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SHORT COMMUNICATION

Leaf age and methodology impact assessments of thermotolerance of *Coffea arabica*

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Abstract

Key message **Mature *Coffea arabica* leaves were more heat tolerant than expanding leaves, longer recovery time yielded more accurate thermotolerance assessments, and photochemistry was more heat sensitive than cell membranes.**

Abstract Given future climate predictions of increased heatwaves in the tropics, suitable habitat to grow ecologically, economically, and socially valuable *Coffea arabica* L. is threatened. Accurate assessments of high temperature tolerance or thermotolerance are critical for understanding *C. arabica* responses to increased temperature. Thermotolerance curves of *C. arabica* leaf discs were constructed by measuring chlorophyll fluorescence (ratio of variable to maximum fluorescence, F_V/F_M; minimum fluorescence, F_O) across three leaf age classes and two recovery times (15 min, 24 h) after 15 min exposure to temperatures from 25 to 58 °C. Thermotolerance measured with electrolyte leakage after 20 min at 25–65 °C was compared with F_V/F_M thermotolerance curves. The temperature corresponding to 50 % damage (T_{50}) was 49.0 and 58.6 °C for the chlorophyll fluorescence and electrolyte leakage methods, respectively. The critical temperature at which the F_O rise began on F_O-temperature curves (T_{crit}) was 46.0 °C. We found that the 24 h recovery time yielded more accurate estimates of T_{50}, and that thermotolerance based on T_{crit} increased with leaf age. Differences between the fluorescence and electrolyte leakage methods showed that photosystem II processes were more sensitive to temperatures above 40 °C than cell membrane stability.

Keywords Chlorophyll fluorescence · Electrolyte leakage · Heat tolerance · Leaf age

Abbreviations

F_V/F_M	Ratio of variable to maximum fluorescence
F_O	Minimum fluorescence
T_{50} (F_V/F_M)	Temperature that caused a 50 % reduction in F_V/F_M
T_{50} (electrolyte leakage)	Temperature that caused a 50 % increase in percent electrolyte leakage
T_{crit}	Temperature at which the slow- and fast-rising portions of the F_O-T curve intersect

Introduction

The shade-adapted species *Coffea arabica* L. originated from Ethiopia and is now grown in 80 countries throughout the inter-tropical zone (DaMatta and Ramalho 2006). Mature *C. arabica*'s optimal mean annual temperature range is 18°–24° (Camargo 1985; Teketay 1999). However, the frequency and intensity of heat waves in the tropics are

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expected to increase through the twenty first century (IPCC 2014) and exceed thermal limits (O'Sullivan et al. 2016). *C. arabica* is also often grown under full sunlight where leaf temperatures of ~40 °C have been reported (Barros et al. 1997; Rodríguez-López et al. 2013), especially in outer canopy leaves (DaMatta 2004). Given these potential threats to *C. arabica* (Davis et al. 2012; Craparo et al. 2015), as well as the potential for increased ambient [CO_2] to mitigate temperature stress (Rodrigues et al. 2016), it is vital to accurately quantify the thermotolerance of *C. arabica* to predict and improve our understanding of its physiological responses to increasing temperatures.

High temperature stress impacts many components of plant function including growth, phenology, membrane integrity, photosynthesis, and respiration, all of which may differ in temperature responses (Wahid et al. 2007; Bita and Gerats 2013). To assess plant responses to increasing temperature, thermotolerance curves are constructed using plant functions such as photosynthesis and photorespiration (Larcher 1995; O'Sullivan et al. 2013), as well as proxies for plant functions including visible foliar damage for permanent damage (Bilger et al. 1984; Cunningham and Read 2006), electrolyte leakage for membrane stability (Whitlow et al. 1992; Bajji et al. 2002), and chlorophyll fluorescence for photochemistry and photosynthetic performance (Maxwell and Johnson 2000). Proxy measurements are simplified by conducting them on leaf discs rather than intact leaves attached to a plant (Buchner et al. 2013). Although diverse plant functions have been used to assess thermotolerance, each plant function or proxy may differ in its response to increased temperature, influencing assessments of overall thermotolerance.

