

# Imaging of Chlorophyll a Fluorescence: A Tool to Study Abiotic Stress in Plants

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## 1. Introduction

Chlorophyll (Chl) fluorescence is a tool which is widely used to examine photosynthetic performance in algae and plants. It is a non-invasive analysis that permits to assess photosynthetic performance in vivo (Baker, 2008; Baker & Rosenqvist, 2004; Chaerle & Van Der Straeten, 2001; Woo et al. 2008). Chl fluorescence analysis is widely used to estimate photosystem II (PSII) activity, which is an important target of abiotic stresses (Balachandran et al., 1994; Baker et al., 1983; Briantais et al., 1996; Calatayud et al., 2008; Chaerle & Van Der Straeten, 2000; Ehlert & Hinch, 2008; Gilmore & Govindjee, 1999; Guidi et al., 2007; Guidi & Degl'Innocenti, 2008; Hogewoning & Harbinson, 2007; Krause, 1988; Lichtenthaler et al., 2007; Massacci et al., 2008; Osmond et al., 1999; Scholes & Rolfe, 1996; Strand & Oquist, 1985).

It is known as the energy absorbed by Chl molecules must be dissipated into three mechanisms, namely internal conversion, fluorescence and photochemistry (Butler, 1978). All of these downward processes competitively contribute to the decay of the Chl excited state and, consequently, an increase in the rate of one of these processes would increase its share of the decay process and lower the fluorescence yield. Typically, all processes that lower the Chl fluorescence yield are defined with the term *quenching*.

Kautsky and co-workers (1960) were the first which observed changes in yield of Chl fluorescence. These researchers found that transferring a leaves from the dark into the light, an increase in Chl fluorescence yield occurred. This increase has been explained with the reduction of electron acceptors of the PSII and, in particular, plastoquinone  $Q_A$ : once PSII light harvesting system (LCHII) absorbs light and the charge separation occurs,  $Q_A$  accepts electron and it is not able to accept another electron until it has been passed the first one onto the subsequent carrier, namely plastoquinone  $Q_B$ . During this time the reaction centers are said to be *closed*. The presence of closed reaction centers determines a reduction in the efficiency of PSII photochemistry and, consequently, an increase in the Chl fluorescence yield.

Transferring the leaf from the dark into light, PSII reaction centers are progressively closed, but, following this time, Chl fluorescence level typically decreases again and this phenomenon is due to two types of quenching mechanisms. The presence of light induced the activation of enzymes involved in  $CO_2$  assimilation and the stomatal aperture that determines that electrons are transferred away PSII. This induced the so-called *photochemical quenching*,  $q_p$ . At the same time, there is an increase in the conversion of light energy into

heat related to the *non-photochemical quenching*,  $q_{NP}$ . This non-photochemical quenching  $q_{NP}$ , can be divided into three components. The major and most rapid component in algae and plants is the pH- or energy-dependent component,  $q_E$ . A second component,  $q_T$ , relaxes within minutes and is due to the phenomenon of state transition, the uncoupling of LHCII<sub>s</sub> from PSII. The third component of  $q_{NP}$  shows the slowest relaxation and is the least defined. It is related to photoinhibition of photosynthesis and is therefore called  $q_I$ .

To evaluate Chl fluorescence quenching coefficients during illumination we must determine minimal and maximal fluorescence yields after dark adaptation,  $F_0$  and  $F_m$  respectively. This is important because these values serve as references for the evaluation of the photochemical and non-photochemical quenching coefficients in an illuminated leaf by using the *saturation pulse method*. The concept on the basis of this method is extremely simply: at any give state of illumination,  $Q_A$  can be fully reduced by a saturation pulse of light, such that photochemical quenching is completely suppressed. During the saturation pulse, a maximal fluorescence  $F_m'$  is achieved which generally shows value lower than the dark reference values ( $F_m$ ). With the assumption that non-photochemical quenching does not change during a short saturation pulse, the reduction of  $F_m$  is a measure of non-photochemical quenching.

In **Figure 1** the calculation of Chl fluorescence parameters by using the saturation pulse method is reported. The photochemical quenching coefficient  $q_P$  is measured as

$$q_P = (F_m' - F_t) / (F_m' - F_0') \quad (1)$$

where  $F_m'$  is the maximum Chl fluorescence yield in light conditions,  $F_t$  is the steady-state Chl fluorescence immediately prior to the flash. For determination of  $F_0'$  in the light state, the leaf has to be transiently darkened and it has to be assured that  $Q_A$  is quickly and fully oxidized, before there is a substantial relaxation of non-photochemical quenching. In order to enhance of oxidation of the intersystem electron transport chain, far-red light is applied that selectively excites PSI. Usually the alternative expression of this quenching coefficient is used and it is  $(1 - q_P)$ , i.e. the proportion of centers that are closed and it is termed *excitation pressure* on PSII (Maxwell & Johnson, 2000).

