Black leaf-clips increased minimum fluorescence emission in clipped leaves exposed to high solar radiation during dark adaptation

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Abstract

Tomato and pepper leaves were clipped with black leaf clips for dark adaptation under solar radiation in the late spring or early summer 2010 in southern Italy. The leaves showed highly variable maximum PSII quantum yield (F_v/F_m = 0.026–0.802) using a continuous-excitation fluorometer *Pocket PEA*. These results were confirmed using the modulated fluorometer *FMS1* on tomato leaves in mid summer, with F_v/F_m as low as 0.222 ± 0.277 due to nearly equal minimum (F_o) and maximum (F_m) fluorescence emission. A significant clip effect on F_v/F_m occurred after only 12 (tomato) or 25 (pepper) min. Increasing the leaf temperature from 25 to 50°C reportedly induced an F_o increase and F_m decrease so that F_v/F_m approached zero. The hypothesis that black leaf clips overheated under intense solar irradiance was verified by shrouding the clipped leaves with aluminum foil. In clipped leaves of pepper, F_v/F_m with the black clip/Pocket-PEA was 0.769 ± 0.025 (shrouded) and as low as 0.271 ± 0.163 (nonshrouded), the latter showing a double F_o and 32% lower F_m. An 8% clip effect on F_v/F_m was observed with the white clip/*FMS1*. To avoid the clip effect in high irradiance environments, F_v/F_m measurements with black clip/*Pocket PEA* system required leaf dark adaptation with radiation-reflecting shrouds. It would be useful if manufacturing companies could develop better radiation-reflecting leaf clips for the Pocket PEA fluorometer.

Additional key words: leaf-clip effect; pepper; photochemical efficiency; radiation; temperature; tomato.

Introduction

Chlorophyll *a* (Chl *a*) fluorescence induction (FI) is a powerful tool to investigate photosynthesis (Lazár 1999, 2006). The maximum quantum yield of photosystem II (PSII) photochemistry is calculated as F_v/F_m, where the variable fluorescence (F_v) is the difference between the maximum (F_m) and minimum (F_o) fluorescence emission in dark-adapted leaves (Kitajima and Butler 1975). In light-adapted leaves, the effective (or actual) quantum yield [$(F_m' - F')/F_m' = \Delta F'/F_m'$; Genty *et al.* 1989] and the maximum (or potential) quantum yield [$F_v'/F_m' = (F_m' - F_o')/F_m'$; van Kooten and Snel 1990] are determined from the steady-state (F'), and the maximum (F_m') and minimum (F_o') fluorescence emission. The latter is obtained by a few seconds dark application of a far red light that excites photosystem I (PSI) preferentially, favouring the oxidation of plastoquinone and primary

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Abbreviations: Chl *a* – chlorophyll *a*; F' – Chl *a* fluorescence emission of leaves at steady-state under actinic light; F_m – maximum Chl *a* fluorescence emission in dark-adapted leaves; F_m' – maximum Chl *a* fluorescence emission in light-adapted leaves; *FMS1* – fluorescence monitoring system fluorometer version 1 or subscript of a fluorescence parameter obtained with an *FMS1* fluorometer; F_o – minimum Chl *a* fluorescence emission in dark–adapted leaves; F_o' – minimum Chl *a* fluorescence emission in light–adapted leaves; F_p – peak Chl *a* fluorescence emission induced by nonsaturating light; FI – fluorescence induction; FTC – fluorescence temperature curve; F_v – variable fluorescence in dark-adapted leaves; F_v' – variable fluorescence in light-adapted leaves; F_v/F_m – maximum quantum yield of PSII photochemistry in dark-adapted leaves; F_v'/F_m' – maximum quantum yield of PSII photochemistry in light-adapted leaves; PAR – photosynthetically active radiation; PEA – plant efficiency analyser fluorometer; PPEA – Pocket PEA fluorometer or subscript of a fluorescence parameter obtained with a PPEA fluorometer; PSII – photosystem II; RC – reaction centres of photosystems; R_g – global radiation; SD – standard deviation; T_c – temperature at start of F_o sharp increase in FTC; T_m – temperature of maximum F_o in FTC; $\Delta F'/F_m'$ – effective quantum yield of PSII photochemistry in light-adapted leaves.

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quinone acceptor of PSII. There are several reviews on FI methodologies (*e.g.* Roháček and Barták 1999, Lazár 1999, 2006; Maxwell and Johnson 2000, Strasser *et al.* 2000, 2004; Baker 2008). Briefly, continuous-excitation fluorometers detect transient light-induced O–J–I–P fluorescence at very short intervals, *e.g.* 10 μs (Strasser *et al.* 2000). Pulse amplitude modulated fluorometers detect fluorescence emission for a low intensity modulated (measuring) nonactinic light that induces a modulated fluorescence emission (Ögren and Baker 1985, Schreiber 1986). However, recent modulated fluorometers (*e.g.* *PAM 2500*, *Heinz Walz, GmbH*, Effeltrich, Germany) are also capable of 10 μs detection interval.

This study measures F_v/F_m, and therefore F_o and F_m, in relation to the leaf-clip used for dark adaption with the (continuous-excitation) *Pocket PEA* or the (modulated) *FMS1* fluorometer, both produced by *Hansatech Instruments, Ltd*. (King's Lynn, Norfolk, England). To speed up sampling of dark-adapted leaves, *Hansatech* has developed light-weight plastic clips, here referred to as *FMS-Type* and *PEA-Type* (Fig. 1). Both types are ~6 g with two arms (upper and bottom) held together by a pivot spring. The bottom arm has a foam pad that adheres to the leaf surface. The upper one has a locating ring (30 mm diameter) with a central circular hole of 7 (FMS-clip) or 4 (PEA-clip) mm diameter (Fig. 1) that can be covered by a metallic (radiation-reflecting) shutter blade during dark adaptation. To minimize the effects of heat build-up on the leaf during dark adaptation, the plastic is almost all white in the discontinued earlier PEA model, the *Handy PEA* fluorometer (not used here), and the FMS fluorometer (*Hansatech Instruments* 1999, 2006). Conversely, the black locating ring in the *Pocket PEA* clip (Fig. 1) assures that measurement is unaffected under high ambient light intensity (*Hansatech Instruments* 2006). The leaf clips are specific for each model of fluorometer with the exception of the *FMS1*, which shares the same leaf clip with the later *FMS2* model (*Hansatech Instruments* pers. comm.).