Both electrolyte leakage and chlorophyll fluorescence are well-established techniques that are widely used to assess tolerance of heat and other stresses (Krause and Weis 1984; Bilger et al. 1987; Lindgren and Hällgren 1993; Schreiber et al. 1995; Baker and Rosenqvist 2004). Although both methods have been used in *C. arabica* (Ramalho et al. 2000; Campos et al. 2003; Ramalho et al. 2003; Gascó et al. 2004; Chaves et al. 2007; Partelli et al. 2009; Batista-Santos et al. 2011), these methods have not been used to generate thermotolerance parameters for quantitative comparisons nor to measure leaf age-related differences in thermotolerance.

Given the relatively long life span of up to 510 days reported for *C. arabica*'s evergreen leaves (Vasudeva and Gopal 1975), and its specific temperature requirements at different ontogenetic stages (DaMatta and Ramalho 2006), thermotolerance likely changes with leaf age in *C. arabica.* Consistent with this expectation, based on chlorophyll fluorescence, Yamada et al. (1996) found that 3.5-month-old leaves of 23 tropical fruit-bearing species were more heat tolerant than 2.5-month-old leaves, suggesting that leaf ageing and life span are related to photosynthetic thermotolerance (Zhang et al. 2012).

In addition to leaf age, assessments of thermotolerance can be particularly influenced by the time between temperature exposure and when measurements are made. Krause et al. (2010) investigated recovery time after temperature exposure in tropical, mature *Ficus insipida* trees and found that a 24 h recovery time yielded more accurate measures of thermotolerance using chlorophyll fluorescence than a 15 min recovery time.

It is unknown how leaf age, the type of method used, and measurement protocol influence assessments of thermotolerance parameters in *C. arabica.* To fill this gap, our main objectives were to quantify thermotolerance curves of *C. arabica* across a wide range of temperatures in three leaf age classes, using chlorophyll fluorescence and electrolyte leakage methods, and two recovery times after temperature exposure. To our knowledge, this is the first study to compare thermotolerance across methods and leaf age classes in *C. arabica*. We addressed the following hypotheses: (1) older leaves are more heat tolerant than younger leaves, (2) a 24 h recovery time results in more accurate thermotolerance assessments than a 15 min recovery time, and (3) photochemistry assessed from chlorophyll fluorescence will be more sensitive to increasing temperature than membrane damage assessed from electrolyte leakage.

Materials and methods

Plant material

C. arabica (Eritrean Mokka) plants of ~6–9 months old, obtained from the Hawaii Agriculture Research Center in January 2014, were grown in a peat–perlite–pumice growing mix (Sunshine LA4 P) in 9.63 L pots in a greenhouse in Corvallis, Oregon. Supplemental metal halide and high pressure sodium lighting (400 watts) was used to maintain a 12-h photoperiod during fall and winter. Measurements were made in May 2014 when average daytime temperature was 24.0 °C and nighttime temperature was 19.7 °C, relative humidity ranged from 42 to 65 %, and average daily maximum photosynthetically active radiation (PAR) was 424 µmol photons m^{-2} s^{-1}; and in May 2015 when average daytime temperature was 22.9 °C and nighttime temperature was 19.5 °C, relative humidity ranged from 53 to 67 %, and average daily maximum PAR was 623 µmol photons m^{-2} s^{-1}. Plants were watered to field capacity three times per week and fertilized once every 2 weeks (12 % N, 4 % P_2O_5, 8 % K_2O). *C. arabica* is a tropical evergreen species that produces new flushes of leaves year-round. Leaf age class

(expanding, fully expanded, mature) was determined by visually dividing a plagiotropic branch into thirds where leaf age sequence increased from the outermost leaves to the base of the branch (e.g., Wright et al. 2006). Leaves in the outer third were the youngest and still expanding (expanding), leaves in the middle third were expanded (fully expanded), and leaves in the inner third were the oldest, fully expanded, and mature (mature). All leaf age classes showed net CO_2 uptake (Online Resource 1) and rates were similar to those previously reported for *C. arabica* (Ramalho et al. 2013).