An other useful fluorescence parameter derived from saturation pulse method is the efficiency of PSII photochemistry, which is calculated as:

$$\Phi_{PSII} = (F_m' - F_t) / F_m' \quad (2)$$

This parameter has also termed  $\Delta F / F_m'$  or, in fluorescence imaging technique,  $F_q' / F_m'$  and it is very similar to the  $q_P$  coefficient even if its significance is somewhat different. The  $\Phi_{PSII}$  is the proportion of absorbed light energy being used in photochemistry, whilst  $q_P$  gives an indication of the proportion of the PSII reaction centers that are open. A parameter strictly related with both  $q_P$  and  $\Phi_{PSII}$  is the ratio  $F_v / F_m$  determined as:

$$F_v / F_m = (F_m - F_0) / F_m \quad (3)$$

This third parameter is determined in dark adapted leaves and it is a measure of the maximum efficiency of PSII when all centers are open. This ratio is a sensitive indicator of plant photosynthetic performance because of it has an optimal values of about 0.83 in leaves of healthy plants of most species (Bjorkman & Demmig, 1987). An other useful parameter which describes energy dissipation is  $F_v' / F_m'$ , an estimate of the PSII quantum efficiency if all PSII reaction centers are in the open state. It is calculated as reported in equation 4:

$$F_v'/F_m' = (F_m' - F_0')/F_m' \quad (4)$$

Since  $\Phi_{PSII}$  is the quantum yield of PSII photochemistry, it can be used to determine linear electron transport rate (ETR) as described by Genty et al., (1989):

$$ETR = \Phi_{PSII} \times PPFD \times 0.5 \quad (5)$$

where PPFD (photosynthetic photon flux density) is the absorbed light and 0.5 is a factor that accounts for the partitioning of energy between PSII and PSI.

The excess of excitation energy which is not used for photochemistry can be de-excited by thermal dissipation processes. Non-photochemical quenching of Chl fluorescence is an important parameter that gives indication of the non-radiative energy dissipation in the light-harvesting antenna of PSII. This parameter is extremely important taking into account that the level of excitation energy in the antenna can be regulated to prevent over-reduction of the electron transfer chain and protect PSII from photodamage. Non-photochemical quenching coefficient is calculated as:

$$q_{NP} = (F_m - F_m') / (F_m - F_0') \quad (6)$$

In some circumstances  $F_0'$  determination is difficult, e.g. in the field when a leaf cannot be transiently darkened. In this case, another parameter can be used to describe non-photochemical energy dissipation NPQ (Schreiber & Bilger, 1993), which does not require the knowledge of  $F_0'$ . The parameter NPQ is derived from Stern-Volmer equation and its determination implies the assumption of the existence of traps for nonradiative energy dissipation, like zeaxanthin, in the antenna pigment matrix (Butler, 1978). NPQ is calculated as reported in equation 7 (Bilger & Bjorkman, 1990):

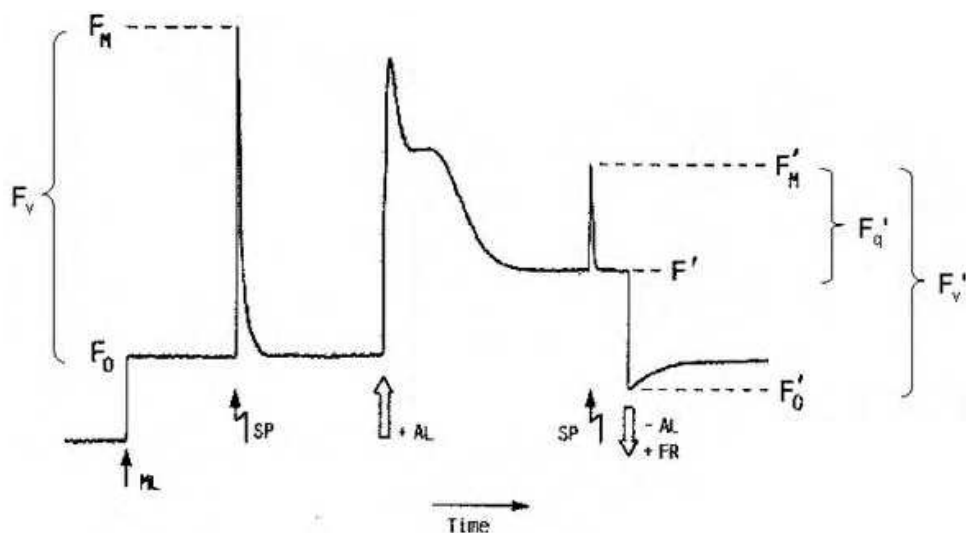


Fig. 1. Measurement of chlorophyll fluorescence by the saturation pulse method (adapted from Van Kooten & Snell, 1990).

$$NPQ = (F_m - F_m') / F_m' \quad (7)$$

NPQ is linearly related to heat dissipation and varies on a scale from 0 until infinity even if in a typical plants value ranges between 0.5 and 3.5 at light saturation level.

Chl fluorescence analysis gives a measure of the photosynthetic rate and for this reason it is extremely useful. Really, Chl fluorescence gives information about the efficiency of PSII photochemistry that, in laboratory conditions, is strictly correlated with CO<sub>2</sub> photoassimilation (Edwards & Baker, 1993; Genty et al., 1989). Under field conditions, this correlation is lost because other processes compete with CO<sub>2</sub> assimilation such as photorespiration, nitrogen metabolism and Mehler reaction (Fryer et al., 1998). In addition to, a complication derives to heterogeneity between samples. To calculate ETR we assume that the light absorbed by PSII is constant, but it is not true. Even if there are some limitations, Chl fluorescence can give a good, rapid and non invasive measurements of changes in PSII photochemistry and then also the possibility to evaluate the effects of abiotic stresses on PSII performance.

