Effect of heat, ultraviolet-B and photosynthetic active radiation stress on apple peel photosystems

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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This dissertation includes two original papers published in peer reviewed journals, one original paper accepted for publication in a peer reviewed journal, and two unpublished manuscrips. I was principally responsible for the development and writing of the papers (published and unpublished).

SUMMARY

The study was undertaken to analyse the response of apple fruit peel photosystems of different cultivars to ultraviolet-B (UV-B) radiation, photosynthetic active radiation (PAR) and heat stresses under laboratory conditions. UV-B, PAR and heat are claimed to be the main fruit sunburn-inducing stress factors. The aim was to identify biochemical, physiological and fruit peel anatomical characteristics that provide photoprotection against sunburn inducing factors and to determine stress threshold levels for photodamage. Previously sun-exposed peels of apple fruits were resistant to photodamage under high UV-B dosage throughout fruit development. However, the shaded peels of mature fruits incurred photodamage under UV-B stress. Furthermore, fruit photosystems at all development stages were equally sensitive to heat stress combined with moderate PAR (500 μmol m^{-2} s^{-1}). Photodamage induced by heat and PAR stress during fruit development was not well correlated to fruit pigments, phenolic levels or fruit peel anatomical characteristics. In addition, repeated heat and PAR stress up to 9 hours did not induce any fruit sunburn symptoms. The photosystems of the less sunburn susceptible ‘Golden Delicious’ and more susceptible ‘Granny Smith’ appeared to be equaly sensitive to heat and PAR stress. The possible involvement of the xanthophyll cycle in fruit sunburn susceptibility needs further investigation as a variation in the dependancy of different cultivars on this cycle for photoprotection under heat and PAR stress was observed. Heat stress alone appears to cause the highest damage to fruit photosystems, while the presence of UV-B and PAR enhances this effect. The results

presented in this document suggest that sensitivity to sunburn browning may not only be related to the heat, PAR and UV-B stress sensitivity of fruit peel photosystems. General non-photoprotective biochemical responses to the experienced stress may also play a role in sunburn symptom development.

OPSOMMING

Hierdie studie is onderneem om die respons van appelvrugskil fotosisteme van verskillende kultivars in reaksie op ultraviolet-B (UV-B) radiasie, fotosintetiese aktiewe radiasie (PAR) en hittestres onder laboratorium toestande te ondersoek. UB-B, PAR en hitte word gesien as die hoof stresfaktore wat sonbrand induseer. Die doelwit was om die biochemiese, fisiologiese en vrugskil anatomiese eienskappe wat beskerming teen die sonbrand induksie faktore verleen asook stres drumpelwaardes vir fotosisteemskade te identifiseer. Son blootgestelde appelvrugskil was strykdeur vrugontwikkeling weerstandig teen fotoskade onder 'n hoë UV-B lading. Oorskadude vrugskil van volwasse vrugte het egter fotoskade ondergaan in reaksie op UV-B stres. Verder was fotosisteme van vrugte by alle ontwikkelingstadiums ewe sensitief tot hitteskade in kombinasie met matige PAR (500 µmol m^{-2} s^{-1}). Hitte- en PAR stres induksie van fotoskade gedurende vrugontwikkeling was nie goed met vrugpigment, fenoolvlakke of met vrugskil anatomiese eienskappe gekorreleer nie. Daarmee saam het herhaaldelike hitte en PAR stres vir tot 9 ure nie enige vrug sonbrandsimptome geïnduseer nie. Die swak korrelasie en die onvermoë om sonbrandsimptome te induseer dui moontlik op die betrokkenheid van addisionele faktore in die manifestasie van vrug sonbrand. Die fotosisteme van die minder sonbrand sensitiewe 'Golden Delicious' en die meer sensitiewe 'Granny Smith' was klaarblyklik ewe sensitief vir hitte en PAR stres. Sonbrand sensitiwiteit hou daarom moontlik nie alleenlik verband met die hitte en PAR stres sensitiwiteit van vrugskil fotosisteme nie. Die moontlike betrokkenheid van die xantofielsiklus in vrugskil sonbrand sensitiwiteit behoort

verder bestudeer te word, siende die variasie wat waargeneem is in die afhanklikheid van die verskillende kultivars op hierdie siklus vir fotobeskerming tydens hitte en PAR stres. Hitte stres opsigself veroorsaak klaarblyklik die grootste skade aan die vrug fotosisteme terwyl UV-B en PAR die effek van hitte versterk. Die resultate wat hier aangebied word, dui daarop dat direkte fotoskade in reaksie op hitte, UV-B en PAR stres nie, soos tans verstaan word, die alleen faktor in die induksie van sonbrand is nie. Die resultate dui verder ook daarop dat die sonbrand sensitiwiteit van verskillende kultivars, d.w.s hul geneigdheid om visuele sonbrandverbruining simptome te onwikkel, nie noodwendig saamhang met hul sensitiwiteit tot die verskillende faktore wat sonbrandverbruining induseer nie. Dit is moontlik omdat sonbrand simptomatologie in die geval van sonbrandverbruining dalk meer verband hou met die reaksie van die kultivar op die stres eerder as die sensitiwiteit daarvan tot die stres.

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I dedicate this work to my wife Ivonn, our son Noah and my late mother Erica Kaveṱu Hengari.

“When you have eliminated the impossible, whatever remains, however improbable, must be the truth” Arthur Conan Doyle

LIST OF ABBREVIATIONS

PAR	Photosynthetic active radiation
UV-B	Ultraviolet – B radiation
OEC	Oxygen evolving complex
ATP	Adenosine triphosphate
ROS	Reactive oxygen species
PS II	Photosystem II
PS I	Photosystem I
NPQ	Non-photochemical quenching
EC	Evaporative cooling
ETR	Electron transport rate
F_o	Minimum fluorescence
F_m	Maximum fluorescence
F_v	Variable fluorescence
F_v/F_m	Maximum light use efficiency of PS II
DAFB	Days after full bloom
EPS	Epoxidation state
AVI	Apple violaxanthin cycle index
PSN	Previously sun-exposed peel
PSH	Previously shaded peel

TABLE OF CONTENT

LIST OF PUBLICATIONS

1. Papers published:

1.1. Paper 1 published as:

Simeon Hengari, Karen I. Theron, Stephanie J.E. Midgley, Willem J. Steyn. 2014. The effect of high UV-B dosage on apple fruit photosystems at different fruit maturity stages. Scientia Horticulturae 170: 103-114.

1.2. Paper 2 published as:

Simeon Hengari, Karen I. Theron, Stephanie J.E. Midgley, Willem J. Steyn. 2014. Response of apple (*Malus domestica* Borkh.) fruit peel photosystems to heat stress coupled with moderate photosynthetic active radiation at different fruit development stages. Scientia Horticulturae 178: 154-162.

2. Paper accepted for publication:

2.1. Paper 4, accepted by the South African Journal of Plant and Soil, pending final corrections:

Simeon Hengari, Karen I. Theron, Stephanie J.E. Midgley, Willem J. Steyn. 2014. Differential dependence of apple (*Malus domestica* Borkh.) cultivars on the xanthophyll cycle for photoprotection. South African Journal of Plant and Soil.

NOTE

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

1. GENERAL INTRODUCTION

1.1. Background

Apple production in the Western Cape Province (33°S; 18°E) of South Africa is challenging because of high temperature associated with the climate of this region. The average summer temperatures between November to March range between 17 °C to 27 °C and can be as high as 30 °C (Climate summary of South Africa, http://reference.sabinet.co.za/sa_epublication/cssa, 31-10-2012). The maximum and minimum temperatures in February, the warmest month, have increased by 1 °C over the last four decades of the 20th century due to climate change (Midgley *et al.*, 2005).

Temperatures above 45 °C damage fruit photosystems and can cause the permanent reduction of photosynthesis (Smillie, 1992; Chen *et al.*, 2009). The temperature of sunexposed fruit peel is generally higher than air temperature by up to 15 °C (Parchomchuk and Meheriuk, 1996; Ferguson *et al.*, 1998). Surface temperature of dark coloured sunexposed fruit peel (dark green or red colour) can even be up to 24 °C above ambient air temperature (Barber and Sharpe, 1971). Therefore fruits can experience temperatures of up to 45 °C at air temperatures of 30 °C. Fruit sunburn is caused by high fruit peel temperatures (45 °C to 49 °C) in combination with photosynthetic active radiation (PAR) and ultraviolet-B radiation (UV-B) (Rabinowitch *et al.*, 1974; Schrader *et al.*, 2003). Sunburned fruits have damaged photosystems (Chen

et al., 2008; Seo *et al.*, 2008). Heat and radiation (PAR + UV) induced photodamage can therefore induce fruit sunburn symptom development by damaging fruit peel photosystems.

The induction of fruit sunburn by heat and sun light stress results in a reduction in fruit peel chlorophyll content and accumulation of phenolic and carotenoid molecules (Felicetti and Schrader, 2009a, b). The loss of chlorophyll and increase in phenolics and carotenoids causes the observed yellow/bronze coloured areas on the sunburned fruits. This yellow or bronze colour change on fruits is referred to in literature as fruit "sunburn browning" (Schrader *et al.*, 2001). Fruit sunburn can be less visible on lightly-coloured apple cultivars such as 'Golden Delicious' or red coloured cultivars like 'Topred' or 'Royal Gala'. However, the yellow/bronze colour associated with sunburn browning is much more easily visible on dark-green fruits such as 'Granny Smith'.

Fruit discolouration due to sunburn has a negative effect on the overall appearance of the fruits and therefore reduce fruit market value. 'Granny Smith' apples for an example, which should be completely green to be marketed as grade 1 fruits. However, the presence of sunburn defects results in fruits having to be downgraded to lower quality classes or even be diverted for processing purposes. Apple fruit sunburn damage results in a loss of up to 18% of the total harvest in South Africa (Gindaba and Wand, 2005). Such loss of top grade fruits results in a reduction of revenue to fruit producers. It is

therefore important to understand the interaction between fruit peel and sunburn inducing factors.

1.2. Research hypothesis, aim and objectives

Research hypothesis:

It is hypothesised that the rate of photodamage and subsequent sunburn development in different apple cultivars can be studied by exposing apples to UV-B, PAR and heat stress in different combination under laboratory conditions.

Research aim:

The aim of this work was to measure the response of apple peel photosystems to heat and light (PAR and UV-B) stress under laboratory conditions in relation to the possibility of peel biochemical, physiological and anatomical characteristics offering photoprotection and subsequently inhibiting sunburn development.

Research objectives:

a. determine whether there is a specific development stage at which fruits become more sensitive to UV-B stress;
b. study the effect of sun light exposure history on UV-B sensitivity of the peel;
c. determine the difference in heat stress susceptibility of photosystems of apple fruit peel at different fruit development stages;

d. determine the correlation between both the biochemical and anatomical characteristics of apple fruit peel and the heat stress-induced changes in the maximum light use efficiency (Fv/Fm) of photosystem II of the peel;
e. determine the critical temperature for photodamage of the photosystems of apple fruit peel;
f. study the difference between the damage to the photosystems of 'Granny Smith' and 'Golden Delicious' apples at maturity by: 1) different heat stress levels coupled with a constant moderate light stress level; and 2) by continuously increasing light stress;
g. determine the difference in the dependency of apple fruit photosystems of different cultivars on the xanthophyll cycle for photoprotection under laboratory conditions of temperature and PAR similar to conditions that induces fruit sunburn on the trees;
h. study the effect of the heat, PAR and UV-B stress in different combinations on the photosystems of apple fruit peels;
i. determine the response of apple fruit photosystems to continuous exposure of different heat stress levels coupled with a moderate PAR level.

1.3. Thesis structure

a. The general introduction and literature review sections introduce the background to fruit sunburn as well as fruit and orchard management practices and factors that can influence sunburn sensitivity

b. The susceptibility of apple fruit photosystems to UV-B radiation stress at different maturity stages was studied in paper 1.
c. The change in Fv/Fm due to heat and PAR stress during fruit development was analysed and correlated to fruit peel biochemical and anatomical features in paper 2.
d. In paper 3, the difference in heat stress sensitivity at moderate PAR levels between ‘Granny Smith’ and ‘Golden Delicious’ fruits was analysed, to establish if purportedly sunburn-sensitive fruits are also more heat sensitive.
e. Paper 4 focused on determining the dependency of apple cultivars on the xanthophyll cycle for photoprotection after exposure to heat and PAR stress.
f. In paper 5, the combined effect of heat, UV-B and PAR stress in different combinations was assessed to determine their photodamaging effects.
g. The findings of the different papers are summarised in the General discussion and conclusion chapter and a general conclusion is drawn from the study about fruit sunburn development.

1.4. Reference

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2. LITERATURE REVIEW

2.1. Properties of solar radiation

"Without the interaction of light with matter the world would not exist. There would be no chemistry and no biology" (Pike and Sarkar, 1995). The earth receives about 5.2 x 10^{21} kJ $year^{-1}$ of energy from the sun (Lawlor, 1993; Ksenzhek and Volkov, 1998). Global organic matter (total 5 x 10^{12} tons) is produced from only 0.05% of 50% of this total energy, which falls within the wavelength used for photosynthesis (Lawlor, 1993). Total global photosynthesis is divided equally between marine organisms and terrestrial plants. Sir Isaac Newton in 1666 discovered that sunlight consists of different colours mixed in certain quantities to produce white light (Porter, 1928). These colours/radiation, commonly referred to as electromagnetic radiation/waves, have different properties. The wavelength, frequency and energy levels of electromagnetic radiation are given in Table 1. The main photosynthetic solar radiation absorbing plant pigments, chlorophyll (a+b), absorb best between wavelengths 400 to 500 nm (blue light) and 600 to 700 nm (red light) (Figure 1; Mader, 1996). The rest of the energy is either reflected, reradiated or emitted as heat.

Philosophers and scientists have answered the question of what light is with various theories and models over time. The models describing light are the ray model, corpuscle model, wave model, and the photon model (Mauldin,

1988). These models are used to describe the various characteristics of light. Light is generally described as an electromagnetic wave with photons that carry energy (quanta), having electrical and magnetic vectors (fields) perpendicular to each other and both being perpendicular to the direction in which the wave travels (Lawlor, 1993). This light wave travels at a speed of 3 x 10^8 m s^{-1} *in vacuo*, taking 8 minutes for it to travel from the sun to earth. Light/energy from the sun is radiated into space by hot gasses in its atmosphere. This energy is produced by the continuous collision of free hydrogen nuclei, released due to the destruction of the electron shells of the atoms under extreme heating (15 million ^oC) at the core of the sun. This transforms hydrogen into helium while releasing excess energy as radiation over millions of years (Ksenzhek and Volkov, 1998).

Table 1. Properties of electromagnetic radiation (Lawlor, 1993).

Type of radiation	**Wave length**	**Frequency (s^{-1})**	**Energy per photon (J)**
Radio wave	10^3 – 10^{-3} m	3 x 10	19.86 x 10^{-26}
Infra-red	800 nm	3.8 x 10^{14}	25.16 x 10^{-20}
Visible red light	680 nm	4.4 x 10^{14}	29.13 x 10^{-20}
Visible green light	500 nm	6.0 x 10^{14}	39.72 x 10^{-20}
Visible violet-blue light	400 nm	7.5 x 10^{14}	49.65 x 10^{-20}
Near ultraviolet	200 nm	1.5 x 10^{15}	9.93 x 10^{-19}
Ultraviolet	10 nm	3.0 x 10^{16}	19.86 x 10^{-18}
X-rays	0.01 nm	3.0 x 10^{19}	19.86 x 10^{-15}

Light energy absorption by plant molecules happens when the electrons in the atoms of the absorbing molecules have a lower vibration frequency than that

of the incoming photon. The electrons of the molecule are then caused to vibrate faster than their natural vibration and energy from the sun is "captured", the molecules are then said to have excitation energy (Mauldin, 1988). In photosynthetic organisms, this excitation energy is transferred via other molecules to the reaction centres where it is converted into chemical energy (Lawlor, 1993).

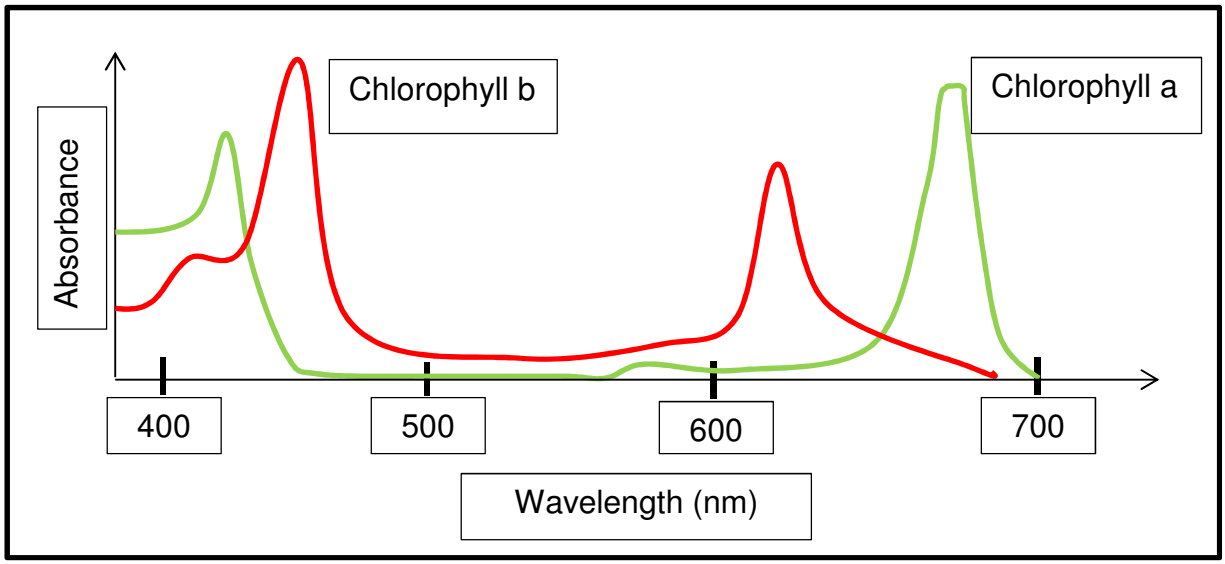

Figure 1. The absorption spectrum of chlorophyll a and b. Redrawn and modified from Mader (1996).

2.2. Photoinhibition

Plants require the sun's radiation for photosynthesis to occur. The quantity of radiation received by plant leaves should be within the ecological limits of the specific plant species. Excess light reaching the chloroplasts can result in damage to the photosynthetic system (Barber and Anderson, 1992). The level

of excess sun radiation that causes photoinhibition (temporal down regulation of the photosystem) or photodamage (irreversible damage to the photosystem) in plants differs between plant types, with shade plants being more susceptible than sun plants (Powles, 1984; Aro *et al.*, 1993). Shade plants have higher photosynthetic rates at low light levels than sun plants (Lambers *et al.*, 1998). Radiation can damage the photosystem by two possible mechanisms (Aro *et al.*, 1993): 1. absorbed radiation energy is transferred to oxygen, generating highly reactive oxygen species (ROS) that can damage the photosystem; 2. the highly activated, radiation absorbing molecules from the photosystem can react with and damage other photosystem molecules. Photoinhibition is a result of the disruption of the balance between the rate of damage to the photosystem and its repair (Takahashi and Murata, 2008, Murata *et al.*, 2012).

Photoinhibition can be caused by various environmental factors, including radiation (PAR + UV), temperature, osmotic, and drought stress (Wong *et al.*, 1985; Sonoike, 1999; Chartzoulakis, 2005; Takahashi and Murata, 2008). Photosynthesis generally increases with increasing PAR levels (Lambers *et al.*, 1998). However, a continuous supply of radiation beyond the utilisation capacity of the affected photosystem can lead to photoinhibition. The response of plant leaves to PAR is influenced by the presence of other environmental stresses that limit photosynthesis (Chen and Cheng, 2009). Environmental stresses contribute to photoinhibition by inhibiting the repair mechanisms of subunits of the photosystem (Takahashi and Murata, 2008). The photosystem of plants contain the following units: light harvesting

complex; photosystem II reaction centre (P680); oxygen evolving complex; electron transport system; and photosystem I reaction centre (P700) (Figure 2). The different photosystems in the chloroplast membrane have been found to respond differently to environmental stresses that cause photoinhibition. Photosystem II (PS II) is reported to be the main target of photoinhibition, with photosystem I (PS I) having its activity reduced to a lesser extent (Critchley, 1981).

Radiation (PAR + UV) and heat-induced photoinhibition of the photosystem occurs via the following activities (Smillie, 1992; Aro *et al.*, 1993; Mishra *et al.*, 1994; Takahashi *et al.*, 2010; Murata *et al.*, 2012; Marthur *et al.*, 2011):

1. Disruption of electron transport
2. Damage to the oxygen-evolving complex
3. Damage to the D1 + D2 proteins
4. Chlorophyll bleaching

The damages to different components of plant photosystems will be discussed in detail in relation to the effect of radiation (PAR + UV) and heat stress. The effects of UV and PAR radiation and heat stress on apple fruit photosystems will also be discussed in the subsequent chapters.

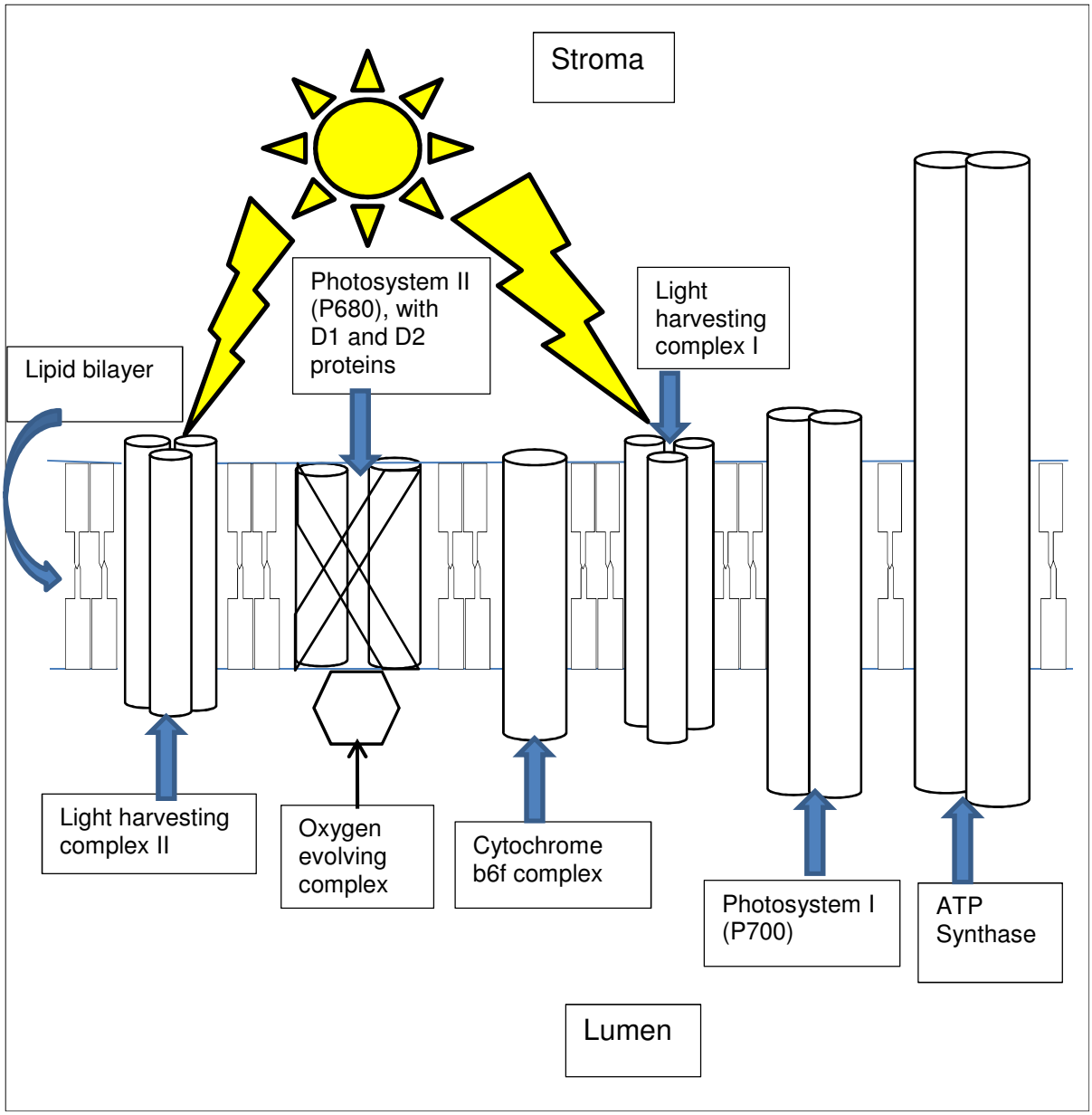

Figure 2. Plant photosynthetic system in the chloroplast thylakoid membrane. Redrawn and modified from Rochaix (2011) and Wollman *et al.* (1999).

2.2.1. Disruption of electron transport

Electron transport in the photosystems is activated by the absorption of radiation energy by PS II and PS I (Hill and Bendall, 1960). The absorption of radiation energy activates the transfer of electrons between the two systems.

Radiation energy is absorbed by the antennae complex of PS II and the energy is transferred to the primary electron donor chlorophyll (P680) of PS II reaction centre (Wollman *et al.*, 1999). The activated $P680^+$ transfers an electron to the primary electron acceptor of PS II, pheophytin, which results in electron transfer through plastoquinones (Q_A and Q_B), the cytochrome complex, plastocyanin, on to PS I and ferrodoxin, up to the final electron acceptor $NADP^+$ to produce NADPH (Wollman *et al.*, 1999; Rochaix, 2011).

The oxygen evolving complex (OEC) of PS II produces molecular oxygen (O_2) and protons (H^+) by splitting water on the lumen side of the thylakoid membrane (Goussias *et al.*, 2002). Proton production by the OEC enables ATP synthesis via a proton pump that pumps protons from the lumen to the stromal side of the thylakoid membrane (Rochaix, 2011). The splitting of water by the OEC also generates electrons that reduce the oxidised $P680^+$ molecules to P680 via a tyrosine radical (Barry and Babcock, 1987; Barber, 2002). The synthesised ATP and NADPH are utilised in CO_2 capture by the Calvin cycle and in other metabolic processes (Bassham and Calvin, 1962; Fridlyand and Scheibe, 1999).

Electron transport in the photosystem can generally be interrupted by either damage to the OEC, resulting in reduced electrons available to reduce the activated P680* of PS II, or by damage to the up-stream events beyond the electron acceptor pheophytin (Ramalho *et al.*, 1999). Heat stress above 45 ℃ disrupts electron transport in PS II by inhibiting the transfer of electrons within the plastoquinone pool and causing back flow of electrons to the OEC (Wen

et al., 2005; Zhao *et al.*, 2008). UV-B causes a reduction in electron transport of the photosystems by decreasing the content of PS II complexes and of ATP hydrolase (Strid *et al.*, 1990). UV radiation also damages cell DNA (Sinha and Häder, 2002), and this can result in reduced replacement of damaged PS II units. Furthermore, absorption of PAR and UV by Manganese (Mn) can cause its release from the OEC, disrupting electron transport directly by reducing electron transfer from the OEC, and indirectly inducing the production of reactive oxygen species (ROS) that damage PS II complexes (Hakala *et al.*, 2005).

2.2.2. Damage to the oxygen evolving complex

The oxygen evolving complex (OEC) of PS II is composed of three major proteins, PsbO, PsbP and PsbQ (Spector and Winget, 1980; Åkerlund and Jansson, 1981; Yamamoto *et al.*, 1981; Kuwabara and Murata, 1982). The OEC is located on the lumen side of the chloroplast membrane and it also has 4 Mn, 2-3 calcium (Ca) and chlorine (Cl) ions (Debus, 1992). The PsbO protein is of critical importance to the stability of PS II and for preserving Mn (Miyao and Murata, 1984; Bricker and Frankel, 2011). The loss or damage to the PsbO protein detrimentally affects the functioning of the OEC. During PAR induced photosynthesis, the Mn atoms are oxidised by the energised $P680^{+}$ chlorophyll of PS II via a tyrosine radical, and Mn in turn oxidises water, splitting it and releasing a proton and O_2 (Barber and Archer, 2001).

PAR stress causes a detachment of OEC proteins (Bertamini and Nedunchezhian, 2003; Chen *et al.*, 2011). Isolated chloroplasts of spinach plants treated with 4000 µmol m^{-2} s^{-1} PAR at 25 ℃ for 3 hours released OEC proteins from their thylakoid membranes (Chen *et al.*, 2011). Additionally, Bertamini and Nedunchezhia (2003) found that the loss of the OEC protein PsbO after PAR stress was greater in younger than in old grape leaves. Bertamini and Nedunchezhian (2004) further reported that the loss of OEC proteins differs between different grape cultivars of similar maturity. This indicates that the sensitivity of the OEC to PAR stress differ with maturity between cultivars and even plant types. However, this can certainly be inferred about all the other photoinhibitory changes caused by different environmental stresses.

Heat stress also damages the OEC by causing a release of its proteins (Enami *et al.*, 1994). Heat stress of 45 ℃ for five minutes induced cyanobacterium (*Spirulina plantensis*) cells to release the PsbO protein of the OEC, resulting in the release of Mn atoms into the lumen (Zhao *et al.*, 2008). Therefore the heat stress induced release of the PsbO protein inhibits the functioning of the OEC. Yamane *et al.* (1998) found that the sensitivity of OEC and other PS II sections to light stress is enhanced when light stress is combined with high temperatures. This could explain the need for high temperature stress for the induction of sunburn browning in fruits in the presence of high sun radiation.

UV damages the OEC complex when UV radiation is absorbed by the Mn ions (Barbato *et al.*, 1995). The absorption of UV radiation by Mn ions decreases the ability of these ions to transfer electrons to the P680 chlorophyll molecules of PS II, resulting in photoinhibition (Vass *et al.*, 1996). Hakala *et al.* (2005) also reported that UV stress results in the loss of Mn ions from the OEC into the chloroplast lumen. They assumed that this loss of Mn from the OEC results in oxidative stress which further damages PS II.

2.2.3. Damage to the D1 + D2 proteins

The D1 and D2 proteins are the major proteins of PS II on which the major components (i.e. P680, pheophytin, quinones) of the system are attached (Wollman *et al.*, 1999). Damage to these two proteins can therefore disrupt photosynthesis. However, other proteins of PS II are also damaged during photoinhibition and contribute towards the disruption of the function of PS II (Wang *et al.*, 1999).The D1 protein has a very high turnover rate, while D2 is comparatively more stable (Barber and Andersson, 1992). This makes the D1 protein susceptible to factors that can disturb its homeostasis. The D1 and D2 proteins can be degraded by ROS produced under a single stress or combinations of PAR, UV and heat stress (Bradley *et al.*, 1991; Anderson and Chow, 2002; Zhao *et al.*, 2008).

Jansen *et al.* (1999) found that PAR levels of 5 µmol m^{-2} s^{-1} resulted in more than 25% degradation of the D1 protein in a duckweed (*Spirodela oligorrhiza*), while 90% degradation was reached at PAR levels of 1600 µmol m^{-2} s^{-1}. This

showed that the degradation of D1 protein is related to the level of PAR irradiation. The D2 protein was also degraded accordingly in the same experiment, but at lower levels than the D1 protein. PAR-induced D1 protein damage occurs via the generation of ROS that cleave the D1 protein into its subunits (Mishra and Ghanotakis, 1994).

Heat and UV-B stress cleaves the D1 protein from PS II, leading to its degradation (Melis *et al.*, 1992; Komayama *et al.*, 2007). Heat stress-induced damage to the D1 and D2 is preceded by damage to the OEC (Zhao *et al.*, 2008). This indicates that heat stress damage to the D1 and D2 proteins is a secondary event after electron transfer from the OEC has been disrupted. The exact mechanism of the UV effect on the D1 protein is not yet clear but it appears that quinones (or quinone radicals) and the Mn ions of the OEC are involved (Barbato *et al.*, 1995; Friso *et al.*, 1995). However, the increased turnover of the D1 protein during UV stress is considered to be part of the protection mechanism for PS II, with decreased turnover leading to increased photoinhibition (Wu *et al.*, 2011). The increased protein turnover can allow for faster removal of damaged proteins and their replacement with repaired ones into PS II.

2.2.4. Chlorophyll bleaching

Radiation (PAR + UV) can cause pigment bleaching from photosystems, resulting in photoinhibition (Jones and Kok, 1966; Mishra *et al.*, 1994). However, the reduction in the content of pigments of the photosystem can be

a photoprotective mechanism to prevent further damage. The breakdown of chlorophyll molecules can help reduce the possibility of energy transfer to molecular oxygen (Hörtensteiner and Kräutler, 2011). Chlorophyll breakdown, as induced by radiation or heat stress, can be initiated by ROS directly or via the ROS-induced activation of plant senescence enzymes (Triantaphylidès and Havaux, 2009). Pigment bleaching can also occur at high temperatures in the presence of high irradiation levels (Mishra *et al.*, 1994; Felicetti and Schrader, 2008a). UV-B stress reduced chlorophyll content in pea plants, while chlorophyll *a* decreased more than chlorophyll *b*, which was reduced at the same rate as carotenoids (Strid *et al.*, 1990). The UV-B stress also decreased the photochemical efficiency of PS II (Fv/Fm) in the pea plants. The effect of irradiation and temperature on pigment bleaching could be via the production of ROS or the cleavage of pigment hosting proteins and their subsequent degradation (Mishra *et al.*, 1994; Jackowski *et al.*, 2003; Lidon and Ramalho, 2011).

The proteins of the light harvesting complex II (LHCII) of PS II are the main pigment binding proteins of plant photosystems; they are larger and more numerous than those of LHCI (Wollman *et al.*, 1999). PAR absorbed by the LHCII is either used in photochemistry or released as heat (non-photochemical quenching – NPQ); and a small amount of absorbed light energy is released as fluorescence (Krause and Weis, 1991). Under stress conditions, NPQ and fluorescence increase while photochemical quenching decreases (Horton *et al.*, 1996). Heat stress can cause irreversible damage to the LHCII (Marthur *et al.*, 2011). PAR and UV stress reduces the amount of

LHCII in plant photosystem (Jackowski *et al.*, 2003; Lidon and Ramalho, 2011). UV radiation stress is also reported to decrease the phosphorylation of the LHCII (Yu and Björn, 1997). Loss/damage of the LHCII can result in significant plant pigment bleaching because of its high pigment content.

The function of the LHCII is to capture light for photosynthesis as well as to protect the photosystem against photodamage. PAR is absorbed by LHCII and LHCI and the energy transferred to the central chlorophyll molecules of PS II and I (Woolhouse, 1978). LHCII is made of three major proteins units Lhcb1, 2 and 3 (Wollman *et al.*, 1999). LHCII is associated with PS II when its proteins are non-phosphorylated, and transfers absorbed energy to PS II causing oxygen evolution from PS II and electron transport through the plant photosystem (Kyle *et al.*, 1984; Larsson *et al.*, 1987). However, phosphorylated proteins of LHC II move from grana to stroma lamellae and become associated with PS I, inducing cyclic electron transport (Kyle *et al.*, 1984). Heat stress induces phosphorylation of the LHCII proteins (Nellaepalli *et al.*, 2011). The phosphorylation of LHCII insure an energy supply balance between PS II and PS I and reduces photoinhibition (Kyle *et al.*, 1983).

2.3. Fruit sunburn

Fruit sunburn is caused by excessive heating of fruits exposed to direct solar radiation (Rabinowitch *et al.*, 1974; Schrader *et al.*, 2001; Wünsche *et al.*, 2004). There are three types of sunburn (Barber and Sharpe, 1971;

Rabinowitch *et al.*, 1974; Rabinowitch *et al.*, 1983; Woolf and Ferguson, 2000; Schrader *et al.*, 2001; Felicetti and Schrader, 2008a):

1. Sunburn necrosis - this sunburn type appears as a dark brown to black area on the fruit (Figure 4). It is caused by the death of cells in the fruit peel due to high fruit peel temperatures above 50 °C. This the most severe type of sunburn.
2. Sunburn browning - this sunburn type appears as a yellow/bronze or golden coloured area on the fruit (Figure 4). It occurs when fruit peel temperatures are between 45 °C to 49 °C while being exposed to high PAR and UV-B radiation levels.
3. Sunburn bleaching (photooxidative sunburn) - this sunburn type appears as a bleached white area on the fruit. It is caused by sudden exposure of fruit peel to high PAR levels at fruit peel temperature below 30 °C.

Schrader *et al.* (2003) found that protection of apple fruits from UV-B solar radiation reduced sunburn browning occurrence. This further confirmed an earlier report by Cline and Salisbury (1966) about the requirement of UV radiation for the development of sunburn browning. Felicetti and Schrader (2008a) reported that although PAR is required for sunburn bleaching at temperatures below 30 °C, UV-B is not required for this type of sunburn. Velitchkova and Picorel (2004) also found that isolated spinach thylakoid membranes exposed to high PAR (1800 µmol m^{-2} s^{-1}) at 22 °C were bleached. They concluded that the observed spinach pigment bleaching was

because of damage to the electron transport from PS II resulting in ROS formation which then caused the pigment bleaching.

Sunburn damage mainly occurs due to sudden exposure of fruits to high temperature and direct sunlight in the orchard (Wünsche *et al*., 2001). This happens when cool cloudy weather conditions change suddenly to warm sunny conditions, and after pruning, which all expose previously shaded plants to heat and light stress. The moving of branches also causes shaded fruits to be exposed to sudden high light levels. Rabinowitch *et al.* (1974) also found that exposure of fruits to a lower temperature of 40℃ for long a period (28 hours at 40 ℃ compared to 18 hours at 45 ℃) resulted in sunburn browning on tomato fruits. Long term exposure of fruit peels to sub-lethal temperatures could therefore lead to damage.

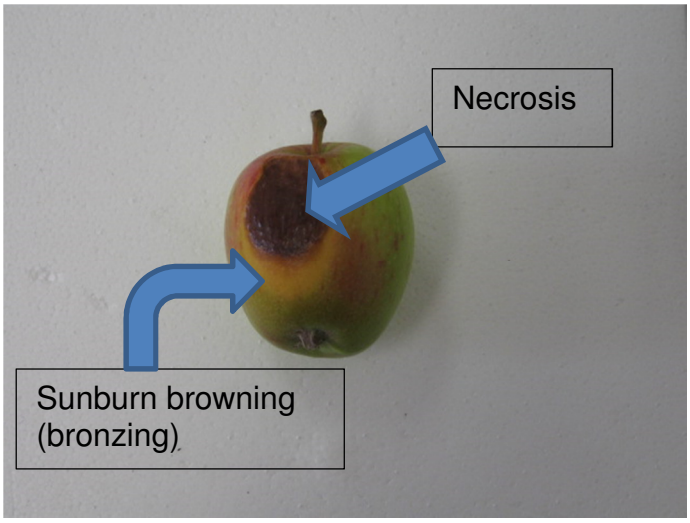

Figure 3. 'Fuji' fruit with sunburn necrotic spot and sunburn browning around the necrosis.

Since surface temperature of exposed fruit is often 10-15 ℃ higher than air temperature (Parchomchuk and Meheriuk, 1996; Ferguson *et al.*, 1998), the risk for sunburn occurring on fruits increases at air temperatures from 30 to 36 ℃. The threshold temperature can be in the lower part of the range when other heat stress inducing climatic factors are present, such as high relative humidity and poor air movement. High relative humidity reduces water loss from fruits (Tu *et al.*, 2000), which can inhibit the ability of fruits to reduce internal temperature through evapotranspiration.

Fruits that are developing sunburn have the following symptoms (Woolf and Ferguson, 2000):

- yellowing or bleaching of fruit peel
- corky or roughened fruit surface
- reduced photosynthesis
- high soluble solids concentration
- advanced starch degradation
- high internal ethylene concentration

In addition, Racskó *et al.* (2005) reported that sunburned fruit have higher fruit firmness than non-sunburned fruits.

2.3.1. Sunburn browning biochemistry

Fruit sunburn is perceived to be a photodamage response, caused by heat and light stress (PAR and UV) and resulting in a reduction of the ability of the photosystems to utilise incoming PAR (Rabinowitch *et al.*, 1974). The

maximum light use efficiency (F_v/F_m) of sunburned fruit photosystems is lower than in non-sunburned fruits (Wünsche *et al.*, 2001; Seo *et al.*, 2008). The OEC of sunburned apple fruits suffer more damage than the Calvin cycle, and increased xanthophyll cycle activity and other antioxidant systems in sunburned fruits do not prevent damage (Chen *et al.*, 2008). The OEC is reported to be the most sensitive component of plant photosystems to heat stress (Allakhverdiev *et al.*, 2008). Damage to the photosystems of sunburned fruit peels is therefore perhaps initiated by high temperature damage to the OEC of the photosystems. The presence of PAR and UV radiation, in addition to heat stress, further increasing the synthesis of ROS and enhances pigment bleaching.

Sunburned fruits have lower chlorophyll content than non-sunburned fruits, while changes in carotenoid content are cultivar specific (Chen *et al.*, 2008; Felicetti and Schrader, 2009a). Felicetti and Schrader (2009b) found that phenolics, specifically quercetin glycosides, increase in sunburned fruit peel compared to non-sunburned fruits. Anthocyanin content was also found to be low in sunburned apple fruit peel (Felicetti and Schrader, 2008b). The loss of chlorophyll and anthocyanin, increase in phenolic content and the relative stability or increase of carotenoid content contribute to the ‘yellow/bronze’ colour of sunburned fruits. Cline and Salisbury (1966) further suggested that the yellow/bronze colour of sunburned plants could be due to polymerised oxidised phenolic compounds.

Sunburned fruits have a higher chlorophyll *a/b* ratio and more xanthophyll cycle carotenoids compared to non-sunburned fruits (Chen *et al.*, 2008). Kleima *et al.* (1999) reported that chlorophyll *a* is more efficient at transferring excitation energy to the xanthophyll cycle than chlorophyll *b*. The reduced loss of chlorophyll a in sunburned fruits can therefore increase the transfer of excitation energy to the xanthophyll cycle. During plant senescence, chlorophyll *b* is converted to chlorophyll a, as chlorophyll is broken down during the plant maturation process (Hörtensteiner and Kräutler, 2011). The higher loss of chlorophyll *b* relative to chlorophyll *a* also leads to a higher chlorophyll a/b ratio. Apple fruits increase ethylene production during their maturation process (Bufler, 1986). Fruits with sunburn symptoms are also observed to have higher ethylene content compared to un-stressed fruits (Woolf and Ferguson, 2000). Ethylene induced chlorophyll breakdown results in an increase in the chlorophyll a/b ratio (Shimokawa, 1990). The generally observed greater loss of chlorophyll *b* in sunburned fruits could therefore possibly be due to ethylene induced chlorophyll breakdown. The higher chlorophyll a/b ratio in sunburned fruits could be a photoprotective action that enhances the transfer of absorbed solar radiation energy to the upregulated xanthophyll cycle, to be further released as heat.

There is wide variability in the apparent susceptibility of apple cultivars to sunburn. ‘Fuji’ and ‘Granny Smith’ appear to be most susceptible to sunburn, with the fully red apples being least susceptible (Personal communication with farmers in the Western Cape region). The loss of chlorophyll is a universal response in sunburned fruit and vegetable peel (Rabinowitch *et al.*, 1983;

Chen *et al.*, 2008; Felicetti and Schrader, 2009a). Rabinowitch *et al.* (1983) even postulated that the presence of chlorophyll is essential for sunburn development on fruit and vegetable peels. Tartachnyk *et al.* (2012) showed that sunburned 'Granny Smith' fruits loose more chlorophyll than 'Fuji' fruits, with 'Braeburn' losing the least amount of chlorophyll. Therefore their experiment showed 'Granny Smith' to be the most sunburn sensitive cultivar of the three cultivars tested.

2.3.2. Orchard management practices contributing to fruit sunburn

The relative degree of exposure to direct sunlight during fruit development is an important determinant of sunburn, which is induced by heat and sunlight. The following orchard management factors play a role in fruit sunburn development: Aspect and row orientation, tree canopy training method, pruning strategy, vegetative growth control mechanisms and cultivar (genetic) factors such as bearing habit (Barber and Sharpe, 1971). Modern orchard practices that maximise tree canopy light penetration to improve fruit red colour development and yield (Saure, 1987), also increase the risk for sunburn.

The peel of fruit that have developed in sunlit positions over the course of the season appear to have higher levels of photoprotection against solar and thermal stress than peel that have developed in the shade (Ma and Chen, 2003). This acclimation process is an important determinant of sensitivity to sunburn. Non-acclimated fruit that are suddenly exposed to solar radiation are

therefore highly vulnerable to photodamage and sunburn development (Wünsche *et al.*, 2004).

