

The Relationship between Chlorophyll *a* Fluorescence and the Lower Oxygen Limit in
Higher Plants

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
June 2011

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DALHOUSIE UNIVERSITY
DEPARTMENT OF BIOLOGY

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DALHOUSIE UNIVERSITY

DATE: June 9, 2011

AUTHOR: A. Harrison Wright

TITLE: The Relationship between Chlorophyll *a* Fluorescence and the Lower
Oxygen Limit in Higher Plants

DEPARTMENT OR SCHOOL: Department of Biology

DEGREE: PhD CONVOCATION: October YEAR: 2011

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Abstract

The lower oxygen limit (LOL) in plants marks the oxygen (O_2) level where the metabolism shifts from being predominantly aerobic to anaerobic; recent work has shown that respiratory-based indicators of this metabolic shift are well-correlated with changes in chlorophyll *a* fluorescence signals. The physiological and biochemical changes at the root of this relationship have not been well-described in the literature. The processes involved are spatially separated: chlorophyll fluorescence is associated with the light-dependent reactions and emanates from the chloroplasts whereas aerobic respiration and fermentation occurs in the mitochondria and cytosol, respectively. Evidences outlined in this thesis are used to suggest the mechanistic link between these three regions of the cell is a fluid exchange of cellular reductant. When mitochondrial respiration is inhibited as a result of inadequate O_2 , used as a terminal electron acceptor, glycolytic reductant in the form of NADH accumulates in the cytosol. Reductant imbalances between the cytosol and organelles can be adjusted indirectly using translocators. Excess chloroplastic reductant is used to reduce the plastoquinone (PQ) pool via NADPH-dehydrogenase, a component of the chlororespiratory pathway, effectively decreasing the photochemical quenching (q_p) capacity thereby inducing a switch from minimum fluorescence (F_0) to a higher relative fluorescence (F) value where $q_p < 1$. Subjecting dark-adapted photosystems to low-intensity light increased F_0 to a slightly higher F value due to a light-induced reduction of the oxidized PQ pool when the O_2 was above the LOL, but decreased F as a result of a PSI-driven oxidation of the already over-reduced PQ pool when the O_2 was below the LOL. Low O_2 was also shown to increase violaxanthin de-epoxidation and non-photochemical quenching (q_N), likely a reflection of the over-reduced state of the photosystems and associated pH decrease.

Dynamic controlled atmosphere (DCA) is a fluorescence-based controlled atmosphere (CA) system that sets the optimum atmosphere for fruits and vegetables based on a product's fluorescence response. Experiments in this thesis on the relationship between O_2 , temperature, light, metabolism, pigmentation and chlorophyll fluorescence were used to interpret the physiology behind fluorescence changes, suggest improved DCA techniques and outline potentially profitable avenues for future research.

List of Abbreviations Used

AA	acetaldehyde
ACP	anaerobic compensation point
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AL	actinic light
AsC ^H	ascorbate
ATP	adenosine triphosphate
CO ₂	carbon dioxide
Cyt <i>b₆/f</i>	cytochrome <i>b₆/f</i> complex
DCA	dynamic controlled atmosphere
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DEPS	de-epoxidation state
DHA	dehydroascorbic acid
DTT	dithiothreitol
EA	ethyl acetate
EP	extinction point
ETC	electron transport chain
EtOH	ethanol
<i>f</i>	frequency
F	relative fluorescence
F _α	pulse frequency modulated-derived minimum fluorescence
F _d	ferredoxin
FNR	ferredoxin-NADP ⁺ reductase
F _o	minimum fluorescence
F _m	maximum fluorescence
FR	far-red light
F _v	variable fluorescence
F _v /F _m	quantum yield
LDH	lactate dehydrogenase
LHC	light harvesting complex
LOL	lower oxygen limit
ML	measuring light
N ₂	nitrogen

NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Ndh	NADPH-dehydrognase
O ₂	oxygen
OAA	oxaloacetate
OEC	oxygen evolution complex
PAM	pulse amplitude modulation
PC	plastocyanin
PCD	programmed cell death
Pheo	pheophytin
PDC	pyruvate decarboxylase
PFM	pulse frequency modulation
PMF	proton motive force
PPFD	photosynthetic photon flux density
PQ	plastoquinone
PS	photosystem
PTOX	plastid terminal oxidase
QBH ₂	plastoquinol
q _N	non-photochemical quenching coefficient
q _P	photochemical quenching coefficient
RC	reaction centre
RQ	respiratory quotient
RQB	respiratory quotient breakpoint
RuBP	ribulose-1,5-bisphosphate
Rubisco	ribulose-1,5-bisphosphate carboxylase oxygenase
SP	saturation pulse
SS	soluble solids
TA	titratable acidity
T ^{1/2}	half time for rise in F _v
Tyr	tyrosine
VDE	violaxanthin de-epoxidase
ZE	zeaxanthin epoxidase

Acknowledgements

I thank my Dalhousie supervisor, Dr. Arunika Gunawardena. Like family, you genuinely care for your students while at the same time hold them to high standards and expectations. To my external supervisors Drs. Robert Prange and John DeLong, thank you for your guidance and for opening up your labs while providing me with everything I needed to do good research. To my committee member, Dr. Tom MacRae, thank you for making yourself available, even in hardship, for my many questions. To Dr. Charles Embree and Doug Nichols, thank you for giving me a job driving a spray truck for the organic spray trials so many years ago and for encouraging me to do more. I would also like to thank my parents, Bob and Marianna, for their support and high expectations and to my loving wife, Aimee Hare, who endured my sometimes long hours at the lab and many, exceedingly dry and one-sided dinnertime conversations about plant physiology.

I gratefully acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) for its support in the form of a Canadian Graduate Studies (CGS) doctoral scholarship.

Chapter 1: Introduction

The lower oxygen limit (LOL) in plants is generally defined as the oxygen (O₂) level where respiration changes from being predominantly aerobic to anaerobic or fermentative. A strong correlation has been found between the switch from aerobic respiration and fermentation and sudden changes observed in chlorophyll *a* fluorescence signals (Prange et al. 2002; DeLong et al. 2004b; Zanella et al. 2005; Burdon et al. 2008; Wright et al. 2008a). The physiological and biochemical changes at the root of this relationship have not been well understood. The processes involved are spatially separated: chlorophyll fluorescence is associated with the light-dependent reactions that take place in the chloroplasts whereas aerobic respiration and fermentation occurs in the mitochondria and cytosol, respectively (Appendix A i). The remainder of this introduction will provide a general biochemical and physiological background and outline the thesis' objectives, while the chapters that follow will present new research, both fundamental and applied, examining 'the relationship between chlorophyll *a* fluorescence and the LOL in higher plants'.

1.1 Chlorophyll *a* Fluorescence and Low-O₂

*1.1.1 Chlorophyll *a* Fluorescence: the Basics*

Certain molecules, termed fluorophores, absorb a photon of light only to release a second photon of lower energy and higher wavelength in a process known as fluorescence. In quantum-mechanical theory, the valence electrons of an atom may be raised from their ground to an excited state if energy, delivered in the form of a photon for instance, is added to the system. The possible excited orbitals of these electrons are quantized, meaning, the probability of absorption is greatest if the energy associated with the incoming photon equals the required excitation energy. Electrons in an excited state are inherently unstable. In some instances it is possible for the excited electron to first de-excite to an interim state through the emission of a photon before reaching the ground state via the release of molecular vibration (i.e. heat). If this occurs in a relatively short period of time ($\approx 10^{-7}$ s), the product is fluorescence (Hecht 1990).

The pigments involved in photosynthesis are found within the chloroplast thylakoid membranes. Chlorophyll *a* is the predominant pigment, and fluorophore, found in higher plants; however, chlorophyll *b* in higher plants, accessory pigments, including several carotenoids, as well as a pigment's association with binding proteins, broadens the absorption spectra. Pigment structures favouring high-energy absorption are located further

away from the reaction centres (RCs) and those favouring low-energy are closer. Since exergonic reactions are favoured (2nd law of thermodynamics), the RCs associated with the two photosystems act as an energy sink and resonance energy transfers gravitate towards them (Taiz and Zeiger 2006). Two photosystems (PSII and PSI), and their corresponding RCs, form the generally-accepted ‘Z-scheme’ of the non-cyclic electron transport chain (ETC) (Appendix A i; Figure 1.1). When a chlorophyll molecule absorbs a quantum of light, the energy stored within the excited, but unstable electron has three fates: 1) it may excite a valence electron on a neighbouring pigment or RC in a process known as ‘resonance energy transfer’; 2) it may be released as heat; or 3) it may be released as a photon (i.e. fluorescence) (Lichtenthaler and Rinderle 1988; Hall and Rao 1999). Under unstressed conditions, approximately 85% of the absorbed energy is eventually transferred to a RC and used in photochemistry, while the rest is released as heat and fluorescence.

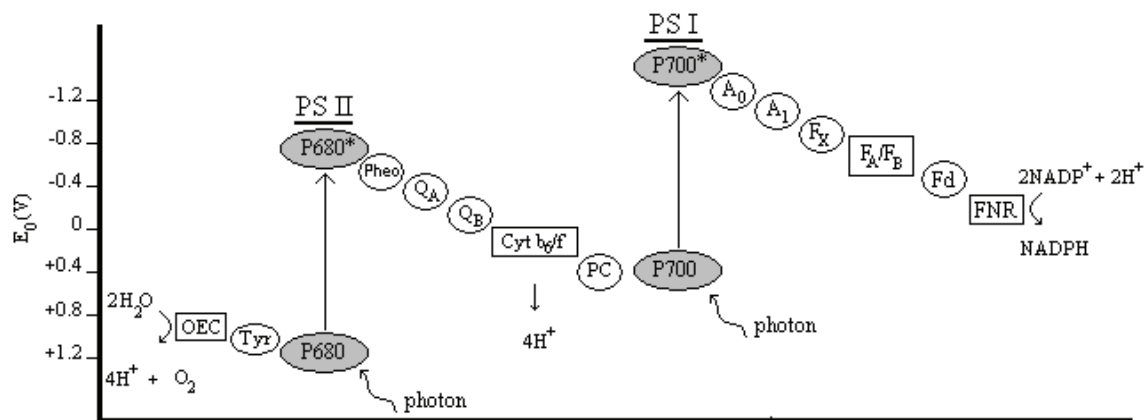


Figure 1.1 PSII and PSI are spatially separated inside the thylakoid membrane. The RC of PSII has a peak absorption of 680 nm (i.e. P680), while the RC of PSI absorbs maximally at 700 nm (P700). Both RCs (modified chlorophyll pigments) have a number of antennae pigments and associated light-harvesting complexes (LHC), primarily comprised of chlorophyll *a*, used to excite the RCs. When P680 becomes excited (P680*) it passes an electron to Pheophytin (Pheo). The oxidized P680⁺ replenishes the lost electron, via the intermediate amino acid Tyrosine (Tyr), from the oxygen evolution complex (OEC), which releases protons (H⁺) into the thylakoid lumen. Pheo⁻¹ passes an electron to the plastoquinone Q_A (attached to the D2 protein); Q_B (attached to the D1 protein) accepts two electrons, one at a time from Q_A, and two H⁺ from the thylakoid stroma before becoming detached from the D1 protein and joining the pool of plastoquinol (Q_BH₂) (i.e. PQ pool). Q_BH₂ passes two electrons to the cytochrome *b₆/f* complex (Cyt *b₆/f*), and releases its two H⁺ to the thylakoid lumen, before being recycled. Cyt *b₆/f* passes electrons to the mobile electron carrier plastocyanin (PC) which it uses to reduce P700⁺ in PSI. The electron transport chain (ETC) described between PSII and PSI, along with the OEC, leads to a build up of H⁺ inside the thylakoid lumen. The potential energy of this proton gradient [referred to as the proton motive force (PMF)] is used to form adenosine triphosphate (ATP) via the enzyme ATP synthase (i.e. photophosphorylation). When P700 is excited (P700*), the electron is passed down a series of carriers: modified chlorophyll A₀, phylloquinone (A₁), three iron-sulphur centres (F_X, F_A and F_B) and Ferredoxin (Fd). The enzyme ferredoxin-NADP⁺ reductase (FNR) facilitates the reduction of NADP⁺ to form NADPH (modified from Govindjee 2004).

1.1.2 Fluorescence as a Diagnostic Tool

Fluorescence intensity may be ‘quenched’ by either photochemical (q_P) or non-photochemical (q_N) means. Quenching coefficients range from 0 (no quenching) to 1 (maximum quenching). The ‘Kautsky curve’ is the basis of many of the primary dark-adapted fluorescence parameters commonly used: minimum fluorescence (F_o) ($q_P = 1$, $q_N = 0$), maximum fluorescence (F_m) ($q_P = 0$, $q_N = 0$), variable fluorescence (F_v) ($F_m - F_o$), maximum quantum yield (F_v/F_m). First reported by Kautsky and Hirsch (1931), the Kautsky curve is the fluorescence trace produced when dark-adapted plant tissue is reintroduced to light. The curve dynamics reflect the light-induced photochemistry that occurs within the chloroplasts (Figure 1.2).

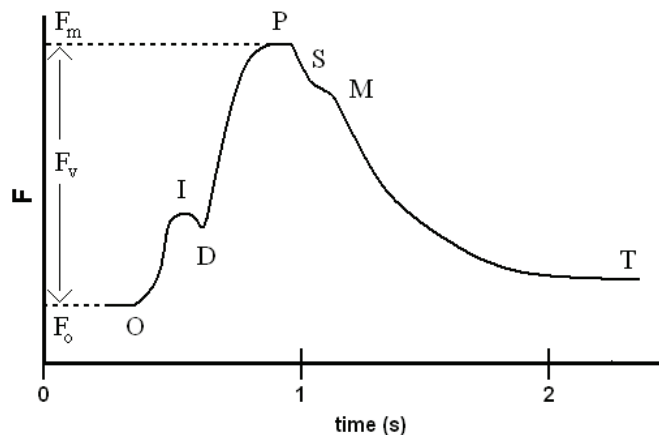


Figure 1.2 The ‘Kautsky curve’, shown here in a plot of time (s) versus relative fluorescence (F), refers to the typical fluorescence trace observed when photosynthetic material is transferred from dark to light. The O, I, D, P, S, M and T points on the fluorescence curve represent changes in photochemistry: O (origin) is the minimum fluorescence (F_o) produced when Q_A is fully oxidized (i.e. $q_P = 1$, $q_N = 0$), fluorescence increases to I (intermediate) as Q_A receives electrons from P680* and then dips (D) when Q_A is again oxidized by

Q_B . Fluorescence levels peak (P) when the plastoquinone pool and Q_A both become highly reduced. This peak will equate to the maximum fluorescence (F_m) in instances where no other quenching mechanisms are activated (i.e. $q_P = 0$, $q_N = 0$). The S (semi-steady state), M (intermediate maxima) and T (terminal) fluorescence levels (F_s – steady state) relate to the induction of CO_2 assimilation and an increase in non-photochemical quenching and electron flow between the photosystems (for a review see DeEll et al. 1999). The variable fluorescence (F_v) is the difference between F_m and F_o and the F_v/F_m ratio, also known as the exciton transfer efficiency, is directly proportional to the quantum yield of PSII (Genty et al. 1989). The $T_{1/2}$ value is the half-time required for dark-adapted fluorescence material to rise from F_o to F_m (DeEll et al. 1999).

The use of far-red, actinic and saturation light sources in a typical fluorescence trace (Figure 1.3) under various stress and control conditions, including various inhibitors and promoters, has greatly aided our understanding of fluorescence quenching, the photosystems and their biochemistry. New fluorescence techniques are always being developed. Established chlorophyll fluorescence techniques are now widely used in the plant sciences and are nearly ubiquitous in the field of plant physiology (Maxwell and Johnson 2000).

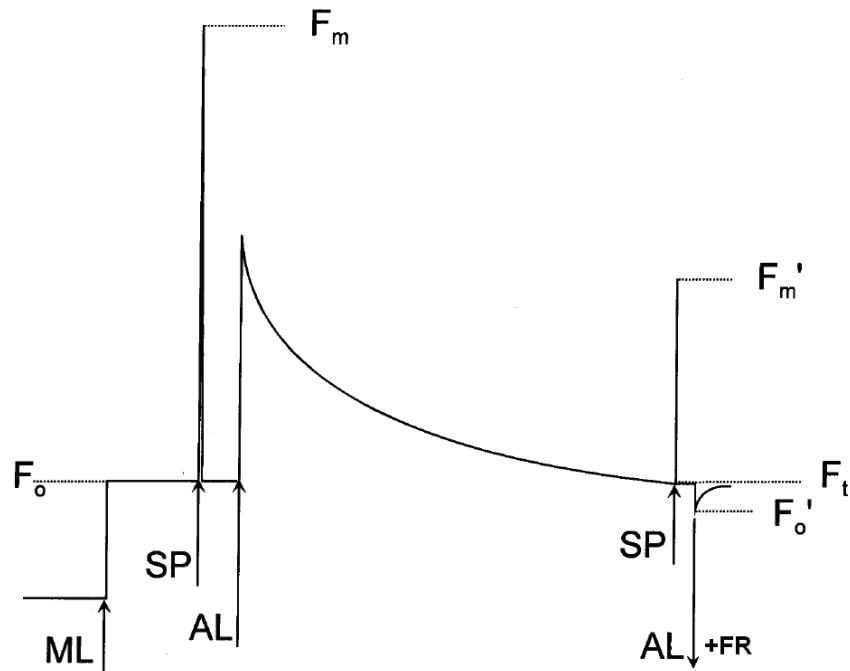


Figure 1.3 A typical fluorescence trace using the 'saturation pulse' method. A weak modulating light (ML) ($\approx 6 \text{ nmol m}^{-2} \text{ s}^{-1}$ at 660 nm) is turned on and the 'dark adapted' minimum fluorescence (F_o) ($q_P = 1$, $q_N = 0$) is measured; the addition of a saturation pulse (SP) ($\approx 10000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$, $400 \text{ nm} < \lambda < 700 \text{ nm}$, duration = 0.5 to 2 s) measure the 'dark adapted' maximum fluorescence (F_m) ($q_P = 0$, $q_N = 0$). A continuous actinic light (AL) is applied to drive photosynthesis until terminal fluorescence (F_t) ($1 > q_P > 0$, $1 > q_N > 0$) is reached. A reapplication of a SP measures the maximum fluorescence in the light (F_m') ($q_P = 0$, $1 > q_N > 0$). Turning the AL off in the presence of a far-red (FR) light ($\approx 6 \text{ nmol m}^{-2} \text{ s}^{-1}$, $\lambda > 700 \text{ nm}$) estimates the minimum fluorescence in the light (F_o') ($q_P = 1$, $1 > q_N > 0$) (modified from Maxwell and Johnson, 2000; van Kooten and Snel, 1990).

1.1.3 Photochemical Quenching

Photochemical quenching refers to a drop in fluorescence because of an increased energy demand for photochemistry. For example, in the Kautsky curve the decrease in fluorescence from the 'P' to 'T' level (Figure 1.2) results from an increase in the photosystems' efficiency to process absorbed excitation energy. Various stresses may also alter a plant's capacity for photochemical quenching. The amount of photochemical quenching may be quantified using fluorescence techniques [e.g. (coefficient of photochemical quenching (q_P) = $(F_m' - F_s) \cdot (F_m' - F_o')^{-1}$) (F_s – steady-state fluorescence; apostrophes denote measurements performed in the light) (Buschmann 1999)]. It is believed the capacity for q_P is lowered once O_2 levels drop below the LOL (Wright et al. 2011a, 2011b; Chapters 4, 5).

1.1.4 Non-Photochemical Quenching

Non-photochemical quenching refers to decrease in fluorescence because of an increased dissipation of excitation energy by means other than photochemistry, typically heat. Non-photochemical quenching can be quantified using fluorescence techniques [e.g. non-photochemical quenching (NPQ) = $(F_m - F_m') \cdot (F_m')^{-1}$; coefficient of non-photochemical quenching (q_N) = $(F_m - F_m') \cdot (F_m - F_o')^{-1}$ (Buschmann 1999)]. Non-photochemical quenching occurs in chloroplasts at low levels as part of the natural inefficiencies inherent to any thermodynamic process; however, some stresses induce increased non-photochemical quenching as a photoprotective mechanism.

The xanthophyll cycle, a non-photochemical quenching pathway, is a key regulator and photoprotective mechanism found ubiquitously in plants. The xanthophyll cycle involves the interconversion of a group of carotenoids known as the xanthophylls. Under excess light conditions, which trigger a high transthylakoid Δ pH, violaxanthin is converted, via sequential de-epoxidations, into the intermediate antheraxanthin and ultimately zeaxanthin (Figure 1.4) (Pfündel and Dilley 1993; Demmig-Adams and Adams, 1996; Munekage et al. 2002). Energy-dependent quenching is linearly related to the transthylakoid Δ pH (Krause and Weis 1991) and employs pH-sensitive enzymes for the interconversion of the xanthophylls (Kramer et al. 2004). When in the violaxanthin state, and possessing nine conjugated double bonds, the carotenoid functions as an antenna pigment and transfers energy, in an exergonic fashion, to chlorophyll *a* pigments and RCs. When in the zeaxanthin state, and possessing eleven conjugated double bonds, the lowest excited singlet state energy level of the carotenoid changes to one that is now below, instead of above, that of chlorophyll *a* and the carotenoid acts as a trap, dissipating energy as heat before it reaches a RC (Frank et al. 1994). Although the xanthophyll cycle is typically associated with high light intensities, research in this thesis examines and documents its activity under low- O_2 conditions even under low-intensity light (Wright et al. 2011a, 2011b; Chapters 4, 5).

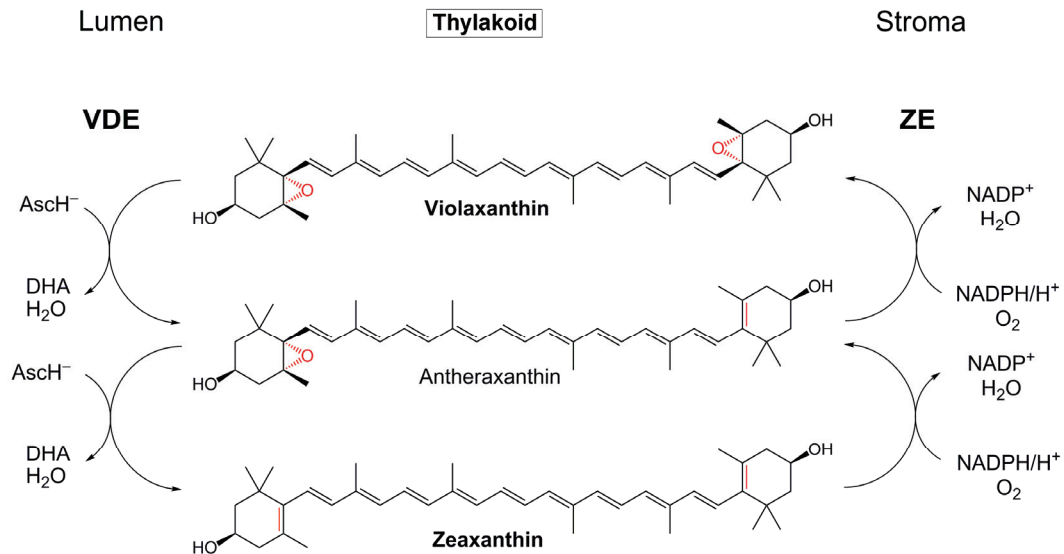


Figure 1.4 The xanthophyll cycle, an interconversion of the xanthophyll carotenoids located in the thylakoid membrane, is a key photoprotective mechanism found in higher plants. When in the violaxanthin state the carotenoid acts as an antennae pigment; when in the zeaxanthin state it acts as a non-photochemical quencher. Violaxanthin de-epoxidase (VDE), found in the lumen side of thylakoid membrane, requires ascorbate (AsCH⁻), which is converted into dehydroascorbic acid (DHA), and low pH. Zeaxanthin epoxidase (ZE), found in the stroma side of the thylakoid membrane, requires O₂, NADPH and a more neutral pH (modified from artwork released into the public domain on Wikimedia Commons, 2011).

1.1.5 The LOL and Fluorescence

A variety of stresses such as osmotic (Wright et al. 2008b), chilling (Smillie et al. 1987b) and heat (Briantais et al. 1996) have been shown to induce fluorescence changes. Work using spinach (*Spinacia oleracea*) plants showed that anoxia enhances the photoinhibition-effect, a light-induced decrease in photosynthesis, and leads to an increase in F_o (Satoh 1971; Krause et al. 1985). DeEll et al. (1995, 1998) first demonstrated the potential application of chlorophyll fluorescence as an indicator of controlled atmosphere (CA) disorders or atmospheric stress in apples (*Malus domestica*). Apples stored under low-O₂ or high-CO₂ have a marked decrease in F_v, F_v/F_m and an increase in T_{1/2} levels, defined as the time required to go from F_o to 1/2F_v on the Kautsky curve. The fluorescence changes occur before off-flavours or fruit discolouration, which manifest days later. More recent work has shown that when O₂ levels are incrementally lowered, F_o remains unchanged until the O₂ levels reaches a critical value that prompts F_o to increase markedly or ‘spike’ indicating the LOL. When O₂ is reintroduced, F_o quickly decreases to pre-stress levels with no apparent permanent damage (Figure 1.5). Such a response has been observed in many plant species (Prange et al. 2003; Burdon et al. 2008; Wright et al. 2008a) and is positively correlated with

temperature and respiration (Wright et al. 2010, 2011b; Chapters 3, 5). All chlorophyll-containing plant tissue tested to date has produced a low- O_2 induced fluorescence response; however, work has primarily focused on commercially viable plant species and similar studies have not been performed using flood-tolerant plants such as rice (*Oryza*) or yellow iris (*Iris pseudacorus*). Future work with such species could be used to increase our understanding of the low- O_2 / fluorescence relationship.

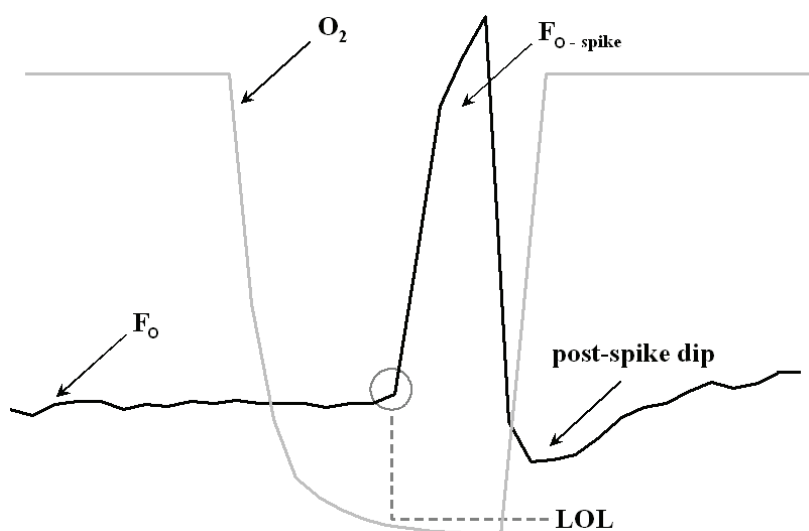


Figure 1.5 A plot showing a typical low- O_2 -induced fluorescence 'spike'. When the O_2 (grey solid) is gradually lowered the minimum fluorescence (F_0) (black solid) does not increase until it reaches a critical threshold referred to as the lower oxygen limit (LOL) which is well correlated with respiratory changes (Gasser et al. 2008; Wright et al. 2011b; Chapter 5). Upon re-oxygenation, F_0 rapidly decreases, sometimes

exhibiting a post-spike dip, before returning to the pre-stress baseline. Shown is averaged data from 4 jars of 'Honeycrisp' apples at 3.5°C measured using a HarvestWatch™ system (an approximation of F_0 referred to as F_a).

1.1.6 Chlorophyll Fluorescence Applications in Industry: Pulse Frequency Modulation

Apparent links between numerous fruit and vegetable physiological and quality parameters and specific fluorescence indicators have motivated countless researchers. However, few established relationships are practical or embraced by industry. Commercial fruit and vegetable producers, storage operators and distributors have a very low tolerance for the often moderate to high amounts of variation inherent in fluorescence studies otherwise showing highly significant correlations. One exception is the fluorescence-based dynamic controlled atmosphere (DCA) system which takes advantage of the sensitivity of F_0 to the LOL to better sustain fruit and vegetable quality in storage (DeLong et al. 2004b; Zanella et al. 2005; Burdon et al. 2008). A specialized DCA fluorometer that uses pulse frequency modulation (PFM) technology, instead of pulse amplitude modulation (PAM), to approximate F_0 (referred to as F_a), was developed in a joint project between Agriculture and

Agri-Food Canada (AAFC) and Satlantic Inc. (Halifax, NS) (Wright et al. 2008a; Chapter 2).

1.1.7 Photoinactivation

Excess excitation energy, as found under high light-intensity conditions or as a result of stress-induced inhibition of the photosynthetic electron flow, increases damaging reactive oxygen species (ROS), such as singlet oxygen ($^1\text{O}_2$), superoxide (O_2^-) or hydrogen peroxide (H_2O_2) produced through the photoreduction of O_2 (Barber and Andersson 1992; Asada 2006). The D1 protein in PSII, which anchors the plastoquinone Q_B , is particularly vulnerable to damage under high-intensity light conditions, a form of photoinactivation known as photoinhibition, and is a likely target of ROS (Lupínková and Komenda 2004). Depriving cells of O_2 exacerbates photoinhibition as indicated by an enhanced decrease in CO_2 assimilation (Sato 1971; Krause et al. 1985). The dynamics of photoinhibition are different under anaerobic compared with aerobic conditions: under the latter, F_0 remains constant while under the former, F_0 increases. Photoinactivation may be divided into two distinct processes: 1) a fast process that occurs under anaerobic conditions or in the presence of a strong reducing agent and, 2) a slow process that occurs under anaerobic and aerobic conditions (Krause et al. 1985; Šetlík et al. 1990). Evidence suggests that an anaerobic environment, even in darkness, reduces the PQ pool, the mobile electron carrier that connects plastoquinone Q_A to the cytochrome *b₆/f* complex (Cyt *b₆/f*) (Figure 1.1) (Harris and Heber 1993; Bennoun 2002; Toth et al. 2007), possibly explaining the link between fluorescence and cell metabolism (Wright et al. 2011a, 2011b; Chapters 4, 5).

1.2 Plant Respiration

1.2.1 Mitochondrial versus the Fermentative Pathway and Volatile Production

Respiration oxidizes organic molecules to generate energy in the form of ATP. During aerobic respiration, glycolysis, which yields two nicotinamide adenine dinucleotides (NADH) and 2 ATP, occurs in the cytosol converting sugars into pyruvate. In the mitochondrial matrix, pyruvate is decarboxylated via pyruvate dehydrogenase to form NADH, CO_2 and acetyl-CoA. Acetyl-CoA enters the citric acid cycle (Krebs cycle) where it is oxidized, generating CO_2 and large amounts of reducing power in the form of NADH and flavin adenine dinucleotide (FADH_2). Oxidative phosphorylation occurs in the inner mitochondrial membrane via a series of protein complexes. NADH dehydrogenase (complex

I) oxidizes NADH and uses the electrons to reduce ubiquinone (UQ), forming ubiquinol, while pumping 4 protons per electron pair into the inner membrane; electrons are also passed to UQ via succinate dehydrogenase (complex II) through the oxidation of succinate. The cytochrome BC1 complex (complex III), similar in structure and function to the chloroplast Cyt *b₆f* complex, oxidizes ubiquinol and passes the electrons to cytochrome *c*, a mobile electron carrier, and pumps 4 protons per electron pair from the matrix to the inner membrane. Cytochrome oxidase (complex IV) receives electrons from cytochrome *c* and uses O₂ as a terminal electron acceptor to form H₂O and pumps 2 protons from the matrix into the inner membrane per electron pair. The ATP synthase protein complex then harnesses the proton gradient formed as a result of the ETC to form ATP in a manner similar to that found in chloroplasts (Taiz and Zeiger 2006).

In a low-O₂ environment, O₂ becomes limiting as a terminal electron acceptor in the mitochondria cytochrome oxidase complex and the plant must resort to fermentation. Lactic acid fermentation is primarily found in mammalian cells, but is sometimes temporarily observed in plant cells before switching to ethanol fermentation, the primary fermentative pathway in plants (Appendix A ii; Perata and Alpi 1993). In lactic acid fermentation, lactate dehydrogenase (LDH) oxidizes NADH making NAD⁺ available for continued glycolysis while converting pyruvate to lactate. In ethanol fermentation, pyruvate decarboxylase (PDC) converts pyruvate to acetaldehyde (AA) while releasing CO₂. The alcohol dehydrogenase (ADH) enzyme oxidizes NADH so it may be recycled in glycolysis while converting AA into EtOH (Perata and Alpi 1993). EtOH may be further converted into ethyl acetate (EA) via alcohol acyltransferase (Defilippi et al. 2005).

Aerobic respiration, which produces a theoretical 38 ATP molecules per glucose molecule oxidized, although likely closer to ≈ 30 ATP/glucose molecule *in situ* (Rich 2003), is much more efficient than fermentation, which only produces a theoretical maximum of 2 ATP/glucose molecules via glycolysis. When aerobic respiration is inhibited, such as in a low-O₂ environment, glycolysis, and its associated ethanol fermentative CO₂ production, sometimes increases, referred to as the *Pasteur Effect*, in an attempt to meet energy demands (Figure 1.6A). However, not all plant materials exhibit a strong *Pasteur Effect* (Figure 1.7A); a re-absorption of CO₂, or an increased reliance on lactic acid fermentation, which, unlike EtOH fermentation, does not emit CO₂, could explain a diminished *Pasteur Effect* in some species. Increased CO₂ production and diminished O₂ consumption leads to a rise in

the respiratory quotient (RQ), the ratio of CO₂ released over O₂ consumed, and is referred to as the RQ breakpoint (RQB). The RQB has been used as a measure of the LOL (Gran and Beaudry 1993b). Alternatively, the anaerobic compensation point (ACP), the O₂ level at which CO₂ production is at a minimum, has also been used as a measure of the LOL (Boersig et al. 1988). Both the RQB and ACP allow for a minimal amount of fermentation (Peppelenbos and Oosterhaven 1998).

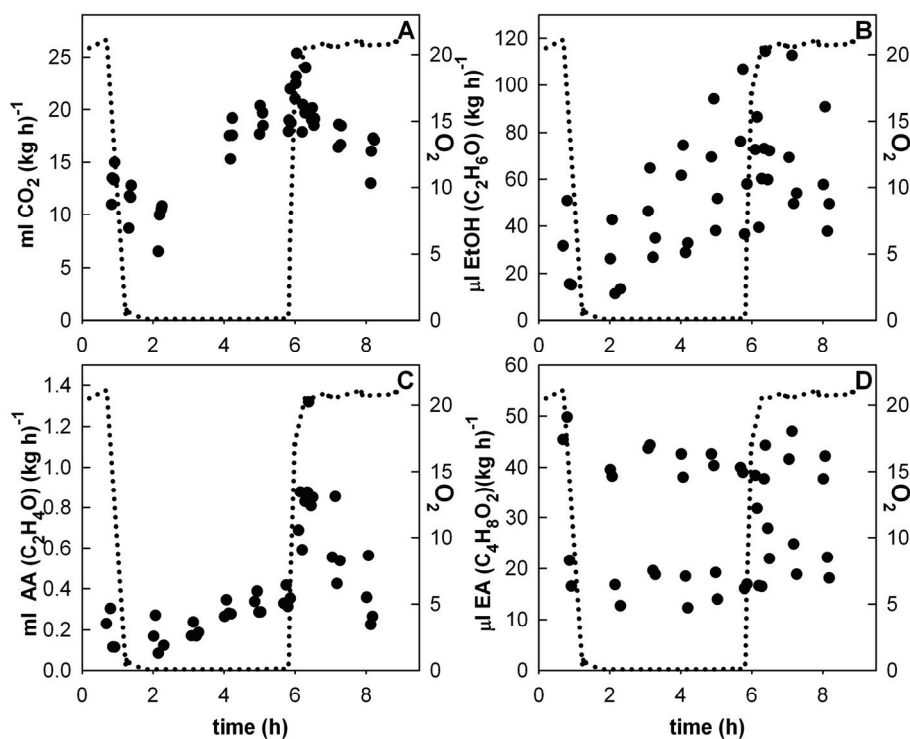


Figure 1.6 The response of 'Honeycrisp' apples held in the dark as the O₂ (.....) was lowered to anoxic levels (at ≈ 2 h) and then O₂ reintroduction (at ≈ 6 h) at 20 °C: (A) carbon dioxide (CO₂), (B) ethanol (EtOH), (C) acetaldehyde (AA) and (D) ethyl acetate (EA) (shown as ●). Data represent measurements collected from four jars of apples (n = 4) over time. Much lower values, with similar trends, were observed when the experiment was

repeated at 3.5 °C. Increased CO₂ under low-O₂ is evidence of the *Pasteur Effect* (A).