## 2. Chl fluorescence imaging

The evolution of Chl fluorescence analysis is represented by Chl fluorescence imaging which can be useful applied into two general areas: the study of heterogeneity on leaf lamina and the screening of a large numbers of samples. This technique has been widely applied in the past during induction of photosynthesis (Bro et al., 1996; Oxborough & Baker, 1997), with changes in carbohydrate translocation (Meng et al., 2001), in response to drought (Meyer & Genty, 1999; West et al., 2005), chilling (Hogewoning & Harbinson, 2007), ozone pollution (Guidi et al., 2007; Guidi & Degl'Innocenti, 2008; Leipner et al., 2001), wounding (Quilliam et al., 2006), high light (Zuluaga et al., 2008) and infection with fungi (Guidi et al., 2007; Meyer et al., 2001; Scharte et al., 2005; Scholes & Rolfe, 1996; Schwarbrick et al., 2006) or virus (Perez-Bueno et al., 2006). With Chl fluorescence imaging is possible to detect an analysis of stress-induced changes in fluorescence emission at very early stage of stress. In addition to, Chl fluorescence imaging technique represents a useful screening tool for crop yield improvement.

The most essential new information provided by Chl fluorescence imaging relates to the detection of lateral heterogeneities of fluorescence parameters which reflect physiological heterogeneities. It is well known that even physiologically healthy leaves are "patchy" with respect to stomatal opening. Furthermore, stress induced limitations, which eventually will lead to damage, are not evenly distributed over the whole leaf area. Fluorescence imaging may serve as a convenient tool for early detection of such stress induced damage. The main difference between the conventional fluorometer and the imaging fluorometer is the possibility of parallel assessment of several samples under identical conditions.

For example we treated plants of *Phaseolus vulgaris* (cv. Cannellino) with a single pulse of ozone (O<sub>3</sub>) (150 nL L<sup>-1</sup> for 5 h) and evidenced upon leaf lamina and evident heterogeneity in some Chl fluorescence parameter as compared to control exposed to charcoal filtered air for the same period (Guidi & Degl'Innocenti, *data not published*) (**Figure 2**).

It is known as in plants exposed to chilling stress, photosynthetic enzymes may be inactivated or degraded and photodamage to PSII may happen, reducing photosynthesis (Dai et al., 2007; Feng & Cao, 2005; Flexas et al. 1999). The reduction in photosynthetic CO<sub>2</sub> assimilation may lead to accumulation of excess energy especially at high irradiance and consequently to

photoinhibition (Feng & Cao, 2005; Hovenden & Warren, 1998). In variegated leaves of *Calathea makoyana* the effect of chilling (5° and 10°C for 1-7 d) on PSII efficiency was studied in order to understand the causes of chilling-induced photoinhibition (Hogewoning & Harbinson, 2007). The individual leaves were divided into a shaded zone and two illuminated, chilled zones. Chilling up to 7 d in the dark did not influence PSII efficiency whereas chilling in the light caused severe photoinhibition. Data obtained from Chl fluorescence imaging were confirmed by visual appearance of symptoms which were evident in the portion of leaves chilled and illuminated. Obtained results showed that photoinhibition was due to a secondary effect in the unchilled leaf tip (sink limitation) as revealed by starch accumulation data. Instead it was a direct effect of chilling and irradiance in the chilled illuminated zones.

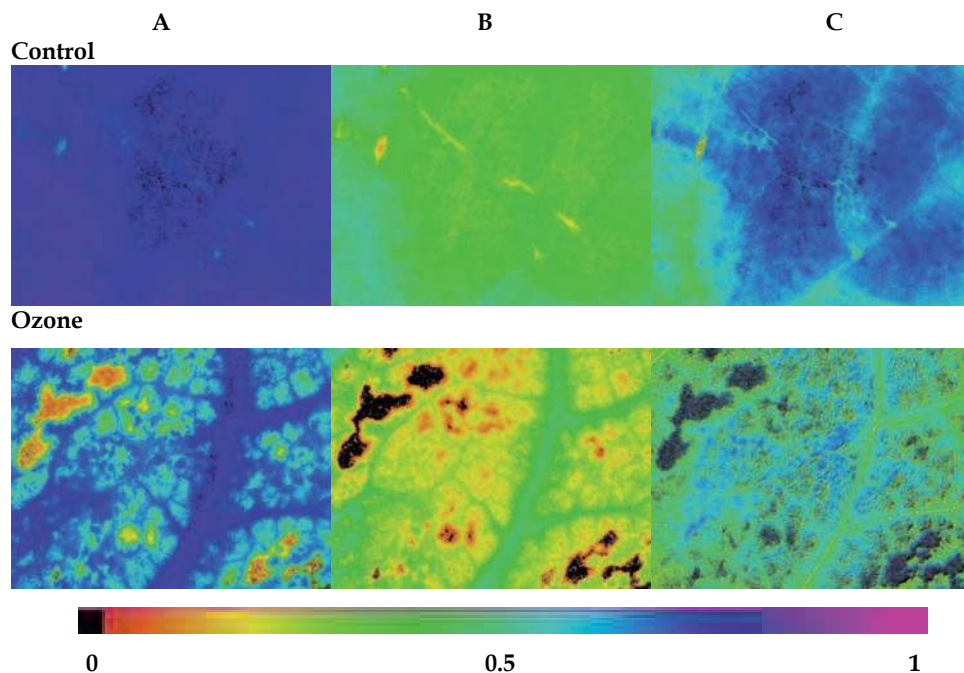


Fig. 2. Chl fluorescence imaging of  $F_v/F_m$  (A),  $\Phi_{PSII}$  (B) and non-photochemical quenching (C) in leaves of *P. vulgaris* cv. Cannellino exposed for 5 h at an  $O_3$  concentration of 150 nL L<sup>-1</sup> (Ozone) or 2 nL L<sup>-1</sup> (Control). All images are normalised to the false colour bar provided. The analyses of  $F_v/F_m$  were carried out on dark-adapted leaves, while  $\Phi_{PSII}$  and  $q_{NP}$  at a light intensity of 500  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . The pixel value display is based on a false-colour scale ranging from black (0.00 to 0.040) via red, yellow, green, blue to purple (ending at 1.00) (from Guidi & Degl'Innocenti, data not published).

