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JIP-test parameters to study apple peel photosystem II behavior under high solar radiation stress during fruit development

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Abstract

Changes in apple (*Malus domestica* Bork.) peel PSII photochemical process during fruit development under high solar radiation field conditions were analysed by the JIP-test to increase understanding on energy dissipation and susceptibility to photooxidative damage. Fruits growing exposed (E), non-exposed (NE) or suddenly exposed (SU) to high solar radiation were evaluated at three developmental stages. Chlorophyll content and chlorophyll *a* fluorescence transient (OJIP), were affected by high solar radiation and development. Minimum fluorescence, maximum fluorescence, maximum quantum yield of photochemistry, and specific fluxes per cross section decreased in E and SU fruits. JIP parameters were a sensitive indicator of high solar radiation stress in apple peel. Peroxidative damage was observed in E fruits at each stage of development and in SU fruits at early and mid-stages. By delineating the photochemical events induced by solar radiation in apple fruits at different developmental stages, our findings might help to increase understanding on susceptibility of apple to photooxidation damage depending on the light environment and developmental stage they are exposed to.

Additional key words: fruit sunburn; high light stress; PSII photochemistry.

Introduction

In fruiting vegetables and fruit crops, high solar radiation stress may lead to photooxidative damage resulting in a physiological disorder known as sunburn (Racskó and Schrader 2012). This physiological disorder is frequently found in apple fruits growing under full and direct high solar radiation. Shaded apple peel may also be susceptible to photooxidative damage when sudden exposure to full sunlight occurs (Ma and Cheng 2004, Li and Cheng 2008). This may happen during hand thinning or summer pruning, and even in harvested fruits exposed to sun radiation during transport (Schrader *et al*. 2003).

Several changes in pigment composition take place as the apple fruit develops. As new pigments (*e.g*., anthocyanin) are synthetized, apple peel chloroplasts become vacuolated and gradually lose their photosynthetic function (Blanke and Lenz 1989, Li and Cheng 2008). Excessive sun radiation also causes significant changes in pigment composition in apple fruits (Racskó 2010). A decline in chlorophyll (Chl) *a* and *b* and in anthocyanin was reported in 'Fuji' apple peel as photooxidation and sunburn severity increased (Felicetti and Schrader 2008).

Photosystem II (PSII) photochemistry and energy flux are altered under environmental stress (Yang *et al*. 2012). This leads to impaired photosynthesis and subsequent

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Abbreviations: ABS/RC – absorption flux per RC; Chl – chlorophyll; CS_0 – excited cross section; DAFB – days after full bloom; DI_0/RC – dissipated energy per RC; E – exposed to solar radiation; ET_0/RC – electron transport flux per RC; F_0 – minimum fluorescence; F_m – maximum fluorescence; F_v – variable fluorescence; F_v/F_m (φ_{P0}) – maximum quantum yield of photochemistry; MDA – malondialdehyde; NE – not exposed to solar radiation; OEC – oxygen-evolving complex; PI_{abs} – performance index on absorption basis; RC – PSII reaction centre; RC_0/CS_0 – density of active PSII reaction centre per CS; ROS – reactive oxygen species; SU – suddenly exposed to solar radiation; t_{Fm} – time (in ms) to reach F_m; TR_0/RC – trapped energy per RC; V_J – relative variable fluorescence at the J-step; δ_{R0} – efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors; φ_{E0} – quantum yield for electron transport; Ψ_{E0} – probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-.
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oxidative damage by generation of reactive oxygen species (ROS). ROS are generated during normal cell metabolism, but high amounts can result in oxidative stress and lead to lipid peroxidation and oxidative destruction of the cell (Mittler 2002).

Chl *a* fluorescence transient measurement allows investigations of various PSII photochemistry parameters that may be altered during environmental stress, such as absorption energy flux, trapped energy flux, electron transport flux, dissipated energy flux, and reduction of end electron acceptors (Strasser *et al.* 2004, Jiang *et al.* 2008, Yang *et al.* 2012). PSII photochemistry investigated using direct time-resolved fluorescence shows a polyphasic rise known as the OJIP transient (Strasser *et al.* 1995, 2004). The O-step reflects the minimum fluorescence (F_0) when all primary quinone electron acceptors (Q_A) are oxidized. The P-step is the peak or maximum fluorescence (F_m) and corresponds to the state when all Q_A are reduced. The rise from the O- to the J-step reflects a reduction of Q_A and is associated with the primary photochemical reactions of PSII. The intermediate I-step and the final P-step reflect the existence of fast and slow reducing plastoquinone (PQ) centres as well as different redox states of the reaction centre (RC) complex (Strasser *et al.* 1995). The analysis of the OJIP transient, derived from complex mathematical models, is known as the 'JIP-test' (Strasser *et al.* 1995, 2004). This test uses OJIP curve inflection points for calculating parameters that describe the photochemical activity of samples (Živčák *et al.* 2014).