The occurrence of tissue disorders in fermenting plant tissue may be a result of either a toxic accumulation of fermentative products or an energy shortage (Peppelenbos and Oosterhaven 1998). The buildup of fermentative products is often rapid once plant material is exposed to a low-O₂ environment (Figures 1.6 and 1.7); however, monitoring volatile production is not a reliable way of detecting significant anaerobic ↔ aerobic metabolic changes and determining the LOL (Prange et al. 2005b). Low levels of fermentative products are tolerable to most plants and the extinction point (EP), the O₂ level where EtOH is first detected, generally occurs at O₂ levels well above the LOL (Gran and Beaudry 1993a). Furthermore, some plant materials can maintain moderate levels of fermentation and EtOH accumulation, with no adverse effects, even under aerobic conditions (Figure 1.6B).

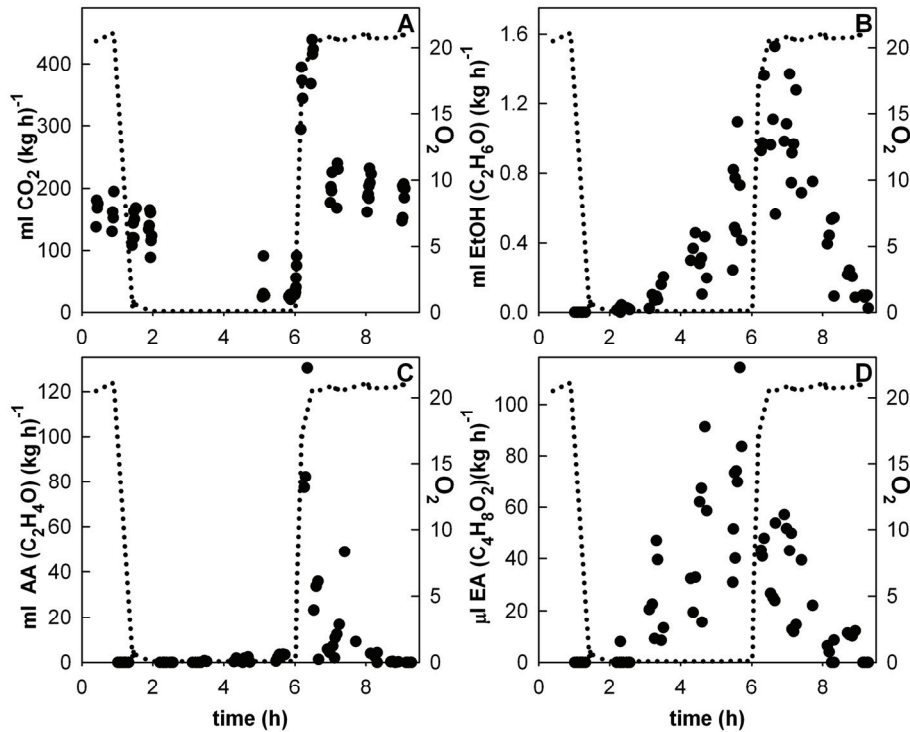


Figure 1.7 The response of spinach leaves held in the dark as the O_2 (.....) was lowered to anoxic levels (at ≈ 2 h) and then O_2 reintroduction (at ≈ 6 h) at $20^\circ C$: (A) carbon dioxide (CO_2), (B) ethanol (EtOH), (C) acetaldehyde (AA) and (D) ethyl acetate (EA) (shown as \bullet). Data represent measurements collected from four spinach leaves ($n = 4$) over time. Much lower values, with similar trends, were observed when the experiment was repeated at $3.5^\circ C$.

The post-anoxic upsurge in AA may be due to ethanol oxidation (Zuckermann et al. 1997; Boamfa et al. 2003) or an H_2O_2 -dependent catalase-mediated peroxidation (Boamfa et al. 2003).

1.2.2 Photorespiration, the Mehler Reaction and Chlororespiration

Oxygen is consumed due to its role as an electron acceptor in the mitochondria during aerobic respiration. In the chloroplasts, O_2 , as well as protons and electrons, are liberated from H_2O as part of the OEC located on the oxidizing side of PSII on the luminal thylakoid surface. However, under certain conditions, such as those favourable for photorespiration, chlororespiration, or the Mehler reaction, O_2 may also be consumed in chloroplasts.

Ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), is capable of binding either CO_2 or O_2 to ribulose-1,5-bisphosphate (RuBP). Although CO_2 is generally favoured, O_2 is fixed if levels are high, CO_2 levels are low or at high temperatures. Fixation of O_2 rather than CO_2 is called 'photorespiration'. Photorespiration, which accounts for as much as 50% of gross photosynthesis in some species, leads to a loss of energy and ultimately to the release of CO_2 from the oxidation of glycine (Hall and Rao 1999). Photorespiration is not simply a waste process but may protect C_3 plants from photoinhibition and ROS (Kozaki and Takeka, 1999; Asada 2006).

In cyclic electron flow, electrons are passed from the reduced electron carrier ferredoxin on the reducing side of PSI back to the Cyt *b₆/f* complex before returning to PSI. The

reduction of the PQ pool by NADPH and Ndh could also facilitate a form of cyclic electron flow. Plants deficient in the genes associated with the mechanism required for cyclic electron flow show an increase in fluorescence (Munekage et al. 2002). In the Mehler reaction (i.e. the water-water cycle or pseudocyclic electron transfer) electrons from reduced ferredoxin react directly with O₂ to form H₂O₂. In both cyclic electron flow and the Mehler reaction ATP is formed via maintenance of the transthylakoid ΔpH, but NADPH is not. Since O₂ evolved at PSII is later consumed during the Mehler reaction, neither it nor cyclic electron flow involve net gas exchange (Heber 2002). Under conditions of low carbon assimilation, the Mehler reaction may help discard potentially destructive excess light energy by maintaining photosynthetic electron flow and reducing photooxidative damage (Rizhsky et al. 2003).

Chlororespiration, a chloroplast respiratory chain that uses O₂ as a terminal electron acceptor (Appendix A iii), was first proposed to account for O₂ consumption, changes in the redox state of the PQ pool and maintenance of the electrochemical gradient across the thylakoid membrane even under complete darkness (Bennoun 1982). Earlier work (Kok 1949) had already noted the ‘Kok effect’ or the presence of lower than expected levels of O₂ evolution per quantum when plants are subjected to low photon flux densities (< 10 μmol·m⁻²·s⁻¹). Although the discovery of an ATP-driven electrogenic pump, capable of maintaining the transthylakoid potential, even under anaerobic conditions, may discount the role of chlororespiration on this account, mounting evidence continues to support the role of a plastoquinone: oxygen oxidoreductase (Bennoun 2002).

1.3 Cytoplasmic Acidosis

Low-O₂ stress induces cytoplasmic acidosis in plant material, including fruits and vegetables (Bertl and Felle 1985; Gout et al. 2001; de Sousa and Sodek 2002; Felle 2005). There is a suspected link between cytoplasmic acidosis and fluorescence changes (Prange et al. 2005b). The relationship between low-O₂, fluorescence changes and cytoplasmic acidosis may be mechanistic or simply correlative. The potential sources of protons that could lead to the cytoplasmic acidification during low-O₂ stress are discussed.

1.3.1 Lactic Acid

In plants, ethanol fermentation, not lactic acid as in animals, is the dominant anaerobic metabolic pathway; however, low levels of lactate accumulation have been documented in

plants (Donaldson et al. 1985; Andreev and Vartapetian 1992). One theory suggests hypoxic conditions first trigger lactate production thereby lowering the pH which in turn triggers the pyruvate decarboxylase enzyme; a short-term lactic fermentation stage thereby precedes the more dominant ethanol fermentation pathway (Davies et al. 1974; Roberts et al. 1984b; Perata and Alpi 1993). However, lactate accumulation is not observed in all plants under low-O₂ conditions (Andreev and Vartapetian 1992). Furthermore, fluctuating lactate levels in plants under low-O₂ stress do not always correlate well with cytosolic pH changes (Saint-Ges et al. 1991; Couldwell et al. 2009).

1.3.2 ATP Hydrolysis

For every molecule of ATP hydrolyzed (i.e. $\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_i + \text{H}^+$) a proton is released into the cytoplasm. Proton accumulation as a result of ATP hydrolysis in the absence of aerobic respiration has gained acceptance as the primary cause for cytoplasmic acidosis found in animal cells with insufficient O₂ (Robergs et al. 2004). Studies using ³¹P-nuclear magnetic resonance analysis show cytoplasmic pH levels correlate well with Cyt P_i, ATP and other nucleoside triphosphates levels in plant cells during anoxic ↔ normoxic events (Saint-Ges et al. 1991; Gout et al. 2001). For example, sycamore cells incubated in P_i-free medium (low initial ATP) experience only slight acidification while cells incubated in adenine-supplied medium (high initial ATP) experience increased acidification of the cytoplasm compared with reference cells during anoxia (Gout et al. 2001).

1.3.3 The 'Energy Crisis'

Cytoplasmic acidosis may also be indirectly induced due to rapid ATP hydrolysis as a result of severely low cell energy levels. Insufficient energy could result in the breakdown of the cellular mechanisms needed to maintain homeostasis. Cold temperature, typical of that found during storage, induces a reversible form of cytoplasmic acidosis in plants (Yoshida 1994; Yoshida et al. 1999). It is suggested that a cold-induced inactivation of vacuolar H⁺-ATPases leads to an acidification of the cytoplasm and an alkalization of the vacuoles. A similar phenomenon is speculated to occur under low-O₂ conditions. Work on maize root tips using nuclear magnetic resonance spectroscopy show pH in the vacuole and cytoplasm increase and decrease, respectively, under anaerobic conditions. This likely occurs as a result of a gradual leakage of protons through the tonoplast and a failure of tonoplast ATPases to maintain the pH gradient due to limited ATP in the absence of aerobic respiration (Roberts et al. 1984a; Saint-Ges et al. 1991).

1.3.4 Acidosis and Chlorophyll Fluorescence

The response of the pH to O₂ level changes in plant cells occur over the course of minutes (Saint-Ges et al. 1991; Gout et al. 2001; Schulte et al. 2006) and so are on the same scale as fluorescence changes observed under similar conditions. Future work should determine whether the acidification of the cytoplasm initiates at O₂ levels above the LOL, and is perhaps correlated with the EP, or if the failure of the cell to maintain cytoplasmic homeostasis is sudden and corresponds to the increase in fluorescence and respiratory-based indicators of the LOL.

The transthylakoid Δ pH is responsible for ATP synthesis and regulating the rate of photosynthesis (Kramer 1999; Kramer et al. 2003). Cytoplasmic acidosis may influence fluorescence parameters due to its effect on the transthylakoid Δ pH. For instance, isolated chloroplast thylakoids transferred from high to low pH medium generate a large transthylakoid Δ pH, even in the dark, and show rapid ATP synthesis (Jagendorf 1967). The F_o measurement is relatively independent of the cytosolic pH within a neutral range; however, low pH has been associated with increased quenching of F_v (Sato 1971; Bruce et al. 1997). pH changes may induce some changes in fluorescence signals as a result of their influence on the xanthophyll cycle (Wright et al. 2011a, 2011b; Chapters 4,5).

1.4 Low-O₂ Signallers, Molecular Changes and Programmed Cell Death (PCD)

Gene expression is strongly altered under hypoxic conditions (Perata and Alpi 1993; Bailey-Serres and Chang 2005); certain forms of programmed cell death (PCD) are attributed to low-O₂ (Gunawardena et al. 2001; Gladish et al. 2006). Feild et al. (1998) speculated that chlororespiration, components of which may be related to the low-O₂-induced F_o increase as a result of an over-reduction of the PQ pool (Wright et al. 2011a; Chapter 4), may require altered gene expression and protein synthesis. Low-O₂ conditions can quickly trigger complex changes in cytosolic Ca²⁺ levels, a known signaller possibly connected with PCD (Sedbrook et al. 1996; Buchanan et al. 2000). Furthermore, elevated levels of cytosolic cytochrome *c* have been correlated with elevated Ca²⁺ and may regulate caspase-like activity and ROS, both associated with PCD (Liu et al. 1996; Skulachev 1998). Elevated cytosolic cytochrome *c* levels, and subsequent caspase activation, has been observed within minutes of ischemia, a localized decrease in the red blood cells used to carry oxygen in animal cells (Soeda et al. 2001). Cytochrome *c* is a heme protein associated with the mitochondrial

terminal oxidase and aerobic respiration thereby implicating a potential role in the low-O₂ / fluorescence relationship.

The switch from aerobic respiration to anaerobic metabolism is sudden, as evidenced by the rapid increase in RQ and F_o in response to low O₂. Determining how low-O₂-induced fluorescence changes may relate to plant metabolism, energy levels, ROS, stress-induced gene expression and the buildup or depletion of cellular products requires more research.

1.5 Purpose and Objectives

Work that strives to better understand the events that surround plant normoxic ↔ anoxic transitions has academic interest as it could increase our understanding of the functionality of various plant metabolic pathways and because hypoxic stress stimulates a form of environmentally-induced PCD (Gunawardena et al. 2001; Xu et al. 2004; Gladish et al. 2006). A better understanding of the relationship between fluorescence, oxygen and temperature also holds the potential for immediate commercial applications. One example would be by broadening the utility and acceptance of the fluorescence-based DCA system, a recent technology increasingly used commercially to non-chemically maintain the quality and storage life of fruits and vegetables. The objectives of the research are as follows: 1. to demonstrate how the pulse frequency modulated (PFM) minimum fluorescence parameter (F_w) is derived and compare it with the pulse amplitude modulated (PAM) minimum fluorescence parameter (F_o); 2. to determine the effect of temperature, scan interval and rate of O₂ decline on chlorophyll fluorescence and the LOL fluorescence spike in apples; 3. to explore the interrelationship between low-O₂, the LOL, mitochondria, chlorophyll fluorescence and the xanthophyll cycle under both dark and low-light conditions; and 4. to describe known fluorescence phenomena in physiological terms while under DCA conditions.

Chapter 2: A New Minimum Fluorescence Parameter, as Generated using Pulse Frequency Modulation (PFM), Compared with Pulse Amplitude Modulation (PAM): F_{α} versus F_o ¹

Published as:

Wright H, DeLong J, Franklin J, Lada R, Prange R. 2008. A new minimum fluorescence parameter, as generated using pulse frequency modulation (PFM), compared with pulse amplitude modulation (PAM): F_{α} versus F_o . *Photosynthesis Research* **97**: 205 – 214

2.1 Abstract

The minimum fluorescence parameter (F_{α}), generated using the new pulse frequency modulation (PFM) technology, was compared with the minimum fluorescence parameter (F_o), generated by pulse amplitude modulation (PAM), in response to a reversible low-oxygen stress in ‘Honeycrisp’™ apples (*Malus domestica*) and an irreversible osmotic stress induced by water loss in two grape (*Vitis* spp.) cultivars (‘L’Acadie’ and ‘Thompson Seedless’). The minimum fluorescence values produced by both fluorometer types in response to a reversible low- O_2 stress in apples were indistinguishable: both F_o and F_{α} increased when O_2 levels were lowered below the anaerobic compensation point (ACP); when gas levels returned to normoxia both parameters dipped below, then returned to, the original fluorescence baseline. The two parameters also responded similarly to the irreversible osmotic stress in grapes: in both cultivars F_{α} and F_o first decreased before reaching an inflection point at approximately 20% mass loss and then increased towards a second inflection point. However, the two parameters were not analogous under the irreversible osmotic stress; most notably, the relative F_{α} values appeared to be lower than F_o during the later stages of dehydration. This was likely due to the influence of the F_m parameter and an overestimation of F_{α} when measuring the fluorescence from healthy and responsive chloroplasts as found in grapes experiencing minimal water loss, but not in grapes undergoing moderate to severe dehydration. An examination of the data during a typical PFM scan reveals this fluorometer system may yield new fluorescence information with interesting biological applications.

2.2 Introduction

A new fluorometer and software system (HarvestWatch™) developed by Satlantic Inc., Halifax, Nova Scotia, in conjunction with Agriculture and Agri-Food Canada (AAFC) uses pulse frequency modulation (PFM) to generate a novel derivation of minimum fluorescence and may be an untapped source of new fluorescence information. The PFM fluorometer was initially developed to determine the anaerobic compensation point (ACP) in fruits and

¹ The portion of this paper on osmotic stress in grapes was from A.H. Wright’s Masters work; the portion on low- O_2 stress in apples (\approx 50%) was his PhD work.

vegetables destined for controlled atmosphere (CA) storage^{II} (Prange et al. 2003; DeLong et al. 2004b; Prange et al. 2007; Burdon et al. 2008) and has found applications ranging from minimizing the incidence of scald in apples (*Malus domestica*) (Zanella et al. 2005) to maintaining kiwifruit firmness (Lallu and Burdon 2007). Many other potential stress physiology applications remain to be explored.

Lowering oxygen (O₂) levels below the ACP in plants induces a stress that may be reversed with little damage, if the O₂ is increased above the ACP within a short period of time (Prange et al. 2003; DeLong et al. 2004b). The physiological changes that occur in grapes (*Vitis* spp.) as they dehydrate over a long period of time induce osmotic stress and irreversible damage to the grape with a decrease in quantum efficiency (Wright et al. 2008b).

Pulse amplitude modulation (PAM) fluorometry has produced a large number of fluorescence investigations and practical applications (Schreiber 2004). Minimum fluorescence (F_o) is measured with a low intensity red light ($\approx 6 \text{ nmol m}^{-2} \text{ s}^{-1}$ at 660 nm) while the maximum fluorescence (F_m) is measured during a high intensity white light saturation pulse ($\approx 10000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ at 400 – 700 nm). For plants measured under dark-adapted conditions, it is assumed that non-photochemical quenching (q_N) is zero for the measurements of both F_o and F_m. The low intensity of the pulsed red light theoretically ensures that photosystem II (PSII) reaction centres are open (i.e. photochemical quenching (q_p) = 1) during the measurement of F_o, while the highly intense saturation pulse used in generating F_m closes all PSII reaction centres (q_p = 0) (van Kooten and Snel 1990). The variable fluorescence (F_v) is the difference between F_m and F_o, while the quantum efficiency of PSII photon capture may be approximated by dividing F_v by F_m (DeEll et al. 1999).

In a PFM fluorometer, the amplitude of the light probe is fixed, but the frequency of the light emission is altered. In each PFM sensor, four red 635 nm light emitting diodes (LEDs) surround a photodiode with a cut-off filter to reject the LED excitation. This arrangement can be used to scan a relatively large surface area capable of detecting small fluorescence changes in more than one leaf, fruit or vegetable simultaneously. During each PFM scan, the duty cycle (D) (i.e. $D = \tau T^{-1}$ where τ = pulse width and T = pulse period) of the four LEDs is gradually increased over a number of increments over a period of time by decreasing T

^{II} The lower oxygen limit (LOL), a more general term, may be more appropriate than ACP in this Chapter as the differences between parameters used to determine low-O₂ stress (ACP, respiratory quotient breakpoint (RQB), extinction point (EP), F_o breakpoint) have not been well studied across different species and circumstances.

while τ remains fixed. The increasingly shorter T translates into a range of pulse frequencies (f) over the course of a scan (Prange et al. 2007). The pulse width, duty cycle, frequency and scan time settings may be manipulated by the user (HarvestWatch™ operator manual). The increase in f results in an increase of the photosynthetic photon flux density (PPFD). Typical scans employ a 9 cm sensor-sample distance and induce a range of $\approx 0.01 - 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light at the sample surface (Prange et al. 2007); however, varying the sensor-sample distance will affect the sample surface PPFD. Contrary to the saturation-pulse PAM method, which uses two distinct PPFDs to measure the F_o and F_m , the PFM fluorometer measures the relative fluorescence (F) over a gradient of PPFDs. The measured F is a function of f . As the f and PPFD increase, the F reaches an “ F_m -like” parameter (it cannot be considered a true measurement of F_m since the conditions required for an F_m measurement (i.e. $q_p = 0$ and $q_N = 0$) are potentially violated; q_N is likely induced due to the length of the scan (58 seconds) and the PPFD achieved by the LEDs most probably falls short of the intensity required to close all the PSII reaction centres). A second-order polynomial ($F = a \cdot f^2 + b \cdot f + F_\alpha$) is fitted to the data, where the extrapolated y-intercept (F_α) value, which has been generated using thousands of readings over the course of a scan and which has been found to be extremely sensitive to stress-induced physiological changes, represents the theoretical value of the fluorescence intensity when the PPFD equals zero (Prange et al. 2007). In other words, F_α represents the amount of fluorescence present when all the PSII reaction centres are open and photochemical quenching is at a maximum; F_α is therefore thought to approximate F_o . However, the question of whether F_α is truly analogous to F_o has not been fully scrutinized and data directly comparing the two systems have never been published. For certain applications, the HarvestWatch™ PFM system has advantages over traditional PAM technology: the fluorometer units are less expensive to manufacture; the scan surface area is far greater than most available PAM fluorometers; several fluorescence sensors may be linked together; measurements may be automated and the PFM F_α parameter often demonstrates a greater sensitivity to physiological stresses than the PAM F_o parameter.

The objectives of this study were to: 1. compare the minimum fluorescence parameter (F_α) (generated by a PFM fluorometer) with the minimum fluorescence parameter (F_o) (generated using a PAM fluorometer) in response to the reversible damage induced by low- O_2 stress in apples, and to the irreversible damage induced by osmotic stress in grapes; and 2. demonstrate how F_α is derived.

2.3 Materials & Methods

2.3.1 F_a versus F_o during Low- O_2 Stress in Apples

Six, 4 L clear plastic respiration jars were each filled with approximately 1 kg (i.e. 5 – 6 apples) of ‘Honeycrisp’™ apples in a two-factor, randomized experiment. Three jars were placed inside each of four PAM fluorometer units (OS-900 prototype, Opti-Sciences Inc., Tyngsboro, Mass., USA) and the fluorescence was measured hourly. The OS-900 prototype was developed specifically to measure a large surface area using PAM technology; it measures F_o and F_m , but is not fitted with an actinic light needed for quenching analysis. The fluorescence in the remaining 3 jars was monitored hourly using 3 PFM sensors. Since both F_o and F_a are relative terms, the values were normalized: normalization = (datum point / dataset average) · 100. The air flow of all 8 jars was controlled; ambient air (20.9% O_2 and 0.03% CO_2) flowed through each jar at $\approx 35 \text{ ml min}^{-1}$. All work was performed in a dark room held at a constant temperature of 3.5 °C. The apples in each jar were dark-adapted for several hours before fluorescence measurements commenced. An exhaust line of equal length was run from each jar through a port leading outside the room, so the flow (digital flow meter J&W Scientific, Inc., Folsom, CA, USA) and gas levels (GCS150 Dual Analyser, Gas Control Systems Inc., Sparta, MI, USA) in each jar could be measured without influencing the fluorescence readings. The fluorescence was measured in all 8 jars for 14 hrs in order to establish a fluorescence baseline, after which the flow of ambient air was turned off and a nitrogen purge ($\approx 100 \text{ ml min}^{-1}$ of 100% N_2) was performed for 7.5 hrs (i.e. hours 14 – 21.5). The N_2 was then turned off and the flow of ambient air through the jars was restored. Fluorescence was monitored for an additional 13.5 hrs (i.e. hours 21.5 – 35) after the low- O_2 stress to establish a post-stress baseline. The average minimum fluorescence for both the PAM and PFM fluorometers were compared over four, key 2 hour periods: pre-low- O_2 baseline (hours 4 – 6), low- O_2 peak (hours 19 – 21), post-low- O_2 recovery (hours 23 – 25) and post-low- O_2 baseline (hours 32 – 34). A two-factor, balanced analysis of variance ANOVA (Minitab ® Release 15) and a least-squared means comparison (SAS Release 8.0) were used to compare normalized fluorescence averages between the fluorometers and the four key periods. Data were checked for constant variance and normality. The average standard deviation (expressed as a %) of the fluorescence baseline prior to the N_2 purge (hours 0 – 14) was compared between the two fluorometers using a t-test. Significance was defined as $p \leq 0.05$.

2.3.2 F_a versus F_o during Osmotic Stress in Grapes

Six clusters of ‘Thompson Seedless’ grapes (*Vitis vinifera*), obtained from the local supermarket and of South American origin, were divided into two treatments in a one-factor, randomized experiment. Each cluster of grapes started with a mass of approximately 240 ± 50 g and were individually suspended from a wire and placed on a digital scale (PK-4, Denver Instrument Company, Arvada, Co., USA). Three cluster/scale systems were placed inside three PAM fluorometer units (Figure 2.1B) (the grapes remained on the scales for the duration of the experiment); a typical point-and-click PAM unit was not used in this study since it had been determined that this design is sensitive to chlorophyll concentration in grapes (Wright et al. 2008b). The remaining 3 clusters were monitored with PFM fluorometer sensors positioned to the left and right of each cluster for a total of six sensors (Figure 2.1A). All 6 systems were dehydrated in a dark room for 32 days ($\approx 60\%$ mass loss) under ambient room conditions (temperature = $23 \pm 1^\circ\text{C}$; RH = $35 \pm 5\%$). The fluorescence on each cluster of grapes was measured hourly. For the PFM system, the left and right sensor measurements were staggered (i.e. left measurement at hour 1, right measurement at hour 2) to avoid light-induced artefacts. The PFM F_a values for each cluster were averaged to partially-imitate the OS-900 PAM fluorometer system, which internally averages 3 sensors to generate one F_o value for each measurement. The mass of each cluster was recorded daily. Both F_o and F_a values were normalized. Individual grape cluster mass loss (%) versus minimum fluorescence plots were created. Regression analyses in conjunction with basic calculus were used to compare the average location of the inflection points (in terms of mass loss) between the two fluorometer systems. A one-factor ANOVA (Minitab ® Release 15) was used to compare the average inflection point locations generated by the two different fluorometer systems. Significance was defined as $p \leq 0.05$.

A second and third repeat of the above experiment was performed with 8 clusters of ‘L’Acadie’ (hybrid) grapes (product of Nova Scotia) per repeat. Each cluster of grapes had an initial mass of approximately 95 ± 15 g. Due to the smaller relative size of the ‘L’Acadie’ compared with the ‘Thompson Seedless’ grapes (individual berries weighed approximately 1.2 g and 5.8 g respectively), the ‘L’Acadie’ fruit dehydrated more quickly (Dreier et al. 2000) and were held in a dark room under ambient conditions (see above) for 18 days ($\approx 70\%$ mass loss).

2.3.3 Dissection of the PFM F_{α} Derivation

To better understand how the F_{α} parameter is derived and influenced, the raw data generated during a PFM fluorometer scan were decoded in order to generate f versus F plots for key points of interest for the apples exposed to low- O_2 stress and the grapes subjected to osmotic stress. The PFM sensor settings were the same for all measurements and used a D range of 0.000366 – 0.05868 and a f range of ≈ 0.10 – 15 kHz with a 58 s scan time.

For the low- O_2 apple trials, three consecutive scans at each of the four points of interest (i.e. 1. pre-low- O_2 baseline, 2. low- O_2 peak, 3. post-low- O_2 recovery and 4. post-low- O_2 baseline) were generated. Regression analysis was performed on these plots (Minitab® Release 15) so that the nature of the second order polynomial response relative to the data, the goodness of fit, and the y-intercept (i.e. F_{α}), for each plot could be analysed.

For the grape dehydration trials, a PFM sensor was placed in a carrier ‘float’ that rested on top of a hamper filled with ‘Thompson Seedless’ grapes as they dehydrated (temperature = 23 ± 1 °C; RH = $35 \pm 5\%$) (Figure 2.1D) in order to generate a stronger, more coherent fluorescence signal; four experimental replications were used. This apparatus was deemed more applicable to a commercial setting; it also ensured that the sensor interacted with a solid surface of drying grapes and the distance between the grapes and the sensor remained constant. Scans were performed every two hours until the grapes achieved a weight loss of 80%. Over the course of dehydration, five key points of interest were identified on the resulting mass loss versus F_{α} plots (i.e. 1. the initial F_{α} value, measured under inadequate dark-adaptation (approximately 10 minutes of dark-adaptation was allowed prior to the first scan), 2. first measurement following adequate dark-adaptation time, 3. first primary inflection point, 4. second primary inflection point and 5. a point after the second primary inflection point). Three consecutive f versus F plots for each of the five points of interest were analyzed as described above for the apple trial.

The f versus F plots for the low- O_2 apple work and the grapes undergoing osmotic stress were used to demonstrate the potential of deriving other new PFM-based fluorescence parameters besides F_{α} . The 2nd derivative ($F''(f)$) (analogous to line curvilinearity), the vertex location ($V[f]$) and the height of the fitted quadratic in the f vs F plots, as well as an “ F_v -like” parameter (vertex height - F_{α}) were discussed as examples of potential physiologically-rich PFM-based parameters that may aid in quantifying changing f versus F curve dynamics.

2.4 Results

A side-scan approach was employed by both the PAM and PFM fluorometers when measuring low-O₂ stress in apples (PAM apple setup not shown) and osmotic stress in grapes (Figure 2.1A – 2.1C). A fluorometer ‘float’ (Figure 2.1D) was employed to collect data used to examine the grape dehydration PFM f versus F plots.

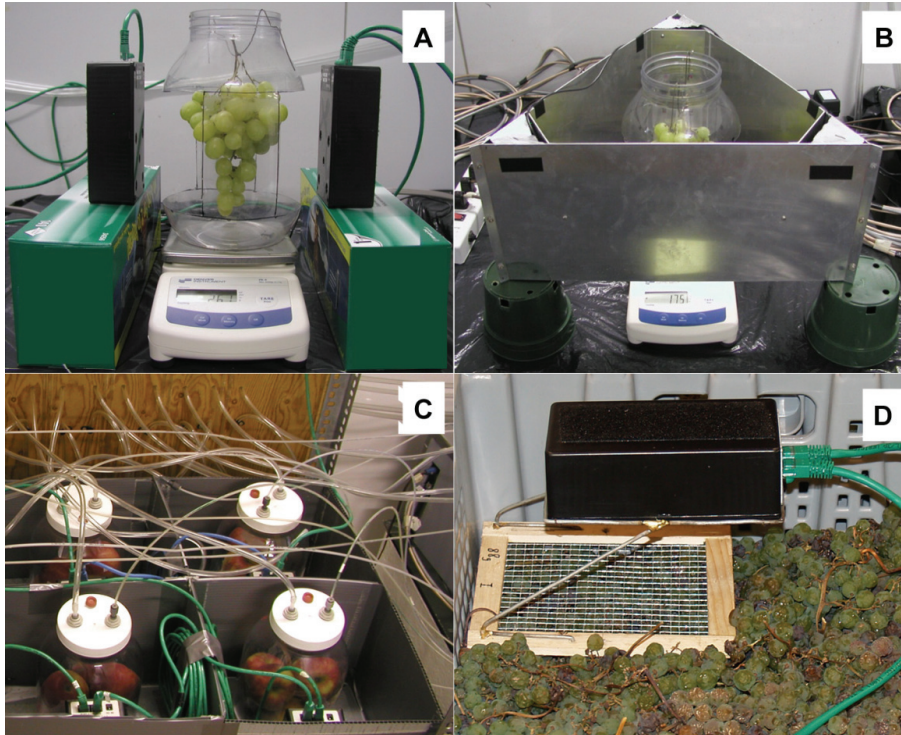


Figure 2.1 (A) dehydrating grapes: PAM side-scan (each unit was an equilateral triangle: length = 44 cm, height = 20 cm; sensor-sample distance was \approx 10 cm), (B) dehydrating grapes: PFM side-scan (sensors were placed \approx 10 cm from sample), (C) low-O₂ apples: PFM side-scan and (D) dehydrating grapes: PFM sensor ‘float’ (‘float’ base = 160 cm²).

2.4.1 F_a versus F_o during Low-O₂ Stress in Apples

There did not appear to be any difference between F_a and F_o in response to low-O₂ stress in apples (Table 2.1): each fluorescence parameter sustained a steady baseline during initial normoxia, showed a slight increase immediately after the N₂ purge began, rose sharply after O₂ levels were lowered below the ACP and then fell below the original fluorescence baseline once normoxia was reestablished before returning to pre-stress levels (Figure 2.2).

Table 2.1 PAM (F_o) vs PFM (F_a). The average normalized minimum fluorescence values over the 4 stages of the low- O_2 spike in ‘Honeycrisp’ apples using both PAM and PFM fluorometers.

stage	PAM (F_o)	PFM (F_a)
1 – pre-low- O_2 baseline	98.9 ^{bA}	99.1 ^{bA}
2 – low- O_2 peak	111.2 ^{aA}	111.3 ^{aA}
3 – post-low- O_2 recovery	96.5 ^{cA}	96.9 ^{cA}
4 – post-low- O_2 baseline	98.8 ^{bA}	99.2 ^{bA}

*Means with different lowercase letters within a column are significantly different ($p \leq 0.05$). Means with different uppercase letters within a row are significantly different ($p \leq 0.05$).

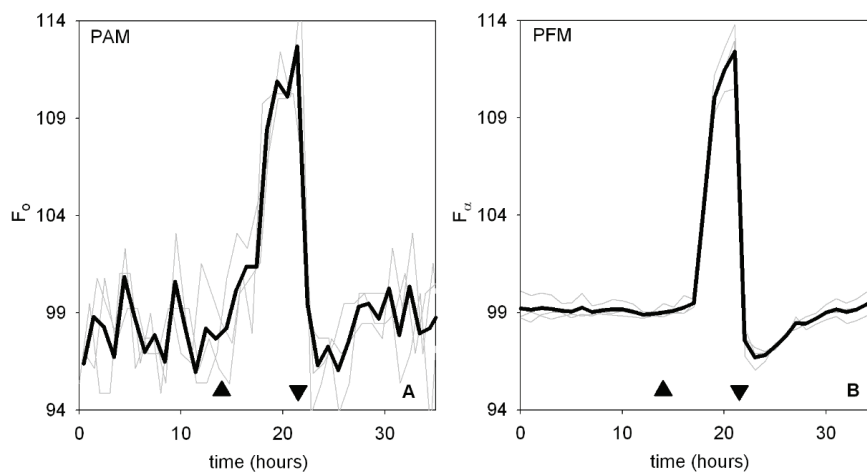


Figure 2.2 The fluorescence spike induced by low- O_2 as by a (A) PAM and (B) PFM fluorometer. Raw data are shown in grey; black lines indicate averaged values. The symbol (\blacktriangle) indicates when the atmospheric air was turned off and the N_2 purge began and the symbol (\blacktriangledown) indicates when the N_2 flow was turned off and the flow of atmospheric air was returned.

The standard deviation of the fluorescence baseline was significantly higher when using the PAM system ($\pm 2.20\%$) compared with the PFM system ($\pm 0.19\%$) ($p \leq 0.001$). The PAM F_m parameter was little-affected by sub-ACP O_2 , but declined slightly before quickly recovering when conditions were returned to normoxia (data not shown).^{III}

2.4.2 F_a versus F_o during Osmotic Stress in Grapes

The minimum fluorescence parameters of both the PAM (Figure 2.3A) and PFM (Figure 2.3B) fluorometers generated similar sigmoidal curves in response to water loss in the ‘Thompson Seedless’ grapes; however, the PFM fluorescence signal appeared to decrease more sharply after the second inflection point compared with the PAM signal. The initial

^{III} More recent work suggests this decline in F_m results from increased zeaxanthin and q_N (Chapters 4 & 5).

increase at the onset of the F_{α} data (Figure 2.3B) was not included in the regression analysis, as it was believed to be an artefact of inadequate dark-adaptation.

