LED Induced Chlorophyll Fluorescence Transient Imager for Measurements of Health and Stress Status of Whole Plants

H. Jalink[a] and R. van der Schoor
Wageningen UR Greenhouse Horticulture
PO Box 644, 6700 AP Wageningen
The Netherlands

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Abstract

We have developed LED (light emitting diode) induced fluorescence transient imaging instrumentation to image the plant health/stress status by calculation of two images: F_v/F_m (variable fluorescence over saturation level of fluorescence) and the time response, τ_{TR}, of the fluorescence time curve. Within a short time interval (≈580 ms) multiple images (typically 20) are captured using the LEDs in the pulsed mode. For each pixel of the fluorescence image F_v/F_m and τ_{TR} are calculated and presented as images that correlate with the quantum yield of PSII photochemistry and the time response of this process, respectively. The advantage of the technology lies in the imaging of photosynthetic parameters within a short time interval, remotely and under light conditions. This was accomplished by the development of a high intensity pulsed LED light source (total 5 kW electrical power) and using the LEDs in the pulsed mode with a pulse width of 15 ms and time between sequential pulses of 14 ms. Using this instrumentation we investigated the effect of herbicide treatment, Sencor, on black nightshade (*Solanum nigrum* L.) plants. Effects of the herbicide on the first fluorescence images could be detected. At the saturation level of the fluorescence this effect disappeared. The effect of the herbicide was visualized on the F_v/F_m image and the time response τ_{TR} image. Healthy and herbicide treated parts of the plant yielded average values of F_v/F_m=0.81±0.03 and 0.06±0.02, respectively. Furthermore, the effect of drought stress was investigated on saintpaulia (*Saintpaulia ionantha*) plants. Under dark conditions no differences in the image of F_v/F_m and τ_{TR} could be detected between the control and the plant with drought stress. Under actinic light of 90 µmol m^{-2} s^{-1} differences were observed in images of $(F_m'-F')/F_m'$ and τ_{TR}'. We conclude that for the first time images of a time response of the photosynthesis of leaves are presented. Furthermore, the proposed instrumentation can be used for high throughput screening, as a sensor in sorting machines and has potential greenhouse applications.

INTRODUCTION

Measurement of chlorophyll fluorescence provides a non-invasive technique to screen the photosynthetic apparatus of plants. Studies of biotic stress using thermal and chlorophyll fluorescence imaging showed heterogeneous responses over the leaf exposed to a pathogen (Chaerle et al., 2007). Several chlorophyll fluorescence imaging systems exist: Imaging PAM (Schreiber et al., 2007), FluorCam (Nedbal et al., 2000) and CF Imager (Lawson et al., 2002), all of them based on rapid flash-modulated excitation originally developed by Schreiber et al. (1986). Flashes of light emitting diodes are used with pulse duration in the µs range and low light intensity, typically <0.5 µmol m^{-2} s^{-1}, too low to enhance the photosynthesis. Actinic light is provided by a second light source. Another methodology to study the photosynthetic apparatus is the fast repetition rate fluorometer, FRRF (Kolber et al., 1998). This instrument performs a spot measurement (non-imaging) using sequential 1 µs pulses at sub-saturating light level to excite chlorophyll-a fluorescence to achieve F_m by driving the yield of PSII photochemistry close to zero. Here we present an imager that in principle is based on the FRRF

[a] henk.jalink@wur.nl

methodology with sub-saturating pulses but now with light pulses of longer duration. It can image whole plants and two images are derived from the fluorescence time response of the multiple fluorescence images at the sequential light pulses, F_v/F_m and τ_{TR} that correlate with the efficiency of photosynthesis and the time response of the photosynthetic system, respectively. The methodology is based on multiple sequential light pulses with variable pulse duration (typically 15 ms) and time between subsequent pulses (typically 14 ms). Here we report the first preliminary details of this instrument and its application to measure the effect of an herbicide treatment and drought stress on plants.

