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# APPLICATION OF MODULATED CHLOROPHYLL FLUORESCENCE AND MODULATED CHLOROPHYLL FLUORESCENCE IMAGING TO STUDY THE ENVIRONMENTAL STRESSES EFFECT

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Abstract – Chlorophyll (Chl) *a* fluorescence is a widely used tool to monitor the photosynthetic process in plants subjected to environmental stresses. This review reports the theoretical bases of Chl fluorescence, and the significance of the most important Chl fluorescence parameters. It also reports how these parameters can be utilised to estimate changes in photosystem II (PSII) photochemistry, linear electron flux and energy dissipation mechanisms. The relation between actual PSII photochemistry and $CO_2$ assimilation is discussed, as is the role of photochemical and non-photochemical quenching in inducing changes in PSII activity. The application of Chl fluorescence imaging to study heterogeneity on leaf lamina is also considered. This review summarises only some of the results obtained by this methodology to study the effects of different environmental stresses, namely water and nutrients availability, pollutants, temperature and salinity.

Keywords: chlorophyll, drought, heavy metals, non-photochemical quenching, ozone, photochemical quenching, photosynthesis, salinity

## Introduction

### Brief description of Chl fluorescence

In 1984, Krause & Weis wrote a review entitled "Chlorophyll fluorescence as a tool in plant physiology" and defined chlorophyll (Chl) as an intrinsic fluorescent probe of photosynthesis. To date, many research works have utilised this technique to determine the effects of environmental stress on photosynthetic apparatus. Certainly, its utilisation has greatly increased in recent few decades, even for relatively simple available instrumentations.

The basic mechanism is light absorption of photons energy by a Chl molecule that induces the excitation of the electron. Consequently, fluorescence represents the light re-emitted by Chl during the return from excited to non-excited states. Kautsky & Hirsch (1931) evidenced for the first time that changes in Chl fluorescence emission are strictly linked to changes in photosynthetic rate. Duysens & Sweers (1963) evidenced how the quencher of fluorescence was the primary quinone electron acceptor of photosystem II (PSII) in the oxidised state, but not in the reduced state. These authors built a simple PSII model in which Chl fluorescence, photochemistry and non-radiative energy dissipation mechanisms all competed for excitation energy within PSII antenna. Consequently, it became clear that Chl fluorescence could be a useful tool for determining changes in the photosynthetic process when considering both Chl fluorescence and the non-radiative process to be stable. However, the non-radiative decay rate is far from being

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stable, but changes in relation to light (Krause & Weiss 1991). As a result, if we able are to relate Chl fluorescence with changes in the photosynthetic system, we must be able to quantify the extent to which Chl fluorescence decreases by photochemistry (photochemical quenching; $q_P$) and non-radiative decay (non-photochemical quenching; $q_{NP}$). A widely used method to determine the relative contribution to $q_P$ and $q_{NP}$ to total Chl fluorescence quenching was that estimated for the first time by Bradbury & Baker (1984). These authors determined photochemical quenching from measurements of the superimposed fast-induction kinetics that occurred in response to applying a second strong illumination at an appropriate time of the underlying induction curve, the so-called light-doubling method. The basic concept that underlies this method is that illumination by strong light causes the transitory complete removal of $q_P$ at any time during induction.

*In vivo* variable Chl fluorescence (i.e., fluorescence increase upon illumination) derives from Chl *a* in PSII. For this reason, changes in the Chl fluorescence yield primarily represent the properties of excitation and energy conversion at the PSII level (Figure 1).

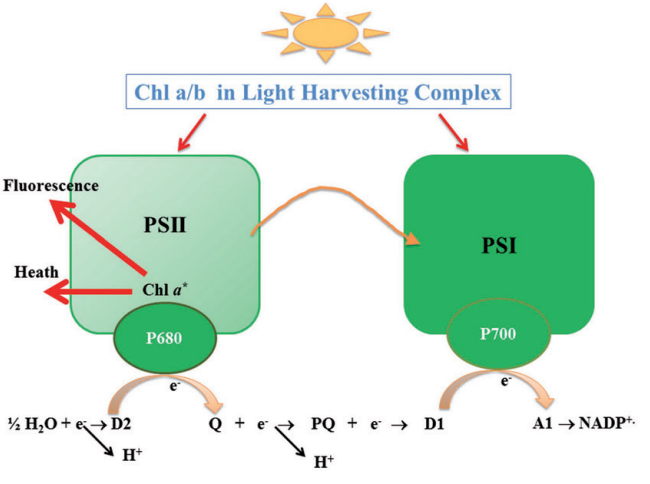


Figure 1. Schematic representation of mechanisms of energy transfer and primary electron transport reactions. Abbreviations: PSII, photosystem II; PSI, photosystem I; PQ, plastoquinone; D2 and D1: proteins D2 and D1; Q: plastoquinone; A1: primary acceptor of PSI.

Clearly the functional connection of PSII to other components of the electron transport chain determines that Chl fluorescence yield provides information on the whole process. Chl fluorescence was studied for the first time during a dark/light transition and emission was connected with photosynthesis induction. This approach had different limits; in fact, while it was possible to obtain an accurate value of the minimal fluorescence, $F_0$ or O (fast kinetics induction), the determination of the maximal values of Chl fluorescence (or P), induced by the rapid re-oxidation of PSII reaction centres, from sunlight- or light-exposed leaves proved difficult.

The revolution in Chl fluorescence use was determined by modulated measuring apparatus (Quick & Horton, 1984). In this application the light source used to determine the Chl fluorescence yield is modulated (switched on and off at a high frequency), and only the fluorescence excited by the measuring light is detected. This permits the determination of Chl fluorescence in the presence of illumination, and even in the presence of full sunlight.

## Modulated Chl *a* fluorescence

The basic technique for determining the Chl fluorescence yield is widely reported in the literature (Schreiber, 2004; Kalaji et al., 2014). Essentially after a period of dark adaptation (30-60 min) when all reaction centres are open, exposure to modulated weak light (< 1.0 µmol photons $m^{-2}s^{-1}$) induces an increase in the Chl fluorescence yield, namely $F_0$. This light intensity is not sufficient to induce the electron transport chain. Afterwards, a strong saturating light pulse (from 8000-15000 µmol photons $m^{-2}s^{-1}$ per millisecond) is applied and induces a strong rapid increase in the Chl fluorescence yield, $F_m$. If the saturation pulse is strong enough, all the reaction centres are completely reduce. Then the Chl fluorescence yield decreases and, in the presence of actinic light, it reaches steady-state values ($F_s$) that correspond to a balance between the reduced and oxidised state of primary electron acceptor $Q_A$. Such Chl fluorescence quenching is attributable to photochemical and non-photochemical dissipation, which can be easily measured by the light-doubling method. Briefly, during Chl fluorescence relaxation in the presence of actinic light, a super-imposed strong pulse completely removes the photochemistry by determining the $F_m$' value, which corresponds to maximal fluorescence under light conditions, and is usually lower than the dark reference value $F_m$. The lowering of $F_m$' is a measure of non-photochemical quenching, assuming that this non-radiative process does not change during a short period. However during curve induction, the $F_0$ value lowers and is attributable to the energy-dependent quenching of $F_0$ (Bilger & Schreiber, 1987). For this reason, the determination of $F_0$' under light conditions requires the use of far-red light to transiently and selectively excite PSI, and to thus enhance the oxidation of the electron transport chain. This induces complete quick $Q_A$ oxidation before complete non-photochemical quenching relaxation. $F_0$ determination is sometimes problematic, for example, in field conditions or by the Chl fluorescence imaging technique. In this case the extent of non-radiative