2.3.3. Orchard management sunburn control mechanisms

The best way to protect fruits against sunburn is to avoid sudden exposure to high temperatures and direct sunlight (Wünsche *et al.*, 2001). This can be achieved by application of reflective films (i.e. kaolin), evaporative cooling and tree shading (Wünsche *et al.*, 2004; Wand *et al.*, 2006; Gindaba and Wand, 2007). Fruit sunburn preventative actions are important since sunburn damage is irreversible (Wünsche *et al.*, 2001). A number of fruit sunburn prevention techniques have been utilised in South Africa, with various degrees of success and side effects on fruit tree physiology. These techniques include foliar application of sunburn preventing substances, over-tree evaporative cooling, shade netting (Gindaba and Wand, 2007), irrigation control (Hartz, 1997), and fertilizer application (Irget *et al.*, 2008).

Processed kaolin based particle film (Surround®WP) and a carnauba wax based (containing kaolin) spray RAYNOX® are used to reduce sunburn development on fruit peels (Glenn *et al.*, 2002; Melgarejo *et al.*, 2004; Wand *et al.*, 2006; Schrader *et al.*, 2008). In a study on the effect of Surround ®WP on sunburn development on pomegranate fruits, sunburn on treated fruits was reduced by 10% compared to the control, while fruit temperature was reduced by 5 ℃ (Melgarejo *et al.*, 2004). On tomatoes, Surround®WP reduced sunburn by 96% and fruit temperature by 4 ℃ (Cantore *et al.*, 2009). The

removal of the kaolin from fruits treated with Surround ®WP can however increase fruit processing cost, requiring additional fruit handling in packhouses. The use of RAYNOX® can bypass this problem, as this product contains much less kaolin than Surround ®WP and therefore require no extra handling at packhouses. Sunburn occurrence on apple fruits treated with RAYNOX® was reduced by up 50% (Schrader *et al.*, 2008). The use of Surround ®WP also causes a reduction in leaf photosynthesis, evapotranspiration and total plant dry biomass (Cantore *et al.*, 2009). The use of the above mentioned sunburn protective sprays or any others must still meet the consumer health concerns in addition to being effective in reducing fruit sunburn. Sunburn protective sprays can be effective at reducing sunburn, thereby reducing production losses.

Evaporative cooling (EC) of fruits to reduce fruit temperature and minimise sunburn damage is achieved by using overhead sprinklers (Parchomchuk and Meheriuk, 1996; Evans, 2004). EC can reduce fruit surface temperature by 3 to 8°C, while reducing sunburn occurrence by up to 15% (Parchomchuk and Meheriuk, 1996; Gindaba and Wand, 2005). EC can also increase fruit anthocyanin synthesis, especially when applied at sunset on warm days (Iglesias *et al.*, 2000, 2005). In an experiment done in Canada, EC reduced fruit soluble solids and increased acidity of 'Jonagold' fruits (Parchomchuk and Meheriuk, 1996). In South Africa, EC has been shown to increase fruit mass in 'Royal Gala' and fruit diameter in 'Cripps Pink' fruits (Gindaba and Wand, 2005), although it had no such effects on 'Jonagold' fruits in Canada

(Parchomchuk and Meheriuk, 1996). The effect of EC on other fruit quality parameters, other than sunburn incidence, is therefore cultivar specific and also depends on the climate at the time of application. Fruits kept under EC become more heat sensitive and therefore the system needs to be kept active continuously and this is especially important on warm days (Gindaba and Wand, 2005).

A good irrigation schedule to prevent water stress can induce vegetative growth which could reduce fruit sunburn through shading (Hartz, 1997), but can also negatively affect total yield. Fruit stomatal density decreases as the fruit matures (Roth, 1977), therefore reducing the possibility for transpiration heat loss from fruits. However, well irrigated trees can increase the relative humidity of the tree canopy which can provide a possibility for evaporative cooling of the fruits.

Shade/hail nets are used to protect plants against sunburn and their main effect is the reduction in solar radiation and heat load (Solomakhin and Blanke, 2008; Solomakhin and Blanke, 2010a). Different coloured shade nets on average reduce fruit temperature by 6°C, incident UV-B (100% = 1.16 Wm^{-2}) by 25%, and PAR by 10% (white and grey nets) to 23% (green/black, red/black, black nets), while increasing relative humidity by 2 to 5% (Solomakhin and Blanke 2010b). However, shade nets can increase fruit tree vegetative growth, reduce yield and inhibit fruit red colour development (Hunsche and Blanke, 2010; Solomakhin and Blanke, 2010a). Shade nets also down-regulate whole tree photosynthetic capacity, stomatal conductance

and day time respiration (Gindaba and Wand, 2007). The positive or negative effects of shade nets on fruit firmness, total soluble solids, starch breakdown, and acidity are cultivar specific (Solomakhin and Blanke, 2010a). Shade nets are significantly more effective at reducing sunburn, when compared to kaolin based particle film sprays and evaporative cooling (Gingaba and Wand, 2005). However, shade nets are expensive and can be more economically viable when used for sunburn protection combined with hail damage prevention (Glenn *et al.*, 2002).

Nutrient deficiency can inhibit cell metabolism and contribute to sunburn development. A standard NPK fertilizer application with additional 280g Ca in a fig orchard resulted in reduced fruit peel cracking and reduced sunburn development (Irget *et al.*, 2008). Iamsub *et al.* (2009) also found that supplying apple trees in the orchard with an abscisic acid (ABA) fertilizer ('MIYOBI'- containing K, P, Mg, Mn and S-ABA) increased fruit antioxidant capacity and led to a reduction in the occurrence of sunburn browning in one cultivar and sunburn necrosis in another cultivar.

2.4. Fruit physiological characteristics influencing sunburn development

Fruit peels have photoprotective mechanisms against radiation and heat stress that can prevent/reduce sunburn development. The sun exposed peels of fruits have a higher photoprotective capacity than the shaded peels (Ma and Cheng, 2003; Chen *et al.*, 2008). Ma and Cheng (2003) reported that the sun exposed peel had more xanthophyll carotenoids and antioxidants of the

ascorbate-glutathione cycle than the shaded peel. Other peel based photoprotective mechanisms that play a role in solar and thermal stress inhibition include:

- Cuticle, peel pigments, epicuticular wax and trichome characteristics that determine reflectance/absorbance ratios and thus energy balance (Lambers *et al.*, 1998; Kakani *et al.*, 2003);
- Stomata and lenticels that reduce fruit heat load via transpiration (Roth, 1977; Ma and Cheng, 2003);
- Fruit water content, fruit size and density, which also influence fruit heat load (Barber and Sharp, 1971; Saudreau *et al.*, 2007).

2.4.1. Fruit peel pigments, trichomes and cuticular waxes

Anthocyanin pigments in plants are responsible for the red, purple to blue colours in many fruits (Lancaster, 1992). Their synthesis is dependent on the level of incident radiation and low fruit temperature (Saure, 1990; Leng *et al.*, 2000). Their functions include the following: Attracting pollinators and seed dispersers (Harborne, 1965), protecting fruits from excess light (Smillie and Hetherington, 1999), and protecting plants against fungal infections (Hipskind *et al.*, 1996). Li and Cheng (2009) also reported that anthocyanins could protect plant photosystems against heat stress in the presence of high radiation levels. Anthocyanins accumulate in epidermal plant tissue and form a protective layer protecting the underlying photosynthetic systems against PAR stress (McClure, 1975; Smillie and Hetherington, 1999). They absorb strongly in blue-green PAR region and reflect in the red region, therefore

reducing the amount of energy reaching the photosystem (McClure, 1975). Feild *et al.* (2001) found that anthocyanin prevented photoinhibition in leaves of red-osier dogwood *(Cornus stolonifera)* when exposed to blue light. The absorption of PAR by anthocyanin can also reduce photosynthesis (Burger and Edwards, 1996), which reduces the formation ROS. Anthocyanin also protects the photosystem against radiation stress by acting as antioxidants (Neill and Gould, 2003). The light absorption and antioxidant capacity of anthocyanin therefore reduce photoinhibition in red fruits under heat and light stress.

Carotenoids are located within plant photosystems and can provide photoprotection to the photosystem and help with light absorption to drive photosynthesis (Cogdell and Gardiner, 1993). Carotenoids protect biological systems against triplet molecular oxygen ($^1O_2$) and act as antioxidants, removing ROS (Krinsky, 1989; Telfer, 2002). The xanthophyll cycle pigments are carotenoids that act to remove excess excitation energy from the photosystem and release it as heat (Lambers *et al.*, 1998). Carotenoids are more stable than chlorophyll during heat and light stress, and they are broken down at a slower rate than chlorophyll during fruit senescence (Merzlyak and Solovchenko, 2002; Camejo *et al.*, 2005). Carotenoids are therefore important pigments that offer photoprotection to plant photosystems during stress conditions.

Trichomes are an extension of the epidermal cell layer on leafs and fruits. They form elongated uni/pluricelluar or glandular structures protruding from

the surface of the tissue (Roth, 1977). Their functions on plant tissue include water balance maintenance, protection against herbivores, gas and water absorption, PAR and UV radiation reflection and absorption, and solute secretion (Uphof and Hummel, 1962; Roth, 1977; Liakoura *et al*., 1997; Lambers *et al*., 1998). Water balance maintenance is achieved by the increased boundary layer (of fruits or leaves) and by reflection of high energy radiation. This reduces the plant organ temperature and in-turn reduces transpiration (Uphof and Hummel, 1962; Roth, 1977; Liakoura *et al*., 1997).

Plants are protected from UV by flavonoids and other UV absorbing phenolics (Middleton and Teramura, 1993). These phenolics are located at the surface of plant tissue, in the epidermis and their cuticular waxes (Skaltsa *et al*, 1994). Light levels affect plant tissue trichome density, with light exposed tissue having higher trichome density than shaded tissue (Liakoura *et al*., 1997). Trichomes are covered with a cuticular wax layer that contains UV-absorbing substances (Uphof and Hummel, 1962; Liakoura *et al*., 1997). They also contain UV-absorbing substances in their cell walls (Liakoura *et al*., 1997). Trichome density varies between different plant tissues, the development period and the season (Uphof and Hummel, 1962). Young plant tissues have a higher trichome density than mature tissue, and their trichomes also have higher flavonoid content than the mature tissue (Liakopoulos *et al*. 2006). Young apple and pear fruits are covered with a dense trichome layer. This breaks off on the surface of mature fruits, while being retained in the calyx cup of these fruits (Roth, 1977).

2.4.2. Stomata and lenticels

Plants take up CO_2 and release water and O_2 through stomatal pores in leaves (Bidwell, 1979). There is a steep gradient of water content from leaves to the surrounding air, while the gradient of CO_2 from the air to the leaves internal space is very low (Bidwell, 1979). Plants, however, still do take up CO_2 and manage to minimize water loss. The loss of water through the stomata is influenced by the availability of water in the soil and the vapour pressure in the air (Lambers *et al*., 1998). Stomata open as the leaves/fruits transpiration increase with the increasing vapour pressure difference between the leaves/fruits and the surrounding air.

Fruit peels have inefficient abilities to utilise and remove excess light energy (Jones, 1981). Fruit peels have low stomatal densities, and these are later mostly replaced by lenticels as the fruit matures (Roth, 1977; Ma and Cheng, 2003). Juvenile pome fruits have a stomatal density of 2 to 10 per mm^2 (Roth, 1977). Stomata and lenticels are blocked by the formation of cuticle over the openings and by suberisation of subepidermal cells as the fruit matures (Roth, 1977). Lenticels are formed from epidermal cracks, old stomata or at the base of trichomes (Roth, 1977). Cracks develop in the epidermal cell layers due to expanding inner tissue. Stomata that cease to function develop into lenticels through cork formation from substomatal cells. In this case stomata guard cells are forced to separate by filling tissue that develops below them during lenticel development. Trichome base originating lenticels are formed by phellogen development at the hair base as the base enlarge and thickens.

2.4.3. Fruit water content, fruit size and density

Most long-wave radiation is absorbed by water in plant parts (Lambers, 1998). Water makes up 80 to 88% of the total weight of apple fruits (Mills *et al.*, 1997; Stevenson *et al.*, 2006). Large fruits have higher water contents than small fruits, giving large fruits a higher heat capacity. Small fruits however have a higher convective heat loss capacity and lower internal temperature than big fruits (Barber and Sharp, 1971). Small, young apple fruits also have a higher stomatal density that is used for conducting heat loss than bigger, older fruits (Roth, 1977). Smart and Sinclair (1976), however, reported that fruit temperature is mainly influenced by solar radiation and wind speed. They found fruit size, albedo, wind direction, fruit transpiration and thermal exchange of long-wave radiation to be less important determinants of fruit temperature. Nevertheless, fruit water content can have a direct effect on heat load capacity and as such fruit temperature, in addition to solar radiation and wind speed.

High density fruits have lower water content (Sessiz *et al.*, 2007), and as such a lower heat capacity than low density fruits. It is also known that during sunburn development fruit firmness increases (Racskó *et al*. 2005). Fruit density at maturity could therefore be used as a fruit sunburn sensitivity criteria.

2.5. Fruit photoprotection against solar radiation and heat

Plant photosystems have different mechanisms to cope with photodamage induced by excess solar radiation and heat stress. These mechanisms include (Aro *et al.*, 1993; Allakhverdiev *et al.*, 1996; Downs *et al.*, 1999a+b Niyogi, 1999):

- repair of damaged reaction centres;
- release of excess absorbed radiation as thermal energy;
- activation of photorespiration;
- cyclic electron transports;
- activation of mechanisms to remove reactive oxygen/radical species;
- accumulation of osmolytes in affected cells;
- synthesis of heat shock proteins.

Photodamage occurs when all the possible prevention mechanisms have been over stressed while excess radiation supply continues to be intercepted by the plant tissue (Powles, 1984). Plant response to heat stress includes the following mechanisms (Wahid *et al.*, 2007): Membrane stability control; removal of ROS, accumulation of osmolytes, synthesis of protein protective enzymes and synthesis of heat shock proteins.

2.5.1. Repair of damaged reaction centres

Photoinhibition or the damage to the photosystem occurs when the balance between photosystem damage and repair cycle shifts towards more damaging

than repairing activities (Takahashi and Murata, 2008, Murata *et al.*, 2012). The components of the photosystem are further degraded after photodamage, recycled into new components and re-fitted back into the photosystem (Aro *et al.*, 1993). PAR is required for complete repair of the photosystem, although it is also involved in its damage (Reisman *et al.*, 1986). However, while the rate of PAR induced photodamage increase with increasing PAR levels, the rate of photorepair increase with decreasing PAR level (Allakhverdiev and Murata, 2004). Post-stress environmental conditions can therefore allow or inhibit repair of the photosystem.

Repair of the damaged photosystem components require *de novo* protein synthesis (Aro *et al.*, 1993). Heat and light (PAR and UV) stress inhibits new protein synthesis and increase photosystem damage (Lurie and Klein, 1990; Murata *et al.*, 2007). Although PAR is needed for repair to the photosystem (Reisman *et al.*, 1986), PAR stress induced ROS production damage the photosystem by inhibiting *de novo* protein synthesis (Murata *et al.*, 2007). Environmental stress induced damage to the Calvin cycle also impair the synthesis of proteins of the photosystem (Takahashi and Murata, 2005), thus reducing photosystem repair mechanisms. Heat and light stress induced photoinhibition is therefore not limited to direct damage to photosystem components as discussed above, but is also extended to the repair mechanisms of the photosystem. Fruit sunburn sensitivity can therefore also be linked to the ability of the fruit's photosystem to continue with new protein synthesis during stress conditions.

The rate of reconstruction and replacement of the D1 protein of PS II is the rate limiting photosystem repair reaction (Melis, 1999). However, other photosystem components, such as the D2 and PSbO proteins are also damaged and replaced during the photodamage-repair cycle (Chi *et al.*, 2012). Damaged D1 proteins are moved from the appressed region of the thylakoid membranes to the non-appressed region for repair (Aro *et al.*, 1993). During the movement the D1 protein is further degraded. *De novo* protein synthesis occurs and co-factors/components are repaired and added at the non-appressed region and the repaired protein system is translocated back to the appressed region (Melis, 1999).

2.5.2. Release of excess absorbed radiation as thermal energy

Plants activate the xanthophyll cycle under conditions of excess light energy (Taiz and Zeiger, 1998). Sun exposed sides of apple fruits have a higher content of xanthophylls than shaded sides (Ma and Cheng, 2003). Chen *et al.* (2008) also found that xanthophyll content per chlorophyll bases was higher in sunburned fruits than in non-sunburned fruits. However, they found that the inverse was true when the content was expressed per peel area. Radiation absorbed by the photosystem results in linear and cyclic electron transport through the photosystem and induces a pH gradient across the thylakoid membrane (Rochaix, 2011). The increase in the pH gradient during exposure to excess radiation activates the enzyme violaxanthin de-epoxidase, which initiates the xanthophyll cycle (Niyogi, 1999). The carotenoid violaxanthin is de-epoxidised via antheraxanthin by violaxanthin de-epoxidase to form

zeaxanthin, while the reverse occurs when radiation levels are reduced (Demmig-Adams and Adams III, 1992). The conversion of violaxanthin to zeaxanthin requires a low pH in the lumen, while the re-conversion of zeaxanthin to violaxanthin requires a high pH (Taiz and Zeiger, 1998). The xanthophyll cycle depends on the water to water cycle and cyclic electron transport system of PS I that maintain the needed pH gradient across the thylakoid membrane under stress conditions (Niyogi, 2000; Johnson, 2011).

The xanthophyll cycle prevents or reduces photoinhibition and eventual photodamage through the release of absorbed radiation energy as heat (Taiz and Zeiger, 1998). About 50 to 70% of absorbed energy from incident PAR is released as heat via the xanthophyll cycle, and the rest is used for photochemical reactions (Osmond *et al.*, 1997). However, Ma and Cheng (2003) reported that the xanthophyll cycle of sun exposed apple peels already function at maximum capacity under non-stress conditions. The capacity of the cycle to reduce radiation stress in apple peels is therefore possibly limited. Xanthophyll carotenoids also act as antioxidants, protecting the photosystem against ROS (Miller *et al.*, 1996).

2.5.3. Activation of photorespiration

Oxygenation of Ribulose-1,5-bisphosphate (RuBP), instead of carboxylation, under condition of high leaf O_2 and low CO_2 partial pressure results in photorespiration (Osmond, 1981; Maurino and Peterhansel, 2010). Photorespiration involves the recycling of glycolate produced during the

RuBisCo oxygenation reaction and its reactions occur in the chloroplast, peroxisome and mitochondria (Maurino and Peterhansel, 2010). Photorespiration only occurs in the presence of PAR (Ludwig and Canvin, 1971). Plant photosynthetic efficiency is reduced during photorespiration (Osmond, 1981).

Photorespiration recycles the toxic carbon by-products from the RuBisCo oxygenation reaction and helps maintain the dark reaction by ensuring further rubisco resynthesis (Osmond, 1981; Bauwe *et al*., 2012). The maintenance of the dark reaction prevents photoinhibition by ensuring that the final electron acceptor of PS I remain available. This maintain the electron flow from PS II to PS I. The maintenance of the electron flow through the two photosystems prevents the formation of ROS that can lead to photodamage.

2.5.4. Cyclic electron transports

Cyclic electron transport involves the transport of electrons around PSI (Johnson, 2011). Heat and PAR stress limits or inhibit linear electron transport, from the OEC to ferredoxin (on PS I), and cause the activation of cycling electron transport (Havaux, 1993; Joliot *et al*., 2004). During cyclic electron transport, electrons are transferred from ferredoxin back to plastoquinones of PS II or directly to the cytochrome complex, and back to ferredoxin via the cytochrome complex and plastocyanin (see Figure 3; Malkin and Chain, 1980; Rochaix, 2011). Light and heat induced phosphorylation of LHC II protein system causes this system to move from grana to stroma

lamellae and become associated with PS I, resulting in cyclic electron transport (Kyle *et al.*, 1984; Nellaepalli *et al.*, 2011). Damage to the CO_2 uptake reactions causes feedback inhibition on the photosystem and can also induces cyclic electron transport (Hald *et al.*, 2008).

The functions of cyclic electron transport include generating ATP, maintaining the pH gradient across the thylakoid membrane, inhibiting over-reduction of electron carriers, reducing the photoactivation of the P680 molecule of the PS II reaction center and reducing the formation of singlet oxygen (Asada, 2006; Johnson, 2011). The generated ATPs are used in various metabolic processes while the thylakoid membrane pH gradient is required for the activation of non-photochemical quenching which prevents photoinhibition (Joliot *et al.*, 2004).

PS I is also involved in the water to water cycle. This cycle occurs when electrons from PS I, instead of being transferred to ferredoxin, are rather transferred to O_2 (Figure 3). The enzymes superoxide dismutase and ascorbate peroxidase are involved in the cycle acting as enzymatic antioxidants (Niyogi, 1999). The cycle ensures the continuation of linear electron transport, produces ATP and maintains the thylakoid membrane pH gradient needed for thermal release of absorbed radiation (Niyogi, 2000). The water-water cycle therefore prevents photoinhibition.

2.5.5. Activation of mechanisms to remove reactive oxygen/radical species

Excess light radiation not used by the photosystem result in the production of ROS (Figure 3). The ROS react with molecules of the photosystem and causes photoinhibition (Nishiyama *et al.* 2006). The scavenging and removal of the photoradicals is essential to minimise damage to the photosystem.

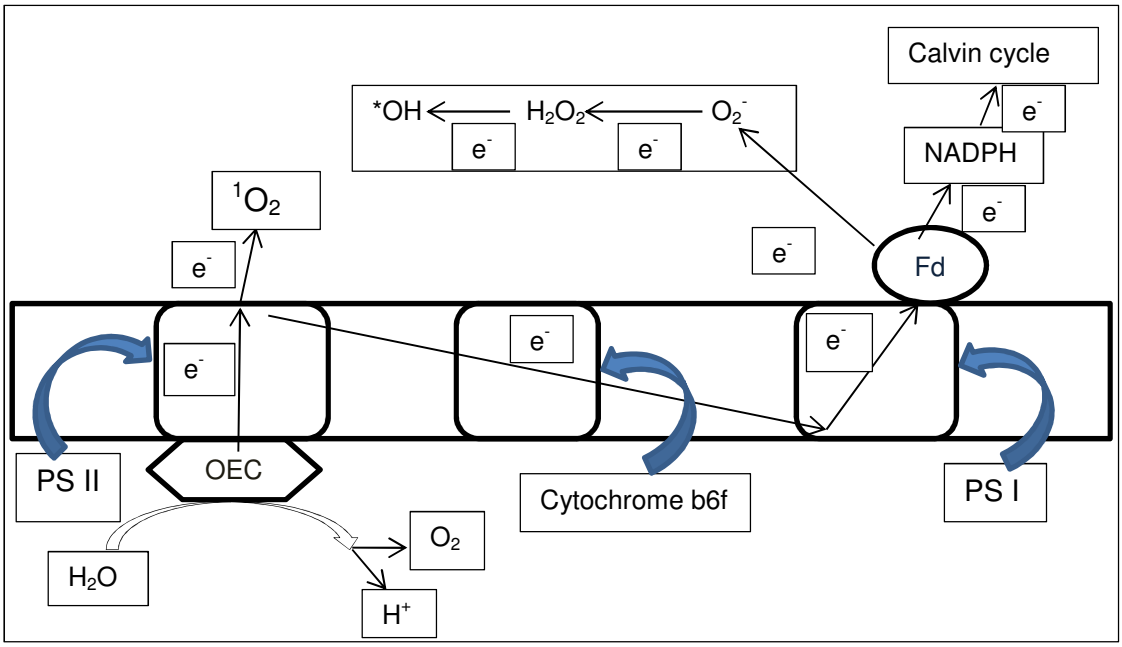

Figure 3. The production of ROS in the photosystems of the thylakoid membrane. Re-drawn and modified from Nishiyama *et al.* (2006). $^1O_2$ = singlet oxygen; $^{\cdot}OH$ hydroxyl radical; H_2O_2 = hydrogen peroxide; O_2^- = superoxide anion radical; Fd = Ferredoxin; OEC = Oxygen evolving complex.

Plants possess different antioxidative mechanisms to protect the photosystem against ROS and other photoradicals. Antioxidants can reduce photoinhibition by scavenging and removing ROS during the repair cycle of damaged photosystems (Inoue *et al.*, 2011). Plant antioxidants can be divided into preventative and chain breaking antioxidants (Ou *et al.*, 2002). Preventative

antioxidants act by preventing or blocking the formation of antioxidants, while chain breaking antioxidants interrupts the ROS synthesis reactions.

Plants produce enzymatic and non-enzymatic antioxidants when under stress to help remove ROS. Enzymatic antioxidants include: ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalases (Drotar *et al.*, 1985; Elstner *et al.*, 1982; Ma and Cheng, 2003). Exposure of tomato leaves to a 35 ℃ heat stress induced the production of H_2O_2 and membrane damage, and the leaves synthesised the enzymatic antioxidants SOD and APX (Ogweno *et al.*, 2009). APX and GPX, and related enzymes from the ascorbate-glutathione cycle, are also up-regulated in sunexposed and sunburned apple peel (Ma and Cheng, 2003; Chen *et al.*, 2008). Non-enzymatic antioxidants include phenolics, ascorbic acid and carotenoids (i.e. beta-carotene and xanthophylls) (Miller et al., 1996; Ju and Bramlage, 1999; Li *et al.*, 2008).

2.5.6. Accumulation of osmolytes in affected cells

Plants experiencing temperature stress can accumulate osmotic compounds to help preserve their growth. These compounds include sugars, proline, glycinebetaine (GB), γ-4-aminobutyric acid (GABA), and glutamate decarboxylase (GAD) (Wahid *et al.*, 2007). GB and sucrose were found to protect the OEC of isolated pea and spinach leave chloroplast from heat stress up to 60℃ (Allakhverdiev *et al.*, 1996). The heat protective effect of GB

and sucrose was understood to stem from the ability of the osmolytes to minimise the protein water interaction.

Osmolytes can be exogenously applied on plants to help protect them from environmental stress (Mäkela *et al.*, 1998). GB, which can be easily extracted from sugar beets, is easily absorbed by plant leaves and translocated to plant parts experiencing stress (Mäkela *et al.*, 1996; Mäkela *et al.*, 1998). GB and proline have been found to also help plants withstand ROS induced oxidative stress (Raza *et al.*, 2007; Kumar *et al.*, 2010).

2.5.7. Synthesis of heat shock proteins

Heat shock proteins (HSPs) are synthesised in organisms during heat stress, but can also be induced by various other factors such as metal toxicity or virus infection (Schlesinger, 1990). There are three major types of HSPs viz HSP70, HSP90, and HSPs with low molecular weights from 16 to 40 KDa (Lindquist and Craig, 1988). HSPs functions include stabilisation of proteins, assisting protein folding, aiding in protein compartmentalisation, and recovering protein function during heat stress (Wahid *et al.*, 2007). Once HSPs have been synthesised they persist in cells and render the cells resistant to various subsequent environmental stresses (Schlesinger, 1990). In a loboratory study by Ferguson *et al.* (1994), heat treatment of 39 ℃ for up to 8 hours increased HSPs in pear cells. A heat treatment of 38 ℃ increased HSPs synthesis and subsequently reduced the firmness deterioration rate of heat treated apple fruits during shelf life when compared to the control fruits

(Lurie and Klein, 1990). HSPs produced during exposure to sub-lethal heat treatment are maintained and can therefore later protect the photosystem from exposure to lethal heat stress.

HSPs have been found to protect the photosystem against heat and light stress (Downs *et al.*, 1999a, b). Downs *et al.* (1999a) reported that the OEC and PS II electrons transport of *Chenopodium album* plants, grown at 25 °C + low light 300 µmol m^{-2} s^{-1}, were protected against an 8 hour 38 °C heat stress by a small HSP found in their chloroplast lumen. However, the small chloroplast HSP did not reactivate damaged PS II systems, but merely protected them against heat damage. Downs *et al.* (1999b) found that the small chloroplast HSP also protected plants against drought stress combined with high PAR levels (1300 µmol m^{-2} s^{-1}), UV-A radiation and H_2O_2 induced oxidative stress. Lee *et al.* (2000) further found that a small HSP protected the photosystem of rice plants against H_2O_2 oxidative stress. The level of chloroplast HSP under non-stressful conditions and/or their rate of synthesis in fruits during stress events could be used as a selection criterion for sunburn resistance.

2.6. References

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PAPER 1

The effect of high UV-B dosage on apple fruit photosystems at different fruit maturity stages

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Abstract

Apple fruits (*Malus domestica* Borkh) of the cultivars Granny Smith, Fuji, Cripp's Pink, Braeburn, Golden Delicious and Topred were harvested at three stages during fruit growth. Previously sun-exposed peels of the apple fruits

were exposed to a high ultraviolet radiation-B (UV-B) dosage for 150 min at each stage. In a second experiment mature ‘Granny Smith’, ‘Fuji’ and ‘Cripps’ Pink’ fruits previously sun or shade exposed were also exposed to the UV-B stress. The effect of UV-B stress on fruits photosystem components was assessed by measuring the change in maximum light use efficiency and light reflection of fruit peels. UV-B induced pigment changes were analysed for ‘Braeburn’, ‘Fuji’ and ‘Cripps’ Pink’. The UV-B stress did not cause photoinhibition to any of the cultivars during fruit growth. However, UV-B stress did cause photoinhibition to previously shaded mature ‘Granny Smith’ and ‘Fuji’ fruits. Previously shaded ‘Cripps’ Pink’ fruits were conversely as insensitive to UV-B stress as the previously sun exposed fruits. ‘Braeburn’ showed no major pigment response to UV-B stress throughout the season. However, in ‘Fuji’ and ‘Cripps’ Pink’ fruits, total phenolic content increased at mid-season and maturity, while decreasing at the juvenile stage. All cultivars appear to have a stronger light reflection response to UV-B stress at the juvenile stage than later in the season. Photosystem II (PS II) units (as indicated by the *Fm* values) and the oxygen evolving complex activity (as indicated by the *Fv* values) in all the cultivars decreased with fruit maturity. Shaded ‘Cripps’ Pink’ fruits seemed to use the xanthophyll cycle as a photoprotective mechanism after UV-B stress. Photosynthetic systems in sun-exposed, therefore acclimatised, apple fruit peel are possibly not sensitive to UV-B stress in isolation. The fruits are probably well screened against UV light. Conversely, shaded peel may be less adapted and therefore more sensitive to high UV-B exposure. The light reflection response to UV-B stress at the juvenile stage could be due to the reduced phenolic content after stress

and the presence of more PS II units at this stage compared to the mature stage.

Keywords: Apple, Fluorescence, UV-B, Photosystem, Fruit maturity, Sunburn

Abbreviations: TBP, thylakoid bound pigments; APX, ascorbate peroxidise; EPS, epoxidation state; AVI, apple violaxanthin index; OEC, Oxygen evolving complex.

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1. Introduction

Fruit appearance is an important quality parameter for marketing horticultural produce. The export of apple fruits (*Malus domestica* Borkh) to the fresh market is of high economic value for South Africa. This export accounted for an average of 41.9% of total apple production from 2002 to 2012 (HORTGRO, 2013). Sunburn reduces the percentage packout and total income from apple fruits. Sunburn damage can affect up to 18% of total production (Gindaba and Wand, 2005). The major sunburn type, sunburn browning, which occurs in fruit production areas around the world, occurs on fruits which are well exposed to the sun. Sunburn browning is caused by light (UV and visible) and heat at temperatures between 46 to 49 ℃ (Schrader et al., 2001; Schrader et al., 2003; Schrader et al., 2008). The transpirational cooling of apple tree leaves is 0.3 to 0.6 ℃ on a clear sunny day (Solomakhin and Blanke, 2010). However, fruit temperatures can be 10 to 15 ℃ higher than air temperature (Parchomchuk and Meheriuk, 1996; Ferguson et al., 1998).

Sunburn develops as a result of direct damage to the photosynthetic apparatus or to the photoprotection mechanism of fruit peel photosystems caused by heat and light stress (Chen et al., 2008). UV-B is involved in the development of fruit sunburn as the exclusion of these light wavelengths reduced the occurrence of sunburn on attached apple fruits (Schrader et al., 2001; Schrader et al., 2003). UV-B causes damage to plant photosystems by damaging the reaction centres of photosystem II (PSII) (Iwanzik et al., 1983). It also leads to the degradation of the D1 and D2 proteins which form the core of the reactions centres of PSII (Jansen et al., 1996). The D1 and D2 proteins degrade much quicker under UV-B combined with photosynthetic active radiation (PAR) than with either one of these stresses alone (Babu et al., 1999). Babu et al. (1999) also found that the degradation in plant leaves by UV-B or PAR alone is not coupled to the redox state of PSII while the degradation under combined UV-B and PAR is. UV-B also has a negative effect on the enzymes of the Calvin cycle leading to reduced CO_2 uptake in plant leaves (Krause et al., 1999; Surabhi et al., 2009).

Fruit producers use different sunburn protection mechanisms, including spraying UV-B protective substances, overhead evaporative cooling and shade netting. It is important to determine the maturity stage at which fruit become sensitive to sunburn inducing factors during fruit development of different cultivars. This can help producers to correctly time their sunburn prevention mechanisms which can reduce waste and minimise operational costs. There is currently no literature regarding the difference in sunburn susceptibility between different apple cultivars in South Africa. The following

apple cultivars can be ranked as follows from high to low sunburn susceptibility, based on personal observation: Granny Smith, Braeburn, Cripps' Pink, Golden Delicious, Fuji, Topred. The seasonal response of apple fruits and the response of apples fruits with different UV-B exposure histories to UV-B stress have not been studied before.

The objectives of the study were to: (1) determine whether there is a specific development stage at which fruits become more sensitive to UV-B stress, and (2) study the effect of sun light exposure history on UV-B sensitivity. The maximum light use efficiency of photosystem II (F_v/F_m) and related parameters were used to measure stress induced damage to the fruit photosystem.

2. Materials and methods

2.1. Plant material and experimental design

Apple fruits were collected from farms in the Grabouw area (34º9'10.55''S; 19º1'47.62''E) of the Mediterranean-type climate Western Cape Province of South Africa. Two experiments were conducted: Experiment 1 analysed the response of apple fruits to UV-B stress at different maturity stages; Experiment 2 analysed the response of apple fruits with different sunlight exposure histories to UV-B stress at maturity.

2.1.1. Experiment 1. UV-B stress on previously sun-exposed peel at different fruit maturity stages

The following apple cultivars were used in this experiment: Granny Smith, Fuji, Cripps' Pink, Braeburn, Golden Delicious and Topred. The apple fruits were harvested at three stages during fruit growth: (1) juvenile stage, at ca. 53 days after full bloom (DAFB); (2) mid-season, at ca. 127 DAFB; and (3) at a late fruit development stage, ca. 155 DAFB. Fruits were stored at –0.5 °C for one night before exposure to UV-B stress the following morning. The apple fruits were kept at room temperature ca. 25 °C for 2 h after removal from cold rooms, before being placed under the lights. Fruits were harvested from mid tree canopy position, from the West and North-West facing side of the row. The sun exposed sides of the fruits were exposed to UV-B stress in the experiment.

2.1.2. Experiment 2. UV-B stress on fruits with different sunlight exposure histories

The following apple cultivars were used: Granny Smith, Cripps' Pink and Fuji. Fruits maturity, DAFB, for 'Granny Smith', 'Cripps' Pink', 'Fuji', were 158, 165, and 151 DAFB respectively. Fruits were treated as explained for Experiment 1 above after harvest. The one set of fruits was exposed to UV-B stress on the sun exposed side, while another set was exposed on the shaded side.

The experimental setup for both experiments was a completely randomized design with 8 UV-B lamp replicates. The fruits from the different cultivars were randomly placed under the 180 cm long UV-B (100 W) fluorescent lamps

(Philips, Amsterdam, Holland), with 5 fruits per cultivar per lamp. The five fruits per cultivar per lamp were arranged such that one fruit was directly under the lamp and it was flanked on both sides by two extra fruits. The fluorescence reading was taken from the one fruit directly under the lamp, while this fruit plus the extra 4 fruits were used for fruit peel chemical analysis after stress. There was a spacing of about 10 to 15 cm between the fruit batches under each lamp, with this spacing changing depending on the specific fruit size through the season. UV-C was removed with a cellulose acetate filter that was placed between the lights and fruit. UV-B radiation was measured with a spectroradiometer (Ophir PD300, Ophir Optronic Solutions, Jerusalem, Israel). The UV-B intensity was 3.9 kJ m^{-2} s^{-1} (290-320 nm) over 150 min. The daily UV-B dosages in the Western Cape can reach 7,59 kJ m^{-2} d^{-1} during summer (Wand et al., 1996). The fruit temperature under the lights was ca. 26 °C. Before the experiment, 10 fruits per cultivar were peeled to determine the initial fruit peel biochemistry. The fruit peels were frozen in liquid nitrogen and stored at -80 °C until pigment extraction. At the beginning of the experiments it was observed that no changes occurred in the fluorescence readings in fruits placed under room condition (20 °C; 15 µmol m^{-2} s^{-1}) for 150 min.

2.2. Pigment analysis

Total phenolics were extracted from 100 mg frozen apple peel samples (which were stored at -80 °C after harvesting) in 80% ethanol using Folin-Ciocalteu's phenol reagent and a standard curve created with gallic acid (Slinkard and

Singleton, 1977). The total concentration was determined by measuring absorbance at 750 nm with a spectrophotometer (UV-visible light spectrophotometer- Cary 50Bio, Varian Ltd, Walton-on-Thyme, London, UK), using the coefficients from the standard curve.

Anthocyanin was extracted from 2 g of milled fruit peel tissue (from fruits not exposed to stress) in methanol (with 1% 3 mol l^{-1} HCl) and kept in the dark at 4 ^{o}C. The solution was stirred for 1 h and the extract was centrifuged at 7840 g for 10 min at 4 ^{o}C. The total anthocyanin concentration was determined by measuring absorbance at 520 nm with a spectrophotometer (UV-visible light spectrophotometer- Cary 50Bio, Varian Ltd, Walton-on-Thyme, London, UK). The anthocyanin absorbance at 520 nm was corrected for the presence of chlorophyll by subtracting absorbance at 653 nm [Abs520nm-(0.24xAbs653nm)] (Murray and Hackett, 1991).

Chlorophyll analyses were also performed fusing milled fruit peel tissue. Chlorophyll was extracted from 0.5 g tissue in 3 ml acetone at 4℃ by stirring with a magnetic stirrer for 24 h. The resulting extract was centrifuged at 7840 g for 15 min, and the supernatant filtered through a 0.45 µm filter. Chlorophyll concentration was determined by measuring absorbance at 470, 645 and 662 nm with a spectrophotometer (UV-visible light spectrophotometer- Cary 50Bio, Varian Ltd, Walton-on-Thyme, London, UK). The concentration of chlorophyll a, b, total chlorophyll and carotenoids were determined according to Lichtenthaler (1987), with 100% acetone as the blank.

2.3. Reflection measurements and irradiation conditions

Reflection analyses were done for Experiment 1 only. Light reflection (percentage reflection) was taken with an USB 2.0 Fiber Optic Spectrometer (Ocean Optics Inc., Dunedin, Florida, USA), with barium sulphate as a standard 100% reflection. The spectrometer is installed with an OFLV4-200-850 Detector, a L4 lens and 25 µm slit. This was attached to a DH-2000-BAL Tunsten Halogen (200 – 1100 nm) light source. Light reflection readings were done on one fruit per replicate per cultivar for each of the 8 lamps.

Light absorption by chloroplast chlorophyll and carotenoids tightly associated with the thylakoid membranes was calculated according to the following formula, as determined by Merzlyak (2006):

$F_T = [R(\lambda)]_1^{-1} - [R(\lambda)]_2^{-1}$ 1

where $[R(\lambda)]^{-1}$ are the reciprocal light reflection curves before and after UV-B stress.

Thermal energy release from the photosystem was determined by assessing the change in light reflection at 531, 530 and 630 nm. There is a positive correlation between the change in the epoxidation state (EPS) of the xanthophyll cycle and light reflection changes at 531 nm (Gamon et al., 1992). A decrease in the EPS of the xanthophyll cycle indicates an increase in the formation of zeaxanthin and thermal energy release from the photosystem (Demmig-Adams and Adams III, 1992). Solovchenko et al. (2010) determined

the apple violaxanthin cycle index (AVI) from apple fruit light reflection. An increase in this index indicates an increase in the de-epoxidation of violaxanthin, as-well-as an increase in non-photochemical quenching (qN). AVI was calculated as:

$$AVI = (1/R_{520} - 1/R_{630}) \times R_{800} \quad \text{..... 2}$$

where $1/R_{520}$ caters for the conversion of violaxanthin to zeaxanthin; while $1/R_{630}$ is not affected by this change; and R_{800} is for reflection changes caused by the physical properties of the fruit peel rather than pigment effects. An increase in R_{520} is therefore expected to lead to a decrease in fruit AVI and vice versa.

2.4. Chlorophyll fluorescence

Fruit Chl *a* fluorescence at room temperature was measured with a fluorescence monitoring system 1 (FSM 1) fluorometer (Fluorescence Monitoring System 1, Hansatech, Norfolk, UK). The fluorometer was connected to one half of a leaf-clip holder with a 6mm hole through which the fluorescence readings are taken. Fruits were dark adapted for 30 min before measuring the maximum (F_m) and minimum (F_o) fluorescence and the maximum light use efficiency of photosystem II ($F_v/F_m = F_m\text{-}F_o/F_m$). Fluorescence readings were done on only one fruit (out of 5) per lamp per cultivar for each of the 8 lights. Readings were taken after fruits were dark adapted for 30 min, after a 30 min UV-B light exposure period.

2.5. Statistical analysis

A linear regression analysis was done with Microsoft Excel (Windows Microsoft Excel 2010, Microsoft Corporation, Redmond, WA., USA). A one way ANOVA was conducted with SAS 9.1 (SAS Institute Inc., Cary, NC., USA) on the UV stress effect during exposure and mean separation was done with LSD at 0.05 where the treatment effect was significant at $p \leq 0.05$. An independent-sample t-test was conducted with SAS 9.1 to compare the chemical change before and after UV stress. When the variances of the *t*-test samples were not equal, the means were compared with a Welch's *t*-test analysis. Means and +/- standard errors are indicated on graphs and figures.

3. Results

3.1. Experiment 1. UV-B stress on previously sun-exposed peel at different fruit maturity stages

The fruit peel maximum light use efficiency (F_v/F_m), minimum fluorescence (F_o), variable fluorescence (F_v), and maximum fluorescence (F_m) had variable responses to UV-B stress (Fig. 1 - 4, respectively). 'Granny Smith' peel showed an increase in F_v/F_m and F_v at the juvenile and mature stages, while their F_o only increased at the juvenile stage in response to UV-B. F_v/F_m increased in 'Braeburn' and 'Cripps' Pink' peel at the mature stage. There was no change in the F_v/F_m, F_o and F_v values in response to UV-B in 'Fuji', 'Golden Delicious', and 'Topred' throughout the season. The F_m generally remained

constant throughout the season in all cultivars, accept for an increase at the juvenile and mature stages in 'Granny Smith' (Fig. 4). The initial F_v/F_m decreased through the season in 'Granny Smith', 'Fuji' and 'Cripps' Pink' fruits. The results were inversed for 'Topred' fruits, while it decreased and then increased in 'Golden Delicious' and 'Braeburn' fruits.

Light absorption by thylakoid bound pigments (TBP) decreased in 'Granny Smith', 'Golden Delicious', 'Topred' and to a lesser extent in 'Braeburn' (Fig. 5 and 6). However, TBP light absorption increased in 'Granny Smith' and 'Braeburn' at mid-season and maturity, respectively. However, in 'Fuji' and 'Cripps' Pink', TBP light absorption increased at maturity in response to UV-B treatment.

'Granny Smith', 'Golden Delicious' and 'Topred' peel showed a reduced epoxidation state (EPS) (Fig. 7) and an increased apple violaxanthin index (AVI) (Fig. 8) after UV-B stress at the juvenile stage. 'Braeburn', 'Fuji' and 'Cripps' Pink' fruits on the contrary had increased EPS and reduced AVI at this stage. At maturity, the AVI of 'Braeburn' and 'Cripps' Pink' fruits was increased after UV-B stress (Fig. 8).

Pigment changes were only analysed for 'Braeburn', 'Fuji' and 'Cripps' Pink'. The chlorophyll a/b ratio increased at the juvenile stage in 'Braeburn' and 'Fuji' (Table 1). This was the only significant UV-B induced pigment change in 'Braeburn' throughout the season. The carotenoid, chlorophyll and phenolic concentration of 'Fuji' peel decreased at the juvenile stage in response to UV-

B. UV-B caused no significant chemical change at mid-season and the mature stage in 'Fuji', apart from an increase in total phenolic concentration. In 'Cripps' Pink', most of the measured parameters increased after UV-B stress at the mid-season and mature stages. However, anthocyanin remained unchanged at mid-season in 'Cripps' Pink'. There are no chemical data for the juvenile stage of 'Cripps' Pink' due to missing samples.