MATERIALS AND METHODS

Plant Material

Plants of black nightschade (*Solanum nigrum* L.) and saintpaulia (*Saintpaulia ionantha*) were used for this study. Black nightshade plants were grown in 11-cm diameter pots filled with a mixture of sand and humus potting soil (1:2 by volume). The pots were placed on sub-irrigation matting, which was wetted daily with half strength nutrient solution. The plants were grown in a growth chamber under 14 h of light at 18/12°C (day/night) temperature and 70/80% (day/night) relative humidity. Light was provided using high-pressure mercury lamps to give 70 μmol m^{-2} s^{-1} at leaf level. The saintpaulia plants were bought at a local plant shop. The flowers were removed, because we were only interested in the photosynthetic response of the leaves.

Treatments

1. Herbicide. The black nightshade plant was treated with Sencor. On one leaf multiple droplets of 2 μl were applied. Another leaf was spot treated with a higher amount of the herbicide (5 μl). The black nightshade plant was measured 6 h after the herbicide treatment.

2. Drought. For the drought stress treatment the plants were not watered for a week and subjected to normal day light conditions and room temperature. The control plants were watered daily with sufficient water.

Chlorophyll Fluorescence Imaging

The LED induced fluorescence transient imager consists of four major components: array of LEDs, CCD camera, LED power supply and a computer. In total 40 LEDs were mounted in good thermal contact on a 30×30 cm^2 aluminum plate of 1 cm in thickness. Two sets of 20 LEDs are electrically connected in series and driven by two identical in-house-built power supplies that control the pulse width and current through the LEDs. A square wave pulse with a width of 15 ms and time between sequential pulses of 14 ms was used resulting in a duty cycle of 15/29=0.52. The LEDs had a center wavelength of 620 nm and the light from the LEDs was non-collimated (each LED: fwhm at a total angle of 120°). This resulted at plant level, at 40 cm from the lens, in an illuminated area of more than a square meter. However, over an area of 30×30 cm^2 an intensity of 1000 μmol m^{-2} s^{-1} was provided. Using the LEDs for 580 ms in the given pulsed mode did not result in a significant increase in temperature of the aluminium plate. The increase in temperature after 20 cycles of 20 pulses (in total 400 pulses) was 2.9°C. The expected life span of the LEDs in this configuration is longer than in the continuous non-pulsed mode since from our design the currents in the pulsed mode do not exceed the specified currents from the factory datasheet for the continuous mode and the temperature rise in the LEDs is for one cycle of 20 pulses very low. In the center of the aluminum plate a hole of 75 mm in diameter was made where the optical lens (f=8 mm) of the CCD camera is located. An interference filter (730 nm, fwhm=10 nm) is mounted between the lens and the CCD-chip of the camera. The CCD camera is of an Electron Multiplier CCD type with 640×480 pixels and was used in the 2×2 binning mode for higher sensitivity and higher frame rate (Hamamatsu C9100-01). The gain factor was set at 50 (on a 0-255

scale with 0 meaning no amplification). The LED power supply and camera were controlled using software written in Delphi. Images were captured in darkness or in light conditions by illuminating the plants with LED lamps (RGB type) with an intensity at leaf level of 90 μmol m^{-2} s^{-1}. Duration of light adaptation of the plant to darkness or light conditions was at least 5 min.

