University of South Bohemia, České Budějovice
Institute of Physical Biology, Nové Hrady

Ph.D. thesis

Potential of chlorophyll fluorescence imaging in plant stress detection

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List of publications

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Movement of viral infection in plant body followed by means of chlorophyll fluorescence imaging.

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Prohlašuji, že Ing. Ladislav Cséfalvay se podílel na společných publikacích přibližně v níže uvedeném rozsahu:

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Movement of viral infection in plant body followed by means of chlorophyll fluorescence imaging. **70%**

Mgr. Julie Olejníčková, Ph.D.

Annotation

Early detection of various kinds of stresses of higher plants can be limited by using appropriate method. Non-invasive methods which making use of change of optical plant properties enables us to observe changes in physiological processes owing to various stress factors in time. Hence, those methods are abundantly used in stress physiology of plants. Autofluorescence of plant tissues, namely chlorophyll fluorescence, is adherent to photosynthetic activity of plants and could offer relevant information about changes under external stress factors. Current status of kinetic imaging fluorometry enables us to obtain information about change of chlorophyll fluorescence emission from million pixels at once, and thus cover wide scale of systemic and non-systemic reactions in plants as an answer to stress factor.

The aim of this Ph.D. thesis was to find out, if within the frame of fluorescence parameters which have or have not physiological interpretation exist the "universal" parameter, which could be used as a main stress identifier. For that purpose, we operated with four various stress factors: nutrient stress, temperature and irradiance changes during whole year, fungal and viral infection. We searched for that parameter in standard fluorescence parameters so as well as in parameters obtained with advanced statistical approach

Our results showed, that for plant stress detection are the best parameters, which could be interpreted as direct answer of photosynthetic apparatus to stress factor: decrease of values of maximal an effective quantum yield and change the rate of reoxidation of plastoquinone pool expressed by changing of fluorescence rate decrease from F_P during fluorescence induction in actinic light.

Using the advanced statistical approach to data analysis of plant fluorescence response leads to earlier stress identification, but obtained parameters are specific to existing host-pathogen system, cultivation conditions etc and could not be used at large. The detection of fungal infection on grapevine showed, that both data evaluating processes (by standard fluorescence parameters or advanced statistical approach) did not showed any difference in timeline of detection. That finding was published in European Journal of Plant Pathology.

Anotace

Včasná detekce různých typů stresu u vyšších rostlin může být limitována použitím vhodné metodiky. Neivazivní metody využívající změny optických vlastností rostlin umožňují sledování změn ve fyziologických procesech vlivem různých stresových faktorů v čase a proto jsou hojně používány ve stresové fyziologii rostlin. Autofluorescence rostlinných pletiv, jmenovitě fluorescence chlorofylu je úzce spjata s fotosyntetickou aktivitou rostlin a může tak poskytovat relevantní informace o její změně vlivem vnějších stresových faktorů. Současná kinetická zobrazovací fluorometrie umožňuje získat informaci o změně fluorescenční emise z milionů bodů současně a pokrýt tak širokou škálu systémových i nesystémových reakcí, které probíhají v rostlině jako odpověď na daný stresový faktor.

Cílem této disertační práce bylo zjistit, zda v rámci fluorescenčních parametrů, které mají či nemají fyziologickou interpretaci, existuje nějaký „univerzální“ parametr, který by se dal použít jako hlavní stresový identifikátor. Pro tento účel, jsme pracovali se čtyřmi různými stresovými faktory: výživový stres, změny teploty a ozářenosti během roku, houbová a virová infekce. Tento parametr jsme hledali jak ve standardních fluorescenčních parametrech, tak i v parametrech získaných pokročilými statistickými metodami.

Naše výsledky ukázaly, že pro detekci stresu u rostlin se nejlépe hodí parametry, které se dají interpretovat jako přímá odpověď fotosyntetického aparátu na stresový faktor: pokles hodnot maximálního a efektivního kvantového výtěžku fotosystému II a změna rychlosti re-oxidace plastochinonového poolu vyjádřená změnou rychlosti poklesu fluorescence z F_P během fluorescenční indukce v aktinickém světle.

Použitím pokročilých statistických metod k analýze fluorescenční odpovědi rostlin lze stres identifikovat mnohem dříve, ale získané parametry jsou specifické pro daný model patogen-hostitel, pro určité kultivační podmínky a nelze je tedy použít obecně. Ale v případu houbové infekce na listech révy vinné jsme nepozorovali žádný rozdíl ve včasnosti detekce stresu pomocí standardních fluorescenčních parametrů a parametrů získaných pokročilými statistickými metodami. Tento poznatek byl publikován v European Journal of Plant Pathology.

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I would like to dedicate this Ph.D. thesis to my dear father *in memoriam.*

Abbreviations

AS - Asymptomatic leaf

AL – Actinic light

ATP - Adenosine 5′-triphosphate

CCD - charged coupled device

Chl-F - chlorophyll fluorescence

Chl-FI - chlorophyll fluorescence imaging

CI - combinatorial imaging

F_0 - minimum chlorophyll fluorescence yield in dark-adapted state

F_M - maximum chlorophyll fluorescence yield in dark-adapted state

F_V - variable chlorophyll fluorescence yield in dark-adapted state

F_P - maximum chlorophyll fluorescence yield measured when the actinic light is switched on

F_S - steady-state chlorophyll fluorescence yield in light-adapted state

F_T - actual chlorophyll fluorescence yield at a particular time

F_V/F_M - maximum quantum yield of photosystem II photochemistry

Φ_{PSII} - effective quantum yield of photosystem II photochemistry

LED - Light Emitting Diode

OEC - Oxygen evolving complex

PSI - photosystem I

PSII - photosystem II

PQ - Plastoquinone

RC - Reaction centre

NADPH - Nicotinamide adenine dinucleotide phosphate

NPQ - non-photochemical quenching of chlorophyll fluorescence

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Motivation

Since the man has started to use the crops for his feed, there was a tendency to increase the yield and to keep the yield maximal as high as possible. But photosynthetic activity of plants and consequent grow and yield in nature is influenced by wide range of stress factors, both abiotic and biotic ones. Decreasing of the yield caused by various external factors leaded to development of wide scale of techniques to evaluate the crop (plants) health status and to detect the plant stress as soon as possible. Since chlorophyll-A as the main photosynthetically active pigment and the most important fluorophore in plant tissue, chlorophyll fluorescence represents a powerful non-invasive reporter signal to monitor changes in photosynthetic activity of plants and to detect plant stress.

This effort has inspired me to work with different kind of plant stresses and look for "typical" chlorophyll fluorescence (Chl-F) parameter suitable for their early detection. In this Ph. D. Thesis I present four studies where we used Chl-F imaging as a principle tool for determination of plant physiological status. We applied complex measuring protocol to find out sensitive Chl-F parameter to reveal early stages of four types of stress: nutrient imbalance (effect of titanium in iron-deficient plants), effect of annual variation of irradiance and air temperature to evergreen temperate plants and fungal and viral infection. In two studies we have applied both standard approach, where stress – sensitive conventional parameters are found empirically, and novel statistical approach combinatorial imaging, which searches for small sets of several contrasting Chl-F images regardless of their potential interpretation that are used to discriminate between the control and stressed/treated leaf tissue.

Overview

The first part ***Theoretical background*** is divided into chapters which are giving short introduction into background of chlorophyll fluorescence and its relationship with plant physiology. Here, we summarize main principles of photosynthesis, Chl-F measurements and instrumental equipment. Introduction to plant stress and relationship between Chl-F and plant stress follows.

In the second part of my PhD. thesis I present four chapters:

First chapter ***Competitive relationships between iron and titanium metabolism in spinach: a hydroponic fluorescence study*** shows the results from hydroponic study where we verified hypothesis that titanium (Ti) can compete with iron (Fe) on the binding sites in roots due to their similar chemical properties and thus caused "apparent" Fe-deficiency. We evaluated gradual changes in Chl-F in iron deficient spinach plants treated by absence and/or presence of Ti and Fe in nutrient solution. In accordance with the hypothesis, we found that spinach plants cultivated in nutrient solution contained both Fe and Ti expressed similar behavior as Fe-deficient plants. Here we demonstrated the capacity of Chl-F to follow changes in plant metabolism under various nutrient treatments.

Second chapter ***Annual variation of the steady-state chlorophyll fluorescence emission of evergreen plants in temperate zone*** deals with the effect of the two major environmental conditions (temperature and irradiance) on the photosynthetic activity and Chl-F emission in overwintering evergreen plants during whole year. Among all standard Chl-F parameters we focused on steady state level of Chl-F (F_S) under ambient light conditions, because it is the sole parameter detectable remotely. F_S was found to be stable along wide range of irradiances. Annual course of field monitored F_S was similar to anual course of the CO_2 assimilation rate It was stable and low in the winter and steeply increased in the spring. During summer, F_S was high and stable and again rapidly decreased in the autumn. These spring and autumn transitions were associated with periods with night intermittent freezing temperatures, and were also marked by a significantly elevated sample-to-sample variability of the F_S signal. We showed that annual variation of F_S carries information about the activation/deactivation of photosynthetic activity in temperate zone evergreen plant species during the year. During this study I was one of the leaders of two B. Sc. thesis directly related to this theme (Šlouf 2007) and (Svidenská 2007).

In last two studies we were dealing with biotic stress of plants.

In the third chapter ***Pre-symptomatic detection of Plasmopara viticola infection in grapevine leaves using chlorophyll fluorescence imaging*** we demonstrate the power of imaging technology to reveal infected tissues earlier than the whole infection is visible by human eye. The aim of this study was to investigate possibilities of the early detection of *P. viticola* infection in grapevine leaves using Chl-F imaging, with particular attention to Chl-F parameters that correlate with leaf photosynthetic activity [F_V/F_M and Φ_{PSII}, maximum and effective quantum yield of photosystem II (PSII)]. Third sensitive parameter was identified by advanced statistical method – combinatorial imaging. Our results showed that Chl-F parameters were able to detect the infection 3 days before occurrence of visible symptoms and 5 days before release of spores. No statistical difference was found between physiological parameters (F_V/F_M and Φ_{PSII}) and CI (combinatorial imaging). Hence, Φ_{PSII} was chosen for validation in future field study. Usage of high resolution imaging could improve the pest management of vineyards – decrease the number of spraying against pathogen, but that method was not validated up to now in the field conditions.

Tracking of viral infection movement within in the whole plant is presented in chapter four. Here, the aim was not only to detect the plant response to viral attack as soon as possible, but to follow the virus movement in plant tissues in time scale of days. Here, we measured the Chl-F variations in three asymptomatic (AS) leaves of infected plant that differed in their age, i.e. the $6^{th,}$ 7^{the} and 8^{th} true AS leaves. We wanted to verify the postulate, that after the inoculation the virus moves first to the apical meristem where it infects the youngest leaves that start to be curly and fragile. Than, the viral particles move down to the older asymptomatic leaves through veins The first symptom of the infection in the youngest symptomatic leaves occurs at 5-7 days after the inoculation (dpi) onwards, which is detectable by curling and fragility of this group of leaves. In accordance with this hypothesis, we observed the first variation in Chl-F in the youngest infected leaves (8^{th} AS leaves) and the later in the older (6^{th}) AS leaves.

THEORETICAL BACKGROUND

1. Photosynthesis

Photosynthesis is the most important metabolic pathway on the Earth, since nearly all living organisms depend on it, either directly or indirectly. Moreover, photosynthesis largely contributes to maintaining stability of atmosphere and mitigating anthropogenic effect on global climate. Oxygenic photosynthesis is driven by light in thylakoids and cytoplasm of green bacteria, cyanobacteria, and in chloroplasts of algae and higher plants. Global title for such organisms is *photoautothrophs*.

During photosynthetic process solar energy is trapped, converted into chemical energy and stored into carbohydrates. Photosynthesis proceeds in two major steps 1) *photoreaction*, where the sunlight energy is captured and stored as chemical energy in bonds of ATP and NADPH; and 2) *carbon dioxide (CO_2) fixation* in Calvin-Benson cycle (Calvin 1948), where the high-energy carriers ATP and NADPH are utilized. The amount of energy trapped by photosynthesis is immense, approximately 100 terawatts per year for whole Earth (Nealson and Conrad 1999). Photosynthesis is also a carbon source for organic compounds in the Earth's trophic chain. Overall photosynthetic organisms convert around 100,000,000,000 tones of carbon into biomass per year (Field 1998). This fact demonstrates the un-substitutable role of photosynthesis in the Earth.

Simple formula of photosynthesis can be described as follows:

$$6CO_2 + 6H_2O \xrightarrow{h\nu} C_6H_{12}O_6 + 6O_2$$

1.1. Light

All the fluxes of energy on Earth are dependent on the closest star – on the Sun.

Sun is the ultimate and uninterruptible source of energy, which propagates the energy to the space. Light has properties of both a) *waves* and b) *particles*.

Ad a) *the wave* is characterized by wavelength (λ), which is a state between two wave crests. Frequency (ν) of the wave is characterized by number of wave crests that pass an observer in given time. Speed of the light, wavelength and frequency relates on simple equation:

$c = \lambda.\nu$;

where c is the speed of the light (~$3{,}0.10^8$ $m.s^{-1}$). The light wave is a transverse (side-by-side) electromagnetic wave, where both magnetic and electric fields oscillate perpendicularly to the direction of wave propagation.