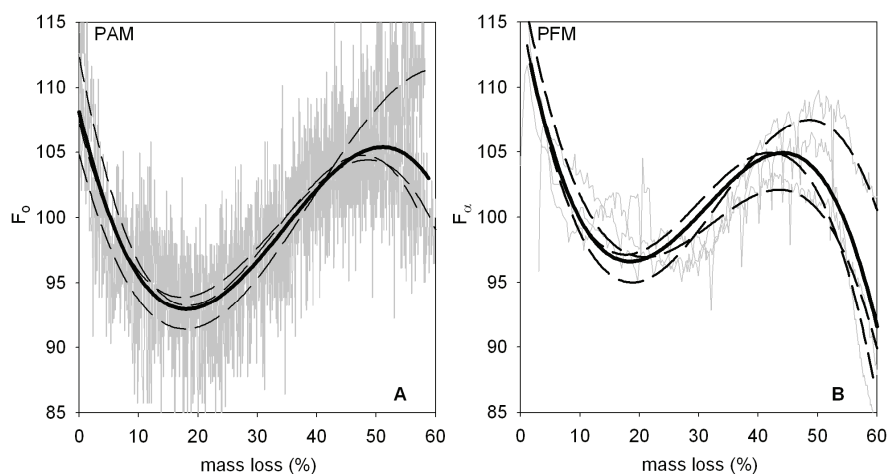


Figure 2.3 The averaged relationship between mass loss ('Thompson Seedless') and the normalized minimum fluorescence values **(A)** F_o ($y = -6.8E-4x^3 + 0.070x^2 - 1.86x + 107$), $R^2 = 0.83$ and **(B)** F_{α} ($y = -1.0E-3x^3 + 0.093x^2 - 2.36x + 115$), $R^2 = 0.89$). Raw data are shown in grey; fitted response curves for averaged data appears as solid line; fitted response curves for individual clusters appear as dashed lines.^{IV}

There was no difference between the two fluorometers in the average location (% mass loss) of both the first and second major inflection points found on the individual cluster response curves ($p = 0.651$ and $p = 0.193$, respectively) (Table 2.2). However, in both cases the statistical assumption of constant variance (general guideline: $0.5 < \text{s.d.} < 2$) was compromised and the small sample size meant conversion factors would be ineffective.

^{IV} The response variables in the regression analysis shown in Figs. 2.3 and 2.4 are repeated measures of the same experimental units (i.e. grapes) over time. Autocorrelation among the measurements is assumed but was not assessed. As 'time' and not 'mass loss' was the actual dependent variable in this experiment, a correlation analysis between F_o (y_1), F_{α} (y_1) and mass loss (%) (y_2) may have been more appropriate. Nonetheless, the data demonstrate a distinct, repeatable and physiologically meaningful relationship between grape 'mass loss (%)' and the minimum fluorescence parameters F_o (Figs. 2.3A, 2.4A) and F_{α} (Fig. 2.3B, 2.4B).

Table 2.2 The average location of the 1st and 2nd major inflection point in the mass loss vs. fluorescence for both PAM and PFM fluorometer systems using ‘Thompson Seedless’ grapes.

	n		1 st inflection (mass loss (%))	2 nd inflection (mass loss (%))
PAM (F_o)	3	avg.	17.6 ^a	54.4 ^a
		s.d.	0.5	10.8
PFM (F_α)	3	avg.	16.8 ^a	44.4 ^a
		s.d.	3.0	2.5

*Means with different letters within a column are significantly different ($p \leq 0.05$).

The minimum fluorescence parameters of the two different fluorometers generated dissimilar curvilinear fitted responses to water loss in ‘L’Acadie’ berries (Figure 2.4). The PAM fluorometer generated a quadratic response curve that appeared to approach, but did not reach, a second major inflection point, within the dehydration range measured (Figure 2.4A). The PFM signal was sigmoidal and reached a well-defined second inflection point at approximately 65% mass loss (Figure 2.4B). There was a greater propensity for the PFM minimum fluorescence signal to decrease compared with the PAM, exhibited by the steeper decline towards the initial inflection point and the occurrence of a second inflection point in the PFM data (Figure 2.4). The two datasets generated using the ‘L’Acadie’ grapes were not combined so the repeatability of the relationships could be observed (Figure 2.4; Table 2.3).

Table 2.3 The average location of the 1st major inflection point in the mass loss vs. fluorescence for both PAM and PFM fluorometer systems using ‘L’Acadie’ grapes.

	n		1 st inflection (mass loss (%)) (rep. 1)	1 st inflection (mass loss (%)) (rep. 2)
PAM (F_o)	4	avg.	22.4 ^a	26.5 ^a
		s.d.	6.0	5.1
PFM (F_α)	4	avg.	24.7 ^a	26.5 ^a
		s.d.	7.7	5.1

*Means with different letters are significantly different ($p \leq 0.05$).

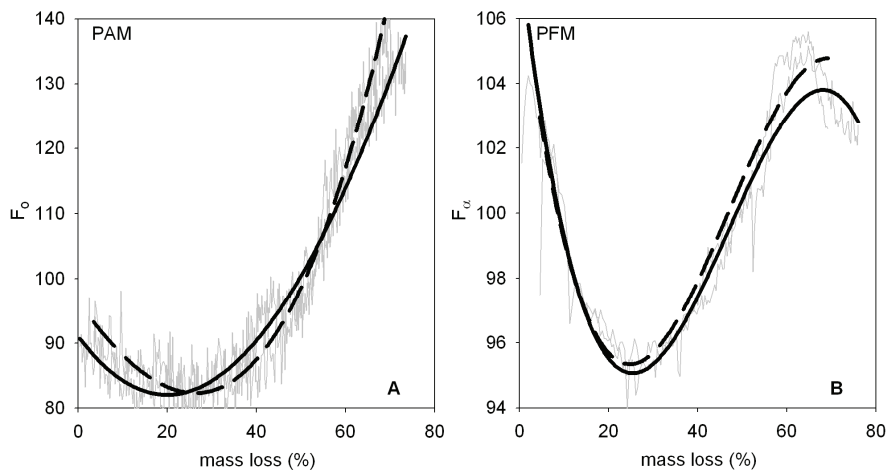


Figure 2.4 The averaged relationship between mass loss ('L'Acadie') and the normalized minimum fluorescence values **(A)** F_0 : rep. 1 (solid) ($y = -4.8E-5x^3 + 0.025x^2 - 0.931x + 91.2$, $R^2 = 0.97$), rep. 2 (dashed) ($y = -1.6E-4x^3 + 0.013x^2 - 1.00x + 96.9$, $R^2 = 0.96$) and **(B)** F_α : rep. 1 (solid) ($y = -2.2E-4x^3 + 0.031x^2 - 1.16x + 108$, $R^2 =$

0.96), rep. 2 (dashed) ($y = -2.6E-4x^3 + 0.036x^2 - 1.33x + 110$, $R^2 = 0.95$). Raw averaged data are shown in faded colour; each represents averaged data from four clusters.

Although the coefficients of determination for the fitted response curves were relatively high for both fluorometer types and cultivars, the minimum fluorescence signal appeared to be more erratic when using the PAM compared with the PFM fluorometer (Figures 2.2 and 2.3). In the PFM data, the few significant deviations of the F_α signal from the fitted response curve can largely be attributed to low-intensity light exposure when the door to the dark-room was momentarily opened slightly (Figures 2.3B and 2.4B). This occurred even though door-openings were consistently timed to occur directly after a scan. Opening the door did not appear to similarly affect the PAM F_0 signal.

The average location of the first primary inflection point in the mass loss versus minimum fluorescence relationship was similar between PAM and PFM systems and between replications when using the 'L'Acadie' grapes (Table 2.3). Although there was no occurrence of a second major inflection point in the PAM data, the location of this point was comparable ($p = 0.701$) between replications for the PFM data: rep. 1 = 67.9%; rep. 2 = 71.5%.

2.4.3 Dissection of the PFM F_α Derivation

For both the low- O_2 apple and grape dehydration trials, F_α values generated using the raw data and regression analysis matched those generated by the HarvestWatch™ software (data not shown). In both the apple and grape work (with the exception of point 1 for the latter), the three fitted response curves at each point of interest were highly similar (Figures 2.5 and 2.6). The mass loss versus F_α plot generated with the sensor 'float' was similar to that of the

side-scan approach; the dynamics of each possessed the same 5 key points of interest (Figures 2.3B and 2.6A).

For the low-O₂-stressed apples (Figure 2.5A), the f versus F plots from the pre and post-low-O₂ baselines (points 1 and 4) showed similar dynamics (Figure 2.5B). F first increased sharply and then continued to increase at a more moderate rate relative to increased f (where f is proportional to PPFD) to form a concave curve (i.e. a negative $F''(f)$). Interestingly, under low-O₂ conditions, F was relatively high under low PPFD and then was severely quenched before increasing moderately under increasing PPFD (Figure 2.5C) to form a convex curve (i.e. a positive $F''(f)$ value). During the post-low-O₂ recovery period (point 3) (Figure 2.5D), the f versus F plots appeared similar to the pre and post-low-O₂ baseline scans, but F was more highly quenched at the higher PPFD values (i.e. a decreased “ F_v -like” parameter).

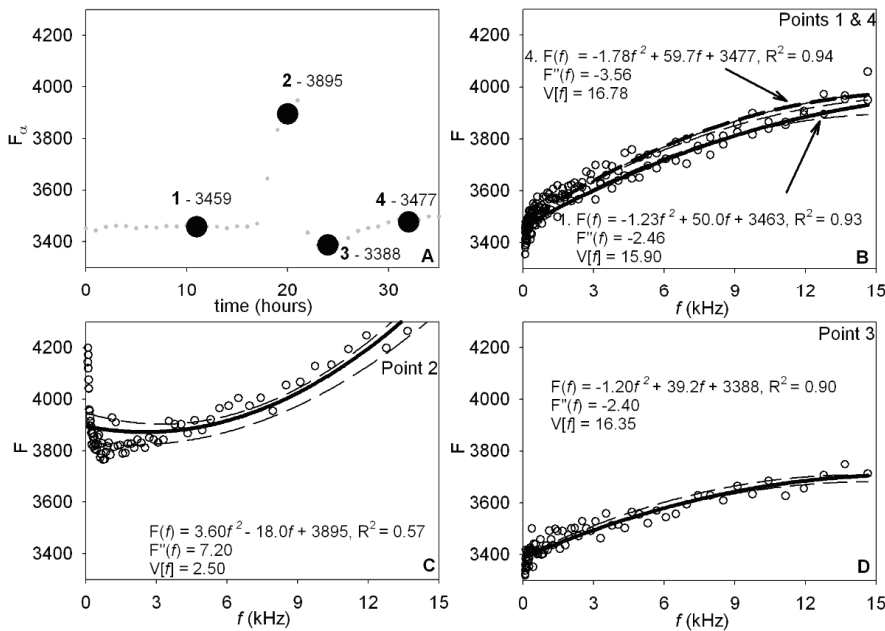


Figure 2.5 (A) a representative ‘Honeycrisp’ PFM time (hours) vs. F_α plot showing 4 points of interest, **(B – D)** corresponding f vs. F plots for points 1 – 4. The individual data points, response curve and equation, $F''(f)$ and $V[f]$ of the first scan (\circ —) at each point of interest is shown, while only the response curve (—) is shown for the second and third scans at each point of interest.

For the grape dehydration work, the f versus F relationships, especially those occurring at points 2 and 3, displayed the dynamics of a typical Kautsky curve (Figure 2.6) (where f is proportional to time). The grape scans revealed increased quenching at the higher f (i.e. increased PPFD) levels, most notable in scans performed under non-stressed conditions, compared with the apple scans (Figures 2.5B and 2.6C). At point 1 (Figure 2.6B), the magnitude of F was much lower in the first grape PFM measurement (time = 0 hrs) compared with the subsequent two at 2 and 4 hrs, respectively, and appeared to be less

curvilinear. After reaching a maximum at point 2 (based on $F''(f)$ values), the data became less curvilinear over the course of dehydration, with the three replications at point 5 showing the lowest degree of curvilinearity (Figure 2.6B – 2.6F).

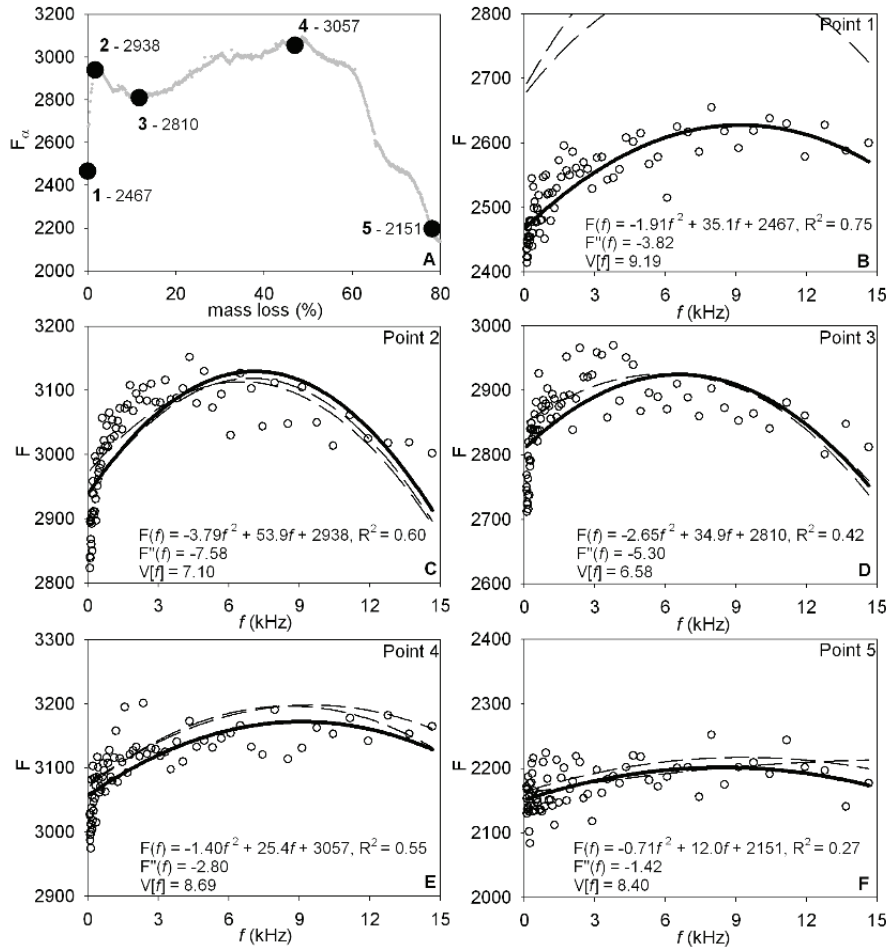


Figure 2.6 (A) a representative ‘Thompson Seedless’ PFM mass loss vs. F_α plot showing 5 points of interest, **(B – F)** corresponding 5 f vs. F plots. Each plot shows the data and response curve for three consecutive scans. The individual data points, response curve and equation, $F''(f)$ and $V[f]$ of the first scan ($\circ \text{---}$) at each point of interest is shown, while only the response curve (---) is shown for the second and third scans at each point of interest.

The y-intercept (i.e. F_α) of the fitted response curve appeared to overestimate the true minimum fluorescence values as observed in datasets exhibiting a high degree of curvilinearity (i.e. grapes measured under minimal water loss) (e.g. Figure 2.6C, 2.6D). The f versus F plots created for the side-scan grapes gave very similar results to those generated using the sensor ‘float’, but showed weaker F values (data not shown).

2.5 Discussion

Although sub-ACP O_2 levels have been shown to increase both F_0 (Prange et al. 2002) and F_α (Prange et al. 2003; DeLong et al. 2004b) in fruits and vegetables, these are the first data showing a direct comparison. For the reversible low- O_2 stress on apples, the PAM and PFM minimum fluorescence parameters were similar (Table 2.1). A slight increase was

observed at the very beginning of the N₂ purge, before the O₂ levels reach sub-ACP levels^V; a post-spike dip was also observed (Figure 2.2). The cause of this was unknown, but may have been induced as a result of the metabolic rate of the fruit adjusting to the O₂ levels.

In the grape dehydration trials, the differences between the two fluorometers did not affect the location of the first inflection point in the mass loss versus minimum fluorescence relationship for either cultivar (Tables 2.2 and 2.3), but did affect the occurrence of a second inflection point when using ‘L’Acadie’ (Figure 2.4). The greater propensity for the PFM minimum fluorescence value to decline relative to grape mass loss compared with the PAM value likely occurred due to: 1) sensitivity to a decreasing F_m factor, and 2) an overestimation of F_α during periods of high curvilinearity in the *f* versus F plots.

Both F_m and F_v have been shown to significantly decrease in both grape cultivars over the course of dehydration (Wright et al. 2008b). If the F_α values were influenced by this decreasing F_m parameter [there is evidence for this based on the similarity of the mass loss versus F_α and mass loss versus vertex height (i.e. “F_m-like” plots) (data not shown)], it could be responsible for lower relative F_α compared with F_o values with respect to mass loss. An ancillary experiment with the PAM fluorometer showed that the F_m parameter decreased sharply under inadequate dark-adaptation conditions, while F_o remained relatively unchanged (data not shown). The F_m value is lower in the presence of light than in a dark-adapted sample as a result of non-photochemical quenching (q_N) (van Kooten and Snel 1990). Under conditions of inadequate dark-adaptation, the grape PFM F_α value was relatively low, resulting in a spike in fluorescence as the grapes became dark-adapted (Figures 2.3B, 2.4B and 2.6B). This sudden increase at the onset of each trial was not apparent when using the PAM fluorometer (Figures 2.3A and 2.4A). The apparent increased sensitivity of the PFM F_α value to inadequate dark-adaptation, compared with the PAM F_o value, may be due, at least in part, to the influence of a decreasing F_m parameter.

In the grape dehydration trials, an overestimation of F_α during periods of high curvilinearity (in the *f* versus F plots) may also influence F_α downwards over the course of dehydration. When grapes were turgid, healthy and measured under adequate dark-adaptation (as shown in Figure 2.6C), the fluorescence rose steeply to a maximum and then quenched slowly, dynamics typical of a classic Kautsky curve. When applying a second-

^V Later Chapters (4 & 5) also speak of a pre-spike dip; the nature of the pre-spike increase or dip is likely influenced by the species, rate of flushing and the activity of the xanthophyll cycle.

order polynomial to these data, the sharp incline in fluorescence observed at the lower f values causes the y-intercept to be overestimated (Figure 2.6C). However, as the grapes dehydrate, the efficiency of their photosystems declines (Wright et al. 2008b), the sharpness of the incline in fluorescence at the low f values is decreased, and the overestimation of the y-intercept also decreases, which may be interpreted (in terms as F_a values) as a factor influencing the minimum fluorescence parameter downwards relative to mass loss (Figure 2.6C – 2.6F). For example, at point 5 (Figure 2.6F) the y-intercept for each of the three respective replications shown is in the middle of the data point clusters observed around the low f values.

The difference in the minimum fluorescence parameter generated by the two different types of fluorometers was more apparent in the ‘L’Acadie’ grapes compared with the ‘Thompson Seedless’. A third factor, chlorophyll degradation, will also affect fluorescence levels. ‘Thompson Seedless’ grapes undergo significant chlorophyll degradation during dehydration, while ‘L’Acadie’ grapes do not (Wright et al. 2008b). Chlorophyll content is positively correlated with minimum fluorescence (Smillie et al. 1987a; Toivonen and DeEll 1998). Therefore, when using the ‘Thompson Seedless’ grapes, the relationship between grape mass loss and the PAM and PFM minimum fluorescence parameters appeared more similar because of the commonality of chlorophyll degradation, a factor that likely influenced both F_0 and F_a downwards. The difference between the two fluorometer types was more apparent when the ‘L’Acadie’ cultivar, which was less affected by chlorophyll degradation, was tested. However, chlorophyll loss would have been a relative non-factor in the low- O_2 apple experiments in which the two relative minimum fluorescence parameters were indistinguishable (Figure 2.2; Table 2.1).

The f versus F plots (Figures 2.5 and 2.6) indicate the potential for the derivation of new PFM-based fluorescence parameters. The difference in these plots for apples and grapes under relatively non-stressed conditions (Figures 2.5B and 2.6C) showed that F was quenched at the higher frequencies in grapes to a greater degree than it was in apples. The location of the curve vertex location ($V[f]$) could be used to quantify this difference (i.e. $V[f]$ occurs at a significantly lower f value in the grapes (≈ 7 kHz) compared with the apples under non-stressed conditions (≈ 16 kHz)). The curvilinearity of the response (approximated by $F''(f)$) may also hold potential as a PFM-based parameter that may show a high correlation with quenching levels or photosystem integrity. A relatively large, negative $F''(f)$

value (as found in both non-stressed grapes and apples (Figures 2.5B and 2.6C)) indicates a strong, concave, relative fluorescence response to increased f (and PPFD). A relatively small, negative $F''(f)$ value may indicate a photosystem with compromised integrity (as observed in dehydrated grapes (Figure 2.6F)). A positive $F''(f)$ value [as found in apple under low- O_2 conditions (Figure 2.5C)] indicates a convex response to increased f and may indicate there is a change in quenching.^{VI} If a third-order polynomial was used on the f versus F plots, the goodness of fit of the fitted curve may be improved and it could increase the potential of developing new PFM-based parameters. Manipulating the PPFD, via the f range and pulse width, and the scan time could be used for finding specific parameters or to calibrate the fluorometer for specific plant species. Future studies should investigate correlations between PAM q_P and q_N analysis (as well as other established PAM-based information) and new PFM-based parameters. The search for new PFM-based parameters may identify fluorescence characteristics analogous to those found using PAM technology or it may yield completely new physiologically-rich information.

Although the PFM F_α does not appear to be completely analogous to the PAM F_o , this study demonstrated that the two parameters shared common characteristics, such as a tight correlation with grape water loss with similar inflection points around the 20% mass loss region and an indistinguishable response to short term, low- O_2 stress in apples. Already used for determining ACP in fruits and vegetables destined for CA storage, the PFM sensor may also find a commercial application in the appassimento-style dessert wine industry. The relative low-cost, multiple sensor, large scan surface area approach of the HarvestWatch™ PFM system makes it an attractive tool for many commercial applications. The data produced by the unique HarvestWatch™ PFM fluorometer probe also hold the potential for the development of new PFM-derived fluorescence parameters capable of monitoring photochemical changes in the photosystem.

^{VI} Later work, discussed in Chapters 4 & 5, suggest this initial decrease in fluorescence in the f versus F plots may result from photochemical quenching due to PSI oxidation of the PQ pool; non-photochemical quenching as a result of zeaxanthin accumulation may also be a factor.

Chapter 3: The Effect of Temperature and Other Factors on Chlorophyll *a* Fluorescence and the Lower Oxygen Limit in Apples (*Malus domestica*)

Published as:

Wright H, DeLong J, Harrison P, Gunawardena A, Prange R. 2010. The effect of temperature and other factors on chlorophyll *a* fluorescence and the lower oxygen limit in apples (*Malus domestica*). *Postharvest Biology & Technology* **55**: 21 – 28

3.1 Abstract

The effects of temperature, scan interval and rate of oxygen (O₂) decline on pulse frequency modulation (PFM) – based minimum fluorescence (F_o) and the F_o-based lower oxygen limit (LOL) were investigated using ‘Honeycrisp’ apples (*Malus domestica*). The effects of temperature and hypoxic stress on pulse amplitude modulation (PAM) fluorescence parameters were also investigated. The PFM scan interval had no effect on the F_o baseline or the measured LOL, but increases in the scan interval decreased the low-O₂-induced fluorescence spike intensity (ΔF_o). Temperature negatively correlated with the F_o baseline while ΔF_o °C⁻¹ was greater at lower than at higher temperatures. When using a PAM fluorometer, the minimum fluorescence (F_o), and to a lesser extent the maximum fluorescence (F_m), were similarly affected by temperature as was F_o. Temperature altered the LOL in fruit. Apples stored at 20, 10, 3.5 and 0 °C spiked at 0.72, 0.33, 0.22 and 0.08 kPa O₂, respectively, under a rapid O₂ decline (i.e. 20.9 to < 1 kPa O₂ in \approx 5 to 6 h). Although the low-O₂ F_o spike apex values did not change with temperature, the spike intensity increased with temperature due to a decrease in fluorescence baseline. A slower O₂ decline rate produced slightly higher LOL and lower spike intensity values. In conclusion, temperature and rate of O₂ decline affected the low-O₂-induced PFM fluorescence spike intensity as well as the LOL, while the PFM scan interval affected the spike intensity.

3.2 Introduction

Decreasing the metabolic rate via controlled atmosphere (CA) can significantly extend the storage life of fruits and vegetables. The efficiency of a CA system may be maximized by lowering the oxygen (O₂) level to near, but not below, the lower oxygen limit (LOL) (i.e. the O₂ level where the fruit or vegetable’s metabolism changes from predominantly aerobic to predominantly anaerobic). The LOL may be differently defined (for a review see Yearsley et al. 1996); dropping O₂ levels below the LOL generally triggers a sudden increase in the respiratory quotient (RQ) and fermentative products. The build-up of the fermentative products (i.e. ethanol, acetaldehyde and ethyl acetate) is to be avoided during storage as they may produce off-flavours and decrease fruit quality.

Chlorophyll *a* fluorescence signals are sensitive to gas levels in fruits and vegetables and may be used to facilitate a form of dynamic controlled atmosphere (DCA) (for a review see

Prange et al. 2005a). The pulse frequency modulation (PFM) and pulse amplitude modulation (PAM)-derived minimum fluorescence parameters (F_a and F_o respectively) are both similarly responsive to low- O_2 stress (Wright et al. 2008a). Work by Gasser et al. (2008; 20010) showed fluorescence and RQ values are highly correlated in response to low- O_2 stress and so yield identical LOL O_2 values in apples (*Malus domestica*).

The LOL of fruit and vegetables may be determined by gradually lowering the O_2 levels while simultaneously monitoring the minimum fluorescence. As the O_2 levels decline, the observed fluorescence baseline remains relatively steady until the LOL is crossed, at which point the fluorescence value increases greatly or ‘spikes’. Once O_2 levels are brought just above the LOL, fluorescence levels return to pre-stress levels (Prange et al. 2003; DeLong et al. 2004b; Wright et al. 2008a). Because the LOL may vary widely amongst different fruits and vegetables (Prange et al. 2003), within a species (DeLong et al. 2004b) or as a result of the growing region or year (DeLong et al. 2004b; Zanella et al. 2005), the product-specific LOL must be determined for each. As a result of this variability, general CA guidelines must be set well above the average LOL for a specific fruit in order to account for such variation. The utility of fluorometry for better maintenance of fruit and vegetable quality via DCA has been widely reported in recent years (Prange et al. 2003; DeLong et al. 2004b; Zanella et al. 2005; Prange et al. 2007; Lallu and Burdon 2007; Burdon et al. 2008).

Other forms of dynamic control monitor CO_2 production (Gasser et al. 2003) or fermentative products such as ethanol (Veltman et al. 2003). Determining proper CO_2 thresholds on a commercial scale is difficult as it is contingent on the room size, product amount, respiration rate and air flow. Basing stress levels on the production of fermentative metabolites is also troublesome as these may be present above the LOL (Boersig et al. 1988; Valle-Guadarrama et al. 2004); it has been shown that the measurement of ethanol is not always a reliable indicator of low- O_2 stress (Prange et al. 2005b).

Although fluorescence-based DCA is primarily used to detect low- O_2 stress, it is also sensitive to other environmental factors. Commercial users of fluorescence-based DCA have occasionally noticed unexplained variability in the strength of the LOL fluorescence spike and shifts in the fluorescence baseline seemingly unrelated to O_2 levels (personal communication from a commercial storage company, 2009). To address this issue, this study was designed to demonstrate the effect of temperature, scan interval and rate of O_2 decline on chlorophyll fluorescence and the LOL fluorescence spike in apples.

3.3 Materials & Methods

All of the experiments in this study were performed using ‘Honeycrisp’ apples (Cornell starch index = 6.6, SSC = 13.9%, firmness = 68 N at harvest (n = 10)) harvested from a 2000 planting of trees (MM111) grown in Queensland, NB, Canada. The apples were stored in CA ($O_2 = 2.0$ kPa, $CO_2 = 2.5$ kPa, relative humidity = 90%, temperature = 3.5 °C) for 3 months before the experiments commenced and were thus acclimated to low- O_2 . As recommended for ‘Honeycrisp’ fruit, they were delay-cooled (6 d at 20 °C) prior to CA storage (DeLong et al. 2004a). The O_2 level at which F_a values begin to spike is regarded as the LOL in this paper. All fluorescence values shown, except for F_v/F_m , were normalized (i.e. = (datum point)·(dataset average)⁻¹·(100)).

3.3.1 Effect of Scan Interval on sub-LOL Fluorescence Spike

Four, 4-L clear plastic jars were each filled with five to six ‘Honeycrisp’ apples (or ≈ 1 kg of fruit); the mass of the contents of each jar was measured using a digital scale (Sartorius, CP4202S, Gottingen, Germany). The exhaust hoses from each jar, which were all of equal length, were vented outside of the room. Flow rates were measured using a digital flow meter (J&W Scientific, Inc., Folsom, CA, USA) with a flow of air (20.9 kPa O_2 and 0.03 kPa CO_2) through each jar set at ≈ 0.53 mL s⁻¹. Oxygen and CO_2 levels were monitored using a gas analyzer (GCS150, Gas Control Systems Inc., MI, USA). The fluorescence of each jar of apples was measured using a PFM fluorometer (HarvestWatch™, Satlantic Inc., Halifax, NS, Canada). Each jar had its own sensor. At 3.5 °C, scan intervals (i.e. the period between subsequent fluorescence measurements) of 0.25, 0.5, 1, 2 and 4 h were compared; scan intervals of 0.25 and 4 h were tested at 20 °C. A sub-LOL event was induced in the apples via a nitrogen flush at a fixed rate [≈ 0.75 mL s⁻¹] with the air supply turned off at each scan interval / temperature combination. Oxygen levels less than 1.0 and 0.2 kPa were achieved within ≈ 5 to 6 h and ≈ 8 h, respectively, for each scan interval / temperature combination. After F_a spiked, sub-LOL levels were held for an additional 1.5 h before they were quickly returned to ambient atmospheric conditions. The experiment was replicated multiple times using both ascending and descending scan intervals; representative data are shown.

3.3.2 Effect of Temperature on Fluorescence Baseline

Five, 4-L jars were filled with approximately 1 kg of ‘Honeycrisp’ apples as described above. The fluorescence was monitored using a PFM fluorometer every 0.5 h; each jar had

its own sensor. The room temperature was set at 20 °C and was lowered by 4 °C steps at 24 h intervals until a temperature of 0 °C was reached. The temperature was then increased on the same fruit to 20 °C using 4 °C increments and 24 h intervals in a similar fashion. The temperature of the room and the respiration jar headspace of all 5 jars were monitored using thermocouples (CR7 Measurement and Control System, Campbell Scientific Inc., Logan, UT, USA). The flesh temperature of a single apple inside each jar was also measured using a thermocouple placed 2 cm below the epidermis. The absolute ΔF_a for every 4 °C increment was calculated by comparing the fluorescence values just prior to the room temperature change to that 15 h later [i.e. after the fruit had become acclimatized to the new temperature (based on apple core temperatures)]. A regression analysis of the temperature changes (20 to 16, 16 to 12, 12 to 8, 8 to 4 and 4 to 0 °C) (x) and the subsequent $\Delta F_a \text{ } ^\circ\text{C}^{-1}$ (y) (i.e. the ΔF_a per °C for every 4 °C step) for the descending, ascending and average [i.e. (descending + ascending)/2] fluorescence changes was performed (Minitab ® Release 15 2007).

In a separate experiment using the same method, the room temperature was set at 3.5 °C and then decreased to -2 °C for 4, 8 and 12 h intervals and to -5 °C for a 12 h interval. A paired t-test was used to compare the $\Delta F_a \text{ } ^\circ\text{C}^{-1}$ between the 3.5 and -2 and between the 3.5 and -5 °C 12 h intervals.

3.3.3 Effect of Temperature on Fluorescence Baseline and LOL

Twelve, 4-L jars were each filled with \approx 1 kg of ‘Honeycrisp’ apples. The flow rates and setup were as described in section 2.2. The fluorescence in jars 1 to 8 was individually measured every 0.5 h using a PFM fluorometer, while the fluorescence in jars 9 to 12 was individually measured every hour using the PAM OS-900 prototype (Opti-Sciences Inc., Tyngsboro, Mass., USA). Each jar had its own fluorometer sensor. All jar/fluorescence monitoring units were shielded to ensure that the measurements would not interfere with each other. Before the experiments, the room temperature was held at 5 °C for 2 d. During the experiment, the room temperature was adjusted to 20, 10, 3.5 and 0 °C and each temperature was held for 3 d. On the second and third day of each temperature regime, the respiration rates were measured under ambient atmosphere on all 12 respiration jars. Respiration rates [CO_2 consumed and O_2 uptake (g (kg s)^{-1})] were calculated from the constituent gas levels measured in the supply air compared with the exhaust air, the flow rate, temperature and the apple mass. Oxygen and CO_2 measurements were obtained using gas chromatography (Gow-Mac Instrument Co., Series 580, Bridgewater, NJ, USA). Oxygen

calibration was performed using 0, 2.98, 5.05, 18.9 and 20.9 kPa O₂ standards and multi-point linear regression; CO₂ calibration was performed using 0.03 and 2.08 kPa CO₂ standards and linear regression. On the third day at each temperature, the LOL was measured in jars 1 to 4 (monitored using PFM). A nitrogen flush at $\approx 0.75 \text{ mL s}^{-1}$ was used to lower O₂ levels. Gas levels were measured in each jar every 0.5 h. After the PFM fluorescence spike, sub-threshold levels were held for at least 1.5 h. The LOL values were obtained by taking the median time between the first PFM F _{α} spike value and the previous baseline value for each jar. The O₂ level at this time was calculated via interpolation. The measurements were duplicated using the same fruit in the same jars

In a separate experiment, the effects of low O₂ on F_o, F_m, F_v and F_v/F_m were tested at one temperature only (3.5 °C); four, 4-L jars filled with apples were subjected to a nitrogen flush while being monitored with the PAM fluorometer as described above.

3.3.4 Temperature-Dependent LOL as Determined Using Gradual O₂ Decline

Five, 4-L jars were filled with $\approx 1 \text{ kg}$ of ‘Honeycrisp’ apples and monitored with the PFM fluorometer using a 0.5 h scan interval as described above. A flow of air blended with nitrogen gas was used to adjust O₂ levels which in jars 1 to 4 were lowered to $\approx 2 \text{ kPa}$ over 24 h; jar 5 was used as a control. Over a period of days, O₂ levels were lowered further in 0.1 kPa increments. Oxygen levels were measured using a paramagnetic O₂ transducer (International Controlled Atmosphere Ltd., ICA40 system, Tonbridge, UK). Calibration and LOL calculations were as described above. The measurements were duplicated for each of two temperature conditions, 3.5 and 20 °C. The LOL and spike intensities found using the gradual O₂ decline described above were compared with those using the more rapid O₂ decline described in section 2.3.

3.3.5 Statistical Analysis

An analysis of variance (ANOVA) was used to determine statistical significance for single-factor models in each experiment performed (Minitab ® Release 15 2007). Individual treatment averages were compared using a least-squares means (LSmeans) mean comparison (SAS Release 8.0 1999). For both the ANOVA and regression analysis, a normal probability plot (NPP) of the residuals tested normality while a fitted values versus residuals plot examined constant variance. When the assumptions of the error term (ϵ_{ij}) were violated, transformations were used; in those instances, averages shown in this paper have been back-

transformed to the original data. Only results significant at $p \leq 0.05$ are discussed, unless noted otherwise.

3.4 Results

3.4.1 Effect of Scan Interval on sub-LOL Fluorescence Spike

The LOL fluorescence spike intensity was proportional to the scan interval used (Figure 3.1). Longer intervals increased spike intensity levels, but resulted in less fluorescence information over a fixed period of time. For apples held at 3.5 °C, the spike intensities (ΔF_α) generated using 0.25, 0.5, 1, 2 and 4 h scan intervals were 2.5, 3.4, 8.0, 12.4 and 13.8, respectively (Figure 3.1). The default scan interval for the PFM fluorometer is 1 h (HarvestWatch™ Operator's Manual 2003). Interestingly, at 20 °C, longer intervals generated higher spike intensities, but to a lesser degree than at 3.5 °C; spike intensities generated using 0.25 and 4 h scan intervals at 20 °C were 14.5 and 21.1, respectively ($n = 4$) (data not shown).

Adjusting the PFM scan interval did not appear to greatly affect the LOL for the intervals tested. In a separate experiment on apples under ambient atmospheric conditions, where the scan intervals were adjusted from 0.25 to 4 h at both 3.5 and 20 °C, no significant change in the fluorescence baseline was observed (data not shown).

