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Early detection of biotic and abiotic stress by kinetic imaging of chlorophyll fluorescence

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Introduction

Although most of the light captured by a plant is directed to photosynthetic energy conversion, a fraction of the absorbed energy is released as chlorophyll fluorescence emission. The energy wasted by chlorophyll fluorescence is only few percent of the absorbed flux. However, because the fluorescence competes with the photosynthetic reaction centers for excitation energy, the chlorophyll fluorescence emitted by a plant provides an effective monitor of photosynthetic activity. Typically, when photosynthesis in a plant is highly efficient, the fluorescence yield is low, whereas when the plant capacity to photosynthesize is saturated, e.g., in strong light, the fluorescence emission yield is high. Thus, measurements of chlorophyll fluorescence emission can be used to monitor non-invasively photochemical yields in plants. Over the past fifteen years the use of chlorophyll fluorescence to investigate plant performance in the lab and field has increased greatly largely due to the introduction of pulse-amplitude-modulated (PAM) instrumentation, which enables the determination of fluorescence yields in plants by measuring key fluorescence parameters (van Kooten and Snel 1990). These include F_0 and F_0', which monitor open reaction centers in Photosystem II representing the maximal photochemical yields in the dark and light-adapted plant states, respectively. F_M and F_M' monitor reaction centers of Photosystem II that are closed by a strong light pulse resulting in minimal photochemical yields. F_S is an instantaneous, steady state fluorescence yield in plants exposed to actinic light that represents an intermediate steady-state level of photochemical yields. The discoveries of the quantitative correlation between the fluorescence-derived parameters and the maximum ($\Phi_{PSII}^{max} \approx (F_M-F_0)/F_M$) and the instantaneous apparent ($\Phi_{PSII} \approx (F_M'-F_S)/F_M'$) quantum yields of Photosystem II provided a theoretical basis for interpreting fluorescence data and relating it to physiological processes as required by plant research and plant biotechnology (reviewed in Govindjee 1995).

The usefulness of chlorophyll fluorescence measurements was greatly enhanced by the introduction of kinetic imaging of chlorophyll fluorescence emission of plants by Omasa et al. 1987. Fluorescence imaging technology produces a two-dimensional map of fluorescence transients that reveals differences in photosynthetic activity over the plant surface. Chlorophyll fluorescence imaging is typically used to investigate variations in photosynthesis within a single leaf to identify regions of damage or between different plants or colonies to select for mutants.

Early studies of biotic stress using chlorophyll fluorescence imaging revealed heterogeneous photosynthetic activities over leaves that had been exposed to a pathogen. For example, heterogeneous fluorescence emission was reported in tobacco leaves and chloroplasts (*Nicotiana tabacum*) infected by mosaic virus TMV (Balachandran et al. 1994), chickpea leaves (*Cicer arietinum*) infected by the fungus *Ascochyta rabiei* (Esfeld et al. 1995), nutmeg cedar needles (*Torreya taxifolia*) with the fungus *Pestalotiopsis spp.* (Ning et al. 1995), bean leaves (*Phaseolus vulgaris*) invaded with rust *Uromyces appendiculatus* (Peterson and Aylor 1995), oat leaves (*Avena sativa*) infected by crown rust *Puccinia coronata* (Scholes and Rolfe 1996), hibiscus (*Hibiscus sabdariffa*) treated with 3 phytotoxins isolated from *Pestalotiopsis spp.* and one from *Drechslera triticirepensis* (Bowyer et al. 1998) and in *Abutilon striatum* leaves infected by mosaic virus AbMV (Osmond et al. 1998, Lohaus et al. 2000). Frequently, biotic stress can be detected by fluorescence emission heterogeneity before any symptoms are revealed visually.

The plant fluorescence emission is dominantly measured using rapid flash-modulated excitation from light emitting diodes, a method developed by Schreiber et al. 1986. Recently, we introduced the rapidly modulated measuring light in kinetic imaging fluorometry of plants in macroscopic (Nedbal et al. 2000a) as well as in microscopic (Küpper et al. 2000) range.

Here, we present two case studies demonstrating the capacity of the kinetic fluorescence imaging in the research of the biotic stress in plants. The imaging of F_0 and F_M is used to visualize the response to fungal toxins and to a fungal infection. In an additional case study, performance of the microscopic version of the imaging instrument is demonstrated in a measurement of Photosystem II activity during a heterocyst formation in a filamentous cyanobacterium. The fourth case study describes a new software technique for segmentation of the fluorescence images that is needed for automated screening operation of the imaging technique.

