Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

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The papers contained in this book report some of the peer reviewed Proceedings of the International Conference "Environmentally friendly and safe technologies for quality of fruit and vegetables", but also other papers related with the subject were included. The manuscripts were reviewed by the Editor and Editorial Board, and only those papers judged suitable for publication were accepted. The Editor wish to thank to all the reviewers and authors for their contribution.

Authors are responsible for content and accuracy of their papers.

Proceedings of the International Conference "Environmentally friendly and safe technologies for quality of fruit and vegetables", held in Universidade do Algarve, Faro, Portugal, on January 14-16, 2009. This Conference was a join activity with COST Action 924.

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06. CHLOROPHYLL FLUORESCENCE IMAGE ANALYSIS FOR NON-DESTRUCTIVE MONITORING OF PHYSIOLOGICAL CHANGES IN FRESH AND FRESH-CUT PRODUCE

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Abstract

Chlorophyll a fluorescence analysis (CFA) is a sensitive indicator of the stability and efficiency of photosynthesis. This fundamental physiological process closely reflects the effects of internal (maturation, aging, senescence etc.) and external (salinity, drought, heat, cold stress etc.) factors on the physiological capacity or vitality of plants. In contrast to many other techniques, CFA can be actually applied remotely and non-invasively. Hence, this method has found widespread applications in plants science. During recent years, CFA has been accepted as a valuable tool to study the metabolic activity of vegetables and salad greens, and some fruits in postharvest. A number of different fluorescence parameters can be used to comprehensively characterize various aspects of photosynthetic performance. For this purpose, commercial chlorophyll fluorescence imaging (CFI) systems may be advantageous because they provided information on both spatial and temporal dynamics of photosynthesis. This yields information on various physiological aspects that are affected by postharvest handling and processing. CFI can, thus, help to objectively, rapidly and non-destructively evaluate and characterize the internal quality of green produce and its changes at many steps of the entire postharvest chain. For a proper application of CFA, a comprehensive knowledge of the physiological background of chlorophyll fluorescence and fluorescence analysis is helpful. Hence, a brief introduction of the physiological basics will be given here. Furthermore, information about alternative approaches to analyse fluorescence data as well as the current technical standard of an available CFI device will be provided. Finally, recent applications of this technique should illustrate the use of this method in fresh food quality research.

Introduction

Effective monitoring of product quality and safety has become an essential demand for growers, packers, distributors, wholesalers and retailers; pre harvest and during the entire postharvest handling chain. For this purpose, instrumental measurements may provide a means to rapidly, objectively and non-destructively characterize and quantify produce defects, overall quality changes and safety problems (Abbott 1999). Many techniques and methods have been developed, improved or adapted for application in this field; most of them are focused on imaging and analysing the optical produce properties. Methods currently investigated or yet in use are conventional RGB image analysis, UV/VIS and NIR-spectroscopy, fluorescence spectroscopy, X-ray- and NMR tomography, and chlorophyll a fluorescence analysis (Abbott 1999). All these techniques rely on the evaluation of physiological or biochemical capabilities of the investigated product. Furthermore, a high actual or potential metabolic activity of a produce also implies a high internal quality.

In chlorophyll-containing, green tissues of fruits and vegetables, photosynthesis is a metabolic pathway of extraordinary importance, comprising many different highly regulated and complex biophysical and biochemical reactions. Hence, measuring the photosynthetic activity and/or its changes offers a promising tool to quantify or predict produce quality and to characterize plant responses to different stressors (von Willert *et al*. 1995). Because photosynthesis is closely integrated into the metabolic network of plant tissue it sensitively responds to many internal (maturation, aging, senescence etc.) and external (salinity, drought, heat, cold stress etc.) stimuli potentially affecting the physiological capacity or vitality.