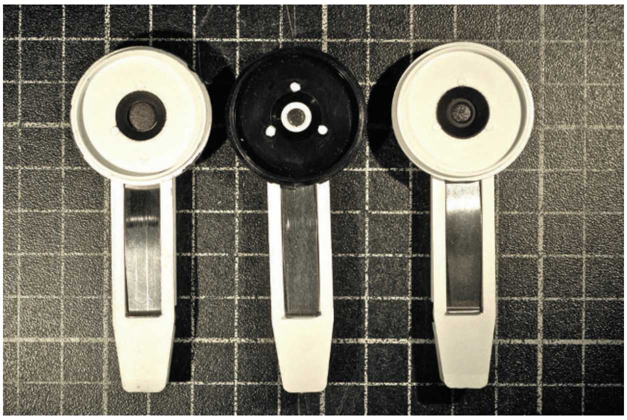

Fig. 1. FMS type leaf clip adopted by *FMS1* or *FMS2* (left) and PEA type leaf clips adopted by *Handy PEA* (*right*) or *Pocket PEA* (*middle*) fluorometers manufactured by *Hansatech Instruments Ltd*. The 30 mm diameter locating ring is white for *Handy PEA* or FMS clips and black for *Pocket PEA* clips. The clips are shown with an open shutter metallic blade, not covering the 7 (FMS clips) or 4 (PEA clips) mm diameter circular hole. The white grid lines show 1 × 1 cm.

The PSII quantum yield should normally improve during dark adaptation mainly due to increased F_m. However, fluorescence temperature curves (FTC) showed F_o sharply increasing above a critical leaf temperature (T_c) (Bilger *et al.* 1984, Kuropatwa *et al.* 1992). F_v/F_m is a suitable parameter to detect short-term heat injury to barley leaves within the first 5-min incubation at 49°C (Nauš *et al.* 1992). The effect on F_o was attributed to light-harvesting complexes and PSII centres (Ilík *et al.* 1995, Yamane *et al.* 1997) or to the PSII electron acceptors (Kouřil *et al.* 2004), whereas the denaturation of chlorophyll–proteins was responsible for a decrease in F_m (Yamane *et al.* 1997). Therefore, if clips cause an increase in leaf temperature above T_c, high F_o and low F_v/F_m would be expected, with T_c depending on species and acclimation (Weng and Lai 2005). Using the *Handy PEA* fluorometer, field-grown rice leaves clipped with white leaf-clips and exposed to 20-min solar radiation showed a 7°C increase over the 40°C critical threshold, leading to high F_o and low F_v/F_m that were not observed in shrouded clipped leaves (Weng 2006). Most incident radiation is absorbed by the black locating ring, which is 55.25 times larger than the central hole (Fig. 1). The clipped leaf-area may have a reduced transpiration because (due to the clip pressure) water vapour accumulates between the clip and leaf where the still air has low boundary layer conductance to heat and water vapour, and because of the progressive dark-induced stomatal closure. Therefore, the absorbed irradiance will dissipate as sensible heat rather than latent heat of transpired water, causing increased leaf temperature. As the clipped-leaf orientation under field conditions is normally not controlled relative to solar position, the absorbed irradiance and built-up sensible heat will vary greatly, and thus also leaf temperature, F_o, and F_v/F_m.

In unstressed tomato or pepper plants using the black clip/*Pocket PEA* fluorometer, high F_o and low F_v/F_m were found in preliminary trials in clipped leaves exposed to intense solar radiation during dark adaptation. The effect was investigated by comparing clipped-only with clipped and shrouded leaves for both the white clip/*FMS1* and the black clip/*Pocket PEA* fluorometers. The spring strength of the different leaf-clips and the reflectance in 350–2,500 nm radiation were also assessed. To my knowledge, there are no reports regarding the clip effect on the fluorescence emission parameters for the black leaf clips adopted by the *Pocket PEA* fluorometer.

Materials and methods

Experimental site and plant materials: Trials were performed in the late spring or summer 2010 on irrigated tomato (*Solanum lycopersicum* L., cv. "*San Marzano*") or pepper (*Capsicum annuum* L., cv. "*Corno di capra rosso*" or "*Papaccella napoletana rossa*") grown under field conditions according to local standard agronomical practices at the European Environmental Company (EURECO, Naples, Italy) experimental station in Acerra (Naples, Italy; 40° 57' 54" N, 14° 25' 43" E, 26 m above sea level). Both species flowered during July and fruited in August, with apparently good nutritional and water status conditions. All leaves were visually green, except for the senescing basalmost tomato leaves in mid August. The soil (pH 6.6) had 48% (w/w) sand, 37% (w/w) loam, 15% (w/w) clay, 278 mg kg^{-1}(P_2O_5 available), ~2,000 mg kg^{-1}(K_2O), 0.29% (w/w) N, 3.1% (w/w) organic matter, and 6.4% (w/w) $CaCO_3$.

Meteorological data: Weather data were provided by the Marigliano (Naples, Italy) meteorological station belonging to the Se.S.I.R.C.A. – C.A.R. of the *Regione Campania*, located 0.8 km from the experimental site.