energy dissipation can be described by the NPQ parameter [$(F_m$-$F_m$')/$F_m$'; Bilger & Bjorkman, 1990], which does not require knowledge of $F_0$'. For NPQ calculations, the existence of non-radiative energy dissipation traps (for example, zeaxanthin) in the antenna matrix should be assumed (Demmig-Adams, 1990; Demmig-Adams & Adams, 1992).

The photochemical quenching $q_P$ coefficient is determined as ($F_m$'-$F_s$)/($F_m$'-$F_0$'), whereas the non-photochemical quenching coefficient is established as 1-($F_m$'-$F_0$')/($F_m$-$F_0$), and both vary between 0 and 1. The $q_{NP}$ coefficient consists of three different components: energy-dependent quenching related to the build-up of the trans-thylakoidal pH-gradient, $q_E$; quenching due to the state II-I transition of the phosphorylated light harvesting complex of PSII, $q_T$; photo-inhibitory quenching, $q_I$ (Horton & Hague, 1988; Walters & Horton, 1991).

Quenching analyses also estimate the quantum yield of energy conversion into PSII. The potential quantum yield, usually determined after the sample's dark adaptation, is well-described by $F_v/F_m$ [= ($F_m$-$F_0$)/$F_m$]. This ratio is widely used as an indicator of plant photosynthetic performance, with optimal values at around 0.83 for most plant species (Bjorkman & Demmig, 1987).

Under light conditions, the PSII quantum yield is reduced by the closure of reaction centres and non-radiative dissipation, and the resulting actual PSII quantum yield for photochemistry is determined by parameter $\Phi_{PSII}$ [=($F_m$'-$F_s$)/$F_m$' or $q_P$x($F_v$'/$F_m$'); Genty et al., 1989]. Under some conditions, i.e. at a saturating $CO_2$ concentration and 1% $O_2$, a linear relationship between $\Phi_{PSII}$ and $\Phi_{CO_2}$ (the quantum yield of $CO_2$ assimilation) is observed (Genty et al., 1989). Harbinson et al. (1990) evidenced that this relationship is non-linear at the 20% $O_2$ concentration, and also indicated, in addition to $CO_2$ fixation, that photorespiration and other electron flows dependent on $O_2$ could be estimated by $\Phi_{PSII}$. The relative electron transport rate (ETR) can be calculated as $\Phi_{PSII}$x0.5xαxPPFD, where 0.5 accounts for the partitioning between the two photosystems, α is the absorption coefficient of the leaf, and PPFD is photosynthetic photon flux density.

## Chl *a* fluorescence imaging

The Chl fluorescence imaging (CFI) technique was pioneered by Omasa et al. (1987) and Daley et al. (1989). The capacity to resolve photosynthetic performance over the whole leaf surface distinguishes CFI from integrative methods, such as gas exchange or non-imaging Chl fluorescence. The molecular and physiological processes that alter the photosynthesis caused by external and/or internal factors can vary their impact on the leaf surface, and give rise to spatial heterogeneity (Nedbal & Whitmarsh, 2004). In other words, CFI presents an immediate overview of the fluorescence emission pattern of cells, leaves or plants, and provides fast, intuitive, visual and precise information on PSII functionality (Omasa, 1990; Calatayud et al. 2006b).

There are many different commercially available Chl image fluorometers with pulse amplitude modulation light. The CFI technique depends on four basic processes according to Nedbal & Whitmarsh (2004): i) image capture through illumination, data capture, image digitalisation and transferring data and images to the computer; ii) defining areas of interest in the sample(s) for the CFI parameters analysis; iii) calculating the CFI parameters in these areas; iv) displaying data and images.

The basics of CFI are the same as those previously described, but modulated measuring light and actinic and saturated light derive from LEDs. The duration of flashes and the intensity of the actinic light produced by LEDs can be controlled down to the microsecond range, and from low to high light (exceeding sunlight), respectively.

A typical charge coupled device camera captures fluorescence emission synchronised with the pulse modulated measuring light. Image digitalisation occurs in the camera and data are transferred to a computer. False colour palettes, where different colours encode for numerical values of different fluorescence parameters, are commonly used to represent heterogeneity in samples, pixel by pixel, in images.

For instance, CFI has been used to assess the dynamics and heterogeneity of leaf stomatal responses, known as stomatal patchiness. This event determines non-uniform stomatal conductance and, in turn, non-homogeneous $CO_2$ fixation and electron transport through PSII (Omasa & Takayama, 2003). CFI is employed to visualise leaf heterogeneity and reveals local effects caused by abiotic stress, like nutrient deficiency (Donnini et al., 2013; Osório et al., 2014), water deficit (Calatayud et al., 2006b), high or low temperatures (Hogewoning & Harbinson, 2007; Mishra et al., 2014), high and low light intensity (Guidi & Degl'Innocenti, 2008; Hughes et al., 2008; Harbinson et al., 2012), herbicides (Barbagallo et al. 2003) and pollutants (Guidi & Degl`Innocenti, 2008). Damage induced by biotic stress, such as fungi, virus and bacteria, is also not often homogeneously distributed in leaf lamina, so its invasion and progression in leaves can be analysed by CFI (Scholes & Rolfe, 1996; Chaerle et al., 2007; Guidi et al., 2007). The extreme sensitivity of this technique allows photosynthesis impairment to be also detected in asymptomatic leaves or non-green plant tissue (low Chl content), such as fruits (Nedbal et al., 2000), and to detect the heterogeneity of pigment accumulation (Agati et al., 2008). Another relevant application of CFI is its capacity to select genotypes with tolerance to different abiotic and biotic stresses (Gorbe &

Calatayud, 2012), and for screening a large number of samples for their photosynthetic performance (Barbagallo et al., 2003).