Chlorophyll fluorescence

In May 2014, one 2.54 cm^2 leaf disc was collected with a 1.8-cm diameter borer from each of three randomly selected leaves within each age class from each of the six plants prior to dawn to ensure sufficient dark-adaptation time. Dark-adapted leaf discs were placed in closed plastic bags and immersed in a preheated water bath (Aquabath 2343, Thermo Fisher Scientific, Marietta, OH, USA) at each temperature (25–58 °C) in 3 °C steps (e.g., Cunningham and Read 2006) for 15 min at each temperature in dim indoor light. Different sets of leaf discs were exposed to each temperature. A fine-wire thermocouple in each plastic bag recorded that discs reached each desired temperature within 1 min. Chlorophyll fluorescence was measured at room temperature with a portable pulse-amplitude modulated chlorophyll fluorometer (Mini-PAM, Heinz Walz GmbH, Germany) 15 min and 24 h after exposure to each temperature. Untreated leaf discs without heating served as controls. During recovery times, leaf discs were stored in the dark (e.g., Cunningham and Read 2006) on petri dishes with moist filter paper.

To measure F_O, a measuring light (red light-emitting diode, 650 nm, 0.15 µmol photons m^{-2} s^{-1} PAR) with a pulse-width of 3 µs was turned on at a pulse modulation frequency of 0.6 kHz to induce the minimal level of fluorescence (F_O). To avoid confounding effects of continuous additional heat exposure time, F_O was measured on leaf discs at each temperature to isolate the effect of a single temperature (e.g., Cunningham and Read 2006; Krause et al. 2010). F_V/F_M was then determined by applying a 0.8 s saturating pulse of white light (18,000 µmol photons m^{-2} s^{-1} PAR), which transiently closed all PSII reaction centers, minimized heat dissipation, and induced maximum fluorescence (F_M) and variable fluorescence (F_V). F_V/F_M was calculated as:

$$\frac{F_V}{F_M} = \frac{F_M - F_O}{F_M} = 1 - \frac{F_O}{F_M}, \quad (1)$$

(Genty et al. 1989). F_V/F_M is a reliable measure of the potential quantum yield of PSII. Declines in F_V/F_M indicate stress-induced changes to photochemistry, damage to the oxygen evolving complex and water splitting system, disruptions in electron donation to PSII reaction centers, shifts in nonradiative (heat) dissipation of excitation energy, photodamage, and/or photoinhibition (Schreiber and Berry 1977; Weis and Berry 1987; Demmig et al. 1987; Krause 1988; Havaux 1993; Maxwell and Johnson 2000; Demmig-Adams et al. 2014).

Electrolyte leakage

In May 2015, one mature, fully expanded leaf (mature age class) was collected before dawn from each of the five plants. Two discs from each leaf were placed in 6 mL of deionized H_2O in 15 mL polycarbonate tubes. Samples were infiltrated under vacuum for 15 min. Tubes were heated for 20 min in a preheated water bath at each desired temperature (30–65 °C) in 5 °C steps. Different sets of leaf discs were exposed to each temperature. Tubes were shaken for 1.5 h and the conductivity of the water in each tube was immediately measured with a conductivity meter (Product 89094-958, VWR International, Radnor, PA, USA). Tubes were then heated for 20 min in a 100 °C water bath and shaken again for 1.5 h. The final conductivity of the solution was immediately measured and represented electrolyte leakage of completely broken leaf membranes. Percent electrolyte leakage or percent damage was calculated as:

$$\%\text{Electrolyte leakage} = \frac{\text{Conductivity after exposure to temperature } (^\circ\text{C})}{\text{Conductivity after exposure to } 100\,^\circ\text{C}} \times 100. \quad (2)$$

To determine whether the 1-year difference in dates on which each method was used to measure thermotolerance curves affected results (F_V/F_M and electrolyte leakage methods were conducted on different plants 1 year apart, May 2014 and May 2015, respectively), three mature fully expanded leaves of the mature age class from each of five plants were collected at pre-dawn on the same day in December 2015. Chlorophyll fluorescence (one disc per leaf) and electrolyte leakage (two discs per leaf) methods were used to measure F_V/F_M and percent damage after discs were exposed to 50 °C, the temperature at which differences between methods were greatest.