Fluorescence measurements: A pulse amplitude modulated *FMS1* fluorometer equipped with white leaf clips, and a continuous-excitation *Pocket PEA* fluorometer equipped with black leaf clips (*Hansatech, Instruments Ltd.*, King's Lynn, Norfolk, England) were used according to Roháček and Barták (1999) and Roháček (2010) for modulated fluorometry, and Strasser *et al.* (2000, 2004) and Lazár (2006) for continuous-excitation fluorometry. The *FMS1* fluorometer adopted a pulsed light source as a very weak exciting modulating (amber) light, peaked at 594 nm (*Hansatech Instruments* 1997), and delivered in "trains" of variable duration. Modulation (MOD) level 2 was used, giving 16 modulating pulses with a 1.5 μs duration delivered during 60 ms every 110 ms (*Hansatech Instruments* pers. comm.). The resulting integrated amount of light incident upon the sample was < 0.01 μmol(photon) of PAR m^{-2} s^{-1}, and can not significantly reduce the primary quinone electron acceptor of PSII (Schreiber *et al.* 1986). The *FMS1* was equipped with a halogen white lamp source to generate a super-saturating light pulse of 17,000 μmol(photon) of PAR m^{-2} s^{-1} applied for 0.7 s for FI, and delivered to the leaf sample through an optical fibre probe inserted at 45° inclination into a closed black dome fitted over the leaf-clip. The same light source could be utilised to generate continuous actinic light up to ~6,000 μmol(photon) of PAR m^{-2} s^{-1} (not used in this study). The exciting red light of the *Pocket PEA* for FI was emitted by a diode source with a dominant 627 nm (peak) with a spectral half width (at half of the peak intensity) of 20 nm (*Hansatech Instruments* pers. comm.). The maximal available intensity of 3,500 μmol(photon) of PAR m^{-2} s^{-1} was applied for 1 s. The first emitted fluorescence detection occurred at 20 μs (data point of 16-bit resolution) after starting the light pulse. *Hansatech PEA Plus 1.02* software was used to estimate F_o by extrapolating the initial raising fluorescence signal to time zero of light pulsing (*Hansatech Instruments* 2006), *i.e.* virtually in the dark. However, it is common to use the fluorescence emission at 50 μs because it is only about ± 10% of the F_o estimated through extrapolation, despite instrumental distortion of FI in the low raising curve (Sušila *et al.* 2004). Because a saturating light pulse is required for F_m measurement, the actual maximum fluorescence emission *Pocket PEA* can induce is the peak value F_p that is normally lower than F_m (Lazár 2006). Thus, the (peak) quantum yield obtained in dark-adapted leaves with *Pocket PEA*, $(F_p - F_o)/F_p$, approaches F_v/F_m only when using saturating exciting light. However, for dark-adapted clipped-only leaves of well irrigated healthy tomato plants grown in a greenhouse, F_m obtained by using the *FMS1* as induced by a supersaturating pulse was not significantly different (p=0.0535) from F_p induced by switching on actinic light at 3,050 μmol (photon) of PAR m^{-2} s^{-1} ($F_p = 1.005^{***} \cdot F_m$; $R^2 = 0.988^{***}$; n = 9, $^{***}p \leq 0.0001$; Giorio unpublished). Similarly, in field-grown pepper plants, F_p measured with *Pocket PEA* in clipped-only dark-adapted leaves induced by an exiting light of 3,500 μmol(photon) of PAR m^{-2} s^{-1} for 1 s was not significantly different from F_m measured immediately after with *FMS1* (*see* Results). Therefore, here $(F_p - F_o)/F_{p(PPEA)}$ will be referred to as $F_v/F_{m(PPEA)}$.

For a minimum of 9 plants per sample, a single young fully expanded and sun-exposed leaf was chosen in the upper plant region. Fluorescence measurements were carried out on the adaxial leaf lamina of (attached, unless otherwise stated) light-adapted leaves or after 30–40 min of dark adaptation. The clipped leaves were either exposed to solar radiation (clipped-only leaves) or shrouded with radiation-reflecting aluminum foil (clipped and shrouded leaves) during dark adaptation.

Preliminary trials: In a few trials carried out on tomato or pepper, the variability of $\Delta F'/F_m'_{(PPEA)}$ and $F_v/F_{m(PPEA)}$ or $F_v/F_{m(FMS1)}$ in clipped-only or clipped and shrouded leaves was preliminary tested.

Verification of $F_v/F_{m(PPEA)}$ with paired $F_v/F_{m(FMS1)}$: In two trials, $F_v/F_{m(PPEA)}$ of tomato or pepper was measured, and each PEA clip was immediately substituted with an FMS clip on exactly the same position on the leaf lamina for paired $F_v/F_{m(FMS1)}$ measurement taken within ~5 s. The monitored leaf portion was exposed to ambient light for less than 1 s during clip switching.

Dynamic of $F_v/F_{m(PPEA)}$ during dark adaptation: In two trials, $\Delta F'/F_m'_{(PPEA)}$ was measured in light-adapted leaves of pepper or tomato. These clipped-only leaves were then carefully laid over nearby foliage in an approximately horizontal position, and $F_v/F_{m(PPEA)}$ was measured at regular intervals during 40 min of dark adaptation under direct solar radiation.

Testing solar radiation effect on $F_v/F_{m(PPEA)}$ and $F_v/F_{m(FMS1)}$: In two trials, F_v/F_m was measured in pepper with *FMS1* or *Pocket PEA* to compare clipped-only with clipped and shrouded leaves, all of them left in a horizontal position as above during dark adaptation.

Caveats for $\Delta F'/F_m'_{(PPEA)}$ or paired $F_v/F_{m(FMS1)}$: Theoretically, the *Pocket PEA* cannot measure either $\Delta F'/F_m'$ or F_v'/F_m'. However, when this fluorometer was fitted to a light-adapted leaf clipped using a clip with an open shutter blade, the leaf tissue was in the dark for just a second, until the exciting light was switched on for FI. In this way, a good approximation of $\Delta F'/F_m'$ was achieved. In fact, the very short darkening time could not lead to an actual fluorescence emission much lower than F', and the relaxation of nonphotochemical quenching mechanisms that are responsible for the (re)increase of maximum fluorescence from F_m' to F_m would have required a much longer time (minutes to several hours) (Maxwell and Johnson 2000). Therefore, the minimum and the maximum fluorescence emissions monitored by the *Pocket PEA* were a good approximation of F' and F_m', respectively. Similar considerations apply to the trials where $F_v/F_{m(PPEA)}$ was verified by the subsequent paired $F_v/F_{m(FMS1)}$. In this case, a good approximation of the true $F_v/F_{m(FMS1)}$ was obtained because the fluorescence induced first by the exciting light of the *Pocket PEA* and then by the ambient light during the quick clip switching, required only a few seconds to decrease back to nearly F_o (*see* Roháček 2010) once the leaf was clipped with the FMS clip.

Strength of the clip spring: The strength of the pivot spring for the different leaf clips was assessed by fixing the bottom side of the clip to the plate of a balance (*Delta Range PC 440*, *Mettler–Toledo S.p.A.*, Novate Milanese (MI), Italy). The counterbalance force [N] of the weight applied over the shorter arm of the clip sufficient to release the upper locating ring from the foam pad was assessed by visual observation of the light passing through the clip.

Radiation reflectance of clips: The reflectance of the locating ring for the black or white clips was measured from 350 to 2,500 nm at 1 nm scanning resolution using a spectroradiometer (*FieldSpec FSP350–2500*, *ASD Inc*., Boulder CO, U.S.A.).

