Application and development of methods for recording fluorescence and reflection signals and images for plant physiology and quality

Ph.D. Thesis booklet

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Introduction

The fluorescence measurement allows a non-destructive study of the material. In the case of plants, it means typically the study of their leaves and fruits. These organs enhance the plant by the utilization of light energy through photochemical processes. The fluorescence light emission usually takes place simultaneously with the photochemical processes of the plant. By its measurement, one can draw conclusions about the plant's physiological functions. Furthermore, it has to be taken into the account, that depending on the excitation and detection wavebands and on the temporal course of the fluorescence measurement protocol, one can gather information about different parts, functionalities and processes of the plant. In my dissertation, I was studying the following phenomena:

Chlorophyll fluorescence

Upon visible excitation, the emitted light is restricted mainly to the waveband of 680-740 nm. The origin of this signal is the fluorescence emission of the chlorophyll molecules of the leaves. The chlorophyll molecules use the absorbed light for photosynthesis, whereas the photochemically unused excess energy is lost by non-radiative dissipation (heat evolution). Thus, the fluorescence light emission of the chlorophyll pigments is one of the complementary processes of the photochemical light usage. Accordingly, the photosynthetic efficiency influences significantly the chlorophyll fluorescence yield. If a plant specimen varies its fluorescence response only slightly and slowly to the changes of the light conditions, for example to an intensive illumination after dark-adaptation, it can be concluded that its photosynthetic activity is damaged or blocked. Hence, one can give estimation to the functionalities of the photosynthetic systems by the examination of the temporal changes of the fluorescence light using illumination conditions with well-defined magnitude and time-course.

UV-excited blue-green fluorescence

Upon UV (ultraviolet) excitation, the leaf emits radiation also in the blue-green range besides the chlorophyll fluorescence. However, the sources of this fluorescence light are not the chlorophyll pigments in the mesophyll, but some other substances in the leaf epidermis. Upon some plant stress conditions, through some processes in the epidermis, this range of the fluorescence can show changes even earlier than the fluorescence of the chlorophyll pigments located in the deeper layers of the leaf tissue.

Relationship of the fluorescence signals, measured at different excitation and detection wavebands

The comparison of the fluorescence signals excited at different wavebands gives information about the absorption of the epidermis, as there are several compounds in the epidermis which use the absorbed light energy not for fluorescence emission, but for some other processes (e.g. thermal radiation). The monitoring of the quantity and quality of these substances has a high importance.

Objectives

My key goal was to study the phenomena mentioned above as well as to define, realize and test novel protocols which give better results – from some aspects – against the existing fluorescence methods used to objectively measure the physiological state and stress conditions of plants.

I. It is known that for the examination of the photosynthesis in plants based on the chlorophyll fluorescence effects (e.g. Kautsky-, and PAM kinetic) several measurement protocols and instruments were developed. These illuminate the plant leaves with pre-defined light levels and utilize pre-defined protocols (e.g. continuous, pulsed or the combination of the two) after dark adaptation of 1 to 20 minutes. The photosynthetic capacity of a plant is obtained from the temporal variation of its fluorescence kinetics by taking several numerical points of the kinetics and calculating their ratios. My goal was to develop a method which illuminates the plant with an initial moderate dose, controllable level excitation so as to keep the resulting fluorescence constant. The variations in the excitation light and the signal of the uncontrolled fluorescence detection channel(s) show the adaptation capability of the leaf to this controlled light environment. I also aimed at testing the suggested protocol and comparing it with the above mentioned, known measurement protocols. The method was realized in a modular fluorometer (FMM, FluoroMeter Module) developed by Opti-Sens Co., Hungary in cooperation with BME in the framework of a GVOP 3.1.1 project (GVOP-3.3.1.-2004-04-0077/3.0, 2005-2007).

II. An opinion is evolved that by recording fluorescence, some biotic stress situations can be recognized earlier than the appearance of its visible symptoms. My aim was to develop the possibility of this testing opportunity and to study this field. I had to realize the ability of the study of the blue-green fluorescence imaging synchronized with the detection of the visual appearance. I implemented it on the fluorescence imaging system of the Botanical Institute of the Karlsruhe Institute of Technology (KIT). After this, I started the systematic research of the application potential of the fluorescence images, which required the automation of the system in order to increase the number of measurements. I chose the virus infection as the examined effect. I wanted to decide, whether the automated image acquisition was suitable to detect the virus infection pre-symptomatically based on UV excited blue-green fluorescence images remotely from a distance of 0.5 meter.

III. The role of the UV absorption layer is the protection of the physiological important leaf tissues below the epidermis from high UV radiation. Based on a simple model this can be analyzed by means of fluorescence rational values. I aimed at monitoring the temporal development of the UV absorption based on fluorescence rational images in the case of leaf samples exposed to different light conditions.