In spite of the goodness and performance of the CFI technique, some technical limitations still remain. One major technical challenge is uniform light illumination over a large sample area since non-homogeneous illumination can impair the distribution of the Chl fluorescence signal over the sample, and can consequently change data interpretation (Roháček et al., 2008; Nebal & Whitmarsh, 2004). As a result, most CFI fluorometers limit signal detection to a relatively small area (approximately 100 $cm^2$), where $F_m$ and $F_m$' can be correctly measured. The calculation of parameters $q_{NP}$ and $q_P$ requires calculating $F_0$', which is a critical point in CFI. Correct $F_0$' determination requires the application of far-red light, which could penetrate the camera detector and seriously disturb fluorescence imaging. Therefore instead of measuring $F_0$', it is estimated by the approach of Oxborough & Baker (1997), where $F_0' = F_0/(F_v/F_m + F_0/F_m')$. This approach relies on the assumption that the same mechanism that causes $F_0$ quenching is also responsible for $F_m$ quenching; for this reason, it is possible to estimate $F_0$' from $F_m$' measurements. Non-flat tissues and samples with strongly reflecting surfaces (like wax or hair) are another problem for the CFI technique. In the first case, a non-homogeneous signal of Chl fluorescence is yielded because of the different distances of the leaf portion to light sources, unlike reflecting surface disturbs and/or altering Chl fluorescence signals.

The blue light emitted by LEDs (frequently utilised in CFI fluorometers) can also be a problem when assessing photosynthesis in pigmented tissues (for example, anthocyanin-rich tissues) since blue light can be partially absorbed by these pigments (Logan et al., 2007). In this way, non-imaging instruments overcome this limitation by utilising red light.

It is clear that imaging techniques offer advantages for detecting photosynthesis on leaf lamina, but also some disadvantages. So, choice of an imaging or a non-imaging technique should be carefully weighed according to plant material, stress symptoms and effects on photosynthetic apparatus.

## Application of Chlorophyll fluorescence in environmental stresses

Chl *a* fluorescence is a good tool to detect the effects of environmental factors on the photosynthetic process. In most works, Chl *a* fluorescence is utilised concomitantly with a gas exchange system to also detect $CO_2$ photoassimilation. By using gas exchange, it is possible to understand the stress-induced limitations to the $CO_2$ assimilation rate, and also the type of limitation to this process; i.e., stomatal, mesophyll and biochemical (Flexas et al., 2004; Chaves et al., 2009). Chl *a* fluorescence provides information on PSII photochemistry. In this way, it is possible to draw a detailed picture of the stress effects on the photosynthetic process. By Chl fluorescence it is also possible to understand mechanisms based on the dissipation of excess excitation energy, which normally occurs under stress conditions (Gorbe & Calatayud, 2012; Guidi & Calatayud, 2014).

This technique is non-invasive and permits the detection of effects of stress on intact leaves/plants. There is no doubt that Chl fluorescence measurements, when carefully applied, can provide useful information about leaf photosynthetic performance. An examination of the literature overwhelmingly reveals more than 400,000 papers on Chl fluorescence, most of which are relevant for eco-physiological performance and can be considered to have implications for agricultural or plant science issues.

Light is the determinant factor in the photosynthetic process and represents the energy by which plants synthesise all organic molecules. However, light intensity and quality are extremely variable in space (geography, leaf canopy and cell position in leaves) and in time terms during the season, but also during the day. So, plants have evolved a wide variety of regulatory mechanisms that aim to accommodate the photosynthetic process to such fluctuations, which also play a key role in plants' ability to assimilate carbon over long time periods and to produce biomass under stressed conditions. These mechanisms are well evidenced by the light curve response of $CO_2$ assimilation in relation to light intensity: the greater light intensity is, the greater photoassimilation remains until a steady-state is reached and photosynthetic $CO_2$ assimilation is saturated. Solar energy is defined in excess when its intensity exceeds the photosynthetic process's ability to use it for $CO_2$ assimilation. To cope with excess light, plants modify their leaf area and leaf exposure by modifying the angle of leaf insertion and chloroplast movements, but by also amending the size of light harvesting complex (LHC) antenna or chlorophyll content (Takahashi & Badger, 2011). Dissipation is an important mechanism by which plants can eliminate excess absorbed light by increasing thermal dissipation or reactions in chloroplast that act as sinks for electrons. Thus, few changes in Chl fluorescence can reflect altered PSII photochemistry. A key point of regulation is represented by the LHCII antenna matrix in which carotenoids, i.e. zeaxanthin, represent the most important accessory pigments. In fact, apart from absorbing light energy and transmitting it to Chl molecules, they are also able to quench the triplet state of Chl by releasing absorbed energy by heat via the well-known xanthophyll cycle (Demmig-Adams & Adams,

1992, 1996). There are reports of a relation between this cycle and NPQ activation (Demmig-Adams & Adams, 1992, 1996; Demmig-Adams et al., 2014). Another important mechanism that plays a key role during short shade/light fluctuations is represented by spillover, i.e. the transition of LHCII to the phosphorylated state, which allows them to move away from PSII to PSI (state II-I), and enables them to transfer excitation energy to PSI (Walters & Horton, 1991; Ruban & Johnson, 2009). In this way, PSII, which is usually more photo-damaged under excess light, can be preserved by overreduction.

Acclimation mechanisms can be divided into two distinct groups: long-term (adaptation) and short-term (regulatory or acclimation) mechanisms. Plants utilise feedback control mechanisms to regulate short-time acclimation responses. Under light conditions, overabsorption of light energy and then overproduction of reducing power cause protons to accumulate in the lumen of chloroplast, which determines the inhibition of a number of key electron transport enzymes; i.e., Cyt $b_6$/f and the oxygen-evolving complex of PSII. The result is lower electron transport rates because of a feedback mechanism for balancing ATP and NADPH production with electron transport. Even photon-to-electron energy conversion needs to be controlled and is represented by a reduced Chl fluorescence yield in PSII antenna (NPQ, Genty et al., 1989; Schreiber, 1986). NPQ is a most efficient process that protects PSII reaction centres from photo-inhibition because of the dissipation of excess energy and the consequential relief of excitation pressure. On the contrary, if leaves are unable to form transthylakoidal ΔpH, the generated NPQ is not photoprotective since nearly half of all reaction centres remain in a reduced state (Ruban, 2015). In conclusion, the NPQ generated in the absence of ΔpH is not photoprotective and is named photoinhibitory quenching, which generally causes permanent damage to reaction centres (for a detailed review, see Ruban et al., 2012).