3.2. Experiment 2. UV-B stress on fruits with different sunlight exposure histories

UV-B decreased F_v/F_m in shaded 'Granny Smith' and 'Fuji' peel, while it remained unchanged in shaded 'Cripps' Pink' fruits (Fig. 9). F_v/F_m of sun exposed peel did not respond to UV-B. The F_o value increased in the shaded 'Granny Smith' and 'Fuji' peel, while it decreased in shaded 'Cripps' Pink' peel (Fig. 9). UV-B decreased the F_m and F_v values of shaded 'Granny Smith', 'Fuji' and 'Cripps' Pink' peel (Fig. 10) but had no effect in sun exposed peel.

4. Discussion

Schrader et al. (2001) showed the involvement of UV-B in apple sunburn development through UV-B exclusion experiments in the orchard. Fruit sunburn is caused by UV-B, high PAR and heat (46 to 49 ℃) stress (Schrader et al., 2001; Schrader et al., 2003; Schrader et al., 2008). We studied the involvement of UV-B in sunburn development by assessing the response of the photoapparatus of detached apples to high dosages of UV-B.

Our study is however based on laboratory conditions and only focus on the effect of UV-B stress on apple fruits photosystems. We excluded the high temperature and PAR stresses which are normally experienced under field conditions in combination with UV-B stress. Chen et al. (2008) studied the effects of high temperature and PAR, Chen et al. (2009) the effect of high temperature while Solovchenko and Schmitz-Eiberger (2003) the effect of low dosage UV-B stress on apple fruit photosystems under laboratory conditions in relation to fruit sunburn development. Our study is in line with these studies and analyses the response of apple fruit photosystems at different maturity stages to high dosage UV-B stress under laboratory conditions. UV-B is known to damage the reaction centres of photosystem II (PSII) (Iwanzik et al., 1983) and also to negatively affect Calvin cycle enzymes (Krause et al., 1999; Surabhi et al., 2009). Hence, chlorophyll fluorescence is a useful tool to study the effect of UV-B on apple peel. A reduction in F_v/F_m indicates a decrease in the maximum light use efficiency of PS II (Maxwell and Johnson, 2000), while the reduction in F_m and F_v indicates a reduction in the amount of undamaged PS II units and increased damage to the OEC, respectively (Govindjee et al., 1981; Pistorius and Schmid, 1984; Lidon and Ramalho, 2011). An increase in F_o indicates damage to the antennae of PS II (Maxwell and Johnson, 2000). A decrease in F_o is an indication of an increase in zeaxanthin synthesis and heat release from the photosystem (Demmig et al., 1987; Krause, 1991). UV-B (0.012 kJ m^{-2} s^{-1} for 80 min) treatment reduced the F_m values while increasing the F_o in spinach (Vass et al, 1996), which caused a reduction in F_v ($F_m - F_o$). Iwanzik et al. (1983) also reported an increase and decrease of F_o

and F_v respectively in isolated spinach chloroplasts after UV-B treatment (0.00047 kJ m^{-2} s^{-1} for 8 h at 15 ℃).

The high dosage UV-B irradiation (3.9 kJ m^{-2} s^{-1} for 150 min at 26 ℃) employed in our research did not affect the F_v/F_m, F_o and F_m fluorescence parameters of previously sun-exposed apple fruit peel throughout the season (Fig. 1 - 4). This is despite the potential effects of unnaturally high UV-B dosages on plant biological system compared to natural solar UV-B radiation dosages, which include oxidative stress and photoinhibition (Ziska, 1996; Brosché and Strid, 2003). In contrast to our findings, much lower UV-B exposure (0.012 kJ m^{-2} s^{-1} for 150 minutes) reduced F_v/F_m of both shaded and sun-exposed 'Granny Smith' fruits, as well as that of shaded 'Braeburn' fruits while having no effect on sun-exposed 'Braeburn' fruits (Solovchenko and Schmitz-Eiberger, 2003). In the same study of Solovchenko and Schmitz-Eiberger (2003), F_o was unchanged or reduced in the sun-exposed fruits of 'Granny Smith' and 'Braeburn', respectively, while it was increased in the shaded fruits of both cultivars. The increased sensitivity of apple peel to UV-B in that particular study may relate to the use of fruit that were stored for 3 months at low temperature and in controlled atmosphere. In addition, the difference in the climate between Germany and South Africa will have affected the biochemical composition of the fruit photosystems and influenced their response to stress. However, while the F_v/F_m of mature 'Gala', 'Gold Rush' and 'Granny Smith' apple fruits was unaffected by an UV-B (0.012 kJ m^{-2} s^{-1} for 10 h) treatment, it was decreased in 'Braeburn' fruits (Glenn et al., 2008). The effect of UV-B on fruit fluorescence parameters is variable, and

can be influenced by a number of factors such as climatic adaptation, fruit maturity and cultivar (Glenn et al., 2008).

Shade-adapted plant leaves are generally more sensitive to UV-B light stress than sun-adapted leaves (Krause et al., 1999; Krause et al., 2003). As evidence of climatic adaptation, the photosystems of shaded 'Granny Smith' and 'Fuji' fruits were damaged by UV-B treatment, while that of 'Cripps' Pink' fruits remained as insensitive as that of sun exposed fruits of all three cultivars. This is indicated by the reduction in F_v/F_m, F_m and F_v combined with an increase in F_o of the shaded 'Granny Smith' and 'Fuji' fruits (Fig. 9 - 10). The increased F_o (Fig. 9) and decreased F_v (Fig. 10) for shaded 'Granny Smith' and 'Fuji' fruits indicate damage to the photosystem of these fruits. The photosystem of shaded 'Cripps' Pink' fruits was possibly well protected against the applied UV-B irradiance as their F_o and F_v remained unchanged (Fig. 9 and 10). Solovchenko and Schmitz-Eiberger (2003) found that the sun-exposed and shaded sides of 'Granny Smith' fruits were equally sensitive to the UV-B irradiance used in that study, while the shaded sides of 'Braeburn' fruits were sensitive and the sun-exposed sides were not.

The resistance of previously sun-exposed apple fruit photosystems to UV-B in our study could be as a result of UV protection mechanisms of these fruits. Sun-exposed apple fruits have higher antioxidants activities and therefore are better photoprotected than shaded fruits (Ma and Cheng, 2003). Phenolic compounds that accumulate in the epidermal cell layer of plant tissue have an UV-B screening and antioxidative function (Winkel-Shirley 2002; Schmitz-

Hoerner and Weissenböck, 2003; Treutter, 2006). Fruit cuticle and wax layer thickness increases during fruit maturity (Ju and Bramlage, 2001), which provides further protection against UV irradiance as the fruit matures. The total phenolic concentration in 'Fuji' and 'Cripps' Pink' fruit peel at mid-season and maturity increased after UV-B stress (Table 1). Hilal et al. (2008) and Huyskens-Kiel et al. (2007) also found an increase in fruit peel phenolic concentration after a 5 min and 3 h UV-B exposure, respectively. In contrast to the increase in phenolics in mid-season and mature 'Fuji' and 'Cripps' Pink', UV-B treatment decreased total phenolics in 'Fuji' fruit peel at the juvenile stage. The reason for this differential effect is unknown. Apple fruits, compared to apple tree leaves, have a more even or random chlorophyll distribution (Blanke and Lenz, 1989). The reduced chlorophyll concentration can also help reduce photodamage in fruits compared to leaves.

The F_v of 'Braeburn'; 'Fuji', 'Golden Delicious', 'Cripps Pink', and 'Topred' remained unchanged by the UV-B treatment while it was increased at the juvenile and mature stages in 'Granny Smith' fruits (Fig. 3). Changes in F_v values are positively correlated to changes in oxygen evolution from the oxygen evolving complex (OEC) of photosystem (PS) II (Govindjee *et al.*, 1981; Pistorius and Schmid, 1984). 'Cripps' Pink' had the lowest OEC activity at maturity while 'Granny Smith' had the highest, as related to seasonal changes of their F_v values (Fig. 3). 'Granny Smith' fruit peel generally has higher chlorophyll concentrations compared to most apple cultivars (Felicetti and Schrader, 2009). Therefore, the high OEC activity observed in 'Granny Smith' peel could be due to their high chlorophyll content. In addition, the F_v of

'Granny Smith' fruits was increased after UV-B treatment at the juvenile and mature stages (Fig. 3). 'Granny Smith' fruits therefore possibly reduced UV-B damage by increasing electron transport through the photosystems at this two growth stages. The observed seasonal decrease in F_v (Fig. 3) in all the cultivars could possibly indicate a decrease in OEC activity as fruits mature. Chlorophyll degradation increases with apple fruit maturity (Ihl et al., 1994). Li and Cheng (2008) also reported that the shaded sides of apple fruits become more sensitive to photoinhibition with maturity.

At the juvenile stage, 'Granny Smith', 'Golden Delicious' and 'Topred' had a reduced epoxidation state (EPS) and an increased apple violaxanthin index (AVI) (Fig. 7 and 8 respectively) after UV-B treatment. The inverse was true in 'Braeburn', 'Fuji' and 'Cripps' Pink'. 'Granny Smith', 'Golden Delicious' and 'Topred' fruits may therefore depend on the xanthophyll cycle for photoprotection after exposure to UV-B stress at the juvenile stage. Apple fruits have a faster chlorophyll a reduction than chlorophyll b with fruit maturity compared to apple tree leaves (Blanke and Lenz, 1989). A high chlorophyll a/b ratio is related to high dependence on the xanthophyll cycle (Kleima et al., 1999), therefore the reduced chlorophyll a/b ratio could indicate a reduced dependence on this cycle with fruit maturity. Although UV irradiation may cause down regulation of genes for proteins involved in photosynthesis, plants respond by increasing the expression of many other genes including those involved in the synthesis of antioxidant enzymes such as ascorbate peroxidase (APX) (Ballaré, 2003). Antioxidant enzymes, including APX, provide photoprotection, which reduce photoinhibition (Niyogi, 1999). The

increased activity of ascorbate peroxidase (APX) subsequently leads to up-regulation of the ascorbate-glutathione cycle which is needed to produce the ascorbate used by APX to convert hydrogen peroxide to water in the Mehler reaction (Mittler et al., 2004). The production of ascorbate is linked to the de-epoxidation of violaxanthin, which result in the eventual activation of the xanthophyll cycle (Müller-Moulé et al., 2003). However, solar UV-B (at daily maximum 0.18 J m^{-2} s^{-1}, for 12 weeks) had variable effects on the leaf xanthophyll cycle of acacia and eucalyptus plants, while it had no effect on their F_v/F_m (Liu et al., 2005). UV-B irradiation therefore can in-directly affect the xanthophyll cycle.

Plants can also increase the turnover rate of D1 proteins during UV stress, thus quickly replacing damaged D1 proteins and reducing photoinhibition (Wu et al., 2011). ‘Braeburn’, ‘Fuji’ and ‘Cripps’ Pink’ fruits may have depended on photoprotective mechanisms which increased or maintained high photosynthetic rates in these fruits after UV-B stress at the juvenile stage. These fruits had an increased EPS at this stage. Demmig-Adams and Adams II (1992) reported that leaves with a high EPS had a high photosynthetic rate. ‘Braeburn’, ‘Fuji’ and ‘Cripps’ Pink’ fruits also had an increased light absorption by thylakoid bound pigments (TBP) (Fig. 5 and 6) at the juvenile stage. This could be due to the observed up-regulated photoprotection mechanisms. Light reflection data (not shown) also showed an increase in the green/red colour ratio after stress at the juvenile stage in all the cultivars accept for ‘Cripps’ Pink’ for which the inverse was true. The increase in the green/red colour ratio could have reduced the masking of chlorophyll by

anthocyanin, therefore contributing to the observed increased light absorption by the TBP. However, the AVI of ‘Braeburn’ and ‘Cripps’ Pink’ fruits was increased after UV-B stress at maturity (Fig. 8), although the EPS (Fig. 7) remained constant. ‘Braeburn’ and ‘Cripps’ Pink’ fruits maintained a higher carotenoid to chlorophyll ratio compared to ‘Fuji’ fruits (Table 1). ‘Braeburn’ and ‘Cripps’ Pink’ fruits therefore possibly use the xanthophyll cycle for photoprotection after UV-B treatment at the mature fruit growth stage.

5. Conclusion

The high UV-B dosage employed in our study caused no apparent damage to the photosystem of previously sun-exposed apple fruit peel. Some cultivars appear to have increased their xanthophyll cycles, while others may have increased their photosynthetic rates. ‘Cripps’ Pink’ fruits seemed to depend more on the xanthophyll cycle for photoprotection after UV-B stress at maturity compared to the other cultivars tested. In contrast to sun-adapted peel, shaded peel of ‘Granny Smith’ and ‘Fuji’ were sensitive to UV-B treatment. Apple fruit that are exposed to sunlight from early development seem to be well protected against UV-B and are unlikely to develop sunburn in response to UV-B exposure. Shaded fruit in contrast are not as well protected and may be damaged by sudden UV-B exposure.

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Table 1

Sun-exposed apple fruit peel carotenoids, chlorophylls, anthocyanin and total phenolics content before and after UV-B treatment. Fruits were peeled 1 hour after exposure to stress. Different letters next to values indicate significant differences between the peel chemistry "Before" and "After" stress, α = 0.05. ND = missing data; ns = not significant

		Harvest 1 (53 DAFB)		**Harvest 2 (127 DAFB)**		**Harvest 3 (155 DAFB)**	
Cultivar	**Pigment content**	Before UV	After UV	Before UV	After UV	Before UV	After UV
'Braeburn'	Carotenoids (µg/g FW)	48.5 ns	45.9	31. 5 b	26.9 a	28.6 ns	27.8
	Chlorophyll b (µg/g FW)	44.6 ns	41.3	23.7 ns	22.2	20.1 ns	22.0
	Chlorophyll a (µg/g FW)	116.8 ns	117.0	72.3 ns	65.3	57.4 ns	65.0
	Total chlorophyll (µg/g FW)	161.4 ns	158.3	96.0 ns	87.6	77.5 ns	87.0
	Chlorophyll a/b	2.6 a	2.8 b	3.1 ns	2.9	2. 9 ns	3.0
	Anthocyanin (µg/g FW)	61.2 ns	50.6	51.8 ns	73.7	ND	ND
	Total Phenolics (mg/100g FW)	856.8 ns	822.8	283.3 ns	249.0	186. 4 ns	201.4
'Fuji'	Carotenoids (µg/g FW)	51.0 b	40.3 a	32.7 ns	34.4	23.2 ns	26.4
	Chlorophyll b (µg/g FW)	53.1 b	40.4 a	30.7 ns	32.0	18.2 ns	22.3
	Chlorophyll a (µg/g FW)	149.4 b	117.6 a	89.0 ns	95.9	54.7 ns	66.6
	Total chlorophyll (µg/g FW)	202.5 b	158.1 a	119.7 ns	127.9	72.9 ns	88.9
	Chlorophyll a/b	2.8 a	2.9 b	2.9 ns	3.0	3.0 ns	3.0
	Anthocyanin (µg/g FW)	ND	ND	42.3 ns	41.7	153.8 ns	178.2
	Total Phenolics (mg/100g FW)	149.7 b	121.8 a	82.8 a	119.6 b	65.7 a	266.0 b
'Cripps' Pink'	Carotenoids (µg/g FW)	ND	ND	19.4 a	25.0 b	17.5 a	21.5 b
	Chlorophyll b (µg/g FW)	ND	ND	16.3 a	21.8 b	11.7 a	15.0 b
	Chlorophyll a (µg/g FW)	ND	ND	47.8 a	70.2 b	37.1 a	50.0 b
	Total chlorophyll (µg/g FW)	ND	ND	64.1 a	92.0 b	48.9 a	65.0 b
	Chlorophyll a/b	ND	ND	2.9 a	3.2 b	3.2 a	3.3 a
	Anthocyanin (µg/g FW)	ND	ND	7.0 ns	9.1	ND	ND
	Total Phenolics (mg/100g FW)	ND	ND	95.5 a	289.7 b	192.7 a	253.9 b

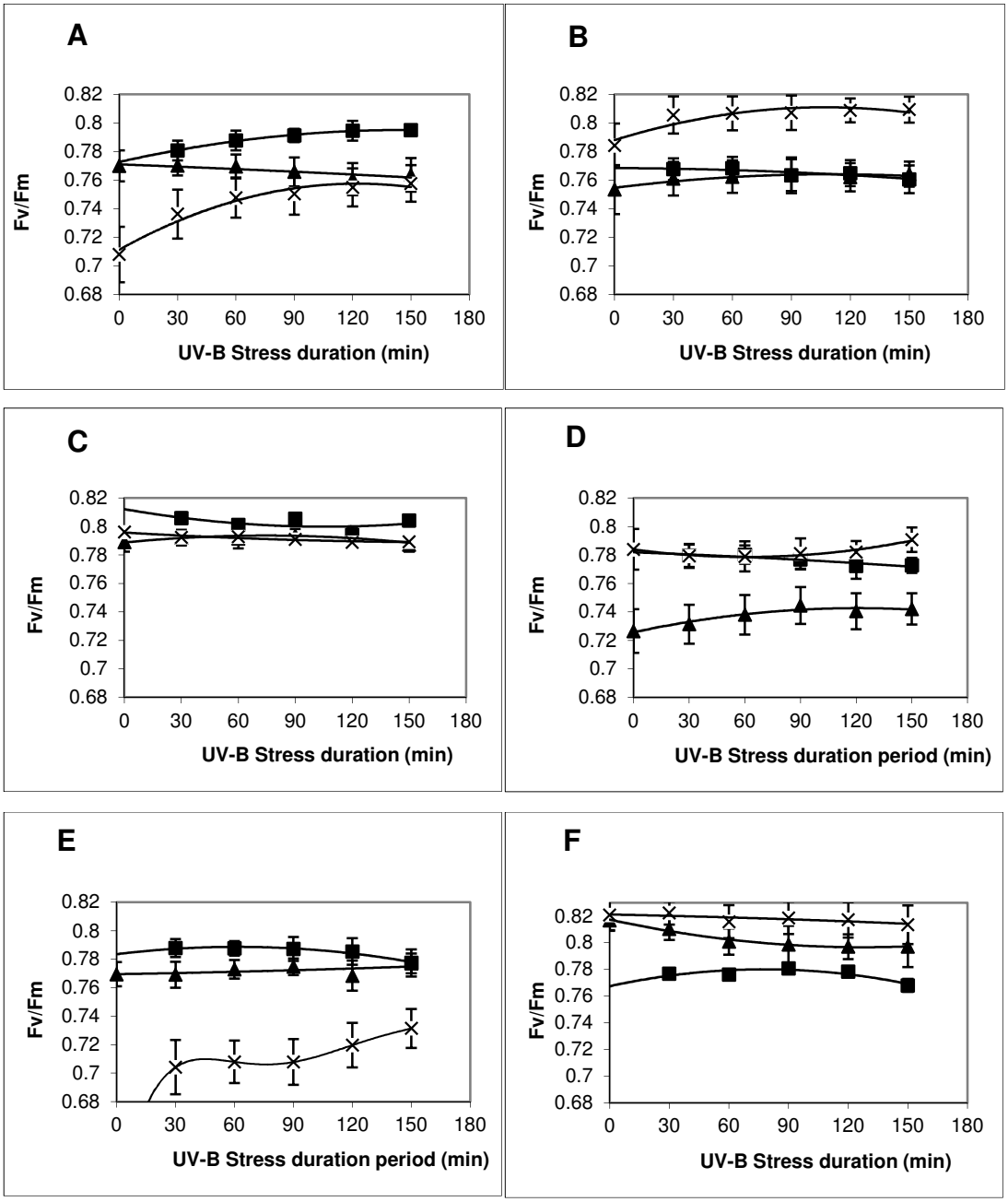

Fig. 1. Changes in the maximum light use efficiency (F_v/F_m) of sun-exposed apple fruit peel during UV-B treatment. Harvest 1 = juvenile stage (■), Harvest 2 = mid-season (▲), Harvest 3 = mature stage (X). A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'; D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'. Means and standard errors are indicated.

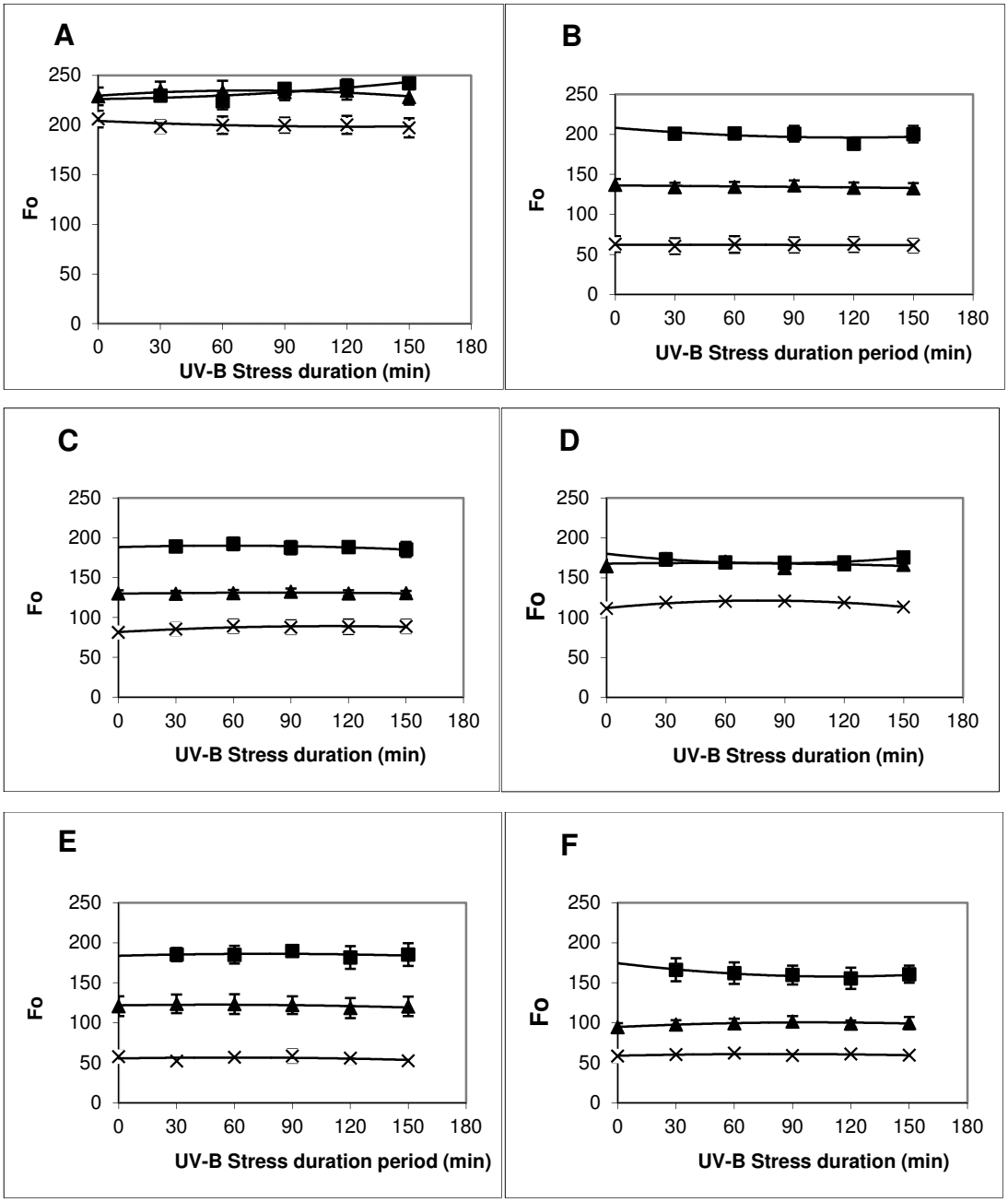

Fig. 2. Changes in chlorophyll a minimum fluorescence (F_o) of sun-exposed apple fruit peel during UV-B treatment. Harvest 1 = juvenile stage (■), Harvest 2 = mid-season (▲), Harvest 3 = mature stage (X). A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'; D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'. Means and standard errors are indicated.

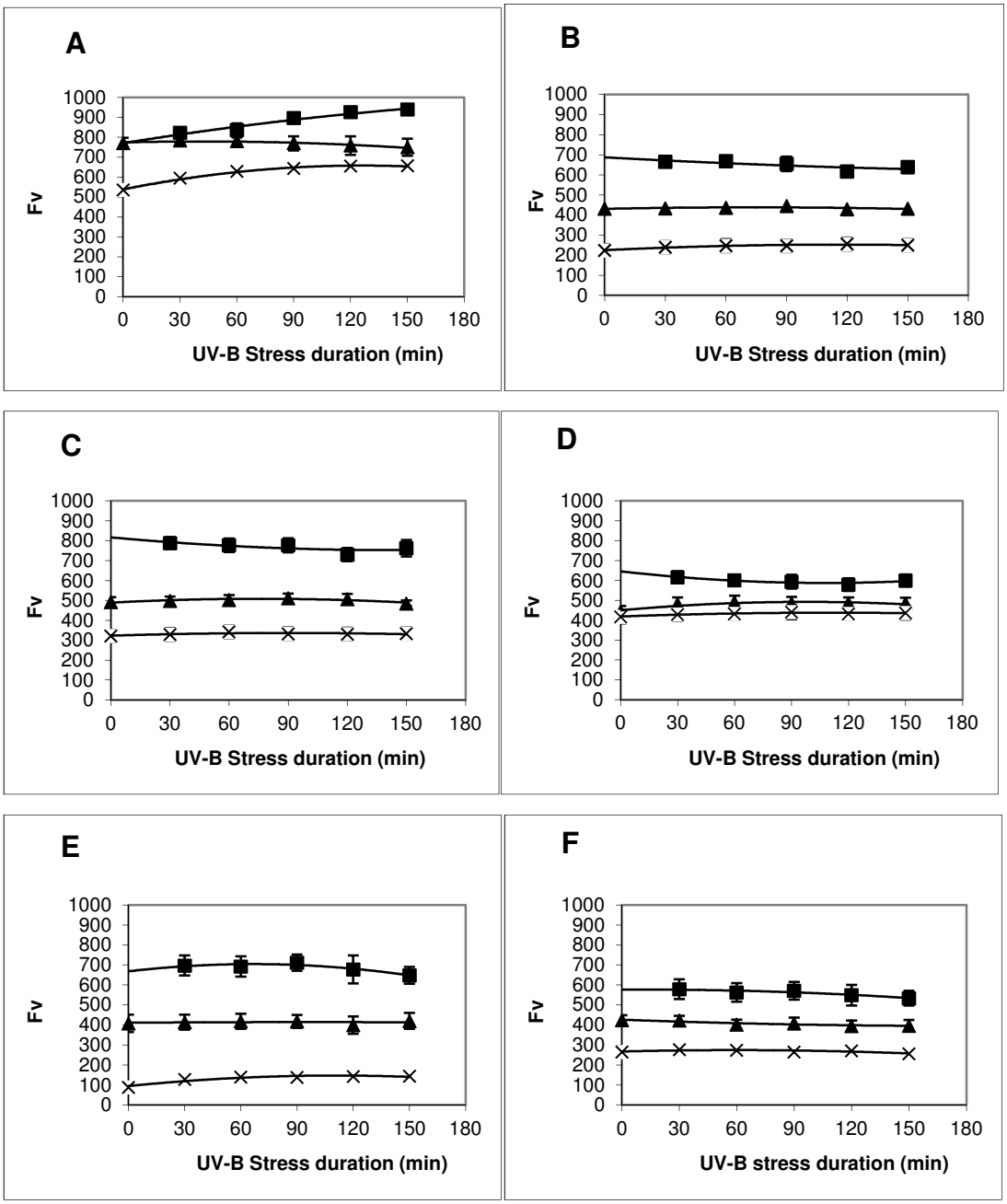

Fig. 3. Changes in chlorophyll a variable fluorescence (F_v) of sun-exposed apple fruit peel during UV-B treatment. Harvest 1 = juvenile stage (■), Harvest 2 = mid-season (▲), Harvest 3 = mature stage (X). A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'; D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'. Means and standard errors are indicated.

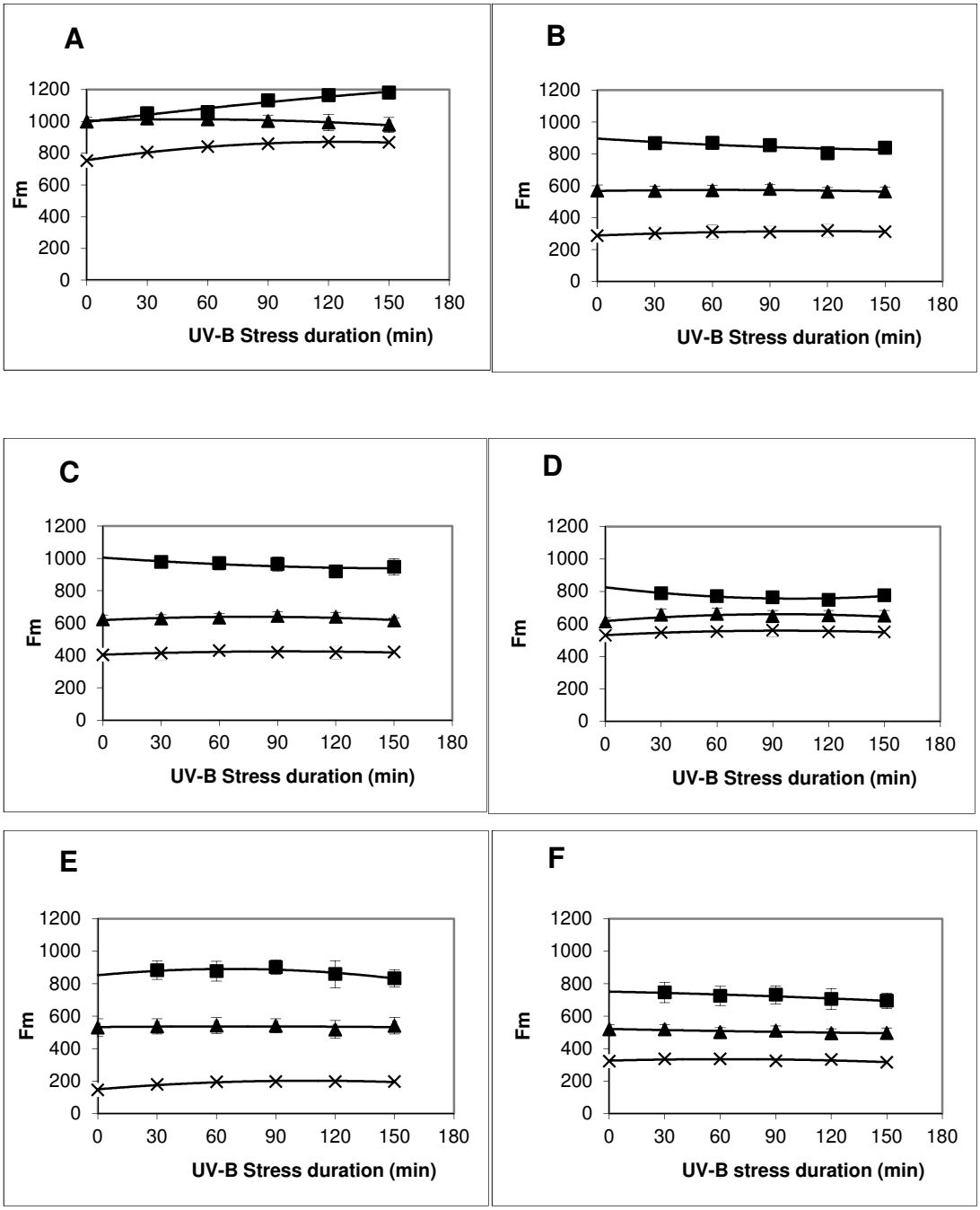

Fig. 4. Changes in chlorophyll a maximum fluorescence (F_m) of sun-exposed apple fruit peel during UV-B treatment. Harvest 1 = juvenile stage (■), Harvest 2 = mid-season (▲), Harvest 3 = mature stage (X). A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'; D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'. Means and standard errors are indicated.

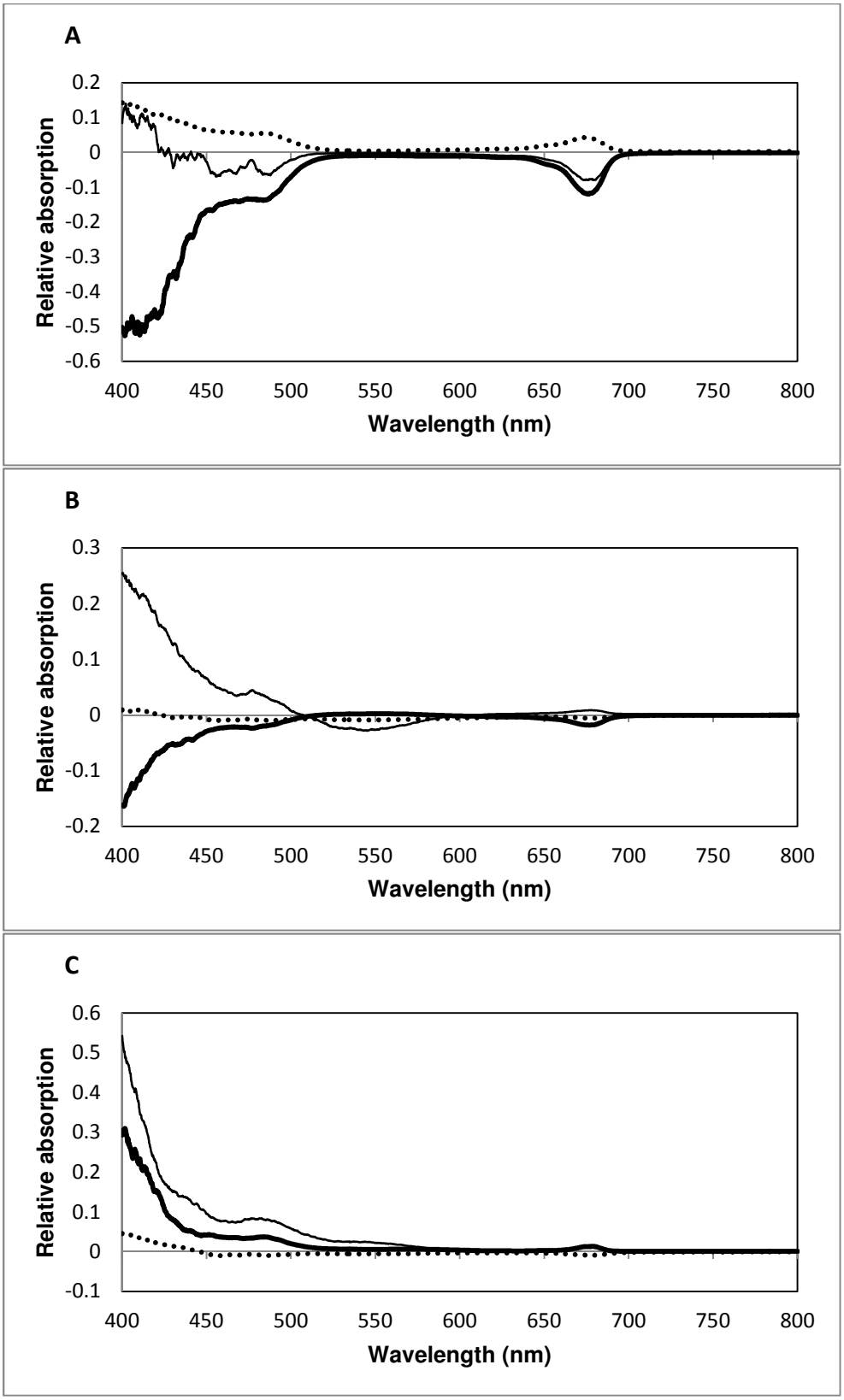

Fig. 5. Light absorption by chloroplast thylakoid bound pigments of sun-exposed apple fruit peel after UV-B treatment. Harvest 1= juvenile stage (Bold line); Harvest 2= mid-season (Dotted line); Harvest 3= mature stage (Solid line). A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'

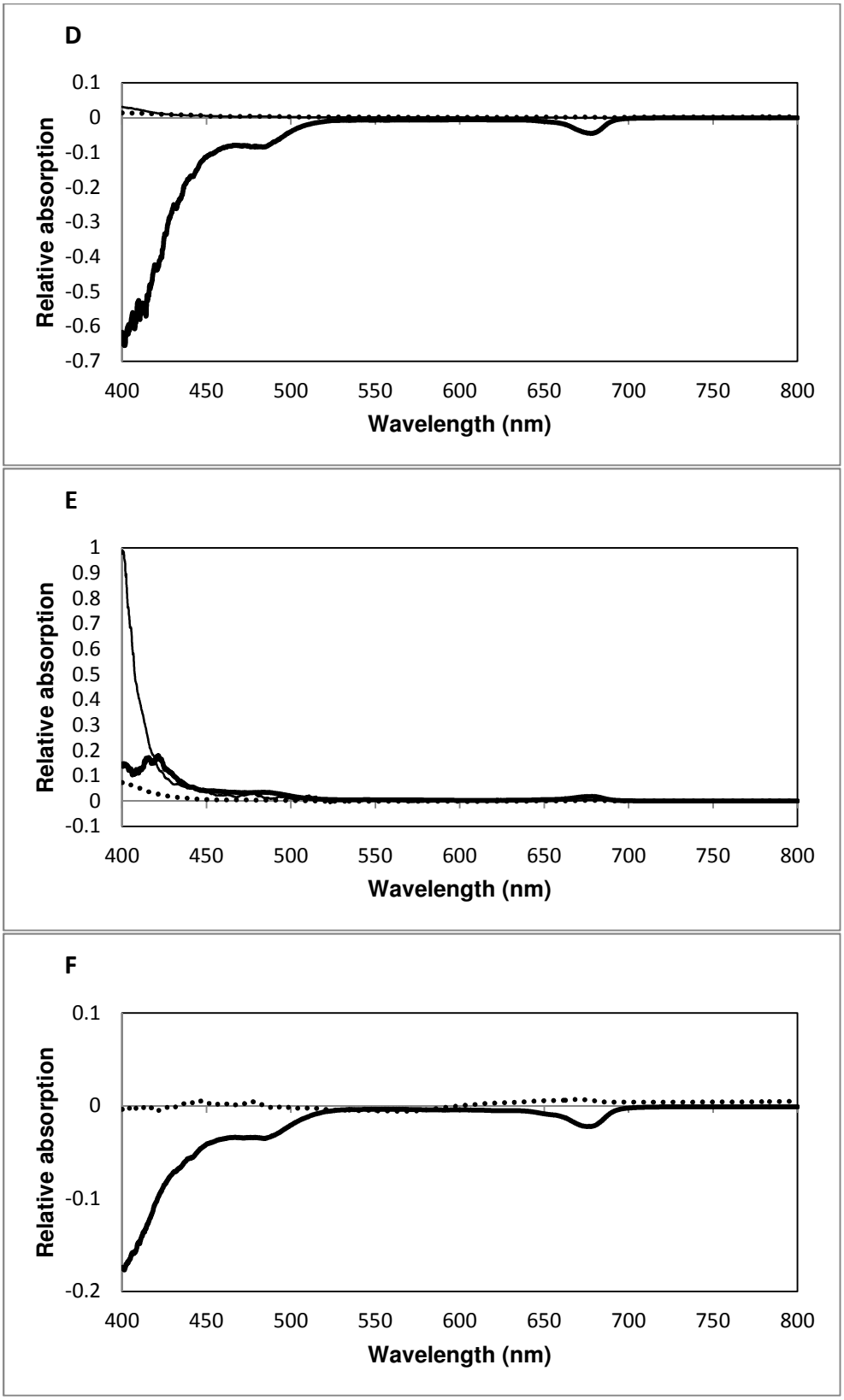

Fig. 6. Light absorption by chloroplast thylakoid bound pigments of sun-exposed apple fruit peel after UV-B treatment. 'Topred' fruits maturity stage data is missing. Harvest 1= juvenile stage (Bold line); Harvest 2= mid-season (Dotted line); Harvest 3= mature stage (Solid line). D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'.

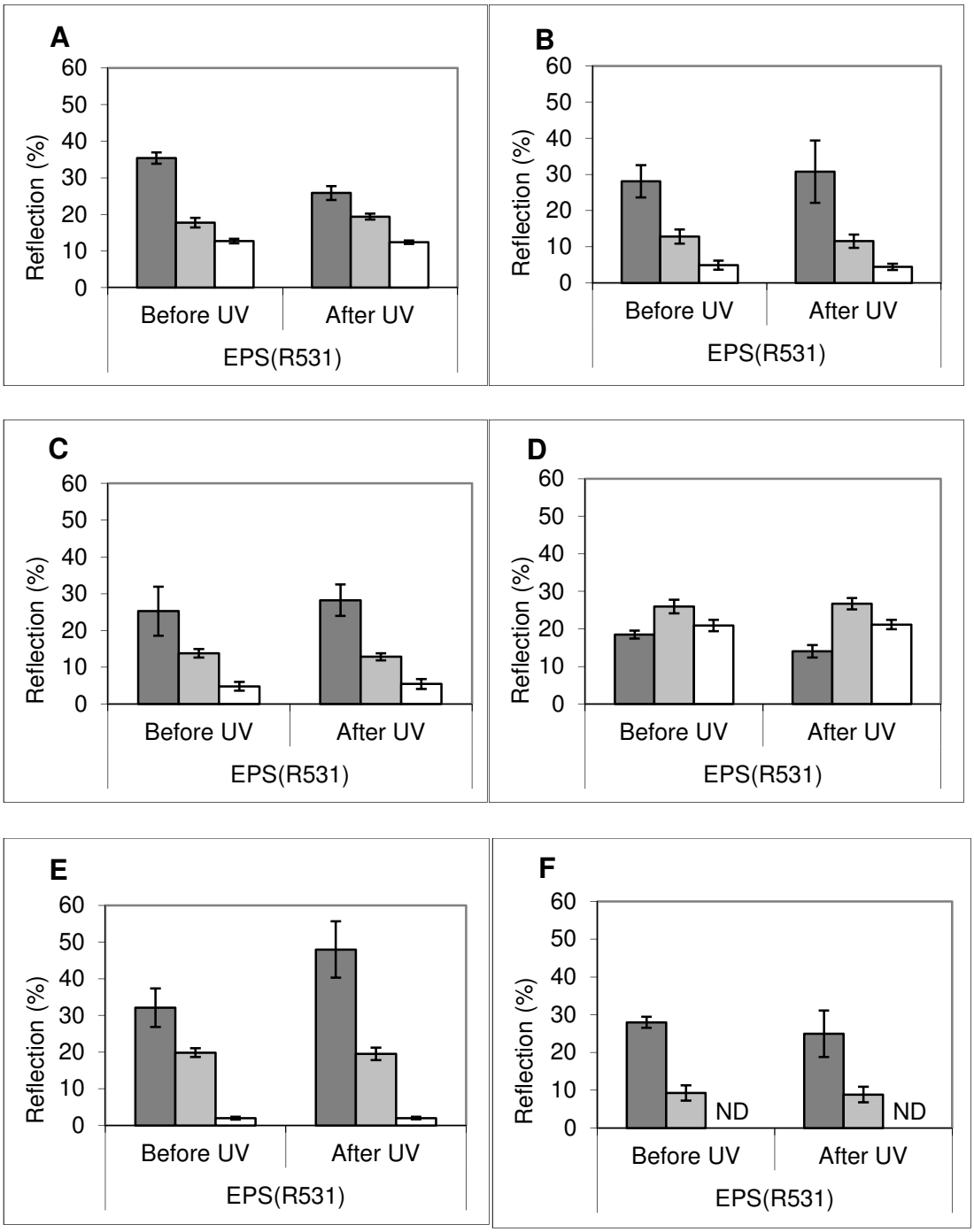

Fig.7. Change in reflection at R531 (EPS) of sun-exposed apple fruit peel after UV-B treatment. Harvest 1 = juvenile stage (Dark grey), Harvest 2 = mid-season (Light grey), Harvest 3 = mature stage (White). A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'; D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'. ND = No data. Means and standard errors are indicated.

