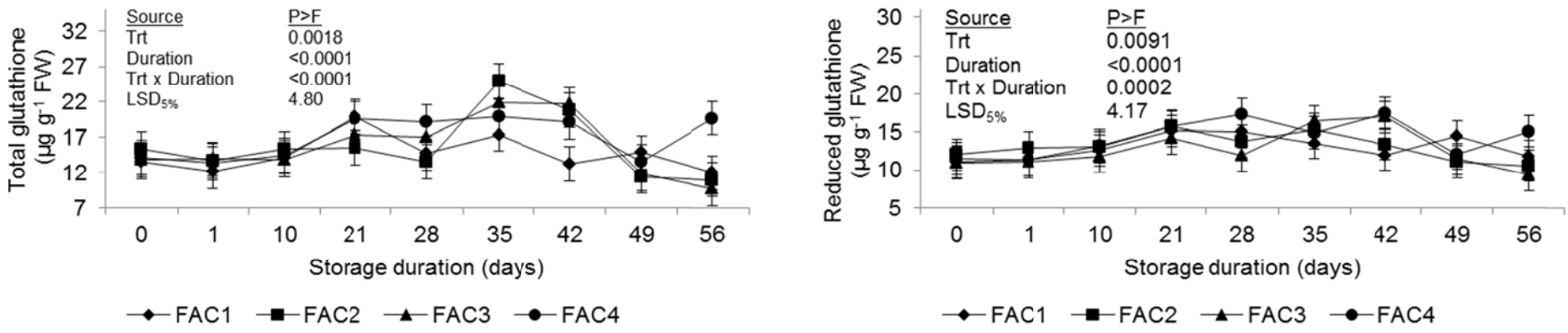


Fig. 5.13. Oxidised glutathione in 'Sapphire' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' plums were stored for 35 days plus a subsequent simulated shelf-life of 7 days at 10 °C.



(A) **(B)**

Fig. 5.14. (A) Total and (B) reduced glutathione levels in 'Laetitia' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Laetitia' plums were stored for 49 plus a subsequent simulated shelf-life of 7 days at 10 °C.

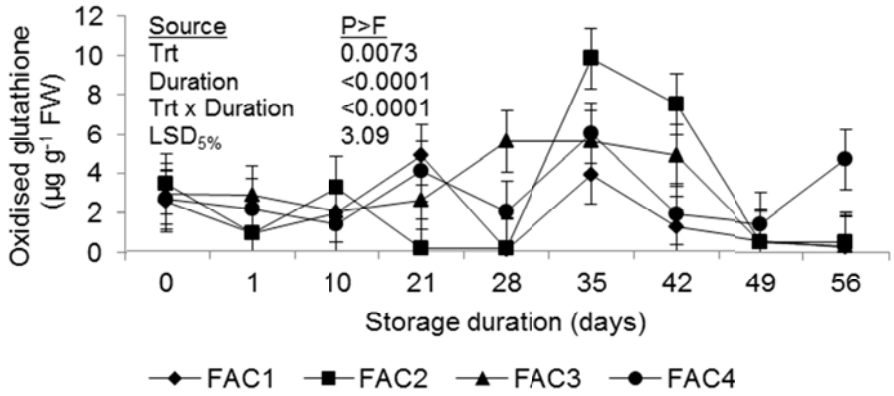
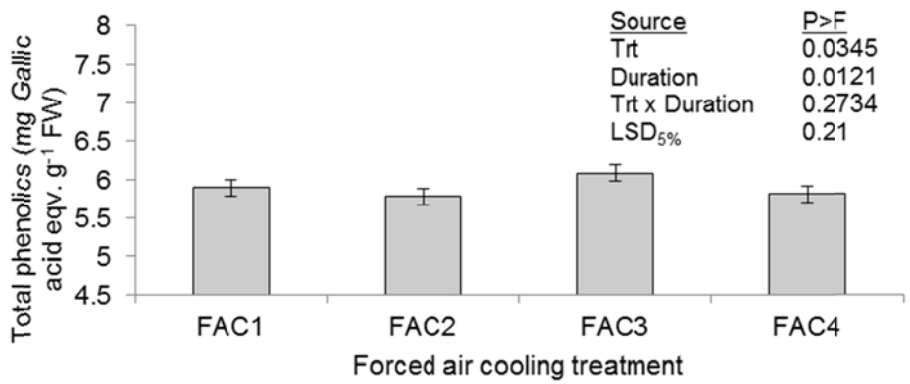
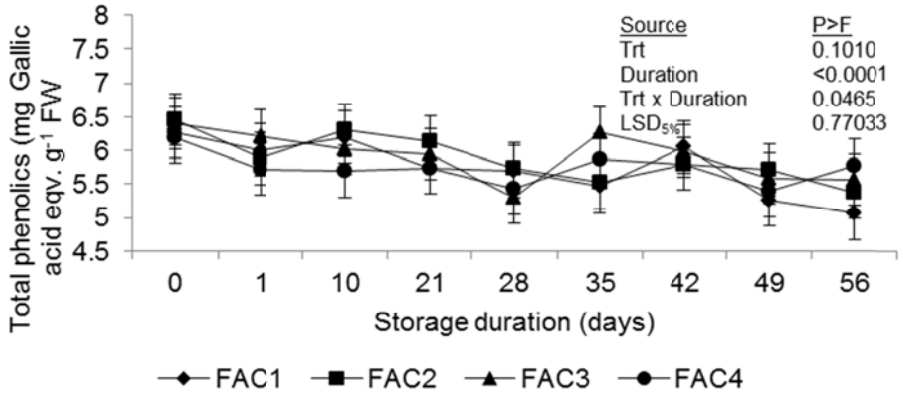


Fig. 5.15. Oxidised glutathione in 'Laetitia' plums as influenced by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Laetitia' plums were stored for 49 days plus a subsequent simulated shelf-life of 7 days at 10 °C.

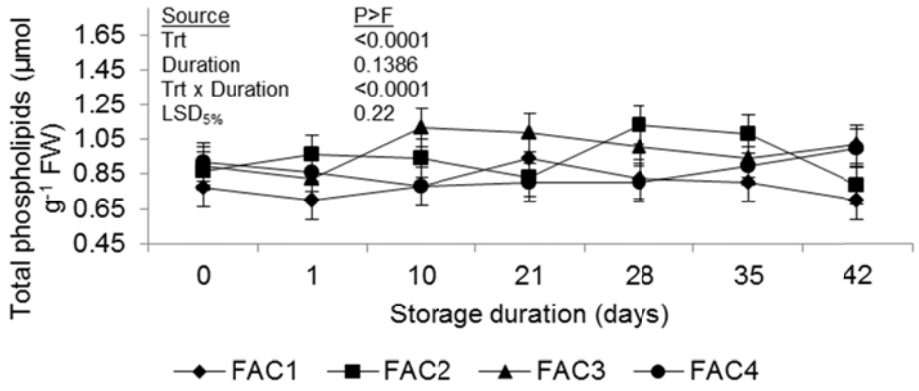


(A)

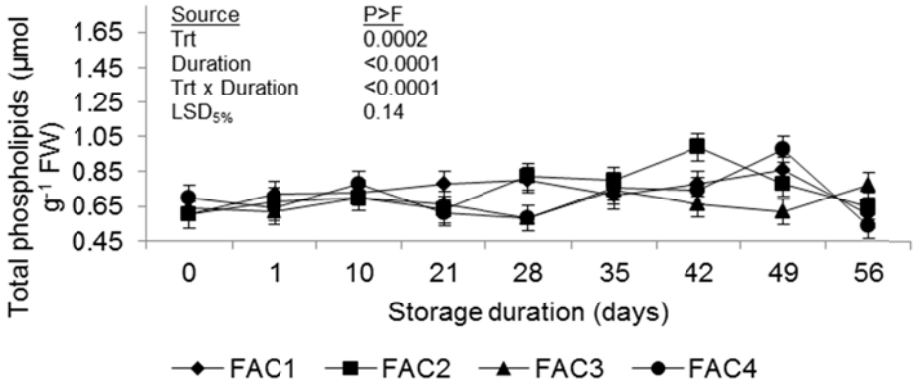


(B)

Fig. 5.16. Total phenolic concentration in (A) 'Sapphire' plums as influenced by forced air cooling (FAC) treatment and in (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

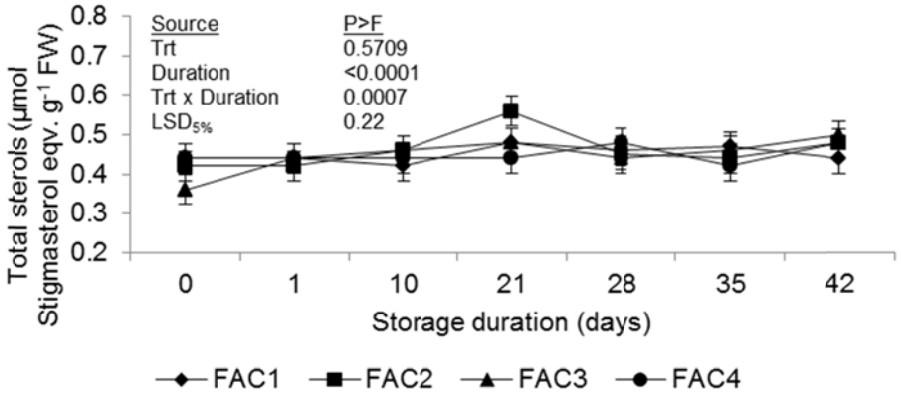


(A)

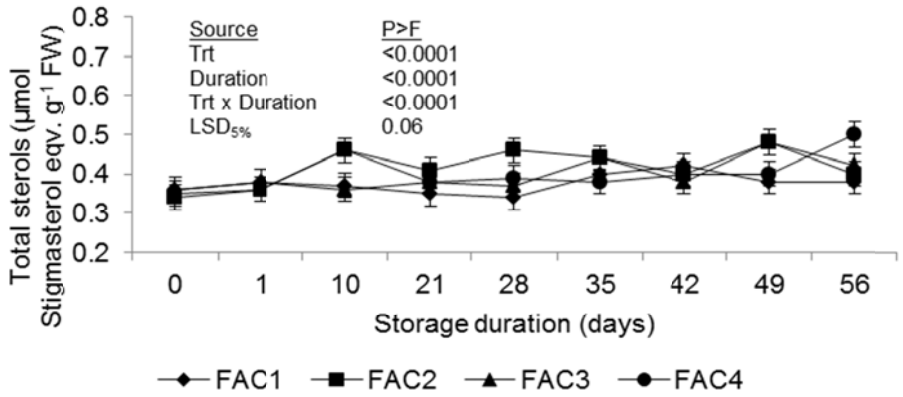


(B)

Fig. 5.17. Total phospholipid concentration in (A) 'Sapphire' and in (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

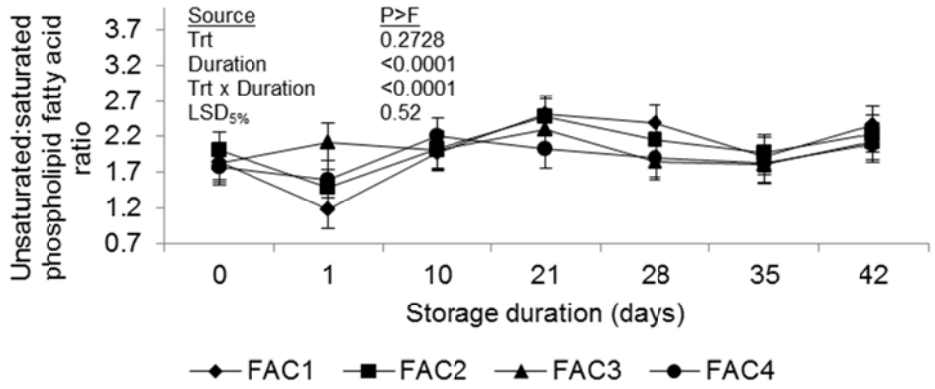


(A)

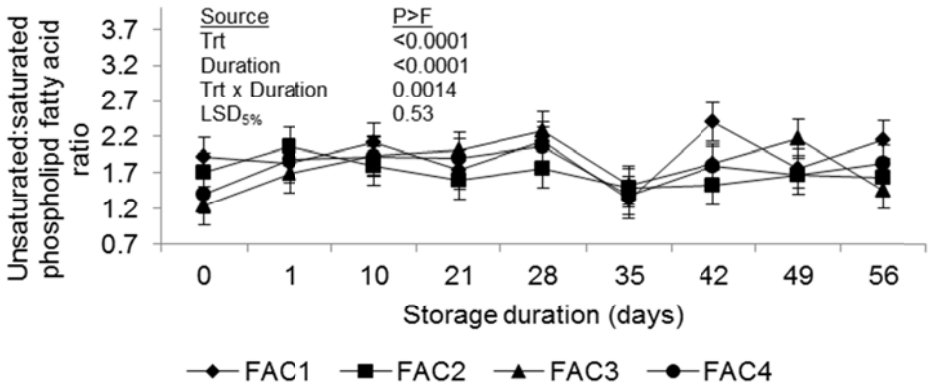


(B)

Fig. 5.18. Total sterol concentration in (A) 'Sapphire' and in (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

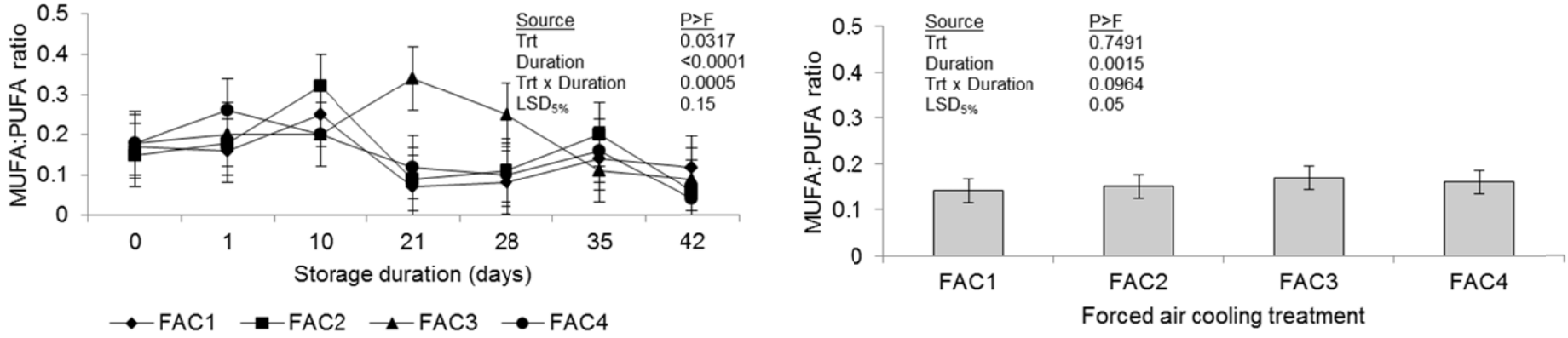


(A)