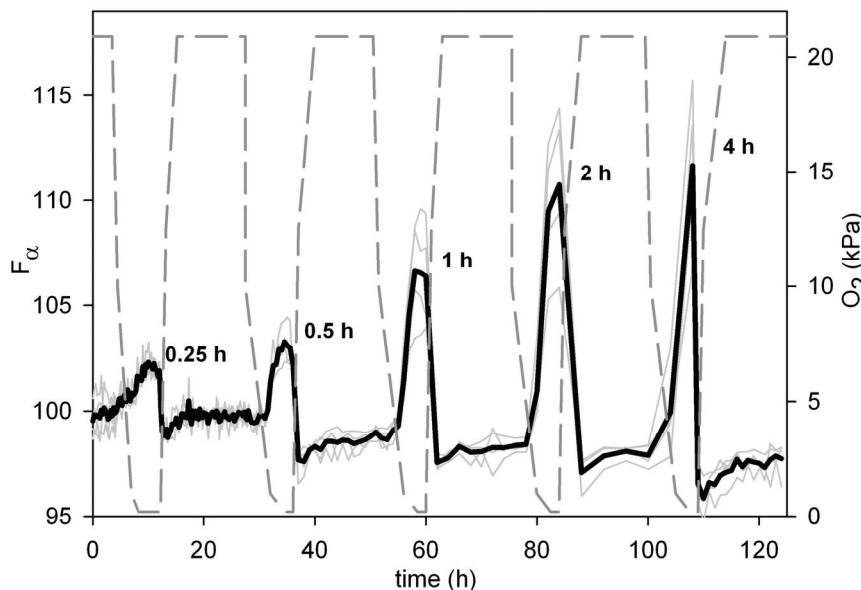


Figure 3.1 A representative plot showing the effect of PFM scan interval (0.25, 0.5, 1, 2 and 4 h scan intervals were each held for 24 h) on the low-O₂ (---) fluorescence response (generated using an ≈ 10 h nitrogen purge) on jars of apples held at 3.5°C under ambient atmosphere. Individual scans are shown as solid grey lines and the averaged response ($n = 4$) is solid black.

3.4.2 Effect of Temperature on Fluorescence Baseline

Decreasing the temperature increased the F_α baseline, while increasing the temperature decreased it (Figure 3.2). The apples required hours to equilibrate to the more rapid room

temperature changes (Figure 3.2B). The fluorescence value changes (Figure 3.3A) mirrored those shifts of the apple core temperature (Figure 3.2B). The $\Delta F_{\alpha} \text{ } ^{\circ}\text{C}^{-1}$ for the 3.5 to -2 $^{\circ}\text{C}$ (12 h) and 3.5 to -5 $^{\circ}\text{C}$ (12 h) temperature changes shown in Figure 3.2 were 1.35 and 1.47, respectively ($p = 0.01$).

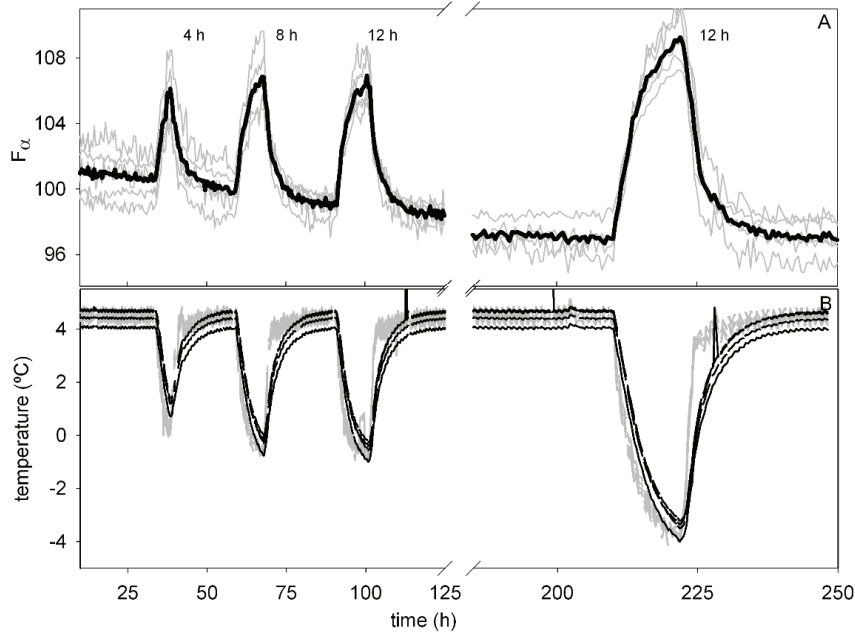


Figure 3.2 The effect of temperature on the fluorescence (F_{α}) response for jars of apples stored under ambient atmospheric conditions: **(A)** F_{α} values in response to -2 $^{\circ}\text{C}$ for 4, 8 and 12 h and -5 $^{\circ}\text{C}$ for 12 h (individual scans are shown in grey) and the averaged response ($n = 5$) in black) and **(B)** the headspace temperature (grey) and apple temperatures (black) over time.

When a gradient of temperatures from 0 to 20 $^{\circ}\text{C}$ was tested, the F_{α} baseline decreased from cold to warm and then increased from warm to cold. Since the fluorescence baseline significantly deteriorates at the warmer, but less so at the cooler temperatures (Figure 3.4), the ΔF_{α} was underestimated when going from warmer to cooler and overestimated from cooler to warmer (Figure 3.3). The $\Delta F_{\alpha} \text{ } ^{\circ}\text{C}^{-1}$ at the cooler was greater than at the warmer temperatures (Figure 3.3). A good approximation of the actual $\Delta F_{\alpha} \text{ } ^{\circ}\text{C}^{-1}$ was calculated using the average values (i.e. descending + ascending) for the 20 to 16, 16 to 12, 12 to 8, 8 to 4 and 4 to 0 $^{\circ}\text{C}$ temperature ranges which were 0.48, 0.85, 0.97, 1.39 and 1.34, respectively (Figure 3.3).

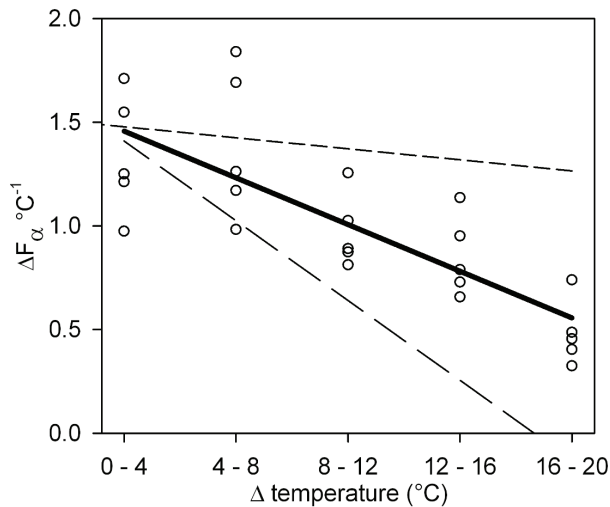


Figure 3.3 Regression (bold) ($p \leq 0.001$; $R^2 = 0.61$) showing the average $\Delta F_{\alpha} \text{ } ^\circ\text{C}^{-1}$ (i.e. (ascending + descending)/2) induced in jars of apples ($n = 5$) stored at ambient atmospheric conditions over a range of 4 $^\circ\text{C}$ temperature changes. Regression lines showing the negative relationship as the temperatures were decreased (20 to 0 $^\circ\text{C}$) (long dash) and increased (0 to 20 $^\circ\text{C}$) (short dash) are also shown.

The possibility that the observed fluorescence changes in response to temperature could be due to the effect of changing temperature on the fluorometer instrumentation was investigated. Ancillary experiments show temperature-induced fluorescence changes predominantly reflect a change in fruit temperature and the sensor/temperature effects are likely minor by comparison (data not shown).

3.4.3 Effect of Temperature on Fluorescence Baseline and LOL

A difference in the LOL due to temperature was noted ($p \leq 0.001$), but not between experimental replicates of the same apples ($p = 0.466$). The LOL and fluorescence spike intensity increased with temperature ($p \leq 0.001$) (Table 3.1; Figure 3.4). There was also a decline in the fluorescence spike intensities, for each temperature, between experiments ($p = 0.002$) (data not shown). At each temperature, the fluorescence signals began to plateau while under the LOL before O_2 levels were raised back above the LOL (Figure 3.4C). The O_2 consumed and CO_2 respired both decreased with lower temperatures ($p \leq 0.001$) (Table 3.1).

Table 3.1 The effect of temperature on the average ($n = 8$) O_2 uptake and CO_2 respired under ambient atmospheric conditions, LOL, fluorescence spike apex and spike intensity.

temperature ($^\circ\text{C}$)	O_2 (g (kg s)^{-1})	CO_2 (g (kg s)^{-1})	LOL (kPa O_2)	spike apex (F_{α})	spike intensity ($\Delta F_{\alpha} \%$)
20	44.4 ^a	45.0 ^a	0.72 ^a	109.2 ^a	18.0 ^a
10	22.1 ^b	23.9 ^b	0.33 ^b	107.2 ^a	10.8 ^b
3.5	15.1 ^{bc}	8.1 ^c	0.22 ^c	107.0 ^a	3.9 ^c
0	5.5 ^c	4.6 ^d	0.08 ^d	108.4 ^a	1.4 ^d

*Means with different letters within a column are significantly different ($p \leq 0.05$).

Similar to the results reported above, absolute changes at the cooler temperatures created a greater response than those at the warmer ones. The change in fluorescence ($\Delta F_{\alpha} \text{ } ^{\circ}\text{C}^{-1}$) induced by the 20 to 10, 10 to 3.5 and 3.5 to 0 $^{\circ}\text{C}$ temperature adjustments were 0.68, 1.15 and 1.32, respectively ($p \leq 0.001$). Although the spike intensity decreased at lower temperatures (Table 3.1) due to higher fluorescence baseline values, the spike apex values were similar regardless of the temperature (Table 3.1; Figure 3.4C). When recovering from the low- O_2 stress, the fluorescence initially dropped below (before returning to) the pre-LOL fluorescence baseline level for each temperature tested (Figure 3.4C).

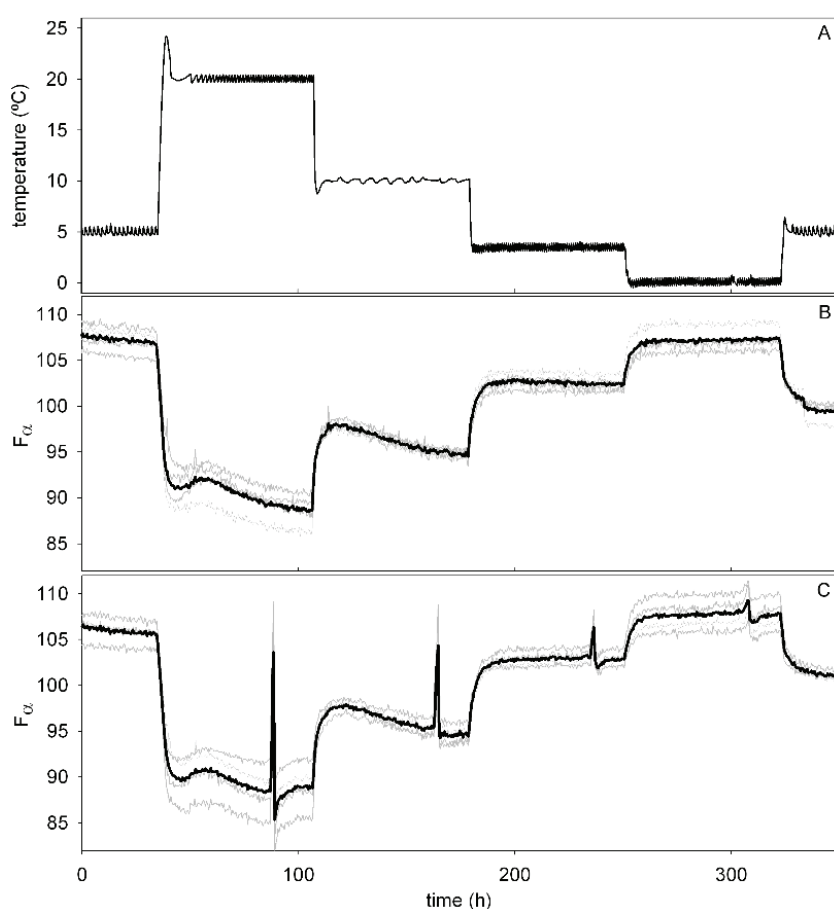


Figure 3.4 The effect of (A) temperature on (B) F_{α} and (C) F_{α} combined with low- O_2 episodes on day 3 following the temperature change on jars of apples. In plots B and C individual scans are shown in grey and the averaged scan ($n = 4$) in black (experimental rep. 2 is shown).

PAM-generated F_o (Figure 3.5A) and F_m (Figure 3.5B) values responded similarly to the temperature changes as did the PFM-derived F_{α} (Figure 3.4B). Both F_v and F_v/F_m only exhibited slight responses to the temperature adjustments with both parameters generally declining over time (Figure 3.5C, 3.5D).

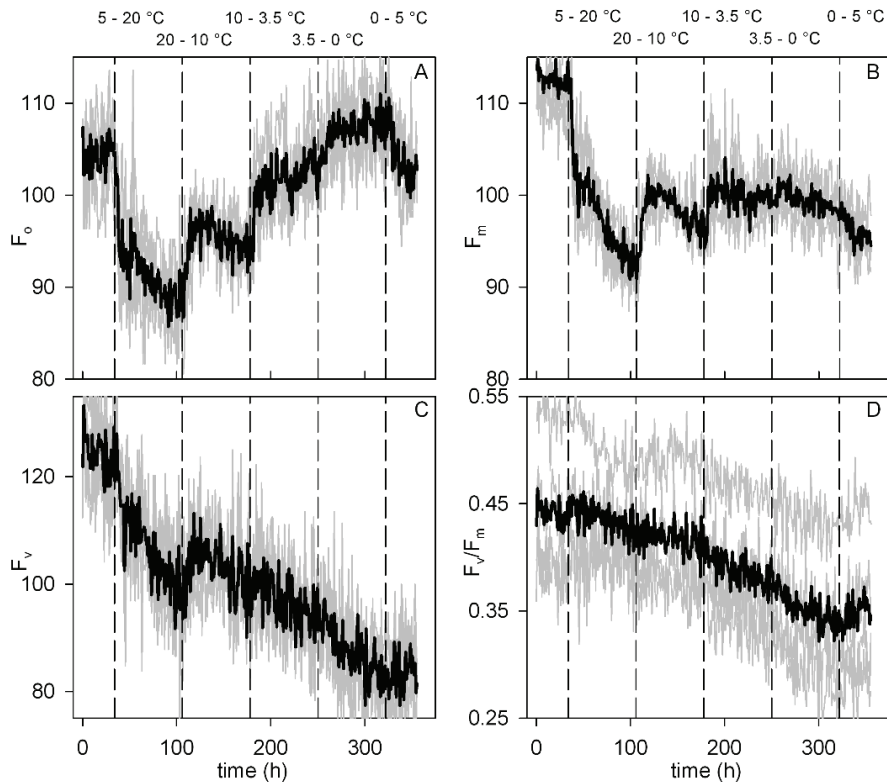


Figure 3.5 The effect of temperature on the PAM fluorescence parameters (A) F_0 , (B) F_m , (C) F_v and (D) F_v/F_m using jars of apples. Black, dashed vertical lines indicate temperature changes of 5 to 20, 20 to 10, 10 to 3.5, 3.5 to 0 and 0 to 5 °C, respectively, from left to right. Individual scans are shown in grey and the averaged scan ($n = 4$) is in black.

The PAM F_0 response to low- O_2 stress, though more variable, resembled the PFM response. Both fluorescence parameters noticeably increased when O_2 levels dropped below the LOL (Figure 3.6A). When the O_2 levels were rapidly returned to normoxia, both fluorescence parameters dropped below, before returning to, the pre-stress fluorescence baseline (Figures 3.4C and 3.6A). There was little change in F_m as the O_2 levels fell below the LOL, but F_m temporarily decreased when levels were returned to normoxia (Figure 3.6B). Primarily as a result of F_0 changes, both F_v and F_v/F_m declined once O_2 levels dropped below the LOL, but returned to pre-stress values after the levels returned to normoxia (Figure 3.6C, 3.6D).

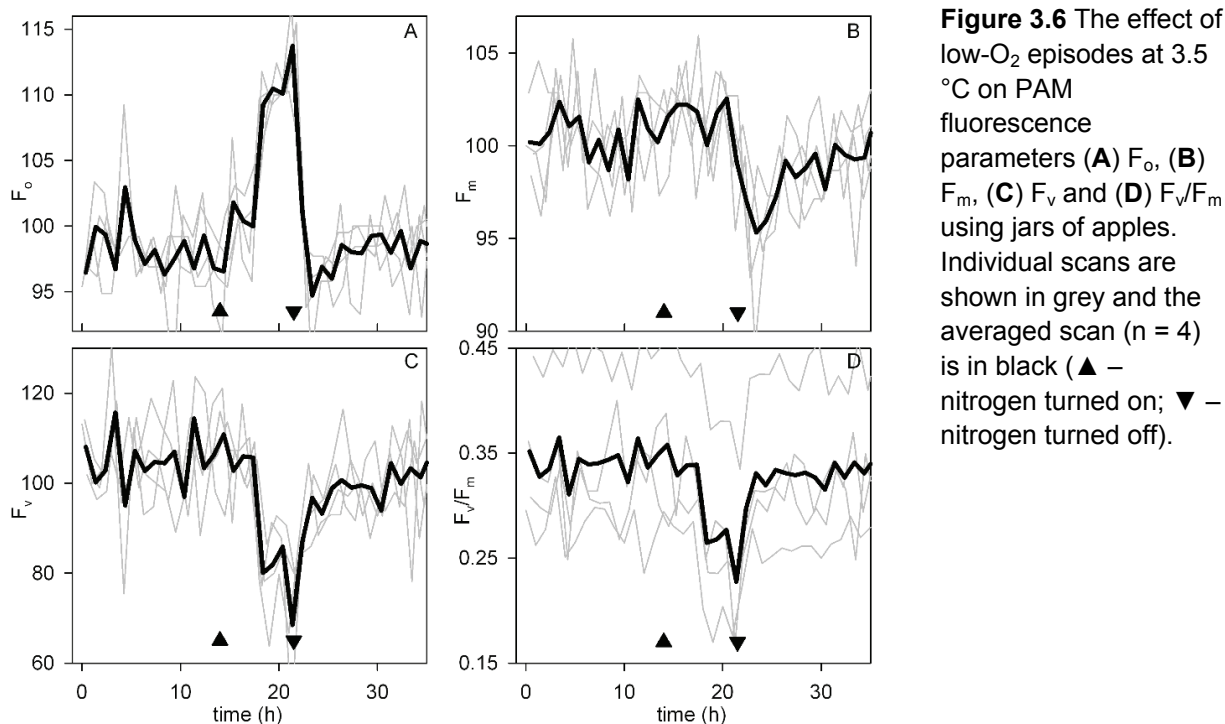


Figure 3.6 The effect of low- O_2 episodes at 3.5 °C on PAM fluorescence parameters (A) F_o , (B) F_m , (C) F_v and (D) F_v/F_m using jars of apples. Individual scans are shown in grey and the averaged scan ($n = 4$) is in black (▲ – nitrogen turned on; ▼ – nitrogen turned off).

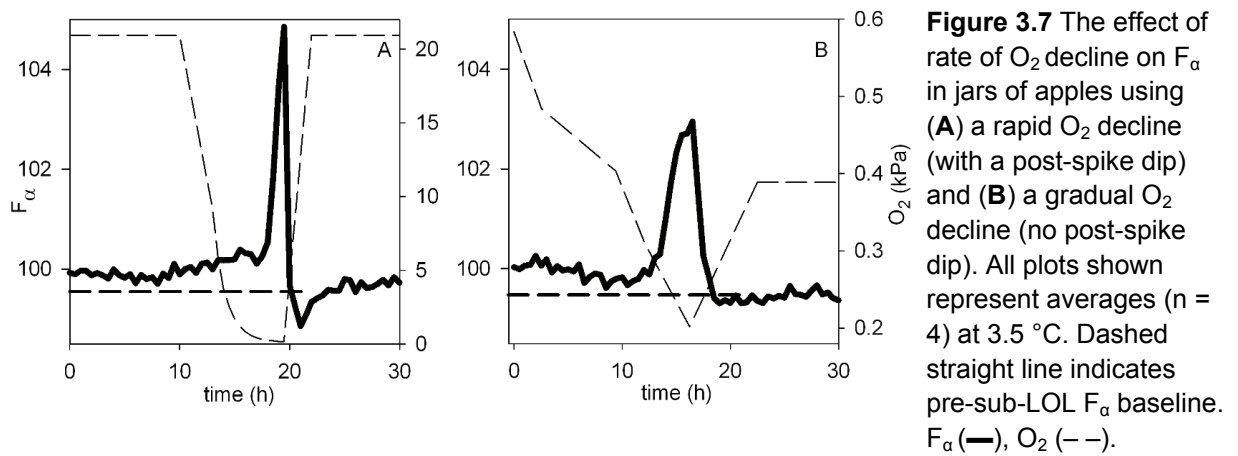
3.4.4 Temperature-Dependent LOL as Determined Using Gradual O_2 Decline

The LOLs determined with the gradual O_2 decline (days) were higher than those observed with the more rapid O_2 decline (≈ 8 h) ($p \leq 0.001$) (Table 3.2). Conversely, the spike intensities measured during the gradual decline were smaller, compared with those of the more rapid decline ($p \leq 0.001$) (Table 3.2; Figure 3.7). The post-spike dip observed in fruit subjected to a rapid change between anoxia and normoxia (Figure 3.7A) was not apparent in the fruit under a more gradual transition (Figure 3.7B).

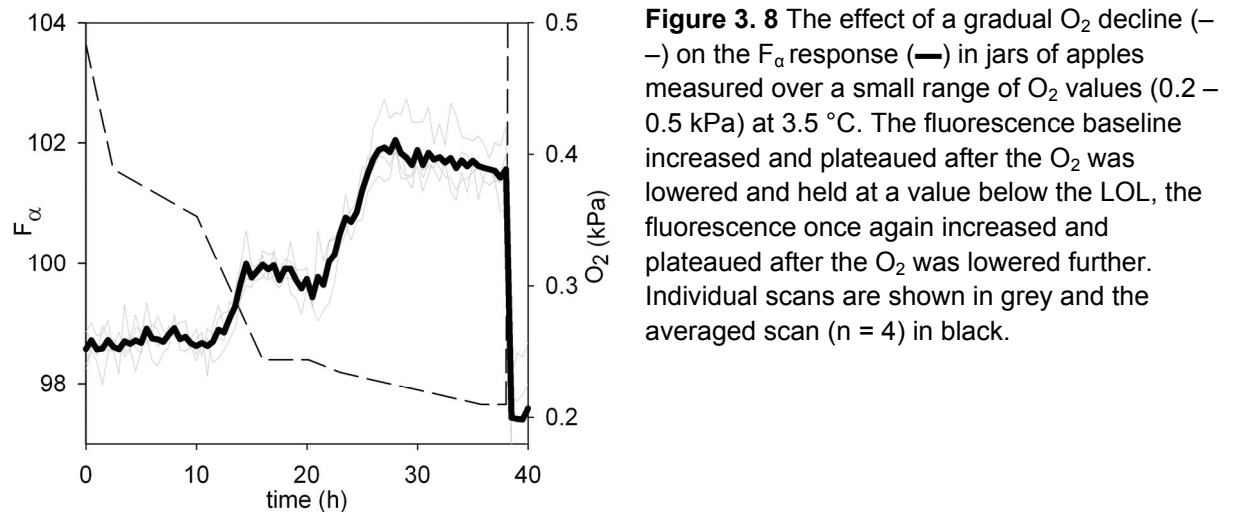
Table 3.2 The effect of temperature and rate of O_2 decline on the average ($n = 8$) LOL and fluorescence (F_o) spike intensity.

O_2 decline	temperature (°C)	LOL (kPa O_2)	spike intensity (ΔF_o)
fast	20	0.72 ^b	18.0 ^a
(≈ 8 h)	3.5	0.22 ^c	3.9 ^c
slow	20	1.01 ^a	10.5 ^b
(days)	3.5	0.27 ^d	3.0 ^d

*Means with different letters within a column are significantly different ($p \leq 0.05$).



The fluorescence increased when O₂ levels were brought below the apparent LOL (≈ 0.27 kPa O₂); additional decreases in O₂ below the LOL caused the fluorescence to increase further (Figure 3.8).



3.5 Discussion

In whole plant tissues, ‘dark-adapted’ fluorescence measurements may be performed once the fluorescence baseline values become stable (a dark-adaptation period of 0.5 to 1 hour is generally recommended (Peeler and Naylor 1988)). When the PFM scan interval was adjusted between 0.25 and 4 hours while monitoring ‘Honeycrisp’ apples, no change in the fluorescence baseline or the temperature-based fluorescence response suggested all scan intervals tested provided adequate dark-adaptation for the sample. However, the fluorescence low-O₂ stress response was severely quenched when using shorter compared with longer scan intervals (Figure 3.1). A PFM scan interval of 2 hours maximized the

strength of the fluorescence low-O₂ stress response and generated a signal approximately 50% greater than at 1 hour (Figure 3.1). These findings suggest that under hypoxic conditions true dark adaptation may still not be achieved when using a dark adaptation period of 2 hours or less. The mechanism of low-O₂ stress is thought to result from either a buildup of toxic fermentative metabolites or an energy deficit (Peppelenbos and Oosterhaven 1998). Increased light exposure from an increased fluorometer scan interval may decrease this deficit or up-regulate non-photochemical means of energy quenching under hypoxic conditions. Further studies are required to determine why the scan interval affected the low-O₂-induced fluorescence spike intensity, but not the fluorescence baseline, whereas temperature affected both.^{VII}

The choice of scan interval will depend on the intended application: a longer interval may be suitable when monitoring fruit in long-term storage, while a short interval can maximize the amount of fluorescence information found within a finite period of time. Preliminary studies suggest that similar results will be obtained using other apple cultivars and fruit and vegetable species; however, results may be cultivar- or species-specific.

It is unknown why the LOL values are higher during a gradual, compared with a more rapid, O₂ decline (Table 3.2). It may be that a more gradual shift in the metabolic rate decreased the stress associated with the low-O₂-induced change due to an unknown acclimation response. It may also be that with the more rapid O₂ decline, O₂ levels in the apple flesh lagged behind those in the jar headspace (i.e. the apple cell solution may have acted as an O₂ reservoir during the O₂ decline thereby enabling the head-space-based LOL measurements to drop below equilibrium values in the apple solution before a fluorescence spike was triggered).

The LOL for ‘Honeycrisp’ apples stored at 3.5 °C (typical storage temperature) in this study (\approx 0.3 kPa O₂) compared favourably with a previous report (\approx 0.4 kPa O₂ at 3.0 °C; DeLong et al. 2004b). The present study is the first to describe the relationship between temperature and LOL using a fluorescence-based DCA system. The increase in the LOL with temperature as measured with fluorescence in this work agrees with studies using other methods. A report by Valle-Guadarrama et al. (2004) showed the LOL (based on anaerobic compensation point (ACP) values) of ‘Hass’ Avocado fruit is higher at higher temperatures:

^{VII} More recent work detailed in Chapters 4 & 5 suggest the low-O₂-induced rise in fluorescence results from a reduction of the plastoquinone (PQ) pool. Increased light (i.e. shorter scan interval) quenches the signal via a PSI oxidation of the PQ pool as well as increased zeaxanthin and q_N.

1.44 kPa and 1.81 kPa at 5.5 and 20 °C, respectively. Similar results were observed using different apple cultivars (Yearsley et al. 1997) and apple slices (Lakakul et al. 1999). Contrastingly, Hong and Kim (2001) showed that temperature has no effect on the LOL of fresh-cut green onion (based on RQ increase).

The positive correlation between temperature and the LOL may be due to several factors. The dissolved oxygen values in apples at the LOL (based on ACP) tend to decrease with increased temperatures (Yearsley et al. 1997). Since gases are more soluble at cooler than at warmer temperatures, it is possible that decreased O₂ may be a contributing factor to higher LOLs measured at higher temperatures. Calculations based on data from Burton (1974) indicate the approximate levels of dissolved oxygen in apple solution at 20 and 3.5 °C are closer than their respective LOLs (data was based on 0.4 M solution (SSC ≈ 13%). The expressed juice from a ‘Honeycrisp’ apple typically has a SSC of ≈ 13% (DeLong et al. 2006)). At ambient atmosphere, ‘Honeycrisp’ apple cells would have an O₂ content of ≈ 7.7 mg L⁻¹ at 20 °C compared with ≈ 11.1 mg L⁻¹ at 3.5 °C, due to temperature-induced solubility changes. During the slow O₂ decline, the LOL in apples stored at 20 °C (LOL = 1.01 kPa O₂) was 3.75 times greater than that at 3.5 °C (LOL = 0.27 kPa O₂). However, if these headspace-based LOLs were converted into their approximate dissolved oxygen concentrations in the apple solution (Burton 1974), the dissolved O₂ within the apples at each temperature would be ≈ 0.38 mg L⁻¹ at 20 °C compared with ≈ 0.15 mg L⁻¹ at 3.5 °C; a factor of 2.5. Fruit skin gas permeance may also be a factor influencing the temperature effect on the LOL. However, in a study by Andrich et al. (1997) on apples there is no change in the skin resistance to O₂ mass-transfer over the temperature range 1 to 21 °C, and only a slight, though significant, change in the resistance to CO₂. Finally, the LOL may have changed with the temperature simply due to changing enzymatic activity and O₂ demand of the fruit.^{VIII} The respiration rate (i.e. O₂ consumed and CO₂ emitted) in the apples was positively correlated with the storage temperature (Table 3.1). Thus, at higher temperatures, the lower O₂ solubility of the apple cell, coupled with an increased demand for the O₂ found within that solution, may both contribute to a higher LOL compared with lower temperatures.

^{VIII} A re-analysis of the data in Chapter 5 that looks at the Q₁₀ coefficients and the implied enzymatic activity appears to confirm this as a significant factor.

The results of this study highlight the importance for commercial users of PFM fluorometers to be aware of fruit temperature, and not just room air temperature. Large volumes of fruit placed in cold rooms may retain ‘field heat’ for several days after the room is closed. Premature measurements of these fruit could lead to incorrectly high LOL values. Alternatively, fruit located in the centre of a large CA room may retain ‘field heat’ for a period of time and these fruit may resort to anaerobic metabolism if the O₂ is brought too low before these fruit reach their steady state temperature.

The novel finding that the fluorescence baseline was sensitive to storage temperature (Figures 3.1, 3.2, 3.4B and 3.5A), likely explains the majority of unexpected and unexplained fluorescence shifts reported from commercial DCA fluorometer users. Although temperature-induced fluorescence baseline shifts could be related to changes in the respiration rate, no major fluorescence baseline change occurred when the respiration rate was altered by manipulating the O₂ level above the LOL.

Prange et al. (2005b) has suggested that cytoplasmic acidosis, as induced by low-O₂ levels, may correspond with the sudden fluorescence rise in fruits and vegetables. Low-O₂ stress is known to induce cytoplasmic acidosis in plant material (including fruits and vegetables) (Roberts et al. 1984a; Bertl and Felle 1985; Gout et al. 2001; de Sousa and Sodek 2002). Cold temperatures, typical of those found during cold storage conditions, also induce cytoplasmic acidosis in plant tissue (Yoshida 1994; Yoshida et al. 1999). The sensitivity of fluorescence to the cytosolic pH could explain the lower spike intensities in apples stored in cooler compared with warmer temperatures (Figure 3.4C) and why the fluorescence intensity, regardless of the stress, appeared to have a maximum (Figure 3.4C; Table 3.1). Whether temperature-induced fluorescence baseline shifts reflect physiological changes in the fruit and are related to O₂-induced shifts is unknown and may be a source of future research.^{IX}

It is likely that the metabolic rate at the time of a low-O₂ event directly influences the resulting spike intensity. Gasser et al. (2003) reported that the CO₂ production in apples undergoing a rapid O₂ decline is greater than in apples undergoing a slow O₂ decline. A lag between metabolic rate and O₂ level, caused by the time required for the metabolic rate of apples to adjust to lowered O₂ values, may also explain why the spike intensities appeared to

^{IX} More recent work in Chapter 5 suggests the predominant factor affecting temperature-based fluorescence baseline shifts is not physiological.

be greater in fruit that experienced a rapid O₂ decline compared with those undergoing a more gradual change (Table 3.2; Figure 3.7). The reason for the post-spike dip in the apples experiencing a rapid O₂ decline (Figure 3.4A) is not known. Thermocouples (capable of measuring ± 0.1 °C) placed in the apple flesh and jar headspace showed no temperature fluctuations associated with the release of compressed gases (data not shown). The post-spike dip may be due to stresses associated with rapidly changing metabolic rates within the apples. Both low-O₂ stress and the re-oxygenation of O₂-deprived plant material are linked with the generation of damaging reactive oxygen species (ROS) (Blokhina et al. 2003).^X

In the present short-term study, a general decline in the fluorescence baseline increased with temperature (Figure 3.4B). Gradual downward and upward fluorescence baseline shifts over the course of many months have been noted in other long-term studies (unpublished data); Gasser et al. (2003) showed that the ACP in ‘Idared’ apples stored in regular air increases with storage duration. This change may be linked with the respiratory climacteric, or it may be due to changes in the efficiency of the photosystems and their respective light harvesting complexes. It is possible that the gradual changes in the fluorescence baseline over time reflect changing energy needs resulting in changing O₂ requirements which affect the LOL of fruits and vegetables.

^X More recent evidence now suggests the post-spike dip in fluorescence may be related to low-O₂-induced zeaxanthin accumulation and q_N and a relaxed rate of zeaxanthin epoxidation upon the return of normoxia (chapters 4 & 5). The finding that zeaxanthin accumulation also occurs above the LOL (Chapter 4), though to a lesser degree, likely explains why the dip was not observed in the fruit that experienced the more gradual O₂ decline, as their O₂ was raised to only just above the LOL and not to normoxia, thereby decreasing zeaxanthin and q_N changes (Figures 3.7B and 3.8).

Chapter 4: The Interrelationship between the Lower Oxygen Limit (LOL), Chlorophyll Fluorescence and the Xanthophyll Cycle in Plants

Published as:

Wright H, DeLong J, Harrison P, Gunawardena A, Prange R. 2011. The interrelationship between the lower oxygen limit (LOL), chlorophyll fluorescence and the xanthophyll cycle in plants. *Photosynthesis Research* **107**: 223 – 235

4.1 Abstract

The lower oxygen limit (LOL) in plants may be identified through the measure of respiratory gases [i.e. the anaerobic compensation point (ACP) or the respiratory quotient breakpoint (RQB)], but recent work shows it may also be identified by a sudden rise in dark minimum fluorescence (F_0). The interrelationship between aerobic respiration and fermentative metabolism, which occur in the mitochondria and cytosol, respectively, and fluorescence, which emanates from the chloroplasts, is not well documented in the literature. Using spinach (*Spinacia oleracea*), this study showed that F_0 and photochemical quenching (q_P) remained relatively unchanged until O_2 levels dropped below the LOL. An over-reduction of the plastoquinone (PQ) pool is believed to increase F_0 under dark + anoxic conditions. It is proposed that excess cytosolic reductant due to inhibition of the mitochondria's cytochrome oxidase under low- O_2 may be the primary reductant source. The maximum fluorescence (F_m) is largely unaffected by low- O_2 in the dark, but was severely quenched, mirroring changes to the xanthophyll de-epoxidation state (DEPS), under even low-intensity light ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). In low light, the low- O_2 -induced increase in F_0 was also quenched, likely by non-photochemical and photochemical means. The degree of quenching in the light was negatively correlated with the level of ethanol fermentation in the dark. A discussion detailing the possible roles of cyclic electron flow, the xanthophyll cycle, chlororespiration and a pathway we termed 'chlorofermentation' were used to interpret fluorescence phenomena of both spinach and apple (*Malus domestica*) over a range of atmospheric conditions under both dark and low-light.

4.2 Introduction

The lower oxygen limit (LOL) in plants represents the oxygen (O_2) level where metabolism changes from being predominantly aerobic to fermentative; O_2 levels below the LOL are believed to be detrimental to the long-term viability of most plants. Determining the LOL, which is species and environment-specific, along with understanding the limitations of aerobic respiration and fermentation control, are commercially relevant to the controlled atmosphere (CA) storage of fruits and vegetables. An increase in the respiratory quotient (RQ) (i.e. ratio of CO_2 respired over O_2 consumed) in response to low- O_2 , referred to as the RQ breakpoint (RQB), and the anaerobic compensation point (ACP) [i.e. the O_2

concentration at which CO₂ evolution is minimum (Boersig et al. 1988)], have both been used to identify the LOL (Gran and Beaudry 1993b; Yearsley et al. 1996; Gasser et al. 2008, 2010). Lowering O₂ levels decreases aerobic respiration, but eventually induces fermentation [i.e. the extinction point)]; lowering O₂ levels below the EP increases fermentation and decreases mitochondrial respiration levels further. Both the RQB and the ACP are below the EP and so allow for minimal amounts of fermentation (Peppelenbos and Oosterhaven 1998). More recently, a fluorescence-based technique has been developed to locate the LOL in plant material (Prange et al. 2003; DeLong et al. 2004b; Wright et al. 2008a, 2010).