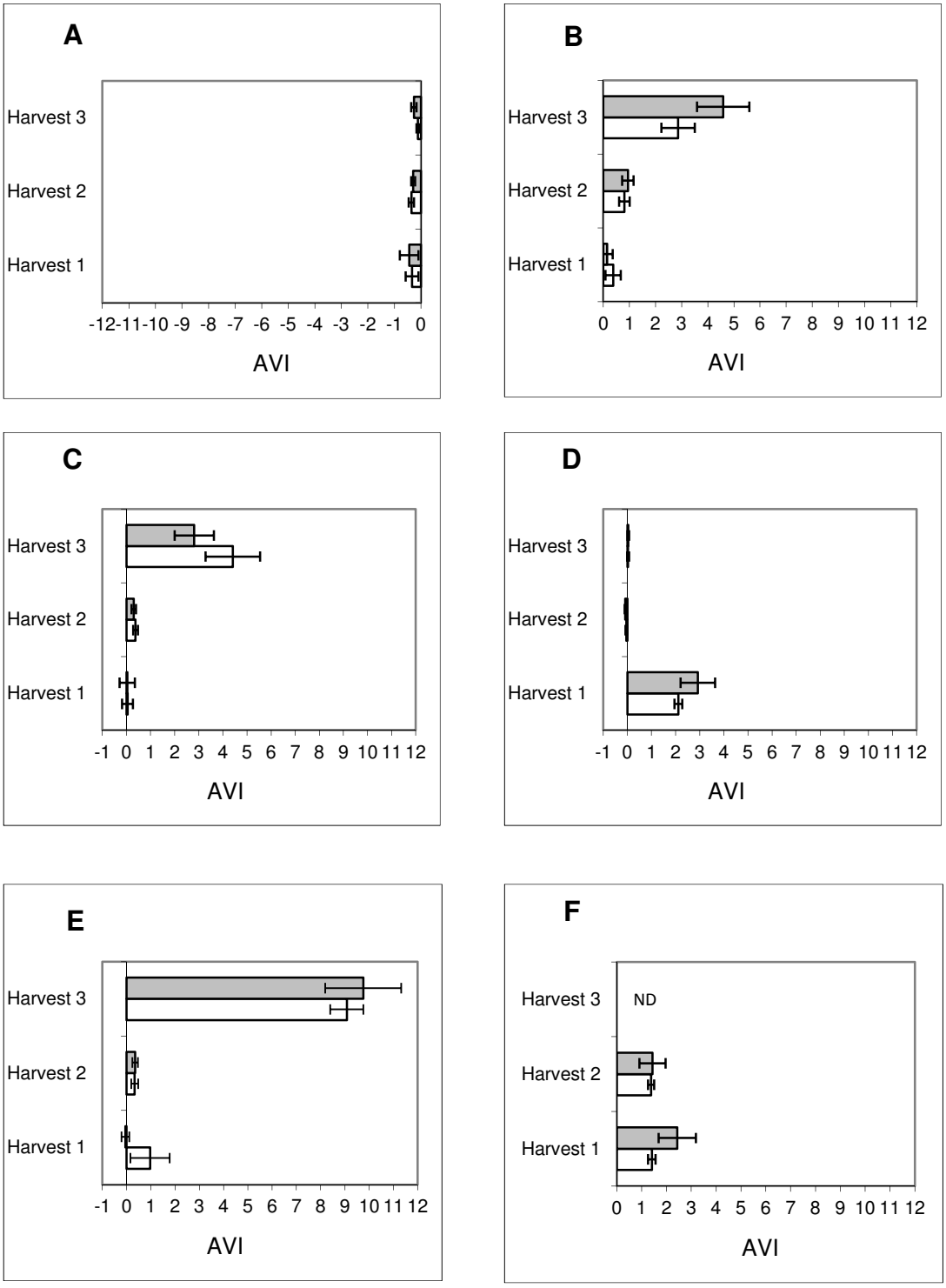

Fig. 8. Change in the apple violaxanthin cycle index (AVI) of sun-exposed apple fruit peel after UV-B treatment. Harvest 1 = juvenile stage, Harvest 2 = mid-season, Harvest 3 = mature stage. A = 'Granny Smith'; B = 'Braeburn'; C = 'Fuji'; D = 'Golden Delicious'; E = 'Cripps' Pink'; F= 'Topred'. White = Before; Dark grey = After, ND = No data. Means and standard errors are indicated.

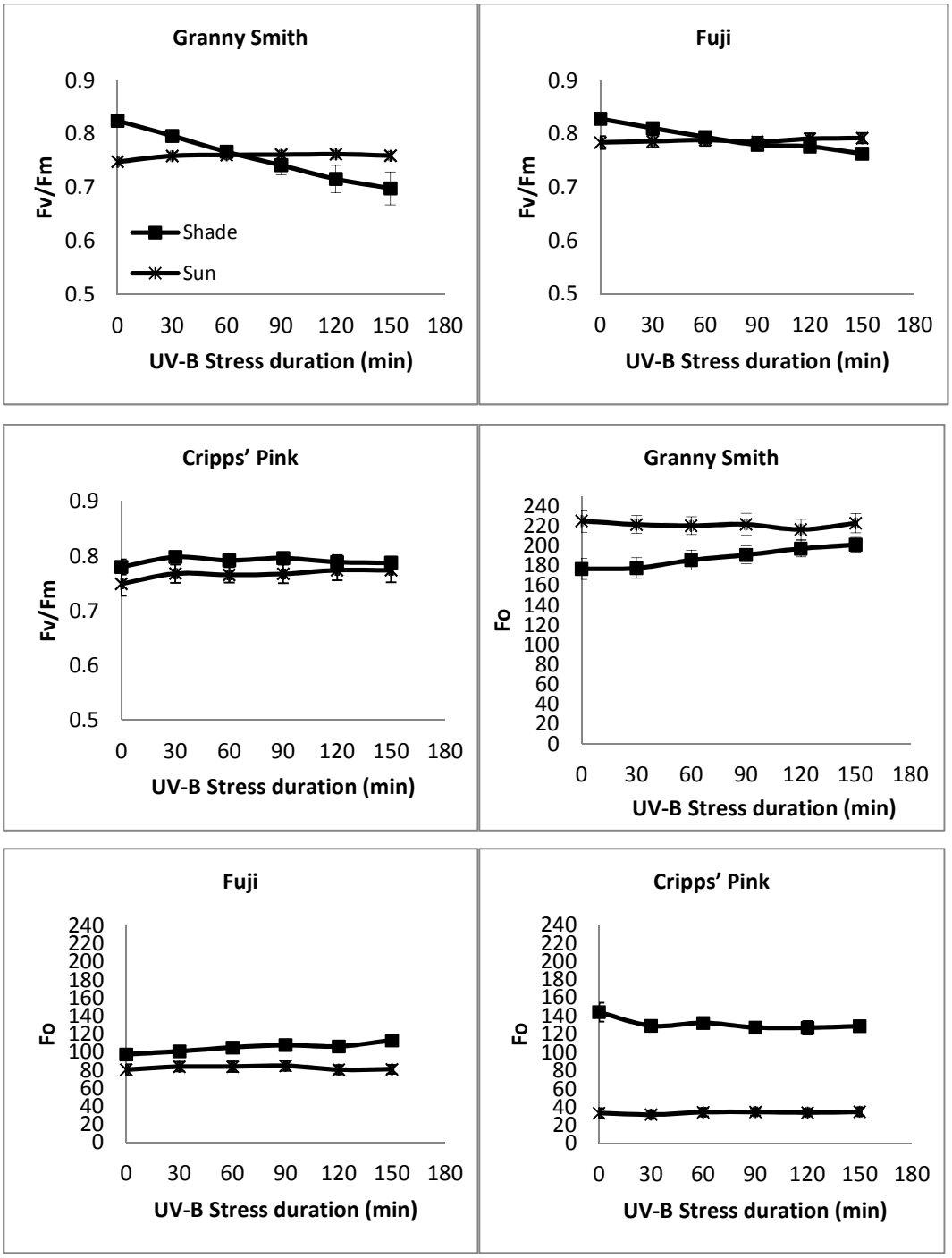

Fig. 9. Maximum light use efficiency of photosystem II (F_v/F_m) and minimum fluorescence (F_o) of shaded and sun-exposed apple fruit peel during UV-B treatment at maturity. Means and standard errors are indicated.

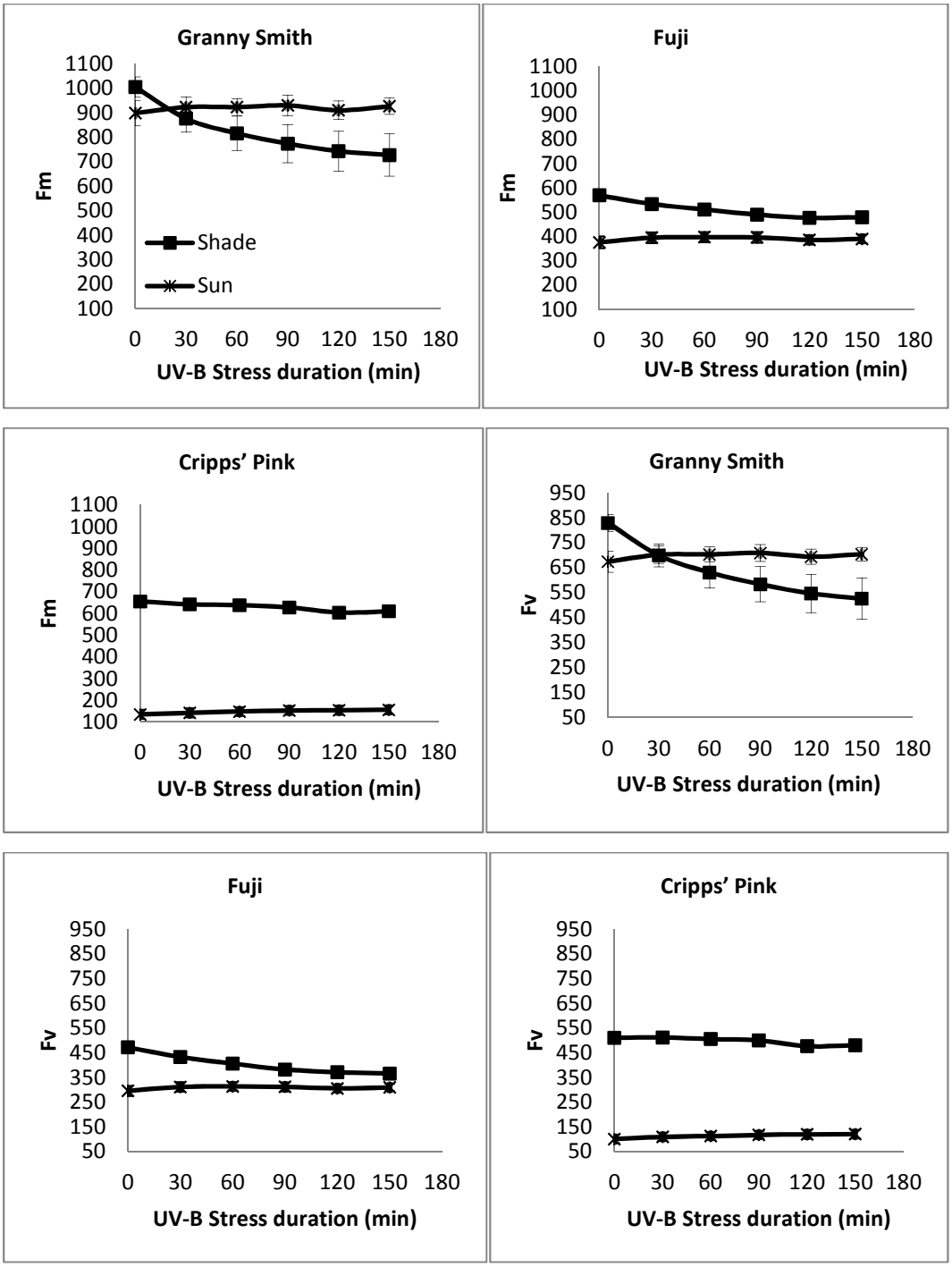

Fig. 10. Maximum (F_m) and variable (F_v) fluorescence of shaded and sun-exposed apple fruit peel during UV-B treatment at maturity. Means and standard errors are indicated.

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PAPER 2

Response of apple (*Malus domestica* Borkh.) fruit peel photosystems to heat stress coupled with moderate photosynthetic active radiation at different fruit developmental stages

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Abstract

Damage to the photosystems of fruit peel of Granny Smith, Fuji and Cripps' Pink apple (*Malus x domestica* Borkh.) cultivars by heat stress of: 30, 35, 40, 45 and 50 ºC, coupled with a 500 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) was analysed under laboratory conditions at these fruit development

stages: 50, 95 and 150 days after full bloom. Photodamage was assessed by measuring the maximum light use efficiency of photosystem II (F_v/F_m) at each fruit development stage. The critical temperature for photodamage was approximately 45 ºC. The initial F_v/F_m before stress of apple fruit peels did not change during fruit development. The thickness of the epicuticular wax and ratio of chlorophyll a/b of the peel increased while stomata density, concentrations of total phenolic, carotenoid and chlorophyll decreased during fruit development. There was no significant correlation between both the biochemical and anatomical features of fruit peel and high temperature stress induced change in F_v/F_m. There was no difference in the susceptibility of photosystems of fruit peel to high temperature stress among all the fruit development stages. These results show that photosystems of apple fruit peel remain equally susceptible to heat stress, and heat stress related damage, throughout fruit development.

Keywords: Heat, Apple, Sunburn, Wax, Stomata, Fluorescence

Abbreviations: F_v/F_m, maximum light use efficiency of PS II; F_o, minimum fluorescence; F_m, maximum fluorescence; F_v, variable fluorescence; PAR, photosynthetic active radiation; CFE, chlorophyll fluorescence excitation; LHCII, Light harvesting complex II; PS II, photosystem II

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1. Introduction

Sunburn on apple fruits is induced by exposure of fruits to both high temperatures (45 – 49 ºC) and sun light during fruit growth (Schrader et al.,

2001; Wünsche et al., 2004; Schrader et al., 2008). Heat and high light stress has been reported to induce fruit sunburn by damaging fruit peel photosystems, which changes fruit colour and results in reduced fruit quality (Wünsche et al., 2001; Chen et al., 2008). Apple fruits exposed to a 38 ℃ heat level showed an increase in respiration, membrane permeability, and chlorophyll degradation (Lurie and Klein, 1990). Heat induced photodamage is generally caused by damage to the Calvin cycle and inactivation of the oxygen evolving complex (Smillie, 1992; Yamane et al., 1998) while light induced photodamage is due to the production of reactive oxygen species (ROS) that damages the photosystems (Aro et al., 1993). The presence of pigments and phenolic compounds such as anthocyanin and other flavonoids, carotenoids including xanthophylls and other antioxidants can protect fruit peel against both heat and light stress (Kondo et al., 2002; Neill and Gould, 2003; Treutter, 2006; Agti et al., 2007; Steyn et al., 2009; Jahns and Holzwarth, 2012). However, red apple fruits, having high anthocyanin concentrations, have been reported to have higher fruit peel temperatures than green-coloured apple fruits (Ferguson et al., 1998). The higher fruit peel temperatures could make red apple fruit varieties more susceptible to photodamage induced by solar radiation stress, although this damage can also be less visible on red than on green fruit varieties. The sun exposed peel of apple fruit also has more photoprotective pigments and antioxidants compared to shaded peel (Ma and Cheng, 2003). Fruit transpiration via stomata can reduce fruit surface temperature, although fruit epicuticular wax may reduce this cooling effect by reducing open stomata density (Blanke and Lenz, 1989; Heredia, 2003). The increase in fruit wax content during fruit

growth combined with decreasing stomata density has been reported to reduce fruit peel permeability (Blanke and Lenz, 1989). This can reduce the ability of the fruit to cool down when exposed to high temperatures, thereby increasing heat stress. The biochemical and anatomical characteristics of apple fruit peels therefore can influence heat stress susceptibility of fruit peel photosystems. However, there are to date limited studies on the correlation between fruit biochemical and anatomical features with changes in their photosynthetic capacity in response to heat and light stress. There are also limited studies on apple fruit peel anatomical features and how they change during fruit development.

Solar radiation induced injury on apple fruit peels is reported to increase with fruit development, with fruits at maturity considered to be most susceptible (Glenn et al., 2002). However, the specific period during apple fruit development when fruit peel photosystems become most susceptible to solar radiation has not been resolved. Furthermore, although the critical temperature for sunburn induction on apple fruit is reported to be 45 ºC (Schrader et al., 2001), literature on the critical temperature for photodamage of apple fruit peel is limited. An understanding of the response of fruit peel photosystem to sunburn inducing factors during fruit development is important in determining which stage of fruit development is most susceptible to the different factors.

The objectives of this study were to: (1) determine the difference in heat stress susceptibility of photosystems of apple fruit peel at different fruit

development stages; (2) determine if there is a correlation between both the biochemical and anatomical characteristics of apple fruit peel and the heat stress-induced changes in the maximum light use efficiency (F_v/F_m) of the peel; and (3) determine the critical temperature for photodamage of the photosystems of apple fruit peel.

2. Materials and methods

2.1. Plant material and treatments

Three apple cultivars, Granny Smith, Fuji and Cripps' Pink, were used to study the seasonal response of photosystems in fruit peel to heat stress. Fruits from each cultivar were harvested approximately at the following development stages: 50 (57±8), 95 (101±4) and 150 (154±7) days after full bloom. Fruits were randomly collected from farms in the Grabouw area (34º9'10.55''S; 19º1'47.62''E) in the Western Cape province of South Africa which has Mediterranean-type climate. Fruits were stored over night at -0.5 ºC and kept at 25 ºC for 2 h after removal from the cold rooms the following day, before the stress treatments. A total of 56 fruits were randomly collected from 14 trees per cultivar at each harvesting period and used as follows: 30 fruits for the heat stress induced photodamage analysis and for subsequent post-stress biochemical analysis (total carotenoids and chlorophyll; six fruits per each of the five heat levels); six fruits for peel anatomical analysis (stomata density and epicuticular wax thickness); 20 fruits for pre-stress biochemical analysis (total anthocyanin, phenolics, carotenoids, chlorophyll and

antioxidants; four replicates with five fruits each). Fruit peels used for the different biochemical analysis were milled and stored at -80 ℃ before the analysis.

A fruit disk of 12 mm diameter and 30 mm height was collected from midway between the stem and calyx ends on the previously sun exposed side of each fruit. Six fruit disks per cultivar were placed under each heat treatment. The disks were randomly placed in distilled water under the lamps (see description in next paragraph) in a white foam cuvette holder directly after being extracted from the fruits. The fruit peel was at least 5 mm above the water level in the cuvette holder. Fluorescence measurements were taken at three stages: before and after exposure, and 12 h after dark adaptation at room temperature (20 ºC).

Peel disks from the previously sun exposed sides of the fruit were exposed to heat stress coupled with a moderate constant photosynthetic active radiation (PAR) of 500±50 µmol m^{-2} s^{-1} (measured with a quantum meter: LI-189; Li-Cor, Lincoln, Nebraska, USA) for 3 h. Measured maximum ambient PAR levels in the orchard were about 1500 – 2000 µmol m^{-2} s^{-1}. The PAR level used in our study therefore represents moderate but non-stressful light levels. The heat stress treatments were: 30 ºC (32 ºC±0.171), 35 ºC (37 ºC±0.250), 40 ºC (42 ºC±0.502), 45 ºC (46 ºC±0.108), and 50 ºC (51 ºC±0.187).

The heat treatments cover the range of temperatures below and above the reported sunburn inducing heat level of 45 ºC (Schrader et al., 2001). The

temperature of fruit peel was measured every 60 min with a hand held infrared thermometer (Ranger MX4, Raytek Corporation, Santa Cruz, USA). It took approximately 10 – 15 min for the fruit peels to reach the target temperatures. PAR was provided by two lamps (50W/12V, 350–1000 nm, 700 nm peak, Titan Halogen Dichroic with a UV filter, OSRAM Gmbt. Augsburg, Germany). The PAR lamps were placed on either sides of a central infrared light lamp (175 W, 300–4000 nm, 1000 nm peak, PAR 38IR175R, Philips, Amsterdam, The Netherlands). The base of the infrared lamps was placed at the following heights above the fruit peels to induce different fruit peel temperatures: 135 cm (30 ºC); 115 cm (35 ºC); 95 cm (40 ºC); 65 cm (45 ºC); 55 cm (50 ºC).

2.2. Chlorophyll fluorescence

Fruit peel Chlorophyll *a* fluorescence at room temperature was measured with a FSM 1 fluorimeter (Fluorescence Monitoring system 1, Hansatech, Norfolk, UK). The fluorimeter was connected to the top half of a leaf-clip holder through a fiber-optic cable. The maximum (F_m) and minimum (F_o) fluorescence and maximum light use efficiency $F_v/F_m = (F_m-F_o)/F_m$ were measured. The initial (before stress) and after stress readings were taken after a 30 min dark adaptation, and the recovery readings were done after a 12 h dark adaptation period at room temperature (20 ºC).

2.3. Pigment and antioxidant activity analysis

Total phenolics were extracted from 100 mg frozen apple peel samples in 80% ethanol using Folin-Ciocalteu's phenol reagent and a standard curve created with gallic acid (Slinkard and Singleton, 1997). The concentration of total phenolics was determined by measuring absorbance of the extract solution at 750 nm with a spectrophotometer (UV-vis light spectrophotometer-Cary 50Bio, Varian Ltd, Walton-on-Thyme, London, UK) and calculated using the coefficients from the standard curve.

Anthocyanin was extracted from 2 g of milled fruit peel tissue in methanol (with 1% 3 mol l^{-1} HCl) and kept in the dark at 4 ^{o}C. The solution was stirred for 1 h and the extract was centrifuged at 10, 000 rpm for 10 min at 4 ^{o}C. The concentration of total anthocyanins was determined by measuring absorbance at 520 nm with a spectrophotometer (UV-vis light spectrophotometer-Cary 50Bio, Varian Ltd., Walton-on-Thyme, London, UK). The anthocyanin absorbance at 520 nm was corrected for the presence of chlorophyll by subtracting absorbance at 653 nm [Abs520 nm-(0.24xAbs653nm)] (Murray and Hackett, 1991).

Chlorophyll and carotenoid of fruit peel were analyzed before and after stress exposure. Chlorophyll was extracted from 0.5 g tissue in 3 ml acetone at 4 ℃ by stirring for 24 h. The extract was centrifuged at 10 000 rpm for 15 min, and the supernatant filtered through a 0.45 µm filter. Chlorophyll concentration was determined by measuring absorbance at 470, 645 and 662 nm with a

spectrophotometer (Cary 50 conc UV-vis spectrometer, Varian Medical Systems, Inc., Palo Alto, CA, USA). The concentration of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid concentrations were determined according to Lichtenthaler (1987), with 100% acetone as the blank. Total antioxidant activity of apple peel was determined as described by Hamadziripi *et al.* (2014).

2.4. Anatomy analysis

Stomata density of the fruit peel was analysed by modifying a method described by Wilson *et al.* (1981). Microscopic slides of previously sun-exposed fruit peel surface were made from an imprint made using a cyanoacrylate clear adhesive ('Super glue''/Bostik Blits Stik, Bostik, Cape Town, South Africa). A 1 cm^2 disk was collected from the previously sun exposed fruit peel for the analysis. The glue was applied onto a microscope slide and the disk placed on it with the peel side down on the slide. The disk was pressed down on the glue for 3 min, peeled off to leave an imprint on the slide. The imprint was used to study fruit peel stomata and trichome density under a light microscope. Three slides (representing one fruit each) with two imprints from each fruit were made for each cultivar per harvest stage. For epicuticular wax thickness analysis, another three fruits per cultivar per harvest stage were used. Two 1 cm long cross sections were cut from each fruit and dyed with Toaline blue for 1 min. The thickness of the epicuticular wax was measured under a light microscope.

2.5. Statistical analysis

Statistical differences in each cultivar between the Fv/Fm before stress (initial), after stress and after recovery from the different heat levels at each harvest stage were analysed using a one way ANOVA with SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The difference in the percentage change from initial to recovery F_v/F_m (%$\Delta F_v/F_m$) between the different harvesting periods for each heat level and cultivar was also analysed using a one way ANOVA with SAS 9.1. The %$\Delta F_v/F_m$ was log transformed for the analysis. Means and +/- standard errors are indicated on the graphs. Linear regression analysis comparing %$\Delta F_v/F_m$ to initial fruit peel pigments and anatomical features were done with Microsoft Excel (Windows Microsoft Excel 2010, Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Effect of heat stress on F_v/F_m during fruit development

There was no significant diferrence in the percentage decrease of the F_v/F_m between the different harvesting periods after the 45 and 50 ºC heat stress (Table 1). However, fluorescence readings of 'Cripps' Pink' fruits after the 45 and 50 ºC stress at maturity could not be recorded due to the low chlorophyll concentration after these heat stress levels. The 30–40 ºC heat stress caused variable effects on the F_v/F_m at the different fruit developmental stages of the

cultivars tested. The percentage decrease in F_v/F_m was lower after the 30–40 ºC heat stress compared to after the 45 and 50 ºC heat stress (Table 1).

The 45 and 50 ℃ heat stress caused irreversible damage to the F_v/F_m at all fruit development stages in all the cultivars tested, while 30–40 ℃ stress did not (Fig. 1A-C). There was no difference between the effects of the 30-40 ℃ heat stress on the recovery F_v/F_m at all the fruit developmental stages (Fig. 1A-C). However, the 40 ℃ stress caused a significantly higher F_v/F_m reduction directly after stress at mid-season, in all the cultivars tested, compared to the 30 and 35 ℃ stress.

3.2. Correlation of biochemical and anatomical characteristics of fruit peel to heat induced change in F_v/F_m

There were only a few significant correlations between the initial biochemical and physical characteristics of fruit peel and the percentage change in F_v/F_m (Table 2), despite the high R^2 obtained in some cases. ‘Granny Smith’ had a significant correlation between total phenolic concentration and $\%\Delta F_v/F_m$ at the 30 ºC stress (p = 0.041). ‘Fuji’ had a significant correlation between anthocyanin concentration and $\%\Delta F_v/F_m$ at 50 ºC stress (p = 0.004), and in ‘Cripps’ Pink’ there was a significant correlation between both anthocyanin and total chlorophyll concentration and $\%\Delta F_v/F_m$ at the 35 ºC stress (p = 0.004). There was also a significant correlation between the chlorophyl a/b ratio and $\%\Delta F_v/F_m$ at 40 ºC for ‘Cripps’ Pink’ (p = 0.045).

3.3. Heat stress induced changes in pigment concentration of fruit peel

There was a significant reduction in the carotenoid and total chlorophyll concentration after the different heat treatments at all the fruit development stages (Fig. 2). The highest reduction occurred after the 50 ºC heat stress for all the cultivars. Total chlorophyll and carotenoid concentrations before stress decreased during fruit development in all the cultivars, although the reduction was higher in 'Cripps' Pink' and 'Fuji' fruit peel compared to 'Granny Smith' fruit peel. The chlorophyll *a/b* ratio generally increased after all the treatments at the juvenile stage for all three cultivars (Fig. 3). At mid-season this ratio was reduced except after 45 ºC in 'Granny Smith' and after 45 and 50 ºC in 'Fuji' fruit peel. At maturity the ratio was also reduced in 'Fuji' and 'Cripps' Pink' fruit peel except after the 50º C stress. However, in 'Granny Smith' fruit peel the ratio was increased after the different treatments at maturity, except after 45 ºC. The chlorophyll *a/b* ratio before stress increased in all the cultivars during fruit development while the total chlorophyll concentration decreased.

3.4. Changes in fruit peel anatomy and biochemistry during fruit development

Stomata density decreased from the juvenile to mid-season fruit development stage, and then remained relatively constant to the mature stage in all cultivars (Fig. 4). Stomata density of 'Cripps' Pink' fruit peel decreased from the juvenile stage to mid-season and maturity by 78 and 89% respectively. For 'Granny Smith' this change was 60 and 80% respectively and for 'Fuji' 75% at both harvesting stages. Epicuticular wax thickness continued to

increase from the juvenile to the mature fruit development stage (Fig. 4). For ‘Fuji’ fruit peel, wax thickness increased from the juvenile stage to mid-season and maturity by 10 and 24% respectively and for ‘Granny Smith’ by 21 and 29% respectively. However, for ‘Cripps’ Pink’ wax thickness was significantly higher at both the mid-season and mature fruit development stage compared to the juvenile stage. The wax thickness on ‘Cripps’ Pink’ fruit was 32% and 29% respectively higher at the mid-season and mature development stages than the juvenile stage.

The total phenolic concentration significantly decreased in all cultivars from the juvenile to the mature fruit development stage (Fig. 5). The anthocyanin concentration of ‘Granny Smith’ fruit peel decreased with maturity (Fig. 5). In ‘Fuji’ and ‘Cripps’ Pink’ fruit peels anthocyanin concentration at the juvenile and mid-season growth stage were similar but significantly increased at maturity. Water soluble antioxidants concentrations in ‘Fuji’ and ‘Cripps’ Pink’ fruits continuously decreased with fruit maturity (Fig. 6). However, lipid soluble antioxidants concentrations decreased from the juvenile to mid-season fruit development stage, but increased again at the mature stage (Fig. 6). No data was available for the antioxidant concentrations of ‘Granny Smith’ fruits.

4. Discussion

The susceptibility of previously sun exposed apple fruit peel photosystems to high temperature stress combined with moderate light stress levels remains similar at all fruit development stages. There was no significant difference in

the percentage reduction of F_v/F_m after the sunburn inducing 45 ℃ heat stress level between the different fruit development stages (Table 1). However, the percentage change in F_v/F_m after the 30–40 ℃ heat stress levels during fruit development varied between cultivars (Table 1). The 45 ℃ heat stress level is involved in the induction of sunburn on fruit peels as it enhances fruit peel photodamage in the presence of light and UV-B radiation (Schrader et al., 2001; Chen et al., 2008). Any differential susceptibility of fruit peel photosystems to the 45 ℃ heat stress during fruit development can therefore potentially indicate the period at which fruits become more susceptible to sunburn. Thermal stability in leaves of Elm seedlings increases with leaf maturity, reaching a maximum in fully expanded mature leaves (Jiang et al., 2006). A change in heat stress susceptibility during fruit development could be related to changes in the sunlight use efficiency of fruit peel photosystems. Greer et al. (1997) found that the F_v/F_m of apple tree leaves does not change during the season. Our results also show that the pre-stress F_v/F_m of apple fruit peel remains constant during fruit development. Therefore the response of fruit peel photosystems to sun radiation induced stress is likely to be similar during fruit development. This is confirmed by the observed similarity in the percentage change of F_v/F_m after high temperature stress at the different fruit development stages. The previously sun exposed apples therefore potentially remain equally susceptible to sunburn development throughout fruit growth.

The critical temperature for photodamage in previously sun exposed apple fruit peel appears to be around 45 ºC, which also has been reported by

Schrader et al. (2001) to be the critical temperature for the development of fruit peel sunburn. Sunburn is reported to be a result of heat and light stress-induced damage to fruit peel photosystems, as sunburned peels are found to have significantly lower F_v/F_m compared to non-sunburned peels (Chen et al., 2008). The critical temperature for damage to barley leaves photosystem II (PSII) is reported to be between 40–50 °C (Lípová et al., 2010) or specifically at 46 °C (Lazár and Ilík, 1997). Wand et al. (2008) also found the critical temperature for damage to apple fruit peel photosystems exposed to heat stress in the dark to be around 48–53 °C. Peel temperatures above 50 °C, or constant long term exposure to temperatures above 45 °C, cause visible damage to apple fruit peel and the affected area turn brown (Lurie, 1998; Racsko and Schrader, 2012). The 45 and 50 °C heat treatment in our study also damaged fruit peel photosystems while the 30–40 °C treatments did not (Fig. 1). However, no visible damage was observed after the 3 h stress period. Our results are in agreement with previous studies in establishing the 45 °C heat stress as the critical temperature for photodamage on apple fruit peels. As the 45 °C photodamaging critical temperature is similar to the reported critical temperature for fruit peel sunburn development (Schrader et al., 2001), it is therefore a further possible indication that heat-induced photodamage is involved in fruit sunburn development.

Fruit peel pigment concentration can modulate the effect of heat and high light stress on the peel photosynthetic systems. Heat stress damage the photosystems by damaging chloroplast membranes (Sharkey, 2005) while also inhibiting the oxygen evolving complexes (Chen et al., 2008) and Calvin

cycle enzyme activities (Salvucci et al., 2001). Light stress induces photodamage by causing the production of ROS that damage molecules of the photosystems (Aro et al., 1993). Heat stress can also cause photodamage through the production of ROS in stressed chloroplasts (Ogweno et al., 2009). Phenolic compounds and carotenoids have photoprotective functions either as screening agents of solar radiation or as antioxidants (Middleton and Teramura, 1993; Telfer, 2002; Drogoudi et al., 2008). The presence of these compounds should therefore help reduce photodamage during heat and high light stress conditions. Red 'Anjou' pears had a high thermal tolerance when exposed to a combined high light and high temperature stress, while the light use efficiency (F_v/F_m) of green 'Anjou' pears was negatively affected under the same conditions (Li and Cheng, 2009). Similarly, the high anthocyanin concentration in 'Fuji' and 'Cripps' Pink' fruits at maturity that we observed could help reduce light stress in these fruits at this fruit development stage. Osmolytes and the lipid composition of thylakoid membranes can further influence the heat stress resistance of plant cells (Sharkey, 2005). Apple fruits also increase their heat shock protein synthesis when their core temperatures are increased (Ferguson et al., 1998). Merzlyak et al. (2008) found that there was a strong negative correlation between chlorophyll fluorescence excitation (CFE) spectra and total flavonols and anthocyanin concentration in non-stressed apple fruits. This correlation indicates that there is a light absorption competition between chlorophyll and both flavonols and anthocyanin which then can affect the light use efficiency of the apple photosystems. In addition, Merzlyak *et al.* (2008) also found no correlation between the CFE spectra and carotenoid and chlorophyll concentrations. In our study, however, there were

few statistically significant correlations between the heat stress induced percentage reductions of F_v/F_m with either biochemical or anatomical features, especially at temperatures >40 ºC (Table 2). Our results show that the ability of fruit peel pigment to reduce heat and light stress could be limited. Chen et al. (2008) also showed that although the xanthophyll cycle pigments and other antioxidants are higher in sunburned apple peel compared to undamaged peel, they were unable to prevent the associated photodamage in sunburned peel.

The peels of previously sun exposed juvenile apples, in contrast to mature apples, have the ability to increase photoprotective mechanisms after heat and light stress. Chlorophyll *a/b* ratio of juvenile fruits was increased by a majority of the heat stress levels, coupled with a moderate light stress, and only by 50 ℃ in mature fruits of all three cultivars tested (Fig. 2). Light stress can cause an increase of the chlorophyll *a/b* ratio (Katajima and Hogan, 2003). In addition, heat stress can affect the susceptibility of plants to light stress and vice versa (Yamane et al., 1998; Chen et al., 2009). An increase in the chlorophyll *a/b* ratio, as also recorded for apples by Li and Cheng (2009), is correlated with a decrease in the amount of light harvesting complexes of photosystem II (LHCII) (Lindahl et al., 1995). Green and Durnford (1996) reported that the LHCII has a higher chlorophyll *b* concentration than LHCI of PSI, and LHCII therefore has a low chlorophyll *a/b* ratio. The chlorophyll *a/b* ratio is also negatively correlated to total chlorophyll concentration (Kitajima and Hogan, 2003). Light stress induced loss of LHCII is correlated with an increase in the photoprotective xanthophyll cycle activities (Polle et al., 2001).

Chlorophyll *a* is more efficient at transferring excitation energy to xanthophylls than chlorophyll *b* (Kleima et al., 1999). The increased chlorophyll *a/b* ratio after heat and light stress in previously sun exposed juvenile fruit peels therefore indicates a loss of LHCII, a decrease in total chlorophyll concentration and an increase in the xanthophyll cycle as photoprotective mechanisms. However, the decreased chlorophyll *a/b* ratio in mature fruit peels after heat and light stress indicate that these fruits do not depend on the xanthophyll cycle for photoprotection and possibly increase light harvesting capacities to minimise the stress effect.

The susceptibility of plant parts to any specific stress will be influenced by biochemical and anatomical characteristics of the specific plant part. Apple fruit cuticle and wax layer thickness increases with fruit maturity (Ju and Bramlage, 2001). However further wax production is reported to decrease during plant maturity (Heredia, 2003). The frequency of stomata on the apple fruit surfaces decreases as the fruit enlarges and the amount of open stomata decrease with maturity as they are converted to open or wax filled closed lenticels (Blanke and Lenz, 1989). Plant epicuticular wax reduces leaf gas exchange, transpiration and water loss (Heredia, 2003). Wax-covered leaves of *Leucadendron lanigerum* had a higher temperature than those without wax (Mohammadian *et al.*, 2007). Fruits at the juvenile stage with the high stomata density (Fig. 3) low wax thickness are therefore likely to have high evapotranspiration rates and maintain peel temperatures below the sunburn inducing temperature level. The observed decrease in fruit stomata density and increasing epicuticular wax thickness during fruit development (Fig. 3)

could therefore increase heat stress on fruit peel photosystems due to reduction in the transpiration potential. This can also increase the potential for light and heat stress induced sunburn development.

5. Conclusion

The critical temperature for heat induced photodamage of previously sun exposed apple peels is approximately 45 ºC. The similarity in threshold temperature for photodamage and sunburn development suggests that photodamage may be contributing or predisposing apple fruit peel to sunburn development. The measured biochemical and anatomical features of fruit peel did not appear to correlate with heat induced photodamage. The difference in sunburn susceptibility during fruit development generally observed under orchard conditions could therefore be related to factors other than heat and light stress induced photodamage. The pre-stress maximum light use efficiency of photosystem II in apple fruit peel remained similar during fruit development. Furthermore, apple fruit peel photosystems were equally susceptible to high temperature stress at all the fruit developmental stages. Sunburn susceptibility of apple fruits could therefore be potentially similar throughout fruit development.

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Table 1

Percentage change in the maximum light use efficiency of photosystem II (F_v/F_m), before stress to recovery, of apple fruit peel after exposure to different heat stress levels coupled with a constant light level of 500 ± 50 µmol m^{-2} s^{-1} for 3 h at different fruit developmental stages. Before stress F_v/F_m readings were taken after a 30 min dark adaptation period and the recovery readings after a 12 h recovery period in the dark at room temperature (20 ℃). There was no interaction between the harvesting periods and the heat treatments for each cultivar. Different letters next to values indicate significant differences between the harvesting periods for each cultivar at each heat level, α = 0.05. DAFB = Days after Full Bloom; ND = missing data.

Cultivars	Harvest period (DAFB)	Heat treatments				
		30 ºC	35 ºC	40 ºC	45 ºC	50 ºC
'Granny Smith'	50	-2 b	-3 a	-4 a	-40 a	-73 a
	95	-3 b	-1 a	-3 a	-57 a	-87 a
	150	-6 a	-2 a	-4 a	-46 a	-88 a
'Fuji'	50	-1 b	-1 b	-3 b	-45 a	-74 b
	95	-8 a	-9 a	-14 a	-56 a	-85 ab
	150	-3 b	-3 b	-4 b	-35 a	-92 a
'Cripps' Pink'	50	-4 ab	-4 a	-10 a	-53 a	-65 a
	95	-2 b	-2 ab	-5 ab	-64 a	-77 a
	150	-6 a	0 b	-2 b	ND	ND

Table 2

Linear correlation and *p* values between fruit biochemical and anatomical features compared to the percentage change in the maximum light use efficiency (F_v/F_m) after exposure to different heat stress levels at a constant light level of 500 ± 50 µmol m^{-2} s^{-1} (α = 0.05).

