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Heat-induced F_0-fluorescence rise is not an indicator of severe tissue necrosis in thermotolerance assays of young and mature leaves of a tropical tree species, *Calophyllum inophyllum*

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Abstract

In heating experiments with leaves, the temperature at which dark-level F_0 chlorophyll *a* fluorescence begins to rise, T_{crit}, is widely used as an indicator of photosystem II thermotolerance. However, little is known about how T_{crit} correlates with irreversible leaf tissue damage. Young and mature leaves of the tropical tree species *Calophyllum inophyllum* were heated stepwise from 30 to 55°C, at 1°C min^{-1}. T_{crit} was 47°C in young leaves and 49°C in mature leaves. Contrary to the higher T_{crit} in mature leaves, heating to 55°C elicited greater tissue damage in mature than in young leaves. Young and mature leaves heated to their respective T_{crit} or T_{crit} + 2°C exhibited no or little tissue necrosis after 14 d of post-culture. It is concluded that measurements of the temperature-dependent F_0 fluorescence rise underestimate the thermal thresholds above which significant irreversible leaf damage occurs.

Keywords: chlorophyll *a* fluorescence; global warming; heat tolerance; necrosis; tropical trees.

Introduction

Tropical rainforests are high-temperature ecosystems. Ongoing global warming is a potential threat as they could be pushed above their upper thermal tolerance limits (Doughty and Goulden 2008, Wright *et al.* 2009). This concern has led to increased research into the thermotolerance of tropical forest vegetation, especially tropical rainforest trees (Krause *et al.* 2010, Tiwari *et al.* 2021, Kullberg *et al.* 2024). Plant physiologists use several methods to examine the heat tolerance of plants.

(*1*) In the traditional necrosis test, leaves heated at different temperatures in a water bath for 15 or 30 min, are assessed for visible damage two to three weeks after treatment (post-culture). The temperature at which 50% of the leaf area becomes necrotic is considered the upper-temperature threshold for plant survival (Sapper 1935, Lange 1961, Larcher and Wagner 1976).

(*2*) Following heat treatments, tissue vitality is determined through the uptake of dyes that stain living and dead cells and cell membrane thermostability is assessed by determining the extent of electrolyte leakage (Lorenz 1939, Didden-Zopfy and Nobel 1982, Yeh and Hsu 2004, Ilík *et al.* 2018).

(*3*) Measurement of chlorophyll *a* fluorescence is currently the preferred method to examine leaf thermotolerance, aided by the availability of small, portable instruments that rapidly determine the activity and integrity of PSII, the most heat-sensitive component of photosynthesis (Berry and Björkman 1980). Non-modulated (Kautsky and Hirsch 1931, Strasser *et al.* 2004) and modulated fluorescence systems (Schreiber *et al.* 1986) are available. Most ecophysiological studies use the fluorescence parameters F_v/F_m or F_0 as indicators of PSII thermal stress. The K peak (or K step) that occurs in fluorescence induction curves of heated leaves (Guissé *et al.* 1995, Lazár *et al.* 1997, Lazár and Ilík 1997) has been proposed as a further indicator of adverse thermal effects on PSII. However, thus far the use of the K peak in ecophysiological research has been limited (Chen *et al.* 2016).

Highlights

- Young and mature leaves of a tropical tree species were heated stepwise to 55°C
- The temperature at which dark level fluorescence began to rise, T_{crit}, was determined
- Leaves heated to T_{crit} showed no or little tissue necrosis after 14 d of post-culture

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Abbreviations: F_0 – dark level fluorescence; F_v/F_m – maximum quantum yield of PSII photochemistry in dark-adapted state.
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The ratio of variable to maximum fluorescence emission, F_v/F_m, is a measure of the maximum quantum yield of PSII chemistry in the dark-adapted state. On a scale from 0 to 1, F_v/F_m is ~0.83 in healthy, non-stressed leaves (Murchie and Lawson 2013). Upon heat stress, the ratio declines. Measurements are usually performed 24 h after heat treatments. The temperature at which F_v/F_m starts to decline is referred to as T_{crit} or T_5, and the temperature at which F_v/F_m has declined by 50% is defined as T_{50}. In a study of 147 tropical forest species, T_{50} averaged 49.9°C (47.8–52.5°C) (Slot *et al.* 2021, *see* also Krause *et al.* 2010, 2013, 2015; Feeley *et al.* 2020). It should be cautioned however that depending upon the species, T_{50} measured 24 h after heat exposure may significantly underestimate the temperature above which leaves become necrotic (Winter *et al.* 2025, *see* also Cunningham and Read 2006).