It has been widely proved that the OJIP transient is a sensitive and reliable method for the detection and quantification of several environmental plant stresses like drought (Li and Ma 2012, Mishra *et al.* 2012, Brestič and Živčák 2013), heat (Chen *et al.* 2008, 2009; Chen and Cheng 2009), mineral (Hermans *et al.* 2004, Yang *et al.* 2012), and high light stress (Chen *et al.* 2008, Kalaji *et al.* 2012, Živčák *et al.* 2014). To our knowledge, only a few studies have applied this technique under high solar radiation field conditions to investigate PSII responses leading to apple peel photooxidative damage. Some studies carried out under laboratory controlled conditions indicate that high light coupled with high temperature damage PSII complexes at both the donor and acceptor sides in apple peel (Chen *et al.* 2008). They also show that partitioning of absorbed light energy differs between the shaded and sun-exposed sides of apple fruits when they are exposed to high light irradiance (Chen *et al.* 2012).

Little is known about apple peel PSII photochemistry (OJIP transient) evolution during fruit development either adapted or not to high solar radiation under field conditions. The aim of this study was to investigate changes in apple peel photochemical processes during fruit development under natural high solar radiation on field conditions.

Materials and methods

Plant material and field conditions during sampling: Ten twenty-year-old apple trees (*Malus domestica* Borkh.) cv. Red Delicious, grown in the Alto Valle of Río Negro, North Patagonia, Argentina (39°04'0.8"S, 67°43'22.8"W), were selected during the 2012–2013 growing season. Spacing among trees was 2.5 × 4.0 m, and they received standard horticultural practices and disease and pest control. At 65, 110, and 140 d after full bloom (DAFB) fruits from these trees, growing either exposed (E) or not exposed (NE) to sun were picked at peak sunlight radiation (between 12:00 and 14:00 h). At 10:00 h on the same day, NE fruits (not adapted to high sun radiation) were selected and suddenly exposed (SU) to sun without being detached from the plant and kept under this condition during three hours before they were picked. Environmental conditions and average fruit size at the different sampling times are shown below. All physiological measurements were performed on the peel of the selected fruits using 4–5 replicates per treatment.

	Days after full bloom		
	65	110	140
Environmental conditions			
Maximum solar radiation [W m^{-2}]	780	790	723
Average air temperature [°C]	34.8	31.1	32.9
PAR at E condition [μmol(photon) m^{-2} s^{-1}]	1,780	1,803	1,796
PAR at NE condition [μmol(photon) m^{-2} s^{-1}]	752	786	795
Fruit size			
Mass [g]	54.6 ± 2.3	145.3 ± 6.0	180.2 ± 6.1
Diameter [mm]	48.1 ± 0.7	68.6 ± 1.1	74.2 ± 1.0

Chl determination: Three peel discs (1 cm in diameter and 1 mm thick) were extracted from each fruit and incubated with 2 ml of dimethyl-sulfoxide at 65°C during 2 h for Chl extraction. Absorbance was measured using a spectrophotometer (*Beckman Coulter DU 800*) at 665.1 and 649.1 nm. Chl *a* and Chl *b* were calculated following Wellburn (1994). Chl *a* [μg ml^{-1}] = 12.47 × $A_{665.1}$ – 3.62 × $A_{649.1}$, Chl *b* [μg ml^{-1}] = 25.06 × $A_{649.1}$ – 6.50 × $A_{665.1}$.

Anthocyanin determination: Anthocyanin extraction and analysis were carried out as described by Giusti *et al.* (2005). Anthocyanins in fruit peel were extracted with a solution of HCl:H_2O:MeOH (0.5:20:79.5, by volume). Absorbance was measured using a spectrophotometer (*Beckman Coulter DU 800*) and expressed as mg of cyanidin-3-glucoside.

Chl *a* fluorescence (OJIP) transient was measured with a portable fluorimeter (*Pocket PEA*, *Hansatech Instruments Ltd.*, Norfolk, UK) according to Strasser *et al.* (1995). Photon flux density was set at 3,500 μmol(photon) m^{-2} s^{-1} during 1 s. Data were sampled at 10-μs intervals for the first 300 μs, and then acquisition rates were lower as the kinetics of the fluorescence signal slowed. All measurements were made on peel discs 1 cm in diameter and 0.5 cm thick, dark-adapted for 30 min at room temperature.

The OJIP transient was analysed according to the JIP-

test (Strasser *et al.* 2004), and calculation and derivation of parameters was performed according to Jiang *et al.* (2008). Measured parameters were fluorescence at time t after onset of actinic illumination (F_t), minimum fluorescence when all PSII reaction centres (RCs) are open (F_0), maximum fluorescence when all PSII RCs are closed (F_m), fluorescence intensities at 50, 100, and 300 µs (F_{50}, F_{100}, and F_{300}, respectively), and fluorescence intensities at the J-step (F_J; 2 ms) and at the I-step (F_I; 30 ms). Selected OJIP parameters were variable fluorescence (F_v), relative variable fluorescence at the J-step (V_J), total complementary area between the fluorescence induction curve and F_m – reflecting the reduced plastoquinone pool size (Area), and time (in ms) to reach F_m (t_{Fm}). Biophysical parameters calculated with basis on the onset of fluorescence induction (time zero) were (*1*) yields or flux ratios: φ_{P0} (or F_v/F_m), maximum quantum yield of primary photochemistry; φ_{E0}, quantum yield for electron transport; Ψ_{E0}, probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; and δ_{R0}, efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors; (*2*) specific fluxes or activities per RC: absorption (ABS/RC); dissipated energy (DI_0/RC); trapped energy (TR_0/RC); and electron transport flux (ET_0/RC); (*3*) phenomenological fluxes or activities per excited cross section (CS): absorption (ABS/CS_0); dissipated energy (DI_0/CS_0); trapped energy (TR_0/CS_0); electron transport flux (ET_0/CS_0); and reduction of end acceptors at PSI electron acceptor side (RE_0/CS_0); (*4*) density of active PSII RC per CS (RC_0/CS_0); (*5*) performance index on absorption basis (PI_{abs}), which reflects overall photochemistry performance up to PSI.