	30		35		40		45		50	
	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*	R^2	*p*
'Granny Smith'										
Phenolics	1.00	0.041*[a]	0.00	0.973	0.08	0.819	0.04	0.876	0.68	0.384
Anthocyanin	0.85	0.253	0.13	0.761	0.33	0.608	0.25	0.665	0.93	0.172
Carotenoids	0.99	0.072	0.02	0.914	0.01	0.932	0.00	0.989	0.51	0.497
Chl.[f] *a/b*	0.84	0.258	0.14	0.756	0.34	0.602	0.26	0.659	0.93	0.167
Total Chl.	0.90	0.203	0.11	0.783	0.01	0.936	0.04	0.879	0.30	0.629
Antioxidants:										
Water soluble	ND[g]	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lipid soluble	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Wax thickness	0.75	0.330	0.23	0.685	0.45	0.531	0.36	0.588	0.98	0.096
Trichome	0.06	0.849	0.95	0.136	0.81	0.290	0.87	0.233	0.18	0.725
Stomata	0.75	0.330	0.23	0.685	0.45	0.531	0.36	0.588	0.98	0.096
'Fuji'										
Phenolics	0.27	0.653	0.27	0.655	0.09	0.805	0.04	0.866	0.01	0.942
Anthocyanin	0.65	0.401	0.66	0.399	0.85	0.249	0.98	0.080	1.00	0.004*[b]
Carotenoids	0.16	0.739	0.16	0.741	0.03	0.890	0.11	0.781	0.05	0.857
Chl. *a/b*	0.02	0.908	0.02	0.906	0.14	0.756	0.61	0.427	0.50	0.503
Total Chl.	0.20	0.708	0.19	0.710	0.05	0.860	0.09	0.811	0.03	0.887
Antioxidants:										
Water soluble	0.15	0.745	0.15	0.747	0.03	0.897	0.12	0.774	0.05	0.850
Lipid soluble	0.82	0.276	0.82	0.278	0.61	0.427	0.14	0.756	0.23	0.680
Wax thickness	0.03	0.884	0.03	0.886	0.00	0.965	0.29	0.636	0.19	0.712
Trichome	0.07	0.831	0.07	0.833	0.00	0.983	0.22	0.688	0.13	0.764
Stomata	0.50	0.498	0.50	0.500	0.27	0.650	0.00	0.978	0.02	0.903
'Cripps' Pink'										
Phenolics	0.61	0.429	0.93	0.167	0.83	0.272	ND	ND	ND	ND
Anthocyanin	0.84	0.260	0.75	0.336	0.59	0.441	ND	ND	ND	ND
Carotenoids	0.42	0.552	1.0	0.045*[c]	0.95	0.150	ND	ND	ND	ND
Chl. *a/b*	0.15	0.746	0.95	0.150	1.0	0.045*[d]	ND	ND	ND	ND
Total Chl.	0.36	0.592	1.0	0.004*[e]	0.97	0.109	ND	ND	ND	ND
Antioxidants:										
Water soluble	0.01	0.938	0.74	0.342	0.87	0.236	ND	ND	ND	ND
Lipid soluble	0.02	0.904	0.50	0.500	0.66	0.395	ND	ND	ND	ND
Wax thickness	0.00	0.992	0.66	0.396	0.81	0.290	ND	ND	ND	ND
Trichome	0.39	0.573	0.07	0.831	0.17	0.726	ND	ND	ND	ND
Stomata	0.05	0.854	0.84	0.258	0.94	0.152	ND	ND	ND	ND

[a] Coefficients: *a* = 41.213; b = -16.793

[b] Coefficients: *a* = -107.431; b = 0.244

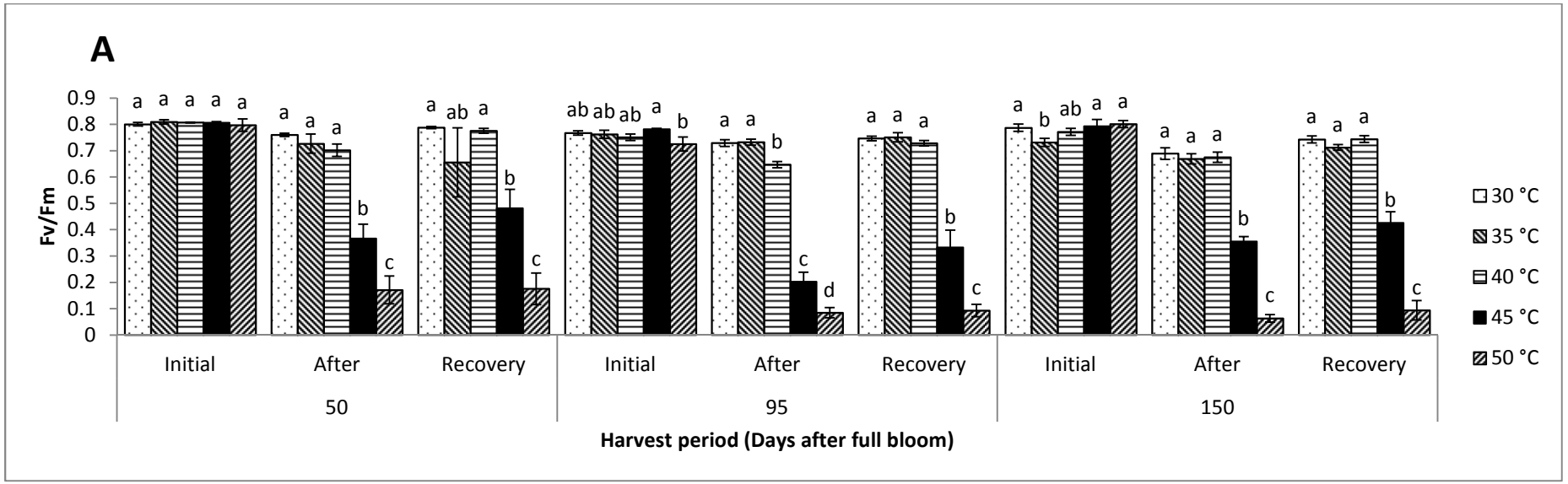
[c] Coefficients: *a* = 3.220; b = -0.143

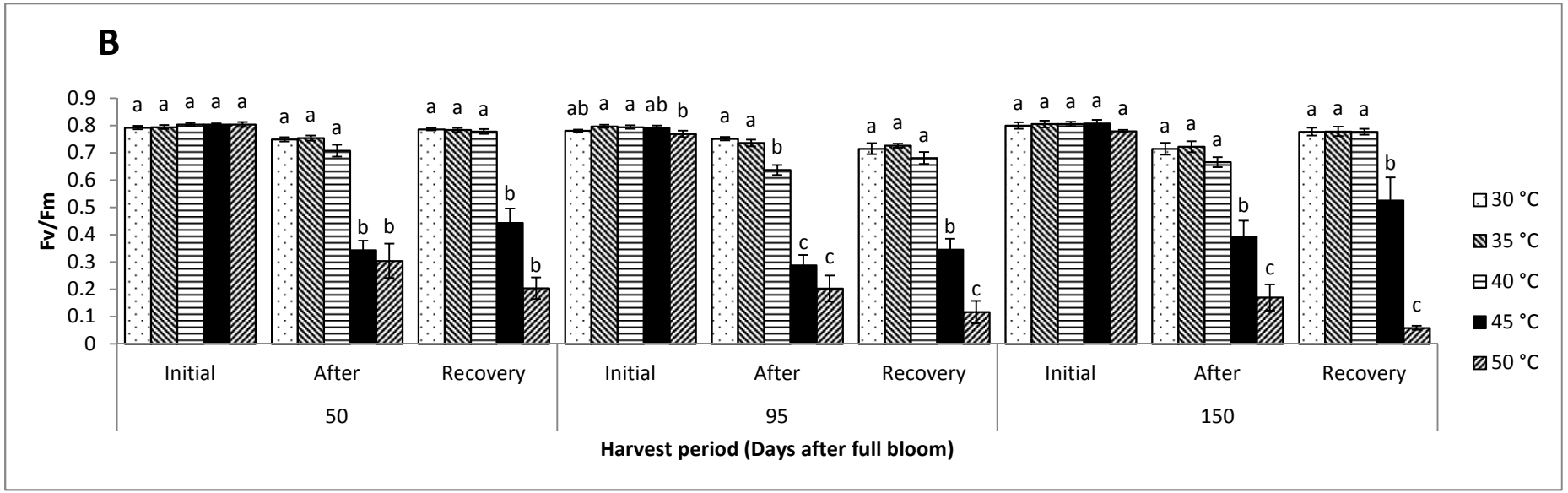
[d] Coefficients: *a* = -51.122; b = 14.110

[e] Coefficients: *a* = 1.532; b = -0.029

[f] Chl. = chlorophyll

[g] ND = No data


A
Fv/Fm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Initial
After
Recovery
50
95
150
Harvest period (Days after full bloom)
30 °C
35 °C
40 °C
45 °C
50 °C


B
Fv/Fm
0.9
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Recovery
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Harvest period (Days after full bloom)
30 °C
35 °C
40 °C
45 °C
50 °C

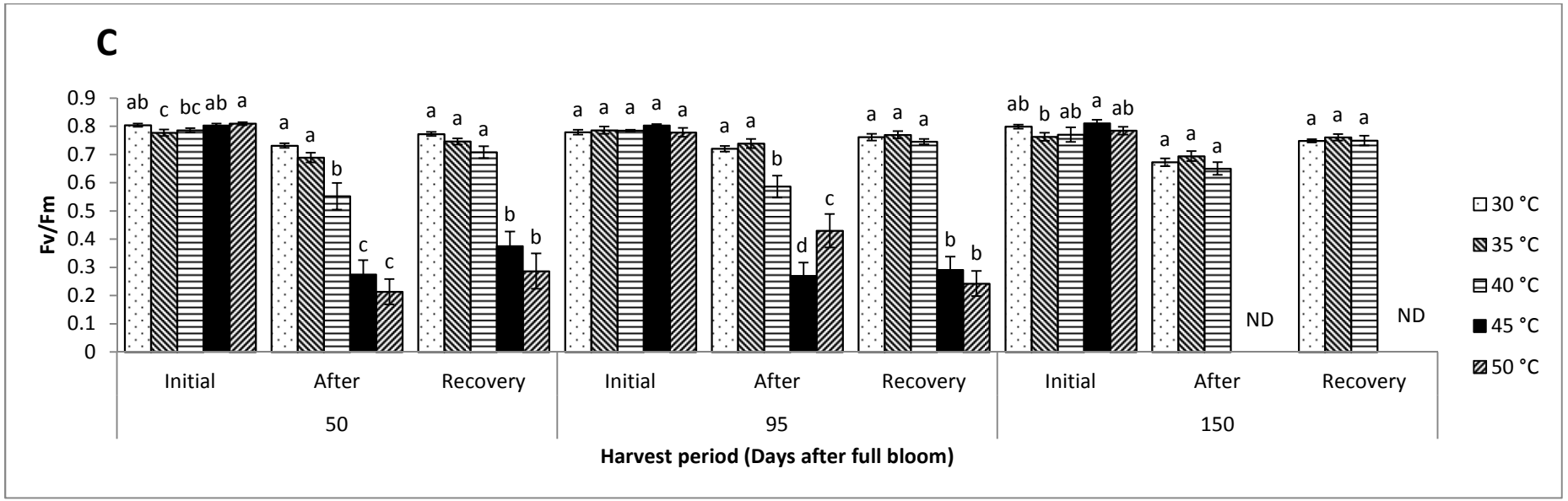

Fig. 1. The effect of different temperature stresses coupled with a constant light level of 500 ± 50 µmol m^{-2} s^{-1} on (A) 'Granny Smith', (B) 'Fuji' and (C) 'Cripps' Pink' apple fruit peel maximum light use efficiency (F_v/F_m) at different fruit developmental stages, stress duration was 3 h. Different letters indicate significant differences in F_v/F_m between different heat levels before stress (Initial), after stress (After) or at recovery (Recovery). ND = missing data (α = 0.05). The 'After' F_v/F_m readings were taken after a 30 min dark adaptation period and the 'Recovery' readings after a 12 h recovery period in the dark at room temperature (20 °C). Means and standard errors are indicated.

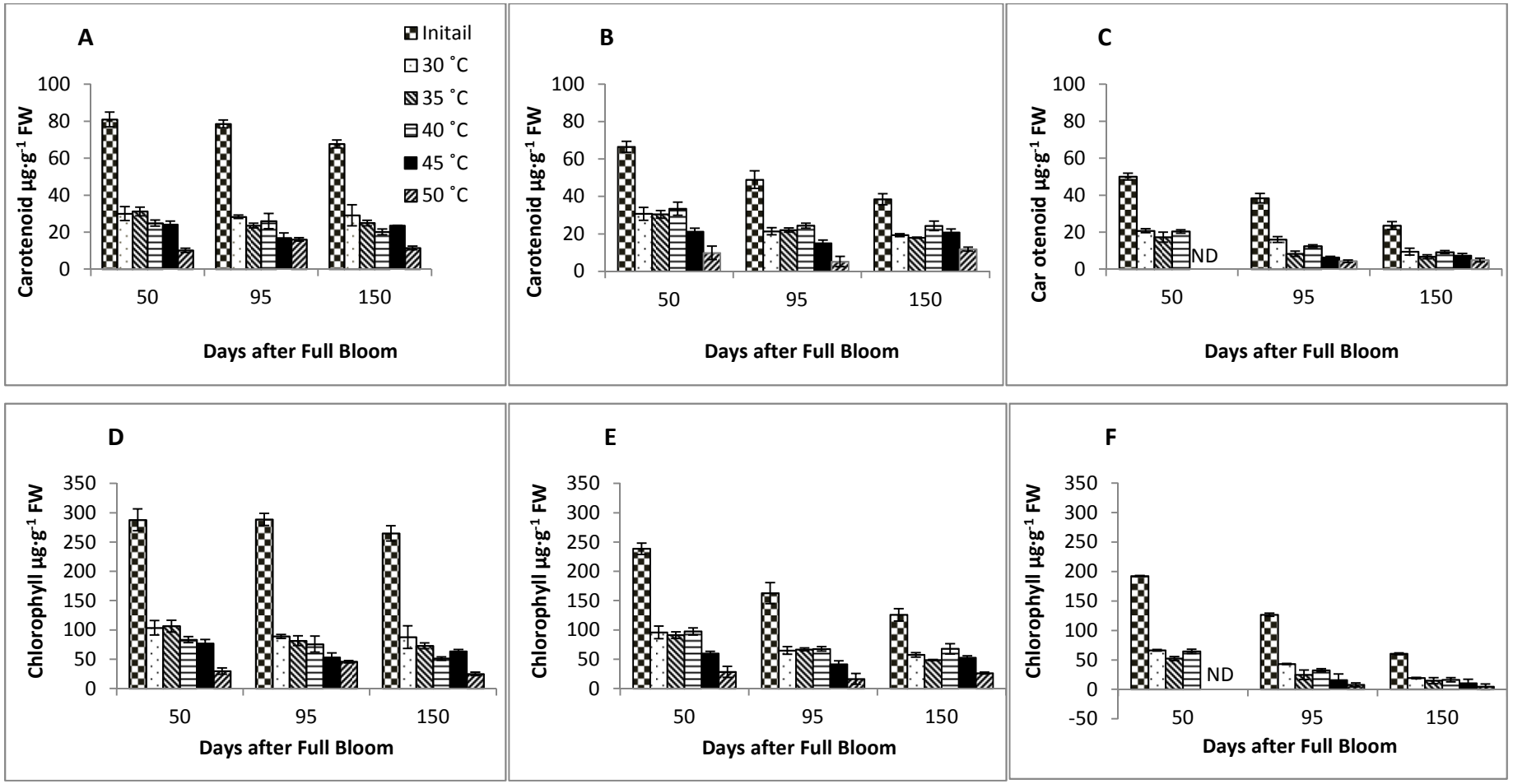

Fig. 2. Apple fruit peel total carotenoid and chlorophyll concentration before and after 3 h heat stress coupled with a constant light level of 500 ± 50 µmol m^{-2} s^{-1} at different fruit development stages. (A and D) 'Granny Smith', (B and E) 'Fuji', (C and F) 'Cripps' Pink'. ND = missing data. Means and standard errors are indicated.

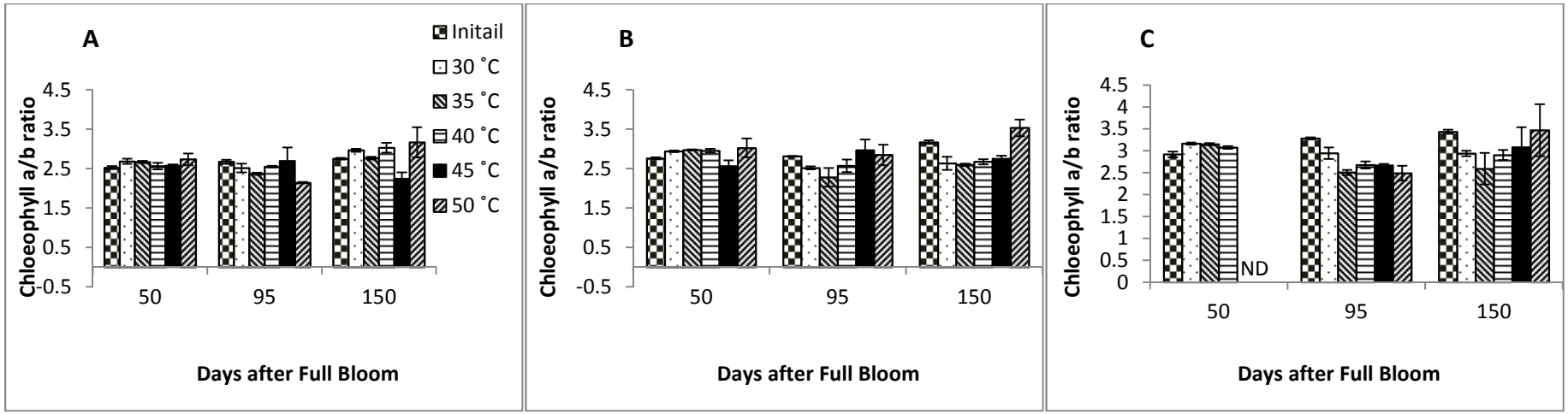

Fig. 3. Apple fruit peel chlorophyll *a/b* ratio before and after 3 h heat stress coupled with a constant light level of 500 ± 50 µmol m^{-2} s^{-1} at different fruit development stages. (A) 'Granny Smith', (B) 'Fuji', (C) 'Cripps' Pink'. ND = missing data. Means and standard errors are indicated.

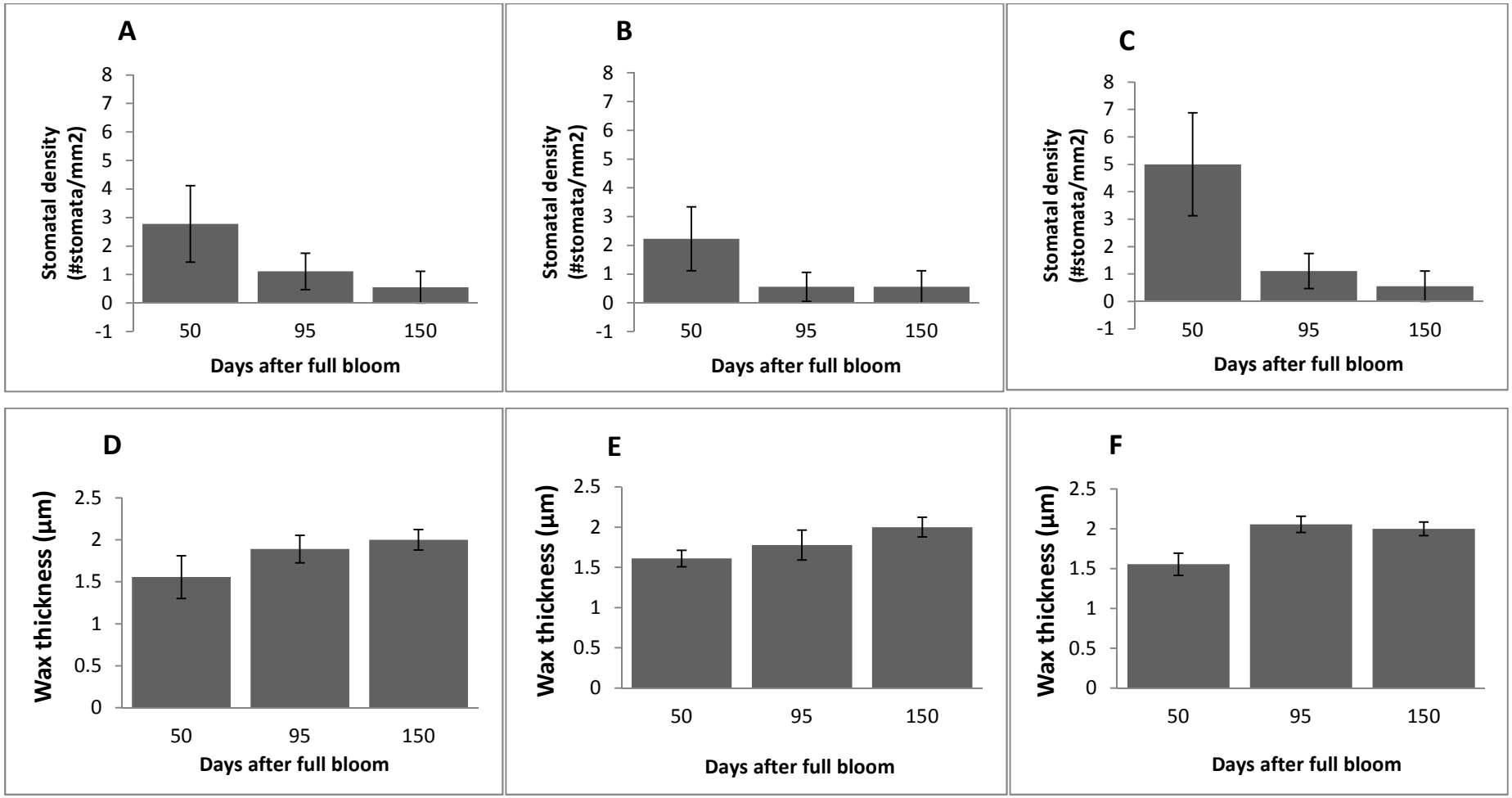

Fig. 4. Apple fruit peel stomata density (# stomata/mm^2) and epicuticular wax thickness (µm) at different fruit developmental stage. (A and D) 'Granny Smith', (B and E) 'Fuji', (C and F) 'Cripps' Pink'. Means and standard errors are indicated.

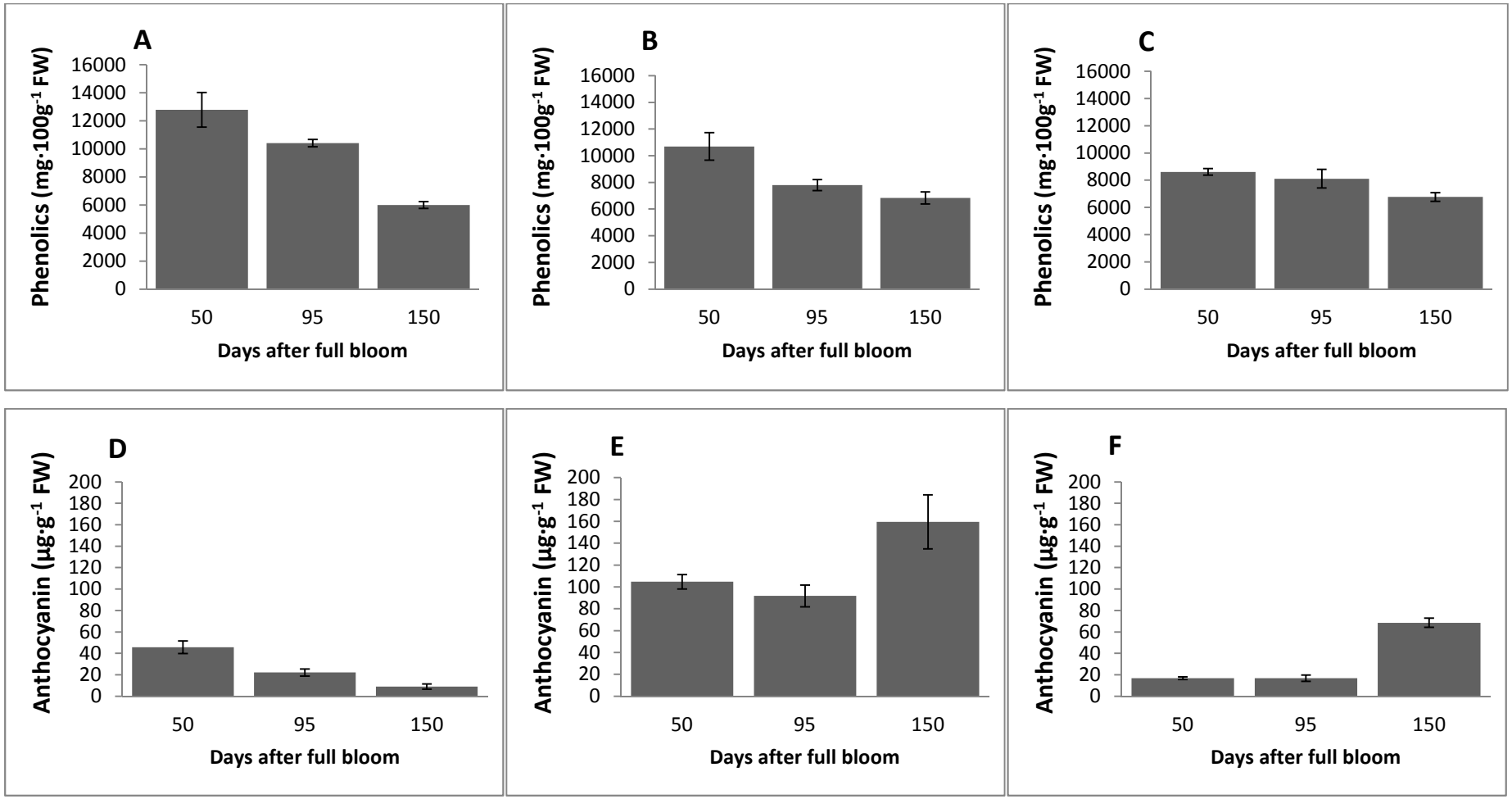

Fig. 5. Apple fruit peel total phenolic and anthocyanin concentrations during fruit development. (A and D) 'Granny Smith', (B and E) 'Fuji', (C and F) 'Cripps' Pink'. Means and standard errors are indicated.

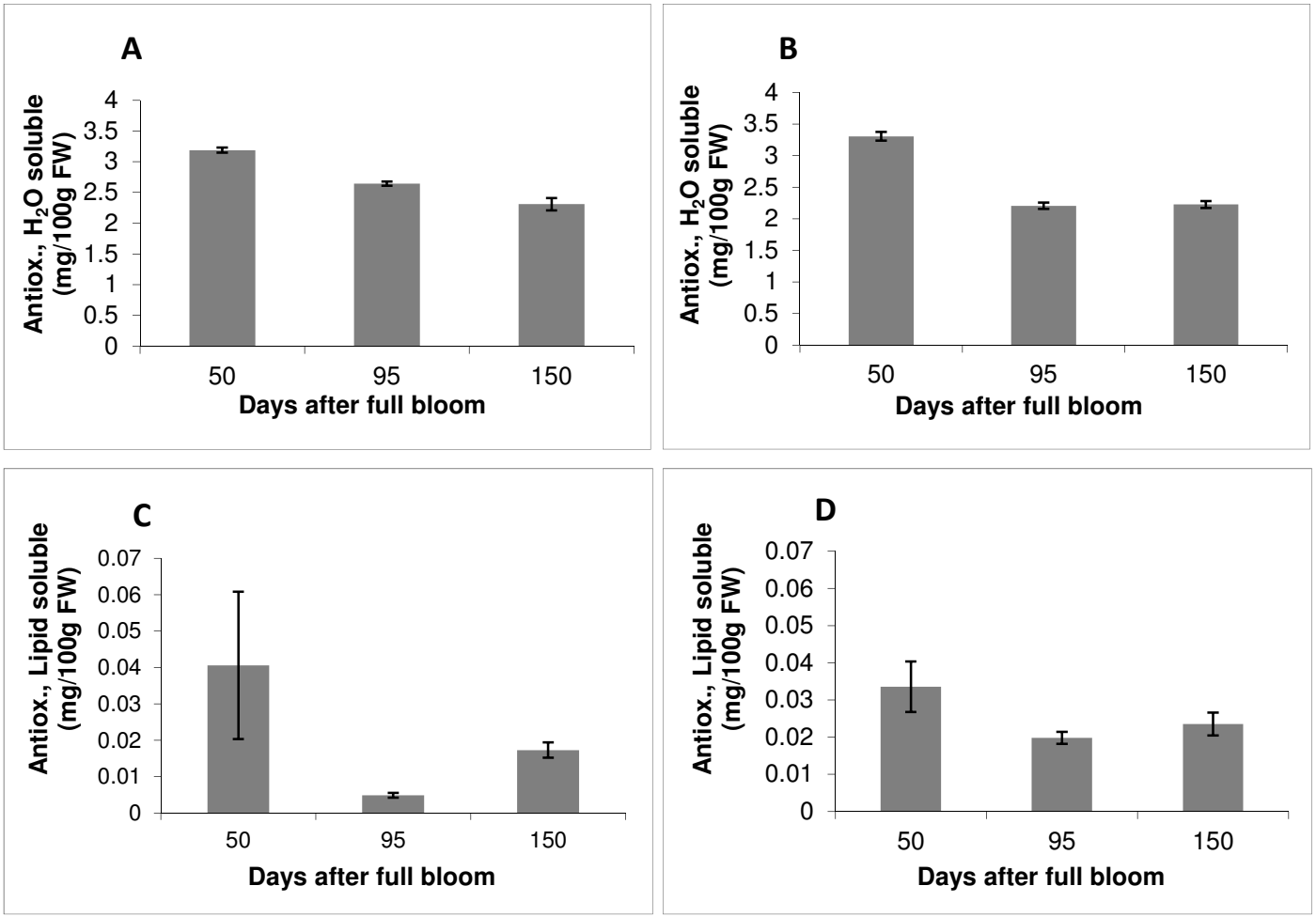

Fig. 6. Apple fruit peel water and lipid soluble antioxidant concentrations during fruit development: (A and C) 'Fuji', (B and D) 'Cripps' Pink'. Means and standard errors are indicated. Data for 'Granny Smith' fruits is missing.

PAPER 3

The apple fruit peel photosynthetic systems of sunburn sensitive cultivars are not necessarily more sensitive to heat and light stress

Abstract

The effects of heat and light stress on apple fruit peel photosystems of the perceived sunburn sensitive Granny Smith and the less sensitive Golden Delicious cultivars at fruit maturity was analysed in three seasons. Two experiments were conducted: In Experiment 1 the fruits were exposed to 30 ºC, 35 ºC, 40 ºC, 45 ºC and 50 ºC heat for 3 hours at a constant photosynthetic active radiation (PAR) level of 550 µmol m^{-2} s^{-1}; in Experiment 2 the fruit peel temperature was kept at 30 ºC while being exposed to increasing PAR levels of 96, 300 and 1220 µmol m^{-2} s^{-1} for 60 min. Different chlorophyll fluorescence parameters were measured to determine the stress effect on fruit peel photosystems. The results were interpreted to explain possible underlying biochemical changes in the fruit peels as influenced by the different treatments. Heat stress caused higher photodamage in 'Golden Delicious' in one season while damage was equal in both cultivars in the second season. This was possibly due to seasonal factors such as orchard temperature regime and maturity development. The photosystems of 'Granny Smith' therefore do not appear to be more sensitive to heat stress compared to those of 'Golden Delicious'. Furthermore, there appear to be no differences in the PAR stress sensitivity between the two

cultivars. The difference in fruit sunburn sensitivity of apple cultivars may therefore not relate to the difference in heat and light stress sensitivity of fruit peel photosystems.

Keywords: heat, light, apple, fluorescence, sunburn

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1. Introduction

High fruit peel temperatures in the presence of light cause apple peel sunburn (Schrader et al., 2003). There are three sunburn types: Sunburn browning = yellow/bronze discoloration of fruit peel (Schrader et al., 2001), photooxidative sunburn (sunburn bleaching) = bleaching of fruit peel pigment (Felicetti and Schrader, 2008), and sunburn necrosis = brown/dark brown discoloration of fruit peel (Schrader et al., 2001). The sunburn discussion in this paper will only focus on sunburn browning, which is the most common type of fruit sunburn (Schrader et al., 2008).

Fruit sunburn symptoms develop due to the heat and light stress-induced damage to fruit peel photosynthetic systems (Chen et al., 2008). High temperature increases the sensitivity of fruit peel photosynthetic systems to light (Chen et al., 2008). Temperature-induced light stress causes the production of reactive oxygen species that damage the photosystem and degrade the associated pigments, resulting in fruit sunburn (Wang et al., 1999). Smillie (1992) reported that heat stress damages the

Calvin cycle activity in fruits more than the electron transport activity of the light reaction and the phosphorylation reaction. However, Chen et al. (2008) found that in apples heat stress combined with high light stress damages both the donor and acceptor sides of the photosystem.

Light stress can damage the D1 protein of photosystem II (PSII), therefore damaging the reaction centres of PSII that are attached to the D1 protein (Yamamoto et al., 2008). This decreases light use in photochemistry while increasing non-photochemical quenching (Horton et al., 1996). Therefore, a measure of changes in fruit peel photochemical and non-photochemical changes under light stress can give a stress sensitivity indication. Light stress has been shown to damage the photosystems of apple fruit peels (Glenn and Yuri, 2013).

Observation in orchards suggests that cultivars differ in their susceptibility to sunburn. The difference in sunburn sensitivity may partially relate to the difference in sensitivity of fruit peel photosystems to sunburn inducing factors. The fruit bearing habits of different cultivars as well as the training systems used in modern orchards can also contribute to fruit sunburn development, i.e. when fruit bearing branches bend during fruit development or after summer pruning. The change in the fruit bearing position or removal of covering foliage after pruning expose previously protected fruits to direct sunlight and leads to sunburn development on fruits. The effect of light stress on apple fruit peel photosystems has been studied before (Chen et al., 2008; Glenn and Yuri, 2013; Merzlyak and Chivkunova, 2000). However, there are still limited studies analysing the direct difference in the sensitivity of fruit peel photosystems to light stress between apple cultivars with purported different sunburn

susceptibilities. The lack of key sunburn sensitivity indicators for selective breeding also hampers the possibility for the elimination of sensitive genotypes during plant breeding.

There is currently no published literature on the difference in susceptibility to sunburn between different apple cultivars. However, ‘Granny Smith’ suffers severe sunburn losses under South African conditions and most fruit in exposed positions in the canopy may show visible sunburn symptoms at harvest (Fouché et al., 2010). It is considered to be the most sunburn sensitive cultivar in the South African industry and much more sensitive than ‘Golden Delicious’ (A. Müller, KROMCO Technical Manager, personal communication). The focus of this research was to study the difference in damage to the photosystems of ‘Granny Smith’ and ‘Golden Delicious’ apples at maturity by 1) different heat stress levels coupled with a constant moderate light stress level and 2) by continuously increasing light stress at a constant moderate temperature.

2. Material and methods

2.1. Plant material and treatments

Granny Smith and Golden Delicious apple cultivars were used in the study. Fruits were randomly collected from a farm in the Grabouw area (34º9’10.55’’S; 19º1’47.62’’E) in the Western Cape province of South Africa, which has a Mediterranean-type climate. The previously sun-exposed fruits were harvested at maturity from the mid-section of the canopy. ‘Granny Smith’ and ‘Golden Delicious’

fruits were harvested, respectively, at 152 days after full bloom (DAFB) and 126 DAFB in 2008/2009 (2009 season), at 148 DAFB and 154 DAFB in 2009/2010 (2010 season) and at 122 DAFB and 112 DAFB in 2010/2011 (2011 season). Fruits for Experiment 1 were harvested during the 2009 and 2010 seasons, while for Experiment 2 fruits were harvested during the 2010 and 2011 seasons. The average maximum summer orchard temperatures (December to March) for the 2009, 2010 and 2011 seasons were 33 ℃ (±1 ℃), 30 ℃ (±1 ℃), 26 ℃ (±1 ℃) respectively, while the highest recorded daily maximum temperatures were 36 ℃, 32 ℃ and 36 ℃, respectively (Data obtained from orchard based weather stations and from an internet weather data site www.weatherspark.com).

The fruits of both cultivars were at similar maturity levels according to the Streif index (DeLong *et al.*, 1999). The Streif index is calculated as: fruit firmness/ (fruit soluble solids content x fruit starch index value). The Streif index values for ‘Granny Smith’ and ‘Golden Delicious’ fruits in the 2009 season were 0.066 and 0.067 respectively, and in the 2010 season were 0.103 and 0.090 respectively. Both cultivars were therefore less mature in the 2010 season than in the 2009 season. No Streif index data were available for the 2011 season because of missing maturity indexing raw data. Fruits were stored over night at -0.5 ºC and kept at 25 ºC for 2 hours after removal from the cold rooms the following day, before the stress treatments.

Fruit peel temperature was measured every 60 minutes with a hand held infrared thermometer (Raynger MX4, Raytek Corporation, Santa Cruz, USA). Fruit Chlorophyll *a* fluorescence was measured with a FSM 1 fluorimeter (Fluorescence

Monitoring system 1, Hansatech, Norfolk, UK). The fluorimeter was connected to one half of a leaf-clip holder through a fiberoptic cable.

2.1.1. Experiment 1: Heat effect on fruit peel photosystems at a moderate light level

A total of 35 fruits were randomly collected from eight trees of each cultivar, with 30 of the fruits used for the five heat stress treatments (six fruits/ treatment) and five fruits used for maturity indexing. Fruit disks of 12 mm diameter and 30 mm height collected from the central part of the sun exposed side of each fruit were exposed to the five different heat stress treatments for three hours while being exposed to a constant photosynthetic active radiation (PAR) level of 500±50 µmol $m^{-2}s^{-1}$ (measured with a quantum meter: LI-189; Li-Cor, Lincoln, Nebraska, USA). The disks were inserted in distilled water in random positions/cavities of a white foam cuvette holder directly after being extracted from the fruits and placed under the lights. The fruit peel was at least 5 mm above the water level in the cuvette holder to prevent direct damage to the peel by the warm water. The temperature ranges for the five heat stress treatments were:

30 ºC (32 ºC ±0.294), 35 ºC (37 ºC ±0.395), 40 ºC (42 ºC ±0.446), 45 ºC (46 ºC ±0.473), and 50 ºC (50 ºC ±0.561). The fruits reached the intended temperatures in approximately 15 min. after the start of the treatments. PAR was provided by two lamps (50W, Titan Halogen Dichroic with a UV filter, OSRAM Gmbt. Augsburg, Germany), placed on either sides of a central infrared light lamp (175 W, PAR 38IR175R, Philips, Amsterdam, Holland). The infrared lights were placed at different heights to induce the different fruit peel temperatures.

The maximum (Fm) and minimum (Fo) fluorescence and maximum light use efficiency Fv/Fm = (Fm-Fo)/Fm were measured. The initial fluorescence measurements were taken before the stress, and the after stress readings were done after a 30 min dark adaptation period. Recovery fluorescence readings were done after a 12 hour relaxation period in the dark at room temperature (20 ℃).

2.1.2. Experiment 2: Light stress effect on fruit peel photosystems

Granny Smith and Golden Delicious apple cultivars were also used in this study. The fruits were harvested at maturity in the 2010 and 2011 seasons. A total of three fruits were used per cultivar for the treatment. The fruits were cut in half, and the flesh of the previously sun-exposed side was further reduced in half. The previously sun-exposed side was then placed on filter paper in a petri dish, with the inside of the fruit facing down on the paper which was moistened with distilled water. The petri dish was then placed in a dark growth chamber. Fruit peel temperature was kept at 30 ºC. A fluorimeter was connected to the fruit with a fiberoptic cable and a leaf-clip. The ambient light level from the fluorimeter was increased every 20 min from 96 to 300 and finally to 1220 µmol m^{-2} s^{-1}. The following photochemical parameters were measured:

ΦPSII = quantum yield of PSII

qP = photochemical quenching

NPQ = non-photochemical quenching

ETR = electron transport rate

2.3. Statistical analysis

2.3.1. Experiment 1: Heat effect on fruit peel photosystems at a moderate light level

Statistical analysis was done with SAS 9.1 (SAS Institute Inc., Cary, NC., USA). The percentage change in Fv/Fm was analysed as a completely randomized factorial design. The percentage change in Fv/Fm was log transformed for the statistical analysis. The difference between the Fv/Fm before (initial), after stress and recovery for the different heat levels and each cultivar was analysed using a one way ANOVA with SAS 9.1. Means and +/- standard errors are indicated on the graphs.

2.3.2. Experiment 2: Light stress effect on fruit peel photosystems

Differences between means of the fluorescence parameters of 'Granny Smith' and 'Golden Delicious' at each light level was analysed with a t-test ($\alpha = 0.05$) in Microsoft Excel 2010 (Windows Microsoft Excel 2010, Microsoft Corporation, Redmond, WA., USA). Means and standard errors are indicated on the graphs.

3. Results

3.1. Experiment 1: Heat effect on fruit peel photosystems at a moderate light level

'Golden Delicious' had a higher percentage reduction of the maximum light use efficiency (Fv/Fm) than 'Granny Smith' after the 30 ºC to 45 ºC treatments in the 2009 season (Fig. 1). However, there was no difference in the Fv/Fm of both cultivars after all the heat treatments in the 2010 season (Fig. 2). The Fv/Fm of 'Golden Delicious' and 'Granny Smith' after the 45 ºC treatment was reduced by 50%

and 10% respectively in the 2009 and by 53% and 46% respectively in the 2010 seasons (Fig. 1, 2). There was a significant difference in the percentage change of Fv/Fm between the two years ($p < 0.0001$). There was also a significant difference between the effect of the treatments on the two cultivars ($p = 0.0009$) and between the effect of the different heat levels ($p < 0.0001$). There was no significant interaction between the cultivars and the temperature levels ($p = 0.0755$), but there was a significant interaction between the years and the treatments (<0.0001). The Fv/Fm of 'Golden Delicious' was reduced by the 30 to 50 ºC treatments in both seasons (Fig. 1, 2). However, the Fv/Fm of 'Granny Smith' increased or remained unchanged after the 30 to 40 ºC treatments in the 2009 season (Fig. 1).

The fruit peel Fv/Fm of both cultivars was only irreversibly damaged by the 45 ºC and 50ºC treatments (Fig. 3, 4). The unstressed Fv/Fm value for most plants is 0.83 (0.7 – 0.8) with a critical value of 0.6 (Maxwell and Johnson, 2000; Ritchie, 2006). However, 'Granny Smith' had a better recovery after stress compared to 'Golden Delicious', especially after the 45 and 50 ºC stress levels (Fig. 3, 4).

The minimum fluorescence (Fo) of 'Granny Smith' was reduced by the 40 to 50 ºC treatments in the 2009 season, while in the 2009/10 season it was increased by the 30 and 40 ºC treatment and reduced by the 35, 45 and 50 ºC treatments (Fig. 1, 2). The Fo of 'Golden Delicious' was increased by the 30 ºC treatment and reduced by the 40 to 50 ºC treatments in the 2009 season (Fig. 1, 2). However, the Fo of 'Golden Delicious' fruits was reduced by all the treatments in the 2010 season. The Fo of 'Golden Delicious' and 'Granny Smith' was reduced by 31 and 20% respectively after 45 ºC in the 2009 season and by 24 and 35% respectively in the

2010 season (Fig. 1, 2). The variable fluorescence (Fv) of 'Golden Delicious' was reduced by all the treatments in both seasons (Fig. 1, 2). There was a variable change in the Fv of 'Granny Smith' in both seasons (Fig. 1, 2). The Fv of 'Golden Delicious' and 'Granny Smith' was reduced by 85% and 32% respectively after 45 ºC in the 2009 season and by 82% and 86% respectively in the 2010 season (Fig. 1, 2).

3.2. Experiment 2: Lights stress effect on fruit peel photosystems

There was no difference between the quantum yield of PSII (ΦPSII) of both cultivars at the 1220 µmol m^{-2} s^{-1} light level in both seasons (Fig.5-6). Similar results were obtained for the photochemical quenching (qP). However, the ΦPSII of 'Granny Smith' fruits was higher than that of 'Golden Delicious' fruits at the 300 µmol m^{-2} s^{-1} PAR level in both seasons.

The non-photochemical quenching (NPQ) and the electron transport rate (ETR) of 'Granny Smith' was higher than that of 'Golden Delicious' at the 1220 µmol $m^{-2}s^{-1}$ light level in the 2010 season (Fig. 4-5). However, the NPQ and the ETR did not differ between both cultivars at the 1220 µmol $m^{-2}s^{-1}$ light level in the 2011 season. The NPQ and ETR at the 300 µmol $m^{-2}s^{-1}$ light level were similar for the cultivars in the 2010 season, but NPQ was higher in 'Golden Delicious' than in 'Granny Smith' in the 2011 season while the inverse was true for the ETR in the same season. NPQ increased with increasing light level while ΦPSII, qP and ETR decreased in both cultivars during the two seasons.

4. Discussion and conclusion

The photosystems of 'Golden Delicious' apples appear to be equally sensitive to heat stress than those of 'Granny Smith'. 'Golden Delicious' incurred greater photodamage at most temperatures than 'Granny Smith' during the 2009 season (Fig. 1, 2). Nonetheless, the photosystems of both cultivars were equally damaged by the applied stress during the 2010 season. The higher heat sensitivity of 'Golden Delicious' experienced during the 2009 season may relate to higher heat stress damage to the oxygen evolving complex (OEC) of this cultivar compared to 'Granny Smith'. Oxygen evolution from the OEC has a positive linear correlation with variable chlorophyll fluorescence (Fv) (Govindjee *et al.*, 1981; Pistorius and Schmid, 1984; Toivonen and Vidaver, 1988). Chen and Cheng (2008) also found that high temperature stress damages the OEC of apple fruits. They further postulated that combining the high temperature stress with high light stress causes a greater damage on the electron acceptor side of photosystem II (PSII). Different apple cultivars display a difference in their response to environmental stress (Lisowa *et al.*, 2002). The photosystems of 'Braeburn' were less damaged by a 2 hour 46 ℃ heat stress than 'Fuji', with 'Cripps' Pink' being damaged the most (Wand et al., 2008). The Fv of 'Golden Delicious' was reduced significantly more than that of 'Granny Smith' fruits by most of the treatments (Fig. 1, 2). The late maturing period of 'Granny Smith' compared to 'Golden Delicious', combined with the higher summer temperatures of the 2009 season compared to the 2010 season may also partly explain the difference in the observed stress responses between the cultivars and between the seasons. 'Granny Smith', compared to 'Golden Delicious', suffered less photodamage in the 2009 season as it could have been more acclimated to the high temperature experienced during that season because of its late or longer maturing

period. In addition, the less mature fruits of both cultivars possibly were less acclimated to heat stress in the cooler 2010 season compared to the warmer 2009 season and therefore suffered more photodamage in the 2010 season. 'Granny Smith' have been observed to be more sunburn sensitive than 'Golden Delicious' fruits in orchards (personal observation and personal communication with apple tree researchers in South Africa). Our data appear to show that the photosystems of 'Golden Delicious' are possibly equally sensitive to heat stress than those of 'Granny Smith'. Fruit sunburn sensitivity is therefore possibly not related to the sensitivity of the photosystem to heat stress, while other factors such as canopy foliage density, bearing habits and fruit colour could play greater roles.

The critical temperature for heat stress-induced photodamage (Tc), specifically when coupled with moderate PAR, to the OEC of 'Golden Delicious' appears to be lower than that of 'Granny Smith'. The lower Tc of 'Golden Delicious' is suggested by the observed changes in Fv, especially during the 2009 season. The Fv of 'Golden Delicious' was reduced after the 30 ºC stress while in 'Granny Smith' it was only reduced after the 45 ºC stress level (Fig. 1). Furthermore, the Fo of 'Golden Delicious' was increased at the 30 ºC and 35 ºC during the same season, indicating possible damage to the PSII, while the Fo of 'Granny Smith' remained unchanged after the same heat stress. An increase in Fo is an indication of damage to the antennae of the light harvesting complex of PSII (Maxwell and Johnson, 2000). Damage to the PSII of 'Golden Delicious' is further suggested by the decrease of Fm in combination with the observed increase in Fo. An increase in Fo combined with a decrease in Fm is reported to be an indication of photoinhibition (Gilmore at al.,

1996). The photosystems of ‘Golden Delicious’ are therefore likely to suffer stress at lower temperature than those of ‘Granny Smith’.

The lower photodamage of ‘Granny Smith’ compared to ‘Golden Delicious’ can also be related to the greater recovery of the former cultivar compared to the later after the imposed stress (Fig. 3, 4). However, both cultivars suffered irreversible photodamage at the 45 and 50 ºC heat treatments. Chlorophyll fluorescence in barley leaves is also irreversibly damaged at temperatures above 45 ºC (Frolec et al., 2008). Irrespective, it appears that ‘Granny Smith’ photosystems are more able to recover from high temperature stress while ‘Golden Delicious’ are less able to recover.

The photosystems of ‘Granny Smith’ and ‘Golden Delicious’ fruit peels appears to be equally sensitive to high photosynthetic active radiation (PAR) stress (Fig. 5, 6). PAR stress has been reported to induce photodamage in ‘Granny Smith’ (Glenn and Yuri, 2013) and in ‘Golden Delicious’ (Chen et al., 2012). However, the responses of these two cultivars to PAR stress have not previously been studied together under similar conditions. Exposure of plants to PAR stress results in reduced photosynthetic efficiency while the NPQ is increased (Yamamoto et al., 2008). Our results also found that ΦPSII was reduced under PAR stress while NPQ was increased in the two cultivars studied. The similarity in the response of these parameters in both cultivars could indicate that these cultivars are equally sensitive to PAR stress. The higher sunburn sensitivity of ‘Granny Smith’ compared to ‘Golden Delicious’ observed under field conditions (personal field observations) may therefore not be related to the difference in PAR stress sensitivity of these two cultivars.