Here, we extend our assessment of the chlorophyll *a* fluorescence approach in studying leaf thermal tolerances to F_0-temperature response curves (Schreiber and Berry 1977); *see* also literature on FTCs (fluorescence temperature curves) by, *e.g.*, Nauš *et al.* (1992a,b) and Lazár and Ilík (1997). F_0 is the minimal fluorescence in darkened leaves when all PSII reaction centers are open. As temperature increases, F_0 sharply rises when a high-temperature threshold is exceeded, reaches a maximum, and falls thereafter. The temperature at which F_0 begins to rise (termed T_c or T_{crit} in the literature) signals the onset of heat-induced disruption of orderly PSII functioning (Frolec *et al.* 2008, Zhu *et al.* 2024).

F_0 monitoring is currently regaining momentum (*e.g.*, Zhu *et al.* 2018, Gauthey *et al.* 2024, Middleby *et al.* 2024), especially because of the recent introduction of high-throughput-imaging fluorometry (Arnold *et al.* 2021, Posch *et al.* 2022). However, there is still little information about how the heat-induced rise in F_0 correlates with visible, irreversible leaf damage. Depending on the heating–cooling protocol, partial reversibility of the F_0 rise has been observed in barley leaves (*e.g.*, Frolec *et al.* 2008), suggesting that T_{crit} is not always a proxy of irreversible damage. Because T_{crit} of F_0 fluorescence is estimated from the intersection of two lines derived from F_0-temperature response curves (*see* Materials and methods), one line of which can only be obtained when leaf samples are heated substantially above T_{crit}, leaf samples used for determining T_{crit} are not suitable for examining the degree of leaf necrosis at T_{crit}. In the classic study by Bilger *et al.* (1984) on a range of species from the Würzburg Botanic Garden, T_{crit} values derived from F_0 measurements of samples heated at a rate of 0.7°C min^{-1} were in relatively good agreement with temperatures at which 50% post-culture necrosis occurred after a separate set of leaves had been exposed to different temperatures in a water bath for 30 min. Although two different heat treatments were applied and two different thermotolerance parameters measured, the authors were able to explain the conformity in results through consideration of a calculated "critical heat dosage" (*see* also Niinemets 2018). However, there was no direct demonstration that leaf samples heated to T_{crit} in a stepwise, F_0-temperature-response fashion would show 50% necrosis during post-culture. In the study of Hüve *et al.* (2011) on *Phaseolus vulgaris*, T_{crit} of F_0 fluorescence rise was ~47°C. Whereas no cellular lesions were observed at this temperature as evidenced by the absence of Evans blue-stained cells, enhanced areas of blue-stained cells (5–20%) occurred above 48–49°C. Likewise, cell membrane thermostability determined through ion leakage measurements was higher than PSII thermostability determined as T_{crit} of F_0 fluorescence rise (Ilík *et al.* 2018).

To more directly explore the significance of T_{crit} in terms of irreversible tissue damage, F_0-temperature response curves were employed to determine T_{crit} in young and mature leaves of the tropical tree species *Calophyllum inophyllum*. Once T_{crit} was established, a separate set of leaves, submitted to the same stepwise increase in temperature, was heated up to T_{crit}, or T_{crit} plus 2°C, for necrosis tests 14 d later. In none of the leaves did significant tissue necrosis occur. The results demonstrate that the temperature at which F_0 begins to rise is not the temperature threshold leading to severe permanent tissue damage.

Materials and methods

Plant material: *Calophyllum inophyllum* L. (Calophyllaceae) is a tropical tree species with a native range from Eastern Africa to Tropical and Subtropical Asia, Australia, and Polynesia (POWO 2024). Trees are up to 30 m tall, and their timber is used for traditional shipbuilding. *C. inophyllum* is an ornamental plant in Panama. Young and mature leaves from a tree growing at the Smithsonian Tropical Research Institute's Tupper complex in Panama City, Republic of Panama were studied in August and September of 2024, *i.e.*, during Panama's rainy season.