Measuring Protocol and Data Handling

First the dark image is stored into the computer. This image is captured by using the same protocol for fluorescence imaging, but now for one image instead of multiple images with the LEDs turned off. During each pulse of the LEDs the image is captured by the camera and directly transferred to the computer. This is a 320×240 pixel image with 14 bit grey values. After the sequence of all the images, typically 20, the dark image is subtracted from the fluorescence images. This yields images for which each pixel contains fluorescence time information (Fig. 1). The first value at t=0 corresponds to the initial, F, fluorescence signal. The maximum value where the curve saturates corresponds to F_m. For the FRRF methodology the fluorescence transient, F(t), can be formally expressed by a function: $F(t)=F+(F_m-F)\{C(t)(1-p)/(1-C(t)p)\}$. Where C(t) is the fraction of closed PSII reaction centers, $0<C(t)<1$, and p is the extent of energy transfer between photosynthetic reaction centers (Kolber et al., 1998). This polyphasic process is mainly determined by a fast photochemical phase that can be completed within several ms and a slower thermal phase (Schreiber and Krieger, 1996; Heredia and De Las Rivas, 2003). Since our system mainly probes the thermal phase due to the larger width of the light pulses and for practical considerations to reduce the number of fitting parameters, the final term with the level of reduction and extent of energy transfer is replaced by an exponential function with one time constant τ_{TR}: $C(t)(1-p)/(1-C(t)p)\approx 1-\exp(-t/\tau_{TR})$. Each pixel is fitted with the exponential expression: $F(t)=F+(F_m-F)(1-\exp(-t/\tau_{TR}))$. An estimation of the maximal quantum yield of PSII photochemistry can be calculated from $\Phi_P=F_v/F_m=(F_m-F_0)/F_m$. Here F_0 denotes the minimal fluorescence at t=0 for dark adapted plants. This calculation is performed for each pixel yielding an image for the estimation of the yield of PSII photochemistry, F_v/F_m, and an image that corresponds with the time response, τ_{TR}, of the photosynthetic system under these experimental conditions. Under actinic illumination the same fitting procedure is followed. In this case the effective quantum yield of PSII photochemistry can be approximated as $\Phi_P(t)=(F_m'-F')/F_m'$, where F_m' and F' denote the maximum value where the curve saturates and the initial fluorescence at t=0, respectively. Under actinic illumination conditions an image that corresponds with the time response, τ_{TR}', can be calculated also. Fluorescence parameters that were measured or calculated under actinic illumination are denoted with a prime.

RESULTS AND DISCUSSION

The first six fluorescence images showed clearly the spots that were treated with herbicide (Fig. 1). The treated spots showed an elevated fluorescence compared with the rest of the leaf. This was due to the inhibition of photosynthesis by the herbicide yielding a maximal fluorescence. After these six images the fluorescence of the untreated leaves increased, resulting in the disappearance of contrast between the treated spots and the non-treated parts. This demonstrates that the LED illuminator is able to saturate the photosynthesis. After 20 sequential pulses (Fig. 2), resulting in a total measuring time of 20×29=580 ms to obtain the necessary saturation level, the measured fluorescence transient F(t) reaches its horizontal asymptote, F_m. A good fit, $r^2=0.973$, is obtained for the exponential expression resulting in an average $F_0=1109$, $F_m=3384$, $\Phi_P=F_v/F_m=0.672$ and $\tau_{TR}=151$ ms. The F_v/F_m image (Fig. 3) shows where the plant is still in good condition. Here the plant is colored false with green. Locations 5, 6, 7 and 8 yielded an average value of $F_v/F_m=0.81\pm0.03$. Where the plant has been treated with herbicide and how the herbicide has spread through the leaf is visualized by a yellow/red/black false color. These parts show a lower value for the variable fluorescence (location 2, 3 and 4 of Table 1). For very low F_v/F_m, location 3, τ_{TR} value is not substantially different from the

healthy locations 1, 5, 6, 7 and 8. However, for low values of F_v/F_m, location 2 and 4, τ_{TR} is higher than that of the healthy locations. The image of τ_{TR} shows about the same features as the F_v/F_m, but with less detail. The individual spots are not as clear and the transport of the herbicide through the vessels cannot be visualized in great detail as observed in the F_v/F_m image.