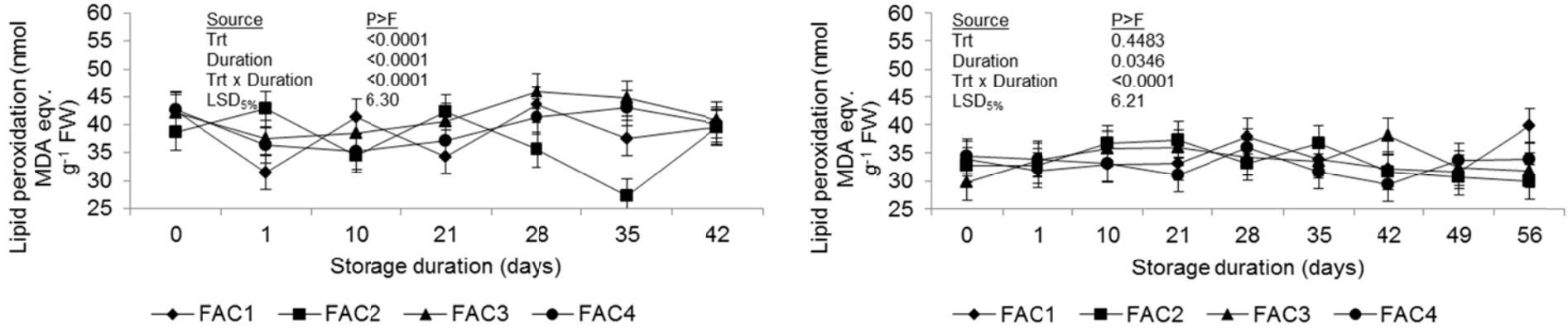
(B)

Fig. 5.19. Unsaturated:saturated phospholipid fatty acid ratio in (A) 'Sapphire' and in (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.



(A) **(B)**

Fig. 5.20. Monounsaturated:polyunsaturated phospholipid fatty acid ratio (MUFA:PUFA) in (A) 'Sapphire' and in (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.



(A) **(B)**

Fig. 5.21. Lipid peroxidation levels in (A) 'Sapphire' and in (B) 'Laetitia' plums as influenced by FAC treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 5.3. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

GENERAL DISCUSSION AND CONCLUSION

Most plum cultivars exported from South Africa are sensitive to storage under low temperatures, which causes chilling injury (CI). These defects usually appear when the fruit is removed from the low storage temperature conditions, i.e. in the fruit basket of the consumer, who subsequently does not buy plums again. According to literature the first physiological response to CI is loss of cell membrane integrity, which is caused, amongst others, by changes in lipid composition (Sevillano et al., 2009; Marangoni et al., 1996). The secondary response to CI is oxidative stress, which further enhances the loss of cell membrane integrity (Sevillano et al., 2009). Therefore, the main aim of this study was to determine how the integrity of the cell membranes and when and how the water and lipid soluble antioxidant activity and the total phenolic, ascorbic acid and glutathione concentrations of plum fruit are influenced during fruit development, forced air cooling and storage under various temperature regimes.

The aim of Paper 1 was to compare antioxidant levels and cell membrane composition during fruit development in a chilling susceptible ('Sapphire') and chilling resistant ('Angeleno') plum cultivar. We found that 'Sapphire' accumulated higher levels of glutathione than 'Angeleno', and that the latter cultivar accumulated much higher levels of ascorbic acid than 'Sapphire'. Glutathione is an important antioxidant in fruit as it scavenges for active oxygen species (AOS), reduces oxidised ascorbic acid back to its reduced (and active) form in the ascorbate/glutathione cycle, maintains α-tocopherol in its reduced state and protects the disulphide bonds in proteins under stress conditions (Hausladen and Alscher, 1993; McKersie and Leshem, 1994; Potters et al., 2002; Gill and Tuteja, 2010). Ascorbic acid, which accumulated to much higher levels in 'Angeleno' than in 'Sapphire', is considered to be the main water soluble antioxidant and an important redox buffer and enzyme cofactor in plants (Smirnoff, 2000; Hancock and Viola, 2005; Ishikawa et al., 2006).

The reason 'Sapphire' accumulated higher levels of glutathione than ascorbic acid may be related to the cooler temperatures during fruit development of this early season cultivar, while the higher temperatures during fruit development of 'Angeleno' caused this late season plum cultivar to accumulate higher levels of ascorbic acid than glutathione. We also found that 'Sapphire' accumulated higher levels of the polyunsaturated fatty acid (PUFA) linoleic acid (C18:2) than 'Angeleno', while the latter cultivar accumulated higher levels of the monounsaturated fatty acid (MUFA) oleic acid (C18:1) than 'Sapphire' plums. PUFAs are the main targets of oxidative stress (Reis and Spickett, 2012). Although antioxidants such as ascorbic acid and glutathione eliminate AOS and the toxic products of lipid peroxidation, levels of AOS produced under severe stress

conditions, such as low temperature storage for extended durations, may overwhelm these reducing agents (Aust, 1986; Spitteler, 2003). Under such conditions lipid peroxidation will cause uncoupling of oxidative phosphorylation in the mitochondria, alteration of the function of the endoplasmic reticulum, increase membrane permeability, alter the fluidity of the membrane, inactivate membrane-bound enzymes and cause polymerization, cross-linking and covalent binding of proteins which may lead to cell death (Stanley, 1991; Blokhina et al., 1999).

Since 'Sapphire' had high levels of glutathione during fruit development, it is suggested that the cultivar's fruit is properly protected against lipid peroxidation while developing on the tree, but that the relatively high levels of PUFAs, which are easily oxidised, may cause this cultivar to be chilling sensitive when stored at low temperatures in the postharvest handling chain. The high levels of MUFAs, which are not easily oxidised, and the high ascorbic acid levels in 'Angeleno' plums probably render this cultivar chilling resistant during long-term cold-storage. It is recommended that future research focus on comparing early, mid and late season cultivars (and especially new cultivars) regarding their MUFA and PUFA ratios and their susceptibility to CI. This knowledge will aid in the choice of the correct postharvest practices to employ (e.g. length of the intermittent warming period, gradual acclimation at lower temperatures, etc.) to ensure the prevention of CI during cold-storage.

In Paper 1 we also found that there were clear differences between seasons for glutathione, ascorbic acid and total phenolic concentrations as well as the hue angle of the fruit. These differences were related to differences in the average temperatures and number of sunshine hours between the two seasons in the study. Glutathione, ascorbic acid and phenolic compounds are linked to the antioxidant status, and hence protection against oxidative stress (that can induce CI), of the fruit. As it is relatively easy to measure daily temperatures and number of sunshine hours in the orchard, it will be interesting to establish if specific threshold values of these two parameters during fruit development can be related to postharvest CI incidence. Such values will aid in preharvest prediction regarding postharvest fruit quality. Since there are also vast differences between plum production areas regarding their average daily temperatures and number of sunshine hours, we also speculate that climatic differences between areas during fruit development may influence postharvest CI incidence in plums. It is, therefore, recommended that future research should also investigate the role of climatic differences between areas in the postharvest manifestation of CI in plums.

In Paper 2 we harvested a known chilling susceptible cultivar ('Sapphire') at two maturities, stored the fruit at -0.5 °C and evaluated subsamples of fruit at weekly intervals to investigate how the cell membrane composition and antioxidant levels change during storage at a chilling temperature and how this temperature influences the severity of CI manifestation. We found that CI incidence escalated with an increase in storage duration, was generally higher at 10 °C (the shelf-life temperature) than at -0.5 °C, and increased at a higher rate in the more mature fruit. These results agree with a number of other studies on various products, including plums (Lyons, 1973; Bramlage, 1982; Morris, 1982; Raison and Orr, 1990; Saltveit and Morris, 1990; Taylor, 1996; Abdi et al., 1997; Sevillano et al., 2009). We also found that the more mature fruit (H2) had higher ethylene evolution rates at 10 °C than the less mature fruit (H1). Ethylene not only increases the respiration rate, but also changes the activity of certain enzymes and increases membrane permeability – all factors that accelerate product senescence and deterioration (Kays and Paull, 2004). We also found that the water soluble antioxidant activity (HAA) and ascorbic acid levels were higher in the H1 'Sapphire' fruit compared to the H2 fruit during storage. Glutathione levels also increased in H1 fruit, but not in H2 fruit during storage at -0.5 °C. This is in accordance with other studies that more mature fruit tend to have lower levels of antioxidants than less mature fruit (Veltman et al., 2000; Toivonen, 2004). It also indicates that the H1 fruit was better equipped to scavenge for AOS and prevent oxidative damage, and hence CI, than the H2 fruit. The H2 fruit also had a lower MUFA:PUFA ratio than the H1 fruit which indicates that the membranes of the H2 fruit were more prone to oxidative stress, since PUFAs are more susceptible to oxidation by AOS than MUFAs (Reis and Spickett, 2012). Therefore, concurrent with the H2 fruit having lower levels of antioxidants to quench the free radicals caused by the chilling stress, their cell membranes were more vulnerable to oxidative stress due to their phospholipid fatty acid composition. The H2 fruit also had higher levels of saturated fatty acids, and hence less fluid cell membranes (a situation closely linked to CI), than the H1 fruit.

This brings us to a very common problem in the South African plum industry, namely mixed fruit maturities packed in the same export carton. Due to the clear differences between fruit maturities demonstrated in Paper 2, proper postharvest treatment to reduce CI will be complex, as the same treatment will not necessarily be optimal for all fruit maturities. A possible solution for this problem is to use near infrared (NIR) spectroscopy on the pack lines to sort the fruit maturities more thoroughly. Louw et al. (2010) found that NIR spectroscopy can be used with great success on South African plums to predict firmness, total soluble solids, titratable acidity and the sugar-to-acid ratio. It is, therefore, strongly recommended that this technology should be included when optimum picking windows are determined for plum cultivars as well as on commercial packlines. According to the results of this Paper, 'Sapphire' plums must be harvested less mature (> 60.0 N) and not be stored for longer than 21 days if they have to be stored at -0.5 °C. However, storage

durations exceeding 21 days are needed to export this cultivar from South Africa and the currently employed intermittent warming (IW) regime fulfils this requirement at this stage. To address the higher levels and earlier manifestation of CI as well as the higher ethylene evolution rates in the more mature fruit, it is recommended that future research should include the use of 1-methylcyclopropene (1-MCP) or other products that inhibit the production or action of ethylene, and hence CI incidence, in these fruit. Although the H1 fruit had higher levels of antioxidants, a higher unsaturated:saturated fatty acid and MUFA:PUFA ratio and lower ethylene evolution rates than the H2 fruit, they still developed CI, albeit at much lower levels than the H2 fruit. It is, therefore, recommended that postharvest treatments such as 1-MCP with or without the conditioning of the fruit at room temperature, or at low, but not chilling temperatures, should be tested in future to establish whether these interventions can prevent or reduce CI incidence in these fruit.

Since Japanese plums produced in South Africa are chilling susceptible, an IW regime has been used the past 60 years to increase the storage potential of these fruit when exported (Taylor, 1996). It is, however, not known by which mechanism the IW regime reduces CI, but a number of hypotheses exist (Sevillano et al., 2009). The aim of Paper 3, therefore, was to determine the effect of a commercially used IW regime and a single-temperature (ST) regime at -0.5 °C on the cell membrane composition, antioxidant status, and CI incidence of two plum cultivars differing in their susceptibility to CI (‘Sapphire’ – more susceptible to CI; ‘Laetitia’ – less susceptible to CI). The IW regime not only delayed the onset of CI symptom appearance, but also reduced CI severity significantly compared to the ST.

‘Sapphire’ stored under the IW regime had higher levels of unsaturated fatty acids, which probably kept the membranes in a fluid, and thus selectively permeable state. The ‘Sapphire’ stored under the ST regime had higher levels of saturated fatty acids which are strongly linked to CI incidence in chilling prone produce (Parkin and Kuo, 1989; Whitaker, 1995). Conversely, the more chilling resistant ‘Laetitia’ plums stored under the IW regime had higher levels of saturated and lower levels of unsaturated phospholipid fatty acids compared to fruit stored under the ST regime. It is suggested that the higher levels of saturated fatty acids were to ensure that the membranes had optimal permeability under the warmer storage conditions of the IW regime. Likewise, the higher levels of unsaturated fatty acids under the ST compared to the IW regime were to ensure that the membranes remained fluid at -0.5 °C. Unfortunately this was not very successful, since the fruit stored under the ST regime developed higher levels of CI compared to the IW regime. Furthermore, it was found that fruit of both cultivars stored under the ST regime had an increase in sterol levels during the shelf-life period, while fruit stored under the IW regime had a decrease. An increase in the total sterol concentration during ripening or senescence is associated with

increased membrane viscosity and an increase in membrane leakiness (O'Neill, 1981; Lurie et al., 1997). It is, therefore, suggested that the decrease in sterol levels during the shelf-life period in the fruit stored under the IW regime caused the cell membranes to remain optimally fluid at the higher storage temperature and prevented excessively high CI levels to develop.