Statistical analysis: Data were analysed with linear regression, Student's *t*-test or 1-way *ANOVA*, and post hoc *Tukey* test for paired or unpaired mean comparisons, with the null hypothesis rejected at $p \leq 0.05$ using the statistical software package *GraphPad Prism ver. 5.0 for Mac* (*GraphPad Software Inc*., San Diego, CA, U.S.A.).

Results

Weather conditions: Summer 2010 in southern Italy was a typically warm season but not particularly hot, with maximum air temperatures averaging 28.0°C (June), 31.6°C (July), and 31.3°C (August), and daily global radiation averaging 17.1, 17.4, and 16.3 MJ m^{-2} d^{-1} in the three months, respectively (data not shown).

Preliminary trials: $F_v/F_{m(PPEA)}$ taken in tomato or pepper (Fig. 2*A*) ranged from 0.802 to as low as 0.026, with an average of 0.560 ± 0.187 (tomato) or 0.645 ± 0.178 (pepper), with an average global radiation during darkening (R_g) of 626 W m^{-2}. Conversely, higher and much less variable F_v/F_m were found on a mid summer day for pepper clipped-only leaves using the *FMS1* (0.796 ± 0.043; R_g = 495.8 W m^{-2}) or in clipped and shrouded pepper leaves using the *Pocket PEA* (0.777 ± 0.022; R_g = 602.8 W m^{-2}) (Fig. 2*B*). In mid August, tomato clipped-only leaves showed stable and high values of both $\Delta F'/F_m'_{(PPEA)}$ (0.665 ± 0.053; R_g = 530.5 W m^{-2}) and $F_v/F_{m(FMS1)}$ (0.767 ± 0.040; R_g = 625.4 W m^{-2}) (Fig. 2*C*). These preliminary data indicated unexpectedly low and highly variable F_v/F_m for clipped-only leaves using the black clip/*Pocket PEA*. Conversely, high F_v/F_m values were observed in clipped and shrouded leaves for the black clip/*Pocket PEA* fluorometer or in clipped-only leaves with the white clip/*FMS1* fluorometer, all data indicating that plants were not suffering any significant stress. The *Pocket PEA* also adequately assessed $\Delta F'/F_m'$ (which did not require leaf dark-adaptation) compared to F_v/F_m for the *FMS1* fluorometer.

Verification of $F_v/F_{m(PPEA)}$ with paired $F_v/F_{m(FMS1)}$: In early August (R_g = 600.3 W m^{-2}), in each clipped-only tomato leaf, $F_v/F_{m(PPEA)}$ was first measured, and the PEA-clip was then carefully substituted by an FMS-clip to carry out an immediate paired measurement of $F_v/F_{m(FMS1)}$ at the same position on the leaf lamina. Data clearly confirmed that the use of the black-clip/*Pocket PEA* fluorometer in clipped-only leaves led to unreasonably low $F_v/F_{m(PPEA)}$ (0.222 ± 0.277) that approached zero in some leaves (Fig. 3*A*). This effect was due to the black clip and not to an artefact of the *Pocket PEA* fluorometer. In fact, the paired $F_v/F_{m(FMS1)}$ (0.224 ± 0.275) was not significantly different from the $F_v/F_{m(PPEA)}$ (p=0.893, data not shown). Low $F_v/F_{m(PPEA)}$ observed in several leaves resulted from low F_v, as F_o was nearly equal to F_m (Fig. 3*B*) or even higher in the subsequent measurement with the *FMS1* fluorometer (Fig. 3*C*), resulting in

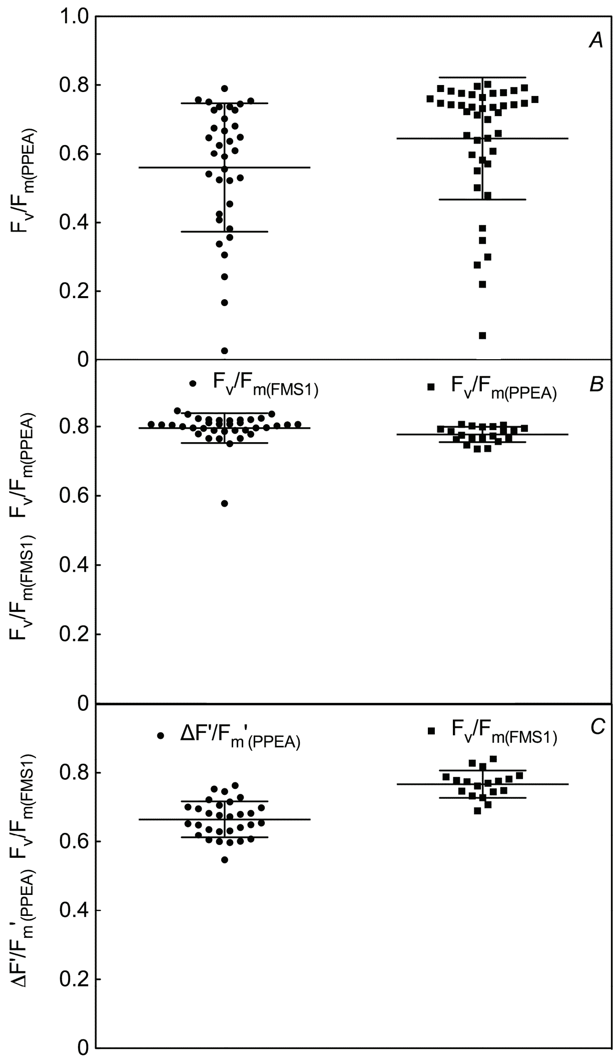

Fig. 2. Vertical scatter plot of maximum [*A*, *B* (left and right), *C* (right)] or effective [*C* (left)] quantum yield of PSII photochemistry measured on (*i*) dark-adapted leaves using the black clip/*Pocket PEA* fluorometer [$F_v/F_{m(PPEA)}$; *A* (left and right), *B* (right)], (*ii*) light-adapted leaves using the black clip/*Pocket PEA* fluorometer [$\Delta F'/F_m'_{(PPEA)}$; *C* (left)], or (*iii*) dark-adapted leaves using the white clip/*FMS1* fluorometer [$F_v/F_{m(FMS1)}$; *B* (left), *C* (right)]. *A*: Clipped-only tomato (left: 16th June at 10:30 h; *n* = 35) or pepper (right: 9th July at 11:00 h; *n* = 43) leaves. *B*: Clipped-only (left: pooled data from 11th August at 09:40 and 10:35; *n* = 36) or clipped and shrouded (right: 11th August at 11:30 h; *n* = 20) pepper leaves. *C*: Clipped-only tomato leaves measured on 9th August at 10:00 h (left: *n* = 30) or 12:30 h (right: *n* = 18). The longer and the two shorter horizontal lines respectively indicate mean and SD of the data set. *See* text for data of global radiation (R_g).