From the image of F_v/F_m and τ_{TR} of the dark adapted saintpaulia plants no conclusion could be derived which plant was stressed by the drought treatment and which plant was watered sufficiently (Fig. 4A,B). Increasing the actinic intensity from zero to 90 μmol m^{-2} s^{-1} showed a small decrease in $(F_m'-F')/F_m'$ of the control plants, but a larger decrease for the drought treated plants (Table 2). The time response for the control plants showed a decrease and for the drought treated plants an increase at increasing actinic light level. At the used experimental set up the maximum achievable actinic light intensity was 90 μmol m^{-2} s^{-1}. $(F_m'-F')/F_m'$ and τ_{TR}', of drought stressed plants at these conditions were respectively eight times lower and five greater than that of control plants. It is expected that an optimum contrast between the two treatments exists as a function of the actinic light intensity. For intensities higher than this optimum value the difference between the two treatments on $(F_m'-F')/F_m'$ and τ_{TR}' will decrease. For dark adapted plants all the reaction centers are open. Performing a measurement in the dark with the described set-up lead to the closure of the reaction centers for both treatments at about the same F_v/F_m values and time responses. Under steady state illumination the electron transport, ETR, of the photosynthetic apparatus is at a constant level. The relative rate of PSII related electron transport can be calculated as $rETR=E_0\ \Phi_P(t)$ with E_0 the irradiance (PAR) (Grunwald and Kühl, 2004). Both plants were exposed to the same actinic illumination. Calculation of rETR resulted for control plants in a factor of eight higher electron transport rate than that of drought stressed plants. For the τ_{TR}' image almost the whole image of the drought stressed plant showed a uniform green false color. The τ_{TR}' image of the control plant showed mainly red and black false colors. Here a green color stands for a slow time response, red being faster and black even faster than red. This implicates that the reaction centers that are open under these irradiance conditions closed for control plants at a faster rate than that for drought stressed plants.

F_v/F_m is calculated from fluorescence intensities of the initial fluorescence value, F, and the maximum value, F_m, where the transient curve saturates. The time response depends on how fast the photosynthetic apparatus is saturated. Therefore, from a mathematical point of view these two parameters F_v/F_m and τ_{TR} are independent of each other. It was observed that for small stresses like drought and salinity (non-published data) the time response increased significantly while the value of F_v/F_m decreased slightly. This can be explained as follows. At the onset of a stress the value of F and F_m will change slightly. The saturation value of the time response curve will still reach its maximum value, but more pulses are needed to complete this process. This results in a larger value for τ_{TR}, but F_v/F_m will almost remain constant. Large stresses on the photosynthetic apparatus, like herbicide treatments with Sencor, will have larger effects on F_v/F_m, because F will increase and F_m will decrease substantially. This results in a small value for $(F_m-F)/F_m$. The value of τ_{TR} will initially increase but at larger stresses suddenly decrease, location 3 Table 1, because the overall photosynthetic time response at our experimental conditions consists of a fast time response which is in the order of several ms (first pulse in Fig. 2) and a slower time response in the order of 10-100 ms. For the investigated plant material we have observed a fast rise during the first pulse of the fluorescence transient curve and a slower increase in fluorescence for the remaining pulses. With the value of the slower time response approaching infinite due to the stress the fluorescence curve will show a flat behavior for pulse number larger than the first. Remains a fast increase from the initial F_0 value until F_m within the first pulse (unpublished data). This phenomenon will be part of future studies.

CONCLUSIONS

The LED Induced Fluorescence Transient Imager was able to capture multiple

fluorescence images. After sequential pulses of the LEDs the photosynthetic apparatus could be saturated. From these images F_v/F_m and τ_{TR} were calculated. Its application was demonstrated on the effect of the herbicide Sencor on the photosynthetic apparatus on a whole plant level and within a measuring time of 0.6 s. Images of F_v/F_m and τ_{TR} showed the effect of the herbicide on the variable fluorescence and the time response of the photosynthesis. Drought stress of saintpaulia plant was not detected for dark adapted plants but after steady state actinic light illumination of 90 μmol m^{-2} s^{-1}. The variable fluorescence over saturation level of fluorescence, $(F_m'-F')/F_m'$, and the time response of drought stressed plants were respectively eight times lower and five greater than that of control plants. For the first time images of a time response of the photosynthesis of leaves was presented. Furthermore, the proposed instrumentation can be used for high throughput screening, as a sensor in sorting machines and has potential greenhouse applications.