Calatayud et al. (2008) studied the effects of two nutrient solution temperatures (10° and 22°C) during the flowering of *Rosa x hybrida* by using Chl fluorescence imaging. The obtained results showed as the nutrient solution temperatures of 10°C induced an increase

in  $\Phi_{\text{PSII}}$  parameters indicating that the majority of photons absorbed by PSII were used in photochemistry and that PSII centers were maintained in an oxidized state.

Water stress is another important abiotic stress that induces reduction of growth and yield of plants. For this reason the development of drought-tolerance is an important target of the researchers. The effects of drought on photosynthetic process have been extensively studied in many plant species and the possible mechanisms involved in the responses have been suggested (Cornic & Fresneau, 2002; Flexas et al., 2002, 2004; Grassi & Magnani, 2005; Long & Bernacchi, 2003). Masacci et al. (2008) took Chl fluorescence images from leaves of *Gossypium hirsutum* to study the spatial pattern of PSII efficiency and non-photochemical quenching parameters. They found that under low and moderate light intensity, the onset of drought stress caused an increase in the operating quantum efficiency of PSII ( $\Phi_{\text{PSII}}$ ) which indicated increased photorespiration since photosynthesis was hardly affected by water shortage. The increase in  $\Phi_{\text{PSII}}$  was caused by an increase in  $F_v'/F_m'$  and by a decrease in non-photochemical quenching. Chl fluorescence imaging showed a low spatial heterogeneity of  $\Phi_{\text{PSII}}$ . The authors concluded that the increase in photorespiration rate in plants during the water stress can be seen as an acclimation process to avoid an over-excitation of PSII under more severe drought conditions.

Qing-Ming et al., (2008) used Chl fluorescence imaging analysis to detect the effects of drought stress and elevated  $\text{CO}_2$  concentration ( $780 \mu\text{mol mol}^{-1}$ ) in cucumber seedlings. They found that electron transport rate and the light saturation level declined significantly with drought stress aggravation in both  $\text{CO}_2$  concentrations. Drought stress decreased maximal photosynthetic ETR and subsequently decreased the capacity of preventing photodamage. At the same time, elevated  $\text{CO}_2$  concentration increased the light saturation level significantly, irrespective of the water conditions. Elevated  $\text{CO}_2$  concentration can alleviate drought stress-induced photoinhibitory damage by improving saturating photosynthetically active radiation.

Sommerville et al. (2010) examined the different spatial response in photosynthesis with drought in two species with contrasting hydraulic architecture. The authors hypothesized that areole regions near primary nerves would show a smaller decline in the maximum efficiency of PSII photochemistry with drought compared with regions between secondary nerves and that the difference between areole regions would be smaller in phyllodes with higher primary nerve density. Indeed, the phyllodes of *Acacia floribunda* were found to have both greater primary nerve density and show greater spatial homogeneity in photosynthetic function with drought compared with the phyllodes of *Acacia pycnantha*. *A. floribunda* phyllodes also maintained function of the photosynthetic apparatus with drought for longer and recovered more swiftly from drought than *A. pycnantha*.

Drought is a type of stress which can induce heterogeneity in leaf photosynthesis that probably occurs when dehydration is rapid as in the case of drought experiments performed on potted plants by withholding water. Using Chl fluorescence imaging, Flexas et al. (2006) showed in herbaceous species that exogenous ABA did not induce patchy stomatal closure even when stomatal conductance dropped to much lower values lower than  $0.05 \text{ mol m}^{-2} \text{ s}^{-1}$ . Even the quality and quantity of light intensity notable influence the photosynthetic apparatus and functioning. Generally, sun- and shade leaves differ in the composition of leaf pigment, electron carriers on thylakoids membranes, structure of the chloroplast and photosynthetic rate (Anderson et al., 1995; Boardman, 1977; Lichtenthaler, 1981, 1984; Lichtenthaler et al., 2007; Takahashi & Badger, 2010). Lichtenthaler et al. (2007) studied the differential pigment composition and photosynthetic activity of sun and shade leaves of

deciduous (*Acer psuedoplatanus*, *Fagus sylvaatica*, *Tilia cordata*) and coniferous (*Abies alba*) trees by using Chl fluorescence imaging analysis. This tool not only provided the possibility to screen the differences in photosynthetic CO<sub>2</sub> assimilation rate between sun and shade leaves, but in addition permitted detection and quantification of the large gradient in photosynthetic rate across the leaf area existing in sun and shade leaves.

Chl fluorescence analysis is used also to characterized photosynthetic process in transgenic plants such as tomato (*Lycopersicon esculentum*) cv. Micro-Tom transformed with the *Arabidopsis thaliana* MYB75/PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1) gene (Zuluaga et al., 2008). This gene encodes for a well known transcription factor, which is involved in anthocyanin production and is modulated by light and sucrose. The presence of a higher constitutive level of anthocyanin pigments in transgenic plants could give them some advantage, in terms of adaptation and defence against environmental stresses. To test this hypothesis, a high light experiment was carried out exposing wild type and transgenic tomato plants to a strong light irradiance for about ten days and monitoring the respective phenotypic and physiological changes. The light intensity used was very high and likely not similar to normal environmental conditions (at least for such a prolonged period). Chlorophyll fluorescence imaging on control and stressed leaves from both genotypes suggest that, in transgenic leaves, the apparent tolerance to photoinhibition was probably not due to an increased capacity for PSII to repair, but reflected instead the ability of these leaves to protect their photosynthetic apparatus.