Membrane peroxidative damage was determined by quantification of thiobarbituric acid (TBA) reactive species produced during lipid peroxidation as described by Hodges *et al.* (1999). Apple peel samples (200 mg of fresh mass) were macerated in liquid nitrogen and homogenized in 0.1% (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × *g* for 5 min at 4°C. Aliquots of the supernatant (500 µl) were added to 2 ml of reaction medium (TBA+) consisting of TBA 0.1% and TCA 20% and incubated at 95°C for 30 min. The reaction was stopped by rapid cooling on an ice bath and samples were subsequently centrifuged at 10,000 × *g* for 10 min at 4°C. Readings were determined using a spectrophotometer (*Beckman Coulter DU 800*) at 440 (to eliminate interference from sucrose), 532, and 600 nm. TBA forms red complexes with low molecular mass aldehydes such as malondialdehyde (MDA), a byproduct of the peroxidation process. To eliminate interference from anthocyanin, aliquots of the same supernatant (500 µl) were added to 2 ml of TCA 20% (TBA–) and processed along with TBA+ samples by measuring absorbance at 532 and 600 nm. The concentration of MDA/TBA complexes was calculated as follows: MDA [nmol ml^{-1}] = $\{[(A_{TBA+532} - A_{TBA+660}) - (A_{TBA-532} - A_{TBA-600}) - (A_{TBA+440} - A_{TBA+600}) \times e]/c\} \times 10^6$, where c represents the extinction coefficient at 532 nm = 157,000 mM cm^{-1} (Hodges *et al.* 1999) and e is the molar absorption of sugar at 532 nm/molar absorption of sugars at 440 nm = 0.057142857 (Hodges *et al.* 1999).

Statistical analysis: Sampling was performed with five replicates per sun-exposed type and DAFB. Data analyses were carried out using one- and two-way analysis of variance (*ANOVA*) (*InfoStat 2013*, Di Rienzo *et al.* 2013). Means were compared using the DGC test (Di Rienzo *et al.* 2002) at a level of $p<0.05$. JIP-test parameters, Chl content, and membrane peroxidative damage were submitted to a principal component analysis (PCA) to get a closer insight in the relation between measured parameters.

Results

Pigment content: As DAFB increased fruit peel Chl content (*a*, *b*, and total) decreased for the three sun exposure conditions (NE, E, and SU; Table 1). On NE, E, and SU fruits degradation rates during development were, respectively, 68, 88, and 60% for Chl *a*; 55, 63, and 57% for Chl *b*; and 64, 79, and 59% for total Chl. No significant difference was observed in SU fruit Chl contents between 110 and 140 DAFB. The Chl *a*/*b* ratio decreased as DAFB

Table 1. Chlorophyll contents in Red Delicious apple fruits grown exposed (E), non-exposed (NE), or suddenly exposed (SU) to sunlight at 65, 110, and 140 d after full bloom (DAFB). Data are means ± SE ($n = 5$). Values followed by *the same letter* within the same column are not significantly different at $p \leq 0.05$.

DAFB	Sun exposure	Chl *a* [µg cm^{-2}]	Chl *b* [µg cm^{-2}]	Chl total [µg cm^{-2}]	Chl *a*/*b*
65	NE	4.70 ± 0.14^e	2.06 ± 0.06^d	6.76 ± 0.20^e	2.28 ± 0.03^c
	E	3.19 ± 0.25^d	1.78 ± 0.09^d	4.97 ± 0.32^d	1.79 ± 0.08^b
	SU	4.20 ± 0.30^e	2.05 ± 0.13^d	6.25 ± 0.42^e	2.04 ± 0.06^b
110	NE	2.39 ± 0.12^c	1.29 ± 0.11^c	3.68 ± 0.22^c	1.90 ± 0.15^b
	E	1.63 ± 0.12^b	1.07 ± 0.06^b	2.70 ± 0.18^b	1.52 ± 0.06^b
	SU	1.89 ± 0.12^b	1.03 ± 0.07^b	2.92 ± 0.18^b	1.86 ± 0.12^b
140	NE	1.50 ± 0.19^b	0.93 ± 0.05^b	2.42 ± 0.21^b	1.61 ± 0.17^b
	E	0.39 ± 0.02^a	0.65 ± 0.08^a	1.04 ± 0.07^a	0.62 ± 0.09^a
	SU	1.67 ± 0.34^b	0.89 ± 0.16^b	2.56 ± 0.48^b	1.90 ± 0.22^b

increased in NE fruits between 65 and 110 DAFB and in E fruits between 110 and 140 DAFB. Solar radiation environment affected the Chl content at each stage of fruit development. NE fruits presented higher Chl concentration (*a* and total) and Chl *a*/*b* ratio than that of E fruits. Chl *b* content was affected by sun exposure at 110 and 140 DAFB. SU fruits showed 20% degradation for both Chl *a* and total Chl at 110 DAFB. At 65 and 140 DAFB, no differences in content were found for Chl *a*, *b*, and total Chl between SU and NE fruits (Table 1).