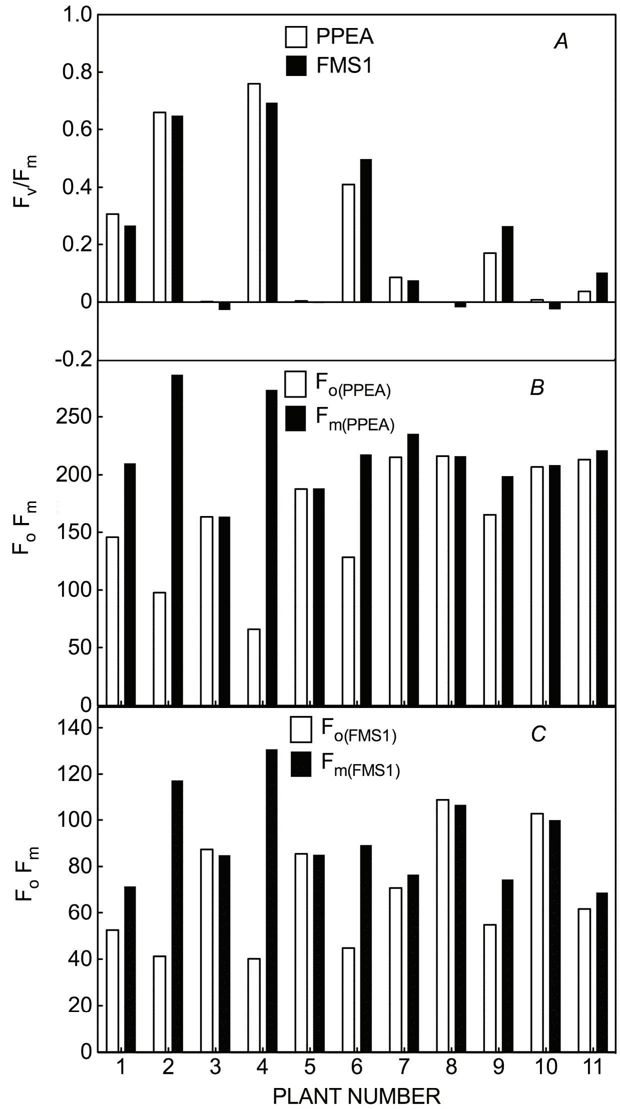

Fig. 3. *A*: Maximum quantum yield of PSII photochemistry in dark-adapted clipped-only leaves (F_v/F_m) measured with black clip/*Pocket PEA* fluorometer (PPEA; open bar) and paired F_v/F_m measured immediately after with white clip/*FMS1* fluorometer (*FMS1*; *closed bar*). Minimum (F_o [r. u.]; *open bar*) and maximum (F_m [r. u.]; *closed bar*) fluorescence emission of Chl *a* in the same leaf measured with a black clip/*Pocket PEA* fluorometer (*B*) or a white clip/*FMS1* fluorometer (*C*). Measurements were done on 11 tomato plants at 11:00 h on 9th August 2010. F_o and F_m are scaled by a multiplier of 0.01 (*Pocket PEA*) or 0.1 (*FMS1*). *See* text for data of global radiation (R_g).

apparent negative $F_v/F_{m(FMS1)}$ (Fig. 3*A*).

An identical trial was carried out on pepper plants the same day (R_g = 625.4 W m^{-2}). After the dark period, each clipped-only leaf with the PEA-clip still on was detached and put in a moistened glass container. Within 2 min, this was brought into a nearby air-conditioned laboratory where under dim light the *FMS1* fluorometer required battery recharging, and $F_v/F_{m(PPEA)}$ and then $F_v/F_{m(FMS1)}$ were measured as above (data not shown). $F_v/F_{m(PPEA)}$ was high (0.712 ± 0.145; *n* = 18) and not significantly different (p=0.0948) from $F_v/F_{m(FMS1)}$ (0.737 ± 0.091; *n* = 18). Moving the clipped leaves indoors likely dissipated the heat build-up and leaves partially recovered from heat stress, although with a probable high variability.

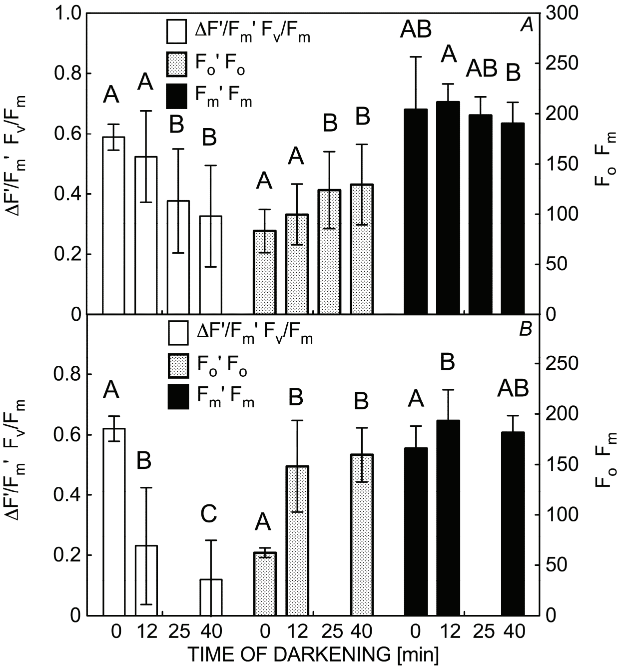

Fig. 4. Black clip/*Pocket PEA* fluorometer measurements of maximum quantum yield of PSII photochemistry ($\Delta F'/F_m'$), minimum (F_o' [r. u.]; *grey bar*) and maximum (F_m' [r. u.]; *black bar*) fluorescence emission of Chl *a* in light-adapted leaves assessed prior to leaf dark-adaptation (time 0) and subsequent paired maximum quantum yield of PSII photochemistry (F_v/F_m; *open bar*), minimum (F_o [r. u.]; *grey bar*) and maximum (F_m [r. u.]; *black bar*) fluorescence emission of Chl *a* in clipped-only leaves after 12, 25, or 40 min of darkening measured on 16th August in pepper at 10:00 h (*A*) or tomato at 12:00 h (*B*). For each parameter, columns with the same letter are not statistically different (*Tukey*'s multiple comparison test; $p \leq 0.05$). *Bars* indicate mean ± SD ($n = 20$). F_o and F_m are scaled by a multiplier of 0.01. *See* text for data of global radiation (R_g).