Certainly among abiotic stress the pollutants can alter the physiology and biochemistry of plants. Ozone is an air pollutant that induces reduction in growth and yield of plants species. The major target of the O<sub>3</sub> effects is represented by photosynthetic process and many works have been reported as this pollutant can impair CO<sub>2</sub> assimilation rate. Plant response depends also on the dose (concentration x time). In fact, it can distinguish chronic exposure to O<sub>3</sub> from acute one. It is termed chronic exposure the long-term exposure at concentration < 100 nL L<sup>-1</sup> whereas the acute O<sub>3</sub> exposure is generally defined as exposure to a high level of O<sub>3</sub> concentration (> 100 nL L<sup>-1</sup>) for a short period of time, typically on the order of hours (Kangasjarvi et al., 2005). Chen et al. (2009) studied the effects of acute (400 nL L<sup>-1</sup>, 6 h) and chronic (90 nL L<sup>-1</sup>, 8 h d<sup>-1</sup>, 28 d) O<sub>3</sub> concentration on photosynthetic process of soybean plants. Although both acute and chronic O<sub>3</sub> treatment resulted in a similar overall photosynthetic impairment compared to the controls, the fluorescence imaging analysis revealed that the physiological mechanisms underlying the decreases differed. In the acute O<sub>3</sub> treatments over the chronic one there was a greater spatial heterogeneity related to several bases. The higher O<sub>3</sub> concentration typically induced oxidative stress and the hypersensitive response within a matter of hours leading to programmed cellular death (PCD). By the end of chronic O<sub>3</sub> treatment, control leaves showed an increase in spatial heterogeneity of photosynthesis linked to the process of natural senescence. Clearly, in this study it has been demonstrated as Chl fluorescence imaging represents a useful tool to study also mechanisms on the basis of plants responses to abiotic stress such as O<sub>3</sub> pollution.

Guidi et al. (2007) used Chl fluorescence analysis to study the effects of an acute O<sub>3</sub> treatment (150 nL L<sup>-1</sup> for 5 h) or artificial inoculation with a pathogen (*Pleiochaeta setosa*) on photosynthesis of *Lupinus albus*. The aim of the work was to compare the perturbations in photosynthesis induced by an abiotic or biotic stress. In addition to, in the work were compared results obtained by conventional Chl fluorescence analysis and the technique of Chl fluorescence imaging. Image analysis of F<sub>v</sub>/F<sub>m</sub> showed a different response in plants

subjected to ozone or inoculated with *P. setosa*. Indeed, in ozonated leaves fluorescence yield was lower in leaf veins than in the mesophyll with the exception of the necrotic areas where no fluorescence signals could be detected. This suggests that the leaf area close to the veins were more sensitive to ozone. The parameter  $\Phi_{PSII}$  decreased significantly in both infected and ozonated leaves, but image analysis provides more information than the conventional fluorometer. In fact, until 48 h after ozone treatment or fungal inoculation,  $\Phi_{PSII}$  tended to decrease, especially in the infected leaves. Afterwards, a distinct stimulation of photosynthesis was observed in the area surrounding the visible lesions induced by the fungus. This did not occur in the ozonated leaves, as suggested also by the higher values of qP (data not shown). This phenomenon was not observed using the conventional fluorometer which recorded a similar reduction in this parameter in both ozonated and inoculated leaves.

In an other work Guidi and Degl'Innocenti (2008) studied the response to photoinhibition and subsequent recovery in plants of *Phaseolus vulgaris* (cv. Pinto) exposed to charcoal-filtered air or to an acute O<sub>3</sub> exposure (150 nL L<sup>-1</sup> for 3 or 5 h). Susceptibility to photoinhibition in bean leaves was determined as changes in the F<sub>v</sub>/F<sub>m</sub> ratio and the images of the ratio are reported in **Figure 3**. Initial values of F<sub>v</sub>/F<sub>m</sub> were 0.796, 0.784 and 0.741 for plants maintained in charcoal-filtered air, or treated with a single exposure to O<sub>3</sub> for 3 h, or for 5 h, respectively. The results indicate that treatment with O<sub>3</sub> for 5 h induced a slight photoinhibition. The exposure of control plants (charcoal-filtered air for 5 h) at a light intensity of 1000 μmol m<sup>-2</sup>s<sup>-1</sup> resulted in a significant reduction in F<sub>v</sub>/F<sub>m</sub> (P < 0.01) (Fig. 2b), while plants treated with O<sub>3</sub> for 3 h showed an increased tolerance to photoinhibition with less reduction in F<sub>v</sub>/F<sub>m</sub> (Fig. 2f). Plants treated with O<sub>3</sub> for 5 h and then exposed to high light showed a reduction in F<sub>v</sub>/F<sub>m</sub> ratio values similar to those recorded in control plants (Fig. 2i and l). However, while control plants or treated with O<sub>3</sub> for 3 h recovered their initial value 24 h after photoinhibition treatment, plants treated with O<sub>3</sub> for 5 h did not show the same ability to recover. In these plants the values of the F<sub>v</sub>/F<sub>m</sub> ratio did not recover and, 48 h after photoinhibition leaves showed visible symptoms of damage over the entire surfaces which precluded further analysis. At the same time, severe wilting did not permit chlorophyll fluorescence imaging.