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Tables

Table 1. Overview of F_v/F_m and τ_{TR} for locations given in Figure 3C. The values are calculated averages from 3×3 pixels with standard deviation from five neighboring positions at the specified location.

Location	F_v/F_m	τ_{TR} in ms
1	0.76±0.002	127±2
2	0.18±0.01	235±11
3	0.06±0.02	184±12
4	0.26±0.01	377±40
5	0.85±0.01	183±10
6	0.82±0.01	156±6
7	0.76±0.01	110±4
8	0.81±0.01	149±9

Table 2. Overview of F_v/F_m, $(F_m'-F')/F_m'$, τ_{TR} and τ_{TR}' for locations given in Figure 4. The values are calculated averages from 3×3 pixels with standard deviation from five neighboring positions at the specified location.

Location	Dark		Light 90 µmol m^{-2} s^{-1}	
	F_v/F_m	τ_{TR} in ms	$(F_m'-F')/F_m'$	τ_{TR}' in ms
1 drought	0.26±0.01	113±7	0.03±0.02	122±39
2 drought	0.41±0.02	53±7	0.02±0.01	132±31
3 drought	0.38±0.01	62±3	0.02±0.01	223±167
4 control	0.29±0.02	105±2	0.21±0.01	42±3
5 control	0.44±0.01	53±2	0.19±0.02	18±5
6 control	0.37±0.01	105±6	0.16±0.01	28±9