Materials and methods

We used commercial instruments (FluorCam, Photon Systems Instruments, Ltd., Brno, Czech Republic, http://www.psi.cz) to obtain images of PAM-standard fluorescence parameters (van Kooten and Snel 1990). Chlorophyll fluorescence was excited by measuring flashes lasting typically only 10-100 microseconds. The instrument software allowed generation of images showing pixel-to-pixel arithmetic operations. The technical details of the macroscopic version of FluorCam as well as of the measuring method are described in Nedbal et al. 2000a. The microscopic FluorCam version used for cyanobacteria is described in Küpper et al. 2000.
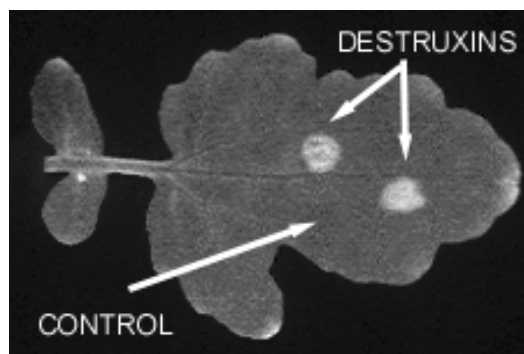
Results

Fluorometric visualization of the plant response to destruxins isolated from Alternaria brassicae.

Brassica blackspot (*Alternaria brassicae*, Berkley, Saccardo) is one of the most damaging fungal diseases of *Brassica* crops (Nacamura et al. 1995). The fungus is producing phytotoxic cyclodepsipeptides called destruxins. Destruxins cause chlorotic and necrotic foliar lesions on diverse *Brassica* species and other cruciferous host plants. Destruxin B, the major

phytotoxin of *A. brassicae*, appears to be the virulence factor, contributing most to the aggressivenes of *A. brassicae* by conditioning the host tissue. It inhibits vacuolar-type ATPase that is present in the membranes of many organelles with a low pH environment (Buchwaldt and Green 1992, Muroi et al. 1994, Pedras et al. 2001). Plants may be resistant to fungal infection if they produce phytoalexins and enzymes that catalyse detoxification of the phytotoxin. Unfortunately, the present *Brassica* crops are highly vulnerable to the infection and, thus, the efforts to produce resistant varieties of crops are of a high importance (Pedras et al. 2001).

The aim of our study was to develop a fluorescence imaging technique for screening of destruxin-resistant plants. In the experiments, we used leaf bioassay with the

Figure 1: The F_0/F_M fluorescence image of *Sinapis alba* leaf exposed for 60 hours to 10μl drop of 1% DMSO without destruxins (control) and with 2 g/l of destruxins. The fluorescence yield was measured using 30 μs flashes from orange light-emitting diodes. The shutter of the CCD camera was open only during the flashes. The F_0 signal was averaged from 10 measuring flashes given 200 ms apart. The F_M signal was measured by flashes given during a 1 s exposure to 2500 μmol (photons) $m^{-2}.s^{-1}$ of white light.

vulnerable *Brassica napus* and with the relatively resistant *Sinapis alba*. Leaves were incubated for 72 hours exposed to 100 μmol (photons).$m^{-2}.s^{-1}$ of white light at 22°C. The FluorCam instrument was used to measure in a single kinetic experiment the fluorescence emission images of F_0, $F_0^{'}$, F_M, $F_M^{'}$, F_S and to generate images showing various arithmetic combinations of the fluorescence images. Out of these combinations, the F_0/F_M ratio (Fig.1) was giving the highest contrast between the destruxin-exposed and untreated leaf surface in both *Sinapis alba* and *Brassica napus*. With a gradual lowering of the destruxin concentration we found that the fluorescence imaging is significantly more sensitive compared to optical microscopy. Detectable contrast between the destruxin treated and untreated *Brassica napus* leaf areas was observed with a concentration of 0.1 mg/l destruxin whereas the lowest concentration detectable with the optical microscopy was 15 mg/l using identical exposure protocols (Buchwaldt and Green 1992).

Chlorophyll fluorescence imaging of a ripe lemon fruit used to detect green mold infection.

Although most applications of imaging fluorometers measure chlorophyll fluorescence from green tissues that are high in chlorophyll content, the extraordinary sensitivity of current instruments enables measurements in non-green plant tissues that have a relatively low chlorophyll content (Nedbal et al. 2000b). This includes many types of ripening fruit that, during development, degrade the chloroplasts that are in the fruit skin. Even non-green fruit that are highly colored contain active chloroplasts that yield a chlorophyll fluorescence signal of sufficient strength that it can be used to probe photosynthetic activity in the fruit skin.

Fig. 2 shows the application of the FluorCam
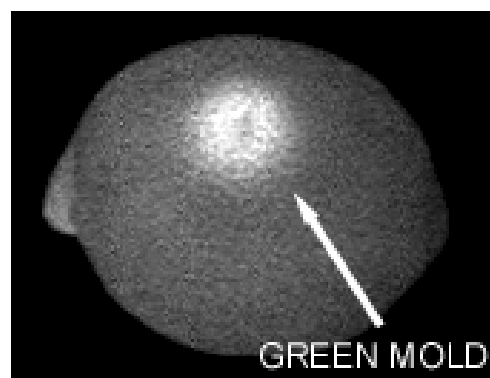
Figure 2: The F_0 fluorescence image of a ripe lemon fruit 50 hours after it was infected by green mold. Five measuring flashes lasting 30 microseconds each were given with a 200 ms period to generate the image.