Most of the abiotic stresses induce in plants an oxidative damage of the cell structure and consequently a loss in the cellular activities. Chloroplast represents the organelle which possesses pigments that absorb light and drive redox reactions of thylakoids but also the site in the cell where O<sub>2</sub> is evolved from water. Clearly, it represents an organelle such as mitochondria, in which the formation of reactive oxygen species (ROS) can occur. On the other hand, chloroplasts are able to produce strong oxidants associated with PSII which are responsible for the splitting of H<sub>2</sub>O molecules, but they can also oxidize pigments, proteins and lipid of the thylakoid membranes as well. This characteristic makes the chloroplast a major stress sensor in green plants (Biswal & Biswal 1999). Even the separation charge and the electron transport rate associated represent another important factor that makes chloroplast sensitive to stress. Using image analysis tools Aldea et al. (2006) observed a statistical relationship between ROS and reductions in photosynthetic efficiency ( $\Phi_{PSII}$ ) in leaves damaged simultaneously by O<sub>3</sub> (80 nL L<sup>-1</sup> for 8 h) and viral infection (soybean mosaic virus). The author by using Chl fluorescence analysis overlapped spatial maps of  $\Phi_{PSII}$  and ROS and found that areas with depressed  $\Phi_{PSII}$  corresponded to areas of high ROS concentration.



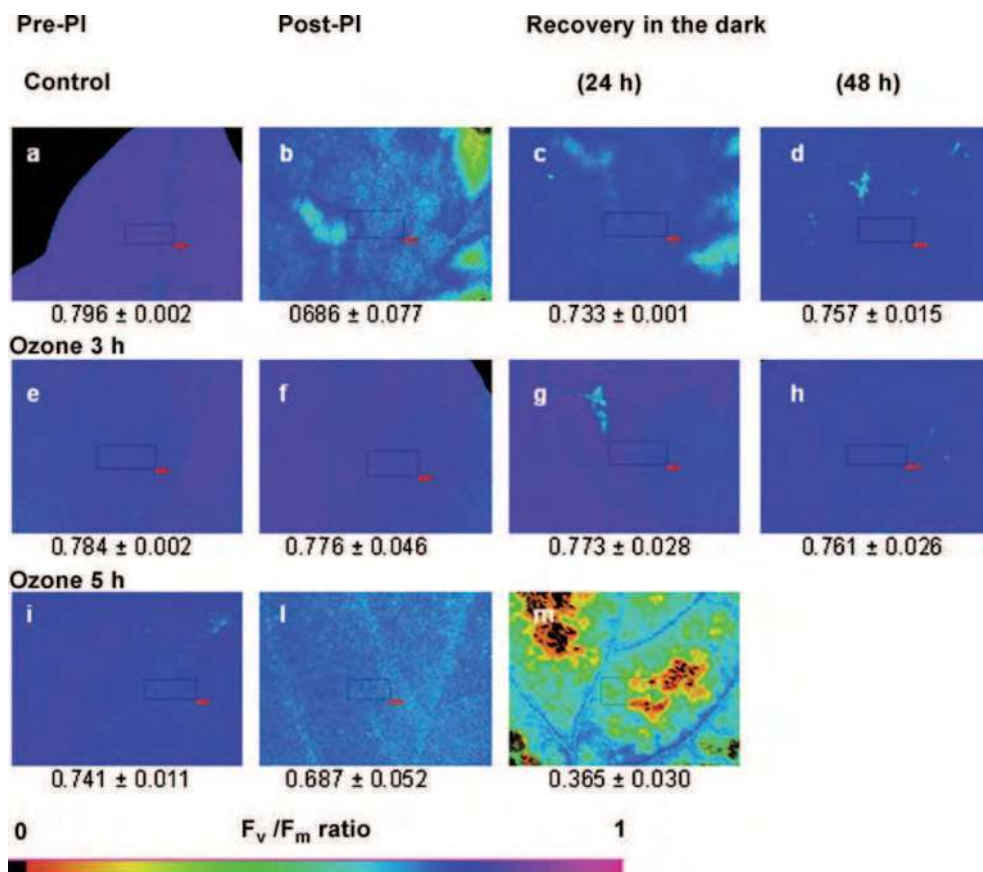


Fig. 3. Representative fluorescence images of the  $F_v/F_m$  ratio in leaves of *Phaseolus vulgaris* L. cultivar Pinto after a single exposure to  $O_3$  (150 ppb) for 3 h (Ozone 3 h; e–h) or 5 h (Ozone 5 h; i–m) or exposed to charcoal-filtered air (control, a–d) (Pre-PI). The images correspond to different measurement times: after charcoal-filtered air or  $O_3$  exposure (a, e and i), after photoinhibitory treatment for 5 h (b, f and l), after recovery in the dark for 24 h (c, g and m) or for 48 h (d and h). All images are normalised to the false colour bar provided. The analyses of  $F_v/F_m$  were carried out on dark-adapted leaves. The pixel value display is based on a false-colour scale ranging from black (0.00 to 0.040) via red, yellow, green, blue to purple (ending at 1.00) (from Guidi & Degl'Innocenti, 2008).