The dark-adapted minimum fluorescence (F_o) parameter is sensitive to low-O₂ stress. When O₂ levels are above the LOL, F_o remains relatively unchanged; however, when O₂ levels drop below the LOL, F_o increases greatly (Wright et al. 2010). When O₂ levels are adjusted back above the LOL, F_o returns to pre-stress levels (Prange et al. 2003; Wright et al. 2008a, 2010). The increase in F_o in response to low-O₂ stress correlates with respiratory changes and so yields comparable LOLs (Gasser et al. 2008, 2010). To date, a detailed explanation describing the relationship between aerobic respiration and fermentative metabolism, which occur in the mitochondria and cytosol, respectively, and fluorescence, which emanates from the chloroplasts, is lacking.

Research indicates that anoxia can lead to an over-reduction of the plastoquinone (PQ) pool even in complete darkness (Harris and Heber 1993; Bennoun 2002; Tóth et al. 2007). The F_o parameter reflects the redox state of the PQ pool; a reduced state leads to an increase in F_o . The potential source of this reductant, why the PQ pool becomes reduced at O₂ levels below, but not above the LOL, and the possible relationship with fluorescence and cell metabolism are discussed in this paper.

The xanthophyll cycle is now recognized as a key regulator and photoprotective mechanism found ubiquitously in plants. It involves the interconversion of a group of carotenoids known as the xanthophylls. Under excess light conditions, violaxanthin is converted, via de-epoxidation, into the intermediate antheraxanthin and ultimately zeaxanthin (for a review see Demmig-Adams and Adams 1996). In the violaxanthin state the carotenoid functions as an antenna pigment and transfers energy to chlorophyll *a* pigments and reaction centres (RCs). However, in the zeaxanthin state the carotenoid acts as an energy trap and dissipates this photochemical energy as heat (Frank et al. 1994). This de-epoxidation of violaxanthin requires a pH gradient across the thylakoid membrane (Pfündel

and Bilger 1994; Munekage et al. 2002) and is generally associated with high light intensity. Work presented here shows the xanthophyll cycle also operates under very low-light intensity conditions in anoxic (below the LOL) and to a lesser degree hypoxic (above or at the LOL) conditions. This relationship could provide insight into the physicochemical relationship between low-O₂, respiration and fluorescence.

This paper examines the interrelationship between low-O₂, the LOL, chlorophyll fluorescence and the xanthophyll cycle under both dark and low-light conditions. The potential roles of the mitochondria, chlororespiration, cyclic electron flow and a pathway that we have termed ‘chlorofermentation’, are discussed.

4.3 Materials & Methods

4.3.1 Plant Materials

Spinach (*Spinacia oleracea*) plants were grown in a growth chamber (Foster Ltd., Model WI-34, Drummondville, QC, Canada) [18°C at 250 μmol·m⁻²·s⁻¹ (unless otherwise noted) on a 12 h dark/12 h light cycle] for at least 8 weeks before sampling. Healthy, mature leaves of a similar age and size were selected for each experiment. The dark-adapted LOL of the spinach at 20 °C, as indicated by an increase in F_a (an approximation of F_o) using pulse frequency modulation (PFM) technology (HarvestWatch™, Satlantic Inc., Halifax, NS, Canada) (Wright et al. 2008a), was ≈ 0.50 kPa O₂ (n = 8) (data not shown)^{XI}. For the purposes of this paper, O₂ levels below 0.5 kPa will be described as anoxic, while those below normoxia (20.9 kPa), but above 0.5 kPa will be referred to as hypoxic.

‘Honeycrisp’ apples (*Malus domestica*) grown in the Annapolis Valley, NS, Canada were stored in CA (O₂ = 2.0 kPa, CO₂ = 2.5 kPa, relative humidity = 90%, temperature = 3.5 °C) for 3 months prior to experimentation. Apples were allowed to acclimate to 20 °C at normoxia for 2 days before treatment.

4.3.2 Fluorescence, Xanthophylls and Volatiles under Normoxic, Anoxic, Dark and Low-Light Conditions

In a two-factor experiment, spinach leaves were placed inside 750 ml glass jars in a room set at 20 °C; the petiole of each leaf was placed directly inside a smaller 5 ml jar filled with water fixed to the inner bottom of the larger jar. Paper wadding was used to position each leaf against the inside of the larger jar. The airflow of each jar was ≈ 0.67 ml·s⁻¹. Using 4

^{XI} This was comparable to a study by (Watada et al. 1996) that found the LOL in spinach to be between 0.2 – 0.4 kPa O₂, based on the RQB and the ACP, at 5 °C.

treatment replicates for each, the following conditions were tested: 1. dark versus low-intensity light, 2. normoxic versus anoxic (N₂) versus anoxic (0.035 kPa CO₂ with N₂ balance). The treatment jars were alternated in a single line 2 m from a ‘cool white’ fluorescent light source. The light intensity from the low-intensity fluorescent light source for the light treatment was $\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ inside the jars as measured using a quantum sensor (LI-188B Integrating Quantum/ Radiometer/Photometer, LI-Cor, Inc., Lincoln, NE, USA). An F_v/F_m fluorescence scan (OS-500, Opti-Sciences Inc., Tyngsboro, Mass., USA – saturation intensity: 200, 1 s; far-red intensity: 120; detector gain: 20) was used to measure the minimum and maximum fluorescence parameters (F_o and F_m) at time = 0 (i.e. normoxic) and time = 17 h. The intensities of the far-red (735 nm) and saturation light (690 nm) sources used by the fluorometer were ≈ 0.55 and $8500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively, as measured using the quantum sensor through the jar glass. The fluorometer had a 0.5 cm² probe and measurements were repeated on the same location on each leaf marked on the exterior of the jar. Pre- and post-treatment fluorescence measurements were used to calculate the coefficient of non-photochemical quenching [$q_N = (F_m - F_m') \cdot (F_m - F_o')^{-1}$] and the coefficient of photochemical quenching [$q_P = (F_m' - F_s) \cdot (F_m' - F_o')^{-1}$]. Headspace volatiles [acetaldehyde (AA), ethanol (EtOH), ethyl acetate (EA)] were measured using 1 ml samples at time = 17 h via gas chromatography (GC) (Varian 3400, Walnut Creek, CA, USA). The xanthophyll content (violaxanthin, antheraxanthin, zeaxanthin) of each leaf was measured using HPLC (see HPLC section) from samples obtained approximately 1 h after the F_v/F_m measurement.

The experiment was also conducted using spinach leaves grown under a higher light intensity ($350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) to test the following conditions: 1. dark versus low-intensity light ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 2. normoxic versus anoxic (N₂).

A similar experiment was performed using ‘Honeycrisp’ apples. Eight fruit were individually placed inside 8, 4 L jars at 20 °C. Four apples were treated with air (20.9 kPa O₂ and 0.035 kPa CO₂) and 4 with N₂ at a flow of 1.25 ml s⁻¹. An F_v/F_m fluorescence scan, volatile measurements (as described above) as well as O₂ and CO₂ measurements (International Controlled Atmosphere Ltd., ICA40 system, Tonbridge, UK) were performed at time = 0 (i.e. normoxic) and again after 19 h of acclimation under low-light intensity ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The xanthophyll content of the apple skin was measured using HPLC.

4.3.3 DTT

A two-factor experiment was designed to compare the effect of normoxia and hypoxia (1.0 kPa O₂) (i.e. an O₂ level above the LOL) on control leaves and leaves treated with dithiothreitol (DTT), an inhibitor of zeaxanthin formation (Yamamoto and Kamite 1972), under low-intensity light ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The petioles of 8 leaves were placed in a 5 mM DTT solution while an additional 8 were placed in pure water and allowed to acclimate for 3 h under low-intensity light. The petioles of each leaf were then placed inside a 5 ml jar filled with similar solution fixed to the bottom of a larger 750 ml jar in a flow-through system as described above. Half the leaves of each treatment were treated with normoxic air and half were treated with hypoxic air. All leaves acclimated for 17 h; the fluorescence and xanthophyll content were measured as described above.

4.3.4 Fluorescence and Xanthophylls Over a Gradient of O₂ Levels Under Low-Light

In a single-factor experiment, 8 spinach leaves were individually placed inside 8, 750 ml glass jars in a flow-through system as described above. Two treatment levels were tested at a time; three sets of treatment levels were tested overall: 20.9 versus 0, 12 versus 7 and 3 versus 1 kPa O₂. The lights were turned off and air (20.9 kPa O₂) flowed through each sealed jar at $\approx 0.67 \text{ ml}\cdot\text{s}^{-1}$. After at least 30 min of dark-adaptation, a fluorescence run was performed on a marked location of each leaf as described above. The lights were then turned back on and the O₂ level in jars 1 – 4 and 5 – 8 were set to their respective O₂ level treatments. After 17 h the fluorescence was re-measured. The xanthophyll content (violaxanthin, antheraxanthin and zeaxanthin) of each leaf was measured using HPLC approximately 1 h after the kinetic run.

4.3.5 Zeaxanthin Epoxidase upon Reintroduction of O₂

In a single-factor experiment, 8 spinach leaves were placed in jars under low-intensity lights ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) as described above. The O₂ level was set to 0 kPa for 17 h. Air (20.9 kPa O₂, 0.035 kPa CO₂) was then reintroduced to the jars at a flow of $\approx 0.75 \text{ ml}\cdot\text{s}^{-1}$. At least two leaves were sampled at the following time intervals: 0.00, 0.083, 0.25, 0.50, 1.25, 2.00 and 3.50 h after O₂ reintroduction. The xanthophyll content of the leaves was measured using HPLC.

4.3.6 F_o upon Reintroduction of O_2

In a single-factor experiment, 4 spinach leaves were placed in jars in the dark as described above. The F_o of each leaf was monitored every 0.25 h with PFM sensors. The O_2 level was set to 0 kPa until F_o increased. O_2 was re-introduced to each leaf and the F_o was measured over several minutes (0.5 to 5 minutes) after O_2 re-introduction. The ratio of the change in F_o relative to the pre-stress value measured after the introduction of O_2 was compared to the initial low- O_2 induced increase in F_o .

4.3.7 Dark Compared to Low-Light Adapted F_o

In a single-factor experiment, 4 spinach leaves were placed in jars in the dark as described above. The F_o of each leaf was monitored every 0.25 h with PFM sensors. The O_2 level was set to 0 kPa for 17 h. The fermentative volatiles of each leaf were measured as described above. A low-intensity light ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was turned on approximately 5 min before a subsequent F_o measurement. The experiment was repeated using 8 leaves of a greater maturity grown under a higher light intensity ($\approx 350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

4.3.8 HPLC

The HPLC protocol was adapted from Thayer and Björkman (1990). Samples (spinach whole leaf or apple peel) were immediately placed into liquid nitrogen. Samples were then ground into a fine powder while frozen using a chilled mortar and pestle and placed in a -80°C freezer (Forma[®]-86C ULT Freezer, Marietta, OH, USA). Under dimmed room lighting, a sample (spinach – 1 g; apple – 2 g) was placed in a centrifuge tube on ice with 5 ml of 85% acetone; 500 μl of 100 μM β -Apo-8'-carotenal (trans) (Sigma-Aldrich, Switzerland) was added as an internal standard. Each extract was thoroughly mixed for 30 s, had N_2 gas blown over it for 2 min and was capped before being mixed for an additional 1 min. The extract was then placed on ice for 15 min before centrifugation (IEC MultiRF, Thermo IEC, Needham Heights, MA, USA) for 4 min at 12,000 x g at 4°C . The supernatant was collected and the pellet was re-extracted in 3 ml of 85% acetone, mixed for 30 s and re-centrifuged, as described above, two more times. The supernatants from each sample were combined and made up to 10 ml with 85% acetone. The supernatants were kept on ice and had a steady stream of N_2 gas blown over them as they were collected. Samples were filtered through a 0.2 μm nylon syringe filter into HPLC vials.

The HPLC system consisted of a K1001 pump, a K1500 solvent organizer, dynamic mixer, and a K2800 diode array detector (Knauer, Berlin, Germany), an autosampler (Basic Marathon, Holland Spark, Emmen, The Netherlands) and a column heater (Alltech, model 330, USA). The xanthophylls were eluted using 100% of solvent A (acetonitrile: methanol 85:15) for the first 14.5 min which was followed by a 2 min linear gradient to 100% solvent B (methanol: ethyl acetate 68:32) for an additional 28 min. The xanthophylls were separated using a Zorbax non-encapped ODS (4.6 x 250mm, C18, 5 μ m particle size) analytical column preceded by a C18 Zorbax ODS guard column (4.5 x 12.5mm, 5 μ m) (Agilent Technologies, IL, USA). Data analysis was carried out using ChromGate Version 3.1.6 (Knauer, Berlin, Germany). Recoveries (> 98 %) and standard curves were generated for violaxanthin, antheraxanthin and zeaxanthin using standards obtained from Carotenature (Lupsingen, Switzerland). Xanthophyll contents were expressed as nmol·g⁻¹ fresh mass as well as the de-epoxidation state (DEPS) = (Z+0.5A)/(V+A+Z).

4.3.9 Statistics

All experiments described were conducted at least twice. Either a balanced analysis of variance (ANOVA) or a general linear model (GLM) was used to determine statistical significance among treatments. Individual treatment averages were compared using a Waller-Duncan k-ratio t test mean comparison (SAS Release 8.0, 1999). Regression analyses were performed using Minitab (® Release 15 2007) and SigmaPlot 11.0 (2008) and used combined datasets. A normal probability plot (NPP) of the residuals tested normality while a fitted values versus residuals plot examined constant variance. When the assumptions of the error term (ϵ_{ij}) were violated, transformations were used; in those instances, averages shown in this paper have been back-transformed. Only results significant at $p \leq 0.05$ are discussed, unless noted otherwise.

4.4 Results

4.4.1 Fluorescence, Xanthophylls and Fermentative Volatiles under Dark + Low-Light and Normoxia + Anoxia

In spinach, the increase in F_o was greatest under dark + anoxic conditions; F_o increased to a lesser extent under low-light + anoxia, but was quenched relative to the dark (Table 4.1). The decrease in F_m was greatest under low-light + anoxic conditions, while leaves subjected to dark + anoxia experienced a slight decline in F_m compared with the normoxic controls.

The F_v/F_m values decreased under anoxia, but were unchanged between lighting treatments due to the contrasting trends of F_o and F_m (Table 4.1). q_p decreased while q_N increased under anoxia; both values were lower in the dark compared with the low-light (Table 4.1). Fermentative volatiles (AA, EA and EtOH) were only present in appreciable amounts under low- O_2 in spinach. Volatile levels were suppressed under low-light compared with the dark (Table 4.1). Violaxanthin levels decreased while zeaxanthin and the DEPS increased under low-light + anoxic conditions compared with normoxic values. Slightly-elevated levels of zeaxanthin and DEPS also occurred under dark + anoxic conditions. The addition of ambient CO_2 during the N_2 purge did not have an effect on any of the results shown (Table 4.1). Stomata closure was not believed to be an issue with gas exchange as fluorescence values quickly responded to increases in O_2 (Figure 4.1A).

Table 4.1 The average values in spinach grown under moderate light ($\approx 250 \mu\text{mol m}^{-2} \text{s}^{-1}$) of F_v/F_m , ΔF_o %, ΔF_m %, acetaldehyde (AA), ethanol (EtOH) and ethyl acetate (EA), violaxanthin (Viol), antheraxanthin (Anth), zeaxanthin (Zea) and the DEPS (Z+0.5A)/(V+A+Z) under normoxic (20.9 kPa O_2 + 0.035 kPa CO_2), anoxic (N_2 + 0.035 kPa CO_2) and anoxic (N_2) atmospheric conditions under both dark and low light ($\approx 4 \mu\text{mol m}^{-2} \text{s}^{-1}$) (17 h acclimation). Values represent combined experimental replicates (n = 8).

Trt		F_v/F_m	ΔF_o (%)	ΔF_m (%)	q_P	q_N
Normoxic	dark	0.830 ^a	2.8 ^c	2.5 ^a	1.01 ^a	-0.03 ^c
	light	0.820 ^a	1.6 ^c	-0.2 ^a	1.00 ^a	0.00 ^c
Anoxic (N_2 + 0.035 kPa CO_2)	dark	0.562 ^b	136.7 ^a	-7.2 ^b	0.69 ^c	0.12 ^b
	light	0.552 ^b	35.0 ^b	-39.9 ^c	0.80 ^b	0.54 ^a
Anoxic (N_2)	dark	0.559 ^b	144.7 ^a	-6.6 ^b	0.68 ^c	0.11 ^b
	light	0.551 ^b	15.7 ^b	-50.8 ^c	0.87 ^b	0.65 ^a
		AA ml (kg h)^{-1}	EtOH $\mu\text{l (kg h)}^{-1}$	EA $\mu\text{l (kg h)}^{-1}$		
Normoxic	dark	0.0 ^c	1.9 ^d	4.2 ^d		
	light	0.0 ^c	0.0 ^d	0.0 ^d		
Anoxic (N_2 + 0.035 kPa CO_2)	dark	75.0 ^a	3990.2 ^a	1579.6 ^a		
	light	0.0 ^c	729.1 ^c	402.1 ^c		
Anoxic (N_2)	dark	78.9 ^a	3423.8 ^a	1284.2 ^a		
	light	0.0 ^c	310.1 ^c	489.4 ^c		
		Viol (nmol g^{-1})	Anth (nmol g^{-1})	Zea (nmol g^{-1})	DEPS	
Normoxic	dark	322 ^a	11 ^b	16 ^c	0.06 ^c	
	light	357 ^a	11 ^b	14 ^c	0.05 ^c	
Anoxic (N_2 + 0.035 kPa CO_2)	dark	346 ^a	15 ^a	72 ^b	0.19 ^b	
	light	232 ^b	10 ^b	257 ^a	0.54 ^a	
Anoxic (N_2)	dark	327 ^a	16 ^a	116 ^b	0.26 ^b	
	light	184 ^b	9 ^b	285 ^a	0.60 ^a	

*Means with different letters within a given parameter column are significantly different ($p \leq 0.05$).

More mature leaves grown under a higher light intensity ($\approx 350 \mu\text{mol m}^{-2} \text{s}^{-1}$) exhibited the same general trends as the less mature grown under the lower light irradiance ($\approx 250 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Table 4.2). However, the increase in F_o , zeaxanthin and the volatiles evolved, under both the dark and low-light anoxic conditions were greater in the plants grown under the higher compared with the lower light intensity ($p \leq 0.001$) (Tables 4.1 and 4.2).

A positive, linear correlation between ethanol fermentation and the DEPS occurred under low-intensity light ($p \leq 0.001$) (Figure 4.1B). Furthermore, the degree to which F_o was

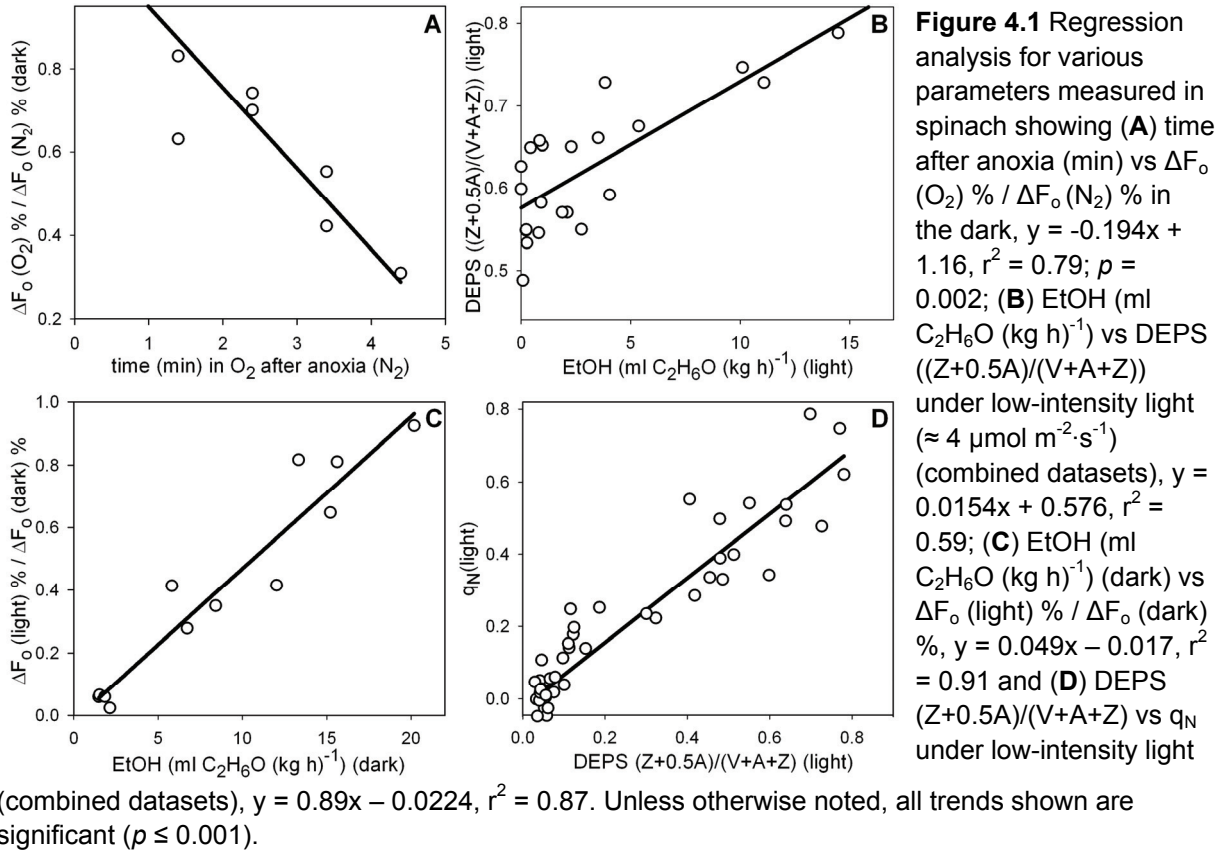
quenched under low-light intensity decreased with increased ethanol fermentation (Figure 4.1C).

‘Honeycrisp’ apples are known to engage in ethanol fermentation even under normoxic conditions at harvest. The CO₂ and fermentative volatiles evolved, with the exception of EA, increased in ‘Honeycrisp’ apples under anoxia (Table 4.3). F_m’, and to a lesser extent, F_o’, were both quenched under anoxia. Similar to spinach, violaxanthin levels decreased and zeaxanthin increased under anoxia relative to normoxia in apples (Table 4.3).

Table 4.2 The average values in spinach grown under high light intensity ($\approx 350 \mu\text{mol m}^{-2} \text{s}^{-1}$) of F_v/F_m, ΔF_o %, ΔF_m %, acetaldehyde (AA), ethanol (EtOH) and ethyl acetate (EA), violaxanthin (Viol), antheraxanthin (Anth), zeaxanthin (Zea) and the DEPS (Z+0.5A)/(V+A+Z) under normoxic (20.9 kPa O₂ + 0.035 kPa CO₂) and anoxic (N₂) atmospheric conditions under both dark and low light ($\approx 4 \mu\text{mol m}^{-2} \cdot \text{s}^{-1}$) (17 h acclimation). Values represent combined experimental replicates (n = 8).

Trt		F _v /F _m	ΔF_o (%)	ΔF_m (%)	q _P	q _N
Normoxic	dark	0.816 ^a	3.3 ^c	4.3 ^a	0.99 ^a	-0.07 ^c
	light	0.821 ^a	2.7 ^c	8.7 ^a	0.99 ^a	-0.12 ^c
Anoxic (N₂)	dark	0.361 ^b	264.8 ^a	0.0 ^b	0.41 ^c	0.07 ^b
	light	0.452 ^b	58.4 ^b	-49.8 ^c	0.64 ^b	0.76 ^a
		AA ml (kg h) ⁻¹	EtOH $\mu\text{l (kg h)}^{-1}$	EA $\mu\text{l (kg h)}^{-1}$		
Normoxic	dark	0.0 ^c	13.7 ^c	0.0 ^c		
	light	0.0 ^c	15.3 ^c	0.0 ^c		
Anoxic (N₂)	dark	97.0 ^a	11205.2 ^a	2058.5 ^a		
	light	10.0 ^b	6571.5 ^b	838.1 ^b		
		Viol (nmol g ⁻¹)	Anth (nmol g ⁻¹)	Zea (nmol g ⁻¹)	DEPS	
Normoxic	dark	394.9 ^a	17.5 ^a	26.7 ^c	0.08 ^c	
	light	382.1 ^a	12.7 ^b	15.6 ^c	0.06 ^c	
Anoxic (N₂)	dark	368.9 ^a	20.1 ^a	49.6 ^b	0.15 ^b	
	light	159.0 ^b	6.9 ^c	343.3 ^a	0.69 ^a	

*Means with different letters within a given parameter column are significantly different ($p \leq 0.05$).



4.4.2 Fluorescence and Xanthophylls Over a Range of Low- O_2 Under Low-Light in Spinach

All of the fluorescence parameters measured responded significantly to changes in the O_2 level under low-intensity light ($\approx 4 \mu mol \cdot m^{-2} \cdot s^{-1}$) ($p \leq 0.001$) (Figure 4.2). F_0' did not increase until O_2 levels dropped below the LOL (i.e. ≈ 0.5 kPa O_2) (Figure 4.2A). Regression analysis on the lower O_2 levels above the LOL (i.e. 7, 3 and 1 kPa O_2) did reveal a slight, though significant, decline in F_0' ($p = 0.006$) (data not shown) (Figure 4.2A). Both F_m' and F_v'/F_m' began to decline at O_2 levels above the LOL (Figure 4.2B, 4.2C, respectively). Correspondingly, q_p decreased only after O_2 levels were lowered below the LOL, while q_N increased at O_2 levels above the LOL and continued to increase as the O_2 was lowered further (Figure 4.2D).

Table 4.3 ‘Honeycrisp’ apple averages of F_v'/F_m' , $\Delta F_o'$ %, $\Delta F_m'$ %, Δ acetaldehyde (AA) %, Δ ethanol (EtOH) %, Δ ethyl acetate (EA) %, Δ CO₂ %, violaxanthin (Viol), antheraxanthin (Anth), zeaxanthin (Zea) and the DEPS (Z+0.5A)/(V+A+Z). The apples were subjected to either normoxic (20.9 kPa O₂ + 0.035 kPa CO₂) or anoxic (N₂) conditions under low light ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (19 h acclimation). Values represent combined experimental replicates (n = 8).

Trt	F_v'/F_m'	$\Delta F_o'$ (%)	$\Delta F_m'$ (%)	
Normoxic	0.518 ^a	0.9 ^a	2.8 ^a	
Anoxic (N₂)	0.342 ^b	-18.0 ^b	-32.7 ^b	
Trt	ΔAA ml (kg h)⁻¹ (%)	ΔEtOH μl (kg h)⁻¹ (%)	ΔEA μl (kg h)⁻¹ (%)	ΔCO_2 ml (kg h)⁻¹ (%)
Normoxic	-23.6 ^b	-8.9 ^b	-5.6 ^a	5.5 ^b
Anoxic (N₂)	227.7 ^a	83.3 ^a	-45.6 ^b	85.3 ^a
Trt	Viol (nmol g⁻¹)	Anth (nmol g⁻¹)	Zea (nmol g⁻¹)	DEPS
Normoxic	0.93 ^a	--	0.26 ^b	0.22 ^b
Anoxic (N₂)	0.34 ^b	--	0.80 ^a	0.70 ^a

*Means with different letters within a given parameter column are significantly different ($p \leq 0.05$).

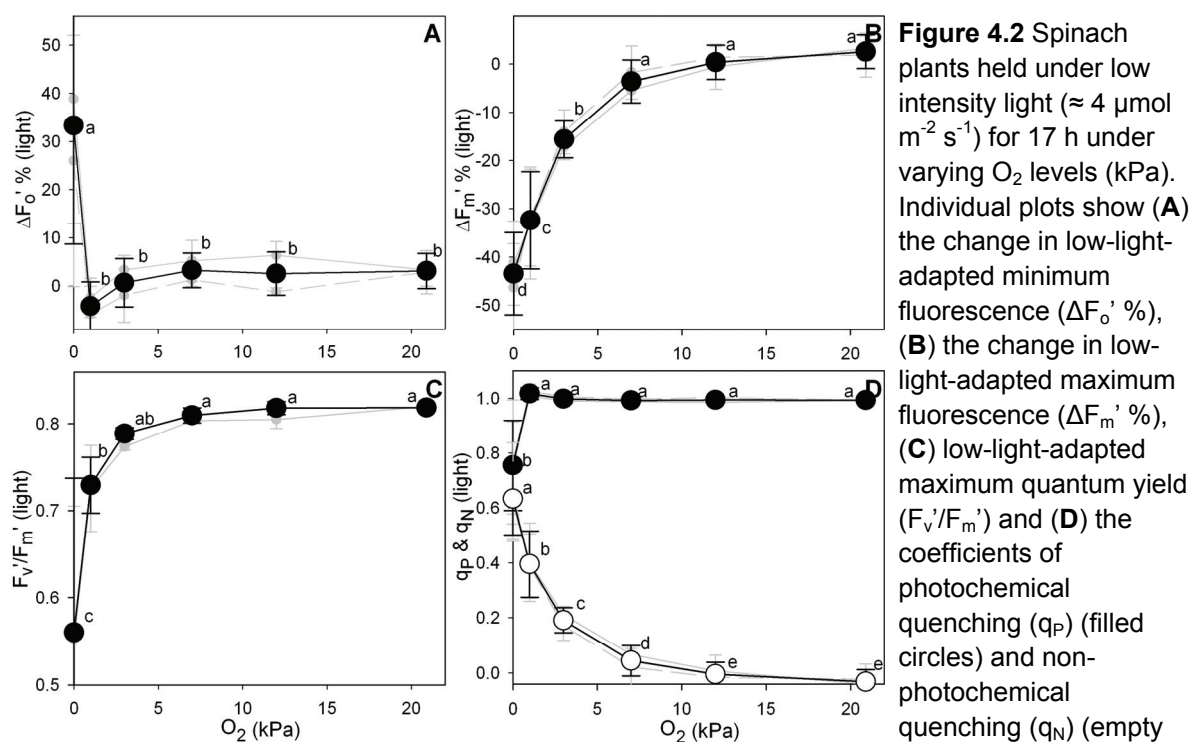
Violaxanthin, antheraxanthin and zeaxanthin levels were affected by O₂ levels above the LOL ($p \leq 0.001$) (Figure 4.3). Violaxanthin levels decreased at the lower O₂ levels (Figure 4.3A). The intermediate antheraxanthin levels were low overall, but increased under the lower hypoxic values (i.e. 1 and 3 kPa) only to decrease under anoxia (Figure 4.3B). Zeaxanthin levels, and the DEPS, increased moderately under the lower hypoxic values and peaked under anoxia (Figure 4.3C, 4.3D). Lutein and neoxanthin levels were unchanged by all treatments (data not shown).

Using data from both this and the previous spinach experiments, it was shown that there is a correlation between the DEPS and q_N in spinach leaves under low-intensity light ($p \leq 0.0001$) (Figure 4.1D). In this and the previous spinach experiments, the low-O₂-induced increase in zeaxanthin was always moderately greater than the decrease in violaxanthin (Tables 4.1 and 4.2; Figure 4.3).

Table 4.4 The average values in spinach leaves treated with 5 mM dithiothreitol (DTT) compared with control leaves under hypoxia (1.0 kPa O₂) for 17 h under low-light intensity ($\approx 4 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$): F_v'/F_m' , $\Delta F_o'$ %, $\Delta F_m'$ %, q_N ; violaxanthin (Viol), antheraxanthin (Anth), zeaxanthin (Zea) and the DEPS (Z+0.5A)/(V+A+Z). Values represent combined experimental replicates (n = 8).

Trt		F_v'/F_m'	$\Delta F_o'$ (%)	$\Delta F_m'$ (%)	q_N
Hypoxic	control	0.730 ^b	1.2 ^c	-22.0 ^c	0.28 ^a
	DTT	0.738 ^b	22.6 ^a	1.5 ^b	-0.03 ^b
Trt		Viol (nmol g ⁻¹)	Anth (nmol g ⁻¹)	Zea (nmol g ⁻¹)	DEPS
Hypoxic	control	223.8 ^b	14.3 ^a	161.9 ^a	0.43 ^a
	DTT	373.9 ^a	3.2 ^b	5.5 ^b	0.02 ^b

*Means with different letters within a given parameter column are significantly different ($p \leq 0.05$).



Each experimental replication is represented by small circles [exp. rep. i (solid), exp. rep. ii (dashed)]. Data points showing the two experimental replications combined are shown as large circles. Each combined data point average represents n = 8. Error bars show the standard deviation. Combined means with different letter groupings are significantly different ($p \leq 0.05$).

4.4.3 Zeaxanthin Epoxidase upon Reintroduction of O₂

The levels of all three xanthophylls (violaxanthin, antheraxanthin, zeaxanthin) and the DEPS changed over time in spinach after being reintroduced to normoxic O₂ after an anoxic event under low-intensity light ($p \leq 0.001$) (Figure 4.4). The trends were the reverse of those

induced by anoxia (Figures 4.3 and 4.4). Upon re-oxygenation, violaxanthin levels increased (Figure 4.4A), while antheraxanthin levels increased before decreasing after half an hour (Figure 4.4B). Zeaxanthin concentrations and the DEPS decreased markedly in the first 1.25 h, then continued to decline at a slower rate, but did not reach their pre-stress levels in 3.5 h (Figure 4.4C, 4.4D).

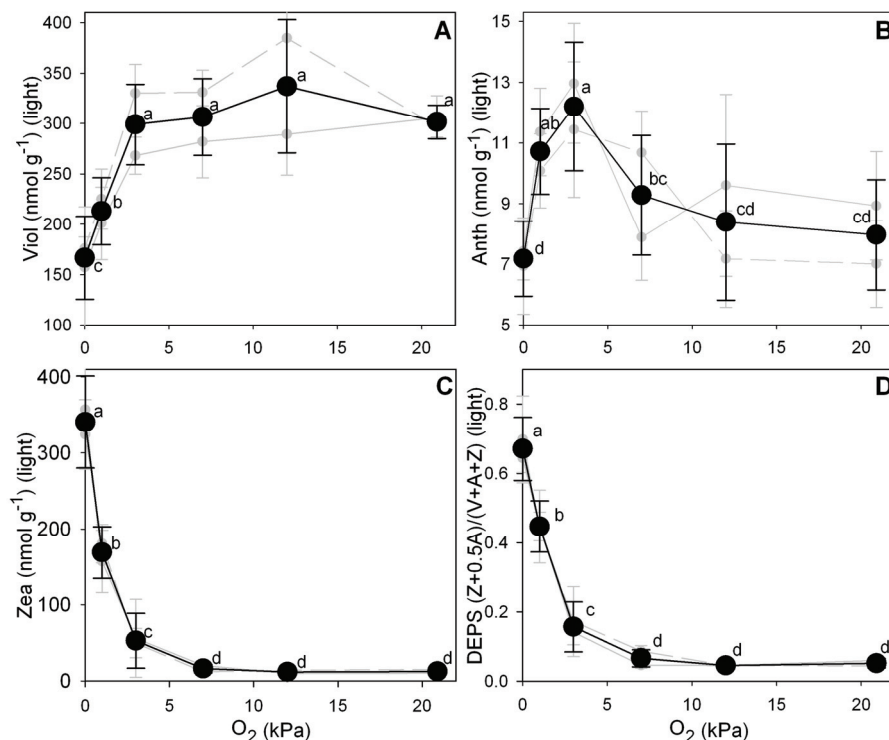


Figure 4.3 The xanthophyll content (nmol g⁻¹) and DEPS (Z+0.5A)/(V+A+Z) found in spinach plants in response to O₂ levels (kPa). Plants were held under low intensity light ($\approx 4 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 17 h. Individual plots show (A) Violaxanthin, (B) Antheraxanthin, (C) Zeaxanthin and (D) DEPS (Z+0.5A)/(V+A+Z). Each experimental replication is represented by small circles [exp. rep. i (solid), exp. rep. ii (dashed)]. Data points

showing the two experimental replications combined are shown as large circles. Each combined data point average represents n = 8. Error bars show the standard deviation. Combined means with different letter groupings are significantly different ($p \leq 0.05$).