(Nedbal et al. 2000a, Nedbal et al. 2000b) to monitor fluorescence emission parameters from lemons. We found that chlorophyll fluorescence from individual lemon fruits (*Citrus limon*) reveals that photosynthesis is active throughout the post-harvest ripening process. Furthermore, because photosynthesis is highly sensitive to biotic and abiotic stress, variations in chlorophyll fluorescence parameters over the surface of a lemon fruit can be used to predict areas that will eventually exhibit visible damage. For example, using images of chlorophyll fluorescence we were able to distinguish between mold-infected areas that eventually spread over the surface of the fruit, and damaged areas that do not increase in size during ripening. We infected a healthy lemon fruit by green mould (*Penicillium digitatum*) that is causing substantial post-harvest damage. The first visual signs of the infection-induced degradation appeared ca. 66 hours after the inoculation whereas the fluorescence signature of the infection was clearly seen already after 48 hours. Fig.2 demonstrates that the mold grow led to an elevated F_0 emission. The F_M emission was largely unaffected by the infection (Nedbal et al. 2000b). The highest contrast between the healthy and infected fruit skin was found in the F_0 / F_V ratio image.

The chlorophyll fluorescence imaging of lemons can serve as the basis for developing an automated machine that removes damaged or infected fruit long before visible symptoms appear. It is likely that this technique can be applied to other ripening fruits and vegetables (see, e.g., Gross and Flugel 1982, Minguez-Mosquera and Hornero-Mendez 1994, Minguez-Mosquera and Gallardo-Guerrero 1995, Smillie et al. 1999, DeEll and Toivonen 1999, DeEll and Toivonen 2000, Nedbal et al. 2000b).
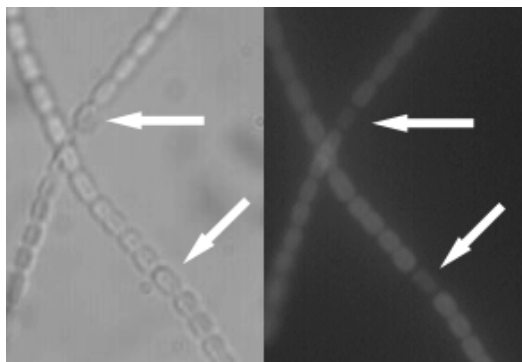
Figure 3: Anabaena filaments after 36 hours of nitrogen starvation with two heterocysts shown by white arrows. The left panel shows the transmission microscopy image and the right panel shows the F_M image.

Kinetic fluorescence imaging of filamentous cyanobacterium Anabaena *sp. strain PCC 7120 reveals a residual Photosystem II activity in young heterocysts.*

Progress of heterocyst formation induced by nitrogen starvation in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 was followed by the microscopic version of FluorCam (Küpper et al. 2000). Heterocysts are the sites of N_2 fixation providing a micro anaerobic environment for the O_2-labile nitrogenase (Adams and Duggan 1999).

Our data demonstrate that, after 36 hours of the nitrogen starvation, the Photosystem II activity in heterocysts was totally suppressed to avoid irreversible deactivation of nitrogenase by the photosynthetically produced oxygen. However, after 24 hours of nitrogen starvation, when the nitrogenase activity can already be detected in mature heterocysts, an unexpected residual activity of PS II was detected. The activity was measured as variable fluorescence ($F_V=F_M-F_0$) that was not much lower than the variable fluorescence in the neighboring vegetative cells within the same filament.

New software tool for image segmentation.

The kinetic fluorescence imaging of plants yields typically fluorescence transients of hundreds of thousands of picture elements (pixels). To obtain physiologically meaningful fluorescence kinetics of sufficiently high signal-to-noise ratio, it is necessary to dissect the image into segments of related fluorescence signatures. These can be individual plants, leaves or photosynthetically homogenous leaf segments as, for example, healthy and pathogen-

Figure 4: Fluorescence kinetics of overlapping plant leaves was measured using 10 μs measuring flashes. During the experiment 37 fluorescence images were captured including the F_0 and F_M emission in the dark-adapted state, the peak fluorescence F_P in a moderate actinic light and the maximal fluorescence F'_M in the light adapted state. The contrast between the neighboring leaves was varying throughout the measurements. All 37 images captured in the experiment were analyzed for gradient in fluorescence signals to find image segments (SG panel) that were enclosed by a steep gradient of the fluorescence signal. Similar but not identical segmentation was achieved when using changes in the kinetics of the individual pixels to find areas of similar or smoothly varying fluorescence signatures.

attacked leaf parts. New software to group neighboring pixels of similar fluorescence signatures is demonstrated here using a model canopy of overlapping plants (Fig.4). The software is able to dissect the image into individual plants and leaves using either gradient in the fluorescence intensity or change in the kinetics of the transients to find edges of leaves.

Discussion

The potential of the kinetic fluorescence imaging of plants has not yet been sufficiently exploited. We predict that the currently dominant application for screening of photosynthetic mutants will be, in a near future, supplemented by applications in the post-harvest quality assessment of fruits and vegetables and in water quality technologies. The selective application of pesticides in precision farming, mapping of algal growth on historical monuments or on-line monitoring of the plant photosynthetic activities in a heterogeneous field canopy represent examples of other potential applications.

Acknowledgements

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