Anthocyanins were measured in E and NE fruits at 140 DAFB. At this late stage of development, NE fruits showed 8.9 mg of cyanidin-3-glucoside per 100 g of fresh mass, 30% higher than that of E fruits.

Chl *a* fluorescence transient and JIP test: Chl *a* fluorescence (OJIP) transients showed a typical polyphasic rise in E, NE, and SU fruits (Fig. 1). Solar radiation exposure and fruit development affected Chl *a* fluorescence intensity with an important reduction at the P-step, and a slight increase at the O-step (Fig. 1*A–C*). Differences in variable fluorescence are visualised by F_0 normalization, plotted as F_t/F_0 (Fig. 1*D–F*). NE fruits showed the highest variable fluorescence during fruit development in comparison to E and SU fruits. The higher reduction in F_t/F_0 was observed at 110 DAFB, mainly in SU fruits with respect to NE fruits (Fig. 1*E*).

In OJIP transients, F_0 and F_m were affected by solar radiation and fruit development. At 65 DAFB, F_0 and F_m were higher in E than that in NE and SU fruits. At 110 DAFB, both F_0 and F_m were higher in NE than that in E fruits, but no differences were found between solar radiation exposure at 140 DAFB (Fig. 2*A*,*C*).

Variable fluorescence (V_J) was higher in E and SU than that in NE fruits at all stages, except at 65 DAFB, where SU was greater than that of NE (Fig. 2*B*). E and NE fruits showed a similar F_v/F_m at 65 DAFB (Fig. 2*D*). However, at 110 and 140 DAFB, F_v/F_m in E fruits decreased by 22 and 32%, respectively, compared to NE fruits. F_v/F_m in SU fruits was 32, 45, and 41% lower at 65, 110, and 140 DAFB, respectively, when compared to NE fruits (Fig. 2*D*). The area under the OJIP curve, indicating the reduced plastoquinone pool (Area), decreased during the fruit development (Fig. 2*E*). At 65 DAFB, E fruits showed the largest Area, with a reduction of 67 and 84% observed at 110 and 140 DAFB, respectively. Meanwhile, in NE fruits, Area was reduced by 66% at 140 DAFB; at this time point, E, NE, and SU fruits showed the similar Area (Fig. 2*E*). The time to reach F_m (t_{Fm}) was similar for all exposure conditions at 65 DAFB. In E and NE fruits, t_{Fm} increased at 110 DAFB, and in E and SU fruits at 140 DAFB.

For both E and NE fruits, phenomenological fluxes per excited cross section were reduced as DAFB increased (Fig. 3). Density of active reaction centres per cross section (RC/CS_0) was also reduced as DAFB increased (Fig. 3). These reductions, when analysed in relative values to 65 DAFB, were faster in E than that in NE fruits. Relative to NE fruits, the effects of sun radiation varied according to the morphological developmental stage (Fig. 4). At 65 DAFB, RC/CS_0, and all analysed phenomenological fluxes were higher in E than that in SU and NE fruits (Fig. 4*A*). SU fruits showed an increase in dissipated energy (DI_0/CS_0) and a reduction in both trapped energy (TR_0/CS_0) and electron transport flux (ET_0/CS_0) (Fig. 4*A*). At 110 DAFB, phenomenological fluxes were reduced by sun exposure despite the higher RC/CS_0 observed in E fruits (Fig. 4*B*). At each morphological developmental stage, an increase in DI_0/CS_0 was observed in SU fruits (Fig. 4*A–C*).

When energy fluxes were analysed per active RC, absorption flux (ABS/RC) and dissipated flux (DI_0/RC) were greater in SU than that in E and NE fruits at all stages. The only exception was at 140 DAFB, when

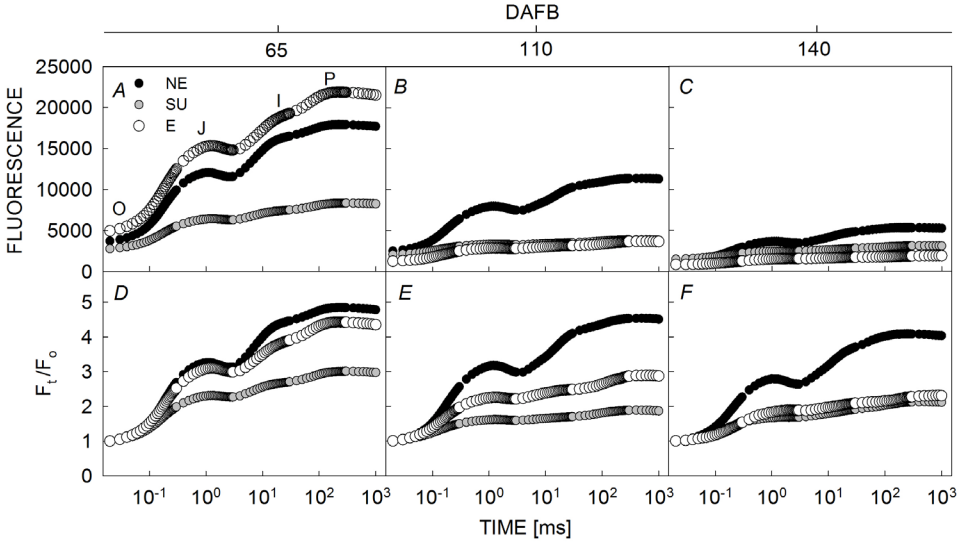

Fig. 1. Chlorophyll *a* OJIP transients at 65, 110, and 140 d after full bloom (DAFB) plotted on a logarithmic time scale in dark-adapted Red Delicious apple fruits exposed (E), non-exposed (NE), and suddenly exposed (SU) to sunlight. (*A–C*) Total chlorophyll *a* fluorescence (F_t). (*D–F*) Normalized fluorescence (F_t/F_0). Each time point is the mean of five replicates.