**Water availability**

Drought represents an environmental factor that impairs photosynthetesis (Chaves et al., 2009; Flexas et al., 2014). In addition, drought stress is frequently coupled with high solar irradiation and high temperature (as in the Mediterranean Region), conditions under which its effects are strongly exacerbated (Misson et al., 2010; Flexas et al., 2014). The first article reported in Scopus was that of Havaux & Lannoye (1983), and it showed that Chl fluorescence is a good tool for investigating effects of drought on plants. By applying this technique, the authors concluded that drought induces irreversible damage to chloroplastic membranes. From this work onwards, much progress has been made and new more sophisticated instrumentation allow us to conclude and highlight changes in chloroplast membranes, whose recording at that time seemed impossible.

It has been established that in plants, drought induces reduced stomatal aperture (Grassi & Magnani, 2005; Pinheiro & Chaves, 2011), which impairs the photosynthetic process for decreased ATP and NADPH consumption for $CO_2$ assimilation. This event inevitably lowers the linear electron transport rate and, consequently, the actual PSII quantum yield for photochemistry. Sometimes the water-water cycle and, in C3 plants, the increase in photorespiration under drought, may maintain similar electron transport rates to those observed in control leaves, despite a lower $CO_2$ assimilation rate (Flexas et al., 1998, 2002; Noctor et al., 2002; Ort & Baker, 2002; Guidi et al., 2008). This results in little or no change in $\Phi_{PSII}$. Flexas et al. (2002) identified such buffering effects of electron sinks, in addition to $CO_2$ assimilation, in field-grown grapevine under mild drought stress, where a 75% decrease in stomatal conductance resulted in a 54% reduction in $CO_2$ assimilation, but the estimated electron transport rate only lowered by 19%.

Differences in the response of Chl fluorescence parameters exist in relation to stress intensity. In fact, if the potential PSII quantum yield for photochemistry $F_v/F_m$ is scarcely affected under mild water stress conditions, reduction in $CO_2$ assimilation due to stomatal restriction can determine alterations in fluorescence induction (Fini et al., 2012; Nogues et al., 2014; Tattini et al., 2015). When water loss in leaves increases, a decline in the photosynthetic potential can be observed, and mesophyll and biochemical limitation can overcome stomatal limitation. Under these conditions, the sharp drop in ATP and NADPH utilisation can induce alterations in $F_v/F_m$, but also in $\Phi_{PSII}$, if alternative electron sinks (water-water cycling, photorespiration and other electron sinks) are not better able to compensate the electron transport chain (Driever & Baker, 2011).

In grapevine, Flexas et al. (2002) reported that NPQ may be gradually activated during steady stomata closure, while drought stress increases, which indicates that NPQ contributes to protect from photodamage. However, Mishra et al. (2012) reported that when drought is severe and the water potential goes below -1.5 MPa, NPQ is significantly suppressed. The same authors also demonstrated that $F_v/F_m$, $\Phi_{PSII}$ and NPQ could be used potentially for routine assays of plant health performance and for the quantification of drought tolerance in different genotypes.

Galmés et al. (2007) reported that some Mediterranean species strongly resist the photo-inactivation of PSII under drought. The obtained results revealed that Mediterranean plants have similar needs and photoprotection capacity when they face episodic water stress, which consists in maintaining or increasing the photorespiration rate under mild stress and in increasing thermal energy dissipation under more severe

stress. Increased thermal energy dissipation due to water stress depends mostly on the de-epoxidation state of xanthophylls. Other studies have reported the role of photorespiration as an alternative electron sink under mild water stress (Bai et al., 2008, Wilhelm & Selmar, 2011; Beis & Patakas, 2012), but also under severe drought stress (Silva et al., 2015).

Other authors (Golding & Johnson, 2003) have observed an increase in NPQ and a drop in the electron transport rate in *Hordeum vulgare* L. subjected to water stress, and an increase in active PSII centres. These authors speculated that active centres may be involved in cyclic electron flow, which generating ΔpH, permits NPQ and protects PSII. They also demonstrated that regulation occurs between the two photosystems; in particular, PSI becomes more oxidised compared to PSII. At the same time, NPQ increases by reducing the excitation pressure to PSII.

Drought stress results in patchy $CO_2$ fixation over small leaf areas (Downton et al., 1988), despite Wise et al. (1992) finding that spatial heterogeneity in photosynthesis in cotton leaves decreases with drought stress severity. Since patches are characterised by uniform stomatal conductance, which is distinct from neighbouring patches (Mott & Buckley, 2000), and as stomatal conductance is closely related to the photosynthetic rate (and therefore also to $\Phi_{PSII}$), the heterogeneity of $\Phi_{PSII}$ along the leaf blade is presumable. Clearly in this context, CFI is a useful technique for evaluating the patchiness of photosynthesis along leaf blades. However, Massacci et al. (2008) did not found the heterogeneity for $\Phi_{PSII}$ and $F_v/F_m$ to be considerable, nor in moderately drought-stressed leaves or in leaves from well-irrigated cotton plants. These authors concluded that photorespiration probably acts as a kind of buffer to keep the spatial and temporal heterogeneities of $\Phi_{PSII}$ low. Similarly, the heterogeneity of $\Phi_{PSII}$ was low in the leaves of rose plants that had been subjected to drought stress (Calatayud et al., 2006b).

## Nutrients

In the last few decades, agriculture has steadily increased because of increased nutrient availability (Marschner, 2012). Macro- and micro-nutrients are essential for plant life, and their deficiency can strongly compromise plant life cycles and yields (Marschner, 2012). Plant growth and mineral nutrients are well described by the dose-response curve (Berry & Wallace, 1981). When the concentration is lower or higher than this optimum, plant growth is stunted, and the general range between optimum and toxicity is extremely narrow for micronutrients.

The photosynthetic process is influenced directly by nutrient deficiency. For example, iron is an essential element for plants and its deficiency causes perturbations in the photosynthetic process for it to play its role as a constituent of the complexes involved in the electron transport rate (Guerinot & Yi, 1994; Msilini et al., 2011; Yadavalli et al., 2012). Reduced iron availability determines reduced actual photosystem II efficiency for steady-state photosynthesis (Belkhodja et al., 1998; Donnini et al., 2013; Osório et al., 2014). The use of CFI has evidenced spatial changes in leaf lamina of cucumber plants grown under Fe-deficiency conditions (Donnini et al., 2013). Osório et al. (2014) reported that Fe stress generates spatio-temporal heterogeneity in the fluorescence response, and that $\Phi_{PSII}$ measured in interveinal mesophyll areas can be used as an early fast indicator of Fe deficiency, and can be applied to fertilisation management.

Even excess nutrients can detrimentally influence the photosynthetic process. Despite the final effect often caused by the luxury availability of nutrients being an impairment of $CO_2$ assimilation, it is sometimes the last event of a series of specifically induced metabolic perturbations caused by different elements available in excess. The toxic effects induced on photosynthesis by different nutrients warrant a detailed description, but are beyond the scope of the present review. This report provides the following results on the use of Chl fluorescence as a diagnostic tool for screening the effect of boron toxicity in plants.