Chlorophyll a fluorescence analysis (CFA) has meanwhile been widely accepted as an elegant and comprehensive method to indicate photosynthetic activity (von Willert *et al*. 1995; Maxwell & Johnson 2000). As a sensitive indicator of the stability and the efficiency of photosynthesis, CFA has also been applied to studying the metabolic activity of vegetables and salad greens, and some fruits in postharvest during recent years (De Ell *et al*. 1999; Herppich 2002). In contrast to many other techniques, CFA can be applied truly remotely and non-invasively. For this purpose, chlorophyll a fluorescence image analysis (CFI), provided by recently commercially available systems, is advantageous over spot measurements with "normal" glass-fibre fluorometers (von Willert *et al*. 1995) because imaging reveals both the potential spatial and temporal dynamics of photosynthesis (Nedbal *et al*. 2000).

For the comprehensive characterization of the various aspects of photosynthesis, the stability and efficiency of the photosynthetic apparatus and the degree of activation, protection and damage, a large number of different fluorescence parameters are available (Baker *et al*. 2007). Although there are also different approaches to measure and to analyse chlorophyll fluorescence data available, for the sake of simplicity and due to its high flexibility, only the 'conventional' fluorescence analysis according to Schreiber *et al*. (1986) using the 'PAM-fluorometry' will be dealt with in the following.

In general, for a proper application of CFA a comprehensive knowledge of the physiological background of chlorophyll fluorescence and fluorescence analysis is indispensable. Hence, a brief introduction of the physiological basics of CFA and the techniques of CFI will be given. Finally, recent applications of CFI should illustrate the current application of CFI in fresh food quality research.

Chlorophyll Fluorescence – Some Basics

In the chloroplasts of green plant tissues, the chlorophyll a molecules are incorporated at fixed positions in specialized protein complexes, the photosystems (PSI and PSII), and several antenna and light harvesting complexes (LHC). Only outer antenna complexes and LHC also contain chlorophyll b. The chlorophylls, which are the most important light harvesting pigments are able to absorb photons in the wavelength range of 400 to 480 nm (blue) and 630 to 700 nm (red). They are accompanied by smaller amounts of carotenoids, either also functioning in photosynthetic energy absorption, as structural or as photoprotective pigment molecules. The photosystems, along with others protein complexes are located within the thylakoids, the inner chloroplastic membrane network. PSII and PSI, some other proteins and electron transport metabolites form the photosynthetic electron transport chain, which primarily helps to convert the absorbed light into metabolically usable energy (ATP) and reduction equivalents (NADPH/H+). These equivalents are mainly used photochemically in the biochemical reactions of photosynthesis for the assimilation of CO_2 into sugars.

On the other hand, in photosynthetically active plants, chlorophyll a molecules of PSII may also radiatively emit part of the absorbed energy as red fluorescence light of - somewhat longer wavelength (mainly between approximately 660 nm and 760 nm) and, hence, lower energy. Total fluorescence can make up 30 to 50% of the total absorbed light energy. However, due to effective re-absorption by other chlorophyll molecules, net-fluorescence will be only 2 to 3% under normal conditions. Furthermore, only the energy equivalent to red is used photochemically. In contrast, the energy of "blue" photons in excess of the "red portion" can be dissipated as heat by chlorophyll molecules. Furthermore, in a concerted action with carotenoides and, especially, xanthophylls, absorbed energy in excess to what can be used photochemically may be safely dissipated as heat as part of complex photosynthetic protection mechanisms.

If in a stress situation (e.g. heat, cold, frost, drought, anoxia, salinity, light,…) light energy supply exceeds its demand in biochemistry, the above mentioned series of protective mechanisms are further activated and energy flow into heat and, to a smaller extent, into fluorescence increases. Because fluorescence light is relatively simple to measure, it may be used as an indicator of many disturbances in photosynthesis.