ABS/RC and DI_0/RC in E fruits reached levels similar to those measured in SU fruits. In contrast, energy fluxes per active RC in NE fruits were not affected during development. Quantum yield for electron transport (φ_{E0}) had similar behaviour as F_v/F_m; it was lower in SU than that in NE fruits at all morphological developmental stages and remained constant in NE fruits throughout the development. However, the efficiency with which an electron is transferred from the reduced intersystem electron acceptors to PSI (δ_{R0}) was highest in SU fruits at all developmental stages, and in E fruits at 110 and 140 DAFB. In turn, both the probability that a trapped exciton moves an electron beyond Q_A^- (Ψ_{E0}) and the performance index (PI_{abs}) were affected by sun radiation at each stage of development (Fig 2*F*). Compared to NE fruits, PI_{abs} in SU fruits was 69, 91, and 77% lower at 65, 110, and 140 DAFB, respectively, while in E fruits, PI_{abs} decreased by 69 and 76% at 110 and 140 DAFB, respectively (Fig. 2*F*).

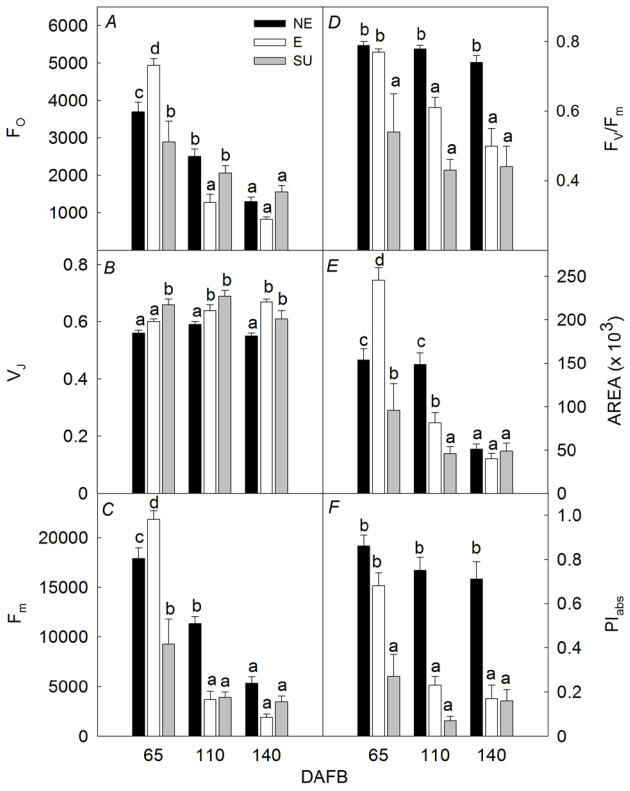

Fig. 2. OJIP transient parameter analysis for dark-adapted Red Delicious apple fruits exposed (E), non-exposed (NE), and suddenly exposed (SU) to sun light at 65, 110, and 140 d after full bloom (DAFB). (*A*) Minimum fluorescence (F_0). (*B*) Relative variable fluorescence at the J-step (V_J). (*C*) Maximum fluorescence (F_m). (*D*) Maximum quantum yield of primary photochemistry (F_v/F_m). (*E*) Reduced plastoquinone pool size (Area). (*F*) Performance index on absorption basis (PI_{abs}). Data are means ± SE (n = 5). Values followed by *the same letter* are not significantly different at $p \leq 0.05$.

Membrane peroxidation: Compared to NE, peel membrane peroxidation was 129, 75, and 229% higher in E fruits at 65, 110, and 140 DAFB, respectively, and 64 and 88% higher in SU fruits at 65 and 110 DAFB, respectively. Membrane peroxidation levels remained constant in E and NE fruits during development, and no differences were found between NE and SU fruits at 140 DAFB (Fig. 5).

Principal component analysis: We used multiparametric analysis to evaluate solar radiation exposure and fruit development effects in fruit peel in order to identify relations between JIP-test parameters, Chl content, and membrane peroxidative damage. Principal component analysis is an exploratory technique that concentrates the information of measured variables in new complex variables not correlated with each other. These new variables, called Principal Components (PC), are linear combinations of the original variables and explain the maximum variation of parameters. The first Principal Component (PC1, Fig. 6) determined about 64% of total changes in apple fruit peel, and is mainly explained by F_v/F_m, PI_{abs}, φ_{E0}, and DI_0/RC, ABS/RC, and δ_{R0}. The second component (PC2) reflected 16% of changes and is sensitive to MDA, F_0, F_m, and Area. Chl concentration is more correlated to parameters like F_0, F_m, and Area, and negatively correlated to t_{Fm}. While MDA is more correlated to V_J and negatively correlated to ET_0/RC and Ψ_{E0}. The sample distribution within PC1/PC2 biplot could be positioned into two relatively good separated clusters (Fig. 6). The first cluster, characterized by higher values of PSII functionality, includes NE fruits at all stages