The young leaves were fully expanded. Their leaf area (68.5 ± 8.6 cm^2, n = 12) was similar to that of mature leaves (73.1 ± 10.2 cm^2, n = 12; P=0.25), but as is typical for young leaves, they had a greater fresh mass: dry mass ratio (3.33 ± 0.10, n = 7, *vs*. 2.63 ± 0.12, n = 7; P<0.001) and a greater specific leaf area (90.6 ± 4.2 $cm^2\ g^{-1}$, n = 7, *vs*. 73.1 ± 4.2 $cm^2\ g^{-1}$; P<0.001) than mature leaves.

Chlorophyll fluorescence: Leaves were excised at different times during daylight hours and kept for 30 min in the dark, with their petioles in water. After the removal of petioles including a small portion of leaf area ~1 cm above petioles, leaf laminas were weighed and placed horizontally into a *3010 GWK1* temperature-controlled gas-exchange chamber (*Walz GmbH*, Effeltrich, Germany). The position of leaf laminas was tightly fixed with nylon strings. After closing the top of the chamber with a custom-made glass plate surrounded by an aluminum frame, the end of the fiber optic cable of a *MINI-PAM-II* was positioned perpendicularly to the glass plate. During heat treatments and chlorophyll fluorescence measurements, leaf laminas were in complete darkness. The internal fan speed (0–5) was set to 4. Leaf temperature was monitored with a fine-wire thermocouple with a stated

accuracy of ± 0.2°C. Using the *Walz GSF-Win* software, the gas-exchange chamber was programmed to increase the leaf temperature in steps of 1°C min^{-1} from 30 to 55°C. Heating rate is an important consideration as the applied heat dose can significantly affect T_{crit} (Arnold *et al.* 2021, *see* also Neuner and Buchner 2023). Relatively slow heating of 1°C min^{-1} was used in Schreiber and Berry (1977) and is employed in many current F_0-temperature experiments, facilitating comparisons across studies. Dark level F_0 fluorescence was continuously monitored with the *MINI-PAM-II* system, and values were recorded at the end of each one-minute interval. After the conclusion of heat treatments, leaf laminas were removed from the gas-exchange chamber, weighed, and placed into humid, transparent plastic boxes for 14 d of post-culture at ambient temperature (~23°C) and continuously low PFD of ~5 µmol m^{-2} s^{-1} in the lab. During heat treatments, fresh mass losses were small: 3.3 ± 2.2% (n = 6) in young leaves, and 5.2 ± 1.5% (n = 6) in mature leaves.

In a second set of experiments, young and mature leaves were exposed to similar heat treatments in the dark as described above, but with final temperatures limited to 47, 49, and 51°C. In these experiments, F_0 fluorescence was not measured. Like before, leaf laminas were removed from the gas-exchange chamber and maintained in humid, transparent plastic boxes for post-culture.

Visual examination and F_v/F_m measurements: After 14 d of post-culture, leaf laminas were examined for visible tissue necrosis (dark areas) and photographed with a *Sony a7 IV* digital camera equipped with a *Sony FE 2.8/24-70 GM II* lens. Using four leaf clips (*Walz GmbH*) placed between the midrib and edge of the upper and lower part of the left and right half of leaf laminas (two on each side), the chlorophyll fluorescence parameter F_v/F_m was determined after 15 min of dark adaptation with the *MINI-PAM-II* system (*Walz GmbH*), *i.e.*, on each leaf four F_v/F_m measurements were performed and averaged.

Calculation of T_{crit}: T_{crit} was determined from the intersection of two lines of F_0-temperature response curves. At least three approaches can be found in the literature to calculate T_{crit}: (a) T_{crit} is the temperature where regression lines extrapolated from the slow and the fast-rising portions of the temperature-dependent F_0 response intersect (*e.g.*, Weng and Lai 2005); (b) T_{crit} is the temperature where regression lines extrapolated from the slow and the fastest rising part of the fast-rising portions of the F_0 response intersect (Bilger *et al.* 1984); (c) T_{crit} is the temperature where the line drawn as extension of the F_0 value at the lowest temperature intersects with the slope of the fastest part of the fast portion of F_0 increase (*e.g.*, Braun *et al.* 2002). Methods (a) and (b) result in slightly lower values of T_{crit} than method (c). We have arbitrarily used method (c) (*see* Fig. 1).