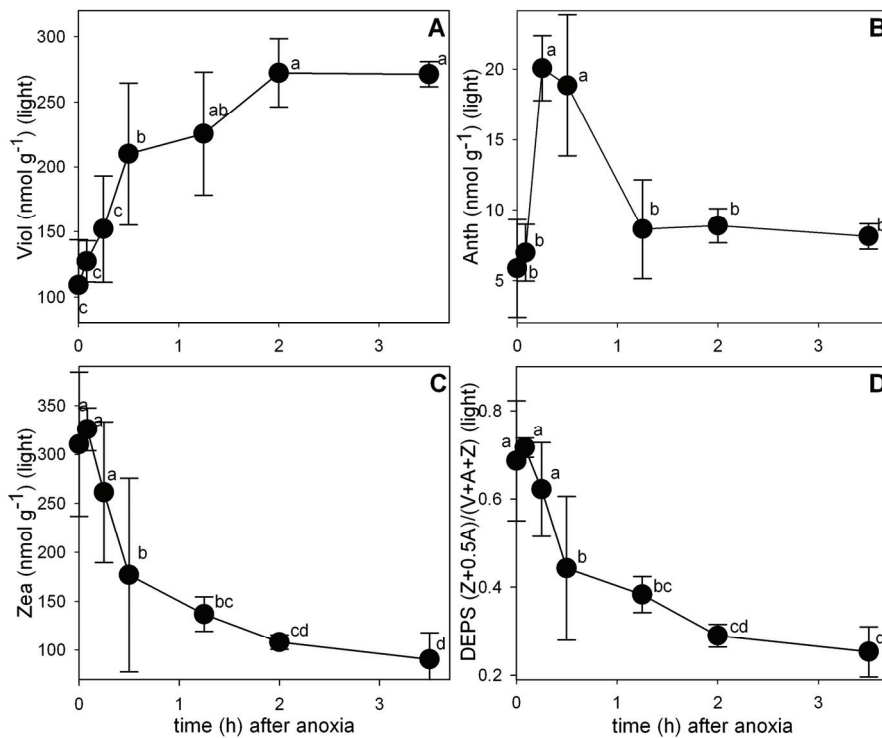


Figure 4.4 The xanthophyll content (nmol g^{-1}) and DEPS ($(Z+0.5A)/(V+A+Z)$) found in spinach plants after an anoxic event. Plants were held under low-intensity light ($\approx 4 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$) with no O_2 for 17 h. The plants were then reintroduced to O_2 and the xanthophyll content was measured at 0.00, 0.083, 0.25, 0.50, 1.25, 2.00 and 3.50 h. Individual plots show (A) Violaxanthin, (B) Antheraxanthin, (C) Zeaxanthin and (D) DEPS ($(Z+0.5A)/(V+A+Z)$). Each data point represents at

least $n = 4$; the 0.00, 0.083 and 0.50 averages contain $n = 5$. Error bars show the standard deviation. Combined means with different letter groupings are significantly different ($p \leq 0.05$).

4.4.4 DTT

There was no difference in F_v'/F_m' between control and DTT spinach leaves under normoxia (0.797 versus 0.787; $p = 0.113$) or hypoxia (Table 4.4) under low-light. The DEPS and q_N increased in control leaves under hypoxia relative to leaves treated with DTT; F_o' increased in DTT leaves relative to control leaves under the same conditions (Table 4.4).

4.5 Discussion

4.5.1 Low O_2 Results in Excess Reductant

Glycolytic reductant in the form of NADH is typically recycled back into NAD^+ via mitochondrial respiration; however, when mitochondrial respiration is inhibited, such as when the terminal electron receptor, O_2 , is limited, NADH accumulates. Ethanol, and to a lesser degree lactic acid fermentation, recycle the elevated reductant levels. The inhibition of mitochondrial oxidative phosphorylation under low- O_2 conditions can be sudden as evidenced by the rapid build-up of cytosolic NADH (≈ 2 min) (Roberts et al. 1984b). The NADH/ NAD^+ ratio is typically unaffected by higher hypoxic values, likely as a result of O_2 not being a limiting factor, but then increases slightly under lower hypoxic values before

increasing greatly under anoxia. In a study on the highly metabolic phloem tissue of *Ricinus communis*, the NADH/NAD⁺ ratio was unchanged between 20.9 and 6 kPa O₂, increased marginally between 6 and 3 kPa, but increased enormously between 3 and 0.5 kPa (van Dongen et al. 2003).

4.5.2 Reductant Transport

Imbalances in reductant levels between the cytosol, mitochondria and chloroplast can be adjusted indirectly using translocators as a conduit. For instance, oxaloacetate (OAA) and malate, found in the cytosol and potential products of glycolysis, can be transported across both mitochondria and chloroplast membranes. OAA is reduced to malate via a malate dehydrogenase using either NADH or NADPH; the reverse reaction can be facilitated via the oxidized forms, NAD⁺ or NADP⁺ (Appendix A iv; for reviews see Nixon 2000; Peltier and Cournac 2002; Heldt 2002).

The addition of glucose to cyanobacteria cells has been used to demonstrate that electrons from the cytosol can be used to reduce the chloroplast PQ pool (Mi et al. 1994; Ma et al. 2008). Conditions that lead to the overoxidation of the PQ pool, such as the addition of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU), were shown to increase the expression and activity of NADPH dehydrogenase (Ma et al. 2008). Antimycin A, an inhibitor of cyclic electron flow, has also been shown to increase NADPH dehydrogenase activity (Quiles et al. 2006). Evidence suggests the transfer of reducing power into the chloroplast's stroma via intermediaries can be highly functional even in higher plants. When DCMU was used to inhibit CO₂ fixation in isolated spinach chloroplasts, the addition of L-malate was found to restore CO₂ fixation by as much as 34%. The addition of OAA did not restore fixation in chloroplasts, but did in protoplasts, suggesting an extrachloroplastic requirement (Rathnam and Zilinskas 1977).

4.5.3 Rise in F_o Results from an Over-Reduction of the PQ Pool

An anaerobic environment, even in darkness, leads to an over reduction of the PQ pool, effectively arresting the photosynthetic ETC (Harris and Heber 1993; Bennoun 2002; Tóth et al. 2007). The existence of a non-photochemical mechanism capable of reducing the PQ pool (e.g. a NADPH dehydrogenase) is supported by numerous studies (Bennoun 1982; Burrows et al. 1998; Carol and Kuntz 2001; Bennoun 2002; Rumeau et al. 2007). The increase in F_o and the decrease in q_p at O₂ levels below the LOL (Tables 4.1 and 4.2; Figure 4.2) mark a shut down of PSII RCs and reflect this previously reported anoxia-induced, over-reduced

state of the PQ pool. Therefore, what is interpreted as a rise in F_o , which requires $q_p = 1$, may be better described as a switch from F_o ($q_p = 1$) to an elevated steady state fluorescence (F_s)^{XII} with $q_p < 1$ (for simplicity and the purposes of this discussion the switch from F_o to F_s will simply be referred to as a rise in F_o). The non-functionality of a small fraction of the RCs as a result of a reduction of the PQ pool will not necessarily lead to a proportional increase in fluorescence, since neighbouring RCs may accommodate excess excitation energy (Schreiber 2004). As a result of this non-linearity, moderate shifts in F_o and q_p may reflect a disproportionate shift in the redox state of the PQ pool.

Endogenous sources of chloroplast reductant exist (e.g. light, pentose phosphate cycle). F_o increases in isolated chloroplasts under photoinhibitory, low- O_2 conditions (Krause et al. 1985), but not in the dark (Harris and Heber 1993). This suggests that an extrachloroplastic reductant source, such as excess cytosolic reductant from impaired mitochondrial activity, is required (Harris and Heber 1993). Under dark + anoxic conditions, the two potential means of oxidizing the PQ pool, light and PSI (photochemical), or O_2 and chlororespiration (non-photochemical), are inhibited. The addition of either O_2 or light, including far-red which only engages PSI, rapidly quenches the elevated F_o value within minutes (Figure 4.1A, 4.1C; Harris and Heber 1993).

4.5.4 Chlororespiration

The existence of a plastid terminal oxidase (PTOX) (i.e. chlororespiration; Appendix A iii) in higher plants is supported by numerous studies (Singh et al. 2002; Harris and Heber 1993; Burrows et al. 1998; Peltier and Cournac 2002; Bennoun 2002; Joët et al. 2002). The function of chlororespiration is largely unknown, but it has been theorized to play a role in starch metabolism and the oxidation of both NADPH and the PQ pool under dark, normoxic conditions (Harris and Heber 1993; Feild et al. 1998). Further speculation suggests chlororespiration could be used to correct a cellular redox imbalance as a result of impaired mitochondrial activity (Harris and Heber 1993; Cournac et al. 2000); low- O_2 creates such an imbalance. Work by Bennoun (1994) suggests the chloroplast oxidase's affinity for O_2 is lower than the mitochondria's and so ceases to function at a higher cellular O_2 level. Thus, a

^{XII} The use of the term F_s in this case may be a misnomer. Although F_s suggests $0 < q_p < 1$ and $0 < q_N < 1$, as is believed to be the case when the fluorescence is measured under low- O_2 , the fluorescence measured under low- O_2 is not necessarily 'steady'. The more general term of 'relative fluorescence', F , which shares the characteristics of $0 < q_p < 1$ and $0 < q_N < 1$ may be more appropriate.

lack of O₂ could inhibit PTOX, but not NADPH dehydrogenase, thereby trapping the PQ pool in the reduced state in the dark.

4.5.5 Low-O₂ and the Xanthophyll Cycle

The concurrent decrease in violaxanthin and rise in zeaxanthin in spinach and apple suggests there is an interconversion of these two xanthophylls under hypoxic and anoxic, low-light conditions (Tables 4.1, 4.2 and 4.3; Figure 4.3). The same has been noted in wheat and rice under anoxic, ambient light (350 μmol m⁻² s⁻¹) (Mustroph et al. 2006), in harvested fiddlehead ferns submerged in water (DeLong, personal communication 2010) and was suggested by Prange et al. (2005b). The increase in zeaxanthin appears to surpass the decrease in violaxanthin in spinach (Figure 4.3; Tables 4.1 and 4.2). A synthesis of zeaxanthin from beta-carotene could explain this discrepancy, but requires further study.

Although typically associated with high light, violaxanthin de-epoxidation may occur at lower light intensities when the energy demand of photochemistry is compromised as a result of stress [e.g. temperature (Jung et al. 1998), water deficits, herbicides (Iturbe-Ormaetxe et al. 1998), low-CO₂ (Gilmore and Björkman 1994)]. CO₂ was not a limiting factor in the present study. Mitochondrial respiration (Appendix A i) and ethanol fermentation (Appendix A ii) would likely provide sufficient CO₂ to meet the carbon fixation demands under the low-light regime. Furthermore, spinach flushed with pure N₂ and N₂ combined with ambient CO₂ levels both exhibited violaxanthin de-epoxidation (Table 4.1). A rapid quenching of the low-O₂-induced elevated F_o value within seconds of re-oxygenation (Harris and Heber 1993; Figure 4.1A) suggests adequate gas exchange through stomata. Convincing evidence is observed in the apple: apples, which also exhibit low-O₂-induced violaxanthin de-epoxidation, elevate endogenous CO₂ levels under anoxia (Table 4.3) and rely on open lenticels for gas exchange instead of stomata.

The induction of a transthylakoid ΔpH and related non-photochemical quenching has been associated with an increase in cyclic electron flow (Munekage et al. 2002; Miyake et al. 2005; Okegawa et al. 2008) and stress conditions (e.g. low-CO₂ or high irradiance) (Johnson 2005; Miyake et al. 2005; Okegawa et al. 2008) or during the induction of photosynthesis when the NADPH/NADP⁺ ratio is high (Joët et al. 2002; Okegawa et al. 2008). Cyclic electron flow pumps protons across the thylakoid membrane without the generation of NADPH and may be employed in an effort to balance the ATP/NADPH ratio. Cyclic electron flow may take the form of the ferredoxin-dependent antimycin-sensitive *proton*

gradient regulator5 (*pgr5*) pathway or through the reduction of plastoquinone via a chloroplast NADPH dehydrogenase (Johnson 2005). A high NADPH/NADP⁺ ratio is known to increase the rate of cyclic electron flow (Hosler and Yocum 1987; Johnson 2005; Okegawa et al. 2008) while inhibiting ferredoxin-mediated NADP⁺ reduction, likely as a result of competition and limited NADP⁺ availability at the ferredoxin-NADP reductase (FNR) binding site (Hosler and Yocum 1987). A surplus of NADPH may also increase cyclic electron flow via an increase in NADPH dehydrogenase activity, as observed in the *Arabidopsis thaliana hcefl* mutant (Livingston et al. 2010). A high NADPH/NADP⁺ ratio and elevated cyclic electron flow under low-O₂, low-light intensity conditions may explain the engagement of the xanthophyll cycle. However, alternative possibilities also exist. Work by Avenson et al. (2004) suggests the sensitivity of energy quenching to low CO₂ and O₂ may not depend on an altered cyclic electron flow, but involve a variable partitioning of the proton motive force into its electric field and pH components.

4.5.6 Low-O₂ and Quenching

Both F_m and the low-O₂-induced elevated F_o were quenched under anoxic, light conditions (Tables 4.1, 4.2 and 3; Figures 4.1C and 4.2B; Harris and Heber 1993). The xanthophyll DEPS and q_N in spinach increases above the low-O₂-induced rise in F_o (Figures 4.2D and 4.3D). The change in the xanthophyll DEPS accounts for the increase in q_N (Figure 4.1D). It is believed violaxanthin de-epoxidation requires some light; the slight decrease in F_m, and increase in q_N and the DEPS under dark + anoxic conditions (Table 4.1) were believed to be an artefact from the fluorescence measurement. Spinach control leaves showed a decrease in F_m and an increase in q_N and DEPS in response to hypoxic conditions (1.0 kPa O₂) compared with leaves treated with DTT, an inhibitor of violaxanthin de-epoxidation. DTT leaves showed an increase in F_o relative to control leaves under the same conditions (Table 4.4). It would be tempting to suggest that this increase is due to the absence of zeaxanthin accumulation; however, as DTT is a strong reductant it could lead to an increased reduction of the PQ pool under certain conditions (Harris and Heber 1993; Atiyeh et al. 2009) or a decrease in dissolved O₂ (Kwong and Rao 1992).

The low-O₂-induced elevated F_o is likely quenched during anoxia by both photochemical and non-photochemical means in the light. Xanthophyll cycle-mediated non-photochemical quenching is believed to occur in the antennae pigments where it quenches both F_o and F_m proportionately (Demmig-Adams et al. 1990; Gilmore et al. 1995). In the highly-

photosynthesizing spinach plants, this decreased the intensity of the low-O₂-induced rise in F_o (Tables 4.1 and 4.2). In the case of the less photosynthetically-active apples, moderate quenching of F_o under low-light + anoxic conditions dropped values below those found under dark-adapted, normoxic conditions (Table 4.3). The low-O₂-induced elevated F_o is also likely quenched photochemically via PSI and the light-driven oxidation of the PQ pool. Work by Harris and Heber (1993) show that the anaerobic F_o signal can be quickly quenched even with a weak far-red light. Far-red light primarily engages PSI and not PSII and the oxygen evolution complex (OEC). Glycolysis in the dark (roughly approximated by measuring ethanol fermentation under dark + anoxic conditions), is correlated with the degree of F_o quenching (Figure 4.1C) and violaxanthin de-epoxidation (Figure 4.1B) observed under light + anoxic conditions. This may be evidence that the rate of glycolysis, and its related reductive pressure on the chloroplast, influences the increase in F_o and the LOL. It was previously shown that the respiration rate, as affected by temperature, is positively correlated with the LOL (Gran and Beaudry 1993a; Wright et al. 2010). If, under low-O₂ conditions, reductant from the cytosol (typically recycled via ethanol fermentation) is transported into the chloroplast and incorporated into photochemistry, a form of ‘chlorofermentation’ may occur: the PQ pool is reduced via non-photochemical means and subsequently oxidized in the absence of O₂ by PSI and light (Appendix A v). An over-reduction of the PQ pool, as observed under low-O₂, is also associated with photosystem state transitions (Hall and Rao 1999). If light harvesting complexes associated with PSII were re-directed to PSI this could potentially increase the efficiency of the ‘chlorofermentative’ pathway, but simultaneously limit O₂ evolution from PSII. The existence of a highly functioning ‘chlorofermentative’ pathway could partially explain the decrease in fermentative volatiles observed in plant tissues rich in chloroplasts, such as a spinach leaf, under low-intensity light compared with dark + anoxic conditions (Tables 4.1 and 4.2). However, this decrease in fermentative volatiles may also be explained by the engagement of the OEC. Future work should endeavour to determine the involvement of these two processes.

Chapter 5: Dynamic Controlled Atmosphere (DCA): Does Fluorescence Reflect Physiology in Storage?

Published as:

Wright H, DeLong J, Harrison P, Gunawardena A, Prange R. 2011. Dynamic controlled atmosphere (DCA): does fluorescence reflect physiology in storage? *Postharvest Biology and Technology* (POSTEC-D-11-00192, *under review*)

5.1 Abstract

A link between the minimum fluorescence (F_0) and a metabolic shift from predominantly aerobic to fermentative metabolism [i.e. the lower oxygen limit (LOL)] is the foundation of dynamic controlled atmosphere (DCA). Current DCA technology uses pulse frequency modulated (PFM) sensors and employs a range of light intensities and extrapolation to measure F_a , an approximation of F_0 . Like fruit mass, colour, sugar or acid levels, the LOL is inherently variable, even between apples (*Malus domestica*) (for example) from a given cultivar and tree or between the sun-exposed and shaded regions of a single fruit. The physiological link between metabolism and fluorescence has not been extensively studied. However, recent work suggests the low- O_2 -induced rise in F_a results from a shut down of mitochondrial function and a buildup of reductant that leads to an over-reduction of the plastoquinone (PQ) pool and a decrease in photochemical quenching. Hypoxic conditions above the LOL can decrease F_a slightly in some species, possibly as a result of zeaxanthin formation and increased non-photochemical quenching. Low-intensity light differentially affects F_a depending on the O_2 level: light increases F_a when O_2 levels are above the LOL due to light-induced reduction of the oxidized PQ pool, but decreases the elevated F_a signal below the LOL as a result of a PSI-driven oxidation of the over-reduced PQ pool. Temperature has a negative, primarily non-physiological correlation with the F_a baseline which seems unrelated to the PQ pool redox state. Understanding how O_2 and other factors affect F_a may improve the utility and commercial application of DCA.

5.2 Introduction

The lower oxygen limit (LOL) can be defined as the environmental oxygen (O_2) level at which cell metabolism changes from being predominantly aerobic to fermentative, and above which the O_2 level should be set in the controlled atmosphere (CA) storage of fruits and vegetables. Plant material stored at O_2 levels below their LOL risk physiological damage, which manifests as various types of storage disorders. Historically, CA guidelines were set for each species and cultivar using experimental trial and error. To discourage losses, these empirically-set guidelines had to be conservatively high to allow for yearly and regional variation.

Respiratory measurements such as the anaerobic compensation point (ACP) - the O₂ concentration at which CO₂ evolution is minimal (Boersig et al. 1988), and the respiratory quotient breakpoint (RQB) - the O₂ level at which the ratio of CO₂ evolved over the O₂ consumed suddenly increases (Gran and Beaudry 1993b; Yearsley et al. 1996; Gasser et al. 2008, 2010), have been used to define the LOL in the past. Both the RQB and the ACP allow for minimal amounts of fermentation (Peppelenbos and Oosterhaven 1998).

The extinction point (EP) is defined as the lowest O₂ level under which no ethanol (EtOH) production can be detected (Thomas and Fidler 1933); however, the presence of fermentative products alone is not a good indicator of the LOL as these products are often evident at O₂ levels above optimal storage conditions or even at normoxia (Fidler 1933; Peppelenbos and Oosterhaven 1998). The Dutch dynamic control system (DCS) determines the LOL by periodically monitoring apple (*Malus domestica*) EtOH levels (Veltman et al. 2003). The nature and tolerance of fruit species and cultivars to EtOH varies (Lidster et al. 1985; Lau 1989; Ke et al. 1991). Since allowable flesh EtOH thresholds vary, empirically-set guidelines are still necessary.

Dynamic controlled atmosphere (DCA) uses fluorometry to detect the LOL (Prange et al. 2003, 2007; DeLong et al. 2004b; Zanella et al. 2005; Burdon et al. 2008). It is used to improve overall fruit quality, most notably as a means of preventing superficial scald and improving post-storage firmness in apples (DeLong et al. 2004b, 2007; Zanella et al. 2005; Watkins 2008). The benefits of DCA on other commodities, such as pears (Prange et al. 2010) and avocados (Yearsley et al. 2003; Burdon et al. 2008) are now being realised. The minimum fluorescence parameter (F_o) has been found to be particularly sensitive to low-O₂ stress (Prange et al. 2003; Wright et al. 2008a, 2010). Pulse frequency modulation (PFM) technology was specially designed for commercial use to be sensitive to low-O₂ stress while increasing the fruit and vegetable surface area measured per scan. The PFM fluorometer measures the relative fluorescence over a range of low-intensity (< 10 μmol m⁻² s⁻¹) photosynthetic photon flux densities (PPFDs) and then uses extrapolation to determine the theoretical F_o, referred to as F_α (Prange et al. 2003; Wright et al. 2008a). To identify the LOL, the O₂ level is slowly lowered over many hours or days while the fluorescence is simultaneously measured using a set scan interval (i.e. time between fluorescence measurements). Once the O₂ levels drop below the LOL the measured F_α intensity rises quickly; as O₂ levels are restored to just above the LOL (or higher), the fluorescence

intensity again returns to pre-stress levels (Prange et al. 2003; DeLong et al. 2004b; Wright et al. 2008a). The DCA-based LOL has been shown to correlate with respiratory-based indicators of metabolic change (Gasser et al. 2008, 2010; Wright et al. 2011b).

In this study, LOL variability, including the difference between the sun-exposed and the shaded side of apple fruit, and the effect of O₂, low-intensity light and temperature on chlorophyll fluorescence levels, are examined. In addition, the physiological bases for fluorescence changes are discussed including the theoretical link between metabolic events occurring in the mitochondria and cytosol, and fluorescence originating from the chloroplasts.

5.3 Materials & Methods

Spinach (*Spinacia oleracea*) plants were grown in a growth chamber (Foster Ltd., Model WI-34, Drummondville, QC, Canada) (18 °C at 250 µmol·m⁻²·s⁻¹ on a 12 h dark/12 h light cycle) for at least 8 weeks before sampling. Healthy, mature leaves of a similar age and size were selected for each experiment. The dark-adapted LOL of the spinach at 20 °C, as indicated by an increase in F_α using PFM technology (HarvestWatch™, Satlantic Inc., Halifax, NS, Canada) (Wright et al. 2008a), was $\approx 0.45 \pm 0.05$ kPa O₂ (n = 8) (data not shown).

‘Granny Smith’ apples (product of USA) were purchased from a local supermarket. ‘Honeycrisp’, ‘Delicious’ and ‘Cortland’ apples were all harvested fresh from local sources unless otherwise noted (Annapolis Valley, NS, Canada).

5.3.1 The F_α Response Rate

Four ‘Granny Smith’ apples were placed singly inside 4 clear, 4 L plastic jars in a room set at 3.5 °C in the dark. Each jar was placed in front of a PFM fluorescence sensor (Satlantic Inc., Halifax, NS, Canada). Each jar and sensor was partitioned to avoid interference. Each sensor performed a fluorescence scan every hour. The apples were allowed to establish a fluorescence baseline under normoxia before a high flow of nitrogen (N₂) (≈ 2.0 mL s⁻¹) was applied to all jars inducing an F_α spike for several hours. After the fluorescence signals had plateaued, the flow of N₂ to the 4 apples was turned off and replaced with a flow of air (20.9 kPa O₂) (≈ 2.0 mL s⁻¹) at 0.5, 1.5, 2.5 and 3.5 min, respectively, prior to the next fluorometer scan. The experiment was repeated using air-flow intervals of 1.5, 2.5, 3.5 and 4.5 min. The entire experiment was replicated (i.e. n = 16). Regression analysis was used to examine the

relationship between the time (min) in O₂ after anoxia and the percent increase in F_α above the pre-stress baseline after O₂ re-introduction, over the percent increase under anoxia (i.e. F_α (O₂) % / F_α (N₂) %). The same experiment was repeated using ‘Honeycrisp’ apples and spinach.

5.3.2 F_α, the ACP and the EP in Apples

Eight clear, 4 L plastic respiration jars were each filled with approximately 1.2 kg of fruit; four were filled with ‘Honeycrisp’ (after 6 days of delayed cooling at 20 °C) and four were filled with ‘Delicious’ apples. The fruit were placed in the dark in a cold room set at 3.5 °C. Each jar was placed in front of a PFM sensor as described above. A continual flow (0.67 mL s⁻¹) of air was supplied to each jar. The O₂ was slowly lowered over several days before being returned to normoxia. O₂ and CO₂ levels were monitored using a gas analyzer (GCS150, Gas Control Systems Inc., MI, USA). Headspace volatiles [acetaldehyde (AA), ethanol (EtOH), ethyl acetate (EA)] were measured using 1 ml samples via gas chromatography (GC) (Varian 3400, Walnut Creek, CA, USA). The EP, the ACP and the F_α breakpoint (i.e. the O₂ level at which F_α first increased) were identified. The experiment was repeated once using new apples from the same source stored in refrigerated air (RA) at 3.5 °C for 3 months.

5.3.3 Sun-Exposed versus Shaded Side of Apple Fruit

Eight ‘Honeycrisp’ apples with distinct shaded and sun-exposed sides (based on colour) were selected at harvest and weighed. The chlorophyll fluorescence (F_o, F_m, F_v, F_v/F_m) (OS-500, Opti-Sciences, Inc., Tyngsboro, MA) and the chlorophyll health (ΔA meter, Sintéleia χ, Bologna, Italy) of each apple was measured on both the sun-exposed and shaded side of the fruit. The apples were then placed inside a partitioned, customized CA cabinet (≈ 0.1 m³); each apple was positioned in front of a PFM sensor. The side of the fruit facing the sensor was staggered (i.e. sun, shade, sun, etc). The O₂ inside the cabinet was lowered from 20.9 to 1.5 kPa O₂ on day 1; on day 2 the O₂ was slowly lowered, approximately 0.1 kPa O₂ per hour, until the F_α signal of each apple began to increase, marking the LOL. The cabinet was then opened and the position of each apple was rotated 180° relative to the PFM sensors (i.e. shade, sun, shade, etc.). The process of determining the LOL was repeated. After removal, the fruit firmness (N) was measured on both the sun-exposed and shaded side of the fruit (Geo-Met Instruments, New Minas, NS, Canada) (DeLong et al. 2000). A slice of apple flesh (≈ 1 cm thick; the outermost peel and a few mm of cortex tissue having been removed)

was obtained from both the sun-exposed and shaded side of each fruit and juiced. The soluble solids content (SS) and titratable acidity (TA) was measured in a juice sample from each side of each apple using a refractometer (PAL-1, Atago Co., Tokyo, Japan) and an automatic titrator (Buret/Dispenser 350, Brinkman, Switzerland) (mg equivalents of malic acid 100 ml⁻¹ juice) (DeEll and Prange 1998), respectively. A paired t-test was used to compare the sun-exposed and shaded measurements (Minitab ® Release 15 2007). The experiment was repeated three more times; once with ‘Honeycrisp’ from the same source and twice with ‘Cortland’ apples from two different local sources (Annapolis Valley, NS, Canada) [source 1 – at harvest; source 2 – unknown local source obtained from supermarket after ≈ 5 weeks in cold storage (3 °C)].

In another experiment, the level of respiration and ethylene production was measured on the sun-exposed and shaded side of ‘Honeycrisp’ apple fruit from the same source used above. A plastic 50 ml Fisherbrand® centrifuge tube fitted with a septum was attached and sealed using putty on both the sun-exposed and shaded side of eight apples. The fruit were left in the dark in a room set at 20°C. The respiration rate (ml CO₂ (cm² h)⁻¹) and ethylene production (µl C₂H₄ (cm² h)⁻¹) were measured after 24 h. The CO₂ levels were measured using a 3 ml sample and a gas analyzer (GCS150, Gas Control Systems Inc., MI, USA); the ethylene was measured using a 1 ml sample and gas chromatography (CARLE™, Anaheim, CA, USA). A paired t-test was used to compare CO₂ and ethylene evolution between fruit sides (Minitab ® Release 15 2007). The experiment was repeated once.

5.3.4 Temperature, Aerobic Respiration, the LOL and Spike Intensity

Previous work published by Wright et al. (2010) showed a positive correlation between aerobic respiration (measured as CO₂ released), the LOL and the low-O₂-induced F_α spike intensity in ‘Honeycrisp’ apples over a range of temperatures. These data are re-analysed as enzyme kinetics using the Q₁₀ constant [$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$], R – rate, T - temperature].

5.3.5 Temperature and the Fluorescence Baseline

The effect of temperature on the fluorescence baseline was tested on two treatments: 1. intact spinach leaves; 2. spinach chlorophyll dissolved in methanol. Four replicates of each treatment were placed in front of PFM sensors with a scan interval of 0.25 h. While the fluorescence was monitored, the temperature was decreased from 20 °C to 1 °C and then increased back to 20 °C. Similar experiments were performed with highly reflective, non-plant materials (e.g. white paper, white vinyl).

In another experiment, four ‘Honeycrisp’ apples stored at 20 °C were quickly placed in front of 4 PFM sensors with a 0.25 h scan interval in a room set at 1 °C; the fluorescence was monitored for several hours as the apples slowly cooled. The experiment was repeated once using apples stored at 1 °C quickly placed in front of sensors held in a room set at 20 °C.

5.3.6 Fluorescence Response to O₂ Changes above the LOL

Four ‘Honeycrisp’ apples were placed inside a 0.34 m³ CA storage cabinet set at 20.9 kPa O₂ at 3.5 °C. Each apple was placed in front of a separate PFM sensor, with a scan interval of 1 h. The storage cabinet was rapidly flushed from 20.9 to 1.5 kPa O₂ (\approx 1 h). The experiment was repeated once.

5.3.7 Low O₂ and the Xanthophyll Cycle

Eight spinach leaves were placed inside eight 750 ml glass jars in a dark room set at 20 °C and were monitored using PFM sensors (10 min scan interval) as described above. The O₂ levels in jars 1 – 4 and 5 – 8 were set at 20.9 and 0 kPa, respectively, with a flow of \approx 0.58 ml·s⁻¹. After 17 h, each leaf was collected in the dark directly after a scan; their xanthophyll content was measured using high pressure liquid chromatography (HPLC) using the protocol of Thayer and Björkman (1990) as modified by Wright et al. (2011). The experiment was replicated once.

5.3.8 Low O₂ Fluorescence and Dithiothreitol (DTT)

Dithiothreitol (DTT) (C₄H₁₀O₂S₂) is a known inhibitor of zeaxanthin formation (Yamamoto and Kamite 1972). Six spinach leaves were divided into 2 treatments: 1. water; 2. 5 mM DTT. The 6 leaves were placed in jars in front of PFM sensors in the dark (20 °C) as described above. The O₂ level was decreased from 20.9 kPa to 1 kPa for 17 h before it was returned to 20.9 kPa. After the fluorescence baselines stabilized for several hours, the O₂ level was decreased to 0 kPa for 17 h before it was again returned to 20.9 kPa. Plots of the fluorescence over time were used to compare the general trends observed in the control and DTT leaves (SigmaPlot 11.0, 2008).

5.3.9 Variable LOL within a Cultivar and the Light Effect

Eight ‘Granny Smith’ apples obtained from the local supermarket were placed in eight 4 L clear plastic respiration jars in a room at 3.5 °C in the dark. An effort was made to select a gradient of apples that varied in size (\approx 125 – 200 g) and apparent maturity (based on

greenness). The respiration rate ($\text{ml CO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) and ethylene ($\mu\text{l C}_2\text{H}_4 \text{ kg}^{-1} \text{ h}^{-1}$) of each apple was measured separately under normoxia. The apples were then transferred to a 0.34 m^3 CA storage cabinet fitted with PFM sensors set to a 1 h scan interval in a dark room set at $3.5 \text{ }^\circ\text{C}$ as described above. The ‘Night Light’ function [a low-intensity ($<0.1 \mu\text{mol m}^{-2} \text{ s}^{-1}$) red background light] was periodically activated 5 min prior to a scan, then turned off directly after the scan, as the O_2 was gradually lowered from normoxia to anoxia over many days. The O_2 was increased to 3 kPa for 10 days and then lowered as before. A paired t-test was used to compare the two LOLs obtained for each fruit.

In a separate experiment, four 0.34 m^3 CA cabinets were each filled with four plastic baskets containing $\approx 15 \text{ kg}$ of ‘Cortland’ fruit (total $\approx 60 \text{ kg}$ per cabinet). Two kennels containing six fruit each and fitted with a PFM sensor were buried within each cabinet. The apples were all stored in a dark room set at $3 \text{ }^\circ\text{C}$. After several days of acclimation at 2.5 kPa O_2 , the O_2 was slowly lowered in all the cabinets and the LOL for each kennel of fruit was tested. The O_2 was reset for all cabinets between 1 and 2 kPa O_2 . After several weeks of acclimation, the O_2 in two of the cabinets was held constant at $\approx 1.5 \text{ kPa O}_2$; the O_2 in the remaining two cabinets was slowly lowered over 2 days until the F_α signal of all four PFM sensors began to increase, marking the LOL. The PFM sensor ‘Night Light’ was turned on to determine the effect on the four kennels of fruit being held above their LOL and the four being held below.

5.3.10 Statistical Analysis

All fluorescence signals were normalized relative to the dataset mean. An analysis of variance (ANOVA) was used to determine statistical significance for single and two-factor models in each experiment performed and regression analysis was used to determine the significance of overall trends (Minitab® Release 15 2007). Individual treatment averages were compared using a Waller-Duncan k-ratio t-test mean comparison (SAS Release 8.0 1999). For both the ANOVA and regression analysis, a normal probability plot (NPP) of the residuals tested normality while a fitted values versus residuals plot examined constant variance. When the assumptions of the error term (ϵ_{ij}) were violated, transformations were used; in those instances, averages shown in this paper have been back-transformed. Only results significant at $p \leq 0.05$ are discussed, unless noted otherwise.

5.4 Results

5.4.1 The F_{α} Response Rate

The fluorescence of low- O_2 -stressed fruit quickly decreased to near pre-stress levels in the presence of O_2 (i.e. < 5 min). After 30 s in O_2 , fluorescence levels showed a noticeable decrease (Figure 5.1). The same experiment performed using ‘Honeycrisp’ apples and spinach yielded similar results (data not shown).

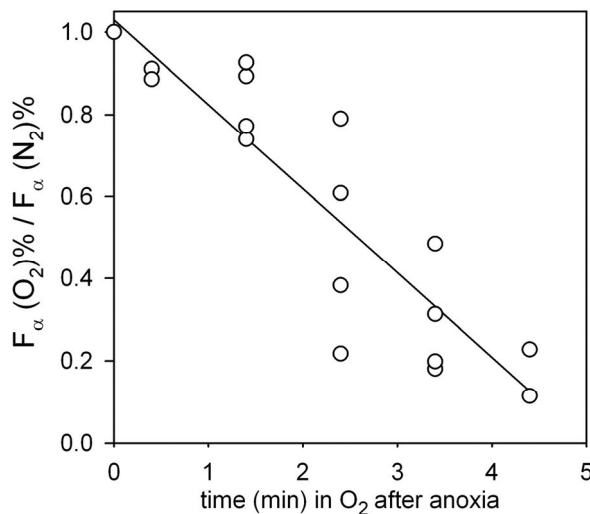


Figure 5.1 Regression analysis of the time (min) in O_2 after anoxia for ‘Granny Smith’ apples vs. the change in fluorescence observed in O_2 compared with N_2 (i.e. the % increase in fluorescence relative to pre-low- O_2 -stress shortly after anoxia divided by % increase in fluorescence relative to pre-low- O_2 -stress during anoxia: $F_{\alpha}(O_2)\% / F_{\alpha}(N_2)\%$; $y = -0.205x + 1.03$, $r^2 = 0.80$, $p \leq 0.001$).