Ad b) Light, it is also *a particle* – a photon. This energy content of photon is not continuous, but it is delivered in discrete packets (quanta). The energy of the photon (E) depends on the frequency of the light (ν) according to a relation known as Planck's law: $E = h.\nu$;
where h is a Planck's constant (6,626. 10^{-34} $J.s^{-1}$). Thus, one can imagine the incident sunlight as a rain of photons with different wavelengths.
Photosynthetically active radiation (PAR) is a range of wavelengths from 400 up to 700 nm. The range of electromagnetic spectrum between these two wavelengths is utilized in photosynthetic process. This range is also very close to part of spectrum, which is specified by sensitivity of human eye ("visible light") - from 360 up to 760 nm (Fig. 1).

Fig. 1. Linear spectrum of visible light (commons.wikimedia.org)

1.2. Pigments

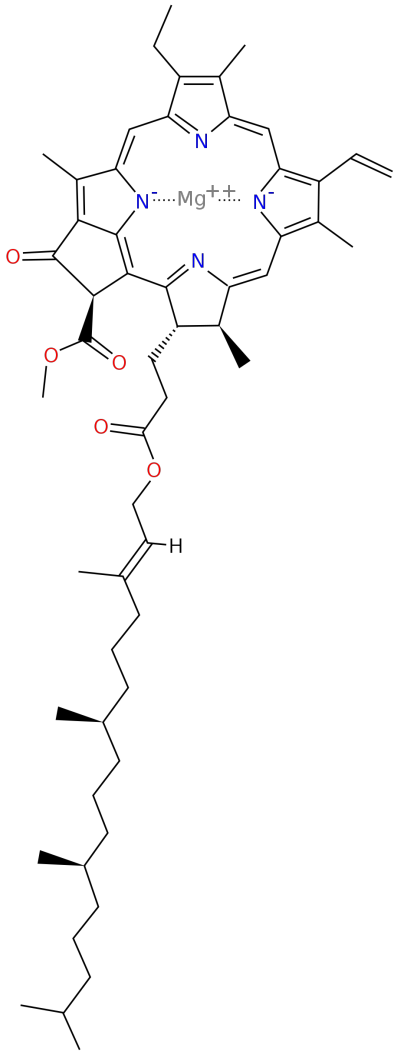
Photosynthetic pigments have three important functions:
1) photochemical transformation of energy in reaction centers, in oxygenic photosynthesis performed by chlorophyll-A.
2) photons capture and transfer the excitons from antennae to reaction centers – performed by chlorophylls and carotenoids (mainly carotenes)
3) protection of the photosynthetic apparatus against unfavorable and/or excessive excitation – performed by carotenoids (mainly xanthophylls)

1.2.1. Chlorophyll

Principal classes of pigments responsible for light absorption in photosynthesis, which can be found in each photosynthetic organism, represent chlorophylls. There are several types of chlorophylls as Mikhail Tswett demonstrated in 1906 using adsorption chromatography (Zechmeister 1946). At 1930 Hans Fischer revealed the structure of various chlorophylls (Fischer 1930). First empirical formula of chlorophyll-A, the principal photosynthetic pigment, was revealed by Richard Willstätter (Aronoff 1966). Richard Willstätter, Hans Fischer and Robert Woodward who was first one successful in chlorophyll synthesis *in vitro,* received the Nobel Prize in chemistry for their studies in this most important pigment of photosynthesizing organism (in 1915, 1930 and 1965 respectively).

Nowadays, ten types of chlorophyll are identified. The various chlorophylls are labeled by letters or by the taxonomic group of the organisms, in which they occur. However, the principal photosynthetically active pigment in photosynthesis is chlorophyll-A (Chl-A), with relative molecule mass off 893.5 and the structure showed in Fig. 2. Chl-A is present in all photosynthetic organisms, except the purple and green bacteria.

Fig. 2. Structure of chlorophyll-A (commons.wikimedia.org)



Chl-A absorbs in blue and red region of the spectrum (Fig. 3), thus the characteristic color of this pigment is green. Absorption band in blue region is well known as Soret band, with peak at λ_{max} = 430 nm. Chl-A has also a major band of absorption in the red region with λ_{max} = 662 nm and minor bands of absorption at 615 nm.

The values of λ_{max} for Chl-A in vivo result from the interactions between chlorophyll molecule and its surrounding molecules - proteins and the lipids in the thylakoid membranes and adjacent water molecules. Chl-A is associated with proteins in chlorophyll–protein complexes. This association causes that the red absorption bands of Chl-A are shifted toward longer wavelengths (with lower energy) than for Chl-A dissolved in ether (λ_{max} at 662 nm), i.e., to λ_{max} at 670 to 680 nm in vivo.

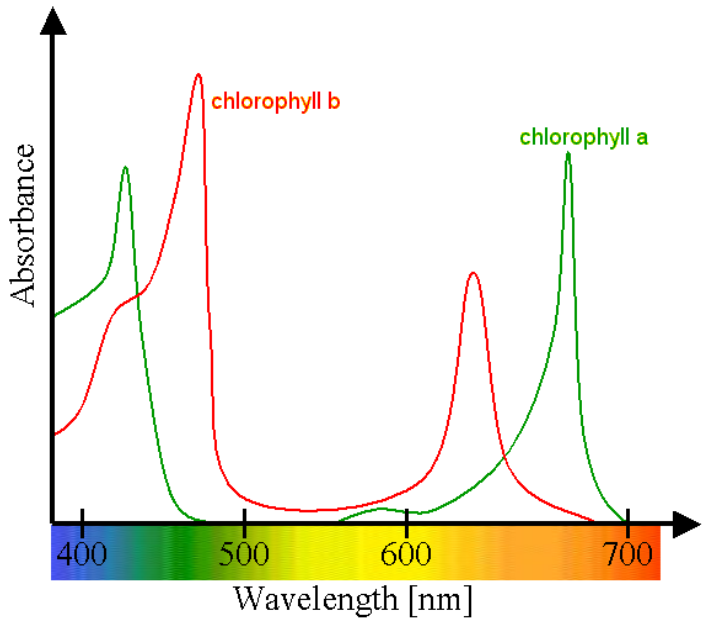
Chl-A is present in reaction centers of photosystems that play a particularly important role in photosynthesis. These Chl-A's have λ_{max}'s at approximately 680 and 700nm and are referred to P680 (in photosystem II) and P700 (in photosystem I), respectively (P indicating pigment). P700 and P680 are dimers of Chl-A molecules (i.e., two Chl-A's acting as a units) in both reaction centers of photosystems (PS).

1.2.2. Accessory photosynthetic pigments

All photoautothrops contain along with Chl-A also other pigments that are capable to capture light, e.g., other types of chlorophylls (B, C, D) and carotenoids. The most abundant

accessory chlorophyll is chlorophyll-B. Main role of Chl-B is the accessory light-absorption in light harvesting complexes and funneling the energy further to Chl-A, which precedes charge separation. The red absorption band of Chl-B in vivo occurs as a "shoulder" on the short-wavelength side of the Chl-A red band (644nm) , and its Soret band occurs at slightly longer wavelengths than for Chl-A (455 nm). Chl-B is present in most eukaryotic photoautotroph excluding red and brown algae.

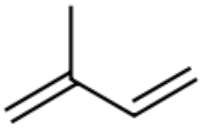
Fig. 3. Absorption spectra of Chlorophyll A and B (www.absoluteastronomy.com/topics/Chlorophyll)



Similarly to Chl-B, Ch-C is an accessory light-harvesting pigment, too. Chlorophyll-D is a main photosynthetic pigment for *Acaryochloris marina*, although this organism also contains Chl-A. Chlorophyll-D is energetically and structurally intermediate between Chl-A and bacteriochlorophyll-A. It can be an intermediate transition between anoxygenic and oxygenic photosynthesis (Blankenship 1998).

The other photosynthetically active groups of pigments are *carotenoids*. Carotenoids are 10-, 20-, and 40-carbon molecules – terpenoids and are present in all known photosynthetic organisms. They are built up from basic five-carbon branched-chain units species isoprene (Britton 1998) (Fig. 4).

Fig. 4. Structure of isoprene (commons.wikimedia.org)



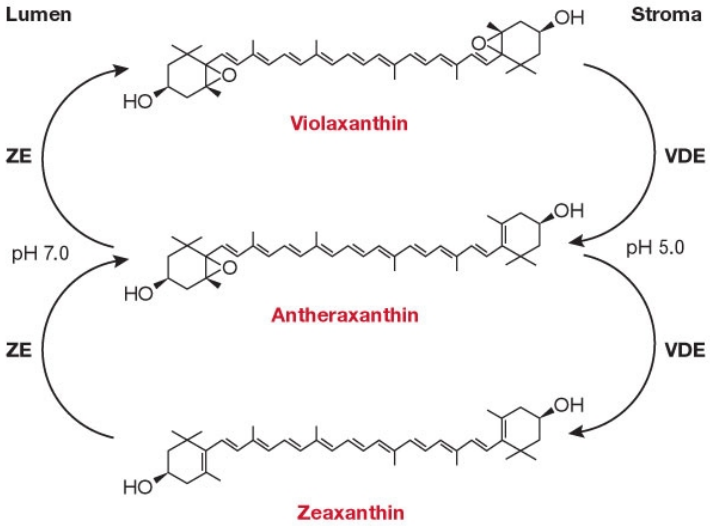
According their chemical structure carotenoids are divided into two groups: *carotenes* (hydrocarbons) and *xanthophylls* (oxygen derivates of carotenoids). Carotenoids have several well-documented essential functions in photosynthetic organisms.

1) They are accessory pigments in the collection and absorbing of light and transferring the energy to chlorophylls. Carotenoids serving as accessory pigments in photosynthesis absorb strongly in blue (425 to 490 nm) and green (490 to 560 nm) part of the spectrum. Absorption

of carotenoids in vivo is shifted about 30 nm toward longer wavelengths (lower energy) compared with absorption in solvent.

2) Second function of carotenes and xanthophylls is a photoprotection. Carotenoids are able to quench rapidly triplet excited states of chlorophylls before they can react with oxygen and form the highly reactive and damaging excited singlet oxygen states. Because such oxidations tend to increase with time, the fraction of xanthophylls generally increases in leaves as the growing season progresses e.g. (Kyparissis 2000). In addition, certain xanthophylls are reduced during the daytime and then oxidized back to the original form at night (xanthophyll cycle (Siefermann-Harms 1987)). Here, epoxidase and de-epoxidase enzymes interconvert violaxanthin and zeaxanthin in response to high light conditions. Excess light activates the de-epoxidation and increases the amount of zeaxanthin, which is the end product of xanthophyll cycle; conversely the low light favors the epoxidation processes and increases the amount of violaxanthin (Fig. 5.).

Fig. 5 Scheme of Xanthophyll cycle (Szabo, Bergantino et al. 2005))



1.3. Light reactions

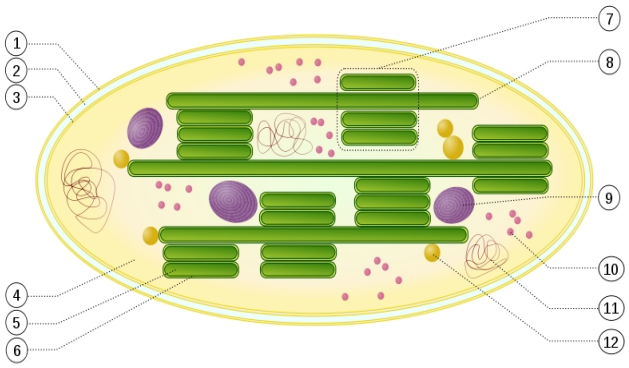
1.3.1. Chloroplasts and thylakoid membrane

The smallest structural and functional units of photosynthesis are chloroplasts. Chloroplasts are specialized organelles found in all higher plant cells containing photosynthetically active pigments. Chloroplasts are vesicles with double outer membrane. Inner space is called stroma. Within the stroma are other membrane structures - the thylakoids. Thylakoids have a form of flattened disk and inside each thylakoid is area called thylakoidal space or lumen. Similarly to the oxidative phosphorylation in mitochondria, also first process of photosynthesis takes place in the membrane, where involves trans-membrane fluxes due to proton electrochemical

gradient. In chloroplasts of higher plants thylakoids create grana – clumps of stacked thylakoids, which are compared to superimposed coins (Fig. 6.).

Fig. 6. Chloroplast ultrastructure (commons.wikimedia.org)

1. *outer membrane*
2. *intermembrane space*
3. *inner membrane (1+2+3: envelope)*
4. *stroma (aqueous fluid)*
5. *thylakoid lumen (inside of thylakoid)*
6. *thylakoid membrane*
7. *granum (stack of thylakoids)*
8. *thylakoid (lamella)*
9. *starch*
10. *ribosome*
11. *plastidial DNA*
12. *plastoglobule (drop of lipids)*



Trapping of photons, consequent photochemistry and ATP synthesis proceed in thylakoid membranes; carbon fixation proceeds in stroma. Some thylakoids are exposed with whole outer area to stroma, some are stacked in grana. Ratio between stromal and granal thylakoids is considerable variable, and mostly depends on light conditions during the chloroplast development (Lichtenthaler 1990).

Membranes of granal thylakoids, which are interacting with each other, are called stacked (or cramped), while the membranes which are "washed" by stroma are called non-stacked.

Light-dependent reactions of photosynthesis that take place in the thylakoid membrane proceed by four macromolecular complexes - Photosystem II (**PSII**), Photosystem I (**PSI**), cytochrome b_6/f complex and ATP – synthase. The complexes are integrated into the membrane, and provide charge separation and electron transfer across the thylakoid membrane (excluding ATP synthase). Transfer of electrons between these complexes is provided by mobile electron carriers: NADP, plastoquinone, plastocyanin and ferredoxin.