IV. My aim was to examine in detail the fluorescence signal and its variation during the ripening process in case of white grape fruit. I expected that the fluorescence imaging signals and its ratios can be linked to the accumulation of substances (phenols and sugar), which determine the fruit quality. The success with imaging equipment opens an exciting possibility for a non-invasive study of the fruits offering an important application potential in the viticulture industry.

Experimental techniques

For my first aim of researching a new fluorescence methodology, I used a custom designed, modular, portable plant fluorometer, which was developed in cooperation between the Opti-Sens Bt and the BME in a GVOP 3.1.1 project. This instrument – depending on the installed modules – has different excitation light sources (laser diodes and LEDs), photodiodes with different spectral filters and one PC/104 form-factor single board computer based control and data processing unit. I chose this instrument for my study, since the Kautsky kinetic measurement protocol had already been implemented in it, and its modular configuration and programming possibility allowed me the rapid development of the novel protocol. Furthermore, the testing and comparing of the protocols could be realized in an optically and instrumentally identical setup (same filters, detectors and light sources). Therefore, there was a difference only in the measurement procedure, which allowed an obvious comparison of the new (plant adapted excitation kinetic) and the former (Kautsky-type kinetic) protocol without disturbing technical issues.

For my fluorescence imaging studies, I used the customized fluorescence imaging system of the KIT. The system is based on a pulsed Xenon lamp (excitation light source) and on a synchronized, gated image intensified camera in combination with different waveband filters (detection unit). I further developed this system with an RGB video camera and with an automated plant rotary table to be able to compare the fluorescence detection method with the visual impression as well as to increase the number of measurements.

New scientific results

The main results of my PhD work are summarized in the following thesis points:

1.) I developed a new chlorophyll fluorescence method and a corresponding feedback technique realized in a fluorometer device for plant physiological studies. I recognized that, after a dark adaptation, forcing the leaf with a given, moderate light intensity to emit fluorescence and, subsequently, changing this excitation light reverse to the trend of the resulting fluorescence change, the temporal behavior of the excitation light gives information about the photosynthetic processes. The proposed feedback technique adapts the excitation light actively during the measurement to the actual photosynthetic capacity of the individual leaf sample. I also that the proposed method provides new, physiologically relevant information against the conventional techniques due to the fact its temporal kinetics is different from the inverse of the traditional Kautsky-curve and displays exceptional sensitivity to the differences between the two chlorophyll fluorescence wavebands. [8, 10, 11]

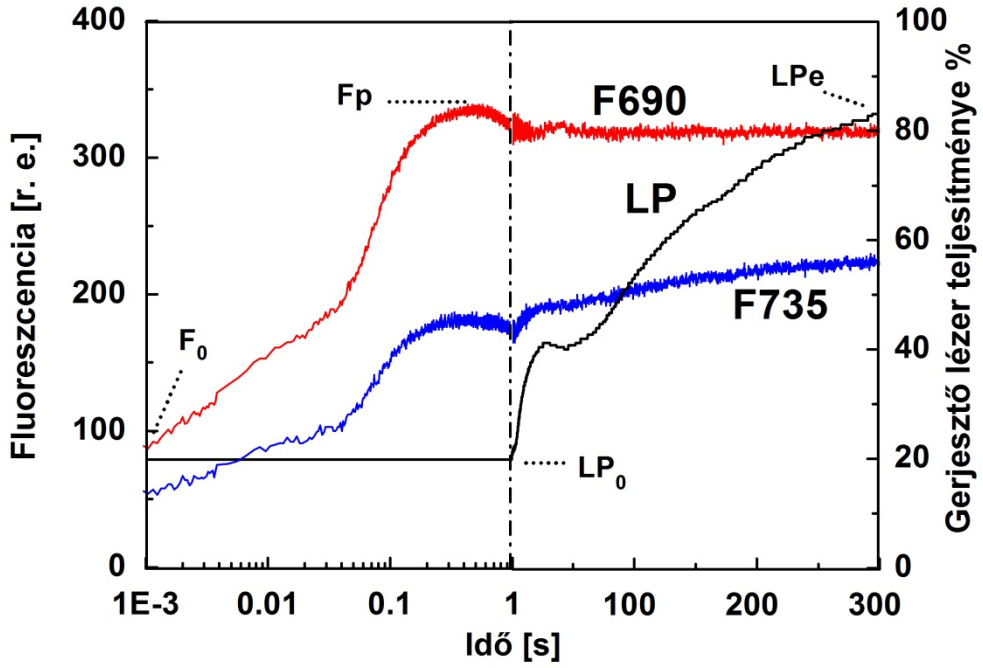

Figure 1.
Example for an excitation kinetics of the Chl fluorescence with a fully green dark adapted primary leaf of a barley seedling (*Hordeum vulgare* L., greening in the light for 48 hours) measured using the FluoroMeter Modul (FMM). It shows the fluorescence in the 690 nm (F690) and 735 nm (F735) bands as well as the LP during 300 s of illumination. The figure based on my publication [11].