5.4.2 F_{α} , the ACP and the EP in Apples

The ACP and the F_{α} breakpoint both occurred at ≈ 0.8 kPa O_2 in both ‘Honeycrisp’ and ‘Delicious’ shortly after harvest. The EP occurred at 20.9 and 5 kPa O_2 in the ‘Honeycrisp’ and ‘Delicious’ fruit, respectively (Figure 5.2). After 3 months in RA storage at 3.5 °C, EtOH was detected in both cultivars at normoxia and the ACP and F_{α} breakpoint for both cultivars dropped to ≈ 0.5 kPa O_2 (Figure 5.3). The fluorescence signals showed a stronger propensity to first increase, but then acclimate in response to a lowering of the O_2 in the ‘Delicious’, compared with the ‘Honeycrisp’ apples. The low- O_2 -induced rise in F_{α} was weaker after storage (Figure 5.3) compared with shortly after harvest (Figure 5.2). There was a slight decline in the fluorescence baseline in both the ‘Honeycrisp’ and ‘Delicious’ apples as the O_2 level was slowly lowered above the LOL in the stored fruit (Figure 5.3) compared with those at harvest (Figure 5.2). All fruit exhibited a post-spike dip in fluorescence below pre-stress levels after the O_2 was returned to normoxia (Figures 5.2 and 5.3).

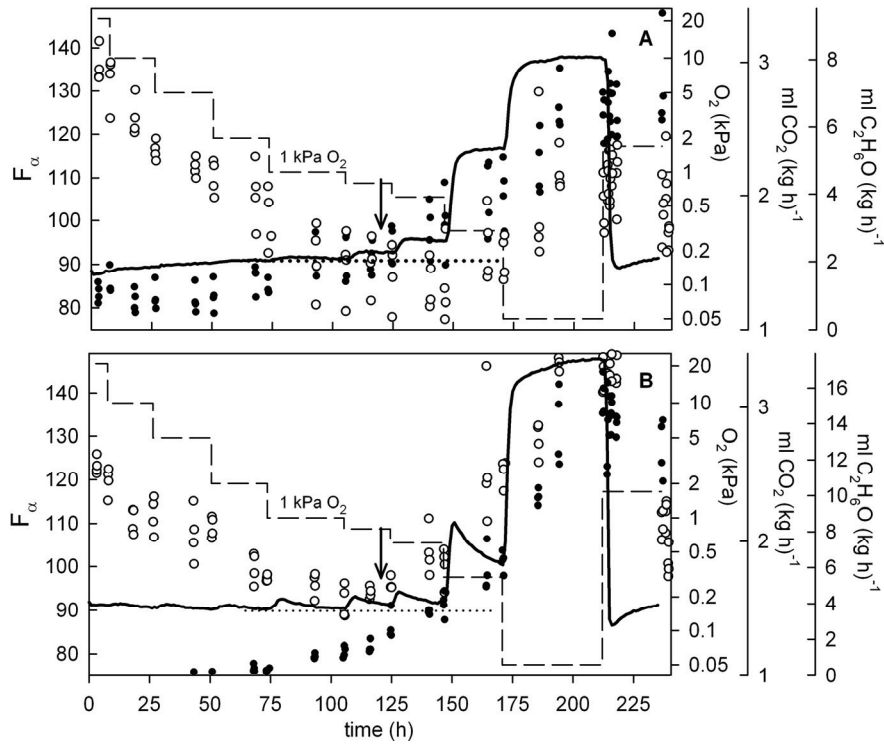


Figure 5.2 The relationship between the averaged fluorescence (F_{α}) response (solid black line) ($n = 4$), O_2 (20.9, 10, 5, 2, 1, 0.8, 0.6, 0.3, 0.05 kPa O_2) (dashed line), CO_2 (empty circles) and ethanol (C_2H_6O) (filled circles) over time (h) in 4 jars, each filled with 1.2 kg of apples stored at 3.5 °C shortly after harvest: **(A)** 'Honeycrisp', **(B)** 'Delicious'. The F_{α} signal was considered 'elevated' when it did not return to pre-stress values after 24 h. The ACP and the first

sustained rise in F_{α} occurred at 0.8 for both cultivars (arrow); EtOH was first apparent at 20.9 and 5 kPa O_2 in the 'Honeycrisp' and 'Delicious', respectively (this figure is modified from data first presented as supplementary material in Wright et al. 2011a).

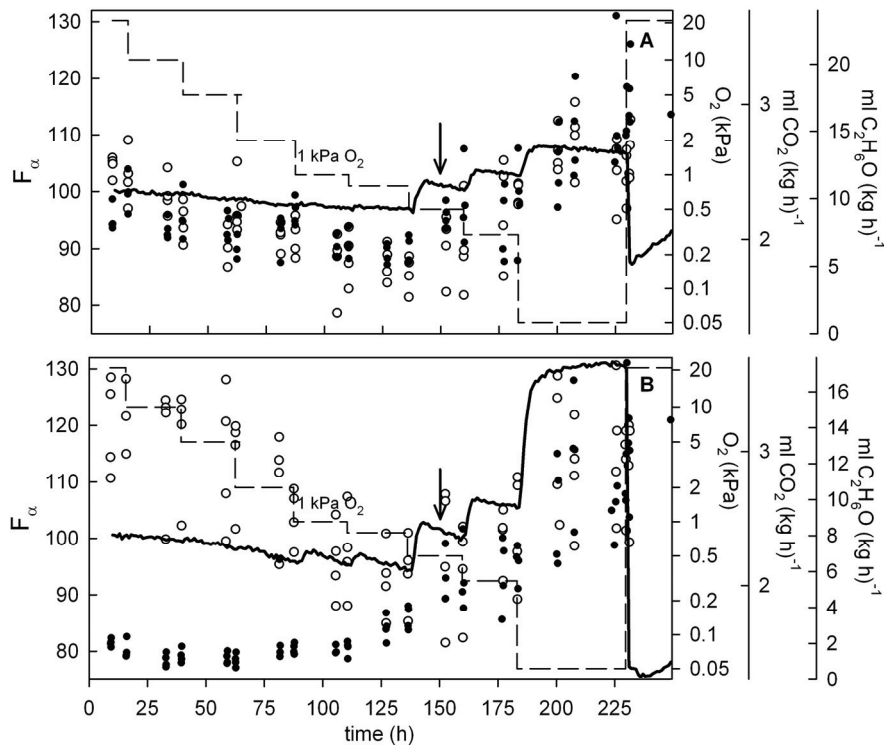


Figure 5.3 The relationship between the averaged fluorescence (F_{α}) response (solid black line) ($n = 4$), O_2 (20.9, 10, 5, 2, 1, 0.8, 0.5, 0.3, 0.05 kPa O_2) (dashed line), CO_2 (empty circles) and ethanol (C_2H_6O) (filled circles) over time (h) in 4 jars, each filled with 1.2 kg of apples and stored at $3.5^{\circ}C$ after 3 months in refrigerated air (RA) at the same temperature: **(A)** ‘Honeycrisp’, **(B)** ‘Delicious’. The F_{α} signal was considered ‘elevated’ when it did not return to pre-stress values after 24 h. In both cultivars, the

ACP and the first sustained rise in F_{α} occurred at 0.5 (arrow), while EtOH was first apparent at 20.9 kPa O_2 .

5.4.3 Sun-Exposed versus Shaded Side of Apple Fruit

The LOL was higher on the sun-exposed compared with the shaded side of the fruit in both ‘Honeycrisp’ and ‘Cortland’ (Table 5.1). The sun-exposed sides of the apples all showed an increase in F_{α} earlier, and consequently at a higher O_2 level, than the shaded sides as demonstrated using the ‘Cortland’ fruit in Figure 5.4. Both cultivars had higher SS and TA in the sun-exposed relative to the shaded side of the fruit; however, only ‘Cortland’ apples were more firm on the sun-exposed compared with the shaded side (Table 5.1).

Table 5.1 The LOL (kPa O₂), firmness (N), SS (%) and TA (mg malic acid 100 ml⁻¹) as measured on the sun-exposed and shaded side of the fruit. Means for both 'Honeycrisp' (n = 16) and 'Cortland' (n = 8) apples are shown.

'Honeycrisp' (n = 16)							
LOL		firmness		SS		TA	
Sun	shade	sun	shade	sun	shade	sun	shade
0.63 ^a	0.51 ^b	70.7 ^a	69.4 ^a	12.7 ^a	11.2 ^b	795.3 ^a	697.2 ^b
'Cortland' (source 1) (n = 8)							
1.06 ^a	0.73 ^b	69.4 ^a	63.2 ^b	13.3 ^a	11.4 ^b	831.6 ^a	723.7 ^b
'Cortland' (source 2) (n = 8)							
1.36 ^a	0.88 ^b	48.5 ^a	44.5 ^b	13.9 ^a	12.1 ^b	648.9 ^a	487.1 ^b

*Means with different letters within a given parameter row are significantly different ($p \leq 0.05$).

The 'Honeycrisp' apples had higher F_o and lower F_v/F_m readings on the sun-exposed compared with the shaded side of the fruit (combined datasets, n = 16) [F_o : 218 vs 173 ($p = 0.008$); F_v/F_m : 0.645 vs 0.741 ($p = 0.008$)] while the F_m and ΔA readings were unchanged. There was no difference in F_o , F_v , F_m , F_v/F_m or ΔA between the sun-exposed and the shaded side of the 'Cortland' fruit. Ethylene levels were 18.5% higher, the O₂ levels were 3.2% lower and the CO₂ was 16.1% higher on the sun-exposed compared with the shaded side of the 'Honeycrisp' fruit (n = 16) ($p = 0.020$, $p = 0.019$; $p = 0.038$, respectively).

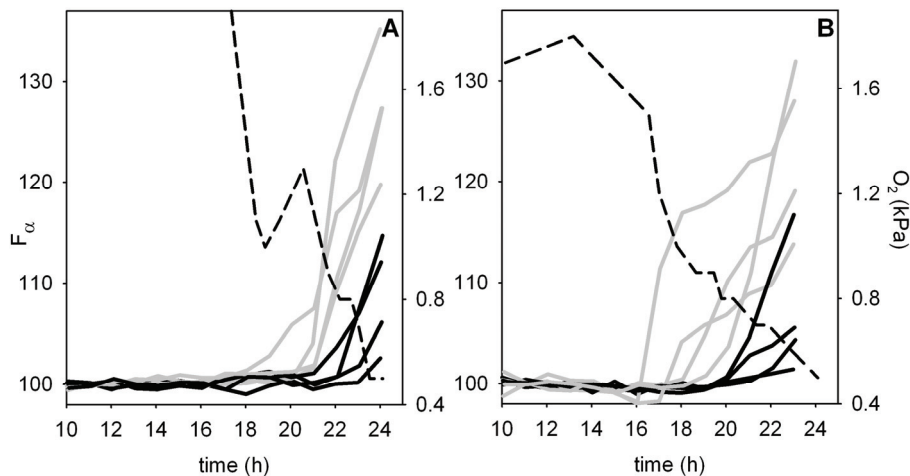


Figure 5.4 The fluorescence (F_{α}) response of the sun-exposed (grey) and shaded (black) side of individual ‘Cortland’ apples ($n = 8$) (source 1) in response to low O_2 (dashed black line). Apples were stored at 3.5°C in the dark. The side of the apple facing the sensors was staggered for the 8 fruit

(i.e. sun, shade, sun...). (A) The O_2 was slowly lowered until the fluorescence of each apple increased; the cabinet was then opened and each apple was rotated 180° relative to their sensor (i.e. shade, sun, shade...). (B) The O_2 was lowered a second time to determine the response of the alternate side of each fruit.

Combined, the two ‘Honeycrisp’ LOL datasets ($n = 16$) had a standard deviation of $\pm 24.3\%$ for the sun-exposed side of the fruit. For comparison, the mass, SS, TA and firmness of the sun-exposed side had standard deviations of $\pm 21.1\%$, $\pm 8.3\%$, $\pm 10.6\%$ and $\pm 14.6\%$, respectively. Also, the F_o , F_m , F_v , F_v/F_m and ΔA of the sun-exposed side of the fruit had standard deviations of $\pm 31.9\%$, $\pm 46.0\%$, $\pm 55.8\%$, $\pm 15.3\%$ and $\pm 39.3\%$, respectively. The respiration rate ($\text{ml CO}_2 (\text{kg h})^{-1}$) and ethylene production ($\mu\text{l C}_2\text{H}_4 (\text{kg h})^{-1}$) from a different set of 16 ‘Honeycrisp’ apples, but from the same source, differed by $\pm 24.6\%$ and $\pm 152.8\%$, respectively.

5.4.4 The Effect of Temperature on Aerobic Respiration, the LOL and Spike Intensity

The rate of aerobic respiration in ‘Honeycrisp’ apples averaged 45.0 , 23.9 and $4.6 \text{ g CO}_2 (\text{kg s})^{-1}$ at 20 , 10 and 0°C , respectively (Wright et al. 2010). The LOL and spike intensity observed at the same three temperatures averaged 0.72 , 0.33 and 0.08 kPa O_2 and 18.0 , 10.8 and $1.4 \Delta F_{\alpha}\%$, respectively (Wright et al. 2010). The Q_{10} coefficients for aerobic respiration, spike intensity and the LOL over the warmer temperature range ($20 \rightarrow 10^{\circ}\text{C}$) were all close to 2, but increased to greater than 4 over the cooler temperature range ($10 \rightarrow 0^{\circ}\text{C}$) (Table 5.2).

Table 5.2 The mean Q_{10} coefficients ($n = 4$) for aerobic respiration, the low- O_2 -induced F_a spike intensity and the LOL in ‘Honeycrisp’ apples over warmer ($20 \rightarrow 10$ °C) and cooler ($10 \rightarrow 0$ °C) temperature ranges.

Temperature range (°C)	<u>Q_{10} coefficients</u>		
	aerobic respiration	spike intensity	LOL
20 → 10	1.9	1.7	2.2
10 → 0	5.2	7.7	4.1

5.4.5 Temperature and the Fluorescence Baseline

The fluorescence baseline of the intact spinach leaves, spinach chlorophyll suspended in methanol and desiccated spinach leaves all reacted similarly showing an inverse relationship with temperature (Figure 5.5A). All trends were reversible (data not shown). All chlorophyll-containing fruits and vegetables tested thus far (e.g. apple, avocado, banana, blueberry, pear, potato, spinach) have exhibited a reversible negative correlation between temperature and their fluorescence baseline (data not shown). However, non-photosynthetic, highly reflective material such as white paper and white vinyl show a similar correlation (Figure 5.6). Experiments in which the sensors were held at a constant temperature and only the apples cooled or warmed, demonstrated the same negative correlation between temperature and fluorescence (Figure 5.5A). Work using thermocouples showed the surface of the fruit likely cooled or warmed a few degrees before the first fluorescence measurement could be obtained (data not shown).

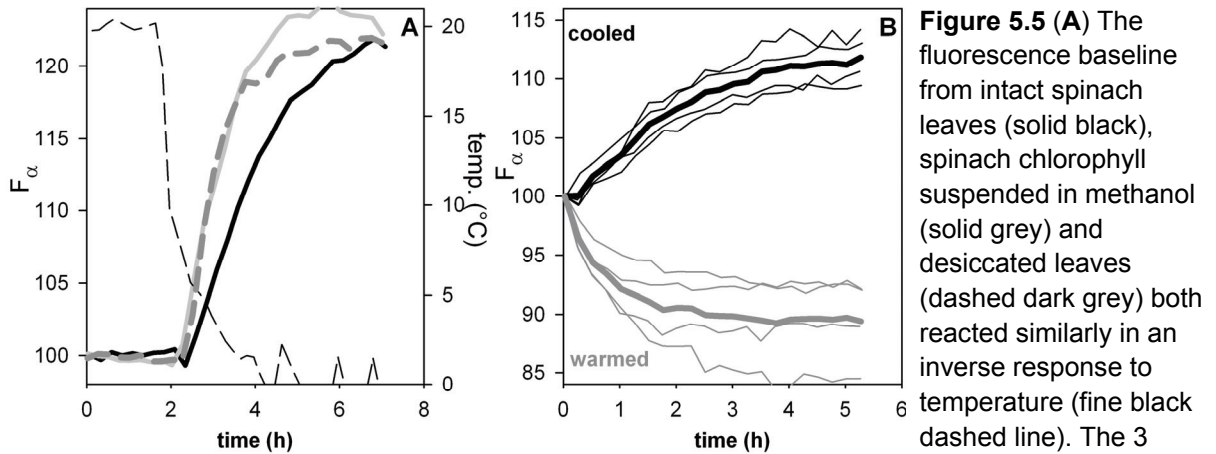


Figure 5.5 (A) The fluorescence baseline from intact spinach leaves (solid black), spinach chlorophyll suspended in methanol (solid grey) and desiccated leaves (dashed dark grey) both reacted similarly in an inverse response to temperature (fine black dashed line). The 3 fluorescence signals shown are means ($n = 4$). **(B)** ‘Honeycrisp’ apples held at 20 °C that were quickly transferred to a room set at 1 °C and allowed to acclimate in front of with PFM sensors (black lines) and vice versa (1 °C to 20 °C) (grey lines) both showed a negative correlation between fruit temperature and fluorescence while the sensor temperature was held steady. Bold lines represent means ($n = 4$).

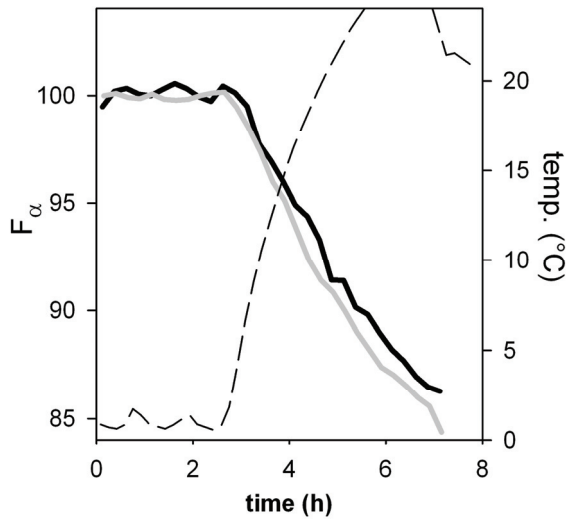


Figure 5.6 The effect of temperature (black dashed line) on the ‘fluorescence’ signals from highly reflective materials such as white vinyl (black, $n = 2$) and white paper (grey, $n = 2$).

5.4.6 Fluorescence Response to O_2 Changes Above the LOL

‘Honeycrisp’ apples underwent a 2 to 4% decrease in fluorescence when O_2 levels were rapidly lowered to a level still above the LOL (Figure 5.7). A similar outcome was observed in spinach (Figure 5.8B).

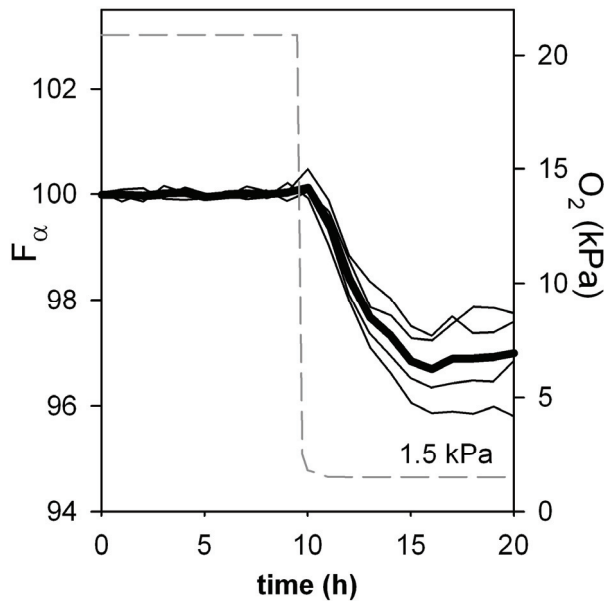


Figure 5.7 The effect of suddenly decreasing the O_2 level (grey, dashed line) in ‘Honeycrisp’ apples to a level still above the LOL (20.9 to 1.5 kPa O_2) on fluorescence. Individual fluorescence scan values are shown as solid lines with the mean scan shown in bold ($n = 4$).

5.4.7 Low O_2 and the Xanthophyll Cycle

The antheraxanthin, zeaxanthin and xanthophyll de-epoxidation state (DEPS) $(Z + 0.5A)/(V + A + Z)$ was greater in the spinach leaves held under anoxia compared with those under normoxia while being monitored via the PFM sensors ($p \leq 0.001$). There was no difference in the violaxanthin levels between treatments ($p = 0.987$) (Table 5.3).

Table 5.3 The xanthophyll content ($\text{nmol}\cdot\text{g}^{-1}$) and the DEPS $(Z + 0.5A)/(V + A + Z)$ found in spinach leaves held in the dark and monitored via PFM ($0 \rightarrow 10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over 58 s every 10 min) at 20.9 and 0 kPa. Each mean value represents $n = 8$.

O_2 (kPa)	Viol ($\text{nmol}\cdot\text{g}^{-1}$)	Anth ($\text{nmol}\cdot\text{g}^{-1}$)	Zea ($\text{nmol}\cdot\text{g}^{-1}$)	DEPS
20.9	381.4 ^a	15.7 ^b	24.0 ^b	0.08 ^b
0	381.8 ^a	23.9 ^a	85.5 ^a	0.20 ^a

*Means with different letter groupings within a column are significantly different ($p \leq 0.05$).

5.4.8 Low O_2 Fluorescence and Dithiothreitol (DTT)

When the O_2 was lowered from 20.9 to 1 kPa (i.e. above the LOL), the F_α signal of the control spinach leaves decreased slightly while DTT-treated leaves increased (Figure 5.8A, 5.8B). When the O_2 was lowered from 20.9 to 0 kPa, the control leaves first decreased slightly (i.e. a pre-spike dip) before increasing greatly, but at a rate less than the leaves treated with DTT (Figure 5.8A, 5.8C). When the O_2 was returned to normoxia, the fluorescence in the control leaves underwent a post-spike dip while the leaves treated with DTT did not (Figure 5.8A, 5.8D).

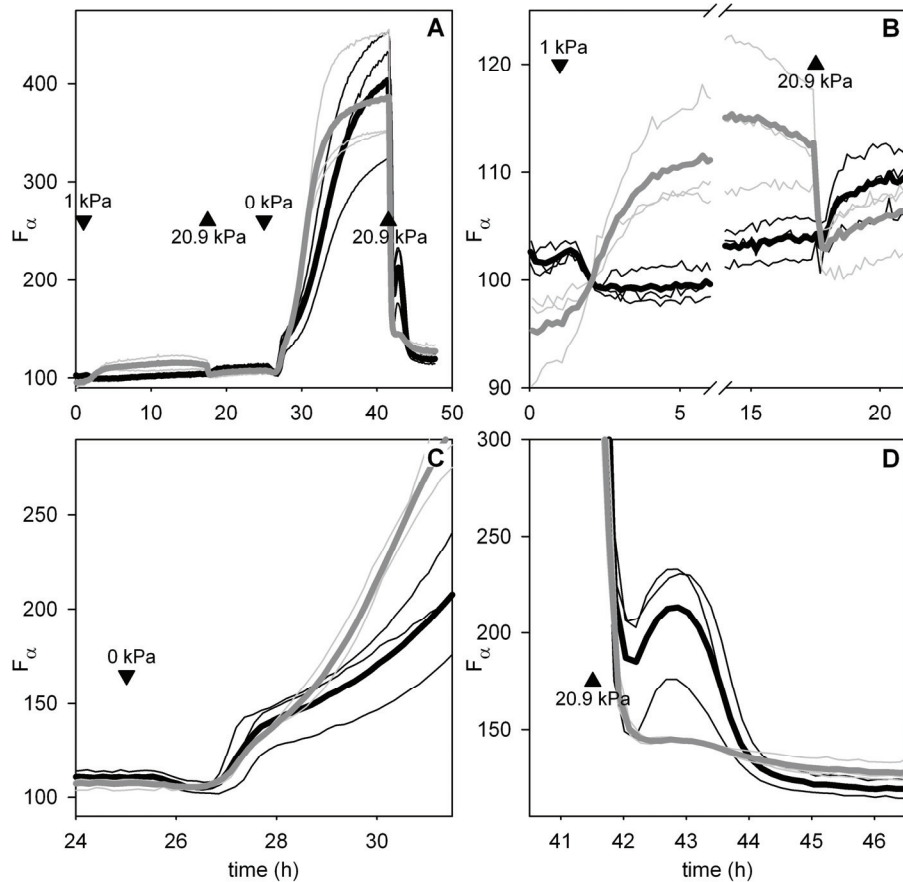


Figure 5.8 Plots showing the fluorescence response to hypoxic and anoxic stress of spinach leaves treated with 5 mM DTT compared with control. **(A)** The O_2 was lowered from 20.9 to 1 kPa, increased from 1 to 20.9 kPa, lowered from 20.9 to 0 kPa and increased from 0 to 20.9 kPa. **(B)** When the leaves were subjected to hypoxic conditions (20.9 to 1 kPa O_2), the fluorescence from the DTT leaves increased moderately while the control leaves decreased slightly; the opposite occurred when the O_2 was returned to normoxia (20.9 kPa). **(C)** When the leaves were subjected to anoxic conditions (20.9 to 0 kPa O_2), both treatments appeared to begin to increase at approximately the same time; however, the rate of incline of the fluorescence given off by the DTT leaves appeared greater and plateaued earlier than the control. **(D)** When the leaves were returned to normoxia after an anoxic event, the fluorescence from the control leaves decreased, but then increased temporarily, before reaching pre-stress conditions; the fluorescence from the DTT leaves simply decreased to pre-stress conditions. Indicated decreases (\blacktriangledown) and increases (\blacktriangle) in O_2 denote the beginning of gas levels changes, which occurred at a rate of $\approx 0.58 \text{ ml}\cdot\text{s}^{-1}$. Control leaves ($n = 3$) are shown in black while DTT leaves ($n = 3$) are shown in grey; the mean scan of each treatment is shown in bold.

5.4.9 Variable LOL within a Cultivar and the Light Effect

As the O_2 was lowered in the CA cabinet, the fluorescence from ‘Granny Smith’ apples, which varied in size and apparent maturity based on greenness, increased over a gradient of O_2 levels (Figure 5.9). When the O_2 was above an apple’s LOL, the ‘Night Light’ caused the fluorescence to increase slightly. However, once the O_2 level dropped below an apple’s LOL and the fluorescence increased, the ‘Night Light’ caused the fluorescence to decrease

significantly (Figure 5.9A). The ‘Night Light’ effect was replicated in numerous apple cultivars as well as in spinach leaves (data not shown). Ethylene evolution was correlated with respiration; the largest 3 fruit had low ethylene levels ($\approx 1 \mu\text{l C}_2\text{H}_4 (\text{kg h}^{-1})$) and were considered post-climacteric, while the smallest 3 fruit were climacteric with very high ethylene levels ($\approx 10 \mu\text{l C}_2\text{H}_4 (\text{kg h}^{-1})$) (data not shown). The LOL of each apple correlated with its respiration rate (Figure 5.9B). When the O_2 was again lowered on the same fruit after 10 days of acclimation at 3 kPa O_2 , the LOL of each fruit decreased by an average of 0.30 ($p = 0.013$).

In the ‘Cortland’ experiment, the ‘Night Light’ induced a relatively homogenous increase in fluorescence ($\approx 2\%$) between replications when the O_2 was held above the LOL (Figure 5.10A). When the O_2 was held below the LOL, the fluorescence increased; in these fruit the ‘Night Light’ caused the fluorescence to decrease if the original low- O_2 -induced increase in fluorescence was greater than that induced by the ‘Night Light’ under unstressed conditions (i.e. 2%). In fruit in which the low- O_2 -induced increase in fluorescence was less than 2%, the ‘Night Light’ induced only a small increase, also less than 2% (Figure 5.10B).

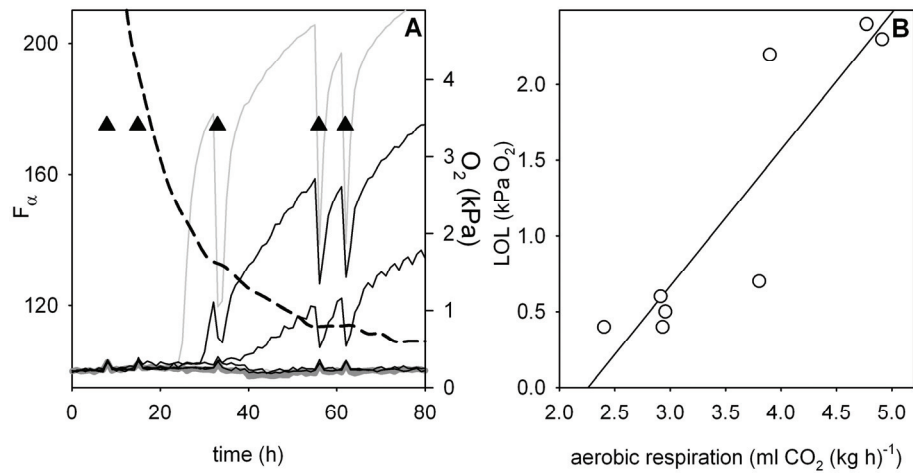


Figure 5.9 (A) The relationship between fluorescence (left axis; each solid line represents an individual ‘Granny Smith’ apple, $n = 8$), O_2 (right axis; dashed line) and light (▲ indicates the ‘Night Light’ was turned on 5 min prior to the next scan and was then turned off after the scan). When the O_2 level was above the LOL for all apples, the ‘Night Light’ induced a small increase in the fluorescence signal from all fruit (e.g. between 0 and 20 h; $O_2 > 2$ kPa). When the O_2 level was lowered further (e.g. between 2 and 0.7 kPa), the fluorescence in the dark from certain fruit began to increase while others remained stable. The ‘Night Light’ significantly decreased the fluorescence in fruit with an elevated F_α value while there was a small increase in the fluorescence of those which had remained stable. **(B)** Regression analysis showing a correlation between aerobic respiration ($\text{ml CO}_2 (\text{kg h})^{-1}$) and the LOL in individual apple fruit ($y = 0.90x + 2.04$, $r^2 = 0.78$, $p = 0.002$).

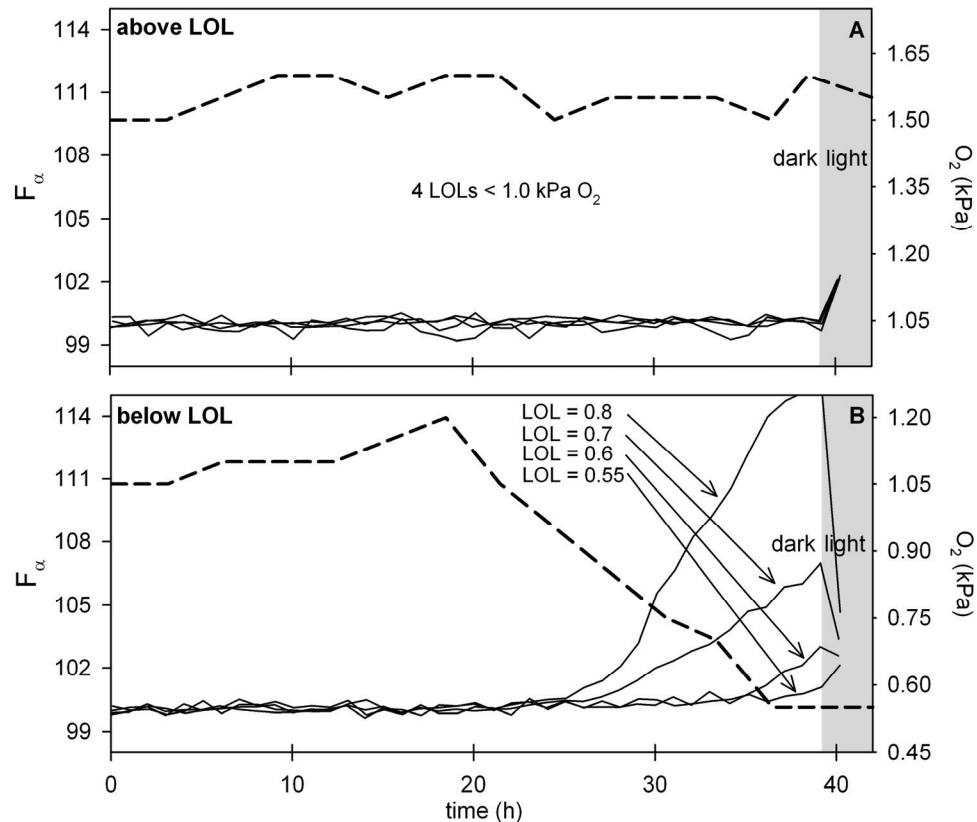


Figure 5.10 Plots showing the relationship between the fluorescence (F_{α}) emitted from dark-adapted ‘Cortland’ apples (solid lines) in CA storage, O_2 (dashed line) and low-intensity light ($< 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$). Each solid line represents the fluorescence emitted from 6 apples inside a kennel fitted with a single PFM fluorometer. The O_2 signal is the mean between the two CA cabinets housing the 4 kennels; the individual LOL values were determined using each PFM sensor’s respective cabinet O_2 value. **(A)** In the cabinets where the O_2 level was held above the LOL (all 4 $< 1.0 \text{ kPa } O_2$), the application of the low-intensity light induced a relatively homogenous increase in the apple fluorescence of $\approx 2\%$. **(B)** In the cabinets where the O_2 was gradually lowered, the apple F_{α} signals exhibited inherent variation by increasing over a gradient of O_2 levels (0.8, 0.7, 0.6 and 0.55 $\text{kPa } O_2$). The increase in the F_{α} signal was proportional to the degree the O_2 was brought below the level where the initial increase in F_{α} was observed (i.e. the LOL). For apples that had a low- O_2 -induced increase in F_{α} greater than 2% (the light-induced increase observed in apples held above their LOL), the application of the low-intensity light quenched F_{α} ; the quenching was proportional to the degree the original low- O_2 -induced increase in F_{α} was greater than 2%. For fruit held only slightly below their LOL, exhibiting a rise of $< 2\%$, the low-intensity light did not quench, but instead increased $F_{\alpha} < 2\%$ so that the total rise in fluorescence (low- O_2 -induced + light-induced) was $\approx 2\%$ [e.g. apples held at 0.55 $\text{kPa } O_2$ induced a small increase in F_{α} ($\approx 1\%$); the addition of the low-intensity light induced an additional increase of $\approx 1\%$ for a total increase of $\approx 2\%$].

5.5 Discussion

5.5.1 Metabolism and Fluorescence

The fluorescence-based DCA system detects the LOL, a critical point in the transition from aerobic to fermentative metabolism, and correlates with respiratory changes (Gasser et al.

2008, 2010; Wright et al. 2011b; Figures 5.2 and 5.3), suggesting an intrinsic relationship between aerobic respiration (which occurs in the mitochondria), ethanol fermentation (occurring in the cytosol) and the rise in F_o (and F_a) (emanating from the chloroplasts). Both the ACP and RQB allow for some fermentative activity (Peppelenbos and Oosterhaven 1998; Figures 5.2 and 5.3) and often occur at a similar O_2 level in apples (Peppelenbos and Rabbinge 1996). Past research suggests that an anaerobic environment, even in darkness, leads to an over-reduction of the chloroplast's PQ pool (Harris and Heber 1993; Bennoun 2002; Tóth et al. 2005; Wright et al. 2011a). Wright et al. (2011a) speculated that low O_2 leads to an overabundance of cytosolic reductant and a loss of homeostasis as a result of a marked decline in the mitochondria's cytochrome oxidase; excess reductant is then transported into the chloroplast where it is used to reduce the plastoquinone (PQ) pool. As a result, photochemical quenching decreases accompanied by a rise in the 'perceived' F_o . The degree to which the measured F_o increases under low O_2 is proportional to the degree the O_2 level is held below the LOL. If correct, what is actually being measured is a transition from F_o to a higher relative fluorescence (F) value where $q_p < 1$. For simplicity, the switch from F_o to a higher F value will be referred to as a rise in the measured F_o (or F_a). The experimental results discussed below will be in the context of this theory.

The effect that a low-intensity light has on the fluorescence signal is consistent with the theory that the F_a signal reflects the redox state of the PQ pool. Under normoxic or hypoxic conditions above the LOL, a low-intensity light induces a small, but significant, increase in the fluorescence baseline (e.g. a $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ light source increased $F_a \approx 4\%$ in 'Granny Smith' apples and $\approx 2\%$ in the 'Cortland' apples used in this study; the amount is likely affected by the species, cultivar, region and product history) (Figures 5.9A and 5.10A). The increase in the dark-adapted F_a value under low-intensity light likely reflects a slight reduction in the PQ pool (and a switch from F_o to F_s). When the O_2 level is well below the LOL and F_a is highly elevated in the dark, as a result of an over-reduction of the PQ pool, the light causes the elevated F_a measurement to decline significantly. The degree of quenching is generally proportional to the original low- O_2 -induced increase in F_a (Figures 5.9A and 5.10B). Under these conditions, it is speculated that light-activation of PSI oxidizes the over-reduced PQ pool thereby increasing the quantum yield of PSII and the measured F_a decreases (Harris and Heber 1993; Wright et al. 2011a). The effect of a low-intensity light on the F_a signal is slightly more complicated when the O_2 level is held at or

very near the LOL. If the O₂ level is set only marginally below the LOL thereby inducing a very small increase in F_α (i.e. < 2%), the addition of a low-intensity light will likely increase, not decrease, the fluorescence as the light-induced effect on the redox state and F_α signal (e.g. ≈ 2% in the case of the ‘Cortland’ fruit used in this study) is greater than the low-O₂-induced effect on F_α (Figure 5.10). The use of a low-intensity light could be used as a tool to help DCA operators determine whether they are above or below the LOL. Protocols for the use of such tools would need to be standardized: varying the duration or the magnitude of the light intensity can vary fluorescence results due to their effects on the PQ pool redox state and non-photochemical quenching (data not shown). The rapid quenching of the low-O₂-induced rise in F_α by a reintroduction of O₂ (Figure 5.1) is believed to restore photochemical quenching capacity by either immediately decreasing cellular reductant levels via increased mitochondrial respiration or through chlororespiration.