Therefore, paired data of $F_{m(PPEA)}$ and the subsequent $F_{m(FMS1)}$ were normalised by the mean and the standard deviation (SD) to perform a linear regression analysis. Results confirmed that a 1-s exciting light intensity of 3,500 μmol(photon) of PAR m^{-2} s^{-1} was suitable to nearly saturate photosystems ($F_{m(PPEA)} = 0.819^{***} F_{m(FMS1)}$; $R^2 = 0.672^{***}$, $n = 18$ $^{***}p \leq 0.0001$; data not shown). Although the goodness of fit was worse than for greenhouse-grown tomato plants, these data are robust enough to indicate that the effect of black leaf clips detected by the *Pocket PEA* was confirmed by the *FMS1* fluorometer.

Dynamics of $F_v/F_{m(PPEA)}$ during dark adaptation: The time course of fluorescence parameters during the darkening of clipped-only leaves was monitored with the *Pocket PEA* in mid August 2010 in pepper or tomato under a global radiation of 533.3 or 598.2 W m^{-2}, respectively. A clear reduction in the PSII quantum yield during dark adaptation was observed. $\Delta F'/F_m'$ assessed just before darkening (time 0) was about 0.6 in both species and it was significantly higher than the F_v/F_m values of pepper (0.327 ± 0.169; Fig. 4*A*) or tomato (0.120 ± 0.129; Fig. 4*B*) measured after 40 min of dark adaptation. These trials indicated a clip effect on $F_v/F_{m(PPEA)}$ caused by a statistically significant F_o increase during darkening, with no clear effect on F_m (Fig. 4*A*,*B*).

Testing solar radiation effect on $F_v/F_{m(PPEA)}$ and $F_v/F_{m(FMS1)}$: On pepper plants in mid August, $F_v/F_{m(PPEA)}$ and $F_v/F_{m(FMS1)}$ for clipped-only leaves were compared with clipped and shrouded leaves. The trial was carried out twice, and all the clipped leaves were exposed during the darkening to R_g of 598.9 or 568.2 W m^{-2}. There was a strong clip effect on F_v/F_m for the *Pocket PEA* fluorometer when (black) clips were not shrouded (Table 1). In the first trial, $F_v/F_{m(PPEA)}$ for clipped-only leaves (0.271 ± 0.163) was statistically significantly lower than in clipped and shrouded leaves (0.769 ± 0.025). $F_v/F_{m(FMS1)}$ in the clipped-only leaves (0.758 ± 0.040) was statistically significantly 8% lower than in the clipped and shrouded leaves. The low $F_v/F_{m(PPEA)}$ in the clipped-only leaves was due to a statistically significantly double F_o and 32% lower F_m as compared with the clipped and shrouded leaves. These results were confirmed in the subsequent trial (Table 1).

Strength of the clip spring: The spring strength was assessed as the counterbalance force sufficient to release the pressure between the upper and bottom clip arms, with an estimated resolution not better than 0.025 N (data not shown). The force for *Pocket PEA* (3.34 ± 0.30 N; $n = 6$), *Handy PEA* (1.74 ± 0.15 N; $n = 6$), and FMS (2.01 ± 0.61 N; $n = 6$) clips was statistically significantly different ($p \leq 0.001$) between *Pocket PEA* and *Handy PEA* or FMS clips. However, the different ages of the clips (1-year old *Pocket PEA* clips, unused 8-year-old *Handy PEA* clips, and ~10-year-old FMS clips) would certainly have affected clip strength.

Radiation reflectance of clips: The reflectance of the locating ring from 350 to 2,500 nm for *Handy PEA* (0.5869 ± 0.0016; $n = 4$), FMS (0.5735 ± 0.0170; $n = 4$), and *Pocket PEA* (0.0145 ± 0.0001; $n = 4$) clips differed from that in the visible waveband for the white (0.8163 ± 0.2720; $n = 8$) and black (0.0237 ± 0.0001; $n = 4$) locating rings (data not shown).

Table 1. Maximum quantum yield of PSII photochemistry (F_v/F_m) and minimum (F_o) and maximum (F_m) fluorescence emission of chlorophyll *a* (Chl *a*) in dark-adapted pepper leaves. Leaves were measured with either black clip/*Pocket PEA* fluorometer (PPEA) or white clip/*FMS1* fluorometer (*FMS1*) for clipped-only leaves or clipped and shrouded leaves in two trials (11:30 or 13:00 h) on 13th August 2010. Mean ± SD (*n* = 10, PPEA; *n* = 9, *FMS1*). Within a row, statistical significance of the mean difference between clipped-only and clipped and shrouded leaves (Student *t*-test) are indicated by * ($p \le 0.05$), ** ($p \le 0.001$), *** ($p \le 0.0001$), or ns (not significant). F_o and F_m are scaled by a multiplier of 0.01 (*Pocket PEA*) or 0.1 (*FMS1*). *See* text for data of global radiation (R_g). r.u. – relative units.