Furthermore, fruit of both cultivars stored under the IW regime generally had higher levels of water soluble and lipid soluble antioxidant activity (LAA), as well as higher levels of ascorbic acid and glutathione than the fruit stored under the ST regime. Therefore, the fruit stored under the IW regime had a much better potential to scavenge for AOS, and hence better protection against CI, compared to the fruit stored under the ST regime. However, although the fruit stored under the IW regime had a more optimal phospholipid fatty acid composition, and maintained their AOS scavenging potential and, consequently, had much lower CI levels compared to the fruit stored under the ST regime, low levels of internal browning still manifested after the shelf-life period in both cultivars. As it would not make commercial sense to recommend a shorter storage duration, other postharvest practices, such as low temperature preconditioning prior to cold-storage, heat treatments and/or the application of 1-MCP should be tested to determine if they can aid in the reduction of CI in Japanese plums intended for long-term storage under the IW regime or at low temperatures.

The commercial use of the IW regime is challenged by the increasing use of integral reefer containers by the South African plum industry. The reasons for this challenge are, firstly that the containers have inadequate capacity to rapidly recool warm cargo because the air-flow system within the container does not allow efficient cooling of the produce (Irving, 1988; Kapp, 2008a), and secondly, that the open vent (to prevent the build-up of ethylene and CO_2 when the IW regime is used) causes the right hand side of the container to be warmer than the left hand side, due to differential frosting on the coils (Kapp, 2008b; PPECB, 2012). However, commercially it is observed that, due to the lack of proper cooling of the produce after the IW period, plums exported from South Africa sometimes arrive overseas with pulp temperatures within the 'killing zone' (between 2 to 7 °C), but without internal disorders. In support of the commercial results, a preliminary trial on elevated storage temperatures indicated that 'Pioneer' plums stored at 2 °C had higher flesh firmness and less internal defects compared to fruit stored under the IW regime (Kapp, 2008c). Therefore, although there is published evidence that elevated storage temperatures are detrimental to plum fruit quality (Mitchell, 1986; Crisosto et al., 1999) there are also indications that higher cold-storage temperatures could possibly be beneficial to plum fruit quality.

Since low levels of CI still manifested in the 'Sapphire' and 'Laetitia' fruit stored under the IW regime tested in Paper 3, and since the commercial use of the IW regime is challenged by the use of integral reefer containers, we decided to investigate the effect of a commercially used IW regime and elevated single storage temperatures of 2.5 °C, 5 °C and 7.5 °C on CI incidence, the cell membrane composition and antioxidant status of 'Sapphire' (more susceptible to CI) and 'Laetitia' (less susceptible to CI) plums in Paper 4. We found that when 'Sapphire' plums were stored at 7.5 °C and 5 °C it caused the fruit to soften quickly and to manifest higher levels of decay and overripeness, apparently due to higher levels of lipid peroxidation in these treatments, compared to the IW regime. On the other hand, storage of 'Sapphire' at 2.5 °C and 5 °C caused the manifestation of higher levels of CI than when the fruit were stored under the IW regime, which agrees with the results of Crisosto et al. (1999) on plums. The 'Sapphire' fruit stored at 2.5 °C had the lowest ascorbic acid levels (and hence poorer protection against oxidative stress), while the fruit stored at 5 °C had high lipid peroxidation levels. The 'Sapphire' stored under the IW regime had a higher LAA than fruit stored under the elevated storage temperatures. Since the IW regime performed better overall, elevated storage temperatures within the 'killing zone' cannot be recommended for the postharvest storage of 'Sapphire' plums. Another solution must, therefore, be found to prevent the CI that still manifests in this cultivar when the fruit is stored under the IW regime, such as low temperature preconditioning prior to cold-storage, heat treatments and/or the application of 1-MCP. However, 'Laetitia' plums stored at 5 °C and 7.5 °C had significantly less CI than the fruit stored under the IW regime, but softened quicker due to higher ethylene evolution rates in these treatments. Generally, the 'Laetitia' fruit stored under the elevated temperatures tended to develop higher shrivel and decay levels compared to the IW regime. Therefore, it seems that the elevated temperature storage regimes could hold promise for the storage of 'Laetitia' plums, but that some adaptions will have to made to the regimes since they enhanced fruit softening, caused slightly higher shrivel and decay levels and did not prevent the development of CI completely. In this regard, it is recommended that the regimes are adapted slightly and/or that 1-MCP should be tested to counteract the negative effects of ethylene resulting from the higher storage temperatures.

Since two prior studies conducted on South African export plums found that rapid pre-cooling exacerbates the development of CI symptoms (Jooste and Khumalo, 2005; Khumalo et al., 2006), it was decided to investigate the effect of the initial cooling rate and total forced air cooling (FAC) duration on the cell membrane composition, antioxidant status and CI incidence of 'Sapphire' (medium susceptibility to injury when rapidly FAC) and 'Laetitia' (susceptible to injury when rapidly FAC) plums in Paper 5. We found that 'Sapphire' plums could tolerate a long and short FAC duration (12 h and 24 h were tested in this study), but that a slower initial FAC rate prevented CI manifestation compared to a faster initial FAC rate. It is suggested that the higher HAA after cold-

storage in the 'Sapphire' fruit cooled with a slower initial FAC rate protected these fruit against oxidative stress, and hence CI development, compared to the other treatments. For 'Laetitia' plums a fast initial FAC rate exacerbated shrivel development (probably due to the cultivar's thin skin which makes it more prone to injuries and moisture loss), while a combination of a slow initial FAC rate and short FAC duration caused significant decay development. The best 'Laetitia' fruit quality was obtained when the fruit were FAC with a slower initial FAC rate and for a longer duration. This treatment had the highest HAA after FAC and after cold-storage, a higher total phospholipid concentration in the cell membranes after cold-storage, and an increased total phenolic concentration and a lower unsaturated:saturated phospholipid fatty acid ratio after shelf-life. It is suggested that the higher HAA and total phenolic concentration protected the fruit of this treatment against oxidative stress, while the high phospholipid and saturated phospholipid fatty acid concentrations caused the membranes to remain optimally permeable under the storage conditions, and hence, protected the fruit against CI. Since Mitchell and Gentry (1963) found that the RH in the FAC facility played an important role in shrivel manifestation in nectarines after FAC, it is recommended that, besides using a slow initial FAC rate and a longer FAC duration, the RH in the FAC facility should be 95% when this cultivar is cooled. An interesting result of this study is that rapid pre-cooling did not result in the best fruit quality of the two plum cultivars tested as is recommended by a number of other studies (Mitchell, 1986; Monzini and Gorini, 1991; Tonini and Caccioni, 1991; Taylor, 1996; Combrink and Visagie, 1997; Brosnan and Sun, 2001). Overall, the findings and recommendations of Paper 5 regarding the most optimal initial FAC rates and total FAC durations for the two tested cultivars support the recommendation of Hortgro Services (2012) for the cooling of South African plums. The recommended FAC procedure for South African plums strongly reminds of the postharvest stepwise temperature conditioning or low temperature conditioning treatments which are recommended for a number of chilling sensitive products such as mangos, bananas, grapefruit, loquat, avocado, papaya and Chinese Duck pears (Bramlage, 1982; Saltveit and Morris, 1990; Wang, 1993; Sevillano et al., 2009; Wang, 2010). It is, therefore, proposed that stepwise temperature conditioning treatments should be investigated in more depth in future research. Such a treatment will be relatively easy to implement commercially since a variant of it is currently employed for the forced air cooling of South African plums. It should also be considered to test the latter treatment with NIR (to reduce mixed maturity in the carton) and/or 1-MCP (to reduce the effect of ethylene on membrane permeability and, hence CI manifestation) to optimise the treatment for different cultivars and harvest maturities within a cultivar. It will be of great benefit if such a pre-storage treatment could replace the current, commercially used IW regime due to the difficulties with temperature management this treatment poses in the integral reefer container as explained earlier.

In conclusion, this study contributed towards our understanding of the differences between early and late season cultivars regarding their cell membrane composition and antioxidant levels and why they differ in their susceptibility to CI. The study also found that there are clear differences between seasons regarding antioxidant levels which are induced by average daily temperatures and number of sunshine hours. This result holds great promise for the development of a future preharvest prediction model to forecast the potential for postharvest CI incidence by simply measuring daily temperatures and sunshine hours in the orchard. Furthermore, the study has clearly shown the changes that take place in the composition of the cell membranes and in antioxidant levels during FAC and storage of Japanese plums when stored at -0.5 °C, under the commercially used IW regime and at elevated storage temperatures, and that these changes are related to the manifestation of CI. There are also clear differences in CI incidence, cell membrane composition and antioxidant levels between fruit harvested at the upper and lower end of the export harvest window and between cultivars differing in their susceptibility to CI. This knowledge will help with a more focused approach in future applied research to establish optimal and robust postharvest treatments to prevent or reduce the manifestation of CI in Japanese plums exported from South Africa.

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APPENDIX A: SELECTED DATA OF PAPER 1

Figure 1. Total glutathione, GSH and GSSG content determined on the sampling dates for (A) ‘Sapphire’ in the 2005 season, (B) ‘Sapphire’ in the 2007 season, (C) ‘Angeleno’ in the 2005 season and (D) ‘Angeleno’ in the 2007 season.

Figure 2. Total ascorbic acid, L-AA and DHA content determined on the sampling dates for (A) ‘Sapphire’ in the 2005 season, (B) ‘Sapphire’ in the 2007 season, (C) ‘Angeleno’ in the 2005 season and (D) ‘Angeleno’ in the 2007 season.

Figure 3. Content of phospholipid oleic acid (C18:1n9c) and linoleic acid (C18:2n6c) determined on the sampling dates for (A) ‘Sapphire’ in the 2005 season, (B) ‘Sapphire’ in the 2007 season, (C) ‘Angeleno’ in the 2005 season and (D) ‘Angeleno’ in the 2007 season.

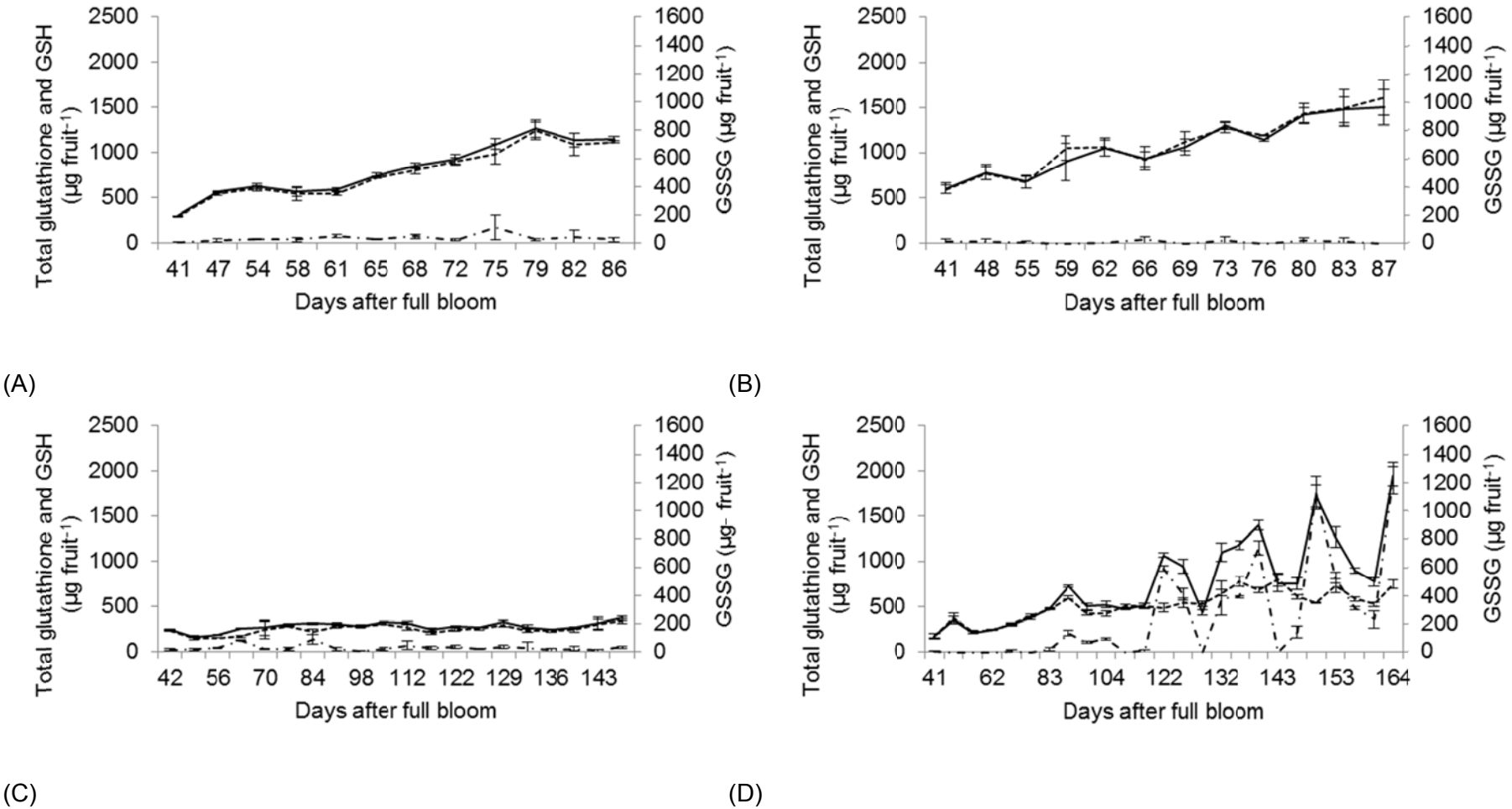


Fig. 1. Total glutathione (———), GSH (- - - - -) and GSSG (— · — · —) content determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.