Statistical analysis

Statistical analyses were conducted in SigmaPlot 13.0 (Systat Software, San Jose, CA, USA) and R version 3.2.3 (2015-12-10, The R Foundation for Statistical Computing). To account for the same individual plants being measured

repeatedly, a two-way repeated measures analysis of variance (ANOVA) extra sum of squares F test was used to determine whether there was a significant interaction between the main effects of leaf age and recovery time on F_V/F_M values of control (untreated) leaf discs, T_{50}, and T_{crit}. The Shapiro–Wilk procedure tested for normality and the Brown–Forsythe procedure tested for equal variance. Simple linear regression was used to compare T_{crit} and T_{50}. To compare the F_V/F_M and electrolyte leakage methods, a two-sample t test compared mean T_{50} derived from each method.

Results and discussion

The mean F_V/F_M of untreated leaf discs of 0.72–0.74 for both recovery times was slightly lower than previously reported values (0.75–0.78, Rodrigues et al. 2016) which may be due to low irradiance (Ramalho et al. 2013) or the nature of the high pressure sodium and metal halide supplemental lighting in the greenhouse that may influence photochemistry (Muneer et al. 2014; Li et al. 2016). F_V/F_M of *C. arabica* began to decline significantly above 40 °C (Fig. 1), consistent with other species (Loik and Harte 1996; Krause et al. 2010), indicating a reduction in maximum quantum efficiency of PSII photochemistry due to high temperature stress (Maxwell and Johnson 2000). Reductions in F_V/F_M have also been attributed to photoinhibition in *C. arabica* (Martins et al. 2016).

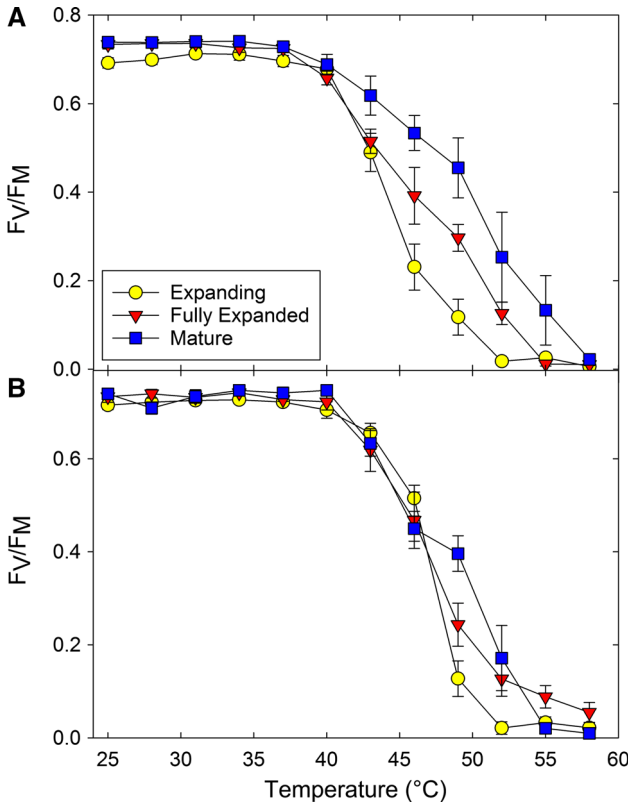

Fig. 1 Thermotolerance curves measured with F_V/F_M as a function of temperature of three leaf age classes (expanding, fully expanded, mature) of *C. arabica* measured 15 min after temperature exposure (**a**) and 24 h after temperature exposure (**b**). $N = 6$. *Error bars* represent SE

There was a marginally significant interaction between recovery time and leaf age on T_{50} ($F = 3.952$, $P = 0.054$). After 15 min of recovery, mean T_{50} differed significantly among all three age classes, with T_{50} increasing with leaf age ($P < 0.05$, Table 1). The greatest difference in mean T_{50} (5.3 °C, $P < 0.001$) occurred between the mature and expanding age classes. After 24 h of recovery, T_{50} also increased with increasing leaf age but differences were not significant between any age classes ($P > 0.05$, Table 1). Significant differences in T_{50} among all leaf age classes at 15 min but not 24 h after treatment demonstrates how the time between temperature exposure and measurement influences apparent thermotolerance and interpretations of T_{50}. T_{50} of the expanding age class only significantly increased after 24 h of recovery ($P = 0.025$), while T_{50} of the fully expanded and mature age classes did not significantly change between recovery times, suggesting that older *C. arabica* leaves are more resistant to temperature-induced damage (Zhang et al. 2012). The significant increase in T_{50} of expanding leaves between recovery times (Krause et al. 2010) from 44.8 to 47.0 °C (Table 1) contrasts with the generalization that damage to PSII is only reversible at temperatures below 40 °C (Teskey et al. 2015), emphasizing the importance of considering recovery time, study species, and light conditions (Demmig-Adams et al. 2014). The increase in T_{50} of the expanding age class between recovery times also decreased the range of T_{50} across leaf age classes after the 24 h recovery (~47–49 °C) compared to the 15 min recovery (~45–50 °C), suggesting that allowing 24 h recovery may reduce leaf age-related variation in thermotolerance curves measured with F_V/Fm and may more accurately assess T_{50} (Krause et al. 2010).