Fluorescence Transients

However, modern fluorometers do not only record the continues steady-state fluorescence. Based on the so-called Kautsky-effect (cf. von Willert *et al*. 1995) and in combination with the saturation-pulse technique (Schreiber *et al*. 1986), information on the maximum and the actual activity, and the integrity of PSII as well as on the relative contribution of photochemical and thermal (non-photochemical) energy dissipation can be obtained. For a comprehensive analysis, the produce must be dark-adapted for a certain time, which "switches off" non-photochemical energy dissipation and sets the photochemical reactions of CO_2 assimilation to a "quiescent state". Illumination with short weak photosynthetically inactive light flashes (approx. 5 μmol m^{-2} s^{-1}) induces the initial fluorescence (F_0) arising from chlorophyll a molecules of PSII antenna complexes (von Willert *et al*. 1995). A short (1 s) saturating light pulse (SP), which rapidly excites all chlorophyll molecules, elicits a maximum fluorescence signal (Fm). Because any photochemical or non-photochemical energy dissipation is prevented, fluorescence is the only pathway of de-excitation.

If the object is afterwards irradiated with a continuous actinic light, driving photosynthesis, the fluorescence signal changes in a characteristic pattern, the so-called Kautsky-curve (von Willert *et al*. 1995). The initial rise from F_0 to the fluorescence peak F_p reflects a complex series of reactions including primary charge separation and electron flow in PSII and in the entire photosynthetic electron chain. The decline of the fluorescence signal from F_p to the terminal steady-state fluorescence (F_t) is partially governed by the initial induction and the final fine tuning of all photochemical processes such as the assimilation of CO_2. Furthermore, the activation of the non-photochemical mechanisms further leads to the quenching of fluorescence. If, in the steady state with fully running photosynthesis, a saturation pulse is given, an intermittent maximum fluorescence (F_m') signal can be recorded. This F_m' is smaller than F_m because of the consisting non-photochemical energy dissipation. On the other hand, the fluorescence rise from F_t to F_m' points out the part of potential fluorescence that is currently quenched by photochemistry. Finally, the fluorescence after switching off the actinic irradiation or after rapidly darkening the object yields F_0', the dark fluorescence emitted from antenna chlorophylls in the 'light-adapted' state.

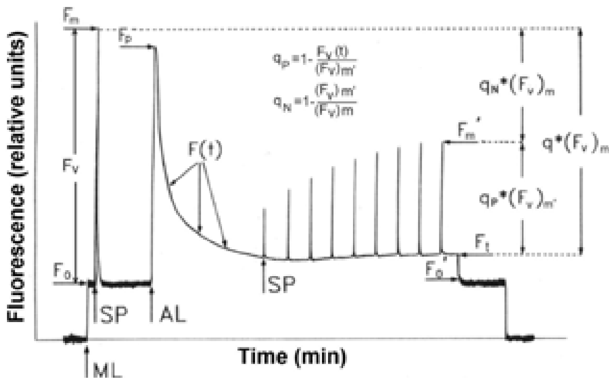

Fig 1. Example of a typical fluorescence transient as recorded with a PAM-fluorometer (ML = measuring light, SP = saturating pulse, AL = actinic light). Additional information is given in the text.

Chlorophyll Fluorescence Parameter and their Meaning

From all these fluorescence signals, quite a (still increasing) number of useful, more or less well defined parameters have been proposed and may be derivated by simple calculations. Very easy to measure is the ratio of the variable fluorescence F_v ($F_v = F_m - F_0$) and F_m of dark-adapted samples. F_v/F_m is an indicator of the potential maximum photochemical quantum efficiency of PSII, and is thus, with some precautions, a valuable tool to determine both capacity and stability of photosynthesis (Krause & Weis 1991; von Willert *et al*. 1995) and its direct response to internal and external constraints.