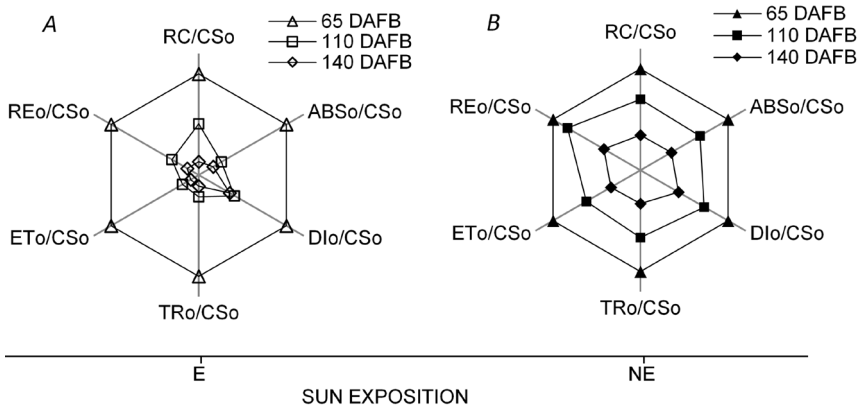

Fig. 3. Phenomenological fluxes per excited cross section in (*A*) sun-exposed (E) and (*B*) non-exposed (NE) Red delicious apple fruits. Radar plot of amount of active PSII RCs per cross section (RC/CS_0), absorption flux per CS (ABS_0/CS_0), dissipated energy flux per CS (DI_0/CS_0), trapped energy flux per CS (TR_0/CS_0), electron transport flux per CS (ET_0/CS_0), and reduction of end acceptors at PSI electron acceptor side per CS (RE_0/CS_0) at 65, 110, and 140 d after full bloom (DAFB). All values are relative to 65 DAFB within each condition.

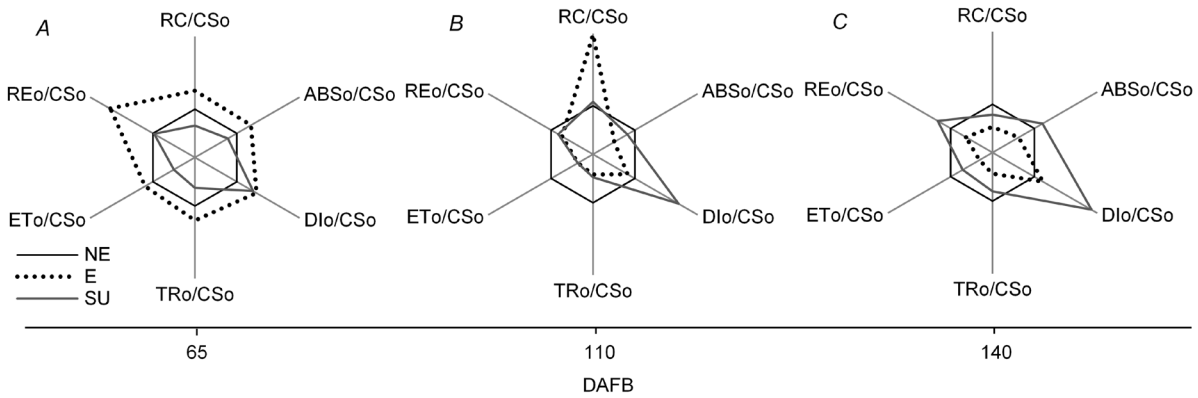

Fig. 4. Phenomenological fluxes per excited cross section at 65 (*A*), 110 (*B*), and 140 (*C*) d after full bloom (DAFB). Radar plot of amount of active PSII RCs per cross section (RC/CS_0), absorption flux per CS (ABS_0/CS_0), dissipated energy flux per CS (DI_0/CS_0), trapped energy flux per CS (TR_0/CS_0), electron transport flux per CS (ET_0/CS_0), and reduction of end acceptors at PSI electron acceptor side per CS (RE_0/CS_0) in Red Delicious apple fruits grown exposed (E), non-exposed (NE), and suddenly exposed (SU) to sunlight. All values are relative to those of NE fruits for each DAFB.

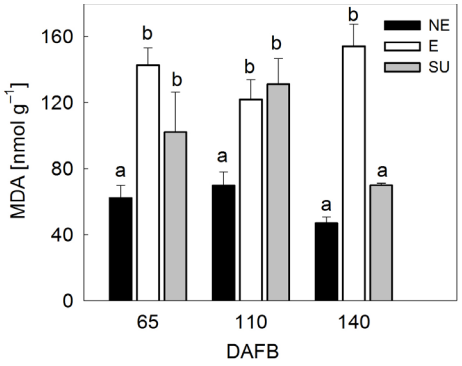

Fig. 5. Membrane peroxidation measured as malondialdehyde (MDA) concentration [nmol g^{-1}] in Red Delicious apple fruits exposed (E), non-exposed (NE), and suddenly exposed (SU) to sunlight sampled at 65, 110, and 140 d after full bloom (DAFB). Data are means ± SE (n = 5). Values followed by *the same letter* are not significantly different at $p \leq 0.05$.

of development and E fruits at 65 DAFB. While the second cluster, characterized by lower PSII functionality, includes AE fruits at all stages of development and E fruits at 110 and 140 DAFB.