There was a statistically significant interaction between recovery time and leaf age on mean T_{crit} ($F = 8.596$, $P = 0.007$). Mean T_{crit} of the mature age class was significantly greater than that of both the expanding and fully expanding age classes for both recovery times (Table 1, $P < 0.001$). Mean T_{crit} did not significantly differ between recovery times for any age class ($P > 0.05$). This contrasts with T_{50}, suggesting that T_{crit} may be a more robust measure of photosynthetic thermotolerance than T_{50}. The T_{crit} results are consistent with the hypothesis that older leaves have greater photosynthetic thermotolerance than younger leaves, an evolutionary adaptation that would protect older leaves from irreversible damage (Yamada et al. 1996). Zhang et al. (2012) also found that leaf lifespan was positively related to T_{crit}, suggesting that longer persistence is

Table 1 Thermotolerance parameters (°C) derived from curves of F_V/F_M, electrolyte leakage, and F_O as a function of treatment temperature for 15 min and 24 h recovery times of three leaf age classes (expanding, fully expanded, mature)

	Expanding	Fully expanded	Mature
T_{50} (F_V/F_M) 15 min	44.8 ± 0.5 Aa	46.9 ± 0.6 Ab	50.1 ± 1.3 Ac
T_{50} (F_V/F_M) 24 h	47.0 ± 0.2 Ba	47.9 ± 0.5 Aa	49.0 ± 0.5 Aa
T_{50} (electrolyte leakage)	–	–	58.6 ± 0.9
T_{crit} (F_O) 15 min	43.1 ± 0.2 Aa	43.7 ± 0.1 Aa	45.1 ± 0.3 Ab
T_{crit} (F_O) 24 h	42.7 ± 0.3 Aa	42.9 ± 0.5 Aa	46.0 ± 0.4 Ab

T_{50} (F_V/F_M) is the temperature that caused a 50 % reduction in untreated (control) F_V/F_M. T_{50} (electrolyte leakage) is the temperature that caused a 50 % increase in minimum percent electrolyte leakage or percent damage

Uppercase letters indicate significant differences between recovery times within each age class ($P < 0.05$)

Lowercase letters indicate significant differences among age classes within each recovery time ($P < 0.05$)

Mean ± SE. $N = 5$–6

Thermotolerance curves assessed with F_V/F_M and electrolyte leakage were determined from third order sigmoidal functions fitted to the data: $f = a/(1 + \exp(-(x - x_0)/b)$, where f is percent of untreated (control) F_V/F_M or percent electrolyte leakage, x is the treatment temperature, and a, $x0$, and b are fitting parameters. T_{50} (F_V/F_M) and T_{50} (electrolyte leakage) were calculated from this equation

related to greater photosynthetic thermotolerance. The leaf age-related differences in T_{crit} and T_{50} may also reflect age-related shifts in the xanthophyll cycle-associated energy dissipation (Demmig-Adams and Adams 1994; Tegischer et al. 2002). Due to the evolutionary tradeoff between metabolism and persistence that governs many leaf traits related to leaf age (Poorter and Bongers 2006), it is expected that thermotolerance also varies with leaf age.