Photosystems use light energy to drive photosynthesis. Photosystem II is located almost exclusively in stacked regions of the thylakoid membranes, while Photosystem I can be primarily found in the stroma lamellae, as well as in on the ends of grana stacks. The cytochrome b_6/f complex is nearly equally distributed between the two types of membranes, and ATP synthase is entirely localized to the stroma lamellae. Thylakoid membrane also

contains the array of light harvesting complexes (antennas), which are responsible for light energy capture at different wavelengths, and for funneling it toward reaction centers using resonance energy transfer.

1.3.2. Light absorption and energy transfer

Photosynthesis starts with the trapping of photons by Chl-A in the antennae. After absorption of the photon, Chl-A reaches its excited state. The energy of the excited state of the molecule is rapidly passed on by neighboring Chl-A molecules of the antenna to RC (Marcus 1985). Such "energy quasi-particle" transferred between Chl-A molecules is called exciton. The energy absorbed by the antennae associated with the reaction center has three possible fates (Govindjee 2004): it can be

1) used by RC to drive the charge separation, photochemistry;
2) dissipated non-photochemically, principally as a heat;
3) re-emitted in form of fluorescence.

1.3.3. Photosystem II and Photosystem I

Energy of excitons funneled from light harvesting complex is utilized in the reaction centers of photosystems. There are two different photosystems, photosystem II (PSII) and photosystem I (PSI) that differ slightly in the wavelength of absorbed light; PSII utilize energy of light with wavelength 680 nm, PSI with 700 nm. **PSII** is the pigment-protein complex, where the charge separation takes place. PSII is composed by complex of integral proteins in thylakoid membrane. Two transmembrane polypeptides carry the prosthetic groups including reaction centre pigment P680, phaeophytin and iron on proteins D1 and D2, plastoquinon Q_A bounded on D2 protein, plastoquinon Q_B bounded on D1 protein, chlorophylls and light harvesting antennas on polypeptides. Oxygen evolving complex (OEC) is attached to PSII on the lumenal site of the thylakoid.

Photosystem I (PSI) particularly with complex of simultaneous reactions can be presented as a plastocyanin-ferredoxin-reductase in thylakoid membranes. PSI complex contains photosynthetic pigments, reaction centre P700, and set of three iron-sulfur (Fe-S) clusters which serve as early electron acceptors (Nugent 1996). After absorption of light quantum by light harvesting complex of PSI occur the handover of excited energy and charge separation in P700 molecule in reaction centre of PSI. Then, electron is gradually forwarded to Fe-S centre. Through next two carriers (FeS_A, FeS_B) ferredoxin F_D is reduced by transferred

electrons. From that step, electrons can be used as reductants for flavoprotein (ferredoxin-$NADP^+$ reductase). At the end with usage of next two protons from stroma also $NADP^+$ to $NADP^+ + H^+$ is reduced. Here are consumed the H^+ ions produced by the splitting of water, leading to a net production of ½ O_2, ATP, and $NADPH+H^+$ with the consumption of solar photons and water. This process is called non-cyclic transfer of electrons and is released when increased depletion of ATP is needed. Cyclic transport starts when the ferredoxin electrons flow back to plastoquinone pool. Reduced ferredoxin is important crossing for reducing other compounds -, not only $NADP^+$ or it can be used in metabolism for reducting sulphates or nitrites.

1.3.4. Charge separation in reaction center of PSII and subsequent reactions

The primary donor of electrons in PSII RC is pigment P680. It consists of four Chl-A molecules. Absorption of photon (λ=680 nm) leads to an excitation of P680. Upon excitation an electron from P680 is passed via phaeophytin - an intermediary acceptor to the primary acceptor quinone (Q_A). Q_A is covalently bound form of plastoquinone (PQ) located in reducing side of PSII. Further, electron passes from Q_A to Q_B, a diffusible form of PQ located on D1. Iron atom bounded on histidin plays a role in the electron transfer between Q_A and Q_B. For reduction of Q_B are necessary two electrons, therefore two excitation cycles are needed - together with two protons, which come from chloroplast's stroma (quinone cycle of electron carrying – Q cycle). When two electron and two protons are accepted, PQH_2 diffuses from the PSII freely.

The electron free $P680^+$ is re-reduced through tyrosine, located on the lumenal site of D1, by manganese ions in the oxygen evolving complex (OEC). Four electrons are needed to split water molecule. The summary of these processes shows following equation:

$$4\ H^+ + 2\ H_2O + 2\ Q_B + 4\ h\nu \longrightarrow O_2 + 4\ H^+ + 2\ Q_BH_2$$

The OEC contains cluster of four bounded atoms of Mn. This cluster changes his quantivalency four times per its oxidation, which is conditioned by four excitations of reaction centre. System for water dissociation is marked S, in that the oxidation of water realizes with gradual conversion from state S_0 through S_1, S_2 and S_3 up to S_4. Evolved electron from water photolysis reduces first the Mn cluster; this subsequently reduces the $P680^+$ despite tyrosine on position 161marked like Y_Z in sequence of D1 protein (Bricker 1996).

PQH_2 carries the electron and the protons from the stroma site to the lumenal site of thylakoid membrane, to the cytochrome b_6/f complex that connects PSII and PSI. This cytochrome contains one heme type C, and two types of heme B and protein Fe-S (Rieske's protein). Protons from PQH_2 are here released to the lumen of thylakoids. In cytochrome complex b_6/f can simultaneously run the Q-cycle (Trumpower 1990), which secures carrying an extra proton along with non-cyclic transfer of electron pairs.

Plastocyanin carries the electron from cytochrome b_6/f complex further to PSI at the lumenal site. In PSI, the energy of light is used for electrons transfer across the membrane and for reduction of $NADP^+$ on the stroma site. The electron transport across the thylakoid membrane is coupled with the transport of hydrogen ions. Generated proton gradient is further used to drive ATP synthesis. Both ATP and NADPH are used in Calvin-Benson cycle for CO_2 fixation.

1.4 CO_2 fixation

Carbon fixation is any process, which leads to conversion of gaseous carbon dioxide into a solid compound. That process is mostly found in photoautothrops, and it is usually driven by photosynthesis.

1.4.1 The carbon fixation reactions

C_3 pathway, also know as Calvin-Benson cycle is a basic process to input CO_2 to organic compound during photosynthesis (Calvin 1948). From evolutionary aspect, C_3 is the simplest and the oldest way how to build atmospheric CO_2 into carbohydrates. The reactions take place in stroma – in the soluble part of chloroplasts.

Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) enzyme is the most important enzyme in this process. It can be described with a simple equation:

$$CO_2 + \text{ribulose 1, 5-bisphosphate} \rightarrow 2^* \text{ 3-phosphoglycerate}$$

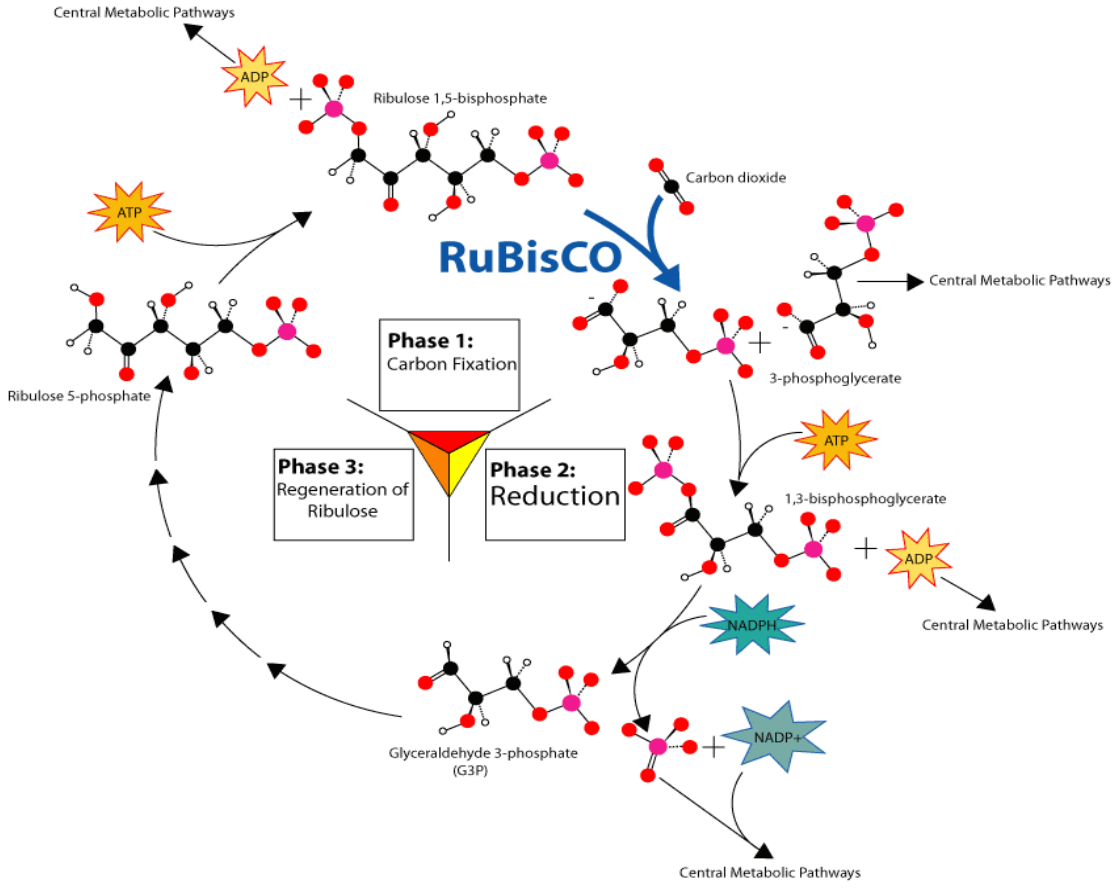
The whole biochemical pathway consists of three major steps. These three steps are:

1) carboxylation of CO_2 and forming the 3-phosphoglycerate as a first intermediate
2) reduction of 3-phosphoglycerate forming glyceraldehyde-3-phosphate and carbohydrates
3) regeneration of CO_2 acceptor ribulose 1,5-bisphosphate from glyceraldehyde-3-phosphate

In that pathway is formed one molecule of triose phosphate from three molecules of CO_2, three molecules of ATP and two molecules of NADPH. The triose phosphates can be further

used for starch synthesis within the chloroplast. Carbon dioxide uptakes to plant cells trough stomata by diffusion. But when the stomata are opened, the plants are losing water molecules. This is one of the reasons, why the C_3 type plants spread mainly in temperate zone, where is not continual water deficiency. Plants in hot and arid zones developed other strategies for carbon fixation, which increases the effectiveness of carbon fixation in higher temperatures - C_4 and minimizes the water loss - CAM photosynthesis (for details see (Blankenship 2002, Taiz 2002).

Fig. 7. Scheme of C3 cycle (Calvin 1948) (commons.wikimedia.org)



2. Fluorescence

2.1. Blue-green fluorescence

Green plants illuminated with UV-radiation (340 nm) emit a blue and green fluorescence (BGF) as well as red and far-red chlorophyll fluorescence with maxima near 440, 520, 690 and 740 nm, respectively (Buschmann 2000) (Fig. 8).

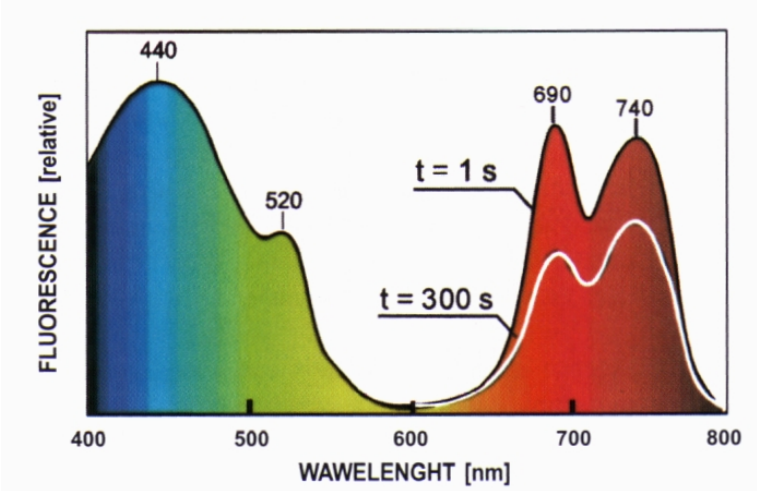


Fig. 8 Fluorescence emission spectrum

A 20 min pre-darkened green tobacco leaf taken via a fluorescence spectrometer with excitation in UV-A (340 nm) directly after onset of irradiation (t = I s) and after of irradiation with "white saturating photosynthesis (t = 300 s light" s). During the light triggered induction kinetics (Kautsky effect) the Chl-F near 690 nm decreases to a stronger degree than the far-red Chl-F near 740 nm. As a consequence the Chl-F ratio F690-F740 decreases from 1.10 (1 s) to 0.85 after 300 s of irradiation. Adopted from (Buschmann, Langsdorf et al. 2000)

BGF derives mainly from ferulic acids covalently bound to cell walls (Lichtenthaler and Schweiger 1998). This phenomenon was firstly measured by (Chappelle 1985). Morales and Cerovic (1998) suggested that flavonoids and other simple phenols, such as p-coumaric acid, contribute to the BGF signal. The initial idea of one major fluorophore for BGF has been challenged by many groups: e.g. (Lichtenthaler`s, Morales`s, Chapelle`s groups). For example, NADPH-dependent BGF was observed in chloroplasts (Hideg 2002) and BGF was also enhanced by flavin accumulation in iron deficient leaves (Buschmann 2000). Finally, comprehensive study of excitation spectra in a large number of species has suggested that several compounds contribute to BGF in addition to cell-wall bound ferulic acid (see Buschmann 2000).

The intensity of the blue-green fluorescence of leaves is constant and the emission spectrum of BGF varied with plant species (Chappelle 1985). Monocotyledonous plants have generally higher values of BGF than dicotyledonous plants, in dependence of cell wall thickness (Buschmann 2000). Ratios between BGF and red and far-red chlorophyll fluorescence have been frequently used for evaluation stress in plants (Buschmann, Langsdorf et al. 2000).

2.2. Red and far-red fluorescence (Chlorophyll fluorescence)

2.2.1. Origin

In detail, absorption of a light quantum by Chl-A in LHC leads to its excitation that changes its electron state:

$$Chl + h\nu \longrightarrow Chl^{*}$$

where Chl is molecule of chlorophyll in ground state, hν is light energy and Chl* is excited molecule of chlorophyll.

Absorption of blue photon (at 430 nm) that has higher energy than red photon (at 662 nm) and excites the chlorophyll molecule to a higher state (S2 excited singlet state, see Fig. 9).

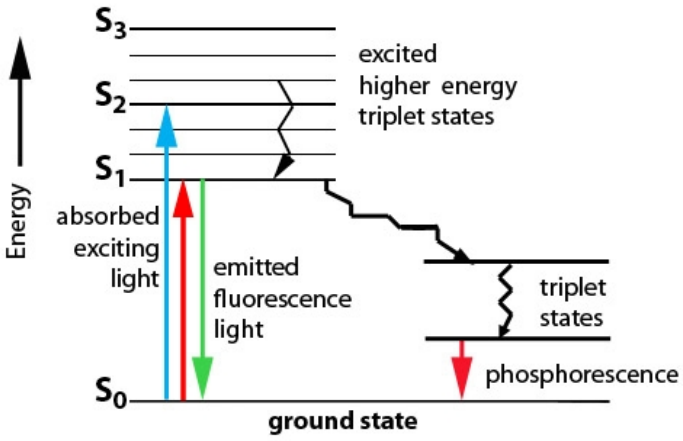


Fig. 9 Jablonski diagram showing resonance energy transfer – blue arrow indicates excitation by blue photon (at 430 nm), red arrow excitation by red photon (662 nm). Excited states in both cases refer to excitation energy of photon at specific wavelength. (http://web.uvic.ca/ail/techniques/epi-fluorescence.html)

In the S2 state is the Chl molecule extremely unstable, and very rapidly (about 10^{-14} s) gives up some energy to surroundings as heat, and enters the lower S1 excited state, where it can be stable about several nanoseconds (10^{-9}). From this lowest S1 excited state, the re-entering to ground state has three possibilities:

1. A first process is called *photochemistry*, in which the energy of the excited state is used for charge separation in photosystem II reaction center. This photochemical reaction of photosynthesis belongs to the fastest known chemical reactions (10^{-15} s). This extreme speed of reactions is necessary for photochemistry to compete with two other possibilities of de-excitation.

2. Excited chlorophyll molecule can re-emit a photon and thereby returns to its ground state this process is known as *fluorescence*; when it does so, the wavelength of fluorescence is slightly longer (and energetically lower) than wavelength of absorption because a portion of excitation energy is converted to heat, before the fluorescence photon is emitted. The fluorescence emission is in red region of the spectrum (690-740 nm).

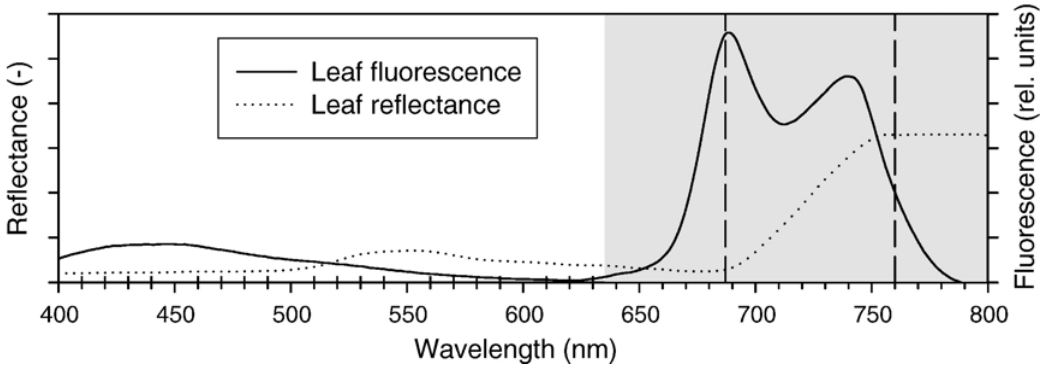
3. The excited chlorophyll can return back to its ground state by direct conversion of this portion of energy into *heat*, with no emission of photon.

Based on the principle of conservation of energy the excitation energy coming into the PSII can not be lost, or fully utilized by the photochemical or non-photochemical processes. Hence the sum of these quantum yields (photochemistry, fluorescence, non-radiative heat deexcitation) equals unity.

$$\Phi_P + \Phi_F + \Phi_D = 1 \qquad (1)$$

The red and far-red Chl-F emission of intact dark green leaves is characterized by two distinct maxima near 690 and 740 nm, usually termed F690 and F740.The Chl-F emission bands at F690 and F740 depends upon the chlorophyll content of the leaves and more than 96 % of the shape of the Chl-F emission spectrum at room temperature (including 740 nm peak) can be explained by re-absorption processes that mainly affect the red Chl-F of PSII in the 690 nm region (Gitelson, Buschmann et al. 1998).The far-red Chl-F near 735-740 nm at room temperature contains a small contribution from PSI ((Schatz, Brock et al. 1987; Gitelson, Buschmann et al. 1998; Pfundel 1998).

Fig. 10 Beech tree leaf emission spectrum expected from solar excitation (continuous line), and green leaf reflectance (dotted line). (Buschmann 2002)



2.2.1. Chlorophyll fluorescence – the basics

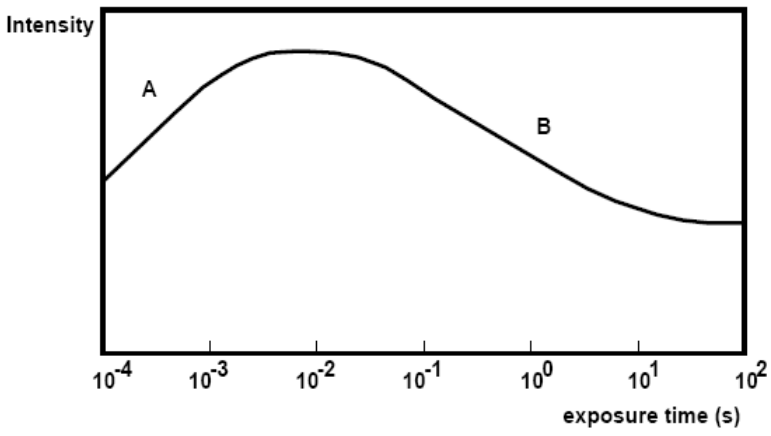
For studying Chl-F in vivo, two basic states of plant have been defined from fluorescence/photochemistry point of view: dark adapted state (DAS) and light adapted state (LAS). Some authors have different terminology: dark-adapted state of the photosynthetic apparatus with open PSII reaction centers is called as “state 1”, whereas the light adapted functional state is defined as “state 2” e.g. (Fork and Satoh 1986; Haldrup, Jensen et al. 2001). However, I will use Dark Adapted State (DAS) and Light Adapted State (LAS) in my Thesis.

In DAS all electron carrier processes in thylakoidal membrane are inactive, and all PSII reaction centers and electron carriers at the acceptor side are oxidized. DAS is characterized by two basic levels of Chl-F: by the basic minimum Chl-F level (F_0) measured in dark adapted plants when all reaction centers are opened and all energy carriers are oxidized, i.e. when the potential photochemistry reaches its maximum, and when the non-photochemical quenching reaches minimal values. F_0 also called initial, basal or minimal fluorescence. Second one is maximum Chl-F (F_M). F_M is measured when all RC are closed, i.e. when the potential photochemistry reaches zero, and when the non-photochemical quenching reaches minimal values. F_M can be measured when dark-adapted plant is illuminated by a short saturating flash that closes all RC, due to a full reduction of the primary quinone-type electron acceptor of PSII (Q_A,(Duysens 1963)) accompanied by a complete saturation of the photochemical processes in PSII. Typically, F_M value is ca five times higher than F_0.

Correct determination of both F_0 and F_M is essential for the subsequent quantification of other Chl-F parameters that have physiological interpretation.

Light adapted state (LAS) is a state when the plant is already adapted to constant actinic irradiation. Here, ca 80% of the absorbed light energy is transferred into photochemistry and also concurrent fixation of CO_2 takes place. Next ca 15% is dissipated in form of heat and approximately 3-5% is emitted as Chl-F (see e.g.(Maxwell and Johnson 2000), Govindjee 1995, Papageorgiou and Govindjee 2004). In situation when the electron transport processes and coupled biochemical reactions in the carbon reduction cycle are equilibrated, the steady-state Chl-F yield level - F_S is reached.

Fig. 11 Fluorescence induction (Kautsky effect)



After exposing a dark-adapted leaf to actinic irradiance, the Chl-F intensity increases first to a peak value within ca 1 s (A) and then decreases to a steady-state level (B). The Chl-F rise to the peak is associated with the reduction of QA, an electron acceptor of PSII. Than, Chl-F emission is modulated not only by photochemical but also by non-photochemical quenching during adaptation of the leaf to actinic irradiation (Govindjee 1995).

When dark adapted plant is exposed to actinic light, chlorophyll fluorescence displays characteristic changes in intensity

accompanying the induction of photosynthetic activity – fluorescence induction or Kautsky effect (Kautsky and Hirsch 1931). First, it increases to a peak level F_P within ca 1s. Than, the Chl-F intensity falls again and finally reaches a steady-state level F_S. The initial „fast“ rise of the Chl-F intensity is attributed to the progressive reduction of the electron acceptors in photosynthetic pathways downstream of PSII, concretely plastoquinone (PQ) and in particular Q_A (Nugent 1996). When the Q_A accepts electron, it is not capable to accept another one. During this time the reaction centre of PSII is “closed”, because it is not able to accept another quantum of light and emit the photon. This closed state decreases the photochemistry efficiency in time; and the absorbed energy is released in form of Chl-F. After the “fast” rise, the yield of Chl-F typically falls again in scale of minutes. This phenomenon, known as *quenching* can be explained in two ways. First, there is activation of PSI and light induced activation of enzymes involved in carbon metabolism and the stomata opening resulting in the increase of electron flow rate out of PSII. This process is called *photochemical quenching* of Chl-F yield. Second one, taking place in parallel is an increase of the heat conversion efficiency, called *non-photochemical quenching*. Typically, these two processes are complete in 15-20 minutes and an approximate steady-state is acquired, although the time taken to reach of that stat can vary significantly from plant to plant species (G.N. Johnson 1990).

2.2.2 Measurement of chlorophyll fluorescence emission

Recently, Chl-F is measured mostly using PAM-fluorometry. PAM – fluorometry is a method based on the principle of the Pulse-Amplitude Modulation (see Schreiber 1986). This approach allows both measurements of photochemical and non-photochemical quenching of Chl-F.

A PAM fluorometer typically consists of four light sources. They provide quantitatively different irradiation that induce changes in a redox state of all components of the electron transport chain and induce Chl-F.

1) Pulsed measuring light (ML): weak red pulses LED used for Chl-F signal determination with various measuring frequencies (for details see Roháček 2008).

2) Actinic light (AL): non-saturating continuous irradiance with duration in minutes. AL is used to drive the photosynthesis. However, intensity of AL should be weak enough not to saturate the photosynthetic process. It should not exceed 30–40 % of the irradiance level saturating net photosynthetic CO_2 assimilation activity - P_N.

3) Saturating light (SL): strong “white light” given as a saturating flash (SL) or as continuous irradiation **(cSL)**. Short saturating flash (typically 1-2 s, ≈ 3000 μmol(photons) m^{-}

^{2}.s^{-1}) is used to close all RC and to measure either F_M in DAS or F_M` in LAS. Or used as continuous irradiation **(cSL**, ≈ 2000 μmol(photons) m^{-2}.s$^{-1,}$ 4 to 5 min) it fully saturates photosynthetic CO_2 fixation.

4) PSI irradiation: far-red radiation LED with peak emission at 735 nm ≈ 10 μmol(photons) m^{-2}.s^{-1}). This low far-red irradiation is absorbed by PSI and takes away the electron from the acceptor side of the reaction center of PSII. Hence, all Q_A molecules at the PSII acceptor side gradually become fully oxidized (the reaction center of PSII is open again). This far-red radiation is used to correctly determine F_0' after AL is removed.

Application of saturating pulses during plant illumination by measuring pulses and/or actinic irradiance allows evaluating photochemical and non-photochemical quenching. Fig. 11 shows typical Chl-F induction kinetics recorded in vivo using combination of above mentioned lights (quenching analysis).

2.2.3 Chlorophyll fluorescence parameters

In general, six primary mutually independent Chl-F levels can be recorded using complex measuring protocol - quenching analysis (Fig. 11) / F_0, F_M, F_P, F_S, F_M`, $F_0$`). These parameters are used to calculate most of Chl-F parameters describing plant physiological status.

DAS is characterized by F_0 and F_M, LAS is characterized by F_M`, $F_S$, $F_0$`.

Table 1 describes the most used Chl-F parameters. For more, see e.g. Roháček (2002).

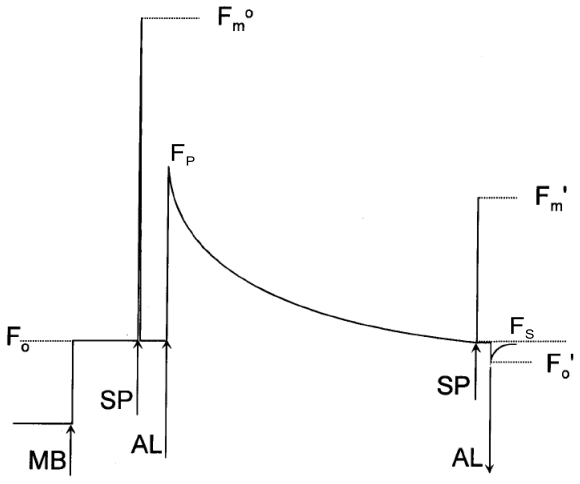


Fig. 12 Chl-F kinetics ((Maxwell and Johnson 2000))

When a measuring light is switched on (MB) is the zero level of Chl-F measured F_0. Applying of saturating flash of light (SP) allows us the measurement of maximal level of Chl-F F_M (here marked as F_m^0). After that, light which drives photosynthesis (photochemistry) is applied – actinic light AL. After a time period next saturating light flash allows us to measure maximum Chl-F in the light F_M` (here $F_m$`). Steady state *Chl fluorescence in light-adapted state F_S. Turning off the actinic irradiance typically in far-red light allows us to determine the zero level fluorescence "in the light" F_0`.*

Tab. 1 Basic Chlorophyll Fluorescence parameters

F_0 initial, also basal or minimal Chl-F yield in the dark-adapted state. All PSII reaction centers are opened and electron carriers of the acceptor side are re-oxidised. Components of the non-photochemical quenching are relaxed to their minimum values.

F_M maximum Chl-F in the dark-adapted state – all PSII reaction centers are closed, primary quinone-type electron acceptor of PSII is fully reduced and non-photochemical quenching components reach their minimal values

F_P transient peak of Chl-F during the transient of plant from DAS to LAS induced by actinic light, the initial „fast“ rise of the Chl-F intensity is attributed to the progressive reduction of the electron acceptors in photosynthetic pathways downstream of PSII.

F_M' maximum Chl-F in the light-adapted state of leaves (measured by application of saturating pulse during actinic light)

F_0' ground Chl-F (minimum yield) in light-adapted state - level of Chl-F yield measured directly after switching off actinic irradiation

F_S steady state Chl-F in light-adapted state - electron transport processes and coupled biochemical reactions are equilibrated

F_V variable Chl-F in the dark-adapted state - difference between F_M and F_0 ($F_V = F_M - F_0$) (Kitajima and Butler 1975, van Kooten and Snel 1990)

F_V' variable Chl-F in the light-adapted state - difference between F_M` and $F_0$` ($F_V` = F_M` - F_0`$)

F_V/F_M maximum quantum yield of photosystem II (PSII) given by $F_V / F_M = (F_M - F_0) / F_M$. In healthy leaves, this value is always close to 0.8, independently of the plant species studied (Kitajima and Butler 1975).

NPQ non-photochemical quenching of Chl-F – quantifies processes leading to the decrease (quenching) of F_M during adaptation of the sample to actinic irradiation. ($NPQ = (F_M - F_M`) - F_M`$) (Bilger 1990)

Φ_{PSII} – effective quantum yield of PSII in LAS, $\Phi_{PSII} = (F_M` - F_T) / F_M`$), overall efficiency of PSII reaction centers in the light and reflects the efficiency of open reaction centers in light (Genty, Briantais et al. 1989).

Rfd – “vitality index” ($R_{FD} = \frac{F_P - F_S}{F_S} = \frac{F_P}{F_S} - 1$) (Lichtenthaler and Meier 1984).

2.2.4 Integrative and imaging fluorometry

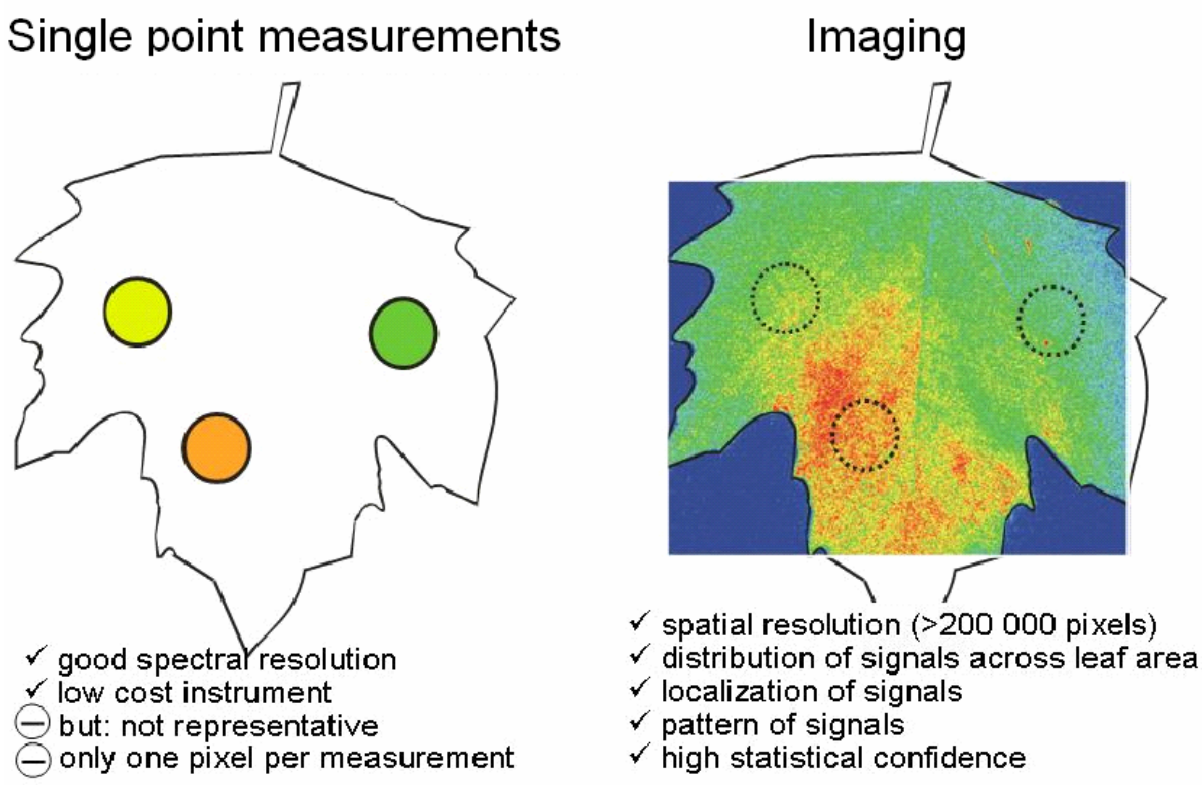
Nowadays two types of fluorometry are used in plant physiology research: integrative and imaging chlorophyll fluorometry.

Integrative fluorometry measures Chl-F signal from larger defined area (typically tens of mm^2). Recently, also recording Chl-F signal on a single cell level is possible. Integrative fluorometry is fast, non-invasive and allows repetitive measurements both in laboratory and in the field.

However, photosynthesis and consequently Chl-F can be altered by various both internal (pigment content, build-up of the pH-gradient, state-transitions, ATP-synthesis regulation, inactivation of RCs II etc) and external factors (water deficiency, light, infection etc) varying over the surface of plant or leaf. Such heterogeneity can be hardly covered by point measurement. Ch-F imaging techniques allow imaging distribution of Chl-F over the leaf/plant area.

Fig. 13 Example of integrative fluorometry and imaging fluorometry approaches.

The advantages and superiority of leaf fluorescence imaging compared to the fluorescence measurement at single leaf spots here shown for a maple leaf. The spatial heterogeneity of the Chl fluorescence signals and ratios shows up via imaging, but not at all or not necessarily via single leaf point measurements, even if several points of a leaf are measured. (Lichtenthaler, 2005)



Chl-F imaging fluorometry enables to examine thousands of image interpreters (pixels in Chl-F image) carrying the Chl-F signal. The Chl-F signal of discrete areas could be analyzed individually, or grouped to image assigned to individual objects e.g. plants, leaves, leaf segments etc. Imaging of Chl-F gives us detailed information about spatial and kinetic heterogeneity of Chl-F over measured sample or allows analyzing a large number of small objects simultaneously in one experiment. Although the performance of Chl-F imaging technique has been improved significantly, it has still some limitations. One should take care if the irradiance (both actinic light and saturation pulse) are homogeneously distributed over measured leaf area. Limitation is also the strength of saturating pulse, which is necessary to fully saturate RCs and to determine F_M and F_M'.

Advantages and disadvantages of both methods are described in detail by Nedbal and Whitmarsh (2004) and Roháček et al. (2007).

2.2.4.1 FluorCam – kinetic imaging of chlorophyll fluorescence

Since I have been mostly using a commercial kinetic imaging fluorometer Open FluorCam 701 MF (Photons Systems Instruments, Ltd., Brno, Czech Republic; Nedbal et al. 2000), I will here briefly describe this instrument.

The kinetic imaging fluorometer FluorCam was primarily designed to capture kinetically resolved chlorophyll fluorescence of leaves, small plants and as well as for imaging algal or cyanobacterial colonies on a Petri dish. FluorCam consists of several parts as shown in Fig. 13.

Fig. 14 FluorCam 701MF imaging fluorometer

Here, Chl-F emission is excited by two panels of light-emitting diodes (1) (LEDs, λmax ≈ 635 nm) that generate short measuring flashes and actinic light. Saturating light pulses (≈ 1500 μmol (photons) $m^{-2}.s^{-1}$, 1-2 s) are generated by a 250 W halogen lamp (2) (λ=400–700 nm). Chl-F transients are captured by a charged coupled device (CCD) camera (3) in a series of images with 12-bits resolution in 512×512 pixels, acquiring 50 images s^{-1}.

Particularly, we have used this instrument to detect an early detection of biotic stress (fungal infection in grapevine (Cséfalvay, Di Gaspero et al. 2009), tracking the viral infection within the *N. benthamiana* plants), to measure annual variation of steady state Chl-F in evergreen overwintering plants (Soukupová et al. 2008), and to compare the plant response to specific nutrient treatment – titanium treated plants (Cígler et al.). In these studies, we inspected images of various conventional Chl-F parameters with known physiological interpretation (e.g., F_0, F_M, F_S, F_V/F_M, Φ_{PSII}, NPQ) over measured leaf samples, mean Chl-F transients in discrete sample areas as well as non-standard parameters of Chl-F counted by advanced statistical approach (*combinatorial imaging*) developed in our laboratory (Matouš 2006)designed for improving the data evaluation of Chl-F measurements and also for finding the earliest symptoms of various stresses.

2.3 Combinatorial imaging

Combinatorial imaging is a new statistical approach for evaluation the measured Chl-F imaging data based on statistical feature selection method (Pudil *et. Al 1994*). It has been developed by Karel Matouš and Ladislav Nedbal. It is in detail described in Ph.D. Thesis of Karel Matouš ((Matouš 2008), in Czech) and in publication Matouš (2006).

Combinatorial imaging and classical evaluation of Chl-F data to find difference between control and infected or treated plant tissue have different approach.

Using classical approach, the Chl-F parameters for stress identification were mostly found empirically: by browsing the images of conventional fluorescence parameters and by finding those that exhibited sufficient contrast between control and stresses leaf tissue (e.g., F_0, F_M, F_P, F_S, F_V, F_V/F_M, Φ_{PSII}, NPQ, R_{fd}). Only seldom, parameters that have no explicit physiological meaning were also included in the search, e.g. F_0/F_V (Nedbal 2000b).

Combinatorial imaging searches in a whole file of recorded Chl-F images recorded through the entire fluorescence transient under various light protocols. It looks for small sets of several contrasting Chl-F images regardless of their potential interpretation that are used to discriminate between the control and stressed/treated leaf tissue. The sole optimization factor is the highest contrast between the stressed and control leaf tissue.

Here, we shall try to describe the method is a simple way:

The algorithm, which is further used to discriminate the treated from control leaf tissue according their Chl-F response, must be trained to recognize the Chl-F transients from controls. Than, the technique sought a set of three Chl-F images that yielded maximum

contrast between the controls and treated tissues using Sequential Forward Floating Search algorithm (SFFS, Pudil 1994, Matouš 2006). In this process, any arbitrarily selected pixel in the testing Chl-F image is compared to homologous training sets of control leaf images. If its Chl-F level is more similar to the control, the pixel is classified as control one, otherwise it is classified as stressed/treated.

Then, we use the a priori knowledge as to what leaf tissue this particular pixel belonged to and decide if the classification was correct or erroneous. The subset of Chl-F images that yields the lowest error rate and, thus, the highest performance is selected for the *combinatorial imaging* and put into optimal linear combination using Linear Discriminant Classifier (Fukunaga 1990).

The final linear combination of the set of Ch-F parameters is usually named as CI parameter (Combinatorial Imaging parameter) for particular stress. Than, the CI parameter over the treated/stressed and control leaves is displayed. Such resulting image calculated pixel-by pixel is further shown in false color scale. First time was this approach used by Berger et al. (2007) to study the interaction between *Pseudomonas syringae* and *Arabidopsis thaliana* and to find Chl-F features for early and late phase of the bacteria infection. Later, we have used this approach to detect spatially determined infection of grapevine by *Plasmopara viticola* and for evaluation of systemic titanium/iron competence in spinach plants.

3. Stress

Plants during their life cycle are exposed to various conditions of environment. The highest importance for plants growth and vitality has the incident irradiance, temperature, humidity, and water and nutrient supply. Limitation of these environmental factors may slow the plant vital functions and harm individual tissues or cause organism death. Specificity of plant is that they can not change the standpoint and escape from the unfavorable conditions. Thus, plants developed mechanisms to cope with the external stress factors (stressors) affecting negatively their vital functions. Terminus “stress” is usually used for complex state of plant under incidence of stressor. This actual state is not static and easily representative condition. It is a highly dynamic complex of reactions, by which the plant defenses against the stressor. If one equates extreme factors of the physical environment with stressors then stress is not the exception from the rule but rather a normal situation of plant life (Larcher 1988; Leshem and Kuiper 1996). However, in laboratory experiments is usually only one stress factor used to study its impact on plant metabolism.

3.1. Types of stress

In plant physiology, two major groups of stress factors are identified:

1. *Abiotic stress*; it covers both *physical* (mechanical damage [e.g. by wind], excess irradiation [UV, visible] or extreme temperatures [heat, cold, frost]) and *chemical factors* (drought, lack of oxygen [hypoxia, anoxia], nutrient deficiency, abundance of salt ions, hydrogen, toxic metals and organic compounds in soil and toxic gases in air, etc.)

2. *Biotic stress* is caused by living beings: herbivory (grazing, harming), pathogenic microorganisms (viruses, microbes, fungi) and mutual impact of other plants (allelopathy, parasitism).

3.2. Response to stress

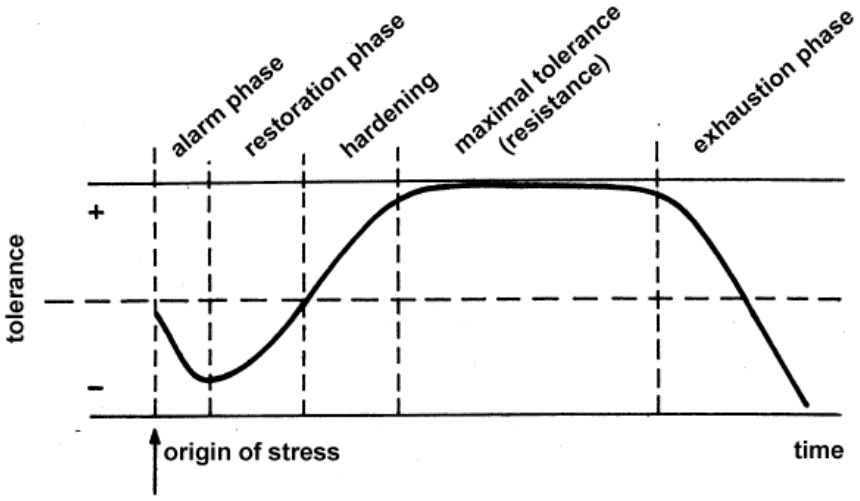
Plant response to stress can be divided into two major groups: 1) *stress avoidance* and 2) *stress tolerance*. *Stress avoidance* (Levitt 1980) is older mechanism and includes all defense structures of the plant, which have to block, or slow down the stress factor progress (thick leaf cuticle, impregnation of cell walls, water reservoirs, reserve of organic compounds etc). The simplest way how to avoid stress is timing of life cycle – to be in dormant state, when the largest pressure of stress occurs. *Stress tolerance* includes younger and much sophisticated mechanisms that are based on mechanisms of active resistance to stress factors. The active mechanism keeps down the negative influence of stressors.

General stress theory (Leshem and Kuiper 1996) calls stress as the sequence of induced states of the plant that generates specific responses on cellular level (general signal responses, adaptations and defense responses). That state leads to new homeostasis; or under strong stress to a dysfunction leading to necrosis of the plant organ or it causes the death of whole plant. That happens when the pressure of stressor is too strong or when several stressors affect the organism and the organism is not able to eliminate stress effect in time. From plant physiology viewpoint are the most interesting mechanisms that of active tolerance, which are able to hamper the negative fall of stressors. In that case starts the chain of changes, which is called stress reaction, or stress avoidance. In general principles we can define a four phases of this process:

1. Response phase – alarm reaction, the main beginning of stress
2. Restoration phase - the stage of resistance during continuous stress
3. End phase – stage of exhaustion in long term non-favorable conditions that can lead to organism death.

4. Regeneration phase – partial or full regeneration – dependent on the stressor strength, plant may return to initial physiological state.

Fig. 15 Idealized course of stress reaction (Larcher 1995)



Stress progress and the end outcome are dependent on intensity of incidence of stress factors and also on genetically bounded presumptions of answer (adaptation skills). Temporary increase of resistance obtained under influence of stressor is called acclimation and could be established on fast changes that have only the short persistency (production of specific metabolites) but also on changes that are continuous (changes in tissues and internal structure of plant organs). Rising of tolerance and re-establishing of homeostasis under long term influence of stressor are commonly reached at the price of certain supplementary energetic costs, mainly for synthesis of specific metabolites. Even some other changes in metabolism ensuring the high stress resistance (for example maintenance of higher concentration of osmolytes in cytoplasm) is usually guided with decrease of gaining speed of new matter sources and with decrease of biomass production. Changes of structures and functions of plant leading to low efficiency of gaining new matter sources can persist long time over the returning to external conditions to optimal values, sometimes whole vegetation state. Incidence of stressors (e.g. low temperature) can qualify complicated morphogenetic processes for example germination or formation of flowers and that way increase reproducibility.

Studying the stress under ambient conditions is always complicated by fact that plants are never impacted only by one stress factor. Usually, the more stressors are affecting the plant at one moment, e.g. high irradiance, water stress, air pollution etc. Hence interaction between

these factors can seriously change the response of stress reaction. (Adopted from Larcher W. 1995)

4. Plant stress detection

The methods for plants stress detection can be either invasive e.g. estimation of leaf relative water content (Barr 1962), estimating the leaf water potential by pressure chamber (Schulte and Hinckley 1985), the cell membrane stability (CMS) test (Tripathy, Zhang et al. 2000) etc, or non-invasive, e.g., monitoring gas exchange parameters or measuring plant optical signals. Using non-invasive measuring methods, enables us to study the plant physiology during whole stressor incidence. Since most stress factors directly or indirectly affect photosynthesis apparatus and/or photosynthetic efficiency, chlorophyll fluorescence is a unique method for early plant stress detection.

4.1 Chlorophyll fluorescence imaging and stress

Chlorophyll fluorescence imaging (Chl-FI) is widely used technique to determine spatial and temporal heterogeneity of leaf photosynthesis under various stresses over a leaf or a plant (reviewed by Nedbal and Whitmarsh 2004). As an example of wide usage of that method is searching result from Web of Science (www.isiknowledge.com), where after submission of keywords *chlorophyll fluorescence and plant stress*, ca 1194 publications from last twenty years occurred. One of the recent is work is from Pieruschka *et al.* 2010, where the photosynthesis in leaf flecks of homobaric and heterobaric leaves were studied. Main parameters used for estimation of Chl-F were F_0, F_M, F_V/F_M and NPQ.

One of the most important abiotic stress factors, which negatively influence the plant metabolism in field, is the temperature - chill or overheats stress. For determination of heat stress as well as drought stress changes in photochemical and non-photochemical Chl-F quenching are frequently used (e.g., Li, Liu et al. 2008; Massacci, Nabiev et al. 2008; Woo, Badger et al. 2008; Pieruschka, Chavarria-Krauser et al. 2010). Chl-F imaging can be also used as an alternative method to accurately determine the freezing tolerance of leaves (Ehlert and Hincha 2008). Inherency of heavy metals in the plant has been determined by F_V/F_M, q_n and Φ_{PSII} changes (Govindjee 2004). Also the iron deficiency has been evaluated from these parameters (Morales, Moise et al. 2001).

Important biotic factors that influence the plant metabolism and decreasing the yield of crops are various infections (bacterial, viral or fungal). Pathogen infections induce dramatic changes in the primary metabolism of the affected plant tissue. Several pathogen have been found to

suppress photosynthesis of the host, some pathogen maintain photosynthesis of the host in green islands of the leaves. However, the carbohydrate metabolism of the infected tissue is substantially modified. Thus, Chl-F offers possibility for non-invasive early detection of infection, since it is closely related to the process of photosynthesis and plant defense reaction.

Recently, Chl-F parameters that are mostly used to describe the effect of particular biotic stress factor to photosynthetic apparatus of the plant are those related with photosynthetic activity - F_V/F_M, Φ_{II}, NPQ. The down regulation of photosynthesis were found in infected areas by *Pseudomonas syringae* and *Botryris cinerea* (Berger, Papadopoulos et al. 2004), the decrease of F_V/F_M were observed by Bonfig, Schreiber et al. (2006) by the comparing the infection of *A. thaliana* by two strains of *P. syringae.* Localised changes in photosynthesis in oat leaves infected with the biotrophic rust fungus *Puccinia coronata* Corda were examined at different stages of disease development by Chl-FI, where the main changes were found in Φ_{PSII} and NPQ (Scholes and Rolfe 1996).

One can recognize that heterogeneous distribution of standard Chl-F parameters, which are in direct conjugation with physiological process in plant tissues have been used for plant stress detection. Particularly those related to photosynthetic activity - as F_V/F_M (Kitajima and Butler 1975) and Φ_{PSII} (Genty, Briantais et al. 1989) (maximum and effective quantum yield of PSII, respectively) and NPQ (non-photochemical quenching, Bilger and Björkman 1990), are the most frequently used approach to identify the altered photosynthetic efficiency over the infected leaf, and as an indicator of a localized plant's stress (Balachandran 1994; Balachandran, Hurry et al. 1997; Lohaus and Moellers 2000; Chou 2000; Chaerle 2004Swarbrick, Schulze-Lefert et al. 2006; Chaerle, Hagenbeek et al. 2006; Chaerle, Hagenbeek et al. 2007; Perez-Bueno, Ciscato et al. 2006; Pineda, Gaspar et al. 2008). Chl-F imaging is frequently combined with other imaging technique (e.g., BGF and thermal imaging) for earlier evaluation of plant stress response e.g. (Chaerle et al. 2004, Hacker, Spindelbock et al. 2008).

Successful combination of Chl-F imaging and combinatorial imaging for very early detection of bacterial infection (*Pseudomonas syringae*) in *A. thaliana* leaves has been demonstrated by Berger et al. (2007). Using the statistical approach, they were able to detect the infection already 6 hours after the inoculation and distinguish the early and late features of the infection. They investigated also the effect of salicylic acid and the oxylipins (12-oxo-phytodienoic acid -OPDA) and jasmonic acid on photosynthesis and compared it with the

effects of infection by virulent and avirulent strains of *P. syringae*. Also other studies (Pineda et al. 2008, Cséfalvay, Di Gaspero et al. 2009) show that combination of Chl-F imaging and combinatorial imaging could be very useful in early detection of plant stress, and also for identification of unique features of particular plant stresses.

RESULTS

Appendix A

1. Competitive relationships between iron and titanium metabolism in spinach: a hydroponic fluorescence study.

Source:

Cígler P, Olejníčková J, Hrubý M, Cséfalvay L, Peterka J, Kužel S (2010) Competitive relationships between iron and titanium metabolism in spinach: a hydroponic fluorescence study. Journal of Plant Physiology, accepted

Abstract:

One of the elements showing strong hormetic beneficial effect on plants at low concentrations and toxic effects at higher concentrations is titanium (Ti). We investigated the interconnection between the Fe uptake and the Ti intoxication in model experiment on Fe-deficient spinach plants to help to elucidate the mechanism of the biological activity of titanium in plants. We compared chemical analysis of Ti and Fe in roots and shoots with the changes of the *in vivo* chlorophyll fluorescence emission. Although Fe and Ti concentration found in shoots of Ti-non-treated Fe-deficient plants was comparable with that in Ti-treated Fe-deficient plants, the soluble form of Ti present in the growth media had a negative effect on photosynthetic activity followed by chlorophyll fluorescence measurements. The presence of Fe in growth medium significantly decreased the Ti content in shoots and increased the photosynthetic activity. Here, we propose that Ti affect components of electron transport chain containing Fe in their structure (particularly photosystem I) and decrease the photosystem II efficiency.

Appendix B

2. Annual variation of steady-state chlorophyll fluorescence emission of field-grown evergreen plants indicates seasonal onset and offset periods of photosynthesis.

Source:

Soukupová J, **Cséfalvay L**, Urban O, Košvancová M, Marek MV, Moersch A, Rascher U and Nedbal L (2008) Annual variation of steady-state chlorophyll fluorescence emission of field-grown evergreen plants indicates seasonal onset and offset periods of photosynthesis. Functional Plant Biology 35, 63-76.

Abstract:

Remotely passive sensed chlorophyll fluorescence emission can become a major reporter signal on global vegetation dynamics. To increase our understanding of this signal, we investigated steady-state chlorophyll fluorescence emission (Chl-F_S) in laboratory and field conditions. Using Chl fluorescence imaging and gas exchange measurements on a leaf level, we explored the modulation of Chl-F_S by irradiance and temperature, as well as changes in Chl-F_S during the year, in three evergreen coniferous and broadleaf plant species. Based on the laboratory measurements, Chl-F_S was found to be stable along wide range of irradiances. Annual course of field monitored Chl-F_S was similar to that of the CO_2 assimilation rate during the year It was low and stable in the winter and steeply increased in the spring. In contrast, high values of Chl-F_S persisted throughout the vegetation season and rapidly decreased in the autumn. These transitions, associated with periods with night intermittent freezing temperatures, were also marked by a significantly elevated sample-to-sample variability of the Chl-F_S signal. In this way, an annual variation of remotely sensed Chl-F_S gives information about the activation/deactivation of photosynthetic activity in temperate zone evergreen plant species during the year.

Appendix C

3. Pre-symptomatic detection of *Plasmopara viticola* infection in grapevine leaves using chlorophyll fluorescence imaging.

Source:

Cséfalvay L, Di Gaspero G, Matouš K, Bellin D, Ruperti B, Olejníčková J (2009) Pre-symptomatic detection of *Plasmopara viticola* infection in grapevine leaves using chlorophyll fluorescence imaging. European Journal of Plant Pathology, 25 (2), 291-302

Abstract:

Plasmopara viticola is an economically important pathogen of grapevine. Early detection of the *P. viticola* infection can lead to improved fungicide treatment. Our study aimed to determine whether chlorophyll fluorescence (Chl-F) imaging can be used to reveal early stages of *P. viticola* infection under conditions similar to those occurring in commercial vineyards. Maximum (F_V/F_M) and effective quantum yield of photosystem II (Φ_{PSII}) were identified as the most sensitive reporters of the infection. Heterogeneous distribution of F_V/F_M and Φ_{PSII} over inoculated leaves was associated with the presence of the developing mycelium three days before the occurrence of visible symptoms and five days before the release of zoospores. Significant changes of F_V/F_M and Φ_{PSII} were spatially coincident with the spots of inoculation across the leaf lamina. Reduction of F_V/F_M was restricted to the leaf area that later yielded sporulation, while area with significantly lower Φ_{PSII} was larger and most probably reflected the leaf parts in which photosynthesis was impaired. Our results indicate that Chl-F can be used for early detection of *P. viticola* infection. However, because the plant did not express systemic reaction against the infection, Chl-F imaging at high resolution is necessary to reveal the disease in the field.

Appendix D Short draft, not submitted

4. Movement of viral infection in plant body followed by means of chlorophyll fluorescence imaging

Cséfalvay L, Pineda M, Olejníčková J and Barón M.

Abstract:

In this study we followed the propagation of two strains of PMMoV virus (Italian and Spanish strains of *Pepper mild mottle virus* – PMMoV-I, PMMoV-S, respectively) within the *Nicotiana benthamiana* plants by means of chlorophyll fluorescence imaging. The infection in asymptomatic leaves (AS) differing in their age was visualized by using measurement of heterogeneous distribution of maximal and effective quantum yield of PSII (F_V/F_M and Φ_{PSII}) and non-photochemical quenching (NPQ). After appearance of visible symptoms in newly developing leaves at 5-8 dpi, we detected the infection in the youngest (8^{th}) AS leaves at 10 dpi. Later, the infection was detected at 12 dpi in older (7^{th}) AS leaves and at 14 dpi in the oldest (6^{th}) AS leaves. This finding confirms the hypothesis that the virus particles move first to the youngest developing leaves that started to be curly and fragile and than the virus propagates down to lower already developed leaves through veins.

INTRODUCTION:

Pepper mild mottle virus (PMMoV) is a member of the genus *Tobamovirus* (Togaviridae). In general, infections with tobamoviruses cause economic losses worldwide in pepper crops (Alonso et al. 1989, Lamb et al. 2001). Pepper mild mottle virus (PMMoV) attacks crops mostly from family *Solanaceae* and it also infects cultivars of pepper carrying resistance genes against tobacco and tomato mosaic viruses' subgroups (TMV, ToMV respectively). Infection by PMMoV induces mild mosaic symptoms on the leaves, malformations and necrosis of fruits and leaves leading to crop loss (Garcia-Luque et al. 1990). These changes of plant tissues are associated with changes in the chloroplast structure and function (e.g., Pérez-Bueno et al. 2004, Sajnani et al. 2007, Pineda et al. 2010).

Several previous studies showed that viruses disturb the donor site of PSII due to lower extrinsic protein content (Rahoutei et al. 2000, Pérez-Bueno et al. 2004) and upset the Calvin cycle (Zhou et al. 2004, Sajnani et al. 2007). In our previous studies (Barón *et al.*

1995, Rahoutei *et al.*1998, 1999, 2000, Pérez-Bueno *et al.* 2004, Pineda et al. 2008) we have proposed the oxygen-evolving complex as the primary target of these tobamoviruses on the electron transport chain. Thus, modulation of photoprotective energy dissipation and photosynthetic activity of infected leaf tissue can be followed by means of chlorophyll fluorescence (Chl-F) measurement.

Chl-F is produced in plant photosynthetic tissues after absorption of light energy as one of the two de-excitation pathways that compete with the photochemical energy conversion leading to CO_2 assimilation; the other pathways is a heat loss (reviewed by e.g.,(Govindjee 2004). The coupling between the photochemistry and fluorescence is the strongest in Photosystem II (PSII), where 90% of fluorescence originates at room temperature (e.g.,(Govindjee 1995). Many studies showed that Chl-F dynamic changes of previously dark adapted plant in actinic light could relate with physiological alterations in plants infected with bacteria ((Berger, Papadopoulos et al. 2004; Bonfig, Schreiber et al. 2006; Berger 2007), Bonfig *et al.* 2006), fungi ((Scholes and Rolfe 1996), (Chou 2000),(Swarbrick, Schulze-Lefert et al. 2006)), and virus ((Balachandran 1994; Balachandran, Hurry et al. 1997), Osmond *et al.* 1998, Lohaus *et al.* 2000, Chaerle *et al.* 2004).

Chlorophyll fluorescence imaging (Chl-FI) is used to follow both spatial and temporal variation in Chl-F over leaf tissue. Modern Chl-FI instruments (Nedbal and Whitmarsh 2004) allow defining complex measuring protocols (see Pineda et al. 2008) and thus measurement of a distribution of wide range of Chl-F parameters over measured plant tissue in one experiment. Chl-FI studies of *N. benthamiana* plants infected with PMMoV revealed that non-photochemical quenching (NPQ, Bilger and Björkman 1990), maximal and effective quantum yield of PSII (F_V/F_M and Φ_{PSII}, (Kitajima and Butler 1975)and(Genty, Briantais et al. 1989), respectively) were good reporters of the infection process in leaves which remain asymptomatic (AS) during the infection process, paralleling the spread of the virus in these leaves along the main veins (Pérez-Bueno *et al.* 2006, Pineda et al. 2008). Moreover Pineda et al. (2008) showed that changes in re-oxidation of plastoquinon pool expressed as changes in Chl-F decrease from peak value (FP) in actinic light during fluorescence induction was also potent in early detecting the PMMoV infection.

In this study, we focused on tracking how the virus infection propagates within the plant body by means of measurement of the above mentioned Chl-F parameters in intact leaves differing in their age. We have used a pathogen – host system plant *Nicotiana benthamiana* and two strains of Pepper mild mottle virus (PMMoV-S - Spanish, PMMoV-I -Italian). These two strains were isolated from Almería (Spain) and Sicily (Italia) (Wetter et al. 1984, Alonso et al.

1989). PMMoV-I is less virulent than PMMoV-S (García-Luque et al. 1993, Rahoutei et al. 2000) and only PMMoV-I-infected plants could recover from their symptoms (Chaerle et al. 2006).

Based on our previous results (see Pineda *et al.* 2008), we proposed that the virus particles move first to the youngest developing leaves (apical meristem) that started to be curly and fragile from 5-7 days after inoculation (dpi) onwards. Than, the virus moves down to lower already developed leaves through veins. Leaves that have been totally developed at the time of infection do not show any symptoms during the whole infection process. They are called asymptomatic (AS). Viral particles in AS leaves were found in the plant tissues surrounding the main veins at 14^{th} dpi and at 17^{th} dpi in case of PMMoV-S and PMMoV-I, respectively (Chaerle et al. 2004). These infected tissue in AS leaves exhibited higher NPQ values and lower Φ_{PSII} values ((Chaerle 2004), Pineda et al 2008). Here, we studied the dynamic changes in Chl-F after inoculation in three AS leaves of infected plant that differed in the age, i.e. the $6^{th,}$ 7^{the} and 8^{th} true AS leaves to verify the above mentioned hypothesis about the viral movement.

MATERIAL AND METHODS:

Plant and virus: *Nicotiana benthamiana* Gray plants were cultivated in a growth chamber at 200 μmol (photons)$\cdot m^{-2}\cdot s^{-1}$ PAR (Photosynthetic Active Radiation), generated by cool white fluorescent lamps, with a 16/8 h light/dark photoperiod, a temperature regime of 24/18ºC (day/night) and a relative humidity of 65%. At the 6-7 fully-expanded-leaf stage, plants were mechanically-inoculated with PMMoV in the three lower leaves, using carborundum powder and 25 μl of inoculum per leaf (50 μg of tobamovirus particles per ml of 20 mM sodium phosphate/biphosphate buffer pH 7.0). Two strains of PMMoV were used in this experiment: Italian (PMMoV-I) and Spanish strain (PMMoV-S). Control plants were treated with buffer only. All the results have been obtained comparing control plants with infected plants. Starting with the lowest inoculated leaf and numbering leaves in ascending order, we have measured AS leaf number 6, 7 and 8, one, i.e. the 6^{th} leaf was the oldest one, while the 8^{th} was the youngest.

Chlorophyll fluorescence measurements:

All chlorophyll fluorescence measurements were performed by commercial kinetic imaging fluorometer FluorCam 701MF (Photon System Instruments PSI, Ltd. Brno, Czech Republic;

www.psi.cz). Two panels of light-emitting diodes excited Chl-F emission from leaf (LEDs) (λmax » 635 nm) that generates measuring light flashes (10 ms) and actinic light. Brief and intense saturating light pulses [1500 μmol·(photons) m^{-2}·s^{-1}, 1s] were generated by 250 W white (λ=400-700 nm) halogen lamp. The Chl-F emission transients are captured by a CCD (charged coupled device) camera in series of images at 12-bit resolution in 512 x 512 pixels, taking fifty images per second. The reflected light is blocked by far-red filter (RG697, SCHOTT, Mainz, Germany). All measurements were carried out in attached leaves.

All plants were dark adapted for 20 min prior the measurement. Than, five 60 ms measuring flashes that yielded images of minimum fluorescence F_0 of the plant with fully oxidized plastoquinone pool (F_0) were applied. A short saturating flash of white light (1 s, 1500 μmol (photons).m^{-2}.s^{-1}) was used to reduce the PQ pool and to measure the maximum fluorescence (F_M). After a short dark relaxation (16s), the leaf was exposed to actinic light (50 μmol (photons).m^{-2}.s^{-1}) for 100s and the corresponding Chl-F induction was recorded. Two saturation pulses (1500 μmol (photons).m^{-2}.s^{-1}, at 2 and 90 s after switching on the actinic light) were applied to measure $F_{M(1)'}$ and $F_{M(2)}$' (respectively), to probe the light-induced quenching. A short dark adaptation (16 s) followed by two other saturating pulses (5 s - $F_{M(1)''}$- and 16 s - $F_{M(2)''}$- after switching off the actinic light) were used to measure NPQ recovery.

Four replicates of each variant (PMMoV-S and PMMoV-I infected leaves) were measured. The measurements were done at 10, 12, 14 and 18 dpi in order to monitor the viral migration in plants. Images of standard Chl-F parameters that were proven in our previous studies to be effective in detecting PMMoV infection were selected: F_V/F_M, Φ_{PSII} and NPQ. Distribution of these parameters over 6th, 7th and 8th AS leaves was displayed in false color scale.

RESULTS:

Visual symptoms:

First visual symptoms of the infection occurred at 5 – 8 dpi in PMMo-V-S and PMMoV-I infected plants, respectively (Fig. 1). Here, the new curly young leaves occurred. Hence we were sure that at 10th dpi the virus particles have already started to move down to the AS leaves through veins. Asymptotic (AS) leaves that were fully developed at the time of inoculation did not show any visible symptoms during the whole experiment.

Growth of the infected plants was inhibited and was evident from 14 dpi onwards. At late phase of the infection, i.e. from 21 dpi, PMMoV-S infected plants were totally senescent while PMMoV-I infected plants started to recover. Here, the new non-symptomatic leaves and elongation of internodia occurred. However, at this late phase of the infection, the selected AS leaves were not longer measured, because the presence of viral particles was already proved at 17 dpi in AS leaves in our previous study (see. Pineda *et al* 2008).

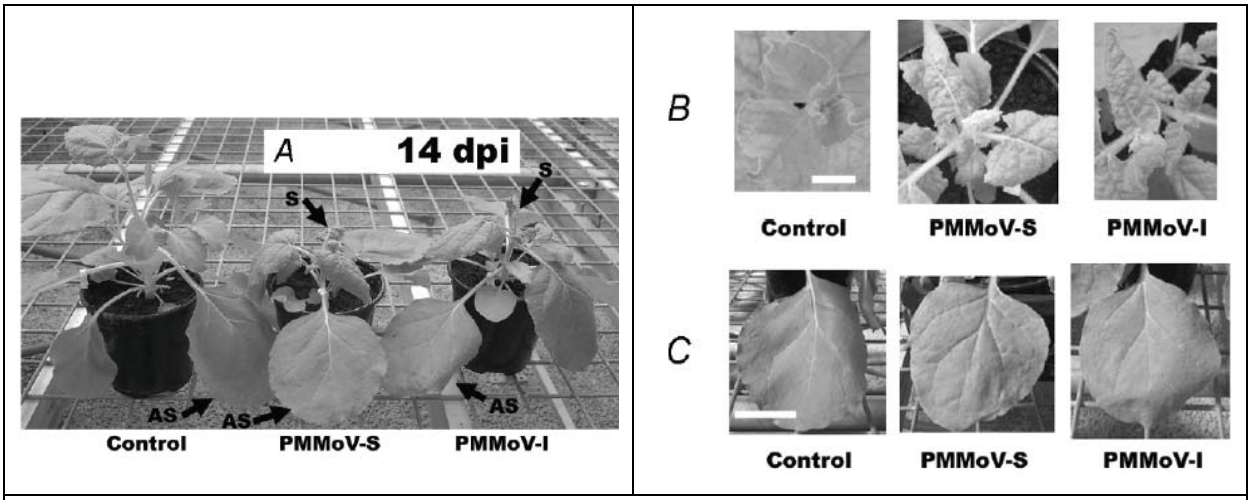


Fig. 1 Visual symptoms of PMMoV infection in Nicotiana benthamiana plant.(A). Representative images of control, PMMoV-S- and PMMoV-I-infected plants at 14 dpi. (B) Detail of the S (symptomatic) curled leaves at 7 dpi (size bar: 10 mm). (C) Detail of the AS (asymptomatic) leaves at 17 dpi (size bar: 40 mm). Adopted from Pineda et al. 2008

Chlorophyll fluorescence:

Based on our previous studies, we have analyzed variations of three standard Chl-F parameters that were identified as highly effective in detecting PMMoV infection in *N. benthamiana* to follow the symptoms movement. We focused on variations of F_V/F_M, Φ_{PSII}, and NPQ parameters. In parallel, we analyzed differences between Chl-F transients of control and infected leaves at different dpi.

Heterogeneous pattern of both F_V/F_M and Φ_{PSII} images was similar; however the differences between Ch-F intensity between the infected plant tissue surrounding the veins and interveinal area were enhanced in Φ_{PSII} images (Fig. 2). While control AS leaves showed homogeneous distribution of Φ_{PSII} over the leaf area up to 18 dpi (Fig.2. right panels), PMMoV infected leaves expressed high heterogeneity both in F_V/F_M and Φ_{PSII} distribution (Fig. 2, left and mid panels). This heterogeneity consisted of different F_V/F_M and Φ_{PSII} values in leaf tissue surrounding the main veins, where the infection was already present, and in the interveinal

tissue. The earliest changes in F_V/F_M and Φ_{PSII} patterns discriminating infected and non-infected tissue started to be detectable at 10 dpi at the youngest AS leaves, i.e. at the 8th leaves of both in PMMoV-S and PMMoV-I infected plants, and more pronounced at 12 dpi as shown in Fig. 2.

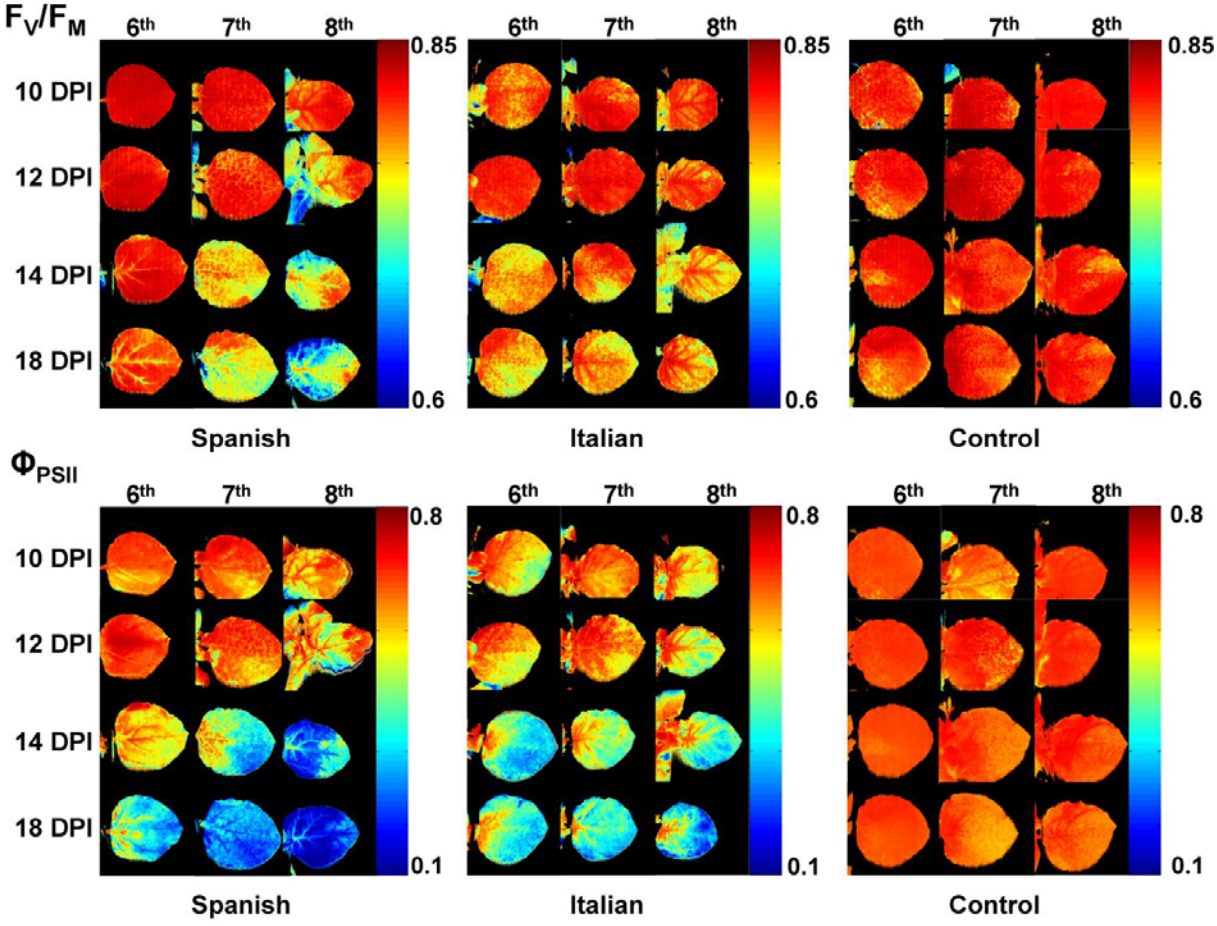


Fig. 2 F_V/F_M and Φ_{PSII} images of AS leaves differing in their age (6th were the oldest, 8th the youngest AS leaves) at different dpi. Φ_{PSII} was measured after 100 seconds of actinic irradiance (50μmol(photons).m^{-2}.s^{-1}). Images are shown for representative samples.

The symptoms at 7th leaves are time shifted and the heterogeneity of F_V/F_M and Φ_{PSII} distribution occurred at 14 dpi both in PMMoV-S and PMMoV-I infected plants. At 18 dpi, all measured infected leaves express the symptoms of PMMoV infection. PMMoV-S infected leaves expressed overall lower Φ_{PSII} value in 8th leaves at 14 dpi and in all AS leaves at 18 dpi compared to PMMoV-I infected plants, in accordance with higher virulence of PMMoV-S strain.

NPQ images of control and infected AS leaves at different dpi are shown in Fig. 3. Similarly to F_V/F_M and Φ_{PSII} images, NPQ heterogeneity caused by the virus infection appeared first at

12 dpi at the youngest 8th AS leaves followed by those at 7th leaves at 14 dpi. 6th leaves of PMMoV-I and PMMoV-S displayed heterogeneous NPQ distribution at 14 dpi and 18 dpi, respectively.

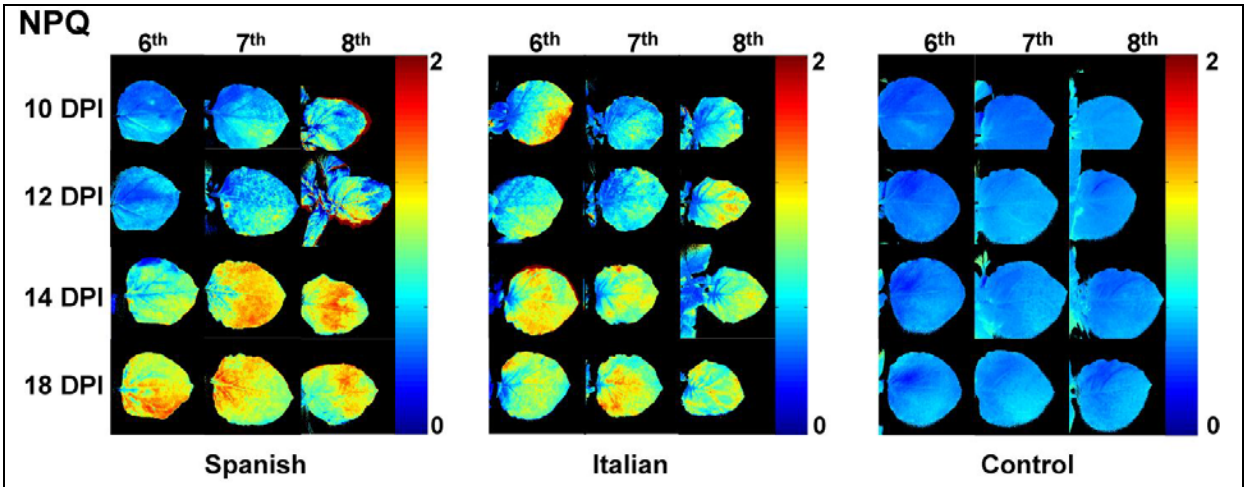


Fig. 3 Images of NPQ distribution over 6th, 7th and 8th AS leaves at different dpi. NPQ was measured after 100 seconds of actinic irradiance (50 μmol(photons).m^{-2}.s^{-1}). Images are shown for representative samples.

DISCUSSION:

Chl-FI has been broadly used cases to detect biotic/abiotic stress and map physiological background of infected plants (reviewed by Nedbal and Whitmarsh 2004). Earlier works mapping the plant-pathogen system PMMoV - *N. benthamiana* by Chl-FI revealed that the Chl-F signal heterogeneity over the leaf correspond with viral distribution in the leaves that, otherwise, remains asymptomatic during the viral infection (Chaerle *et al.* 2006, Pérez-Bueno *et al.* 2006, Pineda et al. 2008).

In this study, we used set of the most potent standard Chl-F parameters (NPQ, Φ_{PSII}, $F_V/F_{M,}$) to track movement of infection by PMMoV-I and PMMoV-S virus within *Nicotiana benthamiana* plants.

In agreement with Pérez-Bueno *et al.* (2006) and Pineda et al. (2008) these three Chl-F parameters were useful in detecting PMMoV-I infection in the AS leaf showing a characteristic Chl-F distribution pattern (Fig. 2 and 3). This pattern is better defined in PMMoV-S infected plants, correlating with the higher virulence of this viral strain.

The time shifted variations in Chl-F distribution in the *N. benthamiana* AS leaves differing in their age confirmed our hypothesis that the viral infection is spreading in descending order,

i.e. from youngest leaves to older ones. The first heterogeneity in Chl-F distribution occurred in the youngest 8th leaves at 10 dpi, more pronounced at 12 dpi. As the viral infection propagated lower to older leaves, the heterogeneity occurred in 7th and later in 6th AS leaves. At 18 dpi, the oldest 6th AS leaves expressed typical NPQ and Φ_{PSII} patterns that were already showed by Pérez-Bueno *et al. (*2006) and Pineda et al. (2008). This pattern corresponds to the viral location in these leaves at the same infection points, already tested by tissue print (Chaerle *et al.* 2006, Pérez-Bueno *et al.* 2006). Changes on the Chl-F pattern in infected leaves reflect a functional response to the infection rather than a viral-induced decrease of the leaf Chl content, as we previously demonstrated (Rahoutei *et al*. 2000, Chaerle *et al.* 2006).
In summary, images of Chl-F parameters provided information on the physiological processes under the viral infection. They are potent in the early detection of PMMoV infection in *N. benthamiana* plants and have also potential to distinguish between PMMoV-I and PMMoV-S infection.

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SUMMARY

In this work we dealt with different kinds of plant stresses. Namely: nutrient deficiency, temperature and irradiance changes during whole year, fungal and viral infection. The main aims were to detect the plant stress as early as possible and to find out if there exists any "universal" chlorophyll fluorescence parameter, which can be found in plant stress response and can be used as a main stress identifier, regardless if it has any physiological interpretation. In all studies we have used complex measuring protocol with two levels of actinic irradiance. For data evaluation we used standard empiric approach (searching for those standard chlorophyll fluorescence parameters that express high contrast between healthy and stresses plant tissue) and novel statistical approach (*combinatorial imaging*).

We found out that decrease of values of maximal an effective quantum yield of PSII and change of the reoxidation rate of plastoquinone pool expressed by the change of fluorescence decrease from F_P during fluorescence induction in actinic light are the main features of the plant-stress response. We also found out that usage of the advanced statistical approach for data analysis can lead to earlier stress identification, but obtained parameters are specific to particular host-pathogen system, cultivation conditions etc and could not be used in general.

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