When a saturation pulse is applied on irradiated samples, the measured fluorescence signals, F_t and F_m', can be used to estimate the fraction of the maximum photochemical quantum efficiency of PSII that is still operating. This actual photochemical quantum efficiency, calculate as $(F_m'-F_t)/F_m'$, is often termed $\Delta F/F_m'$ but also a confusing wealth of other designations can be found (Y, Φ_{PSII}, F_q'/F_m',…). It has been shown that, as the ratio of used to absorbed energy, $\Delta F/F_m'$ is directly proportional to the linear electron flow through PSII. Hence, it can be used to estimate the electron transport rate (ETR) by multiplying it with the amount of absorbed photons I_{abs} (the product of the incident photons and the absorption coefficient) and the relative distribution of photons between PSII and PSI as ETR = $\Delta F/F_m' * I_{abs} * 0.5$. Here, the (over) simplification is generally accepted that the absorption coefficient is 0.84 and the distribution is equal. Under some precautions, ETR is a valuable indicator of the gross photosynthesis.

The comparison of the variable fluorescence measured on a irradiated sample and that of a dark adapted one allows the estimation of the activity of non-photochemical mechanisms because they are the reason for the reduction of F_m to F_m'. Accordingly, a non-photochemical quenching coefficient (Schreiber *et al.* 1986) is defined as $q_N = 1- F_v'/F_v = 1-(F_m'-F_0')/(F_m-F_0)$. Likewise, the activity of photochemical quenching may be estimated from the rise of the fluorescence signal from F_t to F_m', which reflects the transient closure of all (still open) PSII reaction centres, i.e. the transient inhibition of photochemistry. Hence, the photochemical quenching coefficient q_P is defined as $q_P = \Delta F/F_v = (F_m'-F_t)/(F_m-F_0)$. In a different approach based on the Stern-Volmer-equation, non-photochemical quenching (NPQ) is calculated as NPQ = $(F_m-F_m')/F_m'$ (cf. Klughammer & Schreiber 2008). NPQ is directly related to the total amount of quenchers involved in the non-photochemical protection mechanisms, while q_N closely reflects their actual functioning.

Besides many other more or less meaningful fluorescence parameters, calculation of complementary PSII quantum yields has become very popular during recent years (Klughammer & Schreiber 2008). Similar to the terms for dissipation of absorbed energy as heat ($D = 1-(F_m'-F_0')/F_m'$), photochemistry ($P = \Delta F/F_v$) and the unexplained excess ($E = 1-D-P$) proposed by Demmig-Adams *et al*. (1996), more recently quantum yields of photochemical energy conversion ($YII = \Delta F/F_v$), and of regulated ($YNPQ= F/F_m' - F/F_m$) and non-regulated non-photochemical energy loss in PSII ($YNO = F/F_m$) have been developed (Klughammer & Schreiber 2008). The latter approach may simplify the measurements because it does not include the assessment of F_0 and, most important, F_0'. However, it, of course, still demands the determination of F_m, which can be complicated in the field and under conditions of long-term stress effects e.g. due to long-lasting NPQ components (Herppich 2000).

Chlorophyll Fluorescence Image Analysis

CFI was performed with an open system (FluorCAM 640MF, PSI, Brno, Czech Republic) measuring sequences of fluorescence images according to user-defined settings of time intervals and irradiance. Recommended maximum sample size is 10 cm×13 cm. Two sets of 345 orange LEDs (λ_{max} = 620 nm) are used to provide the weak measuring flashes as well as continuous actinic irradiation. Short-term saturation light pulses (max. 2500 μmol photons m^{-2} s^{-1}) are generated by a halogen lamp (24 V, 250 W) equipped with an electronically controlled shutter. Synchronously with the measuring light flashes, a 12-bit CCD camera (with F1.2/2.8-6 mm objective and short-pass filter) records fluorescence images (512x512 pixel) with a maximal frequency of 50 images s^{-1}. Measurements are controlled and data are analysed by a WinXP compatible software (FluorCam 6; PSI). With this system, all the above mentioned fluorescence signal can be obtained. For practical reasons in many investigations, a short standard protocol was used including dark-adaptation (10 min) and illumination of the sample with the measuring light to induce F_0, and then F_m was elicited by a 1 s-saturation pulse. After a short relaxation phase of 20 s, actinic light was provided for additional 30 s followed by a 5 s dark period in which the short-term relaxation of F_0 could be obtained. This protocol was used because only a proper Kautsky-effect can truly indicate the vitality of the sample. Even in a fully inactive e.g. dehydrated sample, fluorescence rises in response to a saturation pulse, simply because of the increased light absorption and, hence, fluorescence emission.