The range of T_{crit} observed in *C. arabica* (42.7–46.0 °C) falls within the broad range of values (35–48 °C) reported for other tropical species (Weng and Lai 2005). In addition to our results showing that leaf age significantly influences T_{crit} in *C. arabica*, other studies show that T_{crit} is highly plastic (Knight and Ackerly 2002) and influenced by environmental conditions such as ambient temperature and drought (Schreiber and Berry 1977; Smillie and Nott 1979; Knight and Ackerly 2002; Ghouil et al. 2003; Daas et al. 2008). To place this study in the context of field grown plants, F_V/F_M of field grown *C. canephora* remained relatively stable throughout the day despite leaf temperatures of 42.8 °C (Rodríguez-López et al. 2013). Similarly, F_V/F_M declines in field grown *C. arabica* did not occur until above 42 °C compared to other exposure temperatures of 25–42 °C (Rodrigues et al. 2016).

T_{crit} derived from F_O-T thermotolerance curves (Fig. 2) was significantly related to T_{50} derived from the F_V/F_M thermotolerance curves (Fig. 1) across all three age classes for both recovery times ($P < 0.001$, $R^2 = 0.69$; $P = 0.003$, $R^2 = 0.43$, Fig. 3). Generally T_{50} derived from F_V/F_M thermotolerance curves was greater than T_{crit} derived from F_O-T curves (Table 1; Fig. 3), consistent with the T_{50}–T_{crit} relationship observed in *Eucalyptus* trees (Lin 2012), implying that significant damage to photochemistry occurs prior to a 50 % reduction in F_V/F_M. The significant

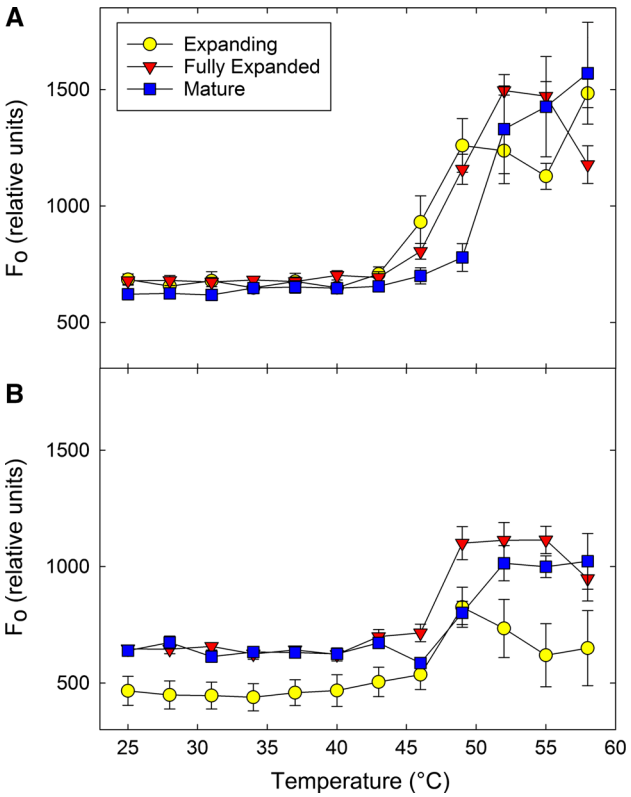

Fig. 2 Thermotolerance curves measured with F_O as a function of temperature of three leaf age classes (expanding, fully expanded, mature) of *C. arabica* measured 15 min after temperature exposure (**a**) and 24 h after temperature exposure (**b**). $N = 6$. *Error bars* represent SE. T_{crit} was determined from the intersection of two regression *lines* (not shown) extrapolated from the slow- and fast-rising portions of the F_O-T curve (e.g., Schreiber and Berry 1977)

relationships between T_{crit} and T_{50} across all leaf age classes for both recovery times (Fig. 3) are consistent with previous observations that T_{crit} was significantly related to