Previous work by Wright et al. (2010) showed that respiration, as affected by temperature, is positively correlated with the measured fluorescence-based LOL in ‘Honeycrisp’ apples. Higher respiration increases demand for O₂ which means that O₂ availability likely becomes critical at a higher O₂ level. A re-analysis of the data published by Wright et al. (2010) showed the Q₁₀ coefficients for the respiration rate, low-O₂-induced rise in F_α and the LOL were approximately 2 between 10 to 20 °C, but increased to >4 between 0 and 10 °C (Table 5.2), i.e. all three parameters reflect temperature-related enzyme kinetics (Graham and Patterson 1982). The results from the present study showed the inherent range of respiration rates for a given cultivar at a particular temperature may also sometimes correlate with the measured LOL (Figure 5.9B). However, further research is needed and the authors caution that this respiration-based explanation for the LOL is likely overly-simplified. Other factors besides respiration, such as epidermis permeability, the rate of gas diffusion within the tissue, energy and enzyme levels and reaction rates likely affect the LOL as well. It is also important to note that the LOL within a product, as observed in this study, often acclimates over time or with extended exposure to hypoxic environments. Thus, future studies on how different fruits and vegetables acclimate to low O₂ over time could be used to improve DCA and CA storage protocols.

Like fruit mass, colour, sugar or acid levels, the LOL is inherently variable, even among apples (for example) from a given cultivar and tree or between the sun-exposed and shaded regions of a single fruit. Within a fruit, the sun-exposed and shaded side of an apple are

physiologically different. For example, the xanthophyll and anthocyanin content are typically higher on the sun-exposed relative to the shaded side of an apple which imparts higher tolerance to photoinhibition (Ju et al. 1996; Ma and Cheng 2003). Past studies have shown light can also affect the distribution of sugars and acids within an apple (Leonard and Dustman 1943; Clijsters 1969) and that the net photosynthetic O₂ evolution is higher on the sun-exposed than the shaded side of the fruit under similar light intensities (Chen et al. 2009). In the present study, the higher SS and TA on the sun-exposed side of the fruit may reflect higher metabolic activity and so explain the elevated LOL (Table 5.1; Figure 5.4). Also, higher ethylene and CO₂ evolution levels on the sun-exposed side of the ‘Honeycrisp’ fruit used in this study supports this hypothesis. However, lenticel density and skin permeance may also be different between the two apple sides and so confound these results. More research should be done to understand why the sun-exposed side of an apple is apparently more sensitive to low-O₂ stress relative to the shaded side. If verified, it should be recommended that DCA operators position the more vulnerable sun-exposed side of the fruit towards the sensors.

The finding that the LOL can differ between the sides of a fruit raises the question: what is the fluorescence measuring? Does it relay the stress of the overall fruit or vegetable, a region of it, or just the chlorophyll-containing epidermis? What is known is that intercellular plasmodesmal transport and membrane permeability increases with anaerobic stress (Cleland et al., 1994; Xu et al., 2004). This response may function in fermentative cells as a means of increasing the import of ATP or products needed for ATP synthesis from more aerobic cells nearby, or to facilitate the export of excess reductant. The chlorophyll-containing cells in the epidermis of the fruit (in the case of apples) may be akin to the “canary in the coal mine”. These cells may signal a metabolic alarm in the form of increased fluorescence when their reductant levels, which may be partially imported from neighbouring cells, are too high. Future research should study reductant transport (i.e. is it active or passive?) and variability within a fruit or vegetable, and within a cell, under low-O₂ stress.

5.5.2 Low-O₂ and the Xanthophyll Cycle

Recent work reported by Wright et al. (2011) shows that low O₂ increases zeaxanthin levels, primarily as a result of violaxanthin de-epoxidation, even in the presence of low intensity light (as demonstrated using spinach and apple skin). The present study showed the intermittent, low-intensity red LEDs of the PFM fluorometer were intense enough to induce

appreciable zeaxanthin accumulation in spinach within hours (Table 5.3). It is speculated that low-O₂-induced violaxanthin de-epoxidation and zeaxanthin accumulation may occur in dark or near-dark storage over time; light, even of a very low intensity, may accelerate or enhance these changes. A slight dip in the fluorescence baseline has been noted in some plant products when the O₂ level is decreased to levels still above the LOL (Figures 5.7 and 5.8B). An accumulation of zeaxanthin and an increase in non-photochemical quenching could account for this decrease in fluorescence; however, other explanations, such as state transitions, may also be a factor. The degree of this response may differ depending on the species, cultivar or the product history. Spinach leaves treated with DTT, an inhibitor of the xanthophyll cycle, showed that fluorescence did not decrease, but instead increased slightly under hypoxic conditions (1 kPa O₂) measured to be above the LOL in control leaves. This suggests the zeaxanthin formation and its associated non-photochemical quenching may be masking a low-level increase in reductant levels associated with a small increase in F_a. The fluorescence-based LOL in control leaves must then indicate a reductant level too great to be masked by increased zeaxanthin and non-photochemical quenching. Under anoxic conditions, the fluorescence increase in DTT-treated leaves was faster with no post-anoxic dip compared with control leaves (Figure 5.8). These results suggest zeaxanthin formation may also be a factor in the post-spike dip sometimes observed in fluorescence studies (Figures 5.2, 5.3 and 5.8D). However, DTT may also affect the fluorescence in other ways due to its reductant chemistry thereby confounding this interpretation; further research is needed.

The xanthophyll cycle may partly contribute to the positive correlation between the scan interval (i.e. the time between successive fluorometer measurements) and the low-O₂-induced spike intensity previously reported by Wright et al. (2010). A shorter scan interval (i.e. increased light intensity per unit of time) increases the level of zeaxanthin accumulation and non-photochemical quenching, and may contribute to the observed decrease in the low-O₂-induced spike (Wright et al. 2010). However, a more dominant factor may be the relationship between increased light intensity and photochemical quenching. As discussed earlier, light decreases an elevated F_a signal (Figures 5.9A and 5.10B), likely the result of a PSI-driven oxidation of the PQ pool and increased photochemical quenching. Employing a longer scan interval therefore increases the chance of detecting a low-O₂-induced rise in fluorescence. An alternative to increasing the fluorometer scan interval would be to simply

turn the fluorometer off for several hours if the O₂ level is believed to be near the LOL. The prolonged absence of the fluorometer's light emission allows reductant to collect in the PQ pool; if the fluorescence increases markedly when the fluorometer is turned back on it may indicate the O₂ level is below the LOL.

The effect of different storage treatments on the pigment and antioxidant levels found naturally within fruits and vegetables could have commercial implications. For instance, the shaded side of apple fruit is often reported as being more susceptible to skin disorders in storage like superficial scald (Bramlage 1988; Ju et al. 1996; Rodikov 2008). Superior scald protection on the sun-exposed side of the fruit may be related to reports of elevated xanthophyll and anthocyanin levels, and a diminished susceptibility to photoinhibition, relative to the shaded side (Ju et al. 1996; Ma and Cheng 2003; Li and Cheng 2008). The development of superficial scald, which has been associated with ethylene levels and the oxidation of α -farnesene, has been effectively treated with the postharvest application of antioxidants, but may also be associated with other factors (Watkins et al. 1993; Du and Bramlage 1994). As DCA advocates below normal O₂ levels which lowers ethylene levels (DeLong and Prange 2003), it is a popular means of scald control, particularly for markets demanding fewer agri-chemical residues. The finding that low O₂ levels induces increased zeaxanthin in the skin of apples under low-light conditions (Wright et al. 2011a) prompts some interesting questions: does low O₂ also protect against some epidermal/hypodermal disorders as a result of a natural increase in antioxidants (i.e. zeaxanthin)? If so, can storage protocols, such as initial low oxygen stress (ILOS), be enhanced further to capitalize on this natural antioxidant activity? Compared with the other xanthophylls, zeaxanthin has high antioxidant activity, even when not bound to PSII (Havaux et al. 2007).

5.5.3 Temperature and Fluorescence

Although cold temperatures can induce stress and account for some fluorescence changes under certain circumstances, cellular physiology does not appear to be the dominant factor in the temperature effects observed in this study. Experiments with intact spinach leaves, spinach chlorophyll suspended in methanol and desiccated leaves showed similar responses to temperature change (Figure 5.5A). Previous work demonstrates a similar relationship exists in apples (Wright et al. 2010). The relationship between temperature and the fluorescence baseline has been observed in both PFM and PAM fluorometers (Wright et al. 2010) and is commonly accounted for in marine algae and plankton fluorescence studies

(Weber et al. 1986). These findings also suggest that the relationship may result from a temperature effect on the fluorometers. Condensation can affect electronics and digital circuitry; however, the fluorescence response to temperature is comparable regardless of whether the temperature changes from warm to cold or cold to warm (Wright et al. 2010). Electrical conductivity is negatively correlated with temperature in metals, but positively correlated in semi-conductors. However, studies where the temperature of the fruit was changed while the temperature of the sensors remained relatively unchanged suggests the primary cause results from the effect of temperature on the fruit and not on the sensors (Wright et al. 2010; Figure 5.5B). An alternative possibility may be that temperature-induced fluorescence baseline changes result from modifications in quantum state populations, which vary exponentially with temperature $[e^{(hc)/(kT\lambda)}]$; T – temperature, h - Planck’s constant, c - speed of light, λ - wavelength of the incident light]. However, highly reflective material (non-plant-based materials included), which some fluorometers can misinterpret as fluorescence, have also shown a negative correlation between temperature and measured “fluorescence” (i.e. reflectance) (Figure 5.6). The reasons for these observations may be in how pulse-based fluorometers interpret infrared light as well as reflectivity, absorptivity and emissivity properties that may change with temperature. None of these suggestions necessarily rule out the possibility that fluorescence may be affected by temperature as a result of physiological changes in some instances; however, physiology does not appear to be the dominant factor in the present study. Further research is needed.

Chapter 6: Conclusions

The interrelationship between aerobic respiration, fermentation and fluorescence appears congruous with the theory proposing the mechanistic link is excess reductant transported between the mitochondria, cytosol and the chloroplasts, respectively. The theory states that as the O_2 level is lowered, eventual limitations at cytochrome oxidase in the mitochondria causes a buildup of glycolytic reductant in the mitochondria and cytosol. At low levels this reductant may be entirely recycled via fermentation and limited mitochondrial respiration. However, at higher levels, the excess reductant is transported into the chloroplast and the plastoquinone (PQ) pool becomes over-reduced. This may result from some inherent redox threshold being met, a more pronounced decrease in mitochondrial respiration combined with an energy crisis or a general loss of homeostasis and increased membrane permeability. Whether this transport is active or passive is for future research to determine.

The reduction of the PQ pool is likely facilitated via chlororespiratory enzymes. If the increase in minimum fluorescence (F_0) is the result of an over-reduced PQ pool, what is often described as a low- O_2 -induced rise in F_0 , would actually be a shift from F_0 to a higher relative fluorescence (F) as $q_p < 1$. A strong correlation between temperature, respiration rate and the lower oxygen limit (LOL), with similar Q_{10} coefficient dynamics, suggests the respiration rate and the LOL are both dependent on enzyme activity. An increased respiration rate may result in a higher LOL and increased spike intensity simply as a result of an increased O_2 demand and potential for generating excess reductant. A decrease in F_0 as a result of a reintroducing O_2 may be evidence of chlororespiration. The finding that even low-intensity light can increase F_0 slightly at O_2 levels above the LOL and strongly quench it a levels below the LOL is consistent with the theory that these changes in F_0 reflect the redox state of the PQ pool and the q_p coefficient of PSII. At O_2 levels above the LOL, light partially reduces the otherwise fully oxidized PQ pool via PSII, the degree of q_p decreases and F_0 appears to increase by transitioning to a higher F level. At O_2 levels below the LOL the PQ pool is over-reduced and a light-driven PSI oxidation of the PQ pool causes q_p to increase and the fluorescence to decrease from an elevated F level to something nearer a true F_0 as the q_p coefficient becomes closer to 1.

The xanthophyll cycle is engaged under low- O_2 conditions in the presence of even low-intensity light. The first evidence of violaxanthin de-epoxidation occurs at O_2 levels above the fluorescence-based LOL; this may coincide with the first signs of increased cytosolic

reductant or a lowering of the pH, or both, but this requires further research. As the O_2 is further lowered below the LOL, violaxanthin and zeaxanthin continue to decrease and increase, respectively. Increases in zeaxanthin are well-correlated with increases in q_N . The engagement of the xanthophyll cycle suggests a photoinhibition-like restriction in PSII, which is in agreement with the theory that low O_2 causes an over-reduction of the PQ pool. Violaxanthin de-epoxidation was found to require some light, though light of a very low-intensity was sufficient. Evidence of violaxanthin de-epoxidation suggests a decrease in the pH, a requirement for the function of the violaxanthin de-epoxidase enzyme; a decrease in pH is also associated with low- O_2 , fermentation and low energy. The xanthophyll cycle is reversed (zeaxanthin epoxidation) when the plant material is reintroduced to O_2 . In spinach, a decrease in zeaxanthin could be observed within minutes of re-oxygenation, but levels continued to decline for hours and may continue to do so for days. Although the xanthophyll cycle is not believed to be associated with the low- O_2 -induced increase in F_o , it may explain other fluorescence phenomena as described below.

A new appreciation of the relationship between metabolism and fluorescence broadens our understanding and the utility of dynamic controlled atmosphere (DCA) in the commercial setting. The pulse frequency modulated (PFM)-based F_a parameter is an approximation of F_o ; however, the parameter may also be mildly influenced by a F component ($q_p \leq 1$, $q_N \geq 0$) in the later part of a typical 58 s scan. The slight decrease in F_a sometimes observed when the O_2 is lowered from normoxic conditions to an O_2 level still above the LOL, as well as the pre- and post-spike dips sometimes observed around a sub-LOL event, may be related to the xanthophyll cycle; however, other explanations, such as a state transition, are possible. The effect that light has on the sub-LOL F_o signals is likely a major factor in the positive correlation between the fluorometer scan interval and the low- O_2 -induced rise in F_a . When the time between fluorescence scans is shortened, the low- O_2 -induced rise in F_a is likely quenched due to PQ pool oxidation via PSI from the increased PPFd per unit time from the fluorometer lights; however, increased violaxanthin de-epoxidation and q_N may also be a factor.

New fluorescence-based storage management techniques may be commercially-adopted based on our improved understanding of the physiological relationship between metabolism, fluorescence and stress. Understanding the inherent variability that exists in a fruit's physiology and LOL from a single source, or even within a single fruit, sheds light on the

need to account for variability when determining what constitutes an appropriate representative sample when using DCA in a storage room. The finding that the sun-exposed side of the fruit is often metabolically more advanced, which is apparently reflected as a higher fluorescence-based LOL, compared with the shaded side of the fruit suggests the sun-exposed side is more vulnerable to low-O₂ stress. Ensuring the sun-exposed side of a fruit is directed towards the fluorometer sensors will assumedly provide a more conservative estimate of low-O₂ tolerance; however, this interpretation demands more research. The use of a periodic background light enhances our ability to pinpoint the fluorescence-based LOL. This technique will be useful for more aggressive forms of DCA management that could increase the benefits of DCA storage. Monitoring the fluorescence response to a periodic background light over time has potential benefits in tracking the gradual deterioration of fruits and vegetables in both CA and regular air storage and to assist in making decisions for timely removals. Although it is now believed that the negative correlation between the temperature and the fluorescence baseline is not solely governed by physiology, monitoring the influence of a periodic background light on the fluorescence trace does hold potential for the improved detection of chilling stress. The finding that low-O₂ improves antioxidant levels in the form of zeaxanthin has the potential to improve the health benefits as well as storability for certain fruits and vegetables. Future work may show the adjustment of storage protocols, such as increased storage light or a pre-withdrawal anoxic or high light period, may capitalize on this relationship. With the rising pressure in the world to decrease its inputs to its food supply management chain, in the form of transport and chemicals, and to decrease waste, work that continues to build upon our understanding of fluorescence, reductant transport, pigmentation, cellular energy levels and their relationship to the storability of fruits and vegetables is increasingly relevant.

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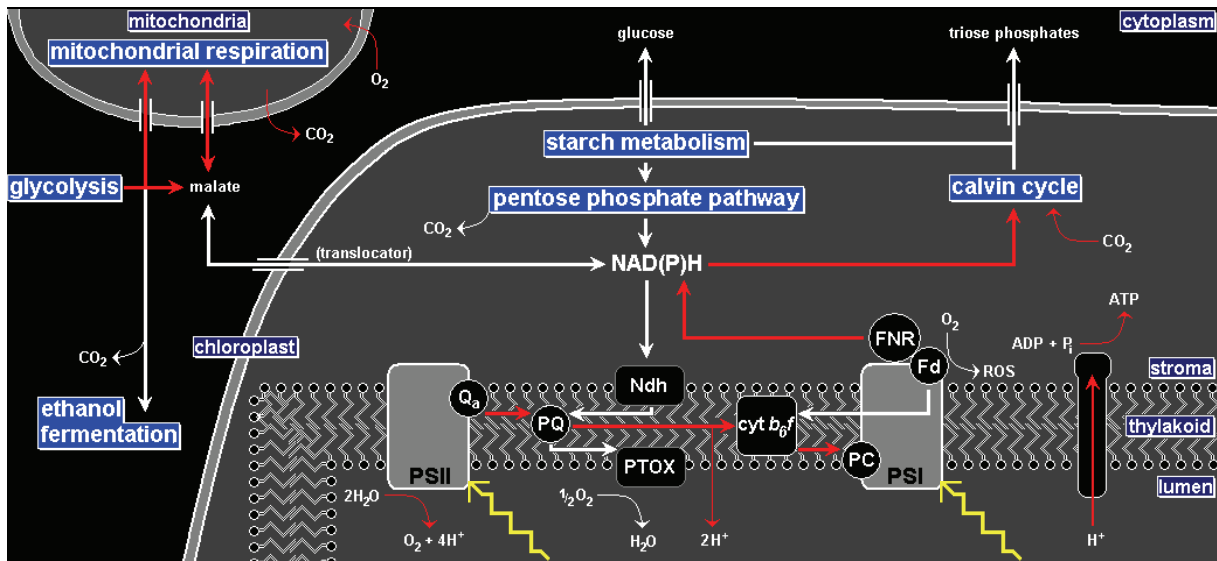
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Appendix A – Metabolic Pathways^{XIII}

Appendix A i – Aerobic Respiration / Non-Cyclic ‘Z Scheme’

Appendix A i – Aerobic Respiration / Non-Cyclic ‘Z scheme’

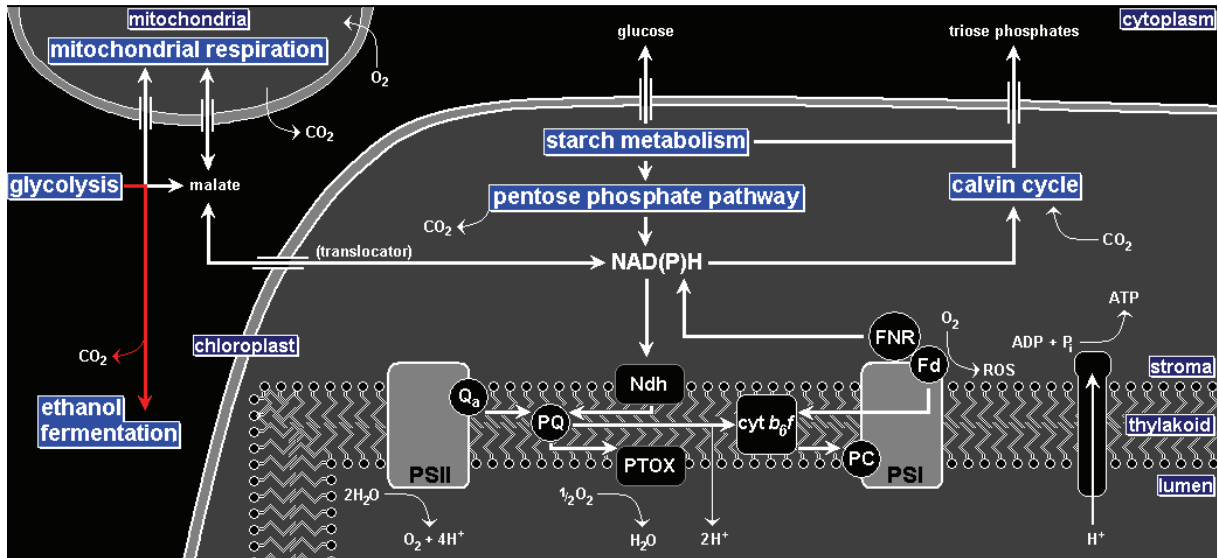


abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt *b₆f*, cytochrome *b₆f* complex; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ reductase; NADH, nicotinamide adenine dinucleotide ; NADPH, nicotinamide adenine dinucleotide phosphate; Ndh, NADPH-dehydrogenase; OAA, oxaloacetate; PC, plastocyanin; P_i, phosphate; PQ, plastoquinone; PTOX, chloroplast terminal oxidase; Q_A, plastoquinone Q_A; ROS, reactive oxygen species

^{XIII} An animated version of each appendix can be found online at <http://dalspace.library.dal.ca/dspace>.

Appendix A ii – Ethanol Fermentation

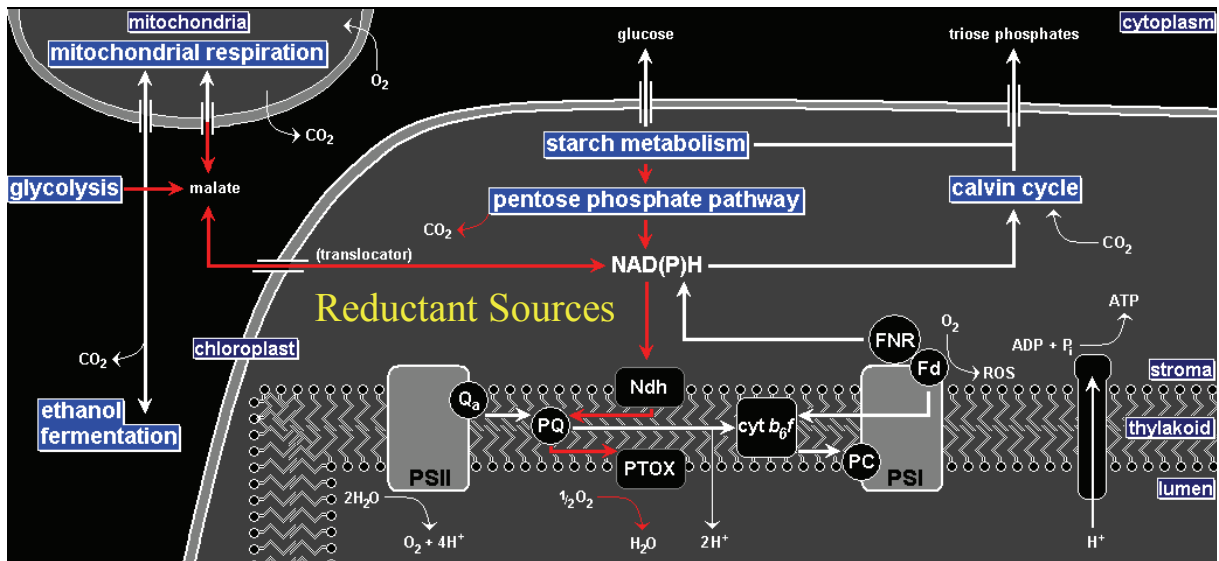
Appendix A ii – Ethanol Fermentation



abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt *b₆/f*, cytochrome *b₆/f* complex; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ reductase; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; Ndh, NADPH-dehydrogenase; OAA, oxaloacetate; PC, plastocyanin; P_i, phosphate; PQ, plastoquinone; PTOX, chloroplast terminal oxidase; Q_A, plastoquinone Q_A; ROS, reactive oxygen species

Appendix A iii – Chlororespiration

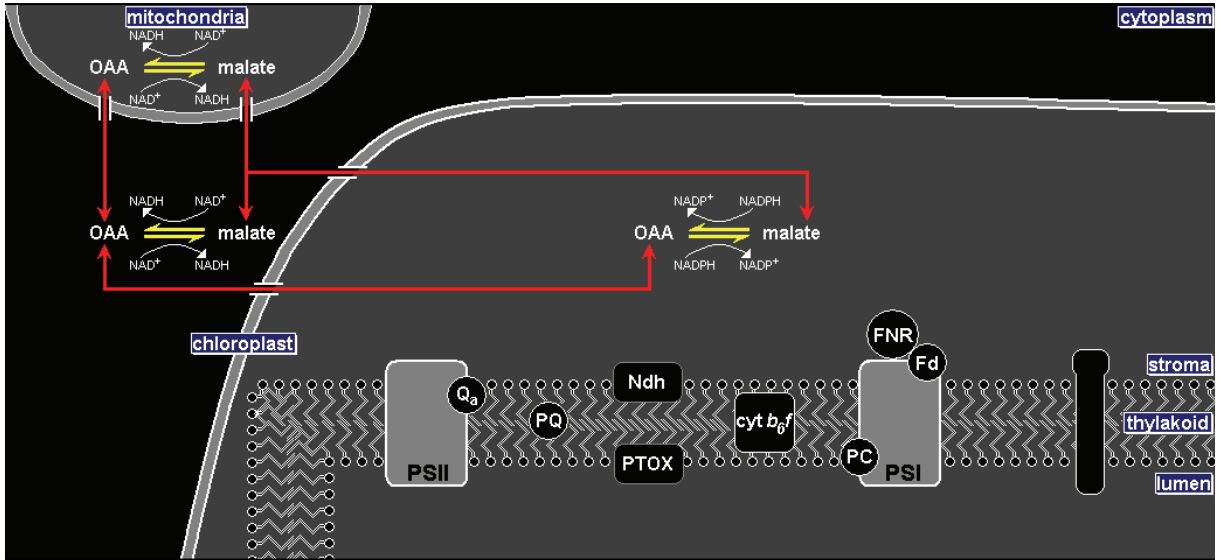
Appendix A iii – Chlororespiration



abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt *b₆/f*, cytochrome *b₆/f* complex; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ reductase; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; Ndh, NADPH-dehydrogenase; OAA, oxaloacetate; PC, plastocyanin; P_i, phosphate; PQ, plastoquinone; PTOX, chloroplast terminal oxidase; Q_A, plastoquinone Q_A; ROS, reactive oxygen species

Appendix A iv – Mitochondria / Chloroplast Interactions

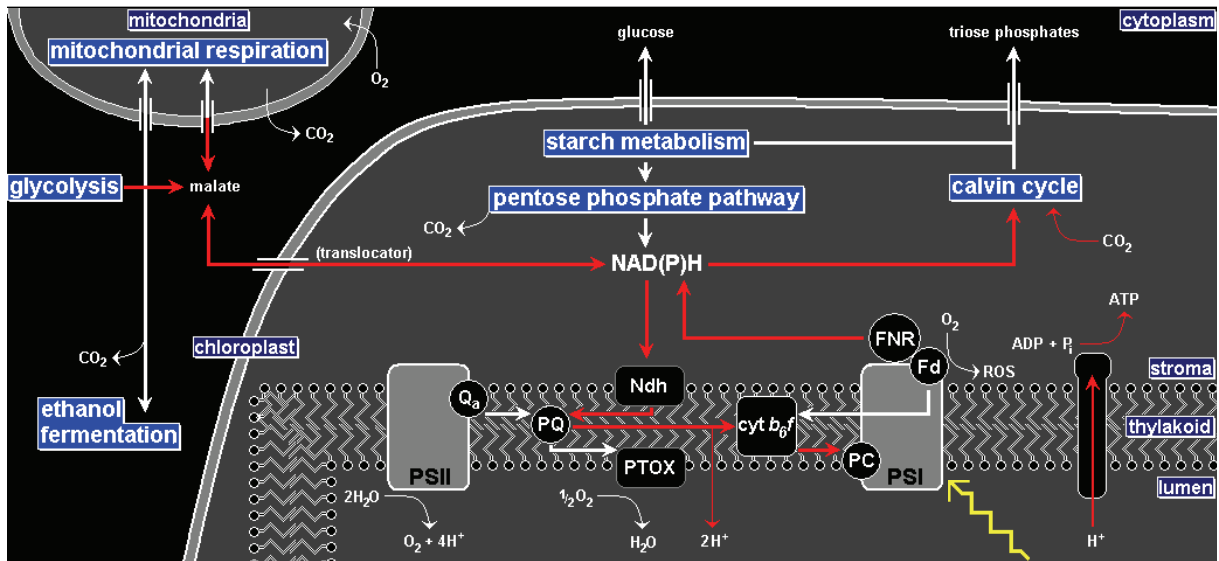
Appendix A iv – Mitochondria / Chloroplast Interactions



abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt *b*₆/*f*, cytochrome *b*₆/*f* complex; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ reductase; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; Ndh, NADPH-dehydrogenase; OAA, oxaloacetate; PC, plastocyanin; P_i, phosphate; PQ, plastoquinone; PTOX, chloroplast terminal oxidase; Q_A, plastoquinone Q_A; ROS, reactive oxygen species

Appendix A v – ‘Chlorofermentation’?

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abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt *b₆f*, cytochrome *b₆f* complex; Fd, ferredoxin; FNR, ferredoxin-NADP⁺ reductase; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; Ndh, NADPH-dehydrogenase; OAA, oxaloacetate; PC, plastocyanin; P_i, phosphate; PQ, plastoquinone; PTOX, chloroplast terminal oxidase; Q_A, plastoquinone Q_A; ROS, reactive oxygen species

Appendix B – PhD-Related Publications

Appendix B i – Journal Articles^{XIV}

- Wright H**, DeLong J, Harrison P, Gunawardena A, Prange R. 2011. The interrelationship between the lower oxygen limit (LOL), chlorophyll fluorescence and the xanthophyll cycle in plants. *Photosynthesis Research* **107**: 223 – 235
- Wright H**, DeLong J, Harrison P, Gunawardena A, Prange R. 2010. The effect of temperature and other factors on chlorophyll *a* fluorescence and the lower oxygen limit in apples (*Malus domestica*). *Postharvest Biology & Technology* **55**: 21 – 28
- Wright H**, van Doorn WG, Gunawardena AHLAN. 2009. An *in vivo* Study of Developmental Programmed Cell Death using the Lace Plant (*Aponogeton madagascariensis*) Leaf Model System. *American Journal of Botany* **96**: 865 – 876^{XV}
- Wright H**, DeLong J, Franklin J, Lada R, Prange R. 2008. A new minimum fluorescence parameter, as generated using pulse frequency modulation (PFM), compared with pulse amplitude modulation (PAM): F_a versus F_o . *Photosynthesis Research* **97**: 205 – 214^{XVI}

Appendix B ii – Journal Articles (*under review & in progress*)

- Wright H**, DeLong J, Harrison P, Gunawardena A, Prange R. 2011. Dynamic controlled atmosphere (DCA): does fluorescence reflect physiology in storage? *Postharvest Biology and Technology* (POSTEC-D-11-00192, *under review*)
- Wright H**, DeLong J, Harrison P, Prange R. 2011. Fermentation during fruit and vegetable storage: friend or foe? *Journal of Horticultural Science and Biotechnology* (*in progress*)

Appendix B iii – Industry

- Prange RK, DeLong JM, **Wright AH**. 2008. Chlorophyll fluorescence takes the ‘pulse’ of a fruit or vegetable. *Rivista di Frutticoltura* **4**: 46 – 49
- Prange RK, DeLong JM, **Wright AH**. 2010. Chlorophyll fluorescence: applications in postharvest horticulture. *Chronica Horticulturae* **50**: 13 – 16.

Appendix B iv – Proceedings

- Lord C, **Wright H**, Rantong G, Dauphinee A, Wertman J, Gunawardena A.^{XVII} 2011. (*oral*) Lace plant: a novel model system to study developmental programmed cell death. *1st International Plant Protease Conference* (Hemavan, Sweden)
- Prange RK^{XVII}, DeLong JM, **Wright AH**. 2011. (*oral*) Storage of pears using dynamic controlled-atmosphere (DCA), a non-chemical method. *Acta Horticulturae* (Rio Negro, Argentina) (*in press*)

^{XIV} Student contribution: Harrison Wright designed and executed the experiments, performed the statistics and wrote all listed co-authored manuscripts with feedback from his PhD supervisors.

^{XV} Article was the product of PhD course work, but was not thesis-related. A cover image for the May 2009 issue of this journal was also accepted.

^{XVI} The portion of this paper on osmotic stress in grapes was from Masters work; the portion on low-O₂ stress in apples (≈ 50%) was PhD work.

^{XVII} Denotes presenter.

- Rantong G, **Wright H**, Gunawardena A.^{XVII} 2010. (*poster*) Developmental Programmed Cell death in the novel lace plant system. *Plant Biology* (Montreal, Canada), Pg. 31
- Wright H**^{XVII}, DeLong J, Gunawardena A, Prange R. 2010. (*oral*) Understanding the relationship between chlorophyll *a* fluorescence and the lower oxygen limit (LOL) in higher plants. *International Society for Plant Anaerobiosis: 10th Conference* (Volterra, Italy) A7-06, Pg. 49
- Wright H**, van Doorn WG, Gunawardena AH.^{XVII} 2009. (*oral*) An *in vivo* study of developmental programmed cell death in lace plant (*Aponogeton madagascariensis*) model system. *Canadian Botanical Association/ L'Association Botanique du Canada* (Wolfville, NS, Canada)
- Wright H**^{XVII}, DeLong J, Gunawardena A, Prange R. 2009. (*oral*) Improving our understanding of the relationship between chlorophyll fluorescence-based F_v/F_m , oxygen, temperature and anaerobic volatiles. *Controlled and Modified Atmosphere Research Conference* (Antalya, Turkey) S1-O2/ CM-CMT 588-OR, Pg. 4
- Prange R^{XVII}, DeLong J, **Wright H**, 2009 (*oral*) A review of dynamic controlled atmosphere (DCA), apples and more. *Controlled and Modified Atmosphere Research Conference* (Antalya, Turkey) S1-O1/ CM-CMT 590-OR, Pg. 4
- Wright H**, Lord CA, Sauerteig KA, Gunawardena AH.^{XVII} 2008. (*oral*) Developmental programmed cell death (PCD) in lace plant (*Aponogeton madagascariensis*). *Plant Development Workshop and the Canadian Society of Plant Physiologists Eastern Regional Meeting* (Toronto, ON, Canada) 2B-7
- Wright H**^{XVII}, Gunawardena A, 2008. (*poster*) An *in vivo* study of developmental programmed cell death (PCD) using the novel lace plant (*Aponogeton madagascariensis*) model system. *Canadian Society of Plant Physiologists Annual Meeting* (Ottawa, ON, Canada) P-128
- Prange RK^{XVII}, DeLong JM, **Wright H**. 2008. (*oral*) Using chlorophyll fluorescence as a dynamic control of postharvest temperature, atmospheres and product water loss in organic temperate and tropical fruit. *ISHS Organic Fruit Conference, IFOAM Organic World Congress* (Vignola and Modena, Italy)
- Prange RK^{XVII}, DeLong JM, **Wright AH**. 2008. (*oral*) Chlorophyll Sensing – A Tool in Postharvest Process Control. *Washington State Horticultural Association 104th Annual Meeting, Postharvest Conference and NW Hort Expo/Trade Show* (Yakima, WA, USA)
- Wright H**, vanDoorn W, Gunawardena A.^{XVII} 2008. (*oral*) *In vivo* cellular – level changes in lace plant (*Aponogeton madagascariensis*) leaves undergoing developmental programmed cell death. *Botanical Society of America (BSA) and Canadian Botanical Association Joint meeting* (Vancouver, BC, Canada) abstract 136
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