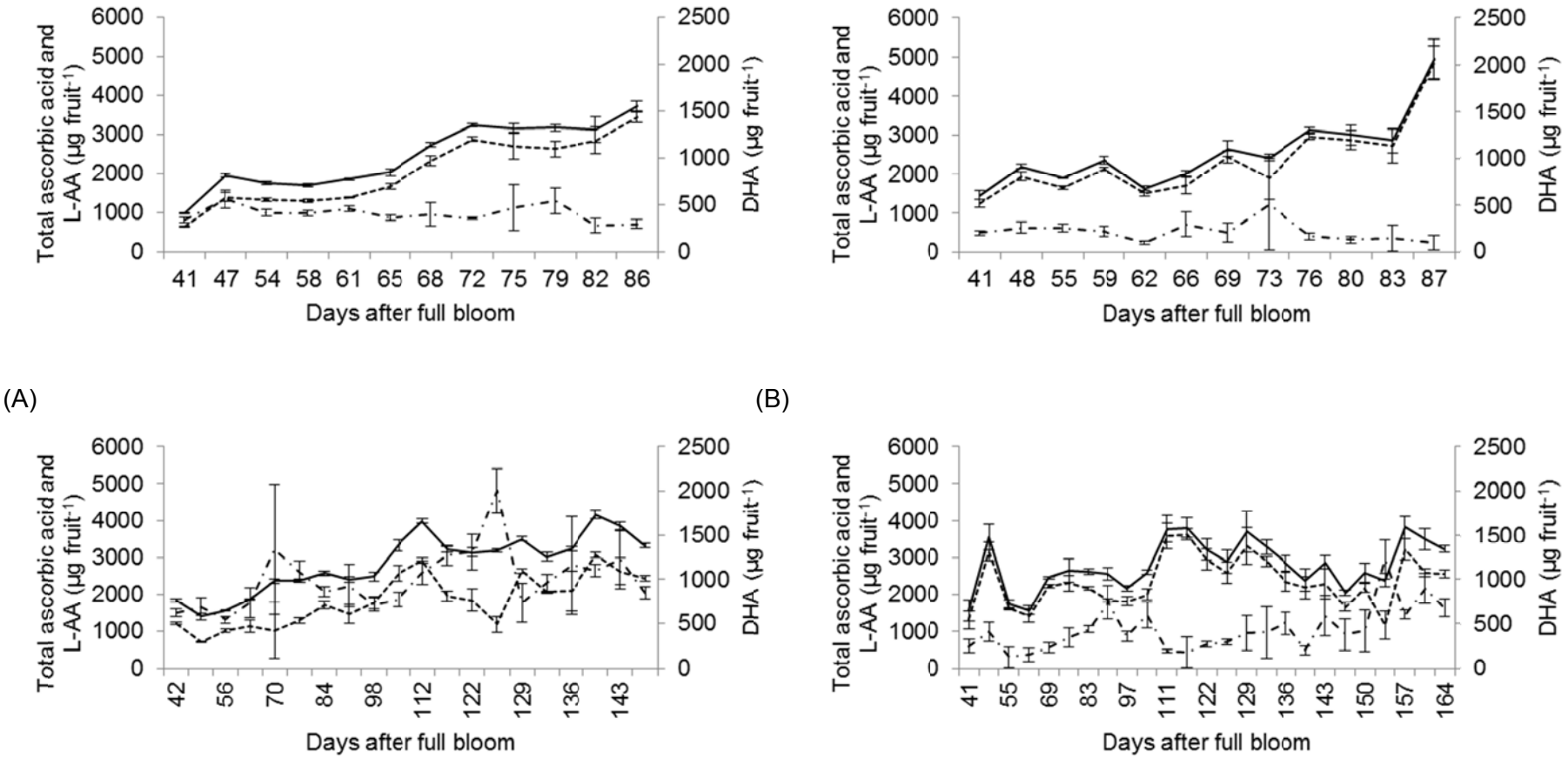


Fig. 2. Total ascorbic acid (———), L-AA (- - - - -) and DHA (—·—·—) contend determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.

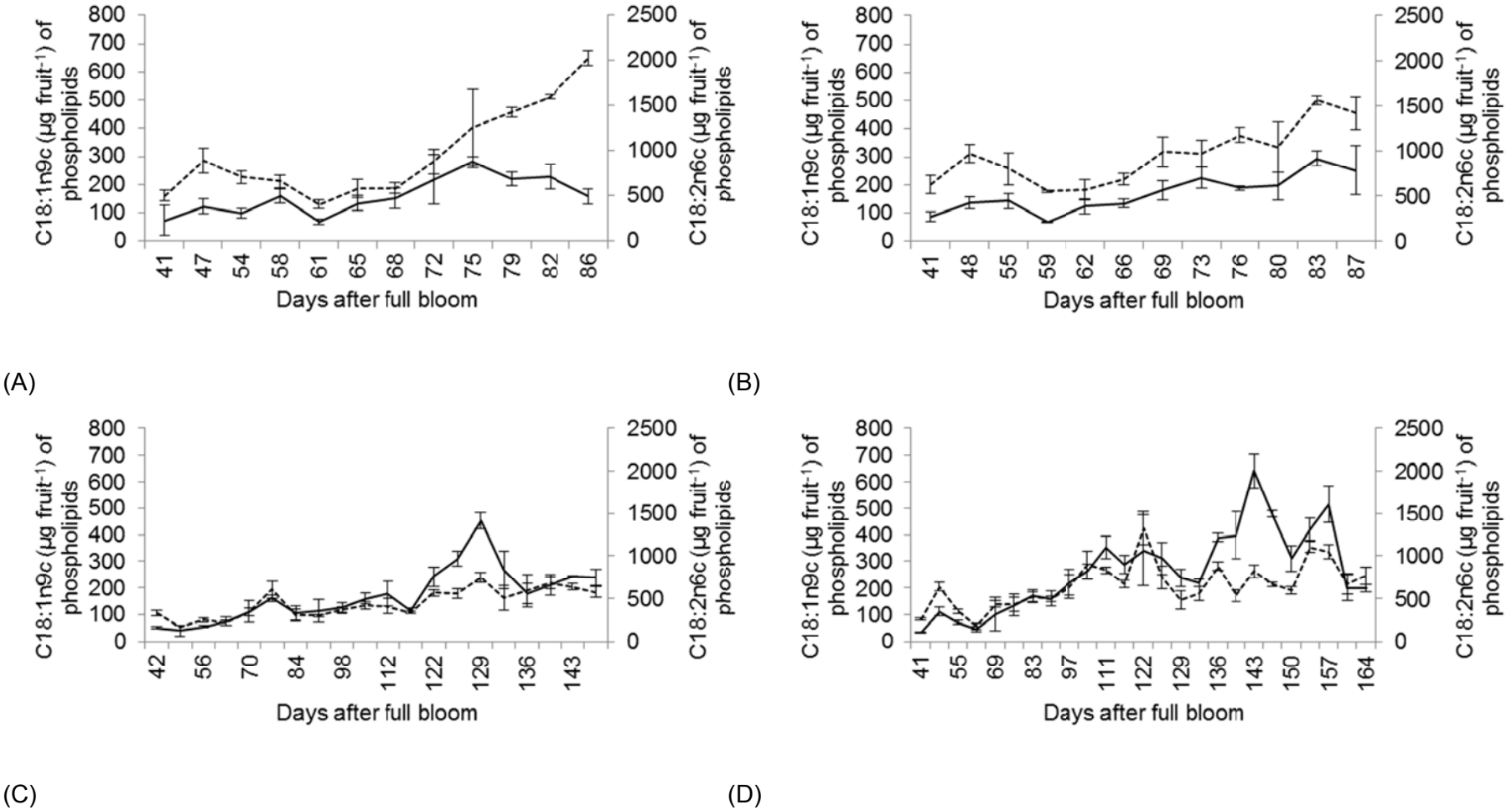


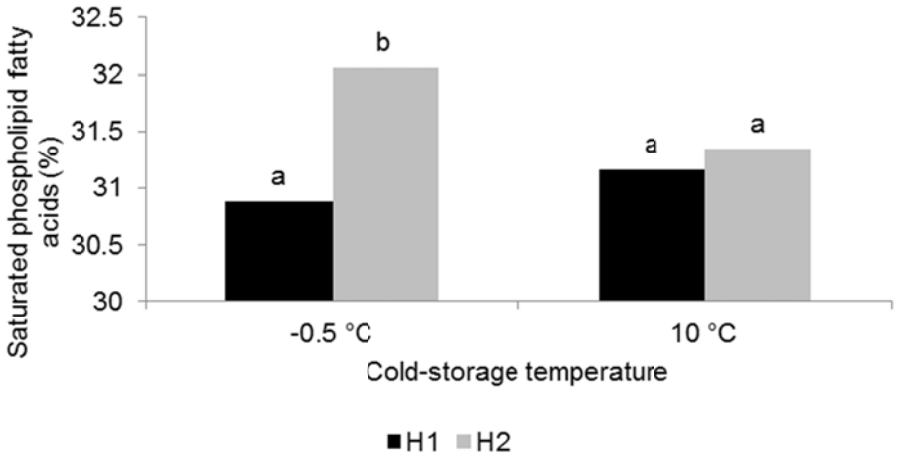
Fig. 3. Content of phospholipid oleic acid (C18:1n9c) (——) and linoleic acid (C18:2n6c) (- - - - -) determined on the sampling dates for (A) 'Sapphire' in the 2005 season, (B) 'Sapphire' in the 2007 season, (C) 'Angeleno' in the 2005 season and (D) 'Angeleno' in the 2007 season. Vertical bars represent S.D.

APPENDIX B: SELECTED DATA OF PAPER 2

Figure 1. Percentage saturated phospholipid fatty acids in ‘Sapphire’ plums as influenced by harvest maturity and cold-storage temperature.

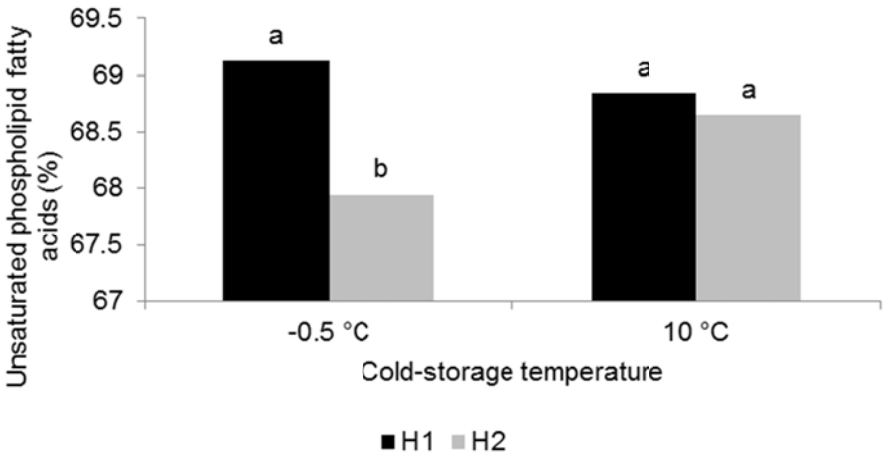
Figure 2. Percentage unsaturated phospholipid fatty acids in ‘Sapphire’ plums as influenced by harvest maturity and storage temperature.

Figure 3. Total sterol:total phospholipid ratio in ‘Sapphire’ plums as influenced by storage duration and harvest maturity.



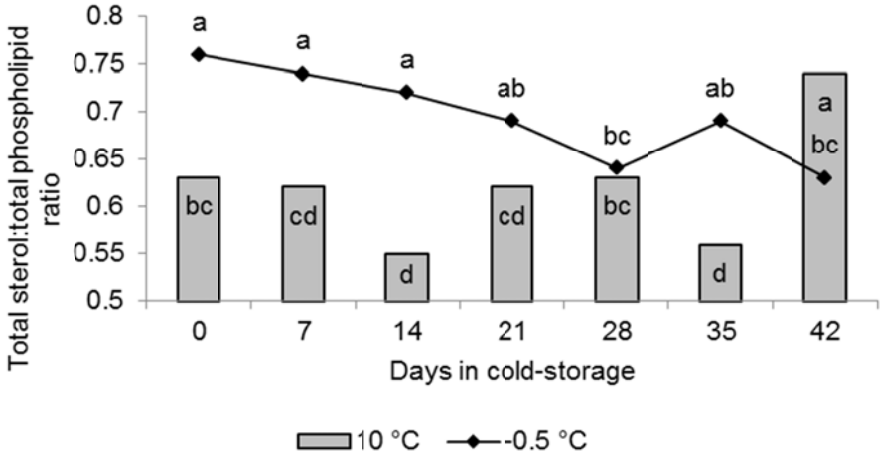
Effect	F	*p*
Harvest maturity	0.90	0.3650
Storage temperature	3.73	0.0032
Storage duration	6.95	0.0248
Harvest maturity x Storage temperature	6.76	**0.0000**
Harvest maturity x Storage duration	4.89	0.0513
Storage temperature x Storage duration	0.55	0.7659
Harvest maturity x Storage temperature x Storage duration	0.42	0.8618

Fig. 1. Percentage saturated phospholipid fatty acids in 'Sapphire' plums as influenced by harvest maturity and storage temperature.



Effect	F	*p*
Harvest maturity	0.90	0.3650
Storage temperature	3.73	0.0032
Storage duration	6.95	0.0248
Harvest maturity x Storage temperature	6.76	**0.0000**
Harvest maturity x Storage duration	4.89	0.0513
Storage temperature x Storage duration	0.55	0.7659
Harvest maturity x Storage temperature x Storage duration	0.42	0.8618

Fig. 2. Percentage unsaturated phospholipid fatty acids in 'Sapphire' plums as influenced by harvest maturity and storage temperature.



Effect	F	*p*
Harvest maturity	20.75	0.0010
Storage temperature	2.52	0.0303
Storage duration	4.84	0.0523
Harvest maturity x Storage temperature	8.14	0.0000
Harvest maturity x Storage duration	0.01	**0.9029**
Storage temperature x Storage duration	2.05	0.0722
Harvest maturity x Storage temperature x Storage duration	1.03	0.4088

Fig. 3. Total sterol:total phospholipid ratio in ‘Sapphire’ plums as influenced by storage duration and temperature.

APPENDIX C: SELECTED DATA OF PAPER 3

Table 1. Effect of storage regime and cold-storage duration on the phospholipid fatty acid composition of ‘Sapphire’ plums.

Table 2. Effect of storage regime and cold-storage duration on the phospholipid fatty acid composition of ‘Laetitia’ plums.

Table 1

Effect of storage regime and cold-storage duration on the phospholipid fatty acid composition of 'Sapphire' plums.