Figures

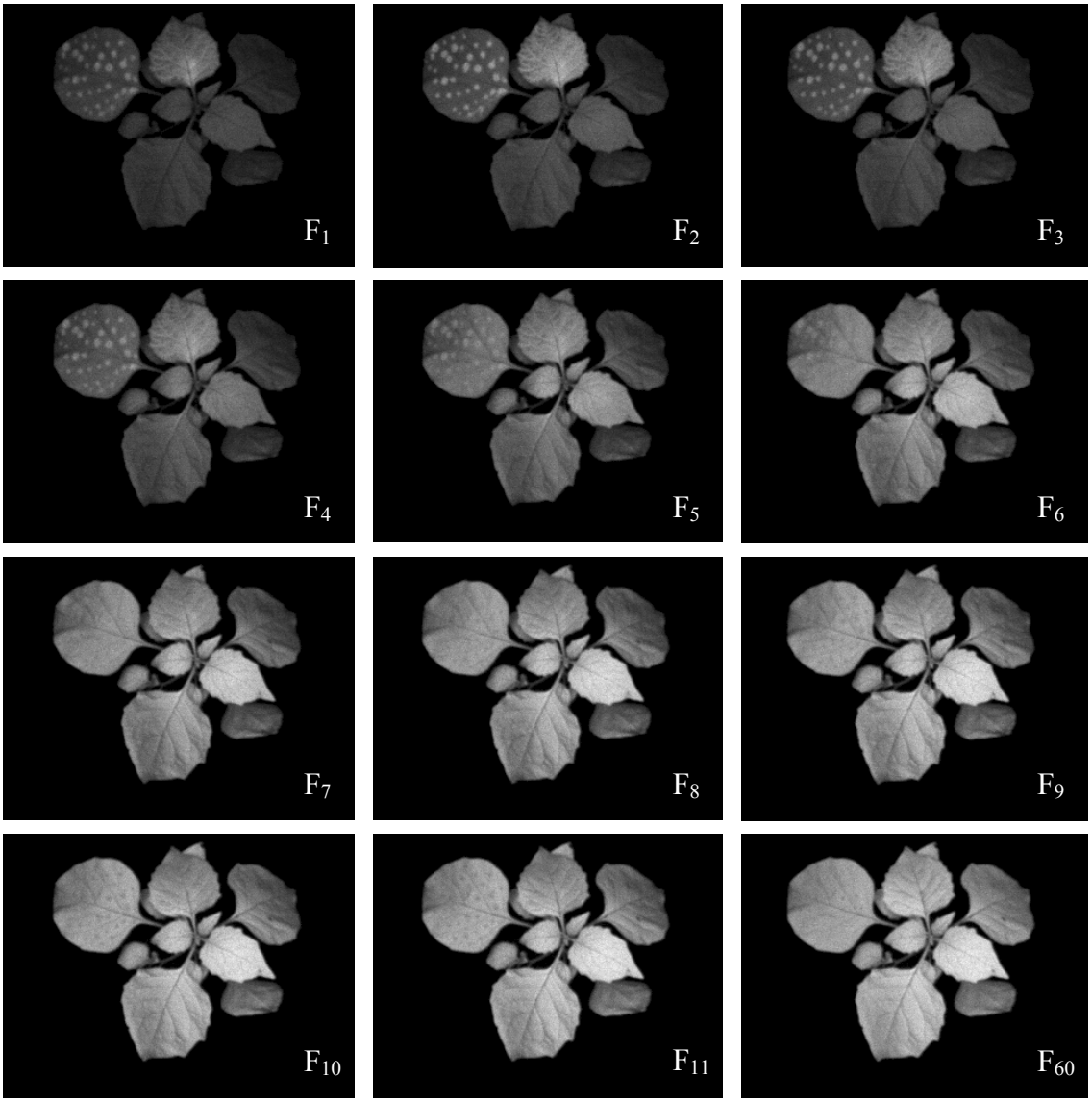

Fig. 1. Fluorescence images, F_i, of a dark-adapted black nightshade plant that has been locally treated with the herbicide Sencor 6 h before. The index i of each image corresponds with the pulse number.

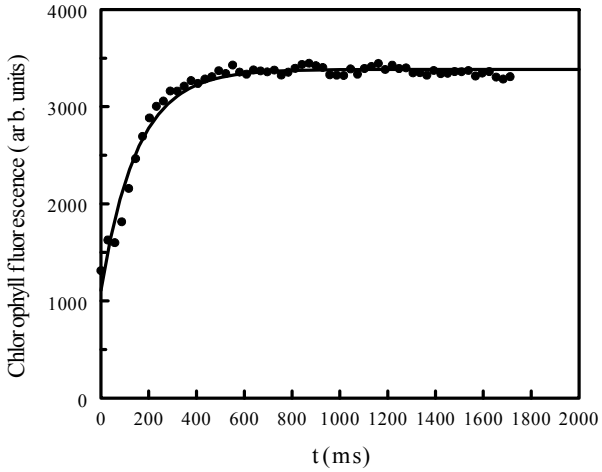

Fig. 2. Measured fluorescence transient, F(t), with fitting curve $F(t)=F+(F_m-F)(1-\exp(-t/\tau_{TR}))$. For a partially herbicide treated black nightshade plant with fitted curve parameters: F_0=1109, F_m=3384 and τ_{TR}=151 ms, yielding for F_v/F_m=0.672 (r^2=0.973).