In conclusion, the photosystems of 'Golden Delicious' fruits appear to be equally sensitive to heat stress than that of 'Granny Smith' fruits. 'Golden Delicious' photosystems also may have a lower Tc than 'Granny Smith'. Both cultivars furthermore appear to be equally sensitive to PAR stress when applied in isolation to heat stress. It is still possible that other factors such as tree training and bearing habit may contribute to the observed differences in sunburn sensitivity between 'Granny Smith' and 'Golden Delicious'. It has been reported that sunburn is caused by heat and light stress-induced damage to fruit peel photosynthetic systems (Chen *et al.*, 2008). However, our results show that the photosystem of sunburn sensitive apple cultivars are not necessarily more sensitive to heat and light stress.

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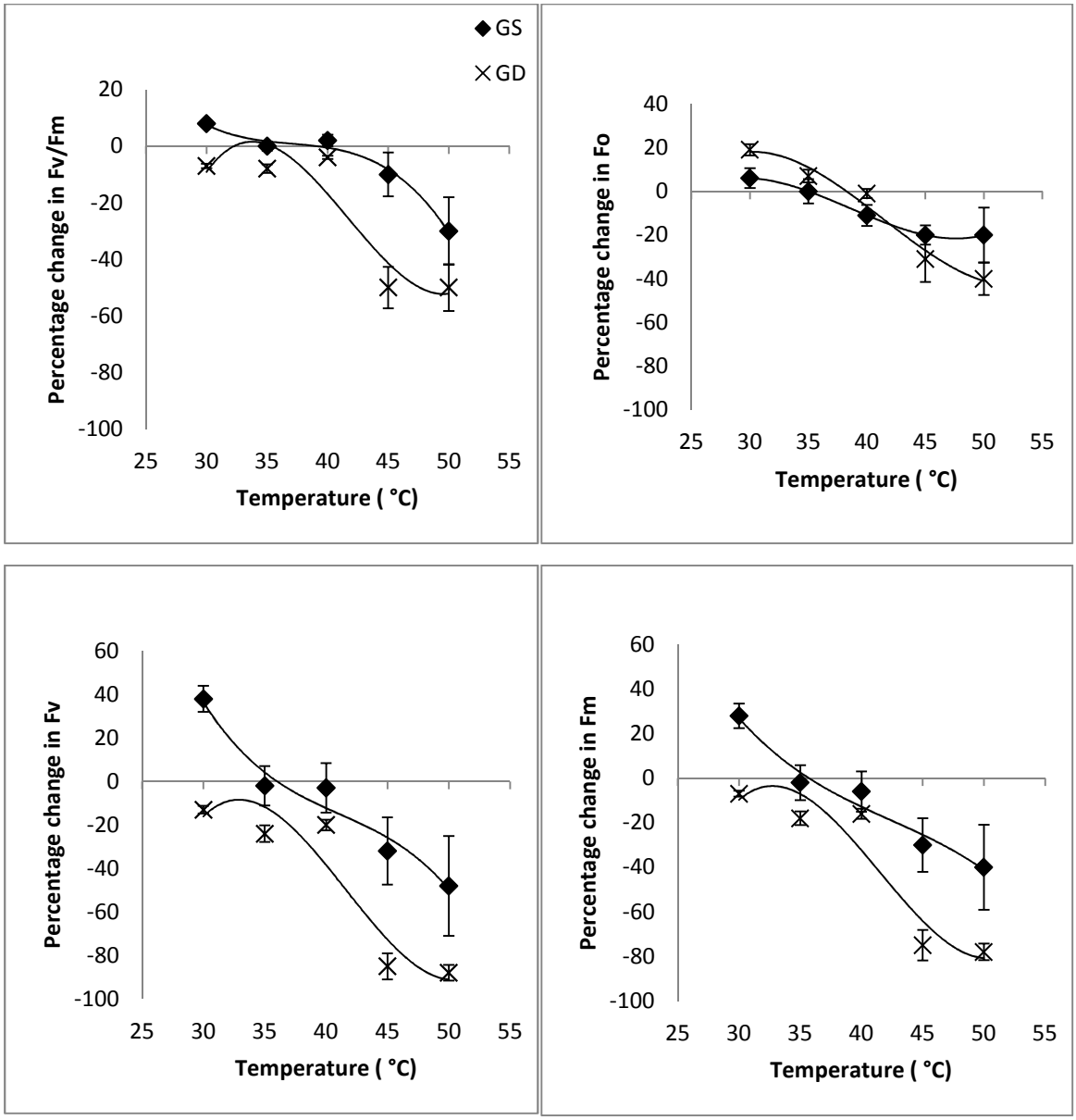

Fig 1. Percentage change of the maximum light use efficiency (Fv/Fm), minimum (Fo), variable (Fv) and maximum (Fm) fluorescence of 'Granny Smith' and 'Golden Delicious' fruit peel from before stress to after the recovery period (12 hours after stress) during the 2009 season. Mature fruits were exposed to different temperature levels for 3 hours at a constant light level of 500 µmol m^{-2} s^{-1} at maturity.

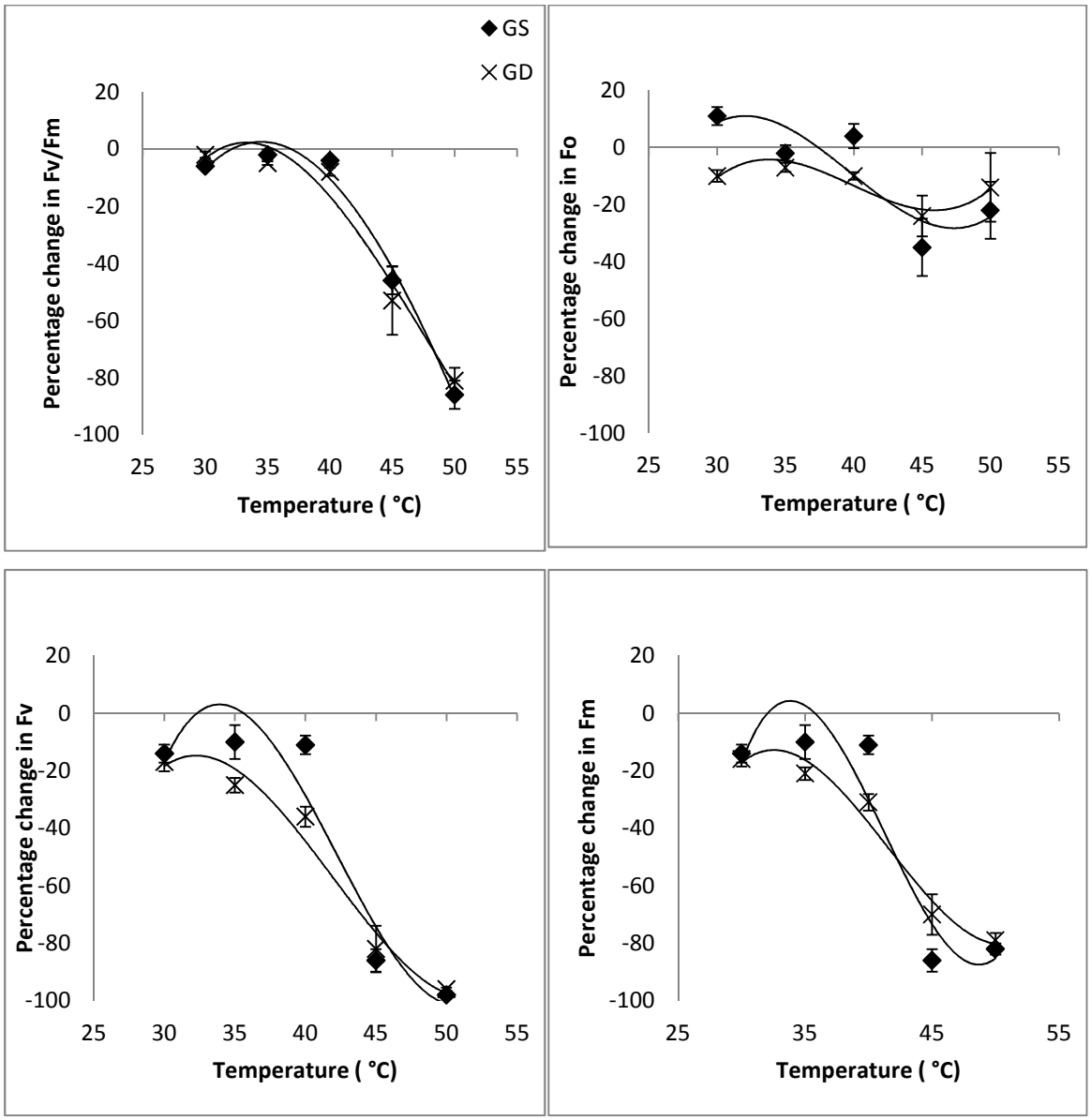

Fig 2. Percentage change of the maximum light use efficiency (Fv/Fm), minimum (Fo), variable (Fv) and maximum (Fm) fluorescence of 'Granny Smith' and 'Golden Delicious' fruit peel from before stress to after the recovery period (12 hours after stress) during the 2010 season. Mature fruits were exposed to different temperature levels for 3 hours at a constant light level of 500 µmol m^{-2} s^{-1} at maturity.

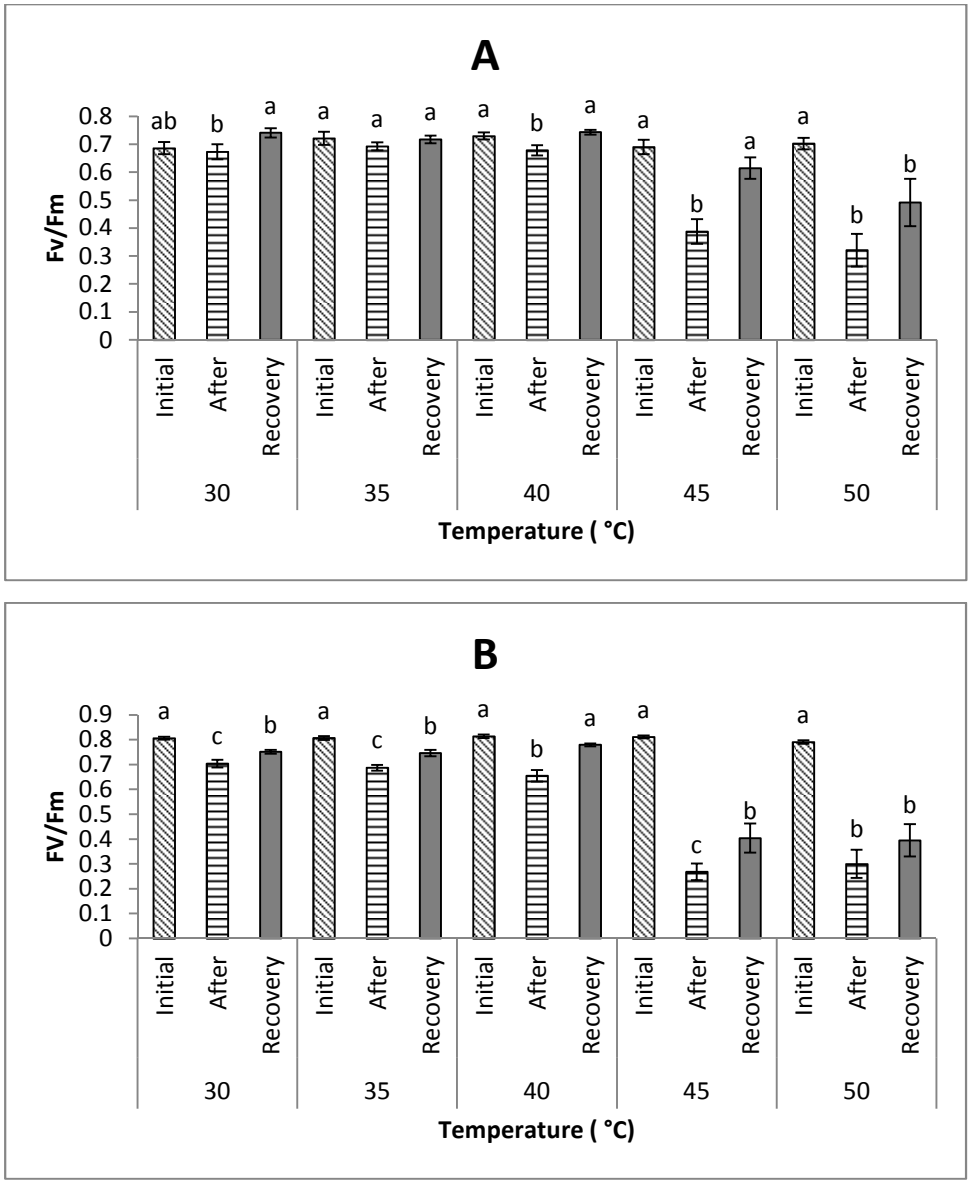

Fig. 3. The effect of different temperature levels on apple fruit peel maximum light use efficiency (Fv/Fm) after 3 hours at a constant light level of 500±50 µmol m^{-2} s^{-1} at fruit maturity of A) 'Granny Smith' and B) 'Golden Delicious' during the 2009 season. Initial = before stress; After = after stress (30 min dark adaptation); Recovery = after a 12 hour recovery period in the dark at room temperature, 20 °C. Different letters indicate differences between the Initial, After and Recovery Fv/Fm at each temperature level. Means and standard errors are indicated.

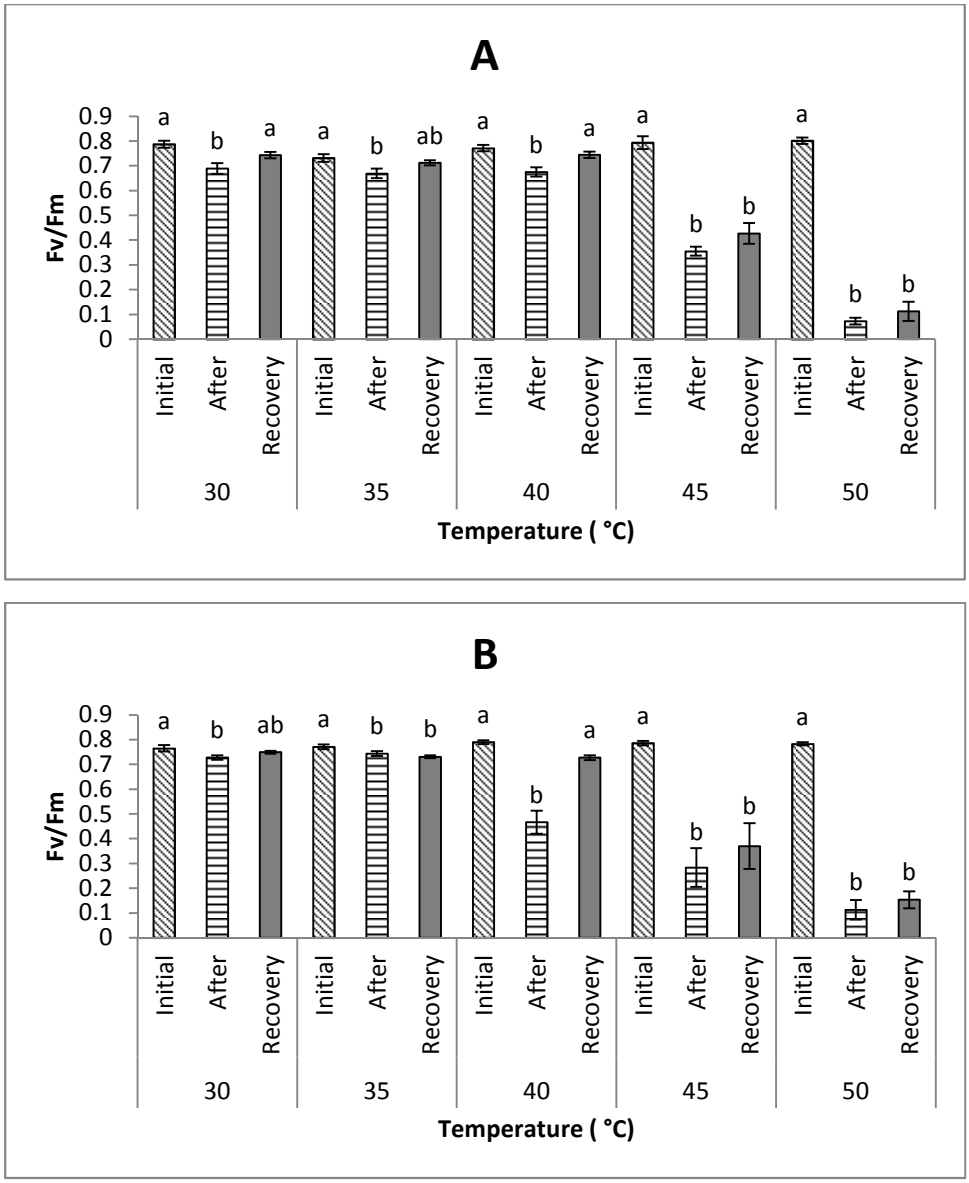

Fig. 4. The effect of different temperature levels on apple fruit peel maximum light use efficiency (Fv/Fm) after 3 hours at a constant light level of 500±50 µmol m^{-2} s^{-1} at fruit maturity of A) 'Granny Smith' and B) 'Golden Delicious' during the 2010 season. Initial = before stress; After = after stress (30 min dark adaptation); Recovery = after a 12 hour recovery period in the dark at room temperature, 20 °C. Different letters indicate differences between the Initial, After and Recovery Fv/Fm at each temperature level. Means and standard errors are indicated.

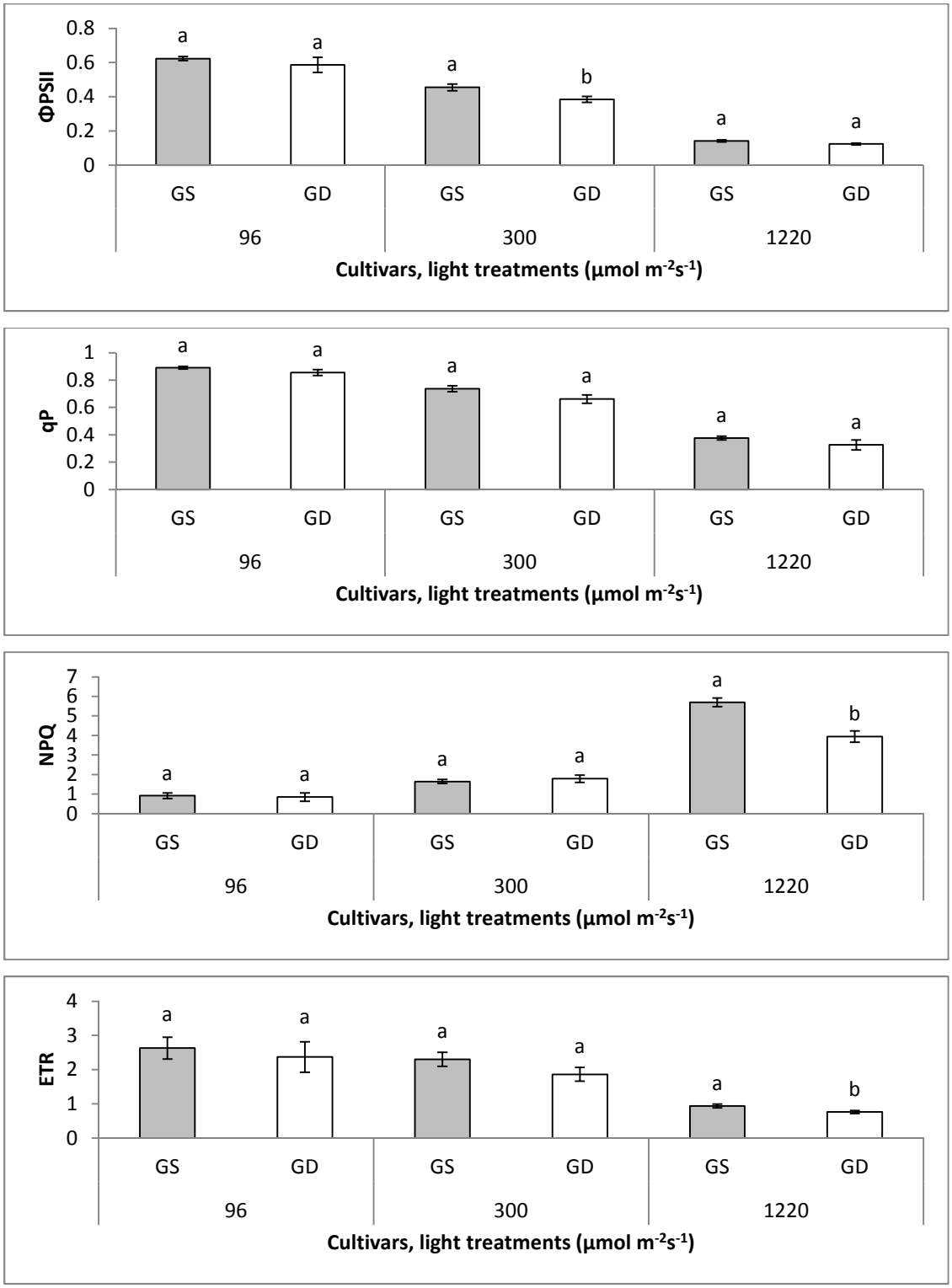

Fig. 5. The effect of continuously increasing photosynthetic active radiation (PAR) on the quantum yield of PSII (ΦPSII), photochemical quenching (qP), non-photochemical quenching (NPQ) and electron transport rate (ETR) of apple fruit peels during the 2010 season. PAR was increased after every 20 minutes while fruit temperature was kept at 30 ℃. Different letters indicate differences between the cultivars at each PAR level. Means and standard errors are indicated.

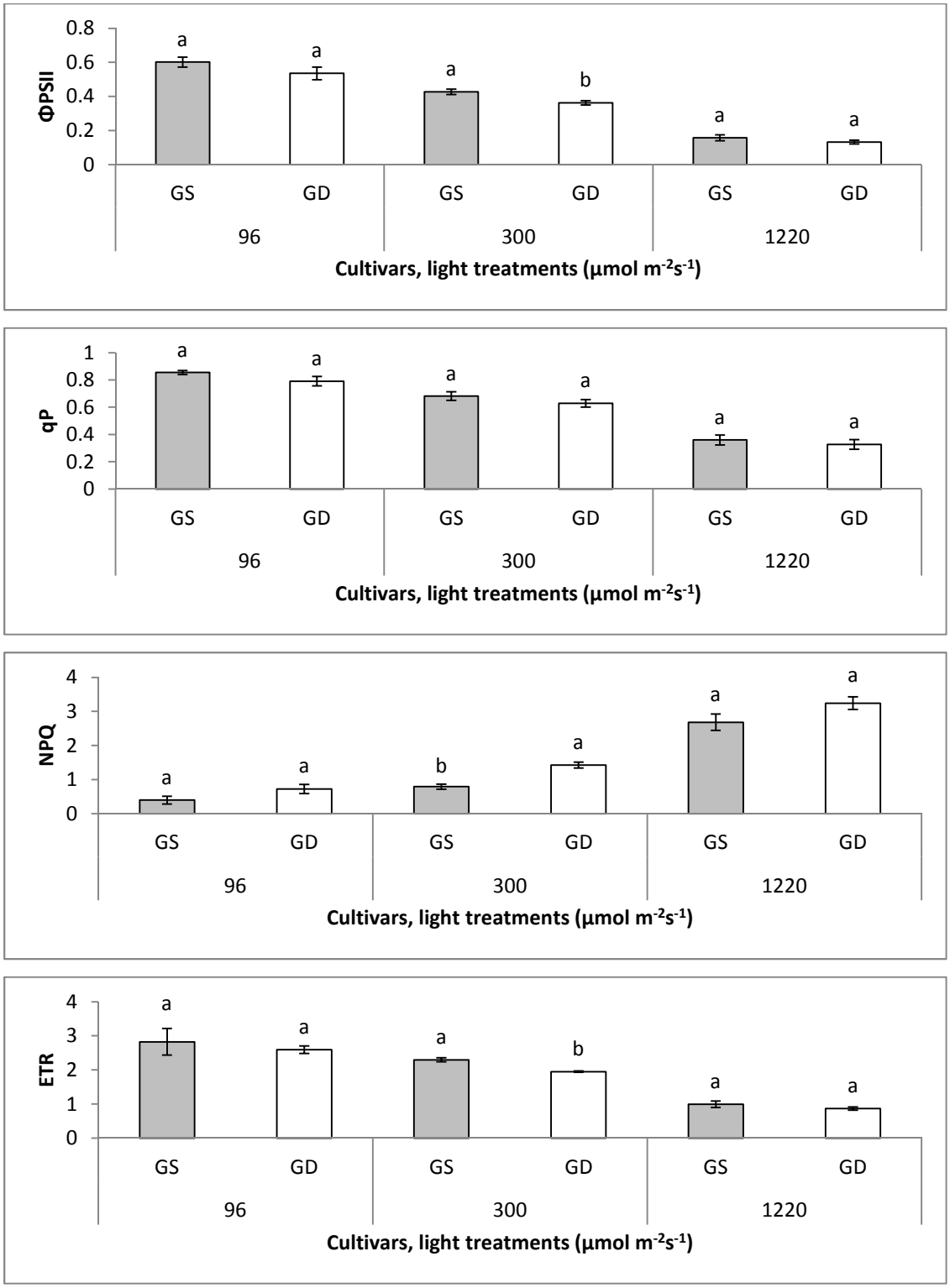

Fig. 6. The effect of continuously increasing photosynthetic active radiation (PAR) on the quantum yield of PSII (ΦPSII), photochemical quenching (qP), non-photochemical quenching (NPQ) and electron transport rate (ETR) of apple fruit peels during the 2011 season. PAR was increased after every 20 minutes while fruit temperature was kept at 30 ℃. Different letters indicate differences between the cultivars at each PAR level. Means and standard errors are indicated.

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PAPER 4

Differential dependence of apple (*Malus domestica* Borkh.) cultivars on the xanthophyll cycle for photoprotection

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Abstract

The dependence of fruit peel photosystems of 'Granny Smith', 'Braeburn', 'Fuji', 'Golden Delicious' and 'Topred' apple (*Malus domestica* Borkh.) peel on the xanthophyll cycle for photoprotection was studied under laboratory conditions. Mature fruit peel were treated or not treated with 1 mM dithiothreitol (DTT) to inhibit the xanthophyll cycle. Fruit peel were subsequently exposed to photosynthetic active radiation (PAR) stress of 1500 µmol m^{-2} s^{-1} combined with heat stress of 45°C for 3 h. Fruit peel photodamage was assessed by measuring the change in the maximum light use efficiency of photosystem II (F_v/F_m). The change in the concentration of

xanthophyll cycle carotenoids zeaxanthin, antheraxanthin and violaxanthin plus lutein and β–carotene were analysed. The F_v/F_m of heat and light stressed DTT treated (+DTT) 'Granny Smith' and 'Braeburn' peel had a low recovery after stress compared to the recovery F_v/F_m of similarly stressed peel not treated with DTT (-DTT). However, there was no difference in the recovery F_v/F_m between +DTT and -DTT 'Fuji', 'Golden Delicious' and 'Topred' peel. The photosystem of 'Granny Smith' and 'Braeburn' fruits therefore appear to have had a higher dependency on the xanthophyll cycle for photoprotection than 'Fuji', 'Golden Delicious' and 'Topred' fruits.

Keywords: *Malus domestica*, xanthophyll cycle, light, sunburn, temperature

Introduction

Plants absorb light energy and convert it to chemical energy through photosynthesis (Lawlor 1993). Excess light reaching the chloroplasts can result in damage to the photosynthetic system (Barber and Anderson 1992). Therefore, the quantity of absorbed light energy should be within the physiological limits of the specific plant species or plant organ. The high solar radiation levels and high summer temperatures that characterise the Mediterranean type climatic fruit growing regions, such as the Western Cape region of South Africa, can be damaging to fruit photosynthetic systems. Fruit photosynthetic rate generally become light saturated at photosynthetic active radiation (PAR) levels above 500 µmol m^{-2} s^{-1} (Aschan and Pfanz 2003), while in apple (*Malus domestica* Borkh.) leaves this happens at 2000 µmol m^{-2} s^{-1} (Mierowska et al. 2002). Apple fruit temperature can be about 10 – 15

℃ higher than air temperature (Parchomchuk and Meheriuk 1996), while leaf temperature is similar to air temperature (Smit et al. 2007). Fruits are therefore likely to experience higher temperatures than leaves, and can suffer heat stress-induced photodamage at lower air temperatures compared to leaves.

Heat and light induced damage to fruit photosystems result in the development of fruit peel sunburn discolouration in apple (Schrader et al. 2001, Chen et al. 2008) and tomato fruit (Rabinowitch et al. 1974). Sunburn browning (bronze colouration) of apple fruits occurs when peel temperatures reach 45 ℃ in the presence of direct sunlight (Schrader et al. 2001). The peel of apples at fruit maturity is considered to be most susceptible to heat and light stress damage compared to earlier fruit development stages (Glenn et al. 2002). Apple fruits grown in the Western Cape region of South Africa develop sunburn, which can reach up to 40% of the total harvest (Hortgro 2013). Sunburn is therefore a major problem in these areas, and indeed, in many apple growing regions of the world.

Plants utilise various photoprotective mechanisms against stress, viz.: adjustment of photosystem I and II (PSI and PSII), scavenging of reactive oxygen species, release of absorbed energy as heat, cyclic electron transport, water-to-water cycle, photorespiration and increased light absorption by photoprotective molecules (Niyogi 1999, Solovchenko and Merzlyak 2008, Takahashi and Badger 2011). Non-photochemical quenching (NPQ), resulting in the release of absorbed light energy as heat, is due to the xanthophyll cycle activated by the ΔpH generated over chloroplast membranes induced by PAR absorption in PSII (Müller et al. 2001, Jahns and

Holzwarth 2012). The cycle entails the conversion/de-epoxidation of the xanthophyll carotenoid, violaxanthin, via antheraxanthin to zeaxanthin, which is then epoxidised back to violaxanthin releasing the absorbed energy as thermal energy in the processes (Demmig et al. 1987, Adams et al. 1990, Jahns and Holzwarth 2012). Inhibition of the xanthophyll cycle results in increased photodamage in plants (Sarry et al. 1994). Sun exposed and sunburned apple fruits have higher xanthophyll cycle activities than shaded or non-sunburned fruits (Ma and Cheng 2003). Felicetti and Schrader (2008) also found that total carotenoid concentration was higher in sunburned fruits compared to non-sunburned fruits. Apple fruits therefore appear to utilise the xanthophyll cycle for photoprotection under sunburn inducing climatic conditions.

An analysis of the ability of different apple cultivars to utilise specific photoprotective mechanisms can help shed more light on fruit sunburn development. The difference in the xanthophyll cycle pool size of previously sun-exposed and shaded apple fruit peel (‘Gala’ and ‘Smoothie’ apples) have been analysed before (Ma and Cheng 2003). However, differences in the dependence of different apple cultivars on the xanthophyll cycle for photoprotection have not been studied. The objective of this study was to determine the difference in the dependency of apple fruit photosystems of different cultivars on the xanthophyll cycle for photoprotection under laboratory conditions of temperature and PAR similar to conditions that induces fruit sunburn on the trees.

Materials and methods

Plant material and experimental design

Apple fruits of the cultivars Granny Smith, Braeburn, Fuji, Golden Delicious and Topred were used in this study. The cultivars Cripps' Pink and Royal Gala were also assessed, but the fluorescence values obtained after stress were too low for reliable assessment of the fluorescence parameters. The fruits were collected from farms in the Grabouw area (34º9'10.55''S; 19º1'47.62''E) located in the Mediterranean-type climate Western Cape Province of South Africa. A total of 103 fruits were randomly harvested from 11 trees per cultivar and used as follows: 60 fruits for the dithiothreitol (DTT) + photosynthetic active radiation (PAR) treatment (10 fruits for both stressed DTT treated and untreated treatments, with each treatment repeated 3 times) with 30 fruits used for the post-stress biochemical analysis and 30 fruits for the fluorescence readings; 28 fruits for initial (pre-stress) biochemical analysis (4 replicates of 7 fruits each); 15 fruits for maturity measurements (10 fruits for fruit firmness and total soluble solutes and 5 fruits for starch breakdown). Sun-exposed fruits were harvested at commercial maturity from mid canopy on the north or west facing side of the tree row in the orchard. The Streif index values of 'Granny Smith', 'Braeburn', 'Fuji', 'Golden Delicious' and 'Topred' fruits were: < 0.000, 0.024, 0.467, 0.018 and 0.031, respectively. The Streif index is calculated as: [fruit firmness/ (fruit soluble solids concentration x fruit starch index value)] (DeLong et al. 1999).

Fruit peel disks of 12 mm diameter and 3 cm long were collected from midway between the stem and calyx ends on the previously sun exposed side of the fruits.

The DTT treatment disks were then placed in a 1 mM DTT (Sigma-Aldrich, Steinheim, Germany) solution to inhibit the xanthophyll cycle during stress exposure, and kept at room temperature (25°C) and 10 µmol m^{-2} s^{-1} PAR for 12 h before initiation of the light and heat exposure. Disks not treated with DTT were placed in distilled water and kept under similar conditions as the DTT treated peel. The fruit disks were then exposed to 1,500 ± 50 µmol m^{-2} s^{-1} PAR (measured at the fruit surface level with a quantum meter: LI-189; Li-Cor, Lincoln, Nebraska, USA) and 45 ± 2°C for 3 h. Temperature was measured with a hand held infrared thermometer (Raynger MX4, Raytek Corporation, Santa Cruz, USA). PAR was provided by two lamps (50W/12V, 350 – 1,000 nm, 700 nm peak, Titan Halogen Dichroic with a UV filter, OSRAM Gmbt. Augsburg, Germany), placed on either sides of a central infrared light lamp (175 W, 300 – 4,000 nm, 1,000 nm peak, PAR 38IR175R, Philips, Amsterdam, Holland).

Chlorophyll fluorescence and pigment analysis

Fruit Chlorophyll a fluorescence was measured with a fluorescence monitoring system (FMS1) fluorometer (Fluorescence Monitoring system 1, Hansatech, Norfolk, UK). The fluorometer was connected to one half of a leaf-clip holder through a fiberoptic cable. The maximum (F_m), variable (F_v) and minimum (F_o) fluorescence, plus maximum light use efficiency $F_v/F_m = (F_m-F_o)/F_m$ were measured. Fluorescence measurements were taken: before and after stress exposure, and again after a 12 h recovery period in darkness at room temperature (20 °C). Peel disks were dark adapted for 30 min before any readings were taken. No changes occurred in the

fluorescence readings in disks placed under room condition (20 ℃; 15 µmol m^{-2} s^{-1}) for 3 h.

Fruit peel was collected before the start of the treatments and directly after the stress treatments for the chlorophyll and xanthophyll cycle pigment analysis. The fruit peel was kept at -80℃ until carotenoid pigment analysis was done using an HPLC method as described by Lashbrooke et al. (2010).

Statistical analysis

The analysis of variance (ANOVA) and covariance (ANCOVA) were done with SAS 9.1 (SAS Institute Inc., Cary, NC., USA). Treatment induced changes in the xanthophyll cycle carotenoids and chlorophyll concentrations for each cultivar were analysed with a one way ANOVA, and the interactions of the main factors (cultivars and treatments) with a two way ANOVA, the means were separated with LSD (α = 0.05). The difference in the recovery fluorescence values between DTT treated and not treated peel was analysed with an ANCOVA using the initial (pre-stress) values as a covariate. An independent sample t-test was done to compare the difference between the fluorescence parameters of DTT treated and non-treated peel at the initial (pre-stress) and directly after stress stages, using Windows Microsoft Excel 2010 (α = 0.05) (Microsoft Corporation, Redmond, WA., USA). Means and +/- standard errors are indicated in the tables and on graphs.

Results

The maximum light use efficiency of photosystem II (F_v/F_m) of the apple cultivars tested decreased irreversibly in response to the applied PAR and heat stress (Figure 1) irrespective of the DTT treatments. The recovery F_v/F_m of the stressed DTT treated peel (+DTT) was significantly lower than that of stressed untreated peel (–DTT) of 'Granny Smith' and 'Braeburn', while there was no differences between +DTT and –DTT peel of 'Fuji', 'Golden Delicious' and 'Topred' (Figure 1, Table 1a). Additionally, although the after stress F_v/F_m values of +DTT peel of 'Granny Smith' was also significantly lower than that of –DTT peel (Figure 1, Table 1b), they recovered during the recovery period. However, the after stress F_v/F_m values of +DTT peel of 'Braeburn' did not recover during the recovery period and it instead deteriorated further (Figure 1). There was no interaction between the treatments and the cultivars for the F_v/F_m values ($p = 0.4123$).

The recovery F_v/F_m values of +DTT peel were 57%, 77%, 37%, 64% and 73% lower than the initial F_v/F_m values in 'Granny Smith', 'Braeburn', 'Fuji', 'Golden Delicious' and 'Topred' respectively (Figure 5). However, the recovery F_v/F_m values of -DTT peel were 44%, 56%, 34%, 60% and 66% lower than the initial F_v/F_m values for the same 4 cultivars respectively. The DTT induced percentage decrease in F_v/F_m of 'Granny Smith' and 'Braeburn' +DTT peel was therefore higher than for 'Fuji', 'Golden Delicious' and 'Topred' +DTT peel.

The minimum (F_o), variable (F_v) and maximum (F_m) fluorescence were also reduced by the applied stress (Figure 2 – 4). However, the F_o of 'Topred' peel had a non-statistically significant increase in both treatments after stress (Figure 2). The -DTT

peel incurred a higher percentage reduction of the F_o from the initial to recovery stage compared to +DTT peel in all cultivars except 'Topred' (Figure 5). However, the –DTT peel had a lower percentage F_v reduction compared to +DTT peel in all cultivars except 'Fuji' (Figure 5). The change in F_m varied between cultivars. Nonetheless, +DTT and –DTT peel of 'Granny Smith' and 'Braeburn', including the –DTT peel of 'Fuji', had an average percentage reduction in F_m of 80%. The average percentage reduction in F_m of 'Golden Delicious' and 'Topred', including the +DTT peel of 'Fuji', was however 70% (Figure 5). There was no significant interaction between the treatments and cultivars for the F_o ($p = 0.2134$), F_v ($p = 0.6924$) and F_m ($p = 0.4573$) values.

The total chlorophyll concentration of 'Braeburn' –DTT peel was significantly higher than +DTT and pre-stress peel (Table 2), while in the +DTT and –DTT peel of 'Topred' it was significantly lower than in pre-stress peel. Chlorophyll a concentration of 'Braeburn' –DTT peel was higher than +DTT and pre-stress peel. There was no significant interaction between the treatments and cultivars for chlorophyll b, chlorophyll a, chlorophyll a/b and total chlorophyll concentrations (Table 5).

The total xanthophyll pool size (zeaxanthin + antheraxanthin + violaxanthin) of the +DTT and –DTT peel was higher than the non-stressed peel in all the cultivars tested (Table 3). No xanthophyll analysis was done for 'Golden Delicious' peel due to missing samples. The total xanthophyll pool size and zeaxanthin concentrations in –DTT peel of 'Granny Smith', 'Fuji' and 'Topred' were significantly higher than in +DTT peel (Table 3, 5). However, the total xanthophyll pool size and zeaxanthin concentrations in –DTT peel of 'Braeburn' did not differ from +DTT peel. There was

no statistically significant difference in the lutein and β–carotene concentrations between the treatments (Table 3, 5).

Chlorophyll a concentration was 14%, 45% and 24% higher in 'Granny Smith', 'Braeburn' and 'Topred' –DTT peel than in +DTT peel respectively (Table 4). In 'Fuji' –DTT peel it was -1% lower than in +DTT peel. -DTT 'Granny Smith', 'Braeburn', 'Fuji', and 'Topred' peel had 29%, 33%, 10% and 0% higher lutein concentration than +DTT peel respectively (Table 4). 'Granny Smith' and 'Braeburn' –DTT peel β–carotene was 19% and 43% higher than +DTT peel respectively, while in 'Fuji' and 'Topred' –DTT peel it was -1% lower and 12% higher than in +DTT peel respectively.

There was a significant interaction between the treatments and the cultivars for the individual zeaxanthin, antheraxanthin and violaxanthin concentrations, and their combined concentration (Z+A+V) (Table 5). However, it is clear from Table 3 that although there was a significant interaction, the effects of the main factors are not obscured by this interaction. The application of DTT clearly reduced the Z+A+V concentration and specifically of zeaxanthin in +DTT peel compared to –DTT peel irrespective of the cultivar (Table 3, Table 4). There was no interaction between the treatments and the cultivars for the neoaxanthin, lutein and β-carotene concentrations (Table 5).

Discussion and conclusion

'Granny Smith' and 'Braeburn' fruits showed a higher dependency on the xanthophyll cycle for photoprotection under light and heat stress compared to 'Fuji', 'Golden

Delicious' and 'Topred' fruits. This is indicated by the low recovery of F_v/F_m values in 'Granny Smith' and 'Braeburn' +DTT peel compared to –DTT peel (Figure 1, Table 1). In contrast, there was no difference between the recovery F_v/F_m of +DTT and –DTT 'Fuji', 'Golden Delicious' and 'Topred' peel. In addition, +DTT 'Granny Smith' and 'Braeburn' peel had a higher percentage photodamage than –DTT peel (Figure 5a), yet, +DTT and –DTT 'Fuji', 'Golden Delicious' and 'Topred' experienced similar photodamage (Figure 5a). DTT interrupts the xanthophyll cycle by inhibiting the de-epoxidation of violaxanthin to zeaxanthin in the xanthophyll cycle (Yamamoto and Kamite 1972). The xanthophyll cycle prevents or reduces photoinhibition and eventual photodamage by removing excess excitation energy from the photosystem and releasing it as heat (Demmig-Adams 1990, Lambers et al. 1998, Cheng 2003). The disruption of the xanthophyll cycle in 'Granny Smith' and 'Braeburn' peel therefore led to a higher photodamage of +DTT peel of these two cultivars under the applied stress than in 'Fuji', 'Golden Delicious' and 'Topred' peel. The results therefore indicate that 'Granny Smith' and 'Braeburn' fruits may have a higher dependency on the xanthophyll cycle for photoprotection than 'Fuji', 'Golden Delicious' and 'Topred' fruits.

The involvement of the xanthophyll cycle in the observed F_v/F_m changes after stress can be revealed by the changes in chlorophyll in post stress peel. 'Braeburn', 'Fuji' and 'Topred' –DTT and +DTT peel had higher chlorophyll a/b ratios compared to pre-stress peel, while the increased ratio in 'Granny Smith' peel was not statistically significantly different from that of pre-stress peel (Table 2). In addition, the percentage chlorophyll a concentrations of 'Granny Smith', 'Braeburn' and 'Topred' –DTT peel was higher than in +DTT peel (Table 4). High chlorophyll a and a/b ratio is

not only associated with reduced light harvesting capacities of plant photosystems (Lindahl et al. 1995) but also with increased xanthophyll cycle activity (Kleima et al. 1999). Consequently, 'Granny Smith', 'Braeburn' and 'Topred' -DTT peel must have had higher xanthophyll cycle activities. It should be noted that 'Granny Smith' peel in general still had higher xanthophyll cycle carotenoid concentrations than 'Braeburn' and 'Topred' peel (Table 3). This observation is in agreement with Chen et al. (2013) who also found that green apples peel have more xanthophyll cycle carotenoids than red apples peel. The potentially higher xanthophyll cycle activity in 'Topred' –DTT peel compared to +DTT peel, however, did not provide sufficient photoprotection as these peel suffered similar photodamage to +DTT peel (Figure 1). In contrast, the observed changes in chlorophyll concentrations and F_v/F_m in 'Granny Smith' and 'Braeburn' –DTT and +DTT peel may indicate that the xanthophyll cycle activities of their -DTT peel was an effective photoprotective mechanism for this peel.

The application of DTT disrupted the xanthophyll cycle in all the cultivars tested. The applied heat and light stress increased zeaxanthin concentration while decreasing the violaxanthin concentration in both the +DTT and –DTT peel (Table 3). However, the zeaxanthin concentration in +DTT peel was lower than that in –DTT peel, indicating the effect of DTT on the xanthophyll cycle. In a study of the tree-fern *Dicksonia antarctica,* Volkova et al. (2009) also found that the xanthophyll pool size was increased by an exposure to PAR (900 µmol m^{-2} s^{-1}) and heat (35 ℃), while increasing the heat level to 47 ℃ had no effect. Chen et al. (2008) further reported that the total xanthophyll pool size is higher in sunburned apple fruits compared to non-sunburned peel. The inhibition of the xanthophyll cycle by the application of DTT

thus possibly resulted in the observed higher photodamage in +DTT than –DTT peel after stress.