Examination parameter	Storage Duration[1]	Storage regime[2] (A)		Storage duration[3] (B)				*Prob. > F*[4]		
		IW	ST	0 days	28 days	35 days	42 days	*A*	*B*	*A x B*
C16:0 (µg g^{-1} FW)	0+28+35+42	66.39	68.64	60.56c	66.05bc	67.64b	78.94a	*0.3201*	***<0.0001***	*0.1518*
C18:0 (µg g^{-1} FW)	0+28+35+42	20.62a	26.01b	22.53	22.53	21.85	28.24	***0.0301***	*0.4090*	*0.0527*
C18:1n9c (µg g^{-1} FW)	0	6.90b	6.91b					*<0.0001*	*<0.0001*	***<0.0001***
	28	1.18cd	9.16a							
	35	0.00d	2.05c							
	42	0.00d	0.08d							
C18:1n9t (µg g^{-1} FW)	0	11.69a	10.69abc					*0.0170*	*0.0018*	***0.0009***
	28	8.58d	10.68abc							
	35	9.76c	11.06ab							
	42	10.47bc	10.55bc							
C18:2n6c (µg g^{-1} FW)	0	96.72cd	93.16d					*<0.0001*	*<0.0001*	***0.0170***
	28	93.34d	109.55bc							
	35	94.32d	117.96b							
	42	115.51b	137.87a							
C18:3n3 (µg g^{-1} FW)	0	24.00e	23.80e					*<0.0001*	*<0.0001*	***<0.0001***
	28	47.84c	30.12d							
	35	70.16b	31.08d							
	42	77.34a	46.01c							
Total fatty acids (µg g^{-1} FW)	0+28+35+42	248.77	257.34	220.05c	243.82b	257.70b	304.64a	*0.3010*	***<0.0001***	*0.2280*
MUFA:PUFA[5]	0	0.16a	0.15ab					*<0.0001*	*<0.0001*	*<0.0001*
	28	0.06d	0.14b							
	35	0.06d	0.08c							
	42	0.05d	0.06d							

[1] Data pooled across storage regimes and storage durations for non-significant interactions, with 0, 28, 35 and 42 indicating number of days in storage.
[2] Values in same row followed by different superscripts indicate significant differences ($P < 0.05$) according to the LSD test. IW and ST indicating intermittent warming regime and single-temperature regime at -0.5 °C, respectively.
[3] Values in same row followed by different letters indicate significant differences where ($P < 0.05$). Simulated arrival of the fruit at the overseas destination is represented by 28 days of storage, the maximum storage time of 'Sapphire' plums is represented by 35 days of storage and 42 days of storage represents the end of the simulated shelf-life period at 10 °C between 35 and 42 days of storage.
[4] Two-way ANOVA table with complete randomised design for Factor A (storage regime) and Factor B (storage duration).
[5] Monounsaturated:polyunsaturated phospholipid fatty acids.

Table 2

Effect of storage regime and cold-storage duration on the phospholipid fatty acid composition of 'Laetitia' plums.

Examination parameter	Storage Duration[1]	Storage regime[2] (A)		Storage duration[3] (B)			*Prob. > F*[4]		
		IW	ST	28 days	49 days	56 days	*A*	*B*	*A x B*
C16:0 (µg g^{-1} FW)	28	54.76a	49.29b				*0.4161*	*<0.0001*	***0.0113***
	49	41.74cd	46.32bc						
	56	40.30d	38.00d						
C18:0 (µg g^{-1} FW)	28 + 49 + 56	19.87a	14.34b	16.52	18.49	16.30	***0.0001***	*0.3151*	*0.8346*
C18:1n9c (µg g^{-1} FW)	28 + 49 + 56	0.002	0.000	0.002	0.000	0.000	*0.3253*	*0.3798*	*0.3798*
C18:1n9t (µg g^{-1} FW)	28	10.94a	10.02ab				*0.7020*	*<0.0001*	***0.0267***
	49	8.66c	9.80b						
	56	8.64c	8.06c						
C18:2n6c (µg g^{-1} FW)	28	63.69a	65.99a				*<0.0001*	*0.0001*	***0.0013***
	49	36.46b	65.04a						
	56	41.18b	60.21a						
C18:3n3 (µg g^{-1} FW)	28 + 49 + 56	61.98a	48.86b	61.16a	54.90b	50.19b	***<0.0001***	***0.0003***	*0.3258*
Total fatty acids (µg g^{-1} FW)	28	217.58a	192.52b				*0.5350*	*<0.0001*	***0.0043***
	49	167.29c	187.54b						
	56	167.11c	162.28c						
MUFA:PUFA[5]	28 + 49 + 56	0.087	0.082	0.083	0.087	0.083	0.1034	0.3408	0.3375

[1] Data pooled across storage regimes and storage durations for non-significant interactions, with 28, 49 and 56 indicating number of days in storage.
[2] Values in same row followed by different superscripts indicate significant differences ($P < 0.05$) according to the LSD test. IW and ST indicating intermittent warming regime and single-temperature regime at -0.5 °C, respectively.
[3] Values in same row followed by different letters indicate significant differences where ($P < 0.05$). Simulated arrival of the fruit at the overseas destination is represented by 28 days of storage, the maximum storage time of 'Laetitia' plums is represented by 49 days of storage and 56 days of storage represents the end of the simulated shelf-life period at 10 °C between 49 and 56 days of storage.
[4] Two-way ANOVA table with complete randomised design for Factor A (storage regime) and Factor B (storage duration).
[5] Monounsaturated:polyunsaturated phospholipid fatty acids.

APPENDIX D: SELECTED DATA OF PAPER 4

Figure 1. Decay levels in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 2. (A) Gel breakdown levels in 'Sapphire' plums as affected by storage regime and storage duration. (B) Internal browning levels in 'Sapphire' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 3. (A) Gel breakdown levels in 'Laetitia' plums as affected by storage regime. (B) Internal browning levels in 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 4. Overripeness levels in 'Sapphire' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 5. Total internal disorders in 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 6. Overripeness levels in 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 7. Water soluble antioxidant activity in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 8. Lipid soluble antioxidant activity in 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 9. Manifestation of internal browning in 'Sapphire' plums as affected by storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 10. Oxidised ascorbic acid levels in 'Sapphire' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 11. Oxidised ascorbic acid levels in 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 12. Total phospholipid concentration in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

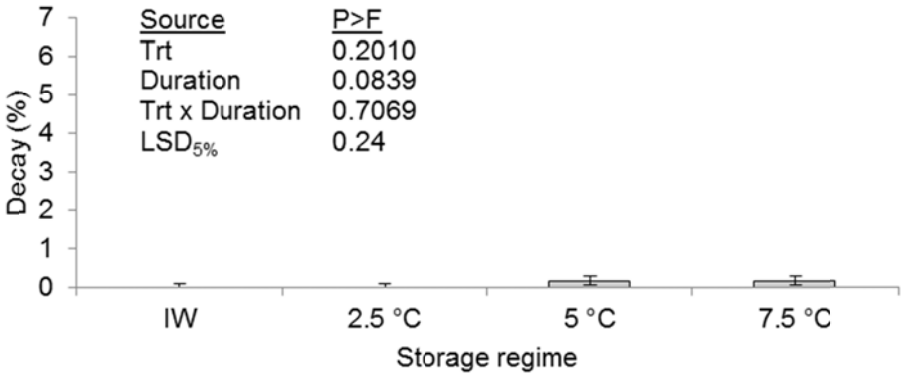
Figure 13. Total sterol concentration in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 14. Total sterol:total phospholipid ratio in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

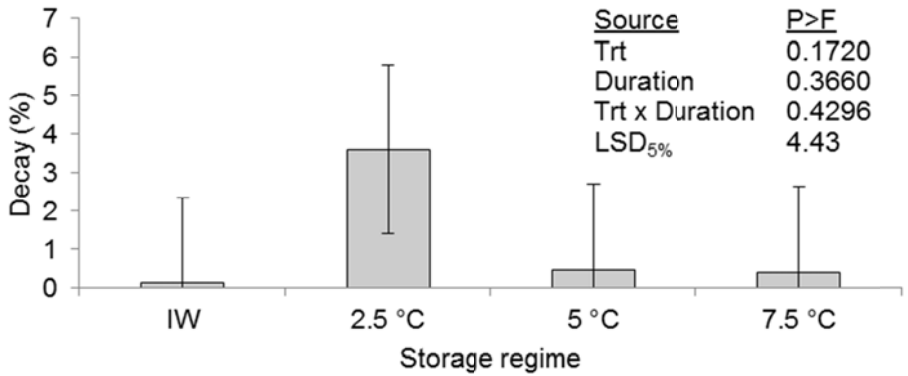
Figure 15. Unsaturated:saturated phospholipid fatty acid ratio in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 16. Linoleic acid concentration in (A) 'Sapphire' plums as affected by storage regime and storage duration, and (B) 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

Figure 17. Linolenic acid concentration in (A) 'Sapphire' plums as affected by storage regime, and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

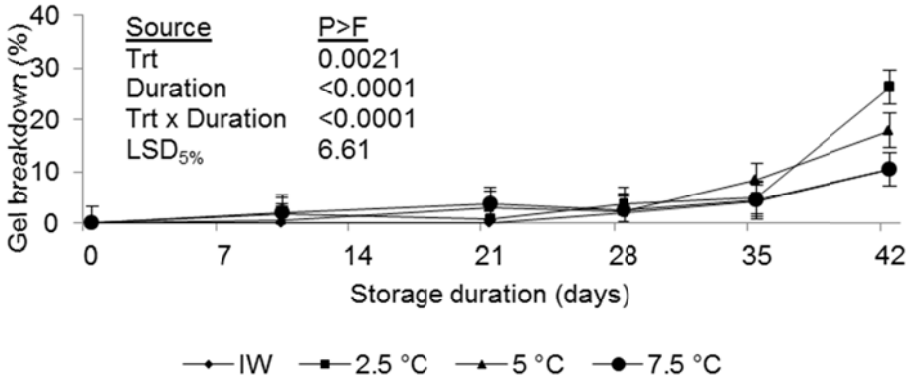


(B)



(B)

Fig. 1. Decay levels in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.



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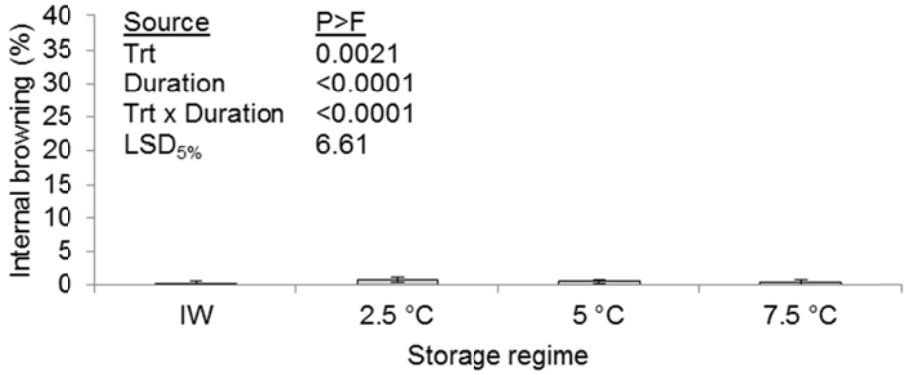
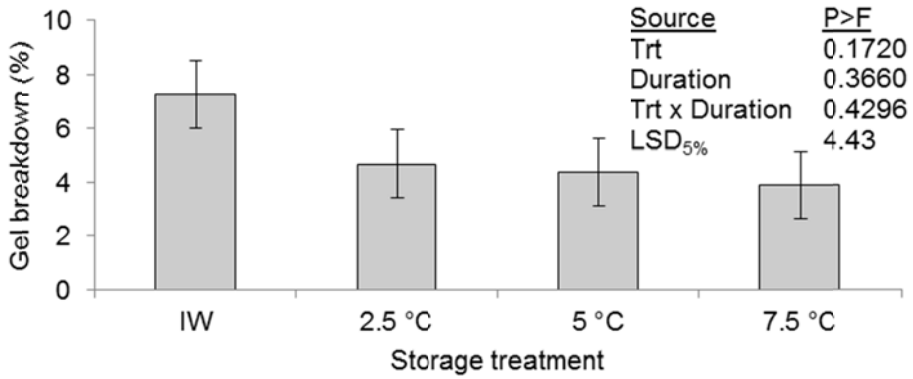
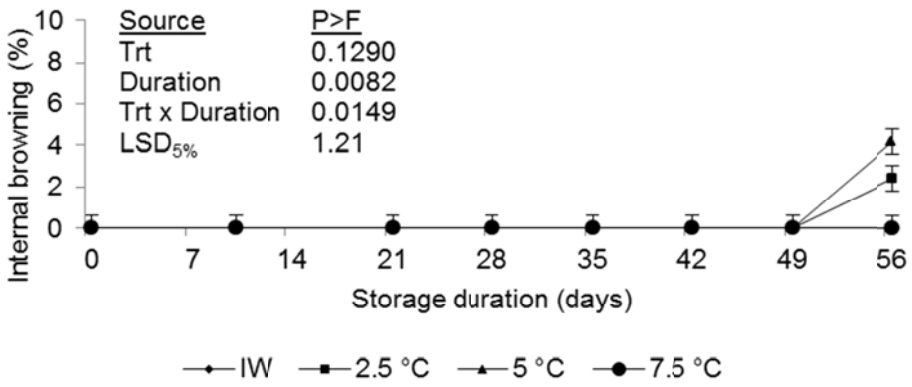


(B)

Fig. 2. (A) Gel breakdown levels in 'Sapphire' plums as affected by storage regime and storage duration. (B) Internal browning levels in 'Sapphire' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.