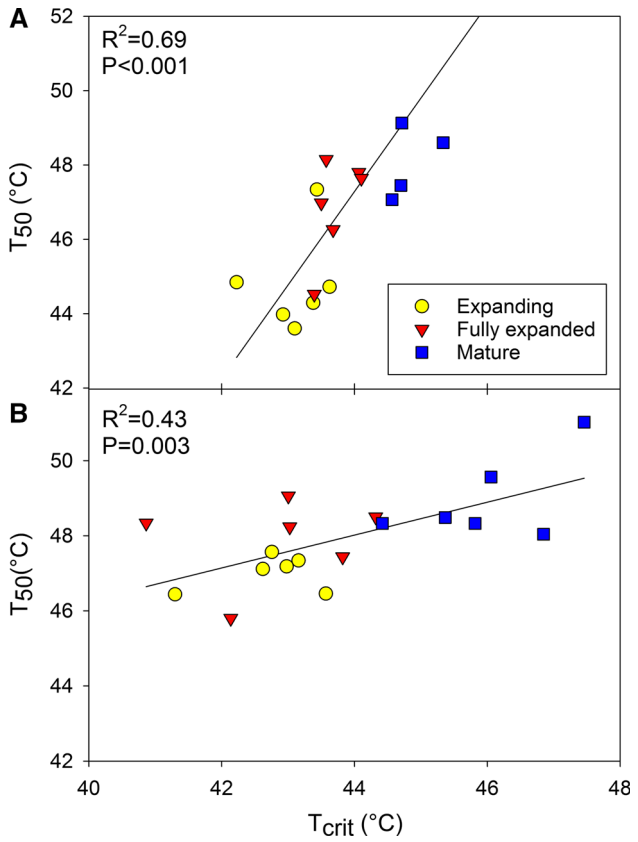

Fig. 3 Relationship between T_{crit} derived from F_O-T thermotolerance curves and T_{50} derived from F_V/F_M thermotolerance curves for all age classes (expanding, fully expanded, mature) measured 15 min after temperature exposure (**a**) and 24 h after temperature exposure (**b**). $N = 6$. Each point is an individual plant. Equation of simple linear regression line: $y = -63.20 + 2.51x$ (**a**) and $y = 28.767 + 0.438x$ (**b**). Non-significant regression *lines* within each age class are not shown ($P > 0.05$)

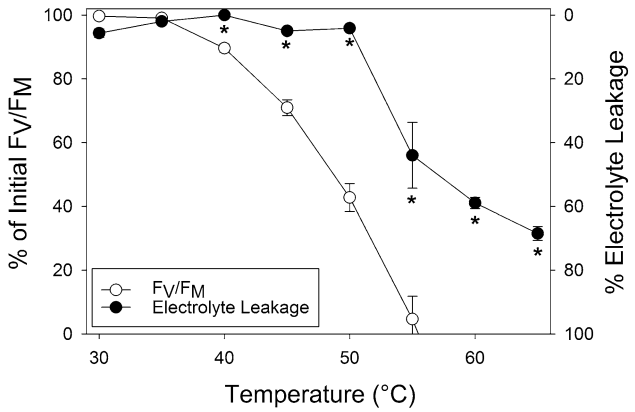

Fig. 4 Thermotolerance curves measured with percent electrolyte leakage and percent F_V/F_M. Percent F_V/F_M data points were estimated from a third order sigmoidal function fitted to the F_V/F_M thermotolerance curve. The equation $f = a/(1 + \exp(-(x - x_0)/b)$, where f is percent of untreated (control) F_V/F_M or percent electrolyte leakage, x is the treatment temperature, and a, $x0$, and b are fitting parameters, was rearranged and used to estimate F_V/F_M percent values at the same temperatures as those used to construct the electrolyte leakage curves (every 5 °C vs every 3 °C). This allowed percent values derived from each method to be directly compared at each temperature. Percent F_V/F_M was normalized so untreated (control) F_V/F_M was 100 % (no damage). Percent electrolyte leakage was normalized so minimum percent electrolyte leakage was 0 % (no damage). Percent electrolyte leakage axis is inverted for easier comparison of curves. *Asterisks* represent $P < 0.05$. $N = 5$–6. *Error bars* represent SE. Because T_{50} derived from F_V/F_M thermotolerance curves of the mature age class did not significantly change with recovery time, the F_V/F_M thermotolerance curve of the mature age class measured after the 24 h recovery time was compared with the electrolyte leakage method

other measures of thermotolerance (Schreiber and Berry 1977; Downton et al. 1984; Bilger et al. 1984). The significant relationship between T_{crit} and T_{50} in *C. arabica* across but not within leaf age classes suggests that considerations of leaf age are important when assessing thermotolerance, and sampling a range of leaf ages would yield the most representative thermotolerance measurements.