CF applications

Pigment Changes in Ripening Sweet Pepper Fruit

For green-ripe sweet pepper cultivars further fruit development, as indicated by chlorophyll degradation and capsanthin accumulation, i.e. the colour change from green to red may reduce produce quality. To optimize postharvest quality maintenance, the dynamics of chlorophyll degradation and carotenoid synthesis and their variation by various storage conditions was non-destructively investigated on green-ripe and full-ripe marketable fruit of the sweet pepper cultivar Kárpia F1. CFI was applied to analyse chances in the photosynthetic activity (F_v/F_m) of whole fruit, while the accumulation of capsanthin was evaluated by the analysis of digital RGB-images. CFI enables the characterisation of the chlorophyll content dynamics independent of the variation in surface colour.

In sweet pepper fruit, stored at 20 °C, capsanthin synthesis is a rapid process, which can be completed within one day (Fig 2). In contrast, chlorophyll degradation occurs more or less continuously and is completed only 4 to 5 d after the fruit was totally red coloured. Digital and chlorophyll fluorescence imaging showed that in ripening sweet pepper chlorophyll degradation and capsanthin accumulation occurred independent but effectively at room temperature in packed or unpacked fruit. Thus, low storage temperature but not modified atmosphere packaging can affect ripening and hence, changes in both pigments (Zsom *et al.* 2010).

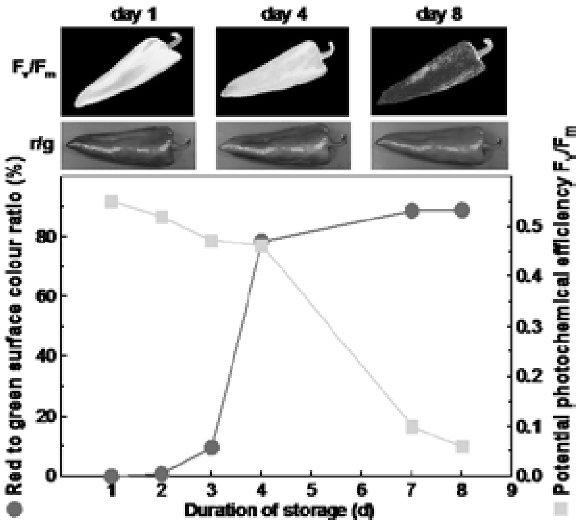

Fig 2. Comparison of the dynamic changes in chlorophyll and carotenoid content of sweet pepper fruit during ripening.

Effects of Continuous Weak Illumination on Packed Fresh Salad Quality

Packaging can maintain the quality of lightly processed fresh salads, reducing mechanical damage, water loss and respiration, and inhibiting browning and microbial decay due to a passively established low O_2 and high CO_2 content of the air (**m**odified **a**tmosphere **p**ackaging, MAP). Lightly processed products retain their physiological activity even in such a specific environment. Illumination and temperature within a display cabinet interactively affect this activity. Optimised illumination may improve quality maintenance of packed photosynthetic active salads, reducing senescence and decay processes. The effects of radiation and temperature on the physiological activity of corn salad and Arugula leaves within the packages can easily and non-invasively be determined using CFI.

The results of long-term (10 d) storage experiments at 20 °C (room temperature) indicated that illumination may better retain salad quality than storage in darkness. It also helps to control the gas composition within the packaging. Even low light intensity effectively improved quality maintenance of the products. Higher photon fluence rates are not necessary but may increase temperature within the

packaging, further reducing product quality. In contrast, illumination, optimised in quality and quantity, can help to retain value-adding substances and reduce senescence of fresh packed products (Fig 3).