(C) **(B)**

Fig. 3. (A) Gel breakdown levels in 'Laetitia' plums as affected by storage regime. (B) Internal browning levels in 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

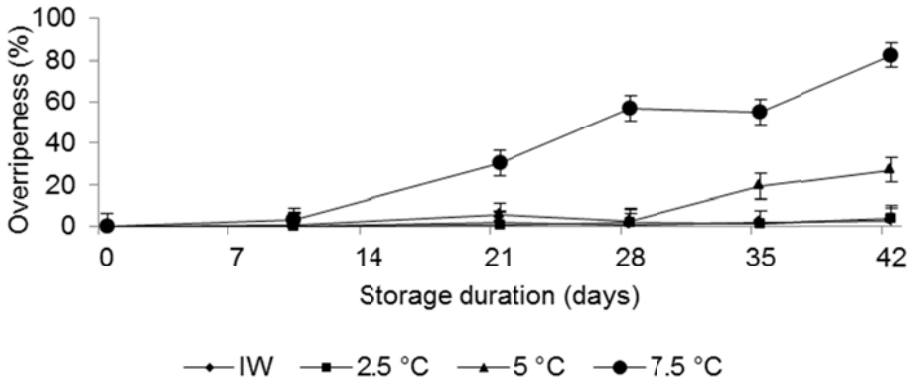


Figure 4. Overripeness levels in 'Sapphire' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

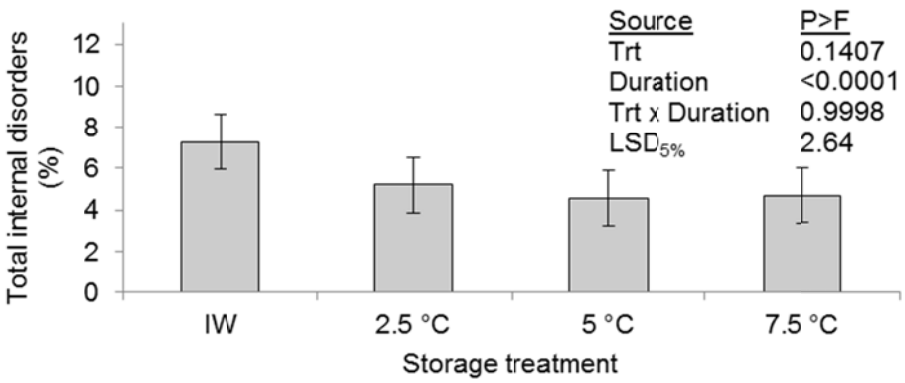


Figure 5. Total internal disorders in 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

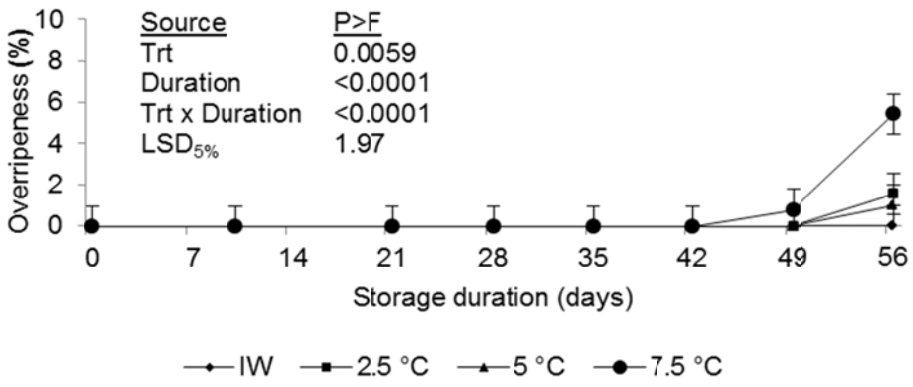
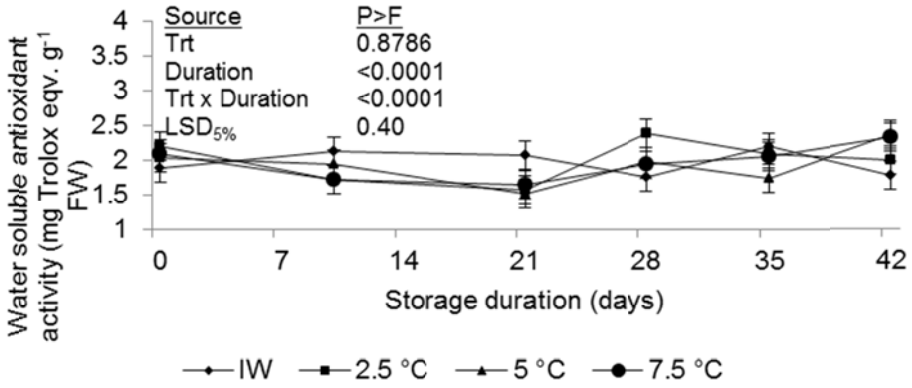
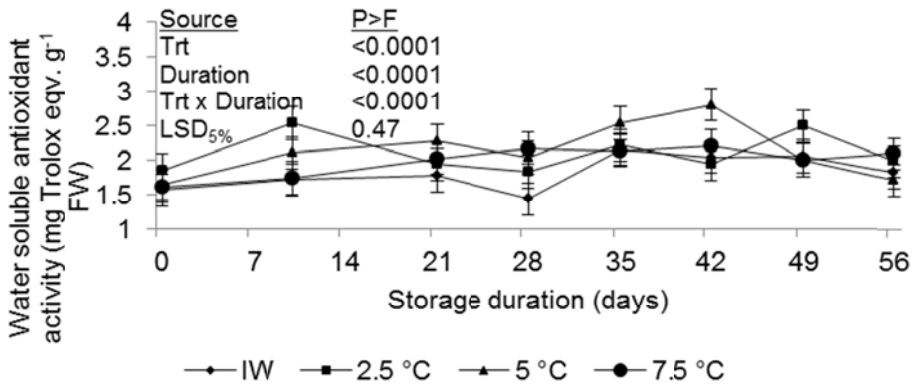


Figure 6. Overripeness levels in 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.



(A)



(B)

Fig. 7. Water soluble antioxidant activity in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

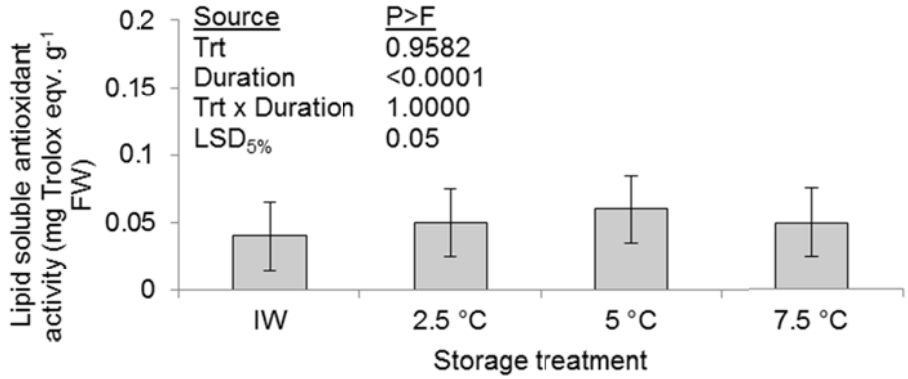


Fig. 8. Lipid soluble antioxidant activity in 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

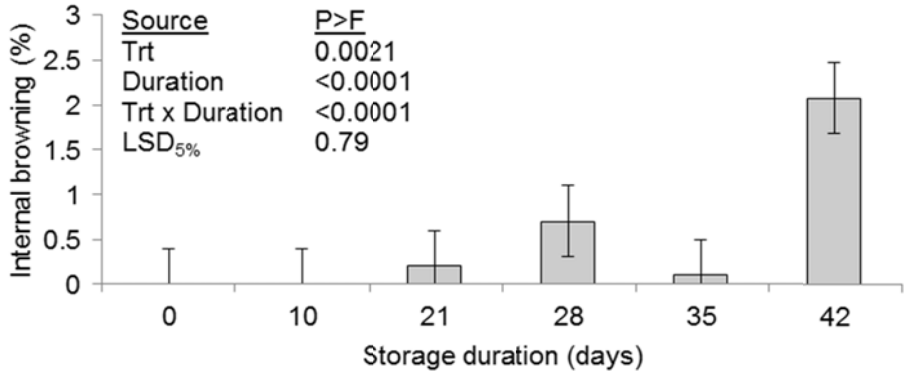


Fig. 9. Manifestation of internal browning in 'Sapphire' plums as affected by storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

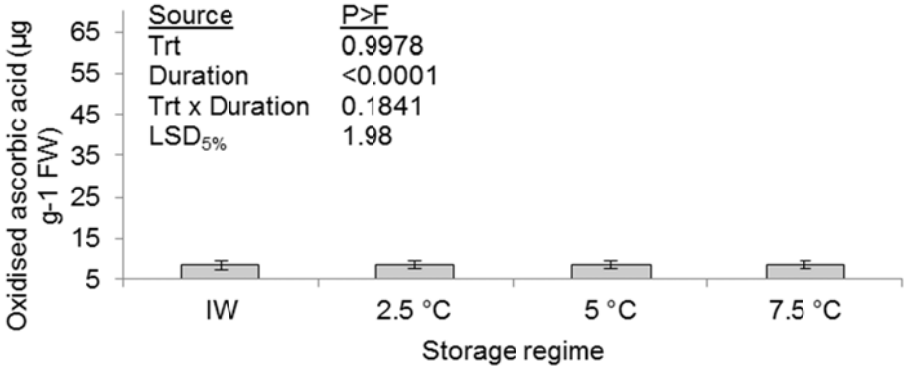


Fig. 10. Oxidised ascorbic acid levels in 'Sapphire' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

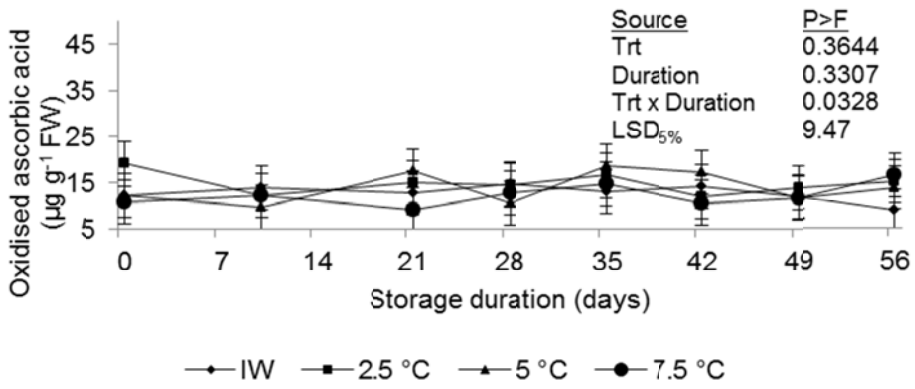
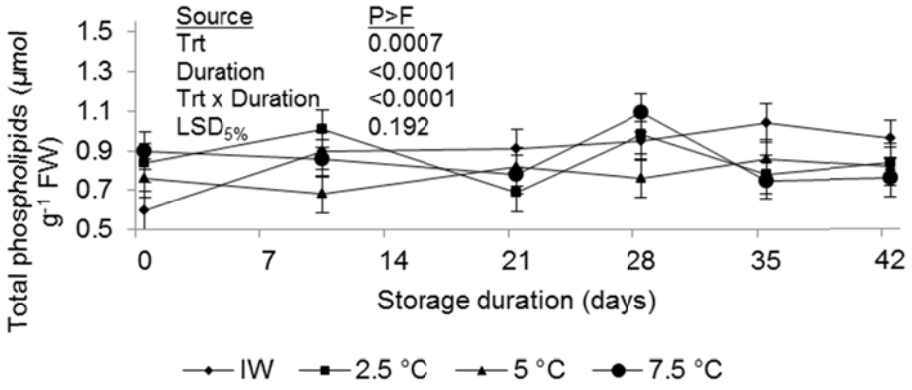
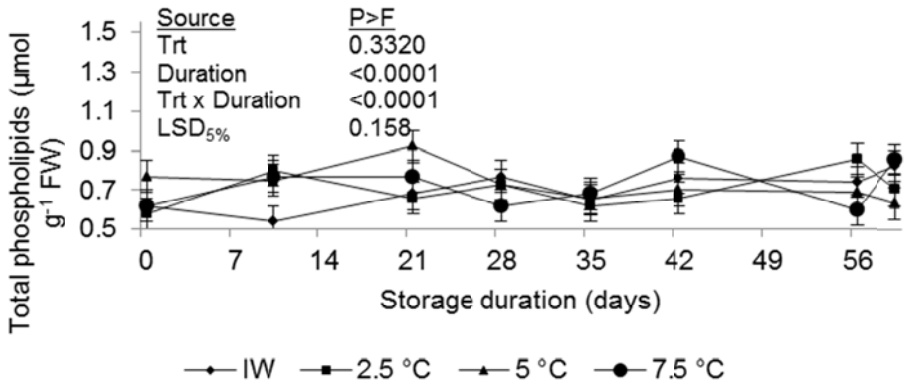


Fig. 11. Oxidised ascorbic acid levels in 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

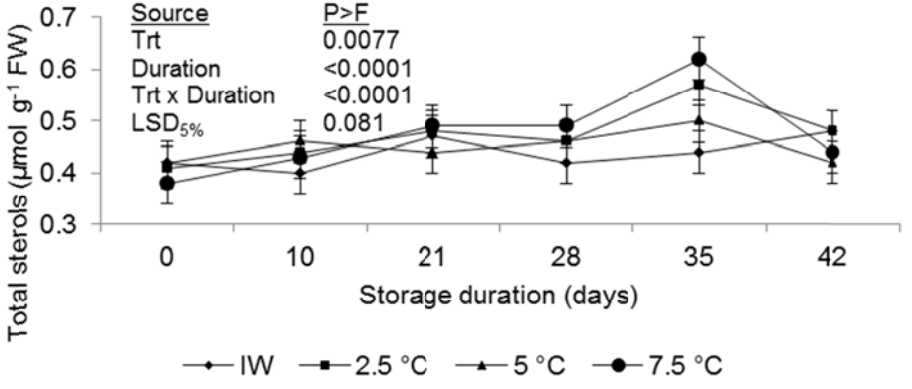


(A)