It has been recently demonstrated that an anthocyanin-rich cultivar of sweet basil (Red Rubin) is less prone to photo-inhibition and maintains higher $\Phi_{PSII}$ compared to a green (anthocyanin-less; Tigullio) counterpart when s ubjected to high B concentrations in the medium (Landi et al., 2013a). By means of CFI, it is now possible to localise the effect of B toxicity on leaf lamina margins, where B tends to accumulate. A similar heterogeneity in B distribution on leaf lamina has also been reported in two *Cucurbitaceae* species (Landi et al., 2013b). It was suddenly demonstrated that another purple cultivar of sweet basil (Dark Opal) displayed a low level of photodamage compared to eight other green basil genotypes (Landi et al., 2013c). These results have allowed the authors to speculate that epidermally-located anthocyanins can limit damage to PSII by absorbing the excess photons that reach PSII when partially compromised by B toxicity.

## Pollutants

Chl fluorescence measurements have been used for decades to determine the effects of environmental pollutants [heavy metals, ozone ($O_3$), sulphur dioxide ($SO_2$), nitric oxides ($NO_x$)] on plants. Plant exposure to heavy metals, mainly Cd,

Cu and Zn, results in alterations in the photosynthetic process due to a single phenomenon or cumulative phenomena of: i) the direct interaction of heavy metals with the protein thioyl-, histidyl- and carboxyl-groups; ii) displacement of essential cations in protein-active centres; iii) formation of reactive oxygen species (ROS) (Nagajyoti et al., 2010; Prasad, 2013). Reduced photosynthetic pigment is another possibility by which heavy metals, particularly Hg, Cu, Cd, Ni or Zn, interfere with photosynthesis. These metals may indeed substitute the central Mg ion in chlorophyll molecule to form chlorophyll-metal complexes, and thus lower PSII quantum efficiency (Sharma & Dietz, 2009; Dietz & Pfannschmidt, 2011).

Of the different heavy metals, Cd is certainly one the most toxic elements (Sanità di Toppi & Gabrielli, 1999; Lin & Aarts, 2012). Most plants show sensitivity to low Cd, which alters the chloroplast ultrastructure and the photosynthesis rate, and disturbs the Calvin cycle, antioxidant enzymes, and the uptake and distribution of macro- and micronutrients (Di Cagno et al., 1999, 2001; Mobin & Khan, 2007; Khan et al., 2007; Anjum et al., 2008; Márquez-García et al., 2010; Degl'Innocenti et al., 2014). Photosynthetic pigment and Chl fluorescence responses of *Quercus suber* L. at high Cd concentrations are impaired $F_v/F_m$, $\Phi_{PSII}$ and qp decrease, and when NPQ increases, which occur with a concomitant increase in the amount of the pigments involved in the xanthophyll cycle (Gogorcena et al., 2011). On the contrary, no alterations in the $F_v/F_m$ ratio have been observed in sunflower plants subjected to Cd stress, even though $q_P$ and $\Phi_{PSII}$ under steady-state conditions were significantly depressed and non-photochemical quenching increased (Di Cagno et al., 1999, 2001). In poplar and willow clones, the use of CFI has allowed areas inside leaves to be visualised, where Cd accumulates and corresponds to the dark area in which $F_{PSII}$ comes close to zero (Pietrini et al. 2010). In general terms, Chl fluorescence has long since been utilised to detect the influence of heavy metal ions on plant species grown in heavy metals-contaminated soil. For example, Zurek et al. (2014) tested perennial grasses as potential phytoremediators, which they grew in contaminated soils. The $F_v/F_m$ ratio lowered in these plants, which indicated reduced PSII efficiency strictly in relation to heavy metal uptake. It was noteworthy that no differences in the $F_v/F_m$ parameter were found for the cultivars that limited ion uptake.

Other metals, like Cu or Zn, cause alterations to Chl fluorescence parameters. Wodala et al. (2012) investigated the effect of moderate Cu and Cd stress on *Pisum sativum* L. plants. Both metals slightly inhibited PSII photochemistry (decrease in $\Phi_{PSII}$) and the electron transport rate. Redondo-Gómez et al. (2011) induced excess zinc in the C4 halophytic species *Spartina densiflora* Brogn. Zinc concentrations > 1 mmol $l^{-1}$ stunted the growth of *S. densiflora* through reduced $CO_2$ assimilation, and a marked overall effect on photochemical PSII efficiency was noted.

In the air pollutants context, tropospheric $O_3$ is one of the most relevant pollutants mainly in the Mediterranean climate (Ferretti et al., 2007; Paoletti, 2009). The first study on the relationship between $O_3$ stress and Chl fluorescence was published by Schreiber in 1978. To date, much research has utilised Chl fluorescence to monitor changes in photosynthetic apparatus in plants subjected to $O_3$ (Guidi & Calatayud, 2014). Clearly, the response of plants to $O_3$ differs among plants, and even among cultivars of the same species (Calatayud et al., 2002a, 2002b; Calatayud & Barreno, 2004; Guidi et al., 1997, 2009). Recently Bussotti et al. (2011) reported the overall reassessment of the results obtained from fluorescence transient and modulated fluorescence analyses on tree species subjected to treatment with $O_3$. Their review aimed to identify the damage mechanisms and repair/avoidance strategies adopted by different plant species that have been reported in the existing literature. In another work, Gottardini et al. (2014) studied the concurrent measurements of Chl fluorescence transient parameters together with $O_3$-specific visible foliar symptoms. They concluded that Chl fluorescence parameters provide a set of valuable diagnostic indicators for the early identification and assessment of $O_3$ effects on native vegetation and, potentially, for phenotyping ozone-sensitive individuals.

Heterogeneities in leaves by $O_3$ can see under CFI, which allows the identification of damaged (localized) areas and provides detailed information about leaf disturbance. An images analysis in *Lupinus albus* L. leaves fumigated with $O_3$ has shown spatial damage distribution (Guidi et al., 2007). In this study, the fluorescence yield was lower in leaf veins than in the interveinal area. This suggests that the leaf area close to the veins is more sensitive to the ozone. CFI has also been used for distinguishing between acute (high level of $O_3$ concentration >100 nL $L^{-1}$ for a short-period, i.e. hours; Guidi et al., 2007) and chronic effects ($O_3$ concentration <100 nL $L^{-1}$ over long-term exposure). Damage by an acute $O_3$ concentration (400 nL $L^{-1}$ for 6 h) in *Glycine max* Merr. has been characterised by small local areas where photosynthesis decreases, most of which are located in the areas near major veins (Chen et al., 2009). In chronic $O_3$ treatment, the worst photosynthetic capacity has been found in the interveinal areas, associated with more variable sizes and shapes of the chlorotic/necrotic area (Chen et al., 2009).