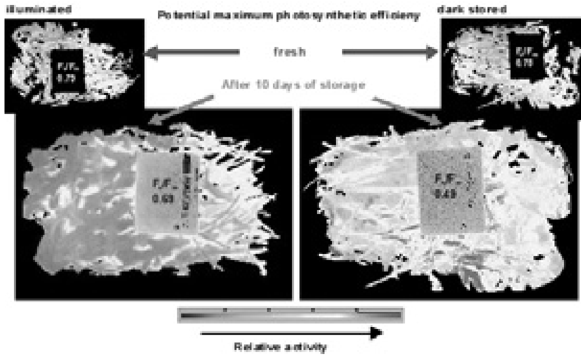

Fig 3. Effect of illumination on potential photosynthetic efficiency of PSII as an indicator of the mean quality of packed Arugula during a 10 d storage at 20 °C.

Evaluation of Heat Treatment Effects

To ensure the distribution of high quality fresh minimally processed products, several preservation methods are currently applied to extend the shelf-life. This may included hot water treatment, which, among other effects, may help to superficial disinfect the produce. However, the window between best effects of the treatment and product damage is often very small. CFI may help to comprehensively characterize the temperature effect on green fruits and vegetables (Schlüter *et al.* 2009).

Even short applications of high, sublethal temperatures (here 45 °C) seriously but reversibly inhibited metabolic activity (F_v/F_m) of intact fresh lamps lettuce leaves (Fig 4). Although applications of 10 and 30 s affected F_v/F_m only temporary and to a minor extent (< 15%), temperature affects the photosynthetic activity, and its ability to recover from heat stress declines with the duration of heat treatment. Furthermore, a small increase of sample temperature to 50 °C resulted in a very pronounced (50%) but still partially (39%) reversible inhibition of F_v/F_m at a 10 s exposure. Any longer treatment causes complete and irreversible damages to the photosynthetic apparatus. Hence, CFI sensitively monitors the short and long-term heat effects and may, thus, help to optimize application of this postharvest treatment.

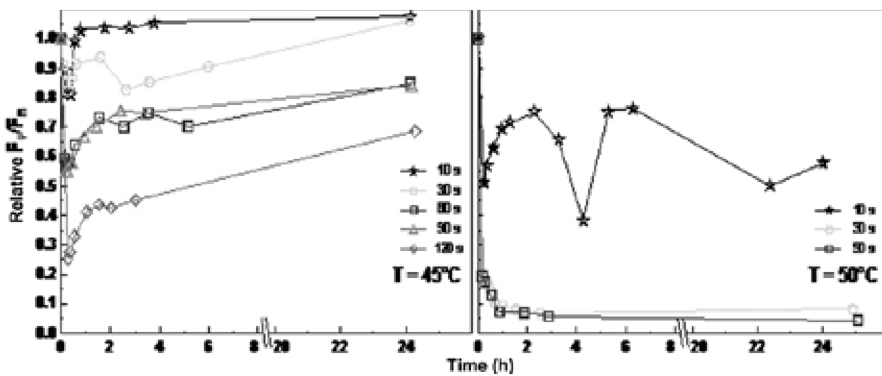

Fig 4. Maximum photochemical efficiency of lamb's lettuce after thermal treatment at 45 °C (a) and 50 °C (b).

Conclusions

Chlorophyll fluorescence analysis is a powerful tool to determine the preharvest and postharvest internal quality of green photosynthetic active produce. However, despite the relative ease of the measurement,

knowledge of the physiological base, and of the actual limits of interpretation of the results are necessary. Chlorophyll fluorescence imaging effectively detects heterogeneity in photosynthetic responses and monitors their local and temporal dynamics.

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