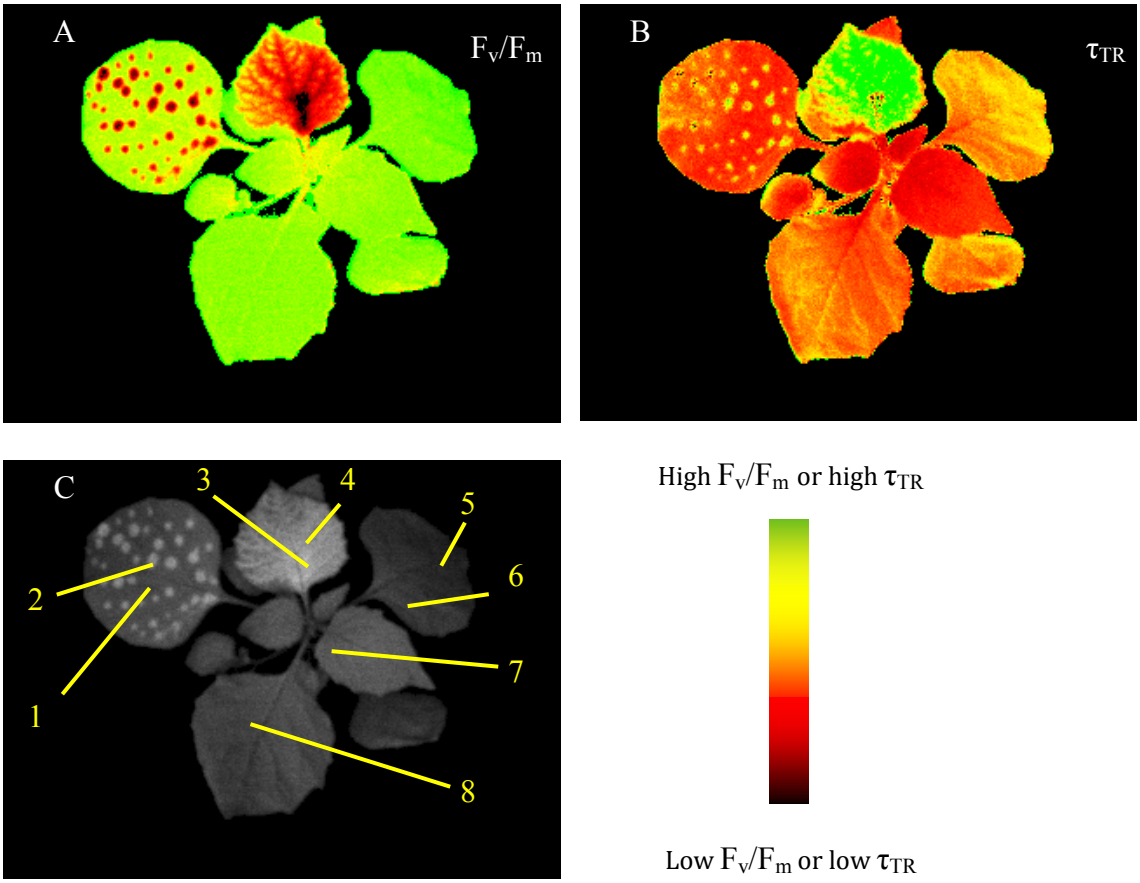

Fig. 3. Images of F_v/F_m (A) and τ_{TR} (B) derived from the 60 fluorescence images of Figure 1 and fitting for each pixel the equation: $F(t)=F+(F_m-F)(1-\exp(-t/\tau_{TR}))$. To enhance the contrast the used false color tables are different for each image. The fluorescence image in panel C shows the location of the fitted parameters of Table 1.