Results and discussion

F_0 responses to stepwise temperature increase from 30 to 55°C yielded T_{crit} of 47.2 ± 0.7°C (n = 6) for young and 49.0 ± 1.0°C (n = 6) for mature leaves (P=0.004), suggesting a higher PSII thermotolerance of mature than young leaves. By contrast, following heating to 55°C, mature leaves developed a significantly greater degree of leaf area necrosis (77%) than young leaves (22%) during post-culture (P=0.002) (Table 1, Fig. 2). Consistent with this observation, F_v/F_m values after post-culture demonstrated an almost complete loss of PSII photochemistry in mature leaves whereas the maximum quantum yield of PSII chemistry was reduced to a much lesser degree in young leaves (P<0.001) (Table 1). At this point, we cannot explain the conflicting results from F_0-temperature response and examination of visible damage after post-culture, except to note that adverse effects of heating on processes other than PSII photochemistry override initial heat effects on PSII in

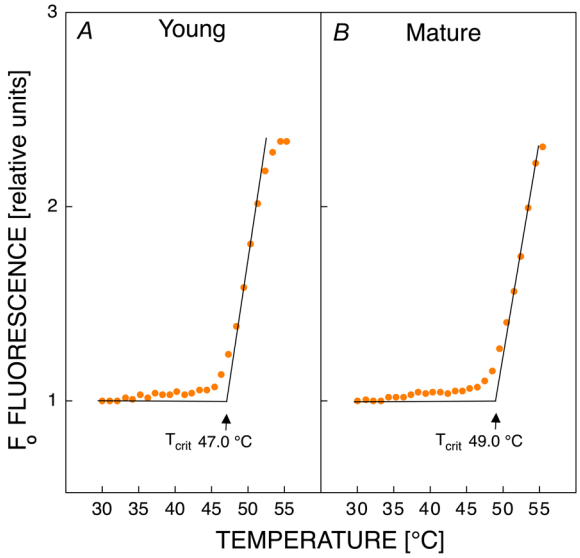

Fig. 1. Representative F_0-temperature response curves of a young (*A*) and a mature (*B*) leaf of *Calophyllum inophyllum*. T_{crit} was calculated from the intersection of the vertical line originating from F_0 at 30°C and the line representing the steepest slope of the F_0 increase.

Table 1. Effect of treatment-temperature range on visible leaf tissue damage (necrosis) and F_v/F_m of young and mature leaves of *Calophyllum inophyllum* after 14 d of post-culture. Errors are standard deviations of six or seven leaf samples. For F_v/F_m, each sample represents the mean of four measurements across a single leaf lamina (*see* Materials and methods).

Leaf type	Treatment	Damage (%)	F_v/F_m
Young	Control	0 ± 0 (7)	0.814 ± 0.004 (7)
	30–55°C	22 ± 15 (6)	0.656 ± 0.096 (6)
	30–47°C	0 ± 0 (7)	0.789 ± 0.009 (7)
	30–49°C	0 ± 0 (7)	0.805 ± 0.009 (7)
Mature	Control	4 ± 3 (7)	0.808 ± 0.009 (7)
	30–55°C	77 ± 27 (6)	0.102 ± 0.188 (6)
	30–49°C	2 ± 4 (7)	0.786 ± 0.010 (7)
	30–51°C	2 ± 2 (7)	0.807 ± 0.008 (7)

the long run. The T_{crit} values obtained with the F_0 method for *C. inophyllum* were within the range of T_{crit} values reported for plants from other tropical sites (O'Sullivan *et al.* 2017).

A second set of experiments demonstrated that T_{crit} was substantially below the temperature which led to significant permanent tissue damage. Whereas in the first series of experiments, leaves were heated 6 or 8°C above their T_{crit}, heating of leaves to exactly T_{crit} (47°C in young and 49°C in mature leaves) resulted in no (young leaves) or very little visible damage (mature leaves) (Table 1, Fig. 3). Even heating of leaves to 2°C above their respective T_{crit} did not alter these findings, which were supported by consistently high F_v/F_m values close to or above 0.8 after post-culture. These F_v/F_m values were identical to or only slightly below those of young and mature control leaves that did not undergo heat treatments (Table 1). In *C. inophyllum*, T_{crit} values derived from the initial rise in F_0 are thus not a reliable proxy of leaf temperatures above which serious irreversible tissue damage occurs. Young and mature leaves of *C. inophyllum* had higher thermotolerances than suggested by their T_{crit}.