Studies into watermelon (Calatayud et al., 2006a) have also shown that the interaction between $O_3$ and N fertiliser can occur. At a high $O_3$ concentration and with strong N fertilisation, a significant drop in the $F_v/F_m$ ratio and in $\Phi_{PSII}$, and an increase in NPQ, were observed.

In addition to the quantum yield of PSII photochemistry

under light conditions, $\Phi_{PSII}$, other useful parameters have been represented by the quantum yield of regulated energy dissipation in PSII, $\Phi_{NPQ}$, and the quantum yield of non-regulated energy dissipation in PSII, $\Phi_{NO}$ (Kramer et al., 2004). Two bean cultivars with different sensitivities to the ozone, i.e., $O_3$-sensitive Cannellino and $O_3$-tolerant Top Crop, have been exposed to acute $O_3$-stress. The ability to dissipate excess energy via regulated $\Phi_{NPQ}$, and unregulated non-photochemical quenching ($\Phi_{NO}$) mechanisms, has been reported to be greater in Top Crop than in Cannellino. These physiological-traits have allowed the $O_3$-tolerant cultivar to compensate for the light-induced declines in $\Phi_{PSII}$, to preserve PSII from excitation-energy, and to likely prevent ROS generation to a lesser extent than the $O_3$-sensitive cultivar (Guidi et al., 2010).

Nitrogen oxides (NOx) are the air pollutants precursors of $O_3$, mainly at high light intensity (Chameides et al., 1992). NOx have been considered less toxic to plants, compared to $SO_2$ and $O_3$, since exposure to NOx does not always lead to deleterious effects on plants (Capron & Mansfield, 1976). $NO_2$ inside leaves can form strong acids, such as $HNO_2$ and $HNO_3$ (Schmidt et al., 1990). Furthermore, cellular acidification causes nitrate reductase to lower (Padidam et al., 1991). The lower activity of this enzyme and the presence of $HNO_3$ and $HNO_2$ might enhance accumulation of nitrates, with negative effects on the photosynthetic process (Qiao & Murray, 1998). However, $NO_2$ exposure on rice has no effect on Chl fluorescence parameters, and only stomatal conductance reduction has been reported compared with the control plants (Maggs and Ashmore, 1998). van Hove et al. (1992) have reported a lower electron transport rate in Douglas firs subjected to chronic $NO_2$ treatment compared to the control shoots (filtered air).

Sulphur dioxide is a primary product of fossil fuel combustion or from refining sulphur-containing minerals. $SO_2$ can easily penetrate chloroplast, which affects plant growth and development (Darrall, 1989; Surówka et al., 2007). It can react with water on the leaf surface to produce bisulphate and enter through the cuticle, where it is converted into sulphite in chloroplast (Laisk et al., 1988), and can be oxidised to sulphate. *Pinus sylvestris* L., a popular Mediterranean pine grown in an industrial polluted area, has shown a reduced $F_v/F_m$ ratio compared to the control area (Pukacki, 2004). These results agree with those of Strand (1995), who showed a lower $F_v/F_m$ ratio after exposing *P. sylvestris* to low concentrations of $SO_2$ and $NO_2$ for four consecutive years.

## Temperature

The effect of high temperature on plants primarily affects photosynthetic functions, and the heat tolerance limit of leaves coincides with the thermal sensitivity of primary photochemical reactions that occur in the thylakoid membrane system (Chen et al., 1982). High temperature modifies structure and damages PSII, and the oxygen evolving complex is particularly deactivated, even at slightly elevated temperatures (Yamane et al., 1998). Furthermore, the separation of LHCII from the core centre induces grana destacking (Gounaris et al., 1984) and induces the migration of LHCII to the non-appressed region.

Chl fluorescence has been used as a biomonitoring technique to test the effects of future warming events or sporadic hot waves on photosynthetic performance in several plant species (see Gorbe & Calatayud, 2012; Guidi & Calatayud, 2014). The optimal temperatures for maximum PSII quantum yields are generally broad and match the average daytime growth temperature (Larcher, 1995). For example, the leaves of *Phalaenopsis* Blume, *Deschampsia antarctica* E. Desv., *Larrea tridentate* (DC.) Coville, *Gossypium hirsutum* L. or *Nicotiana tabacum* L. maintain a constant $F_v/F_m$ over a wide range of temperatures (Salvucci & Crafts-Brandner, 2004). A drop in $F_v/F_m$ occurs at temperatures above those that inhibit a net photosynthetic rate (Georgieva et al., 2000), which indicates that the $F_v/F_m$ ratio is quite a sensitive Chl fluorescence parameter. These results have beene observed in barley (Oukarroum et al., 2009), strawberry (Kadir et al., 2006), tomato (Willits & Peet, 2001), grape (Kadir et al., 2007) and raspberry (Mochizuki et al., 2010; Molina-Bravo et al., 2011). In general, the tolerant varieties to heat temperature have shown a higher $F_v/F_m$, and have maintained higher rates of photosynthesis and $\Phi_{PSII}$ or zeaxanthin accumulation than sensitive varieties (Fracheboud & Leipner, 2003).

However, plants that inhabit different environments have evolved mechanisms to optimise growth within defined temperature ranges. High temperature, in addition to a higher risk of heat damage, also enhances water shortage to plants. In fact, plants increase evaporation of water from stomata to cool leaves to minimise heat damage. Clearly in the era of global change, in which heat stress occurs contemporarily to drought, this event enhances the negative effects induced by water shortage (Allen et al., 2010).

Even low temperature or freezing can decrease, or even stop, biochemical activity in chloroplast. When comparing *Quercus ilex* L. and *Phyllirea latifolia* L. (Ogaya et al., 2011), the effect of cold temperature was stronger in *P. Latifolia,* and was associated with a lower $F_v/F_m$. High irradiance, along with low temperatures, spells overexcitation of the electron transport chain, which produces photo-chilling (Garcia-Plazaola et al., 1999). Photo-chilling occurs when a large amount of energy is trapped by Chls, but cannot be dissipated safely by heat. Consequently, carbon assimilation is limited and overproduction of oxygen free radicals takes place.

Cold-induced limitations on photosynthesis (i.e., reduced photochemical use of absorbed light energy, and an imbalance between the reducing equivalents produced in excess and the consumption capacity of photosynthesis) lead to photosynthesis photo-inhibition (Powles, 1984; Baker, 1994). Increased susceptibility to photo-inhibition has been well established during exposure of photosynthetic organisms to low temperatures in combination with even moderate photon flux density (Öquist & Martin, 1986; Osmond, 1994). It has been suggested that persistent photosynthetic efficiency depression in cold-acclimated maize may also result from the higher proportion of inactive PSII centres and the lesser capacity of repair and/or replacement of damaged PSII centres (Fryer et al., 1995).