Fluorometer	Parameter		Clipped-only	Clipped and shrouded
			11:30 h trial	
PPEA	F_v/F_m	***	0.271 ± 0.163	0.769 ± 0.025
	F_o [r.u.]	***	138 ± 43	64 ± 4
	F_m [r.u.]	***	186 ± 22	277 ± 24
FMS1	F_v/F_m	**	0.758 ± 0.039	0.804 ± 0.017
	F_o [r.u.]	ns	30 ± 5	28 ± 1
	F_m [r.u.]	*	126 ± 14	143 ± 15
			13:00 h trial	
PPEA	F_v/F_m	***	0.339 ± 0.186	0.757 ± 0.039
	F_o [r.u.]	***	124 ± 47	65 ± 43
	F_m [r.u.]	***	185 ± 25	272 ± 32
FMS1	F_v/F_m	**	0.721 ± 0.062	0.798 ± 0.021
	F_o [r.u.]	***	30 ± 2	26 ± 1
	F_m [r.u.]	*	110 ± 23	131 ± 16

Discussion

The use of leaf clips for F_v/F_m assessment can surely increase the number of leaves sampled at a given time by a fluorescence system. For the *Pocket PE*A fluorometer, a clip with a black locating ring (Fig. 1) above the quite small measured leaf surface area is used to avoid interference from ambient light during fluorescence detection. The *Pocket PE*A adequately measured $\Delta F'/F_m'$ in light-adapted leaves of healthy well-irrigated plants, which was high and stable, and lower as expected than F_v/F_m measured with the *FMS1* in clipped-only leaves exposed to high solar radiation during dark adaptation (Fig. 2*C*). The *FMS1* fluorometer was used as a control instrument because it adopts white clips (Fig. 1), and always gave high and stable F_v/F_m (Fig. 2*B*,*C*; Table 1). In contrast, *Pocket PEA* showed apparently inexplicably very low F_v/F_m values in many clipped-only leaves, resulting in low mean values with high variability (Figs. 2*A*, 3*A*, 4*A*,*B*; Table 1). The F_v/F_m obtained with *Pocket PEA* in clipped-only leaves was confirmed in each leaf by the paired F_v/F_m measured immediately after with *FMS1* (Fig. 3*A*). These results indicated that the *Pocket PEA* fluorometer was not malfunctioning, and that the effect on fluorescence parameters could be attributed to the black clip.

The effect on $F_v/F_{m(PPEA)}$ in clipped-only leaves under high radiation was mainly due to increased F_o (Fig. 4*A,B*; Table 1) or almost reached F_m (Fig. 3*B*), and to a substantial decrease in F_m as shown by the comparison of clipped-only with clipped and shrouded leaves (Table 1). This resulted in low or almost zero F_v/F_m values in many leaves, causing very low mean values (Figs. 2*A*, 3*A*, 4*A*,*B*) as confirmed by the paired *FMS1* data (Fig. 3*A*,*C*). Such a strong effect on F_v/F_m was not found when leaves clipped with black clips were shrouded with aluminum foil during dark adaptation (Fig. 2*B*, Table 1).

A sharp increase in F_o starting at T_c and reaching F_m at T_m, with a consequently low F_v/F_m, is a typical feature for FTC of leaves in the dark or under weak actinic light (Bilger *et al.* 1984, Kuropatwa *et al.* 1992, Nauš *et al.* 1992, Kouřil *et al.* 2004, Weng and Lai 2005). Rice leaves clipped with white clips on sunny days showed practically unchanged F_o when the leaf temperature remained below 40°C (Weng 2006) or a drastic increase when this threshold was surpassed. These findings agree with Kouřil *et al.* (2004) FTC curves in barley leaves, which show a sharp increase in F_o above T_c (42°C) and a concomitant decrease in F_m. Their FTC data indicate an abrupt decrease of F_v/F_m down to zero from T_c to the maximum F_o temperature (~50 °C), as also reported by Pospíšil *et al.* (1998). During dark adaptation of the clipped-only leaves under solar radiation, the temperature of the nontranspiring leaf tissue below the black PEA-clip probably strongly increased because the net radiation available over the black locating ring was obviously totally converted to sensible heat and not to latent heat. The black locating rings reflected less than 1.5% of the 350–2,500 nm irradiance, whereas white clips reflected nearly 60%. Therefore, solar radiation probably induced overheating of the leaf area covered by the black locating ring of the *Pocket PEA* clip during dark adaptation, resulting in a remarkably increased F_o.

Lazár and Nauš (1998) showed that the O–J–I–P curve parameters sampled in field-grown winter wheat did not have a Gaussian distribution. Along with such intrinsic deviation from the normal distribution, the clip effect on the fluorescence parameters was highly variable among leaves (Fig. 2*A*). The energy balance of clipped-only leaves will depend on a number of physiological, environmental, and clip variables. However, global radiation during the trials was quite stable (500–600 W m^{-2}). As a consequence, the high variability of the fluorescence parameters for the clipped-only leaves (*e.g.* Fig. 2*A*, Table 1) can be mostly attributed to the clipped leaf position relative to the sun, resulting in a variable incident irradiance. Rice or spinach leaves treated 5 min at high temperature and then cooled down and reincubated for 5 min at room temperature showed a partially reversible F_o increase (Yamane *et al.* 1997). Accordingly, in one trial on 9th August, the clip effect was not observed when after dark adaptation clipped-only pepper leaves with black clips were kept for a few minutes under dim light in an air-conditioned laboratory before performing the measurements, probably because of dissipated overheating.

Nauš *et al.* (1992) reported that 5-min incubation at 49°C was sufficient to observe a F_v/F_m as low as 0.226 in barley leaves, although a slight increase was observed at 30-min incubation. It took only 12 (tomato) or 25 (pepper) min to observe a significant leaf clip effect on both F_o and F_v/F_m during dark adaptation in clipped-only leaves measured with the *Pocket PEA*, but there was no effect on F_m (Fig. 4*A*,*B*). However, a significantly lower F_m for *Pocket PEA* (32%) or *FMS1* (14%) was observed in clipped-only leaves compared to clipped and shrouded leaves (Table 1), in accordance to previously reported FTC data (Pospíšil *et al.* 1998, Kouřil *et al.* 2004). In contrast, increased F_m was observed in clipped-only leaves of field-grown rice after dark adaptation under high irradiance for the white clip/*Handy PEA* fluorometer (Weng 2006). However, similar to our results for the *Pocket PEA* reported in Fig. 4, this author found an increased F_m that did not significantly change with 10–20 min of dark adaptation. Importantly, the clip effect may not be obvious for moderate overheating when the measured F_v/F_m was reasonably high, although underestimated. This probably occurred in many clipped-only leaves for the black clips of *Pocket PEA* showing moderately high F_v/F_m (*e.g.* Fig. 2*A*) while a significant 8% underestimation occurred even in clipped-only leaves for the white clips of *FMS1* fluorometer (Table 1) as found by Weng (2006) for the white clips of *Handy PEA*.

The role of the variable clip spring strength on leaf transpiration, overheating, and fluorescence emission parameters was not evaluated. However, nontranspiring clipped-only leaf tissue would not overheat without absorption of incident radiation. This is clearly demonstrated by the clip effect, which was negligible in clipped-only leaves for the white clips or virtually absent in clipped and shrouded leaves for white or black clips.