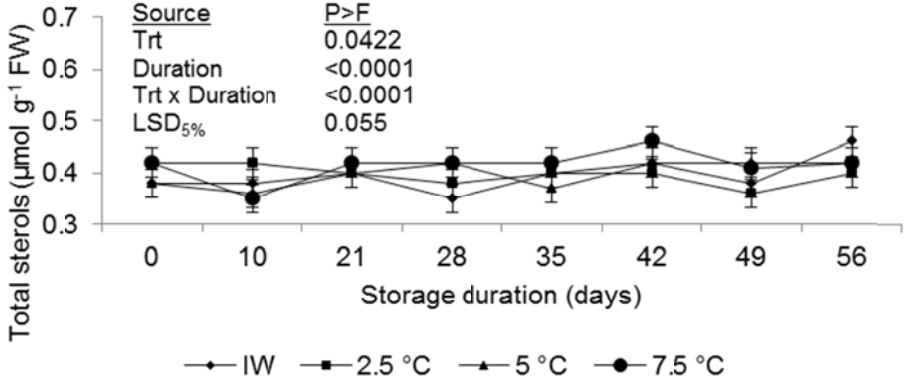


(B)

Fig. 12. Total phospholipid concentration in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

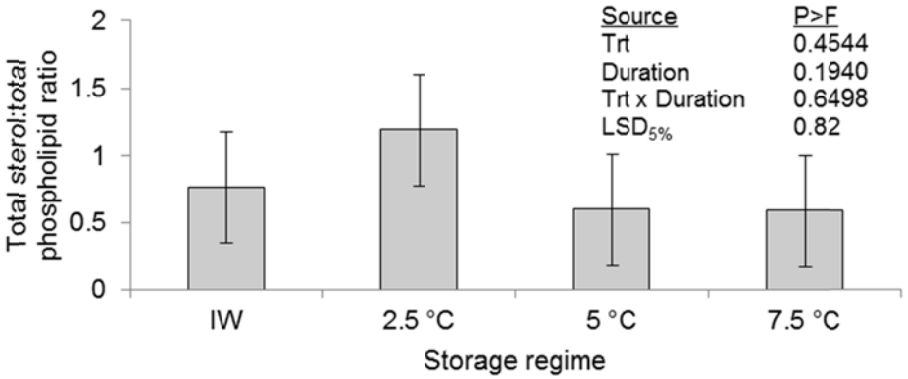


(A)

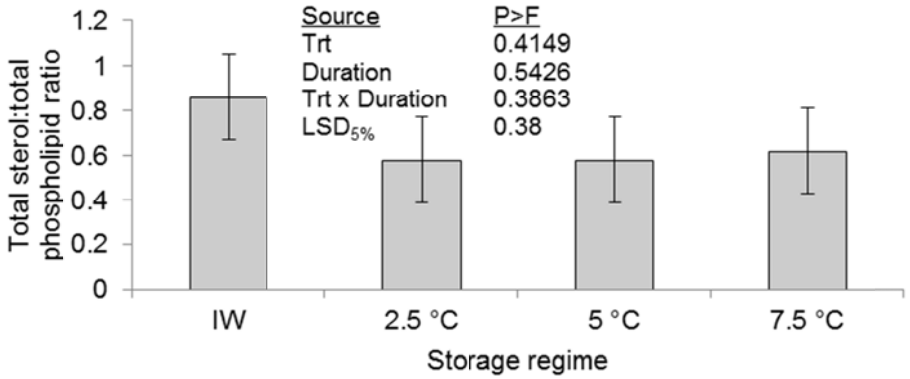


(B)

Fig. 13. Total sterol concentration in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

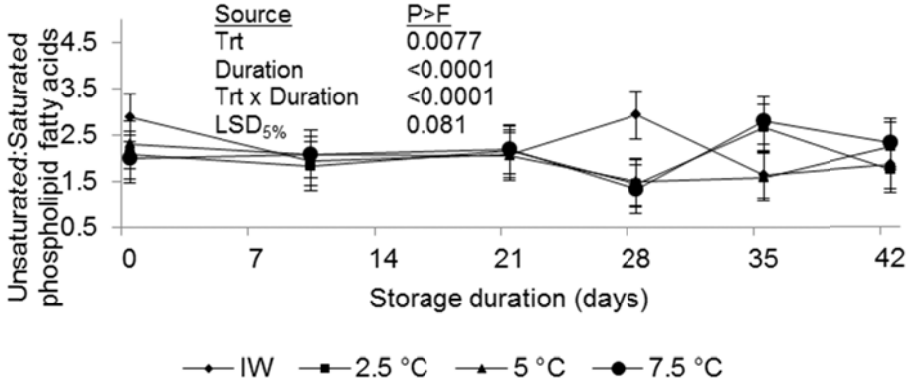


(A)

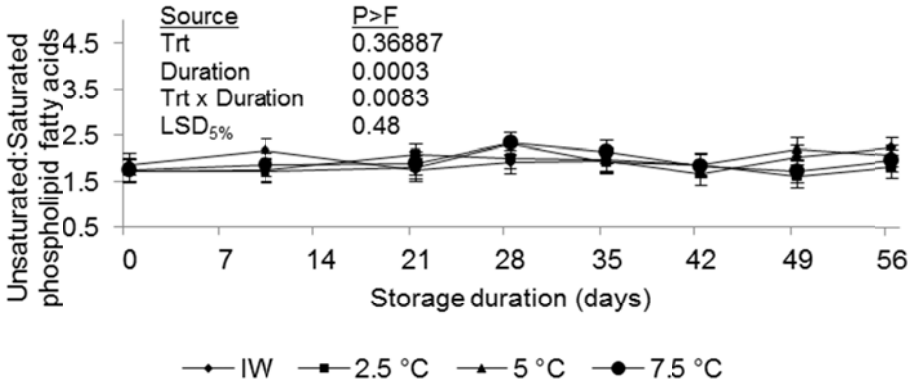


(B)

Fig. 14. Total sterol:total phospholipid ratio in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

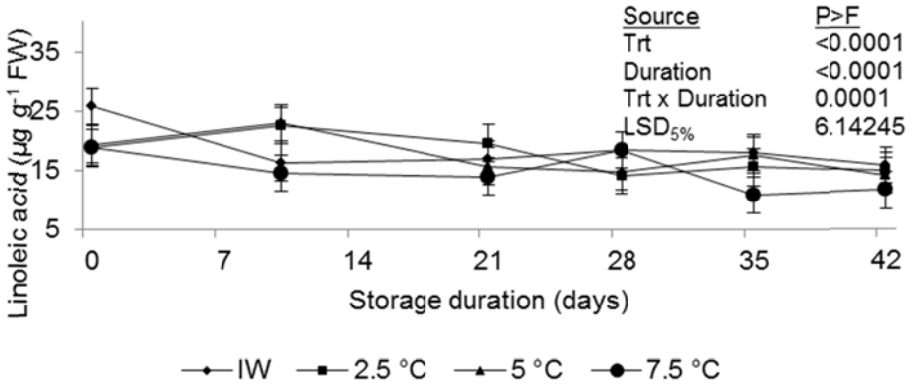


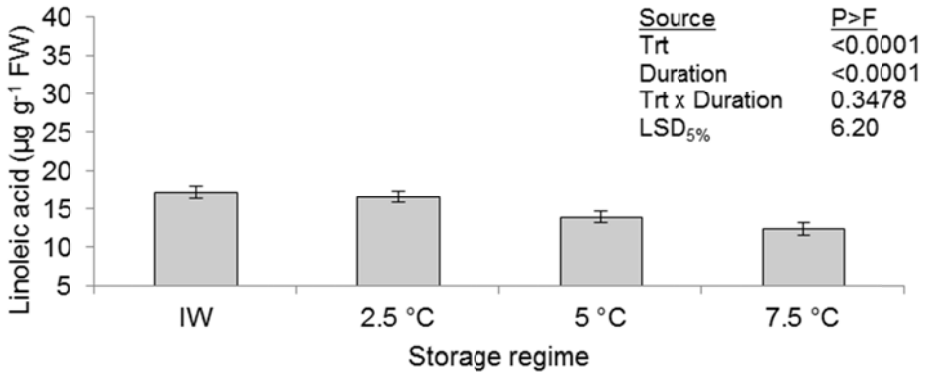
(A)



(B)

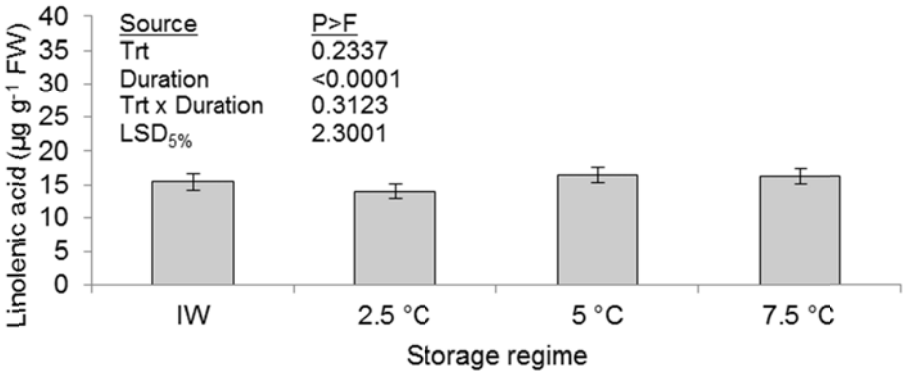
Fig. 15. Unsaturated:saturated phospholipid fatty acid ratio in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

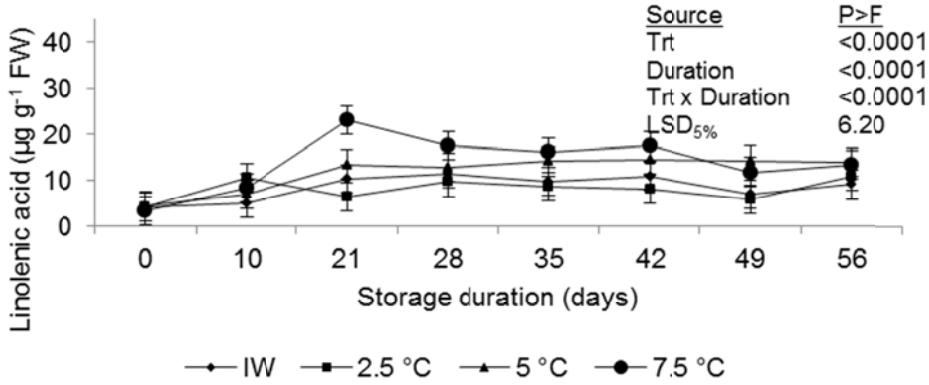




(A) **(B)**

Fig. 16. Linoleic acid concentration in (A) 'Sapphire' plums as affected by storage regime and storage duration, and (B) 'Laetitia' plums as affected by storage regime. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.





(A) **(B)**

Fig. 17. Linolenic acid concentration in (A) 'Sapphire' plums as affected by storage regime, and (B) 'Laetitia' plums as affected by storage regime and storage duration. IW indicates the intermittent warming regime, and 2.5 °C, 5 °C and 7.5 °C single-temperature storage at the named temperatures.

APPENDIX E: SELECTED DATA OF PAPER 5

Figure. 1. Change in flesh firmness of (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by forced air cooling (FAC) treatment. FAC1 = initial FAC to pulp temperature of 5 °C within 3 h; total FAC time to pulp temperature of 0 °C within 12 h; FAC2 = initial FAC to pulp temperature of 5 °C within 6 h; total FAC time to pulp temperature of 0 °C within 12 h; FAC3 = initial FAC to pulp temperature of 5 °C within 3 h; total FAC time to pulp temperature of 0 °C within 24 h; FAC4 = initial FAC to pulp temperature of 5 °C within 6 h; total FAC time to pulp temperature of 0 °C within 24 h. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

Figure. 2. (A) Gel breakdown and (B) internal browning levels in 'Sapphire' plums as affected by forced air cooling treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

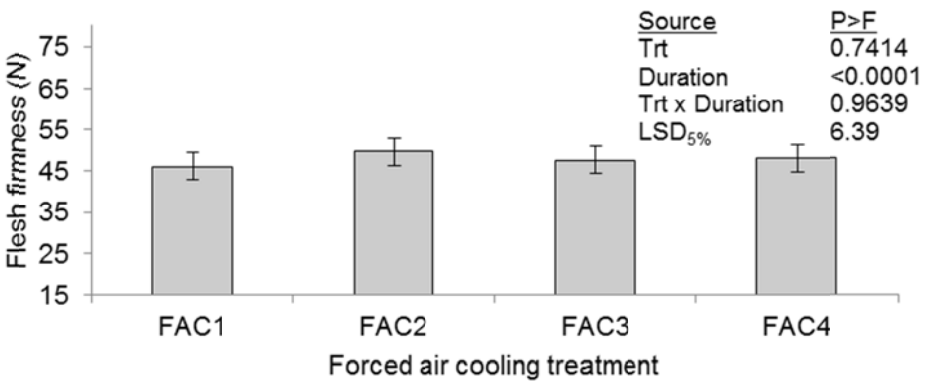
Figure. 3. (A) Gel breakdown and (B) internal browning levels in 'Laetitia' plums as affected by forced air cooling treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

Figure. 4. Overripeness levels in (A) 'Sapphire' plums as affected by forced air cooling treatment and (B) 'Laetitia' plums as affected by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

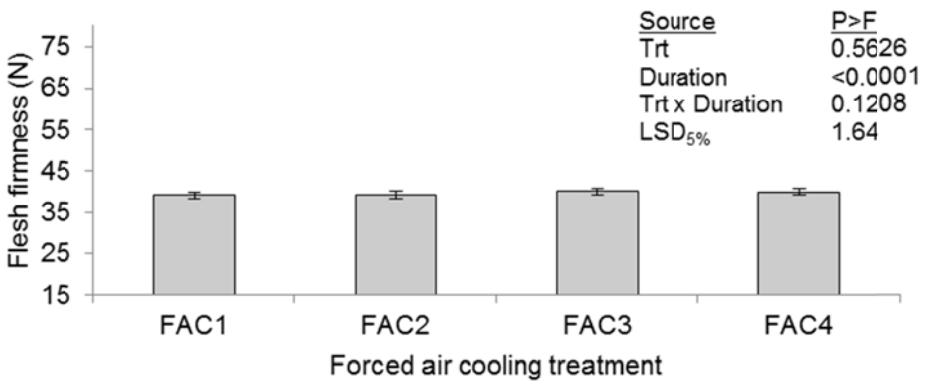
Figure. 5. Levels total internal defects (sum of total chilling injury and overripeness) in (A) 'Sapphire' plums as affected by forced air cooling treatment and (B) 'Laetitia' plums as affected by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

Figure. 6. Total sterol:total phospholipid ratio in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by forced air cooling treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

Figure. 7. Manifestation of chilling injury in 'Sapphire' plums as affected by storage duration. 'Sapphire' plums were treatment with different forced air cooling (FAC) treatments and stored for 35 plus a subsequent simulated shelf-life of 7 days at 10 °C. For definitions of the different FAC treatments, see Fig. 1.