**Salinity**

Salinity is one of the biggest limiting factors for agriculture in semi-arid environments. Nowadays saline soils cover more than 10 million ha only in the 22 countries that surround the Mediterranean Basin (Zdruli, 2014). Soils are classified as saline when electrical conductivity is 4 dS $m^{-1}$ (about 40 mM of NaCl) or higher (USDA-ARS, 2008). This condition makes it harder for roots to extract water from soil, and can also cause hyperaccumulation of $Na^+$ and $Cl^-$ in whole plants (Munns & Tester, 2008). Both these effects can reduce plant growth and photosynthesis (Long & Baker, 1986; Chaves et al., 2009).

Chl fluorescence has been widely utilised for two reasons: firstly, as an applicative tool for screening tolerant *vs.* salt-sensitive genotypes (a few exemplifiable works are: Penella et al., 2013; Hanachi et al., 2014; Khayyat et al., 2014; Sing & Sarkar, 2014); secondly, for more in-depth studies into plant physiology which aim to evaluate the effect of salt excess on photosynthetic machinery (Homann, 1969; Murata, 1969; Gross & Prasher, 1974; Mehta et al., 2010; Guidi et al., 2011; Penella et al., 2015).

For the first aim, simple $F_v/F_m$ measurements suffice in some cases to distinguish salt-sensitive from salt-tolerant genotypes, particularly under severe salinity. For example, Singh and Sarkar (2014) used 12 rice cultivars to find a decline in $F_v/F_m$ in the salt-sensitive, but not in the salt-tolerant, genotypes following 3-day exposure to 80 mM NaCl. Similarly, Khayyat et al. (2014) reported a good correlation between the decline in $F_v/F_m$ and the salt concentration supplied to two pomegranate cultivars characterised by different salt sensitivity, where the decline for sensitive cultivar Shishe Kab was steeper. Conversely, while screening pepper genotypes subjected to mild-prolonged salt stress (40 mM NaCl for 5 months), Penella et al. (2013) evidenced that the widely used $F_v/F_m$ ratio was not a useful parameter alone for assessing damage to photosynthetic apparatus, while gas exchange was more sensitive for this purpose. In addition, Netondo et al. (2004) observed no significant changes in $F_v/F_m$ in response to salt stress between a sensitive and more tolerant cultivar of sorghum, but found a more marked increase in $q_{NP}$ and a less decreased ETR in the salt-sensitive cultivar. The $F_v/F_m$ ratio is a useful parameter, but its reduction can be attributed to regulated or damaged PSII units. Thus in some cases, using Chl fluorescence at the in-depth level (i.e., fast/slow kinetics and/or light curves) could provide a much more informative and detailed picture of PSII status and allow genotypes to be compared (Guidi et al., 1999).

For the second aim, Chl fluorescence has been used in particular to: (i) assess the direct effect of salt excess on photosynthetic apparatus and highlight the special localisation of salt-induced effects on whole leaf lamina; (ii) confirm that the species which adopt different mechanisms to cope with salt excess are healthier than the plant species that lack these mechanisms, or (iii) to evaluate the interaction of the factors that occur with salinity.

Pioneering works into the effect of salt excess on photosynthetic apparatus date back to late 1960s, when it was established that changes in Chl fluorescence in salt-treated thylakoids correlate with an energy spillover from PSII to PSI (Homann, 1969; Murata, 1969). It was suddenly demonstrated that excess cations induce conformation changes in the thylakoid structure (Barber, 1976) and staking (Gross & Prasher, 1974). More recently in detached wheat leaves exposed to high salt stress (0.1-0.5 M NaCl) for 1 h, it has been demonstrated that the number of inactive PSII reaction centres rose with increasing salt concentrations, and the damage caused by a high salt influx was more prominent on the donor side than on the acceptor side of PSII (Mehta et al., 2010). The effects of high salt stress are largely reversible as acceptor side damage completely recovered, while donor side recovery was below 85% (Mehta et al., 2010). As explained above, it has been revealed that $F_v/F_m$ is not a useful indicator of salt stress in some plant species, while other parameters, such as $\Phi_{PSII}$, $q_P$ and NPQ, are more sensitive to salt stress (Bongi & Loreto, 1989; Zribi et al., 2009; Guidi et al., 2011). The latter parameters can also allow the spatial localisation of salt-induced symptoms to PSII centres on the leaf area to be distinguished. For example by CFI, Guidi et al. (2011) found that high salinity did not change the values of either $F_v/F_m$ or NPQ in the whole leaves of tomato irrigated with a saline solution that contained 550 mg $L^{-1}$ NaCl compared to the controls. Conversely, higher $\Phi_{PSII}$ values were found in salt stressed-leaves, and notably the $\Phi_{PSII}$ values in central area of leaves were higher than those found in the marginal area. This finding suggests heterogeneous salt-induced effects on whole leaf lamina.

The adaptive mechanisms adopted by plant species to cope with salinity stress, as well as the effect of the simultaneous concomitance of salinity to other stressors, have been intensively explored in recent years. As many publications on this matter exist, only a few key examples that highlight the versatility of Chl fluorescence are reported below. It has been found that, for example, $\Phi_{PSII}$ and carbon gains remain higher in *Pistacia lentiscus* L. than in *Myrtus communis* L. when exposed to mild-prolonged salt stress either alone or in combination with strong light (Tattini et al., 2006). The better performance of *P. lentiscus* derives from the better ability to use $Na^+$ and $Cl^-$ for osmotic adjustment and by morpho-anatomical traits. Consequently, the biosynthesis of flavonoids and the incremented carotenoids/chlorophyll content ratio are likely adopted to compensate the inherent lower ability of *M. communis* to cope with salt accumulation in leaf tissues, especially when grown under full sunlight. Conversely, *Olea europea* L. leaves have been photo-inhibited; enhanced $q_{NP}$ and reduced PSII efficiency have been reported only when plants were grown with 125 mM NaCl and in partial shade (15% full sunlight), while no differences in any Chl fluorescence parameters have been detected in plants grown in full sunlight (Melgar et al., 2009). In this species, changes in the $Ca^{2+}/Na^+$ ratios have been reported to take place in the root zone, a condition that usually occurs in calcareous soils were *O. europea* is largely cultivated, and which can further increase its salinity tolerance (Tattini et al., 2009).