Discussion

In line with previous investigations in Gala (Li and Cheng 2008) and Golden apple fruits (Chen *et al.* 2012), our findings indicate that Red Delicious apple peel Chl contents decrease during fruit development (Table 1). This trend paralleled the decrease in photosynthetic capacity as fruits become older (Li and Cheng 2008).

At each stage of development NE fruits showed higher Chl contents than that of E fruits (Table 1). These results are also consistent with previous investigations, where changes in Chl content as result of photooxidative stress were reported on leaves and apple fruits exposed to increasing levels of solar radiation (Demmig-Adams 1998, Ma and Cheng 2004, Felicetti and Schrader 2009). In addition, Chl degradation rates were higher in E than that in NE fruits during development. When NE fruits were suddenly exposed to sun (*i.e.*, SU fruits), Chl content decreased only at 110 DAFB. As sun radiation intensity was similar during the three sampling dates, we presume that apple fruits that did not acclimate to solar radiation could be more susceptible to Chl degradation after sun exposure at mid-stages of development.

Chl *a* OJIP transient was affected by natural solar radiation exposure and fruit development (Fig. 1*A–C*). Similar results were reported on studies conducted under controlled conditions, where shaded sides of apple fruits were exposed to artificial high light (Chen *et al.* 2008). The differences found in variable fluorescence (F_t/F_0) show that sun exposure affects maximum fluorescence in E and SU fruits early during development. However, the highest reduction of the P-step produced by solar radiation was observed at 110 DAFB, when NE fruits where suddenly exposed to sun (*i.e.*, SU fruits; Fig. 1*D–F*).

In general, either constancy or a decrease in F_0 indicates a regulatory or protective mechanism (Demmig *et al.* 1987, Chen *et al.* 2008), whereas an increase in F_0 indicates photoinhibition (Uhrmacher *et al.* 1995). The latter was clearly observed in E fruits at the earliest studied developmental stage (Fig. 2*A*). However, the decrease in F_0 in response to solar radiation at 110 and 140 DAFB (Fig. 2*A*) is likely to be associated with irreversible damage to oxygen-evolving complex (OEC), as observed in previous studies in apple peel under high light and temperature stress (Chen *et al.* 2008).

The transitory fluorescence intensity peaks (J- and I-steps) observed in the Chl *a* fluorescence transient represent consecutive kinetic bottlenecks in the electron transport chain, which lead to momentary maximum accumulations of Q_A^- (Strasser *et al.* 2004). In our study, the fact that V_J was enhanced in E and SU fruits (Fig. 2*B*) suggests that the acceptor side of PSII became more reduced under solar radiation. The final I–P phase, affected here by solar radiation and development, might reflect the rate of reduction of ferredoxin considered as a measure of

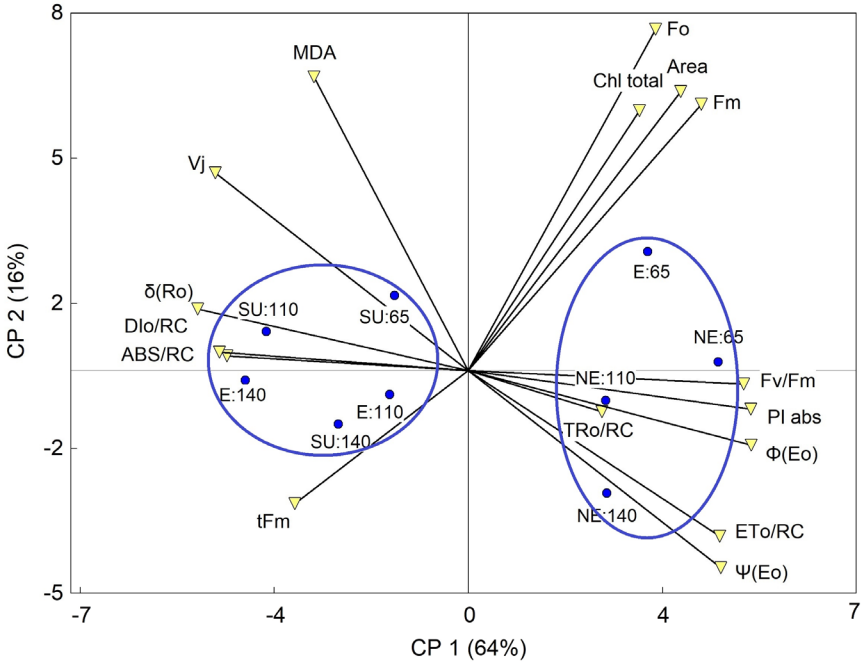

Fig. 6. Principal Component Analysis (PCA) of variability of total chlorophyll content (Chl total), membrane peroxidation (MDA) and JIP-test parameters, on Red Delicious apple fruits exposed (E), non-exposed (NE), and suddenly exposed (SU) to sunlight at 65, 110, and 140 d after full bloom (DAFB).

the relative abundance of PSI with respect to PSII (Bussotti *et al.* 2011, Živčák *et al.* 2014).

Solar radiation affected F_v/F_m (Fig. 2*D*), indicating injury to the PSII complexes (Brestič and Živčák 2013) due to an increase in F_0 and a decrease in F_m. Similar findings were reported by Chen *et al.* (2009, 2008) in apple (*Malus domestica* Bork) peel, and Kalaji *et al.* (2012) and Živčák *et al.* (2014) in barley (*Hordeum vulgare* L.) leaves. F_v/F_m was similar in E and NE fruits at early stages of fruit development (*i.e.*, 65 DAFB; Fig. 2*D*) indicating that PSII susceptibility to solar radiation in E fruits could be developed at later developmental stages.