The involvement of the xanthophyll cycle in the observed photodamage is further suggested by changes in the F_o, F_m and F_v of all the cultivars (Figure 2 – 4, 5). A decrease in the F_o can indicate an activation of the xanthophyll cycle resulting in the release of excess absorbed energy as heat (Demmig et al. 1987, Krause 1991). However, an increase in F_o is an indication of damage to the antennae unit of the light harvesting complex of PSII (Maxwell and Johnson 2000). Müller et al. (2001) and Yang and Yao (2008) also showed that a combined decrease in F_o and F_m correspond with increased photoprotective thermal dissipation via the xanthophyll cycle. In 'Granny Smith', 'Braeburn', 'Fuji' and 'Golden Delicious' peel the percentage decrease in the F_o was higher in –DTT peel than in +DTT peel. The –DTT peel therefore may have had higher xanthophyll cycle activities compared to +DTT peel. In 'Topred' fruits the F_o was increased in both –DTT and +DTT peel, suggesting a damage on the PSII. A decrease in F_v is an indication of damage to the oxygen evolving complex (OEC) of PSII (Govindjee at al. 1981). Therefore, +DTT peel which in general show a higher percentage reduction in F_v than –DTT (Figure 2c), incurred a higher damage to the OEC than –DTT peel. Photodamage in -DTT peel therefore may have been primarily due to the xanthophyll cycle induced increased xanthophyll cycle activities while in +DTT it could have been due to both increased NPQ and direct damages to the photosystem.

The difference in the lutein and β-carotene concentrations of +DTT and –DTT peel may also have contributed to the observed higher photodamage in 'Granny Smith'

and ‘Braeburn’ +DTT peel compared to –DTT peels. ‘Granny Smith’ and ‘Braeburn’ -DTT peel had higher percentage lutein and β-carotene concentrations than +DTT peel, while there was little difference between –DTT and +DTT ‘Fuji’ and ‘Topred’ peel (Table 4). These differences in lutein and β-carotene concentrations may have had a biochemically significant effect, although they were not statistically significant. Lutein is reported to contribute towards light harvesting by the photosystems, stabilizing the antenna proteins and quenching chlorophyll molecules in the triplet state (^{3}Chl*) (Jahns and Holzwarth 2012). β-carotene is reported to transfer an electron to the highly energised P_{680} chlorophyll molecule of PSII, therefore preventing the formation of oxygen radicals (De Las Rivas et al. 1993; Telfer 2002). The stability and efficiency of the photosystems of +DTT ‘Granny Smith’ and ‘Braeburn’ peel therefore may have been lower than those of +DTT ‘Fuji’ and ‘Topred’ peel. This may possibly have contributed to the observed lower recovery F_v/F_m of +DTT ‘Granny Smith’ and ‘Braeburn’ peel compared to –DTT peel of these cultivars.

In conclusion, apple cultivars seem to differ in their dependence on the xanthophyll cycle for photoprotection. The photosystems of ‘Granny Smith’ and ‘Braeburn’ peel appeared to depend more on the xanthophyll cycle for photoprotection than those of ‘Fuji’, ‘Golden Delicious’ and ‘Topred’. The difference in the involvement of the xanthophyll cycle during fruit peel sunburn development on different apple cultivars needs further investigation.

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Table 1a. P values associated with the two way ANCOVA analysis of the difference in the Recovery fluorescence parameters between the DTT treated and non-treated apple peel. Recovery readings were taken after a 12 h recovery period in the dark at room temperature (20 C°). Mean values are presented in Fig. 1 to 4.

	Apple cultivars				
Fluorescence	'Granny Smith'	'Braeburn'	'Fuji'	'Golden Delicious'	'Topred'
F_v/F_m	0.035	0.038	0.988	0.975	0.768
F_o	0.320	0.032	0.182	0.837	0.922
F_v	0.767	0.213	0.190	0.579	0.923
F_m	0.350	0.056	0.035	0.235	0.317

Table 1b. P values associated with the t-test analysis of the difference between the fluorescence parameters of the DTT treated and non-treated apple peel at the Initial and After stress stages. Mean values are presented in Fig. 1 to 4.

Legend: Initial = before stress; After = after the 3 h stress (30 min dark adaptation).

		Apple cultivars				
Period	Fluorescence	'Granny Smith'	'Braeburn'	'Fuji'	'Golden Delicious'	'Topred'
Initial	F_v/F_m	0.455	0.735	0.471	0.610	0.538
	F_o	0.154	0.903	0.453	0.549	0.278
	F_v	0.312	0.637	0.623	0.650	0.383
	F_m	0.117	0.657	0.526	0.697	0.345
After	F_v/F_m	0.017	0.454	0.423	0.372	0.447
	F_o	0.015	0.838	0.111	0.611	0.350
	F_v	0.408	0.785	0.736	0.432	0.569
	F_m	0.048	0.759	0.047	0.344	0.302

Table 2. Chlorophyll concentration of stressed apple fruit peel treated or not treated with DTT and of pre-stressed peel. Stressed fruits were exposed to 1,500 µmol m^{-2} s^{-1} photosynthetic active radiation coupled with 45°C heat stress for 3 h. Pre-stress peel were not exposed to any stress. Different letters indicate differences between the stressed peel treated (+DTT) or not treated (-DTT) with DTT and pre-stressed peel for each cultivar. No data available for 'Golden Delicious' because of missing samples.

Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT.

Chlorophyll	Treatments	Apple cultivars			
(ng/mg FW)		'Granny Smith'	'Braeburn'	'Fuji'	'Topred'
Chlorophyll b	+DTT	50.43 a	17.49 a	27.10 a	22.86 a
	-DTT	53.89 a	23.69 a	28.89 a	25.05 ab
	Pre-stress	53.10 a	20.55 a	25.10 a	31.19 a
Chlorophyll a	+DTT	312.73 a	133.06 a	223.84 a	146.39 a
	-DTT	357.01 a	192.85 b	222.14 a	182.18 ab
	Pre-stress	331.32 a	142.89 a	168.65 a	212.69 b
Chlorophyll a/b	+DTT	6.21 a	7.61 ab	8.26 b	6.40 a
	-DTT	6.67 a	8.22 b	7.72 b	7.29 b
	Pre-stress	6.25 a	6.95 a	6.40 a	6.75 ab
Chlorophyll a+b	+DTT	363.15 a	150.55 a	250.94 a	169.25 b
	-DTT	410.90 a	216.53 b	251.03 a	207.23 b
	Pre-stress	384.42 a	163.45 a	193.74 a	261.52 a

Table 3. Xanthophyll cycle pigment concentration of stressed apple fruit peel treated or not treated with DTT and of pre-stressed peel. Stressed fruits were exposed to 1,500 µmol m^{-2} s^{-1} photosynthetic active radiation coupled with 45°C heat stress for 3 h. Pre-stress peel were not exposed to any stress. No data available for 'Golden Delicious' because of missing samples. Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT. UD = undetectable.

Carotenoids	Treatments	Apple cultivars			
(ng/mg FW)		'Granny Smith'	'Braeburn'	'Fuji'	'Topred'
Zeaxanthin	+DTT	22.89	14.30	17.65	17.07
	-DTT	41.14	19.50	20.63	20.36
	Pre-stress	10.75	2.80	UD	12.08
Antheraxanthin	+DTT	2.00	0.16	0.51	UD
	-DTT	0.77	0.59	0.37	0.05
	Pre-stress	0.52	UD	UD	0.21
Violaxanthin	+DTT	3.16	0.43	0.52	0.08
	-DTT	0.10	0.21	0.00	0.00
	Pre-stress	12.07	3.95	3.68	3.98
Z+A+V	+DTT	28.05	14.89	18.67	17.14
	-DTT	42.01	20.29	20.99	20.40
	Pre-stress	23.33	6.75	3.68	17.08
Neoaxanthin	+DTT	6.13	1.92	4.35	2.89
	-DTT	7.67	3.65	4.25	3.07
	Pre-stress	8.65	3.12	3.08	4.45
Lutein	+DTT	19.74	7.09	10.44	10.39
	-DTT	25.38	9.46	11.49	10.43
	Pre-stress	20.35	6.52	8.07	10.27
β-carotene	+DTT	414.57	313.15	370.29	267.54
	-DTT	494.97	447.34	365.92	298.99
	Pre-stress	482.19	285.25	270.24	345.65

Table 4. Percentage carotenoids and chlorophyll concentration in apple peel not treated with DTT (-DTT) in comparison to peel treated with DTT (+DTT). The numbers indicate how low (negative values) or high (positive values) the percentage pigment concentration in –DTT peel is compared to +DTT peel. ∞ = infinity.

Carotenoids and Chlorophylls	Apple cultivars			
	'Granny Smith'	'Braeburn'	'Fuji'	'Topred'
Zeaxanthin	80	36	17	19
Antheraxanthin	-61	260	-28	∞
Violaxanthin	-97	-52	-100	-100
Neoaxanthin	25	90	-2	6
Z+A+V	50	36	12	19
Lutein	29	33	10	0
β-carotene	19	43	-1	12
Chlorophyll b	7	35	7	10
Chlorophyll a	14	45	-1	24
Chlorophyll a/b	7	8	-7	14
Chlorophyll a+b	13	44	0	22

Table 5. P values associated with the one way ANOVA analysis of the xanthophyll cycle pigment and chlorophyll concentration differences between stressed apple fruit peel treated (+DTT) or not treated (-DTT) and pre-stressed peel. Mean values are in Table 1 and 2.

Carotenoids and Chlorophylls	Apple cultivars				
	'Granny Smith'	'Braeburn'	'Fuji'	'Topred'	Cultivar vs Treatment interaction
Zeaxanthin	0.000	0.002	<0.000	0.001	0.000
Antheraxanthin	0.025	0.380	0.155	0.140	0.010
Violaxanthin	0.002	<0.000	0.001	<0.000	<0.000
Z+A+V	0.008	0.010	<0.000	0.083	0.011
Neoaxanthin	0.345	0.029	0.281	0.308	0.352
Lutein	0.422	0.061	0.065	0.995	0.427
β-carotene	0.652	0.057	0.148	0.264	0.465
Chlorophyll b	0.948	0.133	0.543	0.074	0.961
Chlorophyll a	0.8159	0.027	0.120	0.035	0.745
Chlorophyll a/b	0.107	0.061	0.027	0.042	0.064
Chlorophyll a+b	0.839	0.033	0.145	0.016	0.789

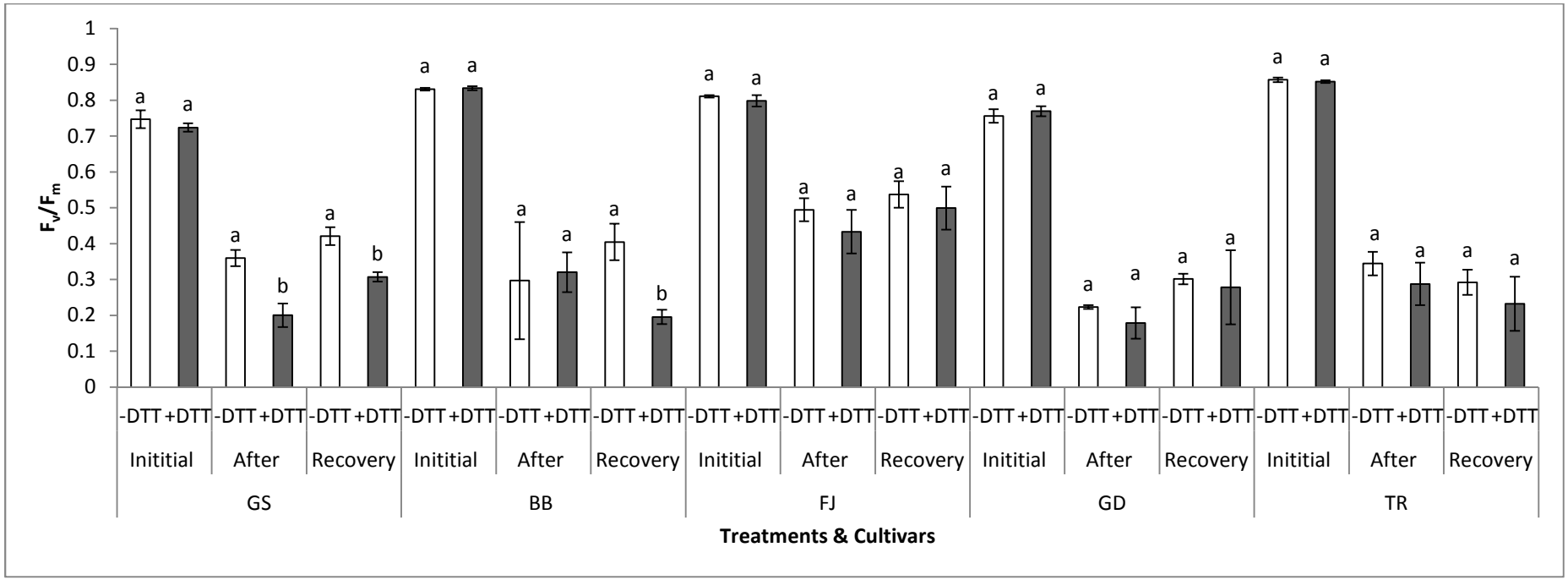

Fig. 1. The maximum light use efficiency (F_v/F_m) of apple fruit peel treated or not treated with DTT and exposed to 1,500 µmol m^{-2} s^{-1} photosynthetic active radiation coupled with 45°C heat stress for 3 h. Means and standard errors are indicated. Different letters indicate statistical differences between the treated and non-treated fruits (α = 0.05).

Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT. GS = 'Granny Smith'; BB = 'Braeburn'; FJ = 'Fuji'; GD = 'Golden Delicious'; TR = 'Topred'. Initial = initial readings; After = readings after stress (30 min dark adaptation); Recovery = photosystem recovery readings after 12 h recovery in the dark at room temperature (20 C°).

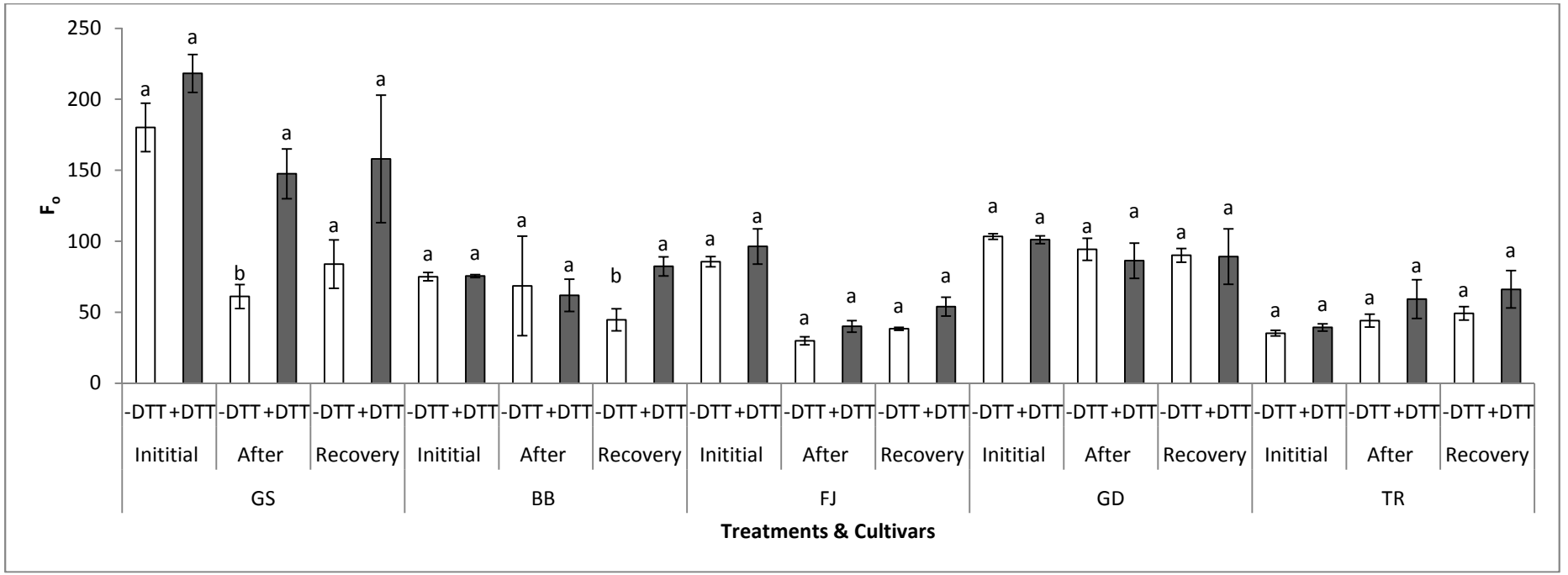

Fig. 2. The minimum fluorescence (F_o) of apple fruit peel treated or not treated with DTT and exposed to 1,500 µmol m^{-2} s^{-1} photosynthetic active radiation coupled with 45°C heat stress for 3 h. Means and standard errors are indicated. Different letters indicate statistical differences between the treated and non-treated fruits (α = 0.05).

Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT. GS = 'Granny Smith'; BB = 'Braeburn'; FJ = 'Fuji'; GD = 'Golden Delicious'; TR = 'Topred'. Initial = initial readings; After = readings after stress (30 min dark adaptation); Recovery = photosystem recovery readings after 12 h recovery in the dark at room temperature (20 C°).

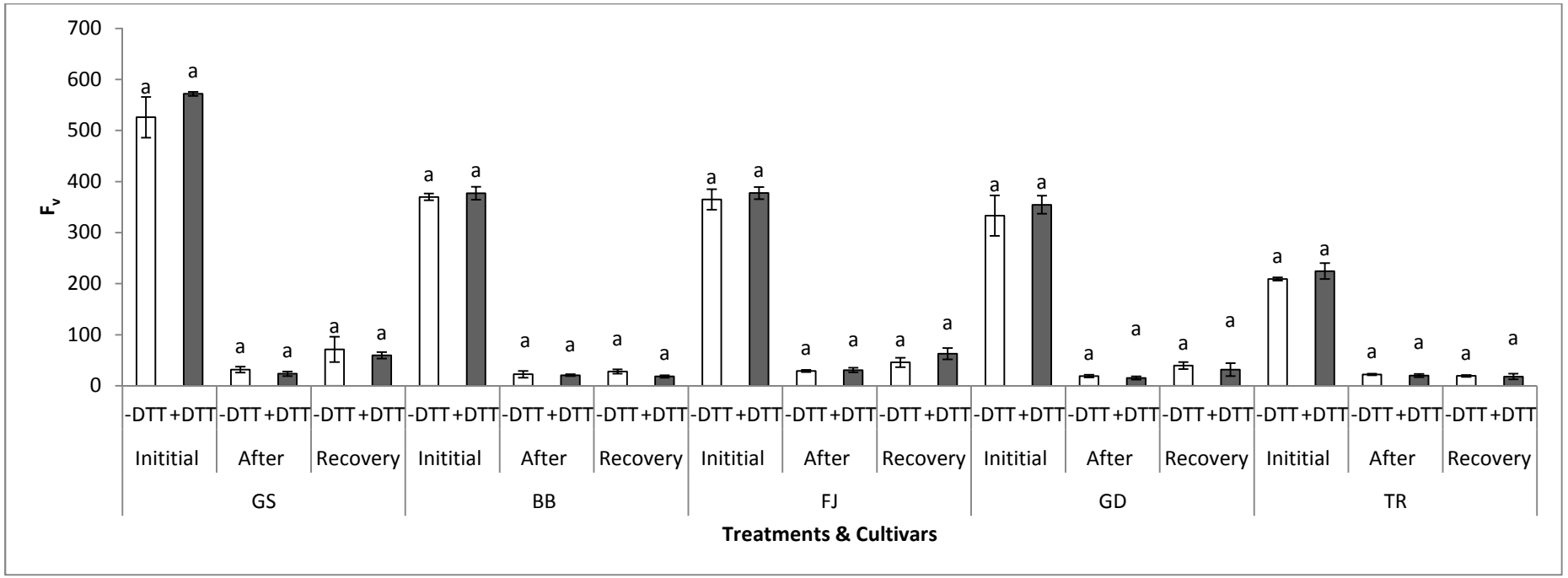

Fig. 3. The variable fluorescence (F_v) of apple fruit peel treated or not treated with DTT and exposed to 1500 µmol m^{-2} s^{-1} photosynthetic active radiation coupled with 45℃ heat stress for 3 h. Means and standard errors are indicated. Different letters indicate statistical differences between the treated and non-treated fruits (α = 0.05).

Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT. GS = 'Granny Smith'; BB = 'Braeburn'; FJ = 'Fuji'; GD = 'Golden Delicious'; TR = 'Topred'. Initial = initial readings; After = readings after stress (30 min dark adaptation); Recovery = photosystem recovery readings after 12 h recovery in the dark at room temperature (20 C°).

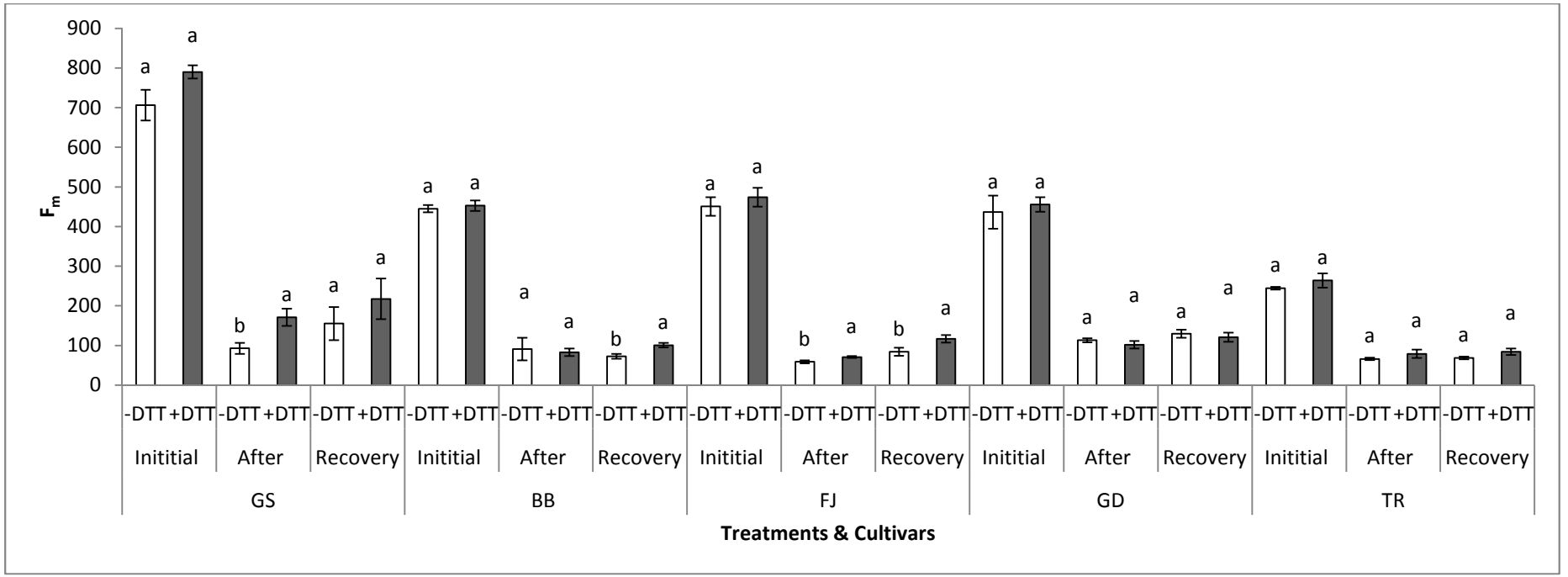

Fig.4. The maximum fluorescence (F_m) of apple fruit peel treated or not treated with DTT and exposed to 1,500 µmol m^{-2} s^{-1} photosynthetic active radiation coupled with 45℃ heat stress for 3 h. Means and standard errors are indicated. Different letters indicate statistical differences between the treated and non-treated fruits (α = 0.05).

Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT. GS = 'Granny Smith'; BB = 'Braeburn'; FJ = 'Fuji'; GD = 'Golden Delicious'; TR = 'Topred'. Initial = initial readings; After = readings after stress (30 min dark adaptation); Recovery = photosystem recovery readings after 12 h recovery in the dark at room temperature (20 C°).

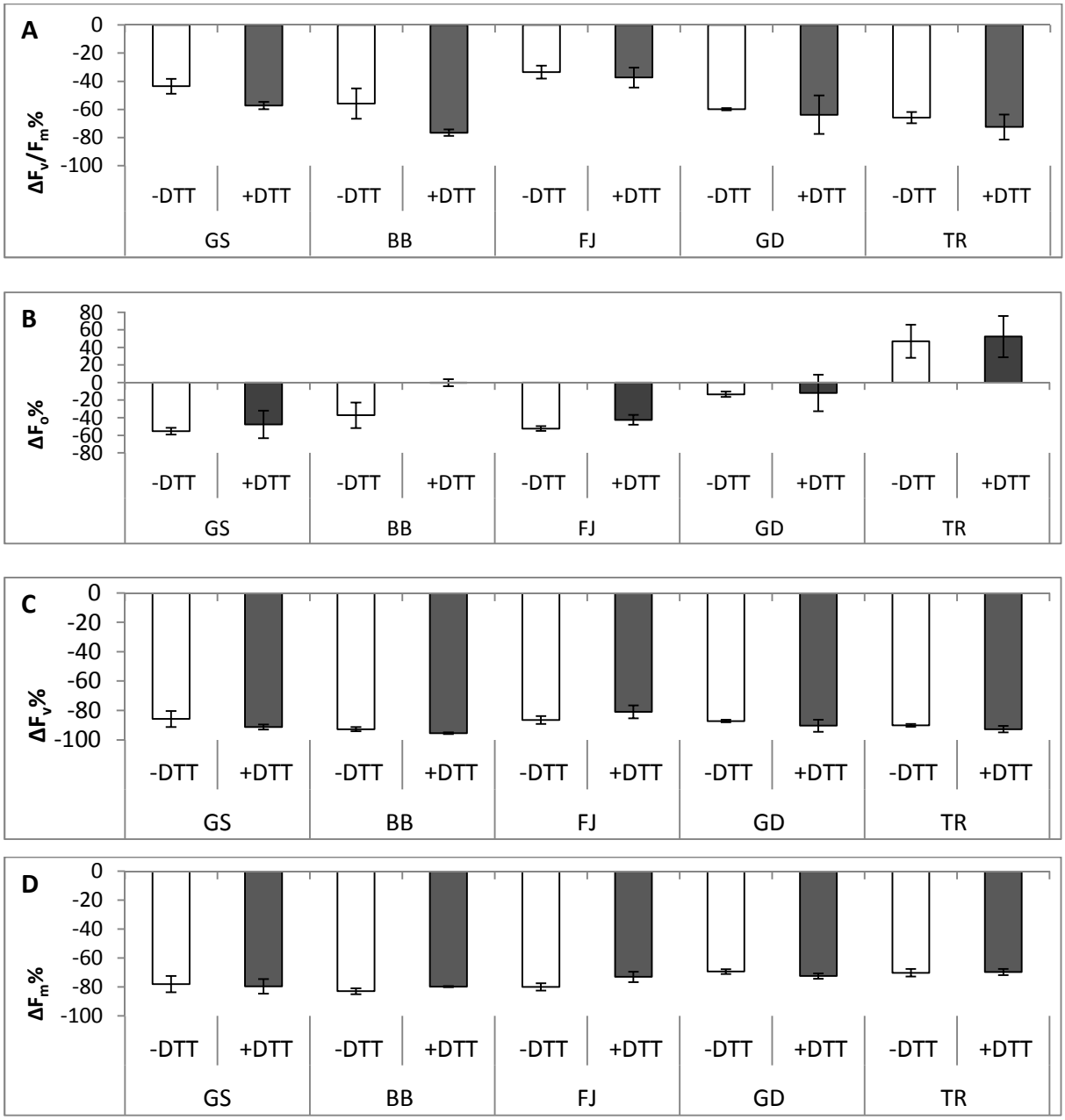

Fig. 5. Percentage change from initial to recovery values of the maximum light use efficiency (F_v/F_m), minimum (F_o), variable (F_v) and maximum (F_m) fluorescence in DTT treated or not treated peels exposed to 1,500 µmol m^{-2} s^{-1} photosynthetic active radiation and 45°C heat stress for 3 h. Initial readings were taken before stress and recovery readings after 12 h recovery in the dark at room temperature (20 C°). Means and standard errors are indicated.

Legend: DTT = dithiothreitol; +DTT = fruit peel treated with DTT; -DTT = fruit peel not treated with DTT GS = 'Granny Smith'; BB = 'Braeburn'; FJ = 'Fuji'; GD = 'Golden Delicious'; TR = 'Topred'.

PAPER 5

The effect of combined ultraviolet-B radiation, heat and photosynthetic active radiation stress on apple fruit photosynthetic systems

Abstract

The response of fruit peel photosystems of 'Granny Smith', 'Fuji' and 'Cripps' Pink' apples to ultraviolet-B radiation (UV-B), heat and photosynthetic active radiation (PAR) stress in different combinations was studied. In Experiment 1 'Granny Smith', 'Fuji' and 'Cripps' Pink' apple peel disks were exposed to the following treatments for 3 hours in the lab: Sub-experiment 1 = UV-B (290 – 320 nm; 3.9 kJ m^{-2} s^{-1}) + Heat (45 ºC) + PAR (1500 µmol m^{-2} s^{-1}); Sub-experiment 2 = UV-B + Heat; Sub-experiment 3 = UV-B alone. The heat, UV-B and PAR levels were the same in all three treatments. In Experiment 2, previously shaded or sun-exposed peel of mature 'Granny Smith' apples were exposed to the following heat levels for 3 hours per day for 3 days (total 9 hours): 30 ºC, 35 ºC, 40 ºC, 45 ºC and 50 ºC plus a constant 550 µmol m^{-2} s^{-1} PAR. The maximum light use efficiency of photosystem II (Fv/Fm) was measured before, after stress and 12 hour after a dark recovery period. Apple fruit photodamage increased with increasing heat stress levels and stress duration period in both previously shaded and sun-exposed peel. Previously shaded fruit peel incurred photodamage after a shorter duration and at a lower temperature than the previously sun-exposed fruit peel. This may relate to photo-oxidative sunburn at relatively low temperatures upon the sudden exposure of previously shaded apples

to stress. The combinational Heat + UV-B + PAR stress treatment caused the greatest damage to fruit peel photosystems compared to individual stresses, while the response to the applied stress varied between cultivars.

Keywords: UV-B, heat, photosynthetic active radiation, apple, sunburn

1. Introduction

Fruits are exposed to heat and light stress while on the trees before harvest under climatic conditions common in apple production areas of South Africa. This exposure can lead to the development of sunburn symptoms on fruit surfaces. Sunburn damage can reduce fruit quality and severe damage can increase susceptibility to other quality risks such as secondary pathogenic attacks. Heat, ultraviolet-B radiation (UV-B) and photosynthetic active radiation (PAR) are reported to be involved in fruit sunburn development (Racsko and Schrader, 2012; Schrader *et al.*, 2001+2003, Wünsche *et al.*, 2004). The combined heat and PAR stress decreases the photosynthetic efficiency of chlorophyllous plant tissue (Chen *et al.*, 2008; Königer *et al.*, 1998). Sunburn symptoms develop on fruit peel after fruits are exposed to temperatures of 45 °C to 49 °C in the presence of sunlight for a period of about one hour (Schrader *et al.*, 2001). The understanding of fruit sunburn symptom development can be improved through the induction of sunburn symptoms, identical to symptoms observed in orchards, under laboratory conditions. To this end, it is important to study the response of fruits to heat stress in the presence of PAR and UV-B. An understanding of the response of fruits with different heat and light exposure histories to heat and light stress can also shed more light on the sunburn

symptom development process. Literature on the study of the response of apple fruits to different long term heat stress levels combined with PAR stress under laboratory conditions is very limited.

Heat stress damage to plant photosystems includes induction of oxidative stress through the production of reactive oxygen species, inhibition of the Calvin cycle and the oxygen evolving complex functions, reduction of the electron transport rate, changes in the chemical components, and denaturation of proteins and chloroplast components (Allakhverdiev, 2008; Chen *et al.*, 2008; Rokka *et al.*, 2000; Wahid *et al.*, 2007). General plant cell response to heat stress includes production of heat shock proteins and other heat stress-tolerance related proteins as well as antioxidants, maintenance of lipid membrane and protein structures and functions, accumulation of osmolytes, reduction of the antenna size of photosystem II (PS II), and increased production of secondary metabolites such as phenolics (Allakhverdiev, 2008; Wahid *et al.*, 2007).

The objectives of this study were to: 1. study the effect of the heat, PAR and UV-B stress in different combinations on the photosystems of apple fruit peels; 2. determine the response of apple fruit photosystems, with different sun light exposure history, to a continuous exposure of different heat stress levels coupled with a moderate PAR level.

2. Material and Methods

2.1. Plant material

Two experiments were conducted for this study. Fruits of Granny Smith, Cripps' Pink and Fuji apple cultivars were used in Experiment 1 while 'Granny Smith' fruits were used in Experiment 2. Sun-exposed fruits were harvested at maturity from the mid-section of the canopy from farms in the Grabouw area (34º9'10.55''S; 19º1'47.62''E) which is located in the Mediterranean-type climate Western Cape Province of South Africa. Fruit peel disks of 12 mm diameter and 3 cm depth were collected from midway between the stem and calyx ends of the fruits. The disks were inserted in distilled water in random positions in a white foam cuvette holder directly after being extracted from the fruits and placed under lamps. The fruit peel was at least 5 mm above the water level in the cuvette holder to prevent direct damage to the peel by the warm water. Fruits were kept at room temperature (20 ºC) in the dark for approximately 4 hours before being used in the study.

2.2. Experiment 1: Effect of UV-B, heat and PAR stress on fruit peel photosystems

The experiment was made up of three sub-experiments to assess the effect of 1) combined UV-B, heat and PAR, 2) combined UV-B and heat, and 3) UV-B on the photosystems of previously sun-exposed apple peel. The individual contributions of PAR and heat stress were calculated as:

PAR effect = (combined UV, Heat and PAR) – (combined UV and Heat);

Heat effect = (combined UV and Heat) - (UV)

2.2.1. Sub-experiment 1: Effect of combined UV-B, heat and PAR on fruit peel photosystems

Fruit peels were exposed to UV-B (290 – 320 nm; 3.9 kJ m^{-2} s^{-1}) + heat (45 ºC) + PAR (1500 µmol m^{-2} s^{-1}) for 3 hours. A total of 39 fruits were randomly collected from 5 trees of each cultivar, with 24 (4 replicates with 6 fruits per replicate) of the fruits used for stress treatments and 15 fruits used for maturity indexing.

2.2.2. Sub-experiment 2: Effect of combined UV-B and heat on fruit peel photosystems

Fruit peels were exposed to heat (45 ºC) + UV-B (290 – 320 nm; 3.9 kJ m^{-2} s^{-}) for 3 hours. A total of 47 fruits were randomly collected from 6 trees of each cultivar, with 32 of the fruits used for the stress treatment (4 replicates with 8 disks per replicate) and 15 fruits used for maturity indexing.

2.2.3. Sub-experiment 3: Effect of UV-B on fruit peel photosystems

Fruit peels were exposed to UV-B (290 – 320 nm; 3.9 kJ m^{-2} s^{-1}) for 3 hours. The same number of fruits was used as in sub-experiment 2.

2.3. Experiment 2: The response of apple fruit photosystems, with different sun light exposure histories, to heat stress combined with moderate PAR

Previously shaded (PSH) and previously sun-exposed (PSN) peel disks were collected from separate fruits. All the fruits were collected from the outer canopy as described for Experiment 1. A total of 90 fruits were randomly collected (45 for PSH and 45 for PSN) from 18 trees, with 60 of the fruits used for the stress treatment (6

disks per 5 heat treatments per exposure history) and 30 fruits used for maturity indexing.

PSH and PSN peel disks were exposed to a constant PAR of 550 µmol m^{-2} s^{-1} and the following five heat stress treatments:

30 ºC (33 ºC ±0.136), 35 ºC (37 ºC ±0.544), 40 ºC (43 ºC ±0.486), 45 ºC (48 ºC ±0.393), and 50 ºC (50 ºC ±0.646).

The heat stress was imposed for 3 hours per day for 3 days, giving a total of 9 hours. The peels were kept in the dark at a room temperature of 20 ºC to 25 ºC between stress exposures.

2.4. Light setup

Photosynthetic active radiation (PAR) was provided by two lamps (50W, Titan Halogen Dichroic with a UV filter, OSRAM Gmbt. Augsburg, Germany), placed on either sides of a central infrared lamp (175 W, PAR 38IR175R, Philips, Amsterdam, Holland). The infrared lamps were placed at different heights to induce the different fruit peel temperatures. Ultraviolet-B radiation (UV-B, 290-320 nm) was provided by UV-B 100 W fluorescent lamps (Philips, Amsterdam, Holland). UV-C was filtered out with cellulose acetate filters placed between the lights and fruit. PAR was measured with a quantum meter: LI-189; Li-Cor, Lincoln, Nebraska, USA. Temperature was measured every 60 minutes with a hand held infrared thermometer (Raynger MX4, Raytek Corporation, Santa Cruz, USA). UV-B radiation was measured with a spectroradiometer (Ophir PD300, Ophir Optronic Solutions, Jerusalem, Israel). The total daily UV-B dosage was 3.9 kJ m^{-2} s^{-1}.

2.5. Assessment of apple peel photosystem dynamics

Fruit chlorophyll *a* fluorescence was measured with an FSM 1 fluorimeter (Fluorescence Monitoring system 1, Hansatech, Norfolk, UK). The fluorimeter was connected to one half of a leaf-clip holder through a fiberoptic cable. The maximum (Fm) and minimum (Fo) fluorescence and maximum light use efficiency of photosystem II Fv/Fm = (Fm-Fo)/Fm were measured. The initial readings were taken before stress, the after stress readings taken after 30 min dark adaptation and the recovery reading after 12 hours dark adaptation at room temperature (20 ºC).

2.6. Statistics

Statistical analysis was done using a one way ANOVA with SAS 9.1 (SAS Institute Inc., Cary, NC., USA). Mean separation was done with LSD at $\alpha = 0.05$. Means and +/- standard errors are indicated on the graphs.

3. Results

3.1. Experiment 1: Effect of UV-B, heat and PAR stress on fruit peel photosystems

All the treatments had a negative effect on fruit peel maximum light use efficiency (Table 1). Combined UV-B + Heat + PAR stress (Sub-experiment 1) caused the greatest damage to fruit peel photosystems in all the cultivars tested compared to the other two sub-experiments, viz. UV-B + Heat stress (Sub-experiment 2) and UV-B stress (Sub-experiment 3) (Table 1; Figure 1). UV-B on its own did not result in significant damage to the photosynthetic capacity in any of the cultivars (Table 1; Figure 1). The calculated effect of PAR and heat on fruit peel photosystems varied

between cultivars, with ‘Cripps’ Pink’ and ‘Fuji’ being most negatively affected by the PAR stress and ‘Cripps’ Pink’ also considerably affected by the heat stress (Table 1).

The recovery minimum (Fo), variable (Fv) and maximum fluorescence (Fm) in all three cultivars was lower than the initial values for Sub-experiment 1 and 2 (Table 1). Although the recovery Fv and Fm in ‘Granny Smith’ peel was reduced in Sub-experiment 3, it was increased in ‘Cripps’ Pink’ peel (Table 1). The recovery Fo was also increased in ‘Granny Smith’, ‘Cripps’ Pink’ and ‘Fuji’ peel (Table 1). The calculated PAR and heat induced change to Fo also showed a reduction in all three cultivars (Table 1).

3.2. Experiment 2: The response of apple fruit photosystems, with different sun light exposure history, to heat stress coupled with a moderate PAR level

The recovery Fv/Fm of PSH peel was lower than the initial value after exposure to the different heat stresses (Table 2). The stress-induced reduction of Fv/Fm was higher in PSH peel than in PSN peel after the three and six hour stress exposure periods. There was however no difference in the Fv/Fm reduction between the PSH and PSN peel after the nine hour stress period (Table 2). There was significant interaction ($p<0.0001$) between temperature and stress duration periods for the Fv/Fm values. The results of changes in Fv/Fm after the 9 hour period showed a lower reduction in Fv/Fm at the 35 ℃ stress level after this period regardless of fruit heat exposure history.

The recovery Fv/Fm value of PSH peel decreased from an initial value of 0.826 to 0.358, 0.435 and 0.220 respectively after the 3, 6 and 9 hours exposure periods to

the 45 ℃ heat stress and moderate PAR (Figure 5). Furthermore, the recovery Fv/Fm value of PSH peel decreased from an initial value of 0.823 to 0.296, 0.0 and 0.0, respectively, after the 3, 6 and 9 hours exposure periods to the 50 ℃ heat stress and moderate PAR (Figure 2). However, the recovery Fv/Fm value of PSN peel decreased from an initial value of 0.741 to 0.500, 0.460 and 0.103, respectively, after the 3, 6 and 9 hours exposure periods to the 45 ℃ heat stress and moderate PAR (Figure 2). In addition, the recovery Fv/Fm value of PSN peel decreased from an initial value of 0.735 to 0.390, 0.0 and 0.0 respectively after the 3, 6 and 9 hours exposure periods to the 50 ℃ heat stress and moderate PAR (Figure 2).

4. Discussion

4.1. Experiment 1: Effect of UV-B, heat and PAR stress on fruit peel photosystems

Combined Ultraviolet radiation-B (UV-B) + Heat + Photosynthetic active radiation (PAR) stress resulted in the greatest damage to fruit peel photosystems compared to the UV-B + Heat or UV-B, PAR and heat stress on their own as indicated by the reduction in the measured and calculated fruit Fv/Fm (Table 1; Figure 1). The unstressed Fv/Fm value for most plants is 0.83 (0.7 – 0.8) and the critical value indicating photodamage is about 0.6 (Maxwell and Johnson, 2000; Ritchie, 2006). UV-B alone did not cause a physiologicaly significant Fv/Fm reduction (Figure 1). This is in agreement with our previous finding that UV-B treatment did not significantly reduced Fv/Fm in previously exposed peel of mature apples (Hengari et al., 2014). The photosystems of ‘Cripps’ Pink’ and ‘Fuji’ appear to be more sensitive to PAR than to heat stress, as PAR stress induced a greater Fv/Fm reduction than heat stress in these cultivars (Table 1). Although UV-B + Heat stress did damage the

photosystems in all three cultivars, UV-B stress alone did not. We calculated the individual effects of PAR and heat stress through substitution. This assumes that UV-B, PAR and heat stress have additive effects when in combination. However, these stresses are likely to have synergistic effects on apple peel photosystems, as suggested by Chen *et al.* (2008) who reported that combined high temperature and PAR treatment had a more damaging effect on apple fruit peel photosystems than high temperature or high PAR stress alone. For example, PAR is well known to have a much greater effect on photosystems at low temperature, while light capture is temperature insensitive, assimilatory enzymatic reactions decrease with decreasing temperature resulting in increased photoinhibition (Huner et al., 1993). Apple fruit peel photosystems are potentially more sensitive to a combined UV-B + Heat + PAR stress than UV-B + Heat or the individual stresses in isolation. Our calculations probably also overestimate the individual effects of heat and PAR stress while UV-B may only become a significant factor in association with other stresses such as heat and high PAR.

The three stress treatments appear to have differed in the way they damaged or negatively effected the fruit photosystems. UV-B caused an increase in Fo while Fo was decreased by the combinational treatments (Table 1). The effect of UV-B on plant photosystems is generally reported to target PS II by various pathways including actions that lead to the displacement of the light harvesting complex (Iwanzik *et al.*, 1983; Hollósy, 2002). A decrease in Fo, combined with a moderate decrease in Fv/Fm is an indication of an increase in the release of absorbed energy as heat (Demmig *et al.*, 1987). Krause (1991) also found a positive correlation between the decrease in Fo and the formation of zeaxanthin in plants. The

combinational stresses as well as the calculated PAR and heat stress, therefore may have increased fruit peel xanthophyll cycle activities, although this did not prevent photodamage. Chen *et al.* (2008) also found that 'Gala' apple fruit peel increased their xanthophyll cycle activities and antioxidant systems when fruits were exposed to a combination of 45 ℃ heat and 1600 µmol m^{-2} s^{-1} PAR. An increase in Fo is an indication of damage to the antenna of the light harvesting complex of photosystem II (PS II) (Maxwell and Johnson, 2000). UV-B therefore seems fruit PS II antenna complex, even though the reduction in Fv/Fm was not of significance. However, despite the seemingly insignificant effect of UV-B on its own, when combined with the effects of heat and PAR, UV-B may become a quite significant factor in damaging apple fruit photosystems.

The combinational treatments and the calculated PAR and heat stress induced a considerable reduction in the Fv and Fm values (Table 1).The activity of the oxygen evolving complex (OEC) in PS II is positively correlated to variable chlorophyll fluorescence (Fv) (Govindjee *et al*., 1981; Pistorius and Schmid, 1984). There is also a positive correlation between the relative concentration of undamaged PS II units in the thylakoid membrane and the Fm values (Lidon and Ramalho, 2011). Therefore, the decrease in Fv and Fm can possibly indicate a decrease in the OEC activities and the number of PS II units respectively in response to the combinational stress treatments. Chen *et al*. (2008) also concluded that the OEC of apple fruit peel is damaged by Heat + PAR stress. PAR stress is reported to cause the degradation of chlorophyll a while heat causes a denaturation of proteins in plant PS II (Wang *et al*., 1999). The induced chlorophyll bleaching in-turn then lead to the reduction in functional PS II units.