To our knowledge, this is the first study to directly quantify and compare thermotolerance assessments using both methods in *C. arabica*. With increasing temperature, percent F_V/F_M declined above 40 °C while percent electrolyte leakage did not increase until a threshold of ~50 °C was reached. T_{50} from the electrolyte leakage thermotolerance curve (58.6 ± 0.9 °C) was significantly greater than T_{50} from the F_V/F_M thermotolerance curve (49.0 ± 0.5 °C; $P < 0.001$; Table 1). The comparison of the electrolyte leakage and chlorophyll fluorescence methods used to measure thermotolerance curves (Table 1; Fig. 4) showed that F_V/F_M was more sensitive to increasing temperature than electrolyte leakage, likely because the photosynthetic apparatus is more sensitive to high temperature stress than cell membranes (Bjorkman et al. 1975; Levitt 1980; Gascó et al. 2004; DaMatta and Ramalho 2006; Xu et al. 2014), visible leaf damage (Cunningham and Read 2006), and tissue necrosis (Bilger et al. 1984).

This contrasts with studies that found close correlations between chlorophyll fluorescence and visible damage assessments (Lindgren and Hällgren 1993; Larcher 1995; Binder and Fielder 1996; Liu and Huang 2000). These discrepancies among studies may be related to differences in species, experimental setup, environmental conditions, and type of stress investigated. Other studies that have used both methods did not directly compare methods (Loik and Harte 1996; Yang et al. 1996). The mixed results when evaluating thermotolerance using chlorophyll fluorescence and tissue damage methods demonstrate that although methods to quantify thermotolerance are often related, response to temperature varies across plant functions (Teskey et al. 2015) and leaf age (Table 1), highlighting the complexity of evaluating thermotolerance and the importance of considering the methodology, plant function, and leaf age. Our results also confirmed that significant differences in assessments of thermotolerance between the

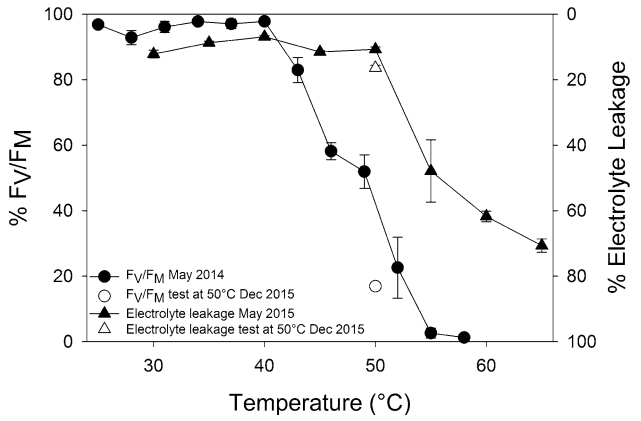

Fig. 5 Thermotolerance curves measured with non-normalized F_V/F_M in May 2014 (*closed circles*) and non-normalized electrolyte leakage in May 2015 (*closed triangles*) compared with mean percent F_V/F_M and mean percent electrolyte leakage values at 50 °C assessed on the same individuals on the same day in December 2015 (*open circle*, *open triangle*, respectively). $N = 5$–6. *Error bars* represent SE. Percent electrolyte leakage axis is inverted for easier comparison of curves. Percent F_V/F_M and percent electrolyte leakage values at 50 °C significantly differed ($P < 0.05$) and were consistent with the original F_V/F_M and electrolyte leakage thermotolerance curves measured in May 2014 and May 2015. This demonstrated that timing of when the method was conducted did not affect the observed differences between methods

F_V/F_M and electrolyte leakage methods (Fig. 5) were not affected by measurement date, demonstrating each method's high repeatability on mature fully expanded leaves and the greater high temperature sensitivity of PSII than cell membrane integrity. Because chlorophyll fluorescence is rapid and non-invasive, it may be a more practical tool for the early evaluation of thermotolerance in different genotypes.

Author contribution statement Danielle E. Marias participated in the experimental design, data collection, data analyses, and writing of the manuscript. Frederick C. Meinzer participated in the experimental design, data analyses, and writing of the manuscript. Christopher Still participated in the experimental design and writing of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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