Wounding is another common abiotic stress which induces a spatial and temporal complex series of responses in plants. In fact, wounding induces by herbivore or mechanical damage determines localized cell death, loss of water and solutes from cut surface which provides a point of entry of bacterial and fungal pathogens and disrupts vascular system. Many responses can be activated following wounding such as defense and repair mechanisms which require a high metabolic demand upon wounded region. These responses determine

the synthesis of new molecules and then energy and carbon skeleton. An interesting work reported the study of the spatial and temporal changes in source-sink relationships which occur in mechanically wounded leaves of *Arabidopsis thaliana* (Quilliam et al., 2006). When the Chl fluorescence imaging analyses was made immediately after wounding there was a localized reduction in the steady-state of  $\Phi_{PSII}$  in cells adjacent to the wound margin and this suggests that these cells were damaged. No changes in  $F_v/F_m$  ratio were observed. Twenty-four hours after wounding, cells proximal to the wound margin showed a rapid induction of  $\Phi_{PSII}$  upon illumination whilst cells more distal to the wound margin exhibited a much slower induction of  $\Phi_{PSII}$  and a large increase of NPQ. The obtained results indicate of an increase in sink strength in the vicinity of the wound.

Chl fluorescence imaging has been used also for particular studies such as the characterization of a mutants with altered leaf morphology that are useful as markers for the study of genetic systems and for probing the leaf differentiation process. In a study carried out by Fambrini et al (2010) a mutant with deficient greening and altered development of the leaf mesophyll appeared in an inbred line of sunflower (*Helianthus annuus* L.). The mutation, named *mesophyll cell defective1 (mcd1)*, has pleiotropic effects and it is inherited as a monogenic recessive. The structure and tissue organization of *mcd1* leaves are disrupted. A deficient accumulation of photosynthetic pigments characterizes both cotyledons and leaves of the mutant. In *mcd1* leaves, Chl fluorescence imaging evidences a spatial heterogeneity of leaf photosynthetic performance. Little black points, which correspond to PSII maximum efficiency ( $F_v/F_m$ ) values close to zero, characterize the *mcd1* leaves. Similarly, the light adapted quantum efficiency ( $\Phi_{PSII}$ ) values show a homogeneous distribution over wild type leaf lamina, while the damaged areas in *mcd1* leaves, represented by yellow zones, are prominent (**Figure 4**).

In conclusion, the loss of function of the *MCD1* gene in *Helianthus annuus* is correlated with a variegated leaf phenotype characterized by a localized destruction of mesophyll morphogenesis and defeat of PSII activity.

Another interesting application of Chl fluorescence imaging is represented by its used to analyze the generation of action potentials in irritated *Dionaea muscipula* traps to determine the 'site effect' of the electrical signal-induced inhibition of photosynthesis (Pavlovic et al. 2011). Irritation of trigger hairs and subsequent generation of action potentials resulted in a decrease in the effective photochemical quantum yield of photosystem II ( $\Phi_{PSII}$ ) and the rate of net photosynthesis (**Figure 5**).

During the first seconds of irritation, increased excitation pressure in PSII was the major contributor to the decreased  $\Phi_{PSII}$ . Within 1 min, NPQ released the excitation pressure at PSII. All the data presented in this work indicate that the main primary target of the electrical signal induced inhibition of photosynthesis is the dark reaction, whereas the inhibition of electron transport is only a consequence of reduced carboxylation efficiency. In addition, the study also provides valuable data confirming the hypothesis that chlorophyll a fluorescence is under electrochemical control.

Chl fluorescence imaging combined with thermal imaging has been used also for monitoring and screening plant population (Chaerle et al., 2006). Rapid screening for stomatal responses can be achieved by thermal imaging, while, combined with fluorescence imaging to study photosynthesis, can potentially be used to derive leaf water use efficiency as a screening parameter.

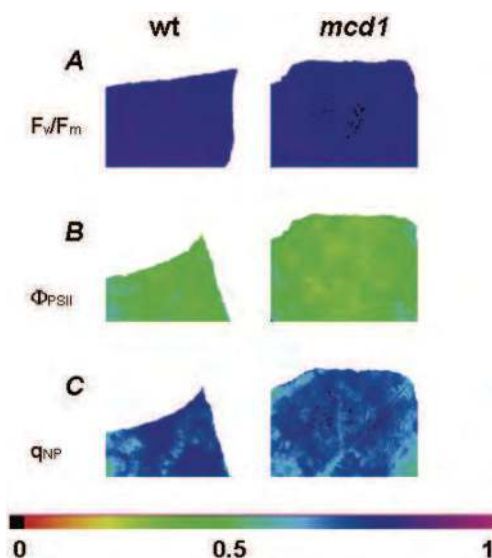


Fig. 4. Analysis of chlorophyll fluorescence parameters in wild type (wt) and *mesophyll cell defective1* (*mcd1*) mutant plants of sunflower (*Helianthus annuus* L.). A–C: Fluorescence images of the maximum efficiency of PSII ( $F_v/F_m$ ; A), the proportion of absorbed light, which is utilized for photosynthetic electron transport ( $\Phi_{PSII}$ ; B), and the nonphotochemical quenching coefficient ( $q_{NP}$ ; C), in representative leaves from wild type (left column) and *mcd1* mutant (right column), are shown (from Fambrini et al., 2010).