## CONCLUSION

Chl fluorescence represents a good useful technique to detect photosynthetic responses of plants grown under environmental stresses. It also provides useful information about the extent to which plant performance is limited by photochemical and non-photochemical processes. When utilised with other non-invasive techniques, such as gas exchange, it can provide insights into limitation to photosynthesis. Its use in plant physiology under environmental stresses has been widely reported. However, care must be taken because it is easy to make mistakes and obtain meaningless results, even though it is easy to utilise Chl fluorometers and to obtain Chl fluorescence parameters (Murchie & Lawson, 2013).

In addition to the above-reported utilisation in plant ecophysiology, Chl fluorescence is widely utilised in other fields, such as studies into the physiology of algae (Roháček et al., 2014) cyanobacteria (Campbell et al., 1998) and lichens (Manrique et al., 1993).

Some Chl fluorescence applications have even focused on the quality, chemistry and physical characteristics of fruits (Nedbal & Withmarsh, 2004; Gorbe & Calatayud, 2012). Indeed Chl fluorescence has been utilised to detect the healthy of flesh kiwi fruits upon storage as fresh-cut produce (Figure 2). Chl fluorescence has also been employed to detect cut flower quality under different storage conditions (Gorbe & Calatayud, 2012).

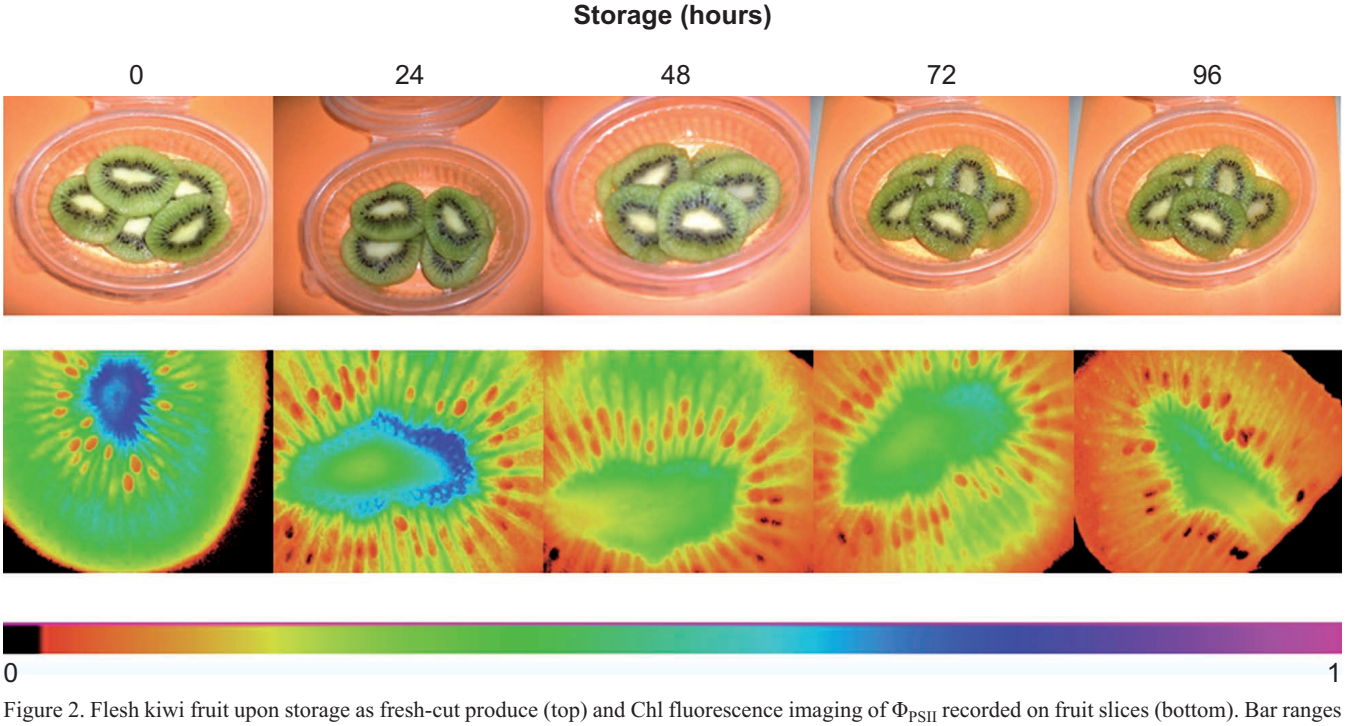


Figure 2. Flesh kiwi fruit upon storage as fresh-cut produce (top) and Chl fluorescence imaging of $\Phi_{PSII}$ recorded on fruit slices (bottom). Bar ranges from 0 to 1, and indicates the false colour palette.

Another recent Chl fluorescence application has been to provide information on the (in)-compatibility between rootstocks and scions in herbaceous plants, such as grafted melon plants (Calatayud et al., 2013) and pepper grafted plants. Figure 3 depicts the greater compatibility between pepper plants grafted onto pepper (compatible union) or onto tomato rootstocks (incompatible combination) measured as an $F_v/F_m$ ratio.

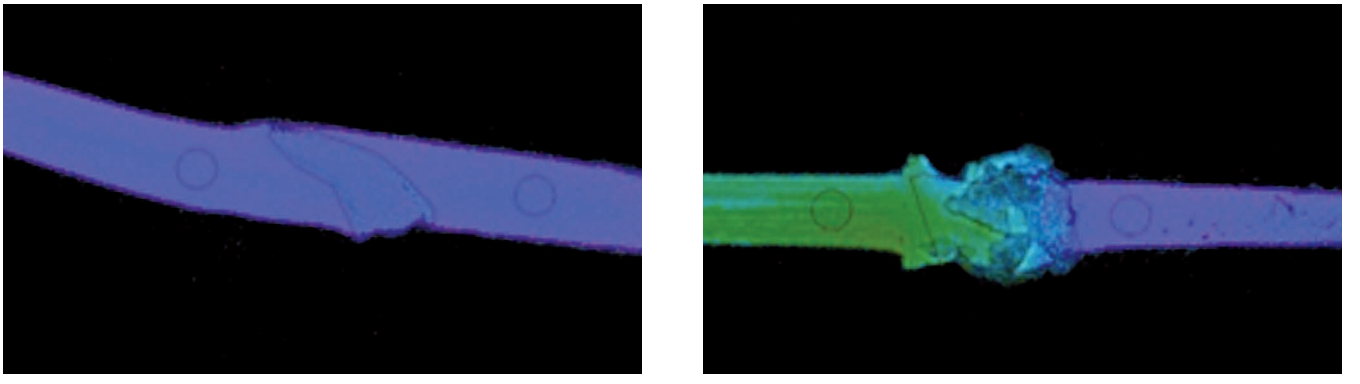

Figure 3. Chl fluorescence images of $F_v/F_m$ recorded in stems of pepper (cultivar Adige) grafted onto pepper rootstock *Capsicum annuum* (left) or onto tomato rootstock Beaufort (right).

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