Conclusions: The use of *Pocket PEA* fluorometers with black clips deserves attention regarding operating conditions. In sunny environments, the risk of clip-effect is reduced if clipped leaves become vertically oriented relative to gravity and the locating ring does not face the sun. However, when black clips are used under open field conditions characterised by intense radiation, such as in southern Italy during the summer, the clipped leaves should not be left under direct solar radiation during dark adaptation because F_v/F_m will be artificially highly variable and underestimated due to probable leaf overheating. It may be difficult, if not impossible, to control the orientation of clipped leaves when high numbers are monitored at a given time. Avoiding direct solar radiation can only be achieved by shrouding the clipped leaves with reflecting material such as aluminum foil, a practice that is strongly recommended. However, this will decrease the ability to sample large leaf-numbers, which *Pocket PEA* fluorometers can handle. Hence, manufactured leaf clips for *Pocket PEA* that adequately reflect solar radiation during dark adaptation would be useful.

References

Baker, N.R.: Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. – Annu. Rev. Plant Biol. **59**: 89-113, 2008.

Bilger, H.W., Schreiber, U., Lange, O.L.: Determination of leaf heat resistance: comparative investigation of chlorophyll fluorescence changes and tissue necrosis methods. – Oecologia **63**: 256-262, 1984.

Genty, B., Briantais, J.-M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. Biophys. Acta **990**: 87-92, 1989.

Hansatech Instruments: Hansatech Fluorescence Monitoring System User Manual. – England, King's Lynn, Norfolk 1997.

Hansatech Instruments: Operating Instructions for Plant Efficiency Analyser Advanced Fluorescence Analysis, Summary Version. – England, King's Lynn, Norfolk 1999.

Hansatech Instruments: Operations Manual, Setup, Installation & Maintenance: Handy PEA, Pocket PEA & PEA Plus Software, version 1.0. – England, King's Lynn, Norfolk 2006.

Ilík, P., Nauš, J., Cikánek, D., Novotný, R.: Chlorophyll fluorescence changes at high temperatures induced by linear heating of greening barley leaves. – Photosynth. Res. **44**: 271-275, 1995.

Kitajima, M., Butler, W.L.: Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. – Acta Biochim. Biophys. **376**: 105-115, 1975.

Kouřil, R., Lazár, D., Ilík, P., Skotnica, J., Krchňák, P., Nauš, J.: High-temperature induced chlorophyll fluorescence rise in plants at 40–50 °C: experimental and theoretical approach. – Photosynth. Res. **81**: 49-66, 2004.

Kuropatwa, R., Nauš, J., Mašláň, M., Dvořák, L.: Basic properties of the chlorophyll fluorescence temperature curve in barley leaves. – Photosynthetica **27**: 129-138, 1992.

Lazár, D.: Chlorophyll *a* fluorescence induction. – Biochim. Biophys. Acta **1412**: 1-28, 1999.

Lazár, D.: The polyphasic chloropyll *a* fluorescence rise measured under high intensity of exciting light. – Funct. Plant Biol. **33**: 9-30, 2006.

Lazár, D, Nauš, J.: Statistical properties of chlorophyll fluorescence induction parameters. – Photosynthetica **35**: 121-127, 1998.

Maxwell, K., Johnson, G.N.: Chlorophyll fluorescence – a practical guide. – J. Exp. Bot. **51**: 659-668, 2000.

Nauš, J., Kuropatwa, R., Klinkovský T., Ilík, P., Lattová J., Pavlová, Z.: Heat injury of barley leaves detected by the chlorophyll fluorescence temperature curve. – Biochim. Biophys. Acta **1101**: 359-362, 1992.

Ögren, E., Baker, N.R.: Evaluation of a technique for the measurement of chlorophyll fluorescence from leaves exposed to continuous white light. – Plant, Cell Environ. **8**: 539-547, 1985.

Pospíšil, P., Skotnica, J., Nauš**,** J.: Low and high temperature of minimum F_0 and maximum F_M chlorophyll fluorescence *in vivo*. – Biochim Biophys Acta **163**: 95-99, 1998.

Roháček, K., Barták, M.: Technique of the modulated chlorophyll fluorescence: basic concepts, useful parameters, and some applications. – Photosynthetica **37**: 339-363, 1999.

Roháček, K.: Method for resolution and quantification of componenets of the non-photochemical quenching (*q*N). – Photosynth. Res. **105**: 101-113, 2010.

Schreiber U.: Detection of rapid induction kinetics with a new type of high–frequency modulated chlorophyll fluorometer. – Photosynth. Res. **9**: 261-272, 1986.

Schreiber, U., Bilger, W., Schliwa, U.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. – Photosynth. Res. **10**: 51-62, 1986.

Strasser, R.J., Srivastava, A, Tsimilli-Michael, M.: The fluorescence transient as a tool to characterize and screen photosynthetic samples. – In: Yunus, M., Pathre, U., Mohanty, P. (ed.): Probing Photosynthesis: Mechanism, Regulation and Adaptation. Pp. 443-480. Taylor and Francis, London 2000.

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A.: Analysis of the fluorescence transient. – In: Papageorgiou, G.C., Govindjee (ed.): Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Pp. 321-362. Springer, Dordrecht 2004.

Sušila, P., Lazár, D., Ilík, P., Tomek, P., Nauš, J.: The gradient exciting radiation within a sample affects the relative height of steps in the fast chlorophyll *a* fluorescence rise. – Photosynthetica **42**: 161-172, 2004.

van Kooten, O., Snel, J.F.H.: The use of chlorophyll fluorescence nomenclature in plant stress physiology. – Photosynth. Res. **27**: 121-133, 1990.

Weng, J.-H.: Underestimate of photosystem 2 efficiency in the field due to high leaf temperature resulting from leaf clipping and its amendment. – Photosynthetica **44**: 467-470, 2006.

Weng, J.-H., Lai, M.-F.: Estimating heat tolerance among plant species by two chlorophyll fluorescence parameters. – Photosynthetica **43**: 439-444, 2005.

Yamane, Y., Kashino, Y., Koike, H., Satoh, K.: Increase in the fluorescence F_o level and reversible inhibition of Photosystem II reaction center by high-temperature treatments in higher plants. – Photosynth. Res. **52**: 57-64, 1997.