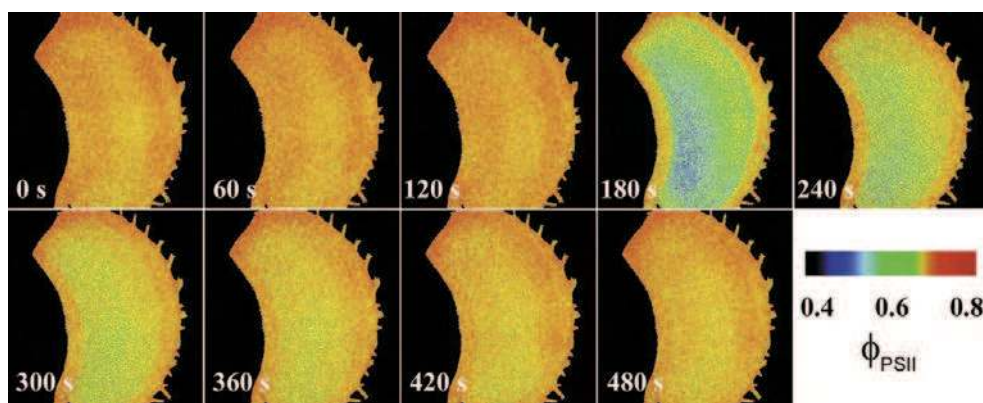


Fig. 5. Spatiotemporal changes of effective photochemical quantum yield of PSII ( $\Phi_{PSII}$ ) in a *D. muscipula* closed trap assessed by chlorophyll fluorescence imaging. The trap was irritated by a thin wire between 162 s and 177 s (image obtained from Pavlovic et al. 2011).

Although Chl fluorescence fluorometers have been developed to measure chlorophyll fluorescence from green tissues, which are high in chlorophyll content, the extraordinary sensitivity of current instruments enables measurements in non-green plant tissues that have relatively low chlorophyll content. This includes many types of ripening fruit that during development degrade the chloroplasts (including chlorophyll) that are contained in the fruit skin. Even non-green fruit that are highly colored (e.g., apples, tomatoes), contain active chloroplasts that yield a chlorophyll fluorescence signal of sufficient strength that it can be used as a probe of photosynthetic activity in the fruit skin (DeEll et al., 1995). In food technology, Chl fluorescence imaging can provide a rapid and non-invasive, post-harvest evaluation of the quality of fruits and vegetables (DeEll et al., 1995; DeEll & Toivonen, 2000). Nedbal & Withmarsh (2004) reported an interesting article on this topic. By applying fluorescence imaging on individual fruit before any symptoms of bitter pit were apparent, lower fluorescence was shown to be associated with bitter pit development in apples in selective cases (Lotze et al., 2006). The authors showed that, using averaged cumulative distribution functions (CDFs) of pitted and non-pitted fruit classes, it was possible to show a difference between these classes with fluorescence imaging. Results of pre-harvest imaging on apples to identify fruit with bitter pit potential at harvest showed that pitted fruit were correctly classified (75–100%). However, misclassification of non-pitted fruit (50% and less) with fluorescence imaging is still too high to be of any commercial.

Obenland & Neipp (2005) used Chl fluorescence analysis in green lemons (*Citrus union*) 30 minutes after immersion of the fruit into 55°C water for 5 minutes to determine if this methodology could be used to identify areas of hot water-induced rind injury before the appearance of visible symptoms. Fluorescence was variable in intensity over the surface of the rind with defined areas of enhanced fluorescence being present that corresponded in shape and location with visible injury that later developed during 24 hours of storage. The authors concluded that imaging of Chl fluorescence has potential as a means to identify areas of incipient rind injury in citrus to facilitate study of the causal mechanisms of postharvest rind disorders. On the other hand, previously Nedbal et al. (2000) demonstrated the potential for using rapid imaging of Chl fluorescence in post-harvest fruit to develop an automated device that can identify and remove poor quality fruit long before visible damage appears.

Meyerhoff & Pfündel (2008) used Chl fluorescence imaging to detect the presence of functioning PSII in fruits of strawberries. From obtained results authors concluded that it is unclear if photosynthesis in strawberry fruits is capable to support seed development.

Chl fluorescence imaging can be conveniently used to study the functioning of PSII in leaves and permits to detect the heterogeneity of photosynthesis which is particularly evident in stressed leaves. However, it has been reported as it can be conveniently used also for particular application such as the study of fruit quality in postharvest. For these reasons Chl fluorescence imaging represents an important and useful tool in ecophysiological and post harvest studies that permits to detect the effects of abiotic stress even at early stages and before the visual appearance of symptoms of damage.

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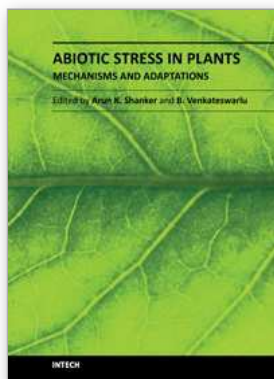
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## **Abiotic Stress in Plants - Mechanisms and Adaptations**

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World population is growing at an alarming rate and is anticipated to reach about six billion by the end of year 2050. On the other hand, agricultural productivity is not increasing at a required rate to keep up with the food demand. The reasons for this are water shortages, depleting soil fertility and mainly various abiotic stresses. The fast pace at which developments and novel findings that are recently taking place in the cutting edge areas of molecular biology and basic genetics, have reinforced and augmented the efficiency of science outputs in dealing with plant abiotic stresses. In depth understanding of the stresses and their effects on plants is of paramount importance to evolve effective strategies to counter them. This book is broadly divided into sections on the stresses, their mechanisms and tolerance, genetics and adaptation, and focuses on the mechanistic aspects in addition to touching some adaptation features. The chief objective of the book hence is to deliver state of the art information for comprehending the nature of abiotic stress in plants. We attempted here to present a judicious mixture of outlooks in order to interest workers in all areas of plant sciences.

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