4.2. Experiment 2: The response of apple fruit photosystems, with different sun light exposure history, to heat stress coupled with a moderate PAR level

Photodamage in apple fruit peel increased with increasing heat stress duration and, heat stress level irrespective of fruit heat and light exposure history (Table 2 and Figure 2). The Fv/Fm values showed a significant interaction between temperature and stress duration periods in that short durations induced significant damage at higher temperatures whereas similar damage levels at lower temperatures required longer exposure periods (Table 2). Apple fruit Fv/Fm is generaly reported to decrease with increasing heat stress duration and heat stress levels (Li and Cheng, 2009; Chen *et al*., 2008; Wand *et al*., 2008). The 9 hours stress period caused significantly greater photodamage compared to the 3 and 6 hour periods, except for the 50 ºC treatment where no recovery already occurred after 6 hours, irrespective of fruit peel heat and light exposure history (Table 2). Our results therefore confirm earlier findings that photodamage to apple fruit photosystems increases with increasing heat stress level and duration. In addition, the level of damage after 9 hours did not seem to relate to the heat and light exposure history of the peel.

Fruit peel photosystems were irreversibly damaged by exposure to 45 ℃ and 50 ℃ for 3 hours irrespective of the fruit heat and light exposure history. The Fv/Fm of fruits peel dropped below 0.6 after these stress treatments (Figure 2). However, the 30 ℃, 35 ℃, and 40 ℃ stress levels only caused the Fv/Fm to drop below 0.6 after a 9 hour stress duration period (Figure 2). Sunburn damage is induced by a 1 hour exposure to a 45 ℃ to 49 ℃ heat stress in the presence of solar radiation, while sunburn symptoms only appear three days after exposure to the critical temperature

(Schrader *et al.*, 2001). Sunburned fruits have Fv/Fm values lower than 0.6 (Seo *et al.*, 2008). In the current study, exposing fruits to potentially sunburn-inducing temperature stress damaged their photosystems, but did not induce sunburn symptoms, even after 9 hours of stress exposure.

Pre-exposure to high temperatures and sun light in the orchard seemed to reduce the rate of photodamage experienced in fruit peel disks upon exposure to heat stress in the presence of PAR. The Fv/Fm reduction in PSH fruit peel appeared to be greater after 3 and 8 hours of exposure than in previously sun-exposed (PSN) fruit peel (Table 2; and Figure 2). Li and Cheng (2008) found that PSH fruits are more sensitive to photoinhibition than PSN fruits. Ma and Cheng (2004) reported that although the Fv/Fm on the shade side of attached apple fruits dropped from 0.835 to 0.341 after a one day exposure to sunlight (PAR of 1850 µmol m^{-2} s^{-1} and air tempearture at 30 ℃), the Fv/Fm recovered to 0.725 after a continuos 10 days exposure period. Chen *et al.* (2009) found that the Fv/Fm of PSN 'Fuji' apples was reduced to a lower extent than in the PSH fruits when detached fruits suffered a 46 to 48 ℃ heat stress in the dark. Our results presented here for 'Granny Smith' apple fruits suggest that PSH apple fruit peel would suffer a higher short term photodamage due to high temperature in the presense of PAR compared to PSN fruit peel.

Shade adapted apple fruit peel photosystems experienced greater photodamage than sun adapted fruits at temperatures lower than 45 ℃. The Fv/Fm reduction in PSN peel after a 3 and 6 hours exposure to 30 ℃ to 40 ℃ stress ranged from 1% to 13%, while in PSH peel it ranged from 8% to 16% (Table 2). The PSH peel also

appeared to suffer greater damage to the OEC and a higher reduction in functional PS II units than the PSN peel at these temperature ranges. These changes in the OEC and PS II units are indicated by the higher reduction in Fv and Fm values, respectively, in PSH compared to PSN peel after the 30 ℃ to 40 ℃ stress levels.. Wand *et al.* (2008) reported that shaded fruits have a lower temperature threshold for heat stress damage. PSN fruits have been found to possess higher heat shock protein and carotenoid concentrations, higher activities of the Calcin cycle enzymes, a faster electron transport rate, and higher xanthophyll and ascorbate-glutathione cycle activities than PSH fruits (Chen and Cheng, 2007; Chen *et al.*, 2009; Ferguson *et al.*, 1998; Ma and Cheng, 2003). However, photoprotective functions, such as the xanthophyll and ascorbate-glutathione cycles, can be upregulated when shaded fruits are exposed to high temperature conditions (Ma and Cheng, 2004). Our results suggests that PSH fruits may have a lower threshold for photodamage than the threshold of 45 ℃ reported for induction of sunburn browning in exposed apple peel. This photodamage at lower temperatures in PSH peel may relate to photo-oxidative sunburn as reported by Felicetti and Schrader (2008).

It is interesting to note that the 35 ℃ (37 ℃$\pm$0.544) heat stress resulted in the lowest reduction in Fv/Fm after the 9 hour stress period irrespective of exposure history when compared to the other temperature levels (Table 2). Wand *et al.* (2008) similarly found that the recovery Fv/Fm in detached apple fruits was higher after exposure to a 36 ℃ than 32 ℃ heat stress for 8 hours in the dark, although not statistically significant, while it was significantly higher than in fruits exposed to 43 to 51 ℃. The synthesis of heat shock proteins in apple fruit cells is reported to be highest at 38 ℃ (Bowen *et al.*, 2002). The lower photodamage experienced after 9

hours of 35 ℃ stress compared to lower and higher temperatures may relate to higher fruit stress tolerance ability over time at this stress level. However, this finding requires further validation.

5. Conclusion

Photodamage in apple fruit peel increased with increasing heat stress level and duration in both previously shaded and sun-exposed apple peel. The 45 ℃ and 50 ℃ stress levels caused irreversible damage to apple fruit peel photosystems after 3 hours. However, neither of these high heat stress levels nor the lower levels did induce externally visible sunburn symptoms on the peel even after the 9 hour exposure period. Shaded peel showed evidence of photodamage even after a short exposure to 30 to 40 ºC. This damage may relate to photo-oxidative sunburn and bleaching of chlorophyll may have resulted at higher PAR.

Combined UV-B + Heat + PAR stress caused the most damage to fruit photosystems. The photosystems of ‘Fuji’ peel appear to be more sensitive to PAR stress than to heat stress, while the inverse seems to be true for ‘Granny Smith’. ‘Cripps’ Pink’ seems sensitive to both PAR and heat stress. The stress combinations did not induce visible sunburn symptoms on the peel. UV-B stress alone appears not to have caused any physiologically significant damage to fruit photosystems. However, the combinational effects of stresses may be greater than the sum of the effect of the individual stressors.

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Table 1. Percentage change (initial to recovery values) in fluorescence parameters of apple fruit peel exposed to heat (45ºC), photosynthetic active radiation (PAR) (1500 µmol m^{-2} s^{-1}) and UV-B (3.9 kJ m^{-2} s^{-1}) in various combinations (Experiment 1). 'PAR' data were calculated as (UV,Heat,PAR) – (UV,Heat) and 'Heat' data calculated as (UV,Heat) - (UV). Each value indicates the percentage change from the initial (before stress) to the recovery values for each cultivar. Negative values indicate that the recovery values were smaller that the initial values while the opposite is true for positive values.

Treatment	Cultivar	ΔFo%	ΔFm%	ΔFv%	ΔFv/Fm%
UV,Heat,PAR	'Granny Smith'	-25	-45	-51	-18
	'Cripps' Pink'	-100	-100	-100	-100
	'Fuji'	-43	-77	-85	-39
UV,Heat	'Granny Smith'	-16	-32	-37	-16
	'Cripps' Pink'	-11	-44	-53	-26
	'Fuji'	-26	-41	-46	-16
UV	'Granny Smith'	7	-3	-6	-3
	'Cripps' Pink'	10	5	4	-2
	'Fuji'	5	-2	-4	-2
'PAR'	'Granny Smith'	-9	-13	-14	-2
	'Cripps' Pink'	-89	-56	-47	-74
	'Fuji'	-17	-36	-39	-24
'Heat'	'Granny Smith'	-22	-29	-31	-13
	'Cripps' Pink'	-21	-49	-57	-24
	'Fuji'	-31	-40	-42	-14

Table 2. Percentage change in fluorescence parameters of 'Granny Smith' fruit peel exposed to different heat stress levels and a constant photosynthetic active radiation (PAR) of 550 µmol m^{-2} s^{-1} (Experiment 2) for nine hours. Each value indicates the percentage change between the initial (before stress) and the recovery (after 12 hours in the dark at 20 ℃) values at each stress period.

	Stress period (hr)	**Shaded peel**					**Sun exposed peel**				
		30 ℃	**35 ℃**	**40 ℃**	**45 ℃**	**50 ℃**	**30 ℃**	**35 ℃**	**40 ℃**	**45 ℃**	**50 ℃**
∆Fo%	**3**	4	5	11	-16	-68	7	-4	-1	-24	-39
	6	8	11	24	-16	-100	15	-3	-4	-4	-100
	9	1	34	24	-47	-100	2	8	1	-25	-100
∆Fm%	**3**	-26	-22	-22	-68	-92	2	-8	-7	-60	-67
	6	-24	-21	-25	-66	-100	1	-14	-24	-53	-100
	9	-68	-35	-56	-88	-100	-56	-32	-53	-78	-100
∆Fv%	**3**	-32	-28	-30	-79	-97	1	-10	-8	-72	-77
	6	-30	-28	-37	-76	-100	-3	-18	-30	-70	-100
	9	-83	-50	-74	-97	-100	-77	-48	-70	-97	-100
∆Fv/Fm%	**3**	-8	-9	-11	-57	-64	-1	-2	-3	-32	-44
	6	-9	-10	-16	-47	-100	-5	-5	-13	-37	-100
	9	-57	-26	-47	-73	-100	-53	-24	-43	-85	-100

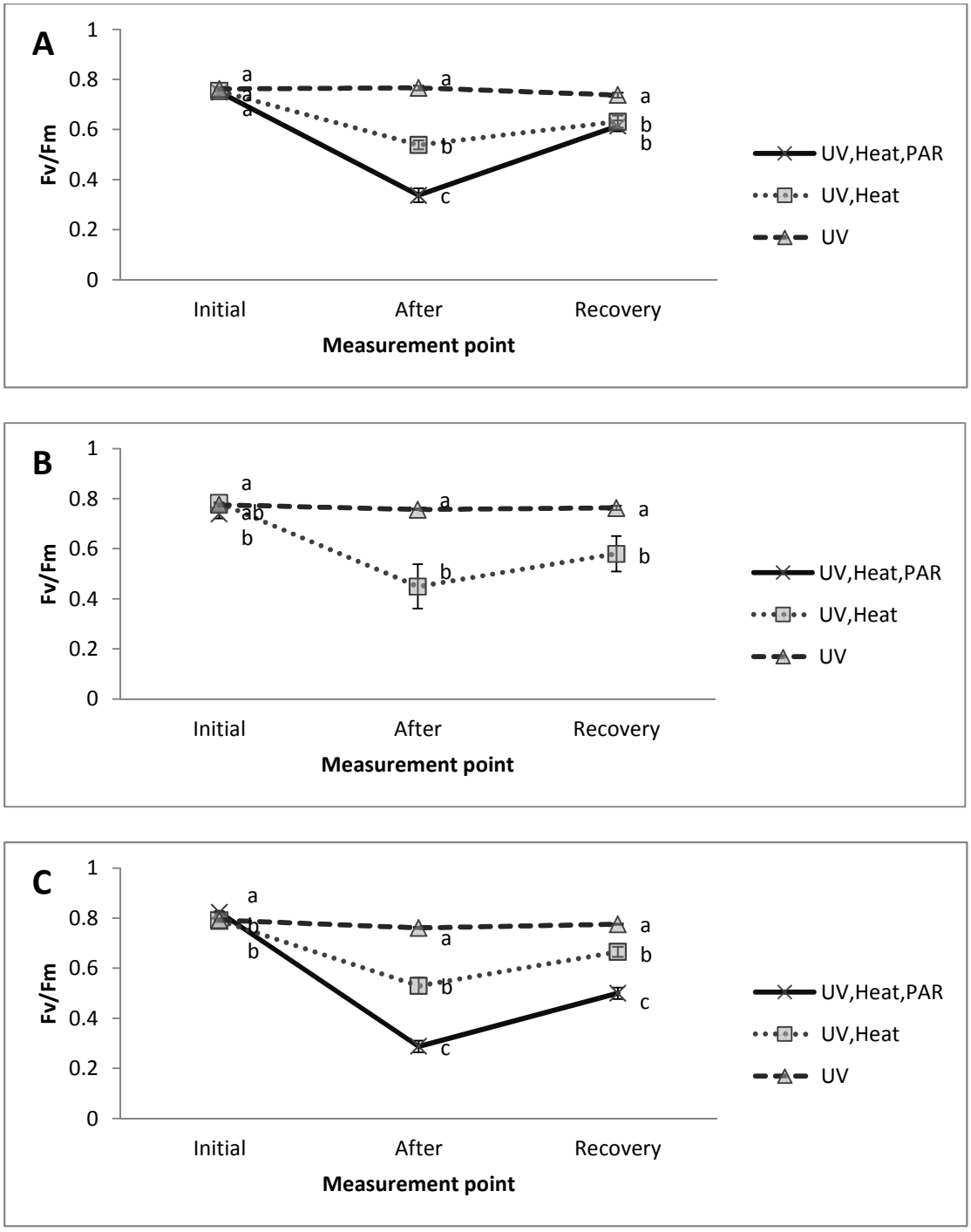

Figure 1. The effect of heat (45ºC), photosynthetic active radiation (PAR) (1500 µmol m^{-2} s^{-1}) and UV-B (3.9 kJ m^{-2} s^{-1}) in various combination on the maximum light use efficiency (Fv/Fm) of mature apple fruit peel (Experiment 1). A = 'Granny Smith'; B = 'Cripps' Pink'; C = 'Fuji'. Means and standard errors are indicated, different letters indicates differences between treatments at each measurement point. There were no readable values after the combined UV, Heat and PAR stress on 'Cripps' Pink'. Initial = Before stress; After = After stress (30 min dark adaptation); Recovery = After 12 hour in the dark at 20 ºC room temperature.

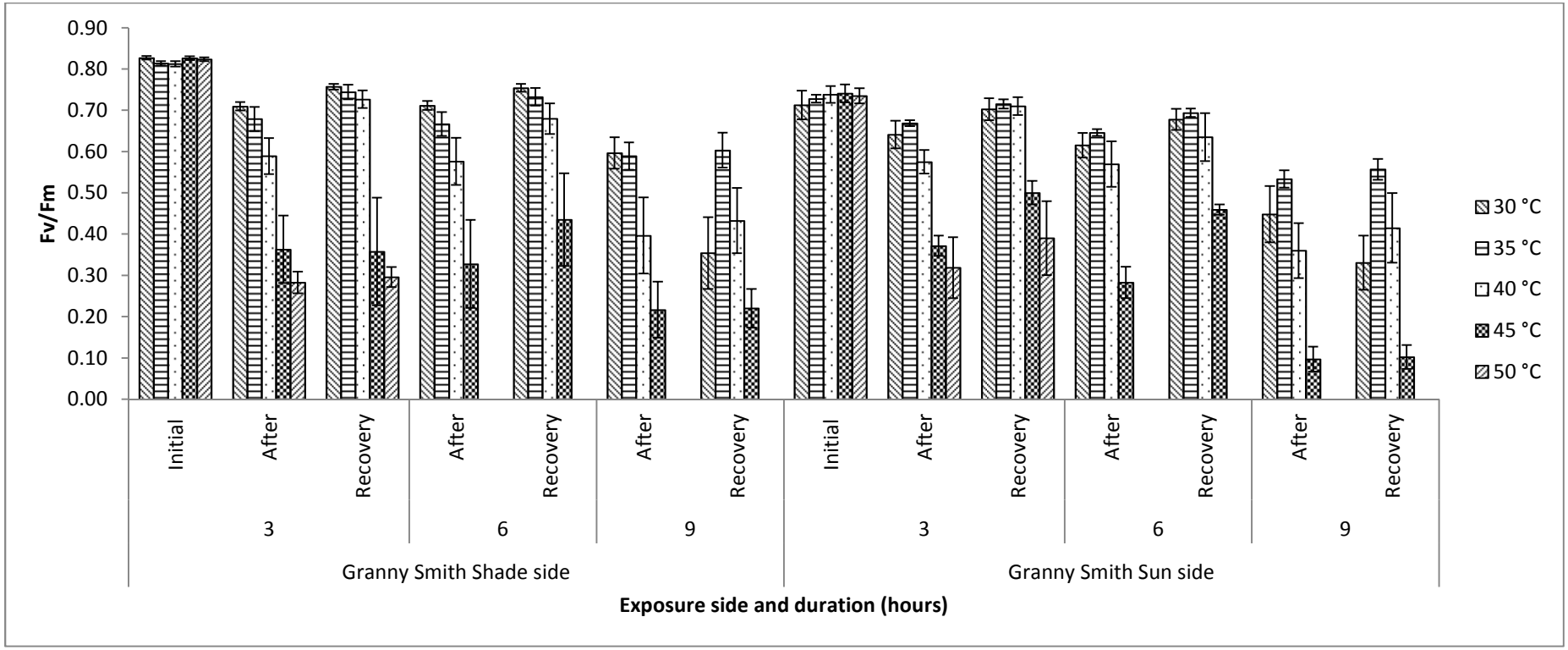

Figure 2. The effect of different temperature levels after 3, 6 and 9 hours (3 hours/day) at a constant photosynthetic active radiation (PAR) level of 550 µmol m^{-2} s^{-1} at maturity on the maximum light use efficiency of photosystem II (Fv/Fm) of previously shaded and sun exposed 'Granny Smith' apple fruit peel (Experiment 2). Means and standard errors are indicated. Initial = Before stress; After = After stress (30 min dark adaptation); Recovery = After 12 hour in the dark at 20 ºC room temperature.

8. GENERAL DISCUSION AND CONCLUSIONS

8.1. Introduction

Apple fruit sunburn affects up to 18% of the total annual production in South Africa (Gindaba and Wand, 2005). The export of apple fruits to the fresh market accounted for an average of 41.9% of the total apple production from 2002 to 2012 while annually a 3rd of total production is sold locally, much of it as 1st class fresh produce (HORTGRO, 2013). The fresh fruit market is therefore of high economic value for South African apple producers. Sunburn reduces the quality of apples which then reduces the first class export volumes and total income. The major sunburn type, sunburn browning, is caused by ultraviolet radiation-B (UV-B), photosynthetic active radiation (PAR) and heat at temperatures between ranging from 45 ℃ to 49 ℃ (Schrader *et al*., 2001). Sunburn browning appears as a yellow-bronze discoloration on apple fruit peels. There are two other fruit sunburn types that also contribute to the loss in fruit quality, i.e., sunburn necrosis (caused by fruit peel temperatures greater than 50 ℃) (Schrader *et al*., 2001) and photooxidative sunburn (caused by sudden exposure to light and fruit peel temperatures >31 ℃) (Felicetti and Schrader, 2008). An understanding of the detailed biochemical processes involved in fruit sunburn development is important for the improvement of current sunburn prevention techniques.

UV-B, heat and PAR cause sunburn by damaging fruit peel photosystems (Rabinowitch *et al.*, 1974). Increasing sunburn severity is found to be exponentially and negatively related to apple fruit chlorophyll fluorescence yield (Glenn and Yuri, 2013). It has also been reported that sunburned apple peel have lower maximum light use efficiency (Fv/Fm) than non-sunburned peel (Chen *et al.*, 2008). Therefore, determining the damage induced by the known sunburn inducing factors, individually or in combination, on fruit peel chlorophyll fluorescence can give an indication of the role of each factor in fruit sunburn development.

The general research hypothesis was that the rate of photodamage and subsequent sunburn browning symptoms in different apple cultivars can be studied by exposing apples to UV-B, PAR and heat stress in different combinations under laboratory conditions. The overall objective of this project was to identify the biochemical, physiological and peel anatomical characteristics that offer photoprotection, and therefore resistance to sunburn, in apple fruit peel. The aim was to characterise the variation between cultivars, and between fruit maturities (seasonal changes) in these characteristics, and to establish the threshold levels for UV-B, heat and PAR stress (in relation to sunburn) to damage fruit photosystems in different apple cultivars and whether these change through the season.

8.2. General discussion

8.2.1. Are there specific development stage at which fruits are more sensitive to UV-B stress, and does fruit light exposure history effect fruit UV-B sensitivity?

The photosystems of previously sun-exposed fruit peels are possibly not sensitive to UV-B stress (Hengari *et al*., 2014a, also see Paper 1). However, shaded 'Granny Smith' and 'Fuji' apples did suffer UV-B induced photodamage. Changes in the variable (Fv), maximum (Fm) fluorescence and the maximum light use efficiency of photosystem II (Fv/Fm) suggest, respectively, that the number of fruit photosystem II units, the activities of the oxygen evolving complex and also photosynthetic efficiency decreased as the fruit matured. Apple fruits also had a greater biochemical response to UV-B stress at the juvenile stage than at maturity, as indicated by reflection readings (see Paper 1). Results for the sun-exposed 'Granny Smith' apples contrast those reported by Solovchenko and Schmitz-Eiberger (2003) who showed a slight physiologically non-significant decrease in Fv/Fm of these fruits after exposure to a lower UV-B dosage than used by us, while 'Braeburn' was not affected. Nevertheless, our results are in agreement with those reported by Glenn *et al.* (2008) who also used a low UV-B dosage. The difference between the different studies could be due to the UV-B doses used, the maturity stages of the fruits as well as the prevailing climatic conditions in the orchards from which the fruits were collected. The UV-B study results and

the cited work demonstrate that the photosystems of sun adapted apple fruits are possibly not sensitive to UV-B stress.

The involvement of UV-B in fruit sunburn development is possibly more related to the induction of phenolic synthesis rather than causing photodamage. This is explained by the observed involvement of UV-B radiation in sunburn development (Schrader *et al*., 2001) and in up-regulation of phenolic synthesis in plants (Solovchencko and Merzlyak, 2008), in addition to the observed higher phenolic content in sunburned compared to non-sunburned fruits (Felicetti and Schrader, 2009a) and our UV-B study results (Hengari et al., 2014a, also see Paper 1). However, the interaction of UV-B with PAR and heat has been reported to enhance the UV-B stress effect and cause photodamage in plant tissue (Herrmann *et al*., 1997; Yamashita and Butler, 1968). Our results do not discount that this may be the case for apple fruit peel photosystems (see Paper 5).

The reported accumulation of phenolic compounds in sunburned fruit tissue (Felicetti and Schrader, 2009a) is possibly enhanced by PAR and could function as direct and indirect photoprotective mechanisms and protect damaged tissues against pathogens. It is known that PAR enhances the effect of UV radiation in phenolic synthesis (Adamse *et al*., 1994; Awad *et al*., 2001). This effect of PAR on phenolic synthesis could partly explain the involvement of PAR in fruit sunburn symptom development as reported by Schrader *et al*. (2001). Hernández and van Breusegem (2010) reported that phenolic compounds can act as possible carbon sinks to stabilize photosynthetic

processes during stress conditions. The synthesis of phenolic compounds therefore indirectly reduces photodamage by allowing the continuation of the light reaction without the possible formation of highly reactive photodamaging molecules. Phenolic compounds also act as antioxidants (Wagner *et al.*, 1988) that directly protect plant photosystems against potentially photodamaging reactive molecules. The necrotic areas that develop on heat damaged fruits can be infected by pathogens (Shane, 2012). Phenolic compounds have antifungal and bacterial properties and they accumulate around wounded plant tissue to provide protection or prevent the spread of the infections (Pourcel *et al.*, 2007; Treutter, 2006). The involvement of UV-B, combined with PAR, in fruit sunburn symptom development could therefore partly be related to the accumulation of phenolic compounds that have photoprotective functions and/or protect damaged tissues against pathogens.

8.2.2. Does the susceptibility of apple photosystems to heat stress change during fruit development, and how does it relate to fruit biochemical and anatomical characteristics?

An understanding of the fruit properties influencing the biochemical response of fruit to sunburn inducing factors can assist with the understanding of sunburn development on fruits. We were, however, unable to establish good correlations between the heat and PAR stress induced changes in Fv/Fm and fruit biochemical as well as anatomical features (see Paper 2). Nevertheless, we found that the r^2 values of these correlations appeared to decrease with increasing temperature for ‘Granny Smith’ and ‘Fuji’, while the inverse was

true for ‘Cripps’ Pink’. It also appeared that apple fruit photosystems remain equally sensitive to heat and PAR stress during fruit development. The results suggest that the sensitivity of apple fruits to the combined heat and PAR stress cannot be easilly identified by analysing changes in fruit peel pigments and anatomical features as other factors such as antioxidants may play a greater role. The difference in cultivar sunburn sensitivity may also not be easilly attributed to differences in their pigmentation and anatomical characteristics.

8.2.3. Do ‘Granny Smith’ and ‘Golden Delicious’ differ in their sensitivity to heat and PAR stress?

The fruit photosystems of the perceived sunburn sensitive apple cultivar Granny Smith were not more sensitive to heat stress than the photosystems of the less sunburn sensitive Golden Delicious and both cultivars showed similar sensitivity to PAR stress (see Paper 3). To the contrary, ‘Golden Delicious’ appeared to be more sensitive to heat stress at low PAR levels than ‘Granny Smith’. The results could indicate that: 1) sunburn sensitivity may not be related to heat stress sensitivity of fruit peel photosystems; and 2) the higher sunburn sensitivity of ‘Granny Smith’, at least compared to ‘Golden Delicious’, could be more related to fruit peel biochemistry and to non-fruit factors such as tree bearing habits. Nevertheless, the data reported by Felicetti and Schrader (2009b) show that the difference in total chlorophyll content between sunburned and non-sunburned tissue was higher in ‘Golden Delicious’ than in ‘Granny Smith’. It appears that sunburned ‘Golden

Delicious' apple tissues lose more chlorophyll than sunburned 'Granny Smith' apple tissues. 'Granny Smith' generally has a higher chlorophyll content than 'Golden Delicious' (Felicetti and Schrader, 2009b) and therefore has a much greener appearance. The loss of chlorophyll in 'Granny Smith' may therefore easily make their yellow-bronze coloured sunburned fruits appear less green and therefore more damaged than dark green non-sunburned fruits. However, there is a lower colour contrast between non-sunburned yellow-green coloured 'Golden Delicious' and their yellow-bronze sunburned fruits.

8.2.4. Do apple cultivars differ in their reliance on the xanthophyll cycle for photoprotection against high temperature and PAR stress?

Sunburned fruits have lower chlorophyll content, a higher chlorophyll a/b ratio and xanthophyll cycle activities than non-sunburned fruits (Felecitti and Schrader, 2009b). To the best of our knowledge, the level to which different apple cultivars depend on the xanthophyll cycle for photoprotection has not been studied before. Our data showed that apple cultivars differ in their dependency on the xanthophyll cycle for photoprotection (see Paper 4). 'Granny Smith' and 'Braeburn' fruits appeared to be more reliant on the xanthophyll cycle for photoprotection under heat and PAR stress than 'Fuji' fruits (Paper 4). Our study is, however, not conclusive on whether cultivars that depend on the xanthophyll cycle for photoprotection may have a higher sunburn sensitivity than cultivars that are less dependent on this cycle, further research is needed to explore these findings. Chen *et al.* (2008) found increased xanthophyll cycle pool sizes in sunburned apple fruit peel.

However, the observed increases in the xanthophyll pool size in sunburned fruits were not sufficient to protect fruit photosystems from photodamage.

8.2.5. What is the effect of heat, PAR and UV-B stress in different combinations on the photosystems of apple fruit peel?

Fruit sunburn is induced by the combined effect of heat and light (UV-B and PAR) (Schrader *et al.*, 2001). The induction of sunburn symptoms under laboratory conditions, that are similar in appearance to those occurring under orchard conditions, is important for further understanding of fruit sunburn development. However, apart from a study by Rabinowitch *et al.* (1974) on tomatoes, no study has been able to induce sunburn symptoms on fruits under laboratory conditions. Glenn and Yuri (2013) recently exposed apples to different heat, PAR and UV levels, but they also did not manage to induce apple fruit sunburn symptoms. We were also unable to induce fruit sunburn on apples by exposing them to heat, PAR and UV-B under laboratory conditions (see Paper 5). Nonetheless, we found that the combined heat + UV-B + PAR treatment caused more damage to fruit photosystems than the UV-B + heat treatment, while UV-B alone had no effect. In addition, we found that previously shaded fruits were more sensitive to the stress treatments than previously sun-exposed fruits. The results for the UV-B treatments reported in Paper 5 are in agreement with the results obtained in Paper 1. Chen *et al.* (2008) also found that high temperature combined with PAR caused greater photodamage on apples than high temperature or high PAR stress alone. It appears therefore that heat plays an the important role in the damage to fruit

photosystems by solar radiation, while the presense of PAR and UV-B may magnify this effect.

8.2.6. Research hypothesis

The results obtained from the different experiments proved that exposing fruit peel to different heat and light stress combinations under laboratory conditions could induce photodamage, but was not able to induce visible fruit sunburn browning symptoms. Therefore, it was incorrect to assume that these stress conditions will be able to induce fruit sunburn browning in addition to causing photodamage. The results indicate that the interaction between climatic stressors and apple peel to induce biochemical changes resulting in fruit sunburn browning symptoms is still not well understood.

Nevertheless, considering that photodamage is the primary injury that occurs in response to high temperature and high irradiance, our study allowed us to study the effects of these sunburn inducing stresses and the role of photoprotective mechanisms in different cultivars separate from the development of the visible sunburn symptoms. The results suggest that susceptibility of different cultivars to sunburn, i.e., their tendency to develop visual sunburn browning symptoms, may not necessarily be related to their sensitivity to the various factors that induce sunburn browning. This is possibly because sunburn symptom development, in the case of sunburn browning, may relate more to the biochemical reactions of the cultivar to the stress than to its susceptibility to the stress.

8.3. Theories on fruit sunburn browning development

The biochemical changes that occur in sunburned fruit peel have been well documented (Chen *et al.*, 2008; Felicetti and Schrader, 2009a+b; Rabinowitch, 1983; Schrader *et al.*, 2009; Yuri *et al.*, 2010). The review on apple fruit sunburn by Racsko and Schrader (2012) also present a good pictorial stepwise process for the external appearance of different sunburn symptoms, including sunburn browning, bleaching and necrosis. However, there is still no clarity about the underlying stepwise biochemical processes that give rise to the visible symptoms. Sunburn necrosis (Racsko and Schrader, 2012) and bleaching (photoxidative sunburn) (Felicetti and Schrader, 2008) symptoms result from processes that completely damage peel biochemical processes while sunburn browning symptoms possibly develop from fruit peel biochemical stress adaptation processes.

Fruits with sunburn browning symptoms have increased phenolic content, reduced chlorophyll content, variable carotenoid content that differs between cultivars (Felicetti and Schrader, 2009a+b) and increased xanthophylls/chlorophyll ratio (Chen *et al.*, 2008) when compared to non-sunburned fruits. The increased phenolic content could function as antioxidants, reduce heat-induced oxidative stress and absorb UV light (Kim *et al.*, 2003; Treutter, 2006). It can also act as a possible carbon sink to help maintain photosynthesis during stress conditions (Hernández and van Breusegem, 2010). In Paper 1, we also observed that high UV-B stress

increased fruit total phenolic content. Previously sun exposed fruit peel did not suffer any photodamage due to the UV-B stress, which could possibly indicate the beneficial effect of phenolics for photoprotection and that high light-adapted fruit photosystems are not sensitive to UV-B stress at moderate temperatures.

Data from literature point to a possibility that oxidative stress and ethylene could be involved individually or synergistically in the development of fruit sunburn browning symptoms. The ethylene and oxidative stress-induced sunburn symptom development theories and the possible sunburn symptom development temperature range are discussed in the next sections. Future experiments are needed to prove or disprove these theories.

8.3.1. Ethylene-based sunburn browning symptom development theory

Sunburned fruits, irrespective of fruit maturity stage, are observed to have higher ethylene content than non-sunburned fruits (Torres *et al.*, 2013; Woolf and Ferguson, 2000). The presence of high ethylene content in sunburned fruits could possibly indicate that this hormone may be involved in the sunburn symptom development. The direct involvement of ethylene in fruit sunburn symptom development has not been explored before. Ethylene induced sunburn development could be indicated by not only the high concentration of this hormone in sunburned tissue, but also by the observed pigment changes in fruit peel with sunburn browning symptoms and the temperature regimes necessary for sunburn browning symptom development.

The pigment changes in sunburned fruits observed by Felecitti and Schrader (2009b) as well as by Chen *et al.* (2008) indicate that sunburned fruits have a greater reduction in chlorophyll *b* content than chlorophyll *a*. Ethylene-induced chlorophyll breakdown is reported to cause an increase of the chlorophyll a/b ratio in plant tissue (Shimokawa, 1990). Purvis and Barmore (1981) reported a positive correlation between chlorophyll degradation in Robinson tangerine fruits exposed to ethylene with increasing chlorophyll a/b ratios and that the loss of green colour as well as the rate of change in the chlorophyll a/b ratio increased with increasing ethylene exposure periods. Chlorophyll a/b ratio can also be increased by other stressors such as light without ethylene involvement (Ballottari *et al.*, 2007). It is therefore also possible that the change in chlorophyll a/b ratio could be due to potential light stress possibly experienced by fruits after the heat stress period (see section 8.3.2).

An increase in the chlorophyll a/b ratio increases the transfer of absorbed excess energy to the xanthophyll cycle (Kleima et al., 1999). High total xanthophylls concentration and de-epoxidation of violaxnthin to zeaxanthin are positively correlated with the chlorophyll a/b ratio (Li and Cheng, 2008; Lu *et al.*, 2001; Štroch *et al.*, 2008). We found that there is a difference in the reliance of different apple cultivars on the xanthophyll system for photoprotection (see Paper 4). The difference in the dependency on the xanthophyll cycle, which in turn is related to the observed changes in chlorophyll a/b ratio in sunburned fruits, could give an indication about the possible susceptibility of different cultivars to sunburn. However, further

studies are needed to confirm this possible correlation. Overall, it is possible that the observed high chlorophyll a/b ratio of sunburned fruits is induced by the observed high ethylene content in these fruits compared to non-sunburned fruits.

Furthermore, the results of Paper 3 show that the photosystems of sunburn sensitive apple cultivars do not necessarily suffer higher photodamage by PAR and heat stress compared to non-sensitive cultivars. Nevertheless, it has been observed that sunburned fruits sustain noticeable photodamage compared to non-sunburned fruits (Seo *et al.*, 2008; Wünsche *et al.*, 2004). This therefore could further indicate that photodamage or the reduced photosynthetic capacity of sunburned fruits could be indirectly caused by other factors, such as ethylene induced chlorophyll degradation, rather than by direct PAR and heat stress damage to the photosystems.

Ethylene synthesis in fruit is inhibited during the high temperature stress period, however, ethylene production of heat treated fruits exceeds that of untreated fruits over time at temperatures below heat stress levels (Ketsa *et al.*, 1999). The high induction temperature regime for sunburn browning development (45 °C) combined with the lower temperature at which symptoms develop could indicate that the temperature stress induces ethylene synthesis which is then up-regulated and is involved in symptom development at the lower temperatures. Furthermore, PAR has been found to enhance ethylene synthesis in plants (Cracker *et al.*, 1973). Therefore, the presence of PAR after heat stress could further enhance ethylene synthesis of

fruits that experience heat stress. Ethylene is also reported to protect plant photosynthetic systems against oxidative stress (Larkindale and Knight, 2002). Therefore, increased ethylene levels in sunburned tissue may not only cause chlorophyll degradation but may also have a photoprotective function.

The following facts therefore all point to the possible involvement of ethylene in fruit sunburn development: 1) the observed high ethylene content in sunburned fruits compared to non-sunburned fruits; 2) changes in chlorophyll *a* and *b* content in sunburned fruits; 3) the need for high induction temperature period followed by a low temperature sunburn symptom development period; 4) the equal sensitivity of the photosystems of sunburn sensitive and non-sensitive apple cultivars to heat and light stress.

8.3.2. Light stress-induced sunburn development theory

It is possible that the previously present but non-stressful PAR and UV-B becomes stressors after the sunburn-inducing heat stress levels. UV-B increases phenolic synthesis in plant tissues (Solovchencko and Merzlyak, 2008), while the presence of PAR enhances this affect (Awad *et al.*, 2001), and sunburned apple peel has higher phenolic content than non-sunburned fruits (Felicetti and Schrader, 2009a). Although heat stress decreases the chlorophyll a/b ratio in previously sun-exposed apple fruit peel (Hengari *et al.*, 2014b; see Paper 2), light stress can also increase this ratio (Ballottari *et al.*, 2007). The observed increased phenolic synthesis and chlorophyll a/b ratio in sunburned peel could therefore be due to the “increased” light stress

experienced by the fruits after the heat stress period. Heat stress damages apple peel photosystems (Chen *et al.*, 2008). The damaged photosystems are then not able to cope with the normal PAR and UV-B levels. PAR and UV-B "stress" then increases the production of reactive oxygen species (ROS) and induces oxidative stress. The observed increased antioxidants and decreased chlorophyll content in sunburned peel could then be due to heat stress-induced PAR and UV-B oxidative stress.

8.3.3. The possible existence of a temperature range for the development of sunburn browning symptoms

Published literature indicates the possible existence of a temperature range in which fruit sunburn browning symptoms develop, which is different from the induction temperature. Sunburn browning symptoms developing on fruits in the orchard are induced by a 1 hour heating period at temperatures between 45 ℃ to 49℃ under direct solar radiation (Schrader et al., 2001). However, the fruit temperature at which sunburn symptoms continue to develop is below this critical temperature. It is also possible that the lower temperature range for sunburn browning symptom development may differ between cultivars. Maintaining fruit temperature above, at or just below the sunburn browning induction temperature (40 to 50 ℃) for periods longer than the reported 1 hour eventually lead to sunburn necrosis (Racsko and Schrader, 2012), or to the appearance of a cooked brown surface (own unpublished observations). A sudden exposure of shaded fruits with a peel temperature lower than 31 ℃ could induce photoxidative sunburn as reported by Felicetti and Schrader

(2008). The temperature range in which sunburn browning symptoms or biochemical changes occurs has to date not been clearly determined.

8.3.4. Stepwise combined sunburn browning theory and schematic representation of the proposed processes

The possible sequence of events that leads to the development of fruit sunburn browning symptoms can be stated as follows (see also Figure 1):

- Heat stress (45 ℃ to 49 ℃ for 1 hour) damages the photosystems, reducing their capacity to processes pre-existing PAR and UV-B, therefore turning these into stressors;
- Heat stress induces ethylene and ROS synthesis;
- A reduction in temperature (> 45 ℃) and continued presence of PAR and UV-B stress leads to increased ethylene and ROS synthesis;
- Ethylene and ROS in turn causes chlorophyll degradation and an increase in the chlorophyll a/b ratio; high ethylene concentration possibly reduces oxidative stress
- PAR and UV-B induces photodamage and cause an increase of xanthophyll cycle pool size, carotenoid content and other photoprotective systems (antioxidants, heat shock proteins etc.);
- UV, in combination with PAR, also enhances phenolic synthesis to:

    1) increase UV absorption capacity,

    2) act as an antioxidant,

    3) protect “wounded” tissue against invading pathogenic infections,

    4) isolate wounded tissue and prevent further decay;

- The combination of decreased chlorophyll content, increased phenolic and carotenoid synthesis leads to the observed bronze like sunburn symptom on the fruit peel surface.

8.4. General conclusion

Apple fruit peel photosystems appear to be well protected against UV-B radiation stress. Fruit sunburn sensitivity can be influenced by many more factors such as photoprotection by the xanthophyll cycle, in addition to fruit pigment content and anatomical features. Sunburn sensitivity also appears not to be directly linked to the sensitivity of the fruit peel photosystems to heat stress. However, it is clear that heat stress plays a major role in inducing photodamage compared to UV-B and PAR stress. More work needs to be done on the induction of sunburn symptoms under laboratory conditions for further understanding of this stress condition. The differences in sunburn susceptibility between apple cultivars may be related to the difference in biochemical changes that occur during stress adaptation in addition to differences in the sensitivity of photosystems to sunburn inducing climatic factors. Sunburn susceptibility may also be related to factors such as tree management methods and cultivar specific characteristics such as pre-sunburn fruit peel colour, fruit bearing habits and foliage levels of different cultivars.

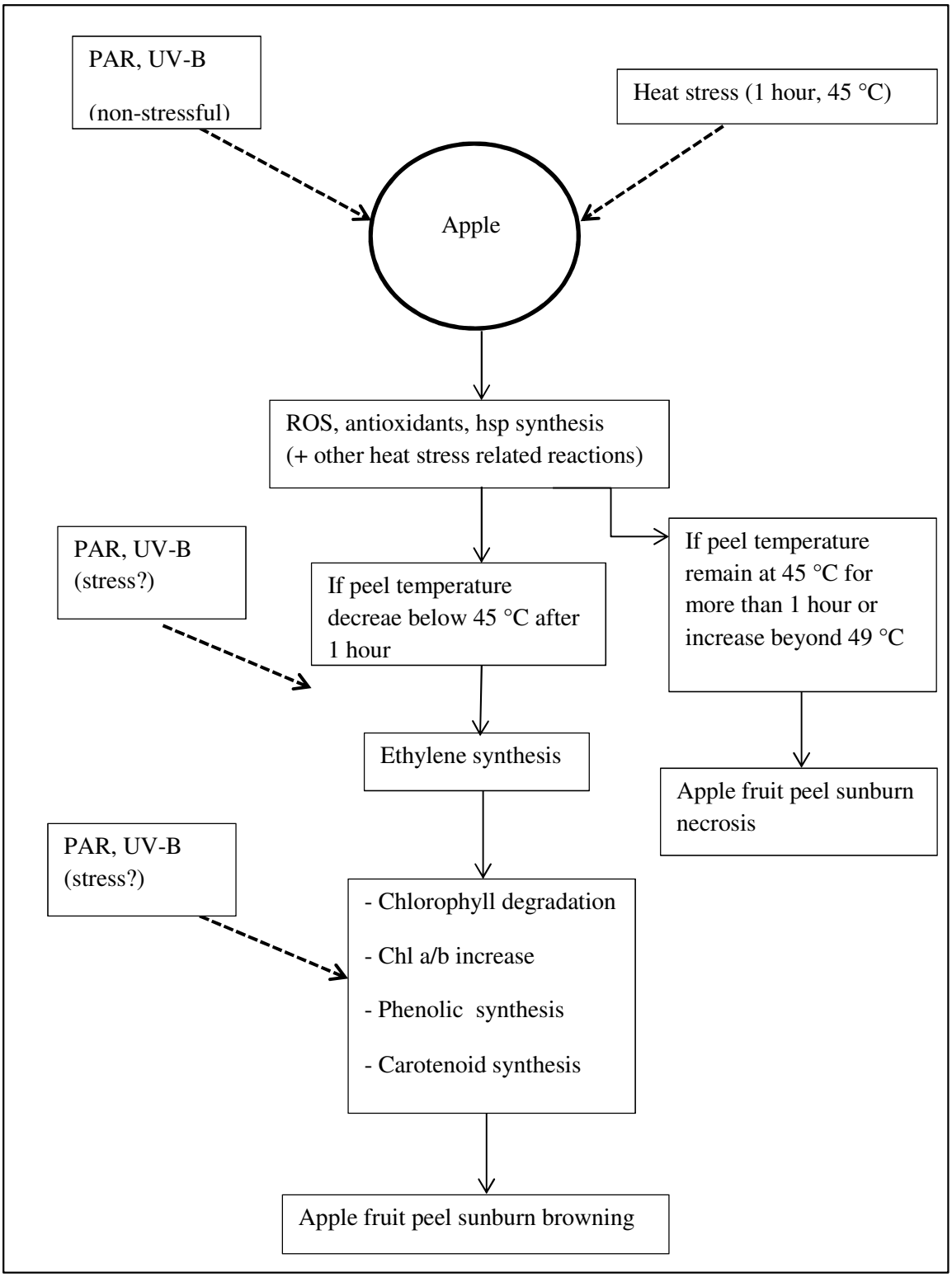

Figure 1. Schematic representation for the proposed sequence of biochemical processes taking place in fruit peel during sunburn browning induction and development. PAR = photosynthetic active radiation; UV-B = ultraviolet radiation-B; ROS = reactive oxygen species; hsp = heat shock proteins; Chl a/b = chlorophyll a/b

8.5. References

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