The reduction of the PQ pool (*i.e.*, Area; Fig. 2*E*) observed in E and NE fruits at mid and end developmental stages, respectively, partially explains the decline in the ability to chemically process light. Reduction of the PQ pool and increases in t_{Fm} under high light exposure are in agreement with previous reports on leaves (Kalaji *et al.* 2012). In addition, the reduction in the ability to chemically process light during fruit development can be clearly appreciated from the decline in the number of active reaction centres per cross section observed at later stages (Fig. 3), which was faster in E than that in NE fruits.

When phenomenological fluxes per excited cross section were compared between sun exposure conditions at each developmental stage, we observed that activities at 65 DAFB were higher in E than that in NE fruits (Fig. 4*A*). However, these values were inverted at 110 DAFB despite the higher number of RC/CS_0. This depression in phenomenological fluxes per excited cross section correlates with the lower F_v/F_m observed in E fruits at this developmental stage.

Sudden exposure to solar radiation mainly affects the PSII antenna but not the PSII reaction centre. This was reflected by the greater increase in both absorption photon flux energy (ABS/RC) and dissipated energy per active reaction centre (DI_0/RC), and the unchanged trapped energy per reaction centre (TR_0/RC) observed in SU compared to NE fruits. A higher absorbance flux per reaction centre (ABS/RC) value seems to indicate an increased antenna size per active reaction centre (Strasser *et al.* 2004, Stirbet and Govindjee 2011, Živčák *et al.* 2014). Photoinhibition is more accurately identified as an increase in DI_0/RC and a decline in Ψ_{E0} rather than by a decline in F_v/F_m (Jiang *et al.* 2008). Exposure to solar radiation increased DI_0/RC and decreased ψ_{E0}. This supports the view that photoinhibition occurred at 65 and 110 DAFB in SU fruits, and at 110 and 140 DAFB in E fruits. However, it was at the latest stage of development (140 DAFB) when a reduction of the activity of the PSII RC was evidenced in E fruits by a decrease in TR_0/RC and ET_0/RC.

Plant vitality, characterised by PI_{abs} (Strasser *et al.* 2004), is a rapid and sensitive stress index used widely to compare the whole primary photochemical reaction (Chen and Cheng 2009). This index reflects the functionality of both PSII and PSI and gives information on the current state of plant performance under stress conditions (Strasser *et al.* 2004). Our findings show that PI_{abs} was affected by solar radiation and developmental stages. In NE fruits, PI_{abs} was constant throughout development (Fig. 2*F*) despite a concomitant decrease in photosynthetic capacity. This confirms that NE fruits were not under solar radiation stress. PI_{abs} decreased on E fruits at mid-stages of development (*i.e.*, 110 DAFB); while in SU fruits this phenomenon was observed at an early stage (*i.e.*, 65 DAFB). A reduction on PI_{abs} and an increase in DI_0/RC upon exposure to solar radiation are indicative of stress due to an excess of excitation energy.

Photoinhibition of both donor and acceptor sides of PSII can increase ROS production (Racskó and Schrader 2012) leading to lipid peroxidation and membrane cell damage. Solar radiation exposure increased membrane peroxidative

damage (Fig. 5) indicating that the antioxidant system was unable to cope with the photooxidation triggered by high solar radiation (Chen *et al.* 2008). These results are in agreement with previous reports in apple fruits (Chen *et al.* 2008). Also, we found that membrane peroxidative damage was not affected by development in E and NE fruits (Fig. 5). SU fruits, in contrast, showed increased peroxidation at each stage of development; however, this increase was only significant at early and mid-developmental stages (Fig. 5) even though several parameters of PSII photochemistry such as F_v/F_m, flux ratios and PI_{abs}, among others, were affected at a late developmental stage. Red Delicious apple fruits synthesize anthocyanin during development, which we presume may play a photoprotective role at a late developmental stage. Anthocyanins photoprotective role is widely known and was previously reported in pome fruits (Merzlyak and Chivkunova 2000, Li and Cheng 2009). The fact that NE fruits at 140 DAFB showed higher anthocyanin content than that of E fruits, may contribute to explain the lower peroxidation damage in SU fruits at 140 DAFB. These observations suggest that NE fruits suddenly exposed to sun (SU) are more resistant to solar radiation stress at late, than at early or mid-developmental stages.

Decreases in Chl content, PQ pool (Area), F_v/F_m, and PI_{abs}, and increases in DI_0/RC in fruits, which grew exposed to sunlight, suggest that apple peel PSII tolerance to high solar radiation decreases during fruit development. This study, carried out under natural high solar radiation on field conditions on fruits not detached from plants, allowed us to confirm previous studies conducted under lab-controlled conditions. Furthermore, we detected that fruits not exposed to sunlight during growth are more susceptible to photooxidative damage when they are suddenly exposed to sun at early and mid-stages of development. Of note, we used a portable fluorimeter that allowed us to evaluate solar radiation stress in apple fruits in natural conditions.

The present comprehensive characterization of apple peel PSII photochemical processes might help to increase understanding of the susceptibility of apple fruits to photooxidation and subsequent sunburn development depending on the light environment and developmental stage they are exposed to.

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