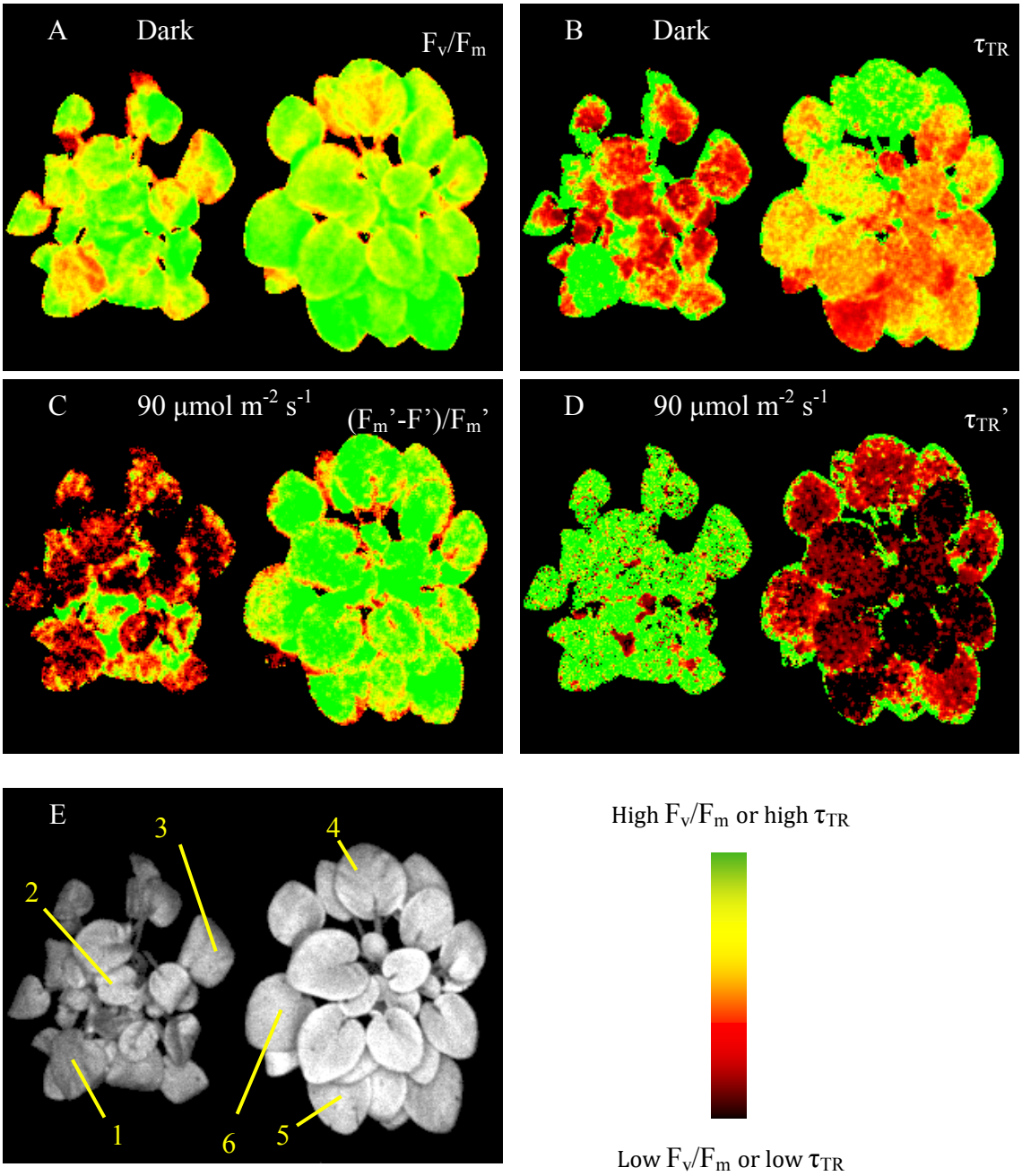

Fig. 4. False color images of saintpaulia plants (left with drought stress, right control) F_v/F_m (A in the dark), $(F_m'-F')/F_m'$ (C in actinic light), τ_{TR} (B in the dark) and τ_{TR}' (D in actinic light) derived from the 30 fluorescence images and fitting for each pixel the equation: $F(t)=F+(F_m-F)(1-\exp(-t/\tau_{TR}))$. To enhance the contrast the used false color tables are different for each image. The fluorescence image in panel E shows the location of the fitted parameters of Table 2.