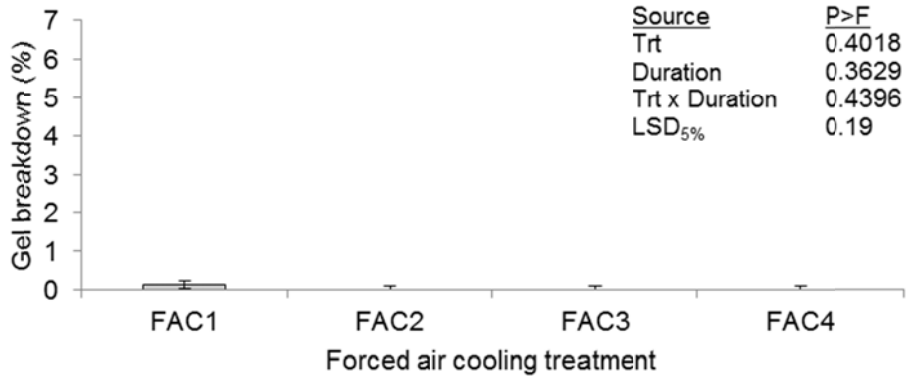


(C)

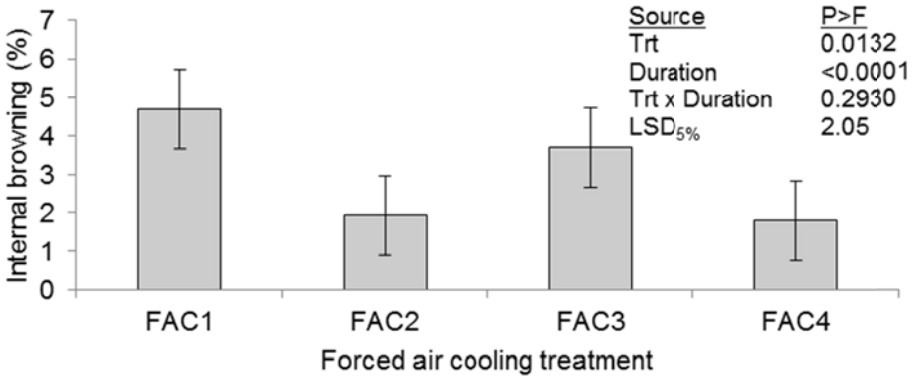


(B)

Fig. 1. Change in flesh firmness of (A) 'Sapphire' and (B) 'Laetitia' plums as influenced by forced air cooling treatment. FAC1 = initial FAC to pulp temperature of 5 °C within 3 h; total FAC time to pulp temperature of 0 °C within 12 h; FAC2 = initial FAC to pulp temperature of 5 °C within 6 h; total FAC time to pulp temperature of 0 °C within 12 h; FAC3 = initial FAC to pulp temperature of 5 °C within 3 h; total FAC time to pulp temperature of 0 °C within 24 h; FAC4 = initial FAC to pulp temperature of 5 °C within 6 h; total FAC time to pulp temperature of 0 °C within 24 h. 'Sapphire' and 'Laetitia' plums were stored for 35 and 49 days, respectively, plus a subsequent simulated shelf-life of 7 days at 10 °C.

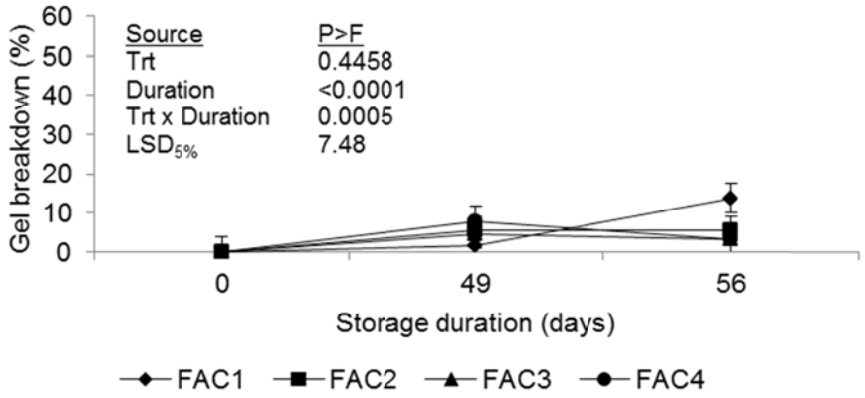


(D)

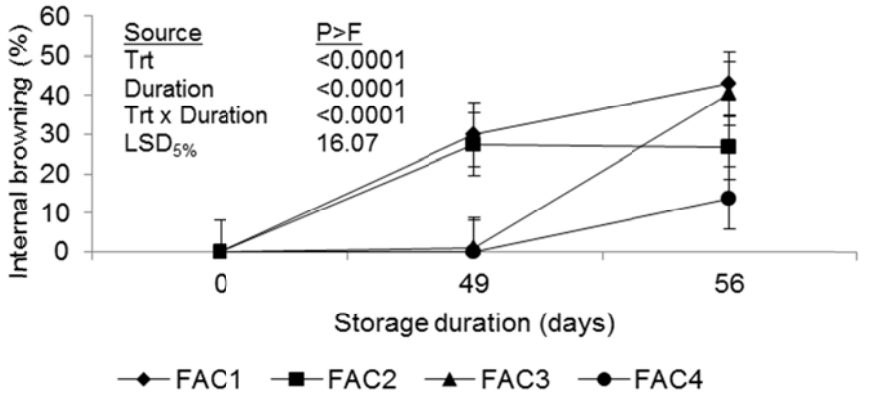


(B)

Fig. 2. (A) Gel breakdown and (B) internal browning levels in 'Sapphire' plums as affected by forced air cooling treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

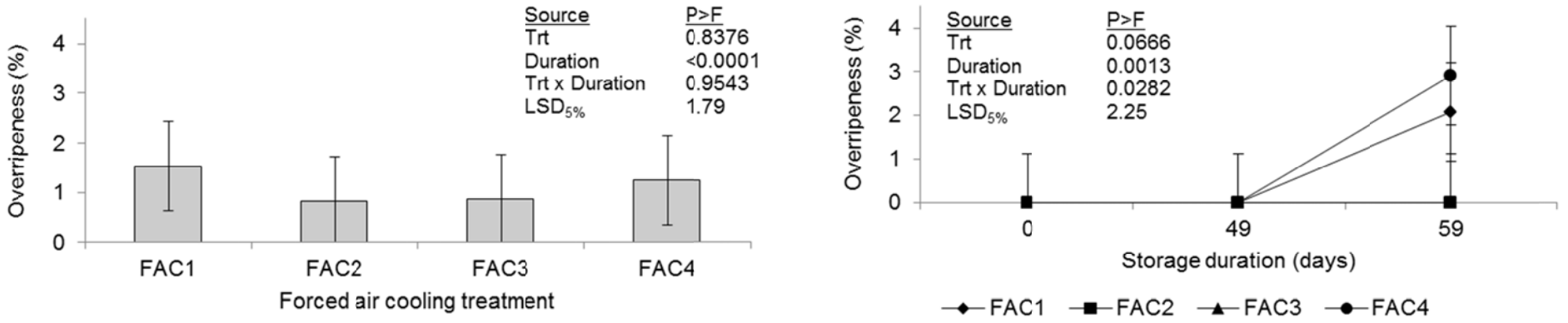


(E)



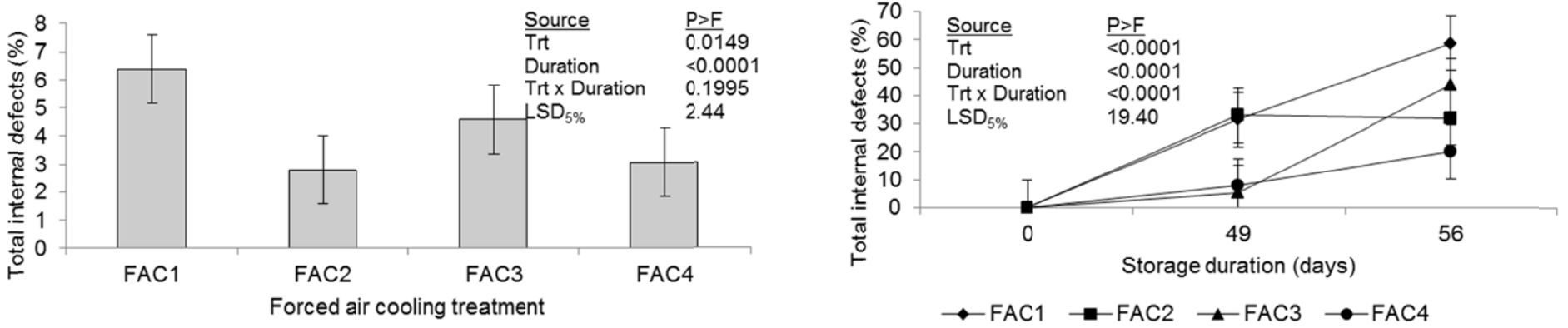
(B)

Fig. 3. (A) Gel breakdown and (B) internal browning levels in 'Laetitia' plums as affected by forced air cooling treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.



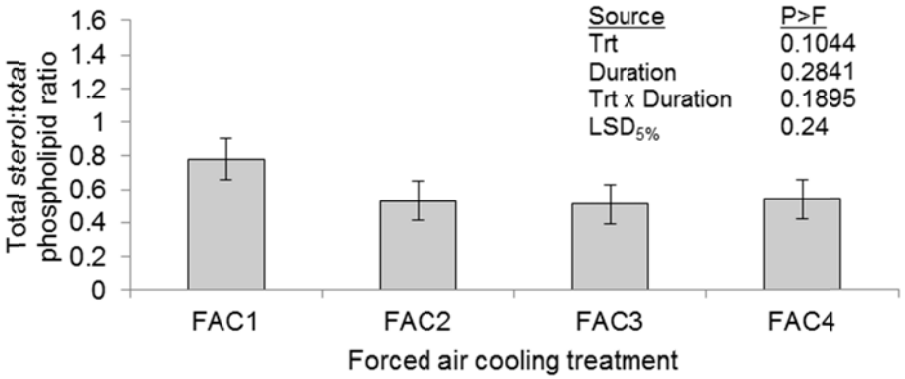
(A) **(B)**

Fig. 4. Overripeness levels in (A) 'Sapphire' plums as affected by forced air cooling treatment and (B) 'Laetitia' plums as affected by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

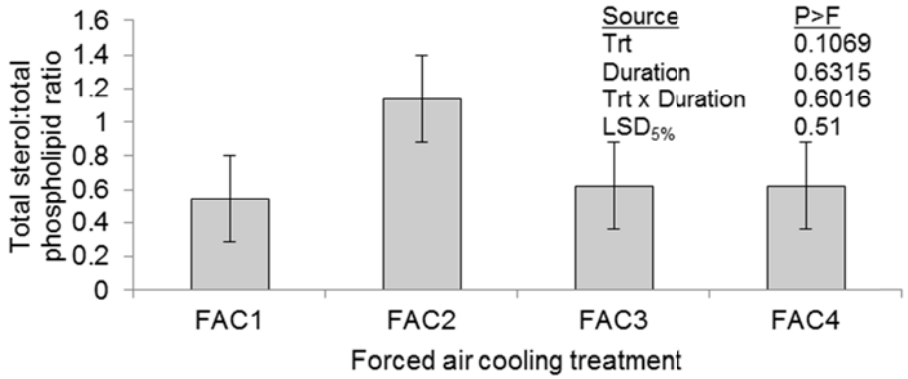


(A) **(B)**

Fig. 5. Levels total internal defects (sum of total chilling injury and overripeness) in (A) 'Sapphire' plums as affected by forced air cooling treatment and (B) 'Laetitia' plums as affected by forced air cooling treatment and storage duration. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.



(B)



(B)

Fig. 6. Total sterol:total phospholipid ratio in (A) 'Sapphire' and (B) 'Laetitia' plums as affected by forced air cooling treatment. For definitions of FAC1, FAC2, FAC3 and FAC4, see Fig. 1.

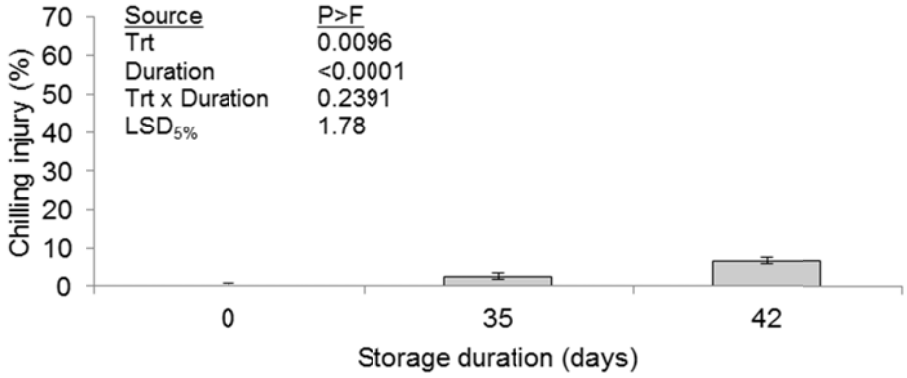


Fig. 7. Manifestation of chilling injury in 'Sapphire' plums as affected by storage duration. 'Sapphire' plums were treatment with different forced air cooling (FAC) treatments and stored for 35 plus a subsequent simulated shelf-life of 7 days at 10 °C. For definitions of the different FAC treatments, see Fig. 1.