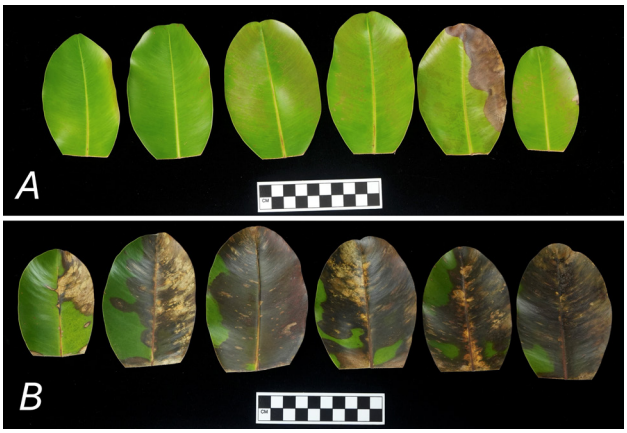

Fig. 2. Photographs of young (*A*) and mature (*B*) leaves of *Calophyllum inophyllum* that had been exposed to stepwise increases in temperature (1°C min^{-1}) from 30 to 55°C, after 14 d of post-culture. Mature leaves are highly necrotic.

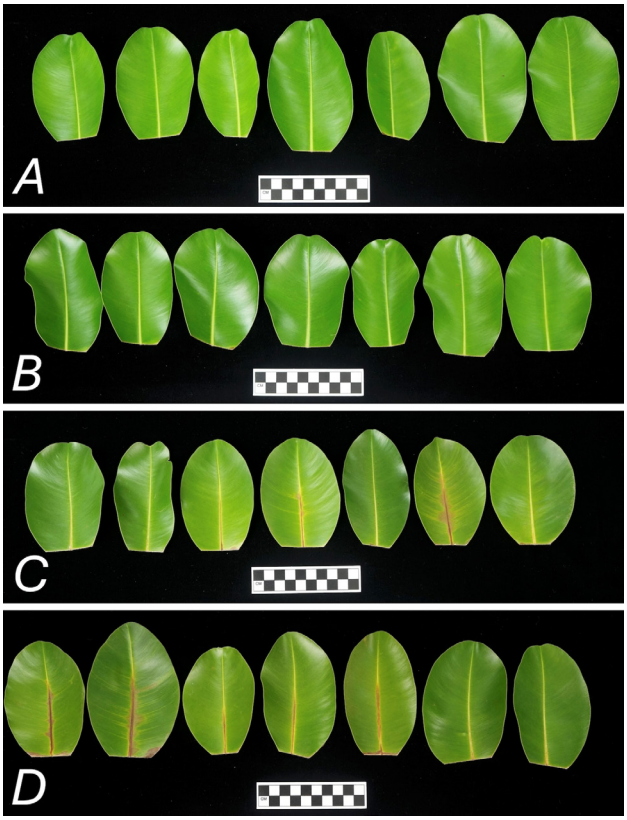

Fig. 3. No or little necrosis in young (*A*,*B*) and mature (*C*,*D*) leaves of *Calophyllum inophyllum* 14 d after they had been heated up to T_{crit} or T_{crit} + 2°C. (*A*) young leaves, temperature increased from 30 to 47°C; (*B*) young leaves, temperature increased from 30 to 49°C; (*C*) mature leaves, temperature increased from 30 to 49°C; (*D*) mature leaves, temperature increased from 30 to 51°C.

Conclusion: T_{crit} derived from measurements of the heat-induced rise of F_0 is not a good indicator of the upper thermal survival limits of leaves of the tropical tree species *C. inophyllum*, because temperatures higher than T_{crit} are necessary to elicit permanent tissue damage. Caution is thus required when considering T_{crit} of the temperature-dependent rise of F_0 to model and predict the future of tropical rainforests facing global warming (*e.g.*, Winter 2024).

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