2.) I verified that the early symptoms of the tobacco mosaic virus on the resistant cultivar of the tobacco can be recognized by UV excited blue-green fluorescence imaging technique before visual symptoms appear. I achieved this by further development of the hardware and the image pre-processing method of the custom fluorescence imaging system of KIT. I proved that the fluorescence method indicates the infection several hours before the appearance of visual symptoms depending on the time course of the hypersensitive response to the virus. I linked the recorded blue-green fluorescence increases to scopoletin accumulation and not to salicylic acid known to be responsible for the pre-symptomatic thermographic signal. [2, 4, 5]

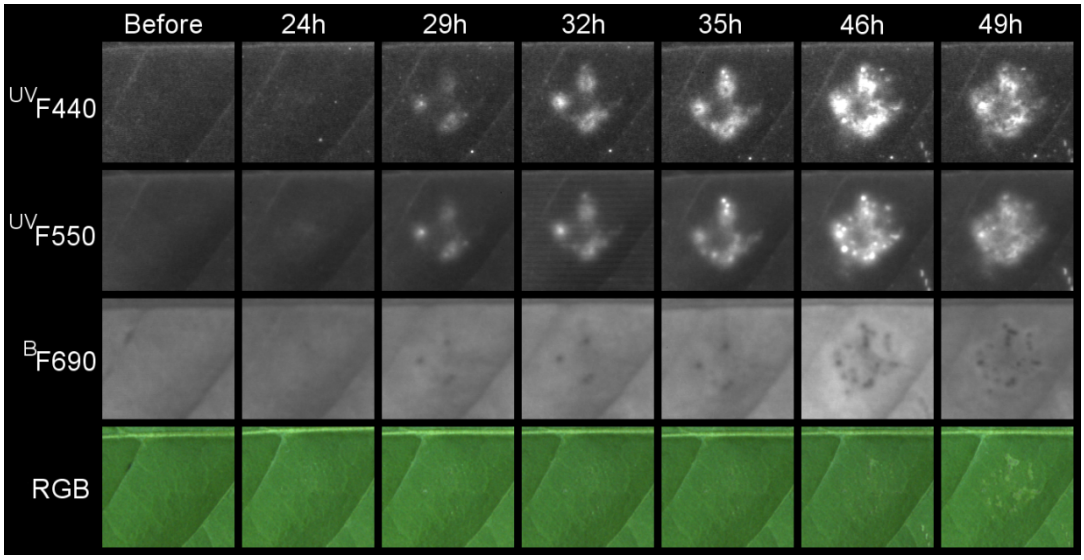

Figure 2.
Images of a tobacco (*Nicotiana tabacum* L.) leaf: before and 24 h up to 49 h after infection with tobacco mosaic virus. Rows 1 to 4, respectively: UV-excited blue fluorescence at 440 nm (^{UV}F440); UV excited green fluorescence at 550 nm (^{UV}F550); blue-excited Chl fluorescence at 690 nm (^{B}F690); video image taken by an RGB camera (= visual impression). The figure based on my publication [5].

3.) I recognized and proved that the image ratios of the blue- and UV-excited chlorophyll fluorescence are adequate to monitor the UV screening of the plant leaves or the whole plant cultivars in a non-invasive way from a remote distance of approximately 0.5 m. I successfully monitored the accumulation of the UV absorbing pigments of barley leaves, which reached its saturation 3 days after transferring the plant from the greenhouse to the outdoor conditions. I found that the carotenoid-mediated photoprotection increased parallel with the UV shielding, and the two processes are mutually dependent so that the resulting response of the assimilatory apparatus to increased UV and PAR radiations depends on the cooperative efficiency of both mechanisms. [3, 5, 7]

4.) I recognized that fluorescence imaging is a suitable method for qualifying grape. I found that, in the case of ripen grape berries (having sugar contents higher than 200 $g \cdot L^{-1}$), their UV screening and the concentrations of phenols, the latter influencing the fruit quality, can be estimated from the ratio of the blue to UV excited fluorescence images. I found a biphasic relationship between the corrected logarithmic parameter $-\log_{10}(({}^{UV}F690-S)/{}^{B}F690)$ and the relative contribution of the spectral compounds kaempferol and quercetin derivatives. I also showed, using the parameter ${}^{B}F740$, that simultaneously with the degradation of the chlorophylls, and thus with the decrease of the chlorophyll fluorescence, the sugar content of the berries increase, which actually occurs during the ripening process of the grapes. [5, 6]

Publications related to the thesis points

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Further publications

[12] P. Maák, **S. Lenk**, L. Jakab, A. Barócsi and P. Richter: Optimization of transducer configuration for bulk acousto-optic tunable filters. *Optics Communication* (2004) **241**, 87-98. IF: 1.581